METHODS IN MOLECULAR BIOLOGY™

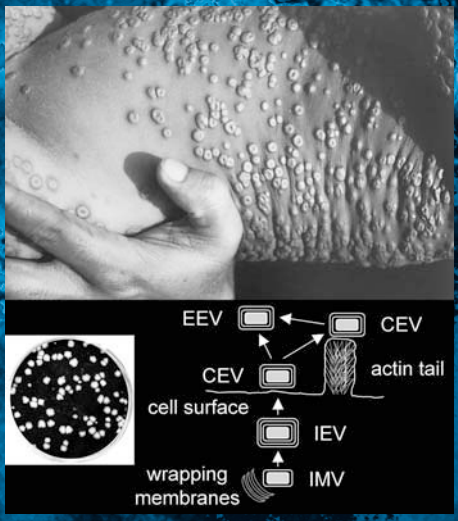
Volume 269

Vaccinia Virus and Poxvirology

Methods and Protocols

Edited by

Stuart N. Isaacs



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Vaccinia Virus and Poxvirology

Methods and Protocols

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Foreword

The Right Book at the Right Time

The poxviruses comprise a family of complex DNA viruses that replicate in the cytoplasm of vertebrate or invertebrate cells. Of the eight recognized genera of vertebrate poxviruses, those belonging to the orthopoxvirus genus have been most intensively studied. This group includes variola virus, the agent of smallpox, as well as cowpox virus and vaccinia virus. Jenner's original smallpox vaccine, described in 1798, consisted of live cowpox virus, but vaccinia virus later replaced it (***1***). There has been speculation as to the origin of vaccinia virus; the most likely idea is that it is a separate species, possibly originally isolated from a horse, and is now extinct or rare in nature (***2***). Recent genome sequencing studies confirm the distinctness of variola virus, cowpox virus, and vaccinia virus and also their very close genetic relationship, which accounts for the cross protection of smallpox vaccines. The novelty of the smallpox vaccine can be readily appreciated by the time it took, about 80 years, before the next live vaccine against rabies was developed, and another 50 years for the yellow fever vaccine. Moreover, the eradication of smallpox in 1977 stands as a unique medical achievement. Because of its historical role, smallpox vaccination contributed greatly to present concepts of infectious disease, immunity, and pathogenesis. Less well known, however, are the many other "firsts" for vaccinia virus. Vaccinia virus was the first animal virus seen microscopically, grown in tissue culture, accurately titrated, physically purified, chemically analyzed, shown to regulate gene expression, and found to encode transcriptase and replicase activities and modulators of host defense (***3***).

Through the early 1980s, a relatively small number of laboratories worked with vaccinia virus. This number increased dramatically following the demonstration of vaccinia virus as a gene expression vector (***4,5***) and the wide dissemination of convenient transfer plasmids for the generation of recombinant vaccinia viruses by homologous recombination (***6***). These recombinant viruses were used for a myriad of purposes by molecular biologists, immunologists, and vaccinologists (***7***). To aid novice poxvirologists, protocols for growing, titrating, and purifying recombinant vaccinia viruses were published (***8–10***). Very recently, there has been public concern regarding the use of orthopoxviruses as agents of bioterrorism. To counter such perceived threats, efforts are

being made to develop new therapeutic agents as well as more attenuated vaccines. This new agenda, and the funds that support it, have resulted in a second influx of novice poxvirologists and a need for a comprehensive compendium of methods for studying many aspects of vaccinia virus. *Vaccinia Virus and Poxvirology: Methods and Protocols*, therefore, comes at an appropriate time and should meet a critical need.

Bernard Moss

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Preface

Vaccinia Virus and Poxvirology: Methods and Protocols provides a single comprehensive source of protocols for work involving poxviruses. Poxvirology has long been important for basic studies in virology, immunology, and cell biology. However, in recent years, poxviruses have become an especially important topic because of the politics involving issues related to smallpox bioterrorism and pre-event vaccinia virus vaccination and the need to develop anti-poxvirus therapeutics and safer smallpox vaccines.

Because recombinant vaccinia viruses are used widely as protein expression vectors, there are multiple chapters covering various approaches for the construction, characterization, and use of such recombinant viruses in immunologic and cell–cell fusion assays. Other chapters focus on methods to study poxvirus gene transcription and DNA replication, and the study of the binding, entry, and movement of the virus in host cells. *Vaccinia Virus and Poxvirology: Methods and Protocols* also covers methods in poxvirus bioinformatics, as well as aspects of viral pathogenesis at both a protein and an animal model level. Finally, protocols involving the study of immune responses to poxviruses are addressed, which is a critical issue given their role in smallpox vaccination and their potential role as vaccine vectors directed against infectious agents and cancer.

Each chapter, written by a leader in the field, contains a short introduction that provides an overall perspective for the topic. The protocols are designed to be easy to follow and the Notes sections include both additional explanatory information and important insights into the protocols. Thus, *Vaccinia Virus and Poxvirology: Methods and Protocols* should be useful to investigators who are new to poxvirology and/or to using vaccinia virus as a research tool. In addition, given the diverse topics covered, those experienced in poxvirology should also find this book useful because it serves as an introduction to new areas of research and novel ways to use poxviruses as scientific tools.

Stuart N. Isaacs

Acknowledgments

I recognize that there are many people who have helped me reach my current position. I would like to specifically acknowledge my parents. Their sacrifices and nurturing were instrumental in setting the stage for my success. I am saddened by the fact that my Father died before seeing me reach my current level of accomplishment at work and at home. I also need to acknowledge Bernie Moss and my former colleagues at the Laboratory of Viral Diseases (NIH). Bernie and my labmates at LVD introduced me to the exciting field of poxvirology and taught me how to become a scientist. They have all become life-long friends. I also want to acknowledge the excellent contributions to this book by each of the authors. It was a pleasure working with them during this process. Finally, I need to acknowledge my family whose support during all stages of this project allowed this book to become a reality.

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1

Working Safely with Vaccinia Virus

*Laboratory Technique and the Role of Vaccinia Vaccination**

Stuart N. Isaacs

Summary

Vaccinia virus, the prototype Orthopoxvirus, is widely used in the laboratory as a model system to study various aspects of viral biology, virus–host interactions, and as a protein expression system and a vaccine vector. The ubiquitous use of vaccinia viruses in the laboratory raises certain safety concerns, because the virus can be a pathogen in individuals with immunological and dermatological abnormalities and, on occasion, can cause serious problems in normal hosts. This chapter reviews standard operating procedures when working with vaccinia virus and issues surrounding the use of prophylactic smallpox vaccination for laboratory workers.

Key Words: Vaccinia virus; biosafety level 2; class II biological safety cabinet; personal protective equipment; smallpox vaccine; vaccination complications.

1. Introduction

Poxviruses are large DNA viruses with genomes of approx 200 kb. Their unique site of DNA replication and transcription ***(1)***, the fascinating immune evasion strategies employed by the virus ***(2,3)***, and the relative ease of generating recombinant viruses that express foreign proteins in eukaryotic cells ***(4,5)*** have made poxviruses an exciting system to study and a common laboratory tool. Although the variola virus, the causative agent of smallpox, is the most famous poxvirus, its eradication as a human disease by the late 1970s and ability to only work with the virus in two World Health Organization–sanctioned sites under biosafety level 4 conditions has made vaccinia virus the more

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studied and prototype member of the Orthopoxvirus family. Vaccinia virus was used as the vaccine to confer immunity to variola virus and helped in the eradication of smallpox. In the United States, routine vaccination with the smallpox vaccine ended in the early 1970s. Since then, the Advisory Committee on Immunization Practices (ACIP) and the Centers for Disease Control (CDC) have recommended that people working with poxviruses continue to get vaccinated ***(6–8)***. This recommendation for those working with vaccinia virus is based mainly on the potential problems that an unintentional infection due to a laboratory accident may cause.

The rationale for this recommendation is furthered by the understanding that the most widely used laboratory strains of vaccinia virus (e.g., Western Reserve [WR]; *see* **Note 1**) are more virulent than the vaccine strain. Also, laboratory workers frequently handle virus at much higher titers than the dose given in the vaccine (*see* **Note 2**). Reports exist of laboratory accidents involving vaccinia virus ***(9–14)***, and more of these incidents likely go unreported. Thus, both the full extent of the problem and potential benefits from the vaccine are unknown. This chapter discusses laboratory and personal safety equipment and procedures that should help prevent accidental laboratory infections and addresses the ongoing controversy of whether the prophylactic smallpox vaccine should be required for all workers that handle vaccinia virus.

2. Materials and Equipment

1. Class II biological safety cabinet (BSC).
2. Personal protective equipment.
3. Autoclave.
4. Disinfectants: 1% sodium hypochlorite, 2% glutaraldehyde, formaldehyde, 10% bleach, Spor-klenz, Expor, 70% alcohol.
5. Sharps container disposal unit.
6. Centrifuge bucket safety caps.
7. Occupational medicine access to the smallpox vaccine (*see* **Note 3**).

3. Methods

3.1. Laboratory and Personal Protective Equipment

Because unintentional vaccinia virus infections most commonly occur through direct contact with the skin or eyes, the most important aspect of safely working with vaccinia virus is to use proper laboratory and personal protective equipment to help prevent accidental exposure to the virus. One primary defense against accidental exposure is to always work with the infectious virus in a BSC. A BSC is a requirement when working with vaccinia virus. The cabinet not only confines the virus to a work area easily defined and cleaned, but the glass shield on the front of the BSC also serves as an excellent

barrier against splashes into the face. A BSC draws room air through the front grille, circulates HEPA-filtered air within the cabinet area, and also HEPA filters the air that is exhausted. Thus, working in a BSC protects the worker and room where vaccinia virus is being handled from the unlikely event of aerosolization of the virus (*see* **Note 4**).

An equally important defense against accidental exposure to the virus is wearing proper personal protective equipment, which includes gloves, a laboratory coat, and eye protection. Vaccinia virus does not enter intact skin, but it gains access through breaks in the skin. Thus, gloves are critical (*see* **Note 5**). Although the front shield of the BSC provides significant protection against splashes into the eyes, it is also recommended that safety glasses with solid side shields be worn when working with vaccinia virus. Depending on the work being done (e.g., handling high titer purified stocks of vaccinia virus), consider additional eye protection like goggles or a full-face shield. This consideration is important because, as an immunologically privileged site ***(15)***, the eye can be susceptible to serious infection even in those previously vaccinated ***(16)***. Finally, a laboratory coat or some other outer protective garment decreases the chance of contaminating clothing. If such contamination occurs, an outer garment can be quickly removed and decontaminated. Furthermore, a laboratory coat prevents accidentally carrying the virus out of the laboratory environment. Because the virus can be stable in the environment, after protective equipment is removed, washing hands efficiently with soap and water is important ***(17)***.

3.2. Laboratory Safety

The CDC recommends that the nonattenuated vaccinia virus be handled at biosafety level 2 ***(18)***. As with all biohazardous agents, routine good laboratory safety practices need to be fully implemented, which includes no eating or drinking in the laboratory. To decrease the chance of introducing the virus into a break in the skin, the use of any sharps or glass should be minimized while working with vaccinia virus (*see* **Note 6**). Syringes and needles still need to be used when performing some experiments in animals; thus, personnel that work with needles to inject animals with the virus perform a higher risk procedure (*see* **Note 7**). If sharps or disposable glassware are necessary, then the proper leak-proof, puncture-resistant sharps disposal container should be conveniently located close to the work area to prevent accidents during the disposal of

eedles and glass (*see* **Note 8**). As discussed previously, a BSC is a requirement when working with vaccinia virus. As a virus that has an outer membrane envelope, vaccinia virus is susceptible to inactivation by a variety of detergents and disinfectants ***(19)***. Thus, after working with the virus in the BSC, surfaces should be wiped down with freshly prepared 1%

bleach. Because experiments with vaccinia virus in tissue culture frequently involve aspirating and discarding virus-contaminated growth medium, one must properly inactive the virus in the media prior to disposal. For aspirating off media from infected cells using the laboratory vacuum system, include a trap to prevent contamination of the house vacuum system with the virus (*see* **Note 9**). The virus contained in the liquid must be inactivated by the addition of disinfectant (*see* **Note 10**). The virus is also susceptible to heat; thus, autoclaving contaminated instruments, dryware, animal cages, and bedding that are exposed to the virus is also required. Properly packaged disposable plasticware for culturing the virus should be sterilized in a humidified autoclave. Although small samples containing the virus have been shown to be inactivated in autoclaves in as little as 15 min ***(20)***, infectious waste should be autoclaved at 121°C for at least 60 min at 15 pounds per square inch (psi) and then disposed of according to the institutional guidelines (*see* **Note 10**). When centrifuging large volumes of media-containing virus, it is best to use centrifuge buckets that have safety lids to contain a spill if a tube should leak during centrifugation. Additionally, such safety caps should contain areosolization of the virus in case of a centrifuge malfunction. Sonication of infected cells, which is frequently done in poxvirus protocols to release the virus from cells and break up virus clumps can also cause aerosolization of the virus (*see* **Note 11**). Therefore, sonication should be performed in a cup sonicator with the virus or virus-infected cells remaining in a closed tube. Larger virus preparations may need to be sonicated with a probe sonicator, which should only be performed if the sonicator device is contained in a proper BSC that properly filters the air and removes any potential aerosolized virus (*see* **Note 4**).

3.3. Smallpox Vaccination

In the United States, the currently licensed smallpox vaccine (Dryvax) is recommended for people who work with poxviruses (***8***; see **Table 1** and **Note 12**). Although there is no debate that this policy should definitely be followed for researchers working with such poxviruses as the variola virus, camelpox virus, and monkeypox virus (*see* **Note 13**), there is considerable debate whether smallpox vaccination should be carried out in all of those in contact with vaccinia virus ***(21–26)***. ACIP and the CDC recommend vaccination every 10 yr of all people who have contact with vaccinia virus ***(8)***. Of note, this recommendation does not apply to those working with highly attenuated strains of vaccinia virus (i.e., MVA, NYVAC, ALVAC, and TROVAC) that do not replicate well in mammalian cells, are avirulent in normal and immunosuppressed animal models, and are therefore considered extremely safe (*see* **Note 14**). The most recent ACIP recommendations for smallpox vaccination of laboratory workers that handle vaccinia virus are nearly identical to recommendations

Table 1
Vaccinia Vaccination of Laboratory Workers 1983–2002 by Vaccine Provided by the CDC (*see* Note 19)

Year	Number of recipients	VIG releases from the CDC[a]
1983	41	–
1984	126	–
1985	300	–
1986	337	–
1987	359	–
1988	500	–
1989	762	–
1990	686	–
1991	1089	1[b]
1992	1140	1[c]
1993	1113	–
1994	1113	3[d,e,f]
1995	458	3[g,h,i]
1996	475	–
1997	516	–
1998	827	1[j]
1999	204	–
2000	408	–
2001	113	–
2002	1153	–
2003[k]	46	
Total	9341	9

[a]The numbers indicated for vaccinia immune globulin (VIG) released by the CDC is unrelated to the smallpox vaccine recipients. It is included on the table merely to indicate the frequency that the CDC released VIG. – indicates no VIG released.

[b]Generalized vaccinia on the neck, back, legs, scalp, and chest that started 10 d after vaccinia immunization in a 36-yr-old American maintenance employee. The patient fully recovered, and no adverse sequela was reported.

[c]Severe local reaction at the site of accidental inoculation with a recombinant vaccinia virus in an unvaccinated American laboratory worker. No follow-up reports were received by the CDC.

[d]Postvaccinia vaccine reaction in American laboratory worker with preliminary diagnoses of generalized vaccinia. No follow-up report was received by the CDC.

[e]Recombinant vaccinia virus eye splash in an American researcher. No adverse sequela was reported.

[f]Vesicular rash over right forehead and severe edema over the right orbital region, causing eye closure 7 d after vaccinia immunization in a 19-yr-old Israeli recruit with a history of disruptive skin disease. VIG was released by the CDC, but not used because the lesions began to improve prior to arrival of VIG.

[g]Gangrenous lesion at inoculation site in an Australian researcher with chronic lymphocytic leukemia working with recombinant vaccinia virus. No follow-up reports were received by CDC.

[h]Eczema vaccinatum on face, neck, and arms starting 9 d after vaccinia immunization in a 27-yr-old Israeli military recruit. No follow-up reports were received by CDC.

[i]Eczema vaccinatum on the face and neck starting 10 d after vaccinia immunization in a previously healthy 19-yr-old Israeli military recruit who never had a known skin disorder. No follow-up reports were received by CDC.

[j]Recombinant vaccinia virus splashed in to both eyes, face, and mouth of a healthy 36-yr-old researcher previously vaccinated during childhood. Both eyes splashed were washed immediately after the accident for more than 10 min. Vidarabine ophthalmic ointment was applied to both eyes for 7 d after accident. No adverse sequela was reported.

[k]Year 2003 data is only until January 9, 2003, a date prior to the start of the pre-event smallpox vaccination program.

given 10 yr earlier ***(7)*** when essentially all adults working with vaccinia virus had previously been vaccinated at least once in childhood. Because routine vaccination in the United States ended in the early 1970s, there are a growing number of workers who have never been vaccinated; thus, the recommendation to vaccinate such workers represents primary vaccinations. The rate of complications from primary vaccination is 10–20 times greater than the rate of complications in those previously vaccinated (***27,28***; *see* **Table 2** and **Note 15**). Thus, the risk–benefit ratio for routine vaccination of all laboratory workers handling vaccinia virus in the United States has significantly changed from the 1980s and 1990s. In addition, as the vaccine can accidentally spread to close contacts, the potential infection of unvaccinated individuals is more problematic now than in previous times when most of the population had been vaccinated (*see* **Note 16**). In contrast to the ACIP guidelines, advisory committees in other countries have reached different conclusions than the ACIP and do not recommend routine vaccination ***(29)***. These committees concluded that the risk of vaccination (i.e., knowingly infecting an individual with vaccinia virus) outweighs the potential benefit of protecting them from accidental exposure, resulting in an infection.

Although some feel strongly that vaccination should be mandatory ***(30)***, an approach implemented at some institutions in the United States is to offer mandatory counseling by occupational medicine regarding vaccination, but to then allow each individual to make an informed personal decision whether or not to be vaccinated ***(21–26)***. The types of procedures being done with vaccinia virus by the worker should enter into this decision-making process (*see* **Notes 7** and **17**). Another important issue related to the counseling session is that the worker should have a complete medical assessment to determine whether the smallpox vaccine can be safely given. Thus, workers may be identified with medical conditions that would preclude them from vaccination. Such conditions include an immunodeficiency, a history of skin conditions like atopic dermatitis/eczema (*see* **Note 18**), pregnancy, and cardiac disease (*see* **Note 15**). Importantly, some feel that the presence of an immunodeficiency, atopic dermatitis/eczema, or pregnancy should preclude someone from working with vaccinia virus in the laboratory setting. Others think that for a properly trained researcher, working with vaccinia virus while pregnant or with mild eczema can be allowed as long as the individual is not performing high-risk procedures with the virus (*see* examples of higher risk procedures in **Note 7**). Laboratory directors and workers need to be aware of the risks and preventative measures, then helped to make wise decisions. Each institution should develop a policy that addresses these concerns.

Table 2
Rates of Reported Vaccinia Virus Vaccine Complications in Adults (Number of Cases/Million Vaccinations)

Vaccination group	Accidental transfer	Generalized vaccinia	Eczema vaccinatum	Progressive vaccinia	Postvaccinial encephalitis	Total[b]
Primary[a]	606	212	30	–[d]	–[d]	1515
Secondary[a]	25	9	4	7	4	114
US Military (2003)[c]	153	80[e]	0	0	2[f]	320

[a]Data from **ref. *28*** for individuals ≥ 20 yr of age. Receiving the vaccine for the first time (primary); receiving the vaccine in those who had previously been vaccinated (secondary).

[b]Includes other complications, such as severe reactions, bacterial superinfection, and erythema multiforme.

[c]Data from **ref. *34*** reporting on 450,293 vaccinations (70% were primary vaccinations).

[d]No report of this complication was included in the 1968 ten-state survey ***(28)***.

[e]All were primary vaccinees.

[f]One case in a primary vaccinee and another case in a secondary vaccinee.

3.4. Future Considerations

With the advent of concerns regarding smallpox bioterrorism, the number of investigators working with poxviruses is increasing. This will be an exciting time, as our knowledge in poxvirology expands and new products are developed. These new products will certainly remedy some safety concerns of working with the nonattenuated laboratory strains of vaccinia virus. For example, the future availability of a smallpox vaccine that is safer than what is currently Food and Drug Administration (FDA) approved in the United States will likely end the controversy of whether or not laboratory workers handling vaccinia virus should be vaccinated. A vaccine with few to no side effects would clearly shift the risk–benefit ratio of vaccination in favor of vaccination to prevent infections that result from a laboratory accident. In addition, the development of antipoxvirus therapeutics (e.g., **ref. *31***) will be beneficial for treatment should an accidental exposure result in a serious infection.

4. Notes

1. Vaccinia virus strain Western Reserve (WR) was selected for its neurovirulence in mice.
2. The smallpox vaccine is administered by scarification with a bifurcated needle. When the needle is dipped into the vaccine stock (approx 10^8 pfu/mL) approx 2.5 μL of solution is taken up, and, thus, approx 2.5×10^5 pfu is delivered.
3. Institutions can obtain the smallpox vaccine from the CDC, Drug Services, and National Center for Infectious Diseases (404-639-3670).
4. If infectious aerosol-generating procedures are performed outside of a BSC, respiratory protection may be required. Individuals requiring respiratory protection must be enrolled in the institution's respiratory protection program.
5. Nitrile or powder-free latex gloves should be used when working with the virus.
6. The plasticware that is now commonly used in the modern-day laboratory has significantly decreased the need for glassware when working with the virus. For example, instead of using sterilized glass Pasteur pipets to aspirate medium while culturing the virus, one can use plastic pipet tips attached to vacuum tubing. A simple system used by many laboratories is to have a P-1000 pipet tip attached to the vacuum tubing. Then, P-200 pipet tips can be used over the P-1000 tip for direct contact with various culture wells, which allows easy changing of the tip to prevent any cross-contamination of specimens.
7. Workers performing higher risk procedures like vaccinating animals, direct handling of infected animals (*see* **Note 17**), or preparing and purifying high-titer virus are individuals who may benefit the most from prophylactic vaccination. Also included in the group for whom vaccination should be strongly considered are those generating or working with recombinant vaccinia viruses that express potentially toxic proteins, proteins that might enhance virulence of the recombinant vaccinia virus, or proteins to which seroconversion may be problematic (e.g., HIV and dengue).

8. Sharps containers (when two-thirds full) should be autoclaved prior to disposal in accordance with the institution's infectious waste policy.
9. Laboratories frequently use a large-volume Erlenmeyer flask placed underneath the BSL-2 cabinet. This flask can be filled with detergent or bleach to inactivate the virus. Bleach should be at a final concentration of approx 10%. The level of fluid in the flask should be monitored to prevent overfilling. It is also recommended that a vent/gas filter with a hydrophobic membrane be placed in-line between the Erlenmeyer flask and the vacuum outlet as an additional safety measure to prevent contamination of the vacuum system if the trap becomes overfilled. Decontaminated liquid waste should be disposed in accordance with the institution's regulatory requirements.
10. Special consideration must be made when working with the virus and radioactivity. The waste stream for all radioactivity must be accounted for and disposed of following institutional guidelines. Thus, the virus-contaminated radioactive waste must first be inactivated. Radioactive liquid waste should be treated with disinfectant, then disposed of with the normal radioactive liquid waste. Because the radioactive waste should not be autoclaved, radioactive contaminated disposable plasticware should be washed down with disinfectant solution to inactivate the virus prior to disposing it as radioactive solid waste.
11. During sonication, use of proper ear protection is recommended.
12. Starting in January 2003, a pre-event smallpox vaccination program began in the United States ***(32)***. This program has significantly increased the number of civilians vaccinated. As of May 9, 2003, 36,217 civilians have been vaccinated ***(33)***. Of note, over approximately the same time period, the US military has vaccinated approx 450,000 personnel ***(34)***. With careful screening of potential vaccine recipients, the rate of the most serious adverse events was less than previously reported (***27,28***; *see* **Table 2**).
13. For those working with these highly virulent poxviruses (e.g., variola virus and monkeypox virus), vaccination every 3 yr should be considered.
14. In fact, these highly attenuated strains of vaccinia virus can now be handled in the laboratory at biosafety level 1 ***(35)***.
15. The potential serious complications from smallpox vaccine (*see* **Table 2**) include eczema vaccinatum, postvaccinial encephalitis, and progressive vaccinia in immunocompromised hosts inadvertently vaccinated or accidentally infected by exposure to a vaccinated individual. Photographs of these types of complications are available ***(8,36)***. During the recent pre-event smallpox vaccination program, myocarditis/pericarditis has also been reidentified as a potential complication from the vaccine ***(33,37,38)***.
16. Because of potential spread of the virus from the vaccination site, close contact with individuals who have the conditions listed in **Note 15** are considered to be a contraindication to elective vaccination. Another potential contraindication for vaccination would be close contact with infants less than 1 yr old. However, a worker who is not vaccinated because of these contraindications should still be able to work with vaccinia virus following the guidelines of personal protection

equipment and efficient hand washing. Alternatively, an individual who still wants to be vaccinated can choose to isolate themselves from contact with such at-risk people until the vaccination site scab has fallen off (typically from 2 to 4 wk). These issues affect the risk–benefit ratio decision.

17. The ACIP recommends that workers who care for vaccinia virus-infected animals also receive smallpox vaccination ***(8)***. This is likely based on the finding that within a cage, vaccinia virus can spread from infected mice to naïve mice ***(39–41)***. But here too, the decision to vaccinate such workers could be personal based on what type of contact the worker has with the infected animals. If, for example, a worker has no direct contact (e.g., use of tongs to transfer animals from cage to cage), then the potential for exposure would be quite small. This type of conclusion is supported by studies that showed no transmission of the virus between subcutaneously vaccinated and unvaccinated guinea pigs ***(42)***, and a recent study ***(41)*** that searched for vaccinia virus transmission to sentinel mice by the soiled bedding from cages housing mice infected by the subcutaneous or intrarectal route with vaccinia virus. None of the mice exposed to the soiled bedding seroconverted.
18. There is increased likelihood of serious complications in those with skin conditions like atopic dermatitis or eczema, whether the skin disease is active or nonactive at the time of vaccination. Although it is unknown why such individuals have increased complications, it is theorized that there is some type of cutaneous immune abnormality ***(43)***.
19. Data presented in **Table 1** is from Christopher K. Allen at the CDC Drug Service, Scientific Resources Program, National Center for Infectious Diseases, Centers for Disease Control and Prevention, and James Braggs at the Immunization Safety Branch, Division of Epidemiology and Surveillance, National Immunization Program, Centers for Disease Control and Prevention.

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2

Construction and Isolation of Recombinant Vaccinia Virus Using Genetic Markers

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Summary

The standard approach for the isolation of vaccinia virus recombinants involves homologous recombination between a transfected plasmid and the replicating viral DNA. In a typical infection/transfection experiment, recombinant viruses only account for a tiny proportion (10^{-4} to 10^{-3}) of the progeny virus; thus, genetic markers are often included in the transfected plasmid to facilitate the selection of recombinant viruses. This chapter describes in detail two different selection procedures: one relies on plaque formation phenotype using the vaccinia virus gene F13L; the other relies on antibiotic resistance using the *Escherichia coli* xanthine–guanine phosphoribosyl transferase gene.

Key Words: Recombinant vaccinia virus; plaque size; F13L; xanthine–guanine phosphoribosyl transferase; *gpt*.

1. Introduction

Procedures for the genetic modification of poxviruses must take into account several aspects of their biology, particularly, the size of the viral genome and that naked poxvirus DNA is not infectious. As a general rule, recombinant vaccinia viruses are generated inside cells by recombination between transfected naked DNA and replicating virus DNA ***(1–5)***. Alternatively, in vitro ligation ***(6–8)*** can be used to generate recombinant vaccinia genomes that can be subsequently recovered as infectious virus with the aid of a helper virus (*see* Chapters 5 and 6).

In the general in vivo recombination procedure and to facilitate proper insertion of the foreign DNA at a given genome position, viral sequences (often referred to as "flanks") are included in the transfected DNA. Virus

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recombinants are subsequently isolated from the progeny virus, where only a small percentage (usually 10^{-4} to 10^{-3}) of the total virus are virus recombinants. Thus, the isolation of a vaccinia recombinant implies the selection of low-frequency viruses, a task that is facilitated by the incorporation of genetic markers in the transfected DNA.

Genetic markers used in the vaccinia virus context include genes that can be:

1. Detected by staining (e.g., β-galactosidase and β-glucuronidase) or fluorescence (e.g., GFP; ***9–13***).
2. Selected by specific drugs (e.g., neomycin, puromycin, or mycophenolic acid [MPA]; ***14–17***).
3. Required for virus spread (e.g., viral genes, A27L or F13L; ***18,19***).
4. Required for virus replication in particular cell lines (e.g., host range genes; ***20–22***).

One important consideration is the difference between markers intended for the detection of virus recombinants and selectable markers that allow a true selection of the virus recombinants. In both cases, isolation of virus recombinants can be achieved by repeated virus plaquing, but generally selection systems permit a faster and more efficient isolation process.

Most selection systems rely on the use of specific antibiotics or cell lines. One notable exception is the selection system that takes advantage of the nonplaquing phenotype of certain vaccinia deletion mutants. From one such mutant, reintroduction of one particular viral gene results in the restoration of the plaque phenotype. This chapter focuses on the use of one antibiotic resistance system (gpt selection) and one plaque selection system (based on the F13L gene). Alternative methods based on fluorescent protein or β-galactosidase detection are slight variations of the methods described are addressed as **Notes**.

2. Materials

1. Six-well tissue culture plates.
2. 25-cm^2 and 150-cm^2 tissue culture flasks.
3. FuGENE 6 transfection reagent (Roche).
4. Complete Eagle's minimum essential medium (EMEM; Gibco BRL) supplemented with 2 m*M* glutamine, 0.1 μg/mL penicillin, and 0.1 μg/mL streptomycin.
5. Cell growth medium: complete EMEM containing 5% fetal bovine serum (FBS).
6. Cell infection medium: complete EMEM containing 2% FBS.
7. Cell lines: CV-1 (ATCC, cat. no. CCL-70) and BSC-1 (ATCC, cat. no. CCL-26) cells.
8. Calcium phosphate transfection buffer (HBS): 0.14 *M* NaCl, 5 m*M* KCl, 1 m*M* $Na_2HPO_4{\cdot}2H_2O$, 0.1% dextrose, 20 m*M* HEPES. Adjust pH precisely to 7.05 by adding 0.5 *M* NaOH, and filter-sterilize.
9. Water bath sonicator.
10. 2.5 *M* $CaCl_2$.

11. Humidified incubator, 37°C, 5% CO_2.
12. Disposable sterile scraper.
13. 5 mL Polypropylene tubes.
14. 5 mL Polystyrene tubes.
15. Complete 2X EMEM medium: 2X EMEM, supplemented with 4 m*M* glutamine, 0.2 μg/mL penicillin, 0.2 μg/mL streptomycin, and 4% FBS.
16. 2% low-melting point (LMP) agarose in water, autoclaved.
17. 4% X-gal in dimethylformamide.
18. 10 mg/mL neutral red in water.
19. Sterile Pasteur pipets.
20. 10 mg/mL MPA in 0.1 *N* NaOH.
21. 10 mg/mL xanthine in 0.1 *N* NaOH.
22. 10 mg/mL hypoxanthine in water.
23. GPT selection medium: complete EMEM containing 1/400 vol of 10 mg/mL MPA, 1/40 vol 10 mg/mL xantine, and 1/670 vol of 10 mg/mL hypoxanthine.
24. 45ºC water bath.
25. 5 mg/mL crystal violet staining solution.

3. Methods

F13L selection system is based on virus plaque formation and does not require the use of drugs or antibiotics. The system is based on the observation that vaccinia virus mutants in which most of the F13L coding sequence is deleted are severely impaired in virus transmission, producing tiny virus plaques only after prolonged incubation times ***(23,24)***. The F13L selection system described here relies on the use of one such mutant (virus vRB12) and a donor plasmid (pRB-21 or derivatives) that restore the complete F13L open-reading frame (ORF) and can simultaneously introduce a foreign gene ***(19)***. In the plasmid, the F13L coding sequence is flanked by vaccinia virus DNA that directs homologous recombination into the natural F13L locus, placing the foreign gene (under a vaccinia virus promoter) in an intergenic region immediately downstream of the F13L stop codon. Recombinant plaques (normal size plaques) can be distinguished easily from parental plaques (tiny plaques) after a standard 2 d vaccinia-plaque assay.

gpt selection is based on the incorporation in the vaccinia genome of *Escherichia coli gpt* gene and its expression under the control of vaccinia virus promoters. *gpt* expression confers resistance to MPA, an inhibitor of the enzyme inosine monophosphate dehydrogenase ***(17,25)***. MPA treatment results in depletion of purine nucleotide pools and, consequently, in the inhibition of virus growth. Recombinant viruses expressing *gpt* can be isolated in a selective medium containing MPA and the nucleotide precursors, xanthine and hypoxanthine (*see* **Note 1**).

3.1. Plasmids

3.1.1. Plasmids for Use by Plaque Selection System

Two different plasmids (**Fig. 1**) have been designed for F13L selection and are used in conjunction with virus vRB12.

1. pRB21 ***(19)*** is designed to mediate constitutive expression of the foreign gene from a synthetic vaccinia early/late promoter (***26***; *see* **Fig. 1** and **Note 2**). However, occasionally the expression of the foreign protein interferes with the replication of vaccinia. In such cases, or when the expression of the foreign protein is not desired during the isolation of the recombinant, the donor plasmid pTIH can be used (*see* **Note 3**).
2. pTIH is designed to place the foreign gene under the control of a T7 promoter (***27***; *see* **Fig. 1** and **Note 4**). This plasmid was derived from pRB21 and contains the T7 promoter, a sequence to generate a hairpin loop structure at the 5' end of the T7 transcript and an internal ribosomal entry site (IRES) element to facilitate translation initiation. The plasmid contains a N-terminal tag sequence including 10-histidine codons to allow the easy purification of expressed proteins.

3.1.2. Plasmids for Use by gpt Selection System

A number of plasmids have been designed for *gpt* selection ***(17,25)***. From those, a *gpt* expression cassette, including a vaccinia promoter and the gpt-coding sequence, can be easily excised (e.g., *see* plasmid pTK61-gpt in **ref.** ***17*** or plasmid pGEM-gpt in **ref.** ***28***). The *gpt* cassette has been used extensively for insertional mutagenesis of vaccinia virus genes to generate knockout virus mutants. When expression of a foreign gene is desired, plasmids including (in addition to the *gpt* cassette) strong promoters and convenient restriction sites, can be used (*see* plasmids pTKgpt-F1-3s in **ref.** ***17***).

Fig. 1. *(opposite page)* Plasmids for F13L selection. Schematic diagram showing relevant features of plasmids pRB21 and pTIH. Relative locations of F13L-coding sequence, vaccinia virus early–late promoter (VV), T7 promoter (T7), T7 terminator (T7t), internal ribosomal entry site (IRES), and unique restriction sites are shown. Vaccinia flanking sequences for recombination in the vaccinia genome are drawn as gray boxes. The lower part shows the sequences downstream of the promoters and the location of restriction sites useful for insertion of foreign genes. TERM, vaccinia virus early transcription termination signal. Note that the polyhistidine tag is positioned at the N-terminus of proteins expressed from open-reading frames (ORF) cloned into the *Nde*I, *Xho*I, or *Bam*HI sites. Foreign genes can be subcloned between pRB21 and pTIH by using restriction sites, *Eco*RI and *Bam*HI.

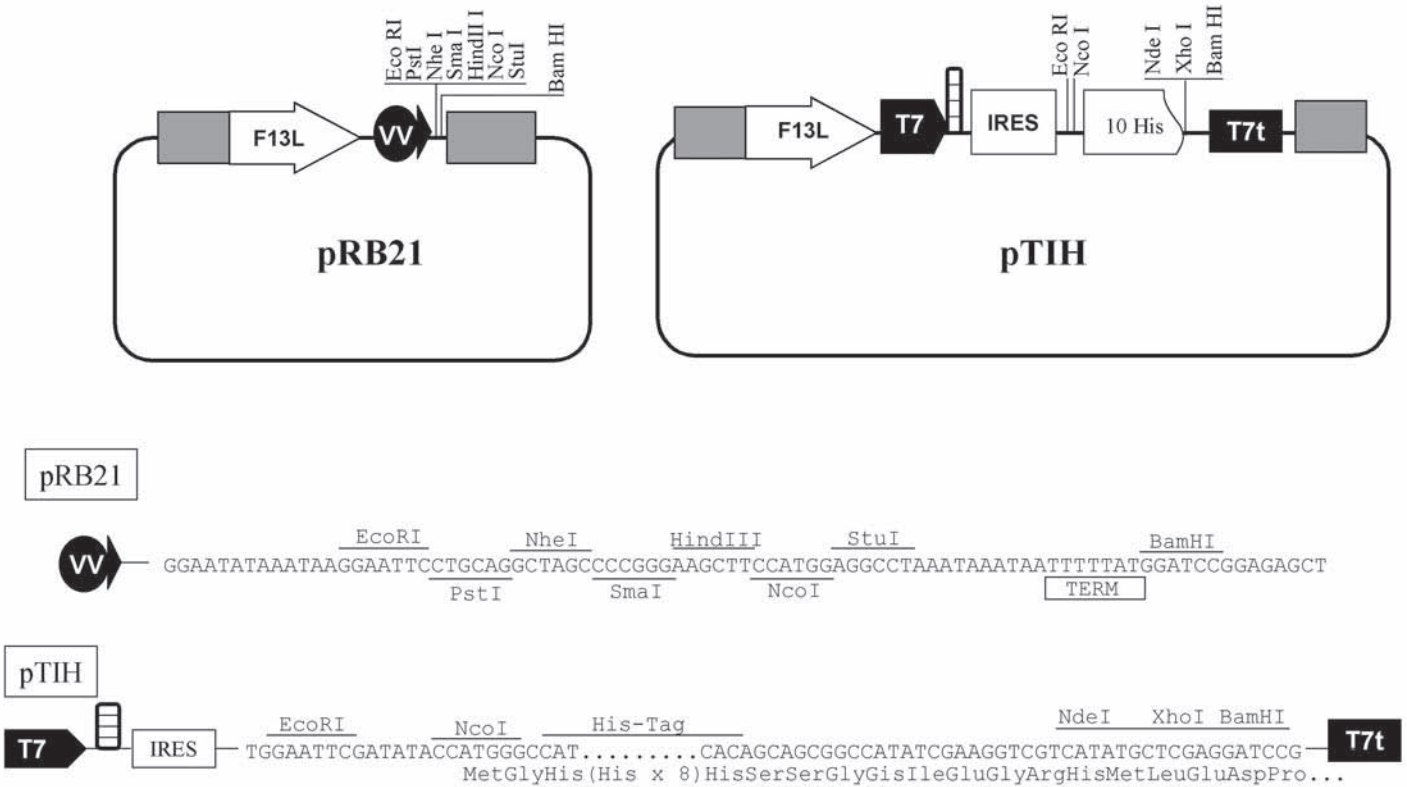
Eco RI
PstI
Nhe I
Sma I
HindIII I
Nco I
StuI
Bam HI
F13L
VV
pRB21
Eco RI
Nco I
Nde I
Xho I
Bam HI
F13L
T7
IRES
10 His
T7t
pTIH
pRB21
VV
EcoRI
NheI
HindIII
StuI
BamHI
GGAATATAAATAAGGAATTCCTGCAGGCTAGCCCCGGGAAGCTTCCATGGAGGCCTAAATAAATAATTTTTATGGATCCGGAGAGCT
PstI
SmaI
NcoI
TERM
pTIH
T7
IRES
EcoRI
NcoI
His-Tag
NdeI
XhoI
BamHI
T7t
TGGAATTCGATATACCATGGGCCAT.....CACAGCAGCGGCCATATCGAAGGTCGTCATATGCTCGAGGATCCG
MetGlyHis(His x 8)HisSerSerGlyGisIleGluGlyArgHisMetLeuGluAspPro...

Fig. 1.

3.2. Infection/Transfection Procedure

To allow recombination between the plasmid and the replicating virus DNA, cells infected with the parental virus are transfected with a plasmid that carries the selective marker. For the F13L selection system, parental virus must be a F13L deletion mutant, such as vRB12 ***(19,29)***. The nonplaquing phenotype of F13L(⁻) viruses should be taken into account when growing and titering the virus (*see* **Note 5**). When using *gpt* selection or β-galactosidase screening, the parental virus can be any normal plaquing virus devoid of those genes.

3.2.1. Transfection with Calcium Phosphate Precipitate

1. About 24 h before infection, split 1:3 a confluent culture of CV-1 cells and seed in a six-well tissue culture plate, in 2 mL cell growth medium per well. Incubate the cells at 37°C in a CO_2 incubator for 20–24 h when the cells should be 60–80% confluent.
2. Prepare virus inoculum (1 mL/well) by diluting a crude virus stock (*see* **Note 6**) in cell infection medium. The amount of virus should be adjusted to give a multiplicity of infection of 0.05 plaque-forming units cell.
3. Remove cell growth medium from the cultures and immediately add virus inoculum. Place in a CO_2 incubator at 37°C for 1–2 h.
4. About 45 min before transfection, prepare calcium phosphate precipitate as follows. For each transfection, dilute 5 μg plasmid DNA in 0.5 mL HBS in a 12 × 75-mm (5 mL) polystyrene tube. Add 25 μL 2.5 *M* $CaCl_2$ droplet by droplet, mixing between each droplet. Leave at room temperature for 20–30 min to allow for a fine DNA–calcium phosphate precipitate to form. The solution should turn opalescent.
5. Aspirate virus inoculum from the CV-1 cell cultures, and add the precipitate on top of the monolayer. Maintain 30 min at room temperature, rocking the plates every 5 min to prevent drying of the monolayers.
6. Add 2 mL per well of cell growth medium. Incubate 4 h in a CO_2 incubator at 37°C.
7. Remove medium from the cultures, and replace with 2 mL fresh cell growth medium. Incubate 72 h in a CO_2 incubator at 37°C.
8. Harvest the infected cells from the well with a disposable rubber scraper or by pipetting repeatedly on top of the monolayer. Collect the cell suspension in a sterile 5-mL polypropylene tube.
9. Release progeny virus from cells by repeated freeze–thawing of the harvested cells by first placing them in dry ice/ethanol bath and then thawing them in a 37°C water bath and vortexing. Repeat the freeze–thaw cycling three times. The cell lysate can be stored at –80°C until plaque isolation is done (*see* **Subheading 3.3.**).

3.2.2. Transfection with FuGENE

1. Follow **Subheading 3.2.1.**, **steps 1–3**.
2. About 45 min before transfection, prepare transfection mix, in 1.5-mL polypropylene tubes. For each transfection, dilute 6 μL FuGENE in 94 μL serum-free EMEM and incubate at room temperature for 5 min. Transfer the EMEM–FuGENE mix to a tube containing 3 μg plasmid DNA, mix gently by pipeting up and down two or three times. Leave at room temperature for 30 min.
3. Aspirate virus inoculum from the CV-1 cell cultures and wash the cells once with 2 mL serum-free EMEM.
4. Immediately add the DNA–FuGENE solution drop-by-drop on top of the monolayer. Add 2 mL cell infection medium. Incubate 72 h in a CO_2 incubator at 37°C.
5. Then, follow **Subheading 3.2.1.**, **steps 8** and **9**.

3.3. Plaque Isolation

The virus stock obtained at the completion of **Subheading 3.2.** includes mainly parental virus and some recombinant viruses. To isolate the recombinant virus away from the parental virus, individual recombinant virus plaques are picked and characterized. To ensure complete removal of the parental virus, several consecutive rounds of plaque purification are performed. It is advisable to pick and amplify several (e.g., four is a reasonable number) independent plaques in the first plaquing step until an initial characterization is made (*see* **Subheading 3.5.**). The plaque isolation protocols described here for F13L and *gpt* selection can be used with slight modifications for β-galactosidase (**Note 7**) or fluorescent protein (**Note 8**) detection.

3.3.1. Plaque Isolation: F13L Selection

F13L selection is based solely on virus plaque size and therefore does not require the use of drugs or special cell lines. Any cell line giving good-size wild-type vaccinia plaques, such as BSC-1 cells, can be used to isolate recombinant plaques. It is advisable to perform control transfections with pRB21 and a negative control plasmid (lacking F13L gene) to monitor the presence of recombinant large-virus plaques (*see* **Fig. 2**).

1. Plate BSC-1 cells in six-well tissue culture plates and incubate until the cells are 90% confluent.
2. Thaw the cell lysate obtained at the completion of **Subheading 3.2.**, remove 0.5 mL, and place in a sterile 5 mL polystyrene tube.
3. Sonicate this in an ice/water bath sonicator for at least three cycles of 15 s until the material in the suspension is dispersed (*see* **Note 9**).
4. Make 10-fold serial dilutions of the infection/transfection cell lysate in cell infection medium (*see* **Note 10**).

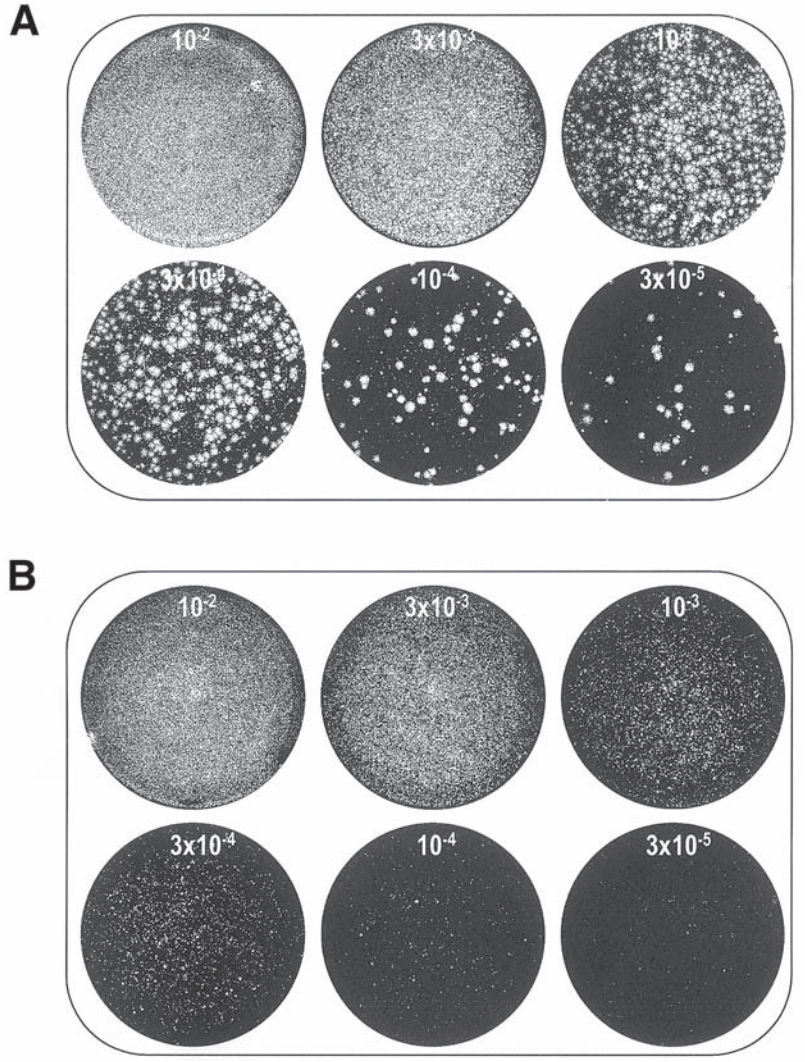

Fig. 2. Plaquing of the infection/transfection progeny virus in F13L selection. An example of a first plaquing step after the infection/transfection with virus vRB12 and a plasmid derived from pRB21 **(A)** or a control plasmid not carrying gene F13L **(B)**. The dilutions used to infect monolayers of BSC-1 cells in six-well plates are indicated inside each well. Recombinant viruses that have incorporated the F13L gene produce large vaccinia plaques. Note the diffuse cytopathic effect produced by the parental virus in dilutions 10^{-2} and 3×10^{-3}.

5. Aspirate off growth medium from the BSC-1 cell monolayers and add 1 mL of the corresponding dilution per well. (Use at least dilutions 10^{-2}, 10^{-3}, and 10^{-4}.) Incubate 1–2 h at 37°C.
6. Melt sterile 2% LMP agarose in water by heating in a microwave oven and cool in a 45°C water bath. Warm complete 2X EMEM medium at 37°C.
7. Immediately before use, prepare EMEM–agarose overlay by mixing equal volumes of complete 2X EMEM medium and 2% LMP agarose solution.
8. Remove virus inoculum from each well and add 3 mL EMEM–agarose overlay medium. Allow overlay to solidify at room temperature (*see* **Note 11**). Then, place the plates in the 37°C incubator for 48 h to allow for vaccinia plaques to develop.
9. Stain the monolayer by overlaying the EMEM agarose with the addition of 2 mL EMEM–agarose containing neutral red. This overlay is prepared by mixing equal volumes of complete 2X EMEM containing 1/100 vol of 10 mg/mL neutral red and 2% LMP agarose (agarose has been melted and cooled at 45°C). Add 2 mL of this cell-staining overlay to each well and allow to solidify at room temperature. Then, place plates in the 37°C incubator until vaccinia plaques are visible (5–16 h).
10. When plaques are clearly visible, pick well-separated plaques by plunging a sterile Pasteur pipet through the agarose medium all the way to the plastic. Rock the pipet tip slightly to scrape and detach cells in the plaque. Gently aspirate (*see* **Note 12**) the agarose plug and transfer to a microcentrifuge tube containing 0.5 mL cell growth medium. Freeze–thaw three times and sonicate.
11. Repeat **steps 1–10** three to five times, each time using the material from the last plaque selected.

3.3.2. Plaque Selection: gpt *Selection*

This selection system is based on plaque isolation in the presence of MPA, an inhibitor of purine metabolism ***(17,25)***. Virus recombinants incorporating and expressing the *E. coli* xanthine–guanine phosphoribosyl transferase gene (*gpt*), but not normal *gpt*(⁻) virus, can form plaques in medium containing MPA and the nucleotide precursors xanthine and hypoxanthine (*see* **Fig. 3**).

1. Seed BSC-1 cells in six-well tissue culture plates and incubate until the cells are approx 90% confluent.
2. Twelve to twenty-four hours before infection, prepare GPT selection medium. Remove cell growth medium from wells and replace with 2 mL/well of freshly prepared GPT selection medium.
3. Thaw the cell lysate obtained at the completion of **Subheading 3.2.**, remove 0.5 mL, and place in a sterile 5-mL polystyrene tube.
4. Sonicate in an ice/water bath sonicator for at least three cycles of 15 s until the material in the suspension is dispersed (*see* **Note 9**).
5. Make 10-fold serial dilutions of the infection/transfection cell lysate in cell infection medium. Aspirate GPT selection medium from the BSC-1 cell mono-

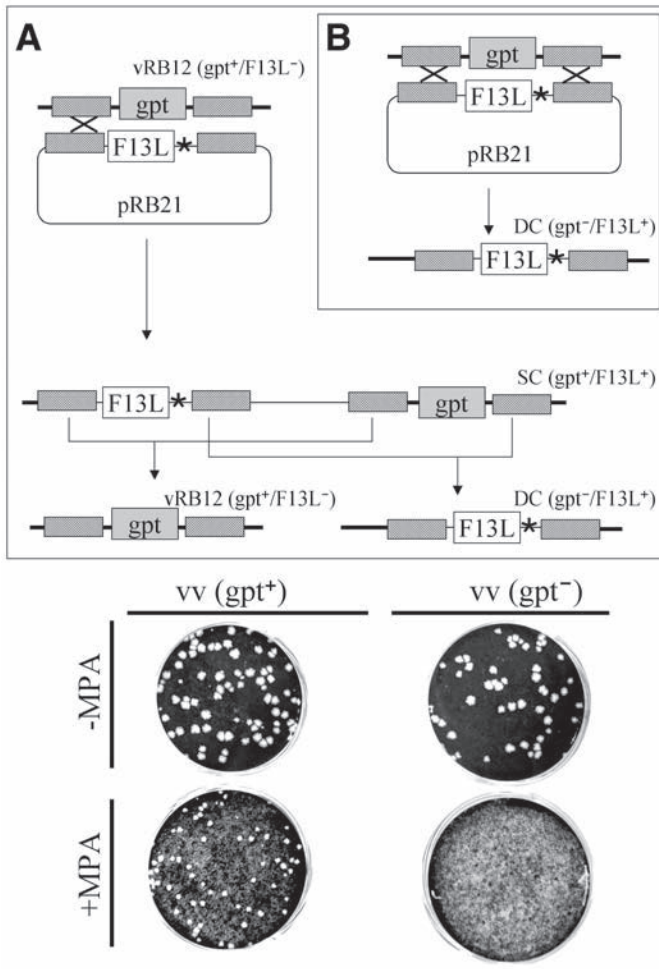

Fig. 3. gpt screening assay. The recombination of the plasmid with the vaccinia genome may occur at only one flanking sequence **(A)** or at both flanking sequences **(B)**. To distinguish between the unstable simple crossover (SC) products (gpt+, F13L+) and the stable double crossover (DC) products (gpt–, F13L+), a gpt assay can be done on the growth of plaque-purified recombinant viruses. The lower panel shows the virus plaques obtained with gpt– and gpt+ viruses in nonselective (–MPA) or selective (+MPA) medium. (The position of the promoter/foreign gene DNA sequence in A and B is indicated by an asterisk.)

layers and add 1 mL of the corresponding dilution per well. (Use at least dilutions 10^{-2}, 10^{-3}, and 10^{-4}.) Incubate 1–2 h at 37°C to allow virus adsorption.

6. Before the end of the adsorption period, melt the 2% LMP agarose in water by heating in a microwave oven and cool in a 45°C water bath. Supplement com-

plete 2X EMEM medium with 1/200 vol of 10 mg/mL MPA, 1/20 vol 10 mg/mL xantine, and 1/335 vol of 10 mg/mL hypoxantine, and warm at 37°C.
7. Immediately before use, prepare EMEM–agarose overlay by mixing equal volumes of the solutions prepared in **step 6** (selective EMEM–agarose overlay medium).
8. Remove virus inoculum from each well and add 3 mL selective EMEM–agarose overlay medium. Allow to solidify at room temperature (*see* **Note 11**). Place the plates in the 37ºC incubator for 48 h to allow for vaccinia plaques to develop.
9. Stain the monolayer and pick plaques as described in **Subheading 3.3.1.**, **steps 9–11**.

3.4. Virus Amplification

After the three to five consecutive rounds of plaque purification, virus recombinants from the last plaques are amplified by infection of monolayer cells to make a first-virus crude stock.

1. Seed BSC-1 cells in a six-well tissue culture plate and incubate in a CO_2 incubator until the cells are confluent.
2. Infect one well with 250 μL of the material from a plaque diluted to 1 mL with cell infection medium. Place the plates in a CO_2 incubator for 2 h at 37°C.
3. After the 2-h adsorption period, remove virus inoculum and add 2 mL fresh cell infection medium. Place at 37°C in a CO_2 incubator for 48–72 h (i.e., until cytopathic effect is complete).
4. Detach the cells from the plastic with a disposable rubber scraper or by repeatedly pipeting on top of the monolayer. Transfer the cell suspension to a tube. Freeze–thaw three times and sonicate (*see* **Note 9**).
5. Use half of the cell lysate, sonicated and diluted to 4 mL in cell infection medium to infect a confluent BSC-1 monolayer culture in a 75-cm^2 flask. After 2-h adsorption in a CO_2 incubator at 37°C, remove virus inoculum, add 12 mL cell infection medium, and place in the incubator at 37°C for 48–72 h (i.e., until cytopathic effect is complete).
6. Detach infected cells from the flask with a disposable scraper and transfer to a 15-mL centrifuge tube. Centrifuge 5 min at 1800*g*, discard supernatant, and resuspend the cells in 1 mL complete EMEM 2% FBS. Titrate virus stock (*see* Chapter 8).

3.5. Initial Screening of Resulting Recombinant Viruses by Growth Under gpt Selection

A first step in the characterization of the viruses isolated by F13L selection and amplified is to check whether they are "single" or "double" recombinants. Single recombinants are highly unstable as they contain direct repeats, producing by recombination both the parental virus and the desired recombinant virus (*see* **Fig. 3**). Because parental vRB12 virus contains a *gpt* cassette that replaces most of the F13L gene, single recombinants maintain the gpt(+) phenotype.

Thus, the presence of gpt(+) viruses in the amplified virus stock is an indication that single recombinants were picked in the plaque isolation process. Conversely, complete absence of gpt(+) viruses in the stock indicate that a desired recombinant has been successfully isolated. Thus, virus plaquing under gpt-selective conditions provides a rapid method in determining if the stock is contaminated with any single recombinants (*see* **Note 13**).

1. Seed BSC-1 cells in cell growth medium in six-well tissue culture plates and incubate until the monolayers reach a 80–90% confluency.
2. Twelve to twenty hours before plaquing, half of the wells are preincubated in 2 mL GPT selection medium. When cells are pretreated with GPT selection medium, change the medium of the remaining wells (control wells) to 2 mL cell infection medium.
3. Thaw the virus stock (*see* **Note 14**) and make serial dilutions in 2 mL cell infection medium.
4. Remove medium and infect monolayers with the virus dilutions using 1 mL/well. Infect one well with each dilution that had been pretreated with GPT selection medium and one control well. Incubate at 37°C for 1 h.
5. Aspirate virus inocula and add 2 mL GPT selective medium to the pretreated wells and 2 mL cell infection medium to the control wells. Incubate 48 h in the 37°C incubator.
6. Stain the monolayers by adding 0.5 mL/well of 5 mg/mL crystal violet solution. Mix with the medium by moving the plate carefully. Leave at room temperature for at least 10 min.
7. Aspirate stained medium from the wells. Monolayers and virus plaques should be clearly visible. Let the monolayers dry at room temperature. Confirm that there are no plaques in the wells with GPT selective medium on them.

4. Notes

1. Recombinant viruses that have *gpt* inserted into the viral genome can be used as parental viruses to introduce other mutations into the virus using reverse *gpt* selection ***(30)***.
2. Plasmid pRB21 ***(19)*** is designed for expression of a foreign gene from a synthetic early–late vaccinia promoter ***(26)***. Many proteins can be expressed to high levels with the resulting recombinants. Note that if the foreign sequence contains a TTTTTNT (an early transcriptional stop signal; *see* Chapter 11) within the ORF, truncated proteins may be expressed at early times during infection.
3. It is likely that before constructing a virus recombinant, the toxicity of the foreign protein may not be predicted. In those cases, it is advisable to make constructs in both plasmids pRB21 and pTIH, and transfect them in parallel. The insert can usually be transported between those plasmids by using the *Eco*RI, *Nco*I, or *Bam*HI sites present in both plasmids. After the infection/transfection, the number of virus plaques obtained with both constructs is a good indication of the toxicity of the protein in the vaccinia virus context.

4. pTIH allows one to carry out the isolation of the virus recombinant in the absence of expression of the foreign gene. Eventually, expression is induced by providing the T7 RNA polymerase *in trans* ***(27)***. This can be achieved by infecting cell lines that stably express T7 RNA polymerase or by coinfection with vaccinia vTF7-3 ***(31)***, a recombinant in which the T7 RNA polymerase gene is placed under the control of a vaccinia promoter (*see* Chapter 4).
5. vRB12 growth and titration. Because vRB12 is not readily exported to the medium or transmitted between cells, efficient amplification of the virus requires mechanical release of the virus replicated within cells and consecutive rounds of amplification. For amplification, it is advisable to use moi more than 1 pfu/cell to ensure an efficient infection of the monolayer (and no requirement for cell-to-cell virus spread in the culture). It is difficult to accurately determine the titers of VRB12 virus stocks, as the virus does not form virus plaques after the standard vaccinia 2-d plaquing assay. However, virus titers can be estimated either by inspection of the cytopathic effect caused by different dilutions of the virus stocks or, preferably, by counting the number of small plaques that form after a 5–7-d plaquing assay.
6. For F13L selection, use virus vRB12 or a suitable F13L deletion mutant as parental virus. For *gpt* selection, use any vaccinia virus that does not contain the *gpt* gene as the parental virus.
7. Isolation of β-galactosidase-expressing viruses involves a normal plaque isolation protocol (e.g., described in **Subheading 3.3.**), in which the chromogenic β-galactosidase substrate X-gal is added in the overlay medium. Thus, in **Subheading 3.3.**, **step 9**, supplement the EMEM 2X medium with 1/120 vol of a solution of 4% (w/v) X-gal in dimethylformamide. After a few hours, blue color for the recombinant plaques will start to develop. Note that β-galactosidase detection is not a true selection, and usually parental virus is usually carried along with the recombinant for at least two or three plaquing rounds. Thus, in this case, a minimum of five to six rounds of plaque selection (i.e., until all the plaques appear blue) is recommended.
8. Detection of recombinant plaques expressing fluorescent proteins is optimally carried out using an inverted fluorescence microscope. Direct inspection of the plaques enables marking the position of fluorescence-positive plaques that can be subsequently picked. We have routinely used a standard FITC filter set (excitation: 465–495 nm; dichroic mirror: 505; emission: 515–555 nm) for GFP visualization and a standard Rhodamine filter set (excitation: 515–560 nm; dichroic mirror: 575 nm; emission: BA590) for dsRed visualization.
9. Sonication seems to work best when carried out on a small volume of lysate in rigid-wall tubes. We find that sonication of the virus in 5-mL or 15-mL tubes works better than when done in microcentrifuge tubes. Care must be taken not to overheat the specimen during sonication because high temperature inactivates the virus.
10. In the F13L system, the first plaquing of the progeny virus from the infection/transfection is a crucial step. Usually, large plaques are clearly visible in the

monolayers, but keep in mind that many more cells infected by the parental virus are present and replicate in the cultures. If the amount of parental virus is high, a diffuse cytopathic effect appears, which makes it difficult to see large-sized plaques. Consequently, making two- or threefold dilutions is recommended for the first plaquing cycle (*see* **Fig. 2**).

11. Optionally, place plates at 4°C for 10–15 min to ensure proper solidification of the agarose overlay.
12. When aspirating the agarose plug into the Pasteur pipet, it is best to try to aspirate the agarose plug only as high as the pipet tip rather than into the body of the pipet. This will make the transfer into the tube easier and minimize potential loss of the plaque.
13. This initial type of screen is only useful to perform when the parental virus contains the *gpt* gene. For recombinant viruses that have been isolated by inserting the *gpt* gene into the virus (**Subheading 3.3.2.**), this type of screening will not work. Because looking for a difference in the number of plaques grown in the presence or absence of GPT selection medium will not identify if parental wild-type virus is contaminating the recombinant stock, as titering under gpt selection typically results in a lower plaque count than when titering without selective medium.
14. This initial check can be done using a small amounts of virus that are from earlier steps of the amplification of the purified plaque (e.g., **Subheading 3.4.**, **step 4**).

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3

Construction of Recombinant Vaccinia Virus

Cloning into the Thymidine Kinase Locus

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Summary

Vaccinia virus (VV) has proven to be a very useful tool for the expression and analysis of foreign gene products. The most common method used to produce recombinant viruses involves the insertion of foreign genes into the thymidine kinase (TK) gene of the VV via homologous recombination. This is accomplished through the construction of a recombination plasmid containing the VV TK gene into the middle of which the gene of interest is inserted, appended to an efficient VV promoter element of the desired temporal class. Confluent monolayers of cells are infected with wild-type VV and transfected with the plasmid DNA to allow homologous recombination to occur. This inactivates the endogenous TK gene-producing TK-negative virus that can be biochemically selected, and recombinants can be identified by a variety of screening methods.

Key Words: Homologous recombination in vivo; recombinant DNA; eukaryotic cloning vector; transfection; vaccinia virus; thymidine kinase; vaccine vehicles.

1. Introduction

An extensive history exists in using vaccinia virus as a eukaryotic cloning and expression vector. Recombinant viruses have shown great utility for studying the expression of genes in eukaryotic systems as well as vehicles for the expression and delivery of antigens in therapeutic or prophylactic vaccines.

Vaccinia virus (VV) is a large double-stranded DNA virus and a member of the poxvirus family, which replicates primarily in the cytoplasm of susceptible host cells ***(1)***. This virus has several unique features that make it useful as a vector, including its large size, extensive host range ***(2)***, retention of infectivity after insertion of an exogenous gene ***(3)***, along with the ability to hold up to

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25,000 bp of foreign DNA ***(4)*** and modify the synthesized gene products ***(5)***. Homologous recombination in VV DNA has been shown by one group to occur with near-perfect fidelity 99% of the time ***(6)***.

Various methods have been developed for the introduction of foreign DNA into the VV genome. For example, one approach to the construction of these recombinant viruses has been to insert a foreign gene into a nonessential region within the *Hin*dIII-F fragment of the vaccinia virus genome ***(3)***; however, this approach does not allow for easy selection of these recombinants. Another approach is to insert a foreign gene into the thymidine kinase (TK) gene of vaccinia virus, inactivating the TK gene and allowing for selection of recombinants due to the TK-negative phenotype ***(7)***.

The TK gene has been mapped to the middle of the *Hin*dIII-J fragment and spans a unique *Eco*RI cleavage site. It contains no intervening sequences and has an open-reading frame of 531 nt capable of encoding a protein of 177 amino acids with a molecular weight of 20,077 ***(8)***. The protein, which functions as a tetramer ***(9)***, is expressed early during infection and is switched off after 4 h by a translational repression mechanism mediated by the protein product of one or more viral genes ***(10)***. This gene is not essential for VV replication in cultures of either TK-positive or TK-negative cells, allowing the isolation of mutants or recombinants due to 5-bromodeoxyuridine (BrdU) selection during virus replication in TK-negative cells ***(11)***.

Several schemes for the design of plasmid vectors have been described in detail to help facilitate the construction and selection of recombinant viruses ***(12–15)*** to express anything from viral antigens ***(16)*** to neuropeptides ***(17)***. VV is particularly useful as a vaccine delivery vehicle due to the observation that inactivation of the VV TK gene attenuates the in vivo replication of the recombinant virus without decreasing its ability to replicate in tissue culture ***(18)***.

2. Materials

1. VV (Western Reserve [WR] strain, *see* **Note 1**).
2. Ltk– cells (ATCC, cat. no. CCL-1.3).
3. Cell growth medium: Eagle's minimal essential media (EMEM) supplemented with 2 m*M* L-glutamine, 25 µg/mL gentamicin sulfate, 10% heat-inactivated fetal calf serum (FCS).
4. Cell infection medium: EMEM supplemented with 2 m*M* L-glutamine, 15 µg/mL gentamicin sulfate, 5% FCS.
5. BrdU: stock solution can be made at 10 mg/mL in dH_2O stored at –20°C.
6. Luria broth (LB) medium.
7. DMRIE-C liposome reagent (Invitrogen).
8. Low-melting agarose (e.g., SeaPlaque agarose).
9. Nitrocellulose filters.

10. Whatman no. 1 9-cm filters.
11. PBS-M: Phosphate-buffered saline (PBS) supplemented with 1 m*M* $MgCl_2$.
12. 1 *M* Tris: 1 *M* Tris-HCl, pH 7.6.
13. Blotting buffer: 50 m*M* Tris-HCl, pH 7.6, 150 m*M* NaCl.
14. 2X Plaque medium: 2X EMEM supplemented with 10% (v/v) FCS, 4 m*M* L-glutamine, and 30 µg/mL gentamicin sulfate.
15. Neutral red/agarose stain: For every 100 mL 1% agarose in water (sterilized by autoclaving), add 2 mL 1% neutral red solution.

3. Methods

The methods outlined here describe the (1) construction of the expression plasmid, (2) transfection of this plasmid into virus infected cells, and (3) selection of recombinant viruses. The time required for this process can range from 3 to 5 wk depending on the ease of cloning the initial gene.

3.1. Construction of the Expression Plasmid

Plasmid design can vary to suit the needs of the project, but each should contain an efficient VV promoter element, such as the VV 7.5-kDa gene promoter, embedded within the VV TK-coding region. The 7.5 kDa promoter is a constitutive early/late promoter, so the gene of interest will be expressed both early and late during a VV infection (*see* **Note 2**). Other promoters may be used to achieve selective expression during only part of the life cycle if desired (*see* **Note 3**). The plasmid should also contain an antibiotic resistance gene, such as ampicillin, for selection in *Escherichia coli* during cloning. **Figure 1** is a schematic diagram of the steps that lead to the construction of the recombinant VV. A single crossover occurs between the homologous TK sequences to insert the plasmid into the VV genome, then a second crossover occurs to excise the plasmid backbone, leaving the expression cassette in the middle of the TK gene.

An example of the design of a theoretical plasmid used to make VV recombinants ***(15)*** is shown (*see* **Fig. 2**). This plasmid was designed with the VV 7.5 kDa gene promoter region embedded within the VV TK-coding region. Downstream of the promoter is a multiple cloning site, which can be used to ligate in the gene of interest. A selectable or screenable marker gene (*see* **Note 4**) can also be inserted under the direction of another promoter to allow the expression of both gene products.

3.2. Cloning and Transfection

After the gene of interest has been cloned into the expression plasmid behind a VV promoter with flanking sequences from the TK gene on either side, the next step is to prepare the DNA and transfect it into VV-infected cells.

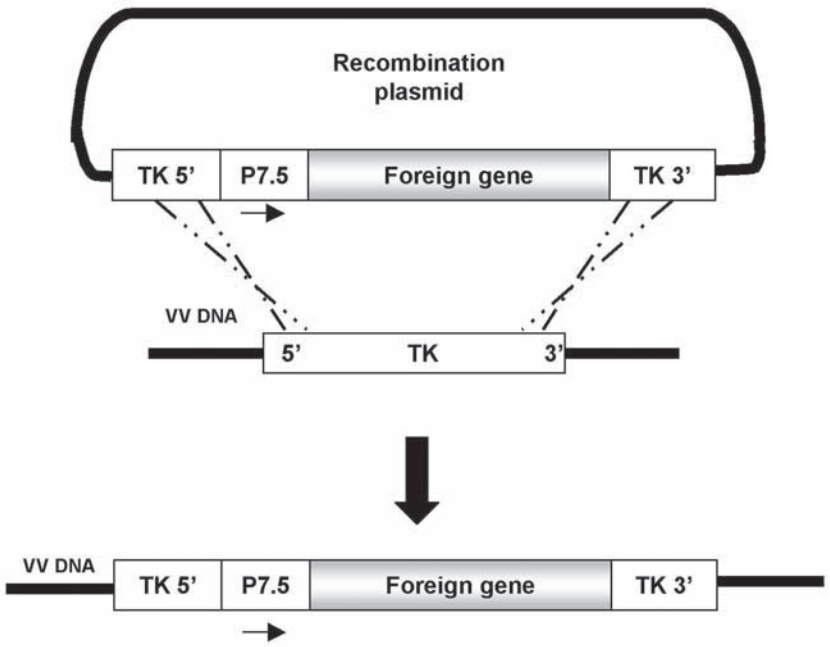

Fig. 1. Diagram of the events leading to construction of recombinant VV. TK-5' and TK-3' represent the left and right portions of the TK gene. P7.5 is a VV early/late promoter.

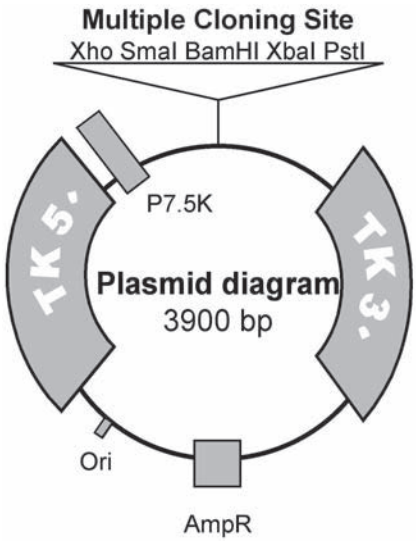

Fig. 2. Schematic drawing of a theoretical expression plasmid designed for the insertion of foreign genes. TK-5' and TK-3' represent the left and right portions of the TK gene. P7.5 is a VV early/late promoter. AmpR is the *E. coli* ampicillin resistance gene. Ori is the plasmid origin of replication.

3.2.1. E. coli Transformation

1. Transform competent *E. coli* cells (*see* **Note 5**) with the plasmid DNA using standard molecular biology methods ***(19)***.
2. Plate cells on LB plates with the appropriate antibiotic (e.g., ampicillin at 50 μg/mL) and incubate overnight at 37°C.
3. Select single colonies and grow overnight in LB media containing 50 μg/mL ampicillin.
4. Make a glycerol stock of a portion of the culture by adding 10% glycerol (i.e., 900 μL culture + 100 μL glycerol), and freeze at –70°C.
5. Use the remaining culture to purify the plasmid DNA by standard methods ***(19)***.
6. Screen the plasmids by restriction digest to verify that the insert is present.

3.2.2. Transfection

1. Grow confluent monolayers of Ltk– cells in 35-mm plates using cell growth medium at 37°C in a humidified atmosphere with 5% CO_2.
2. Preheat cell infection media (*see* **Note 6**).
3. Take plates out of the incubator and aspirate off the media. For controls, keep a plate of cells alone and a plate that will only be infected with the virus and not transfected with DNA (*see* **Note 7**).
4. Infect cells by adding a sufficient amount of the virus to 1 mL infection media to achieve a multiplicity of infection (moi) of 0.05 (*see* **Note 8** for calculating moi).
5. Allow infection to proceed for 2 h by incubating the plate at 37°C in a 5% CO_2 incubator.
6. Set out one 15-mL polystyrene conical tube per transfection and add 1 mL infection media.
7. Add 30 μL DMRIE-C (*see* **Note 9**) liposome reagent to each polystyrene tube.
8. Add 5 μg desired plasmid DNA to the polystyrene tube containing the infection media and DMRIE-C. Vortex at maximum speed for 30 s.
9. Let sit at room temperature for 10–15 min to allow liposomes to form.
10. Aspirate off the infecting inoculum and gently add DNA inoculum to virally infected cells, being careful not to disrupt the cell monolayer.
11. Rock the plate back and forth and place in a 37°C incubator with 5% CO_2.
12. Let the infection proceed for 48–72 h or until all the cells are exhibiting cytopathic effects typical of vaccinia infection (cells rounding up).

3.2.3. Harvest Recombinant Virus

1. Remove the cells from the plate by pipetting the medium up and down.
2. Transfer contents into a microfuge tube and pellet cells by centrifuging at the highest speed (e.g., 21,000*g*) in a microcentrifuge for 20 min at 4°C.
3. Aspirate off the supernatant.
4. Resuspend cell pellet in 1X PBS.
5. Freeze-thaw three times to release the virus from the cell.
6. Centrifuge at low speed (e.g., 500*g*) for 2 min and transfer the supernatant to a fresh tube (the pellet contains cell debris).

7. Prior to performing plaque picks or selecting for recombinants, the virus contained in the supernatant can be passaged through Ltk– cells in the presence of 25 μg/mL BrdU to amplify the number of TK-negative VV in the population (*see* **Note 10**).

3.3. Selection of Recombinants

Several methods for the detection of recombinant viruses have been developed, including the use of coexpression selection or screening markers. Examples include the use of neomycin (neo) resistance as a selectable marker ***(15)***, e.g., coexpression of the luciferase gene as a detectable marker ***(20)***, and coexpression of β-galactosidase for visual identification of recombinants ***(14)***. The cotransfer of selectable or screenable markers can greatly simplify the time and effort necessary to isolate recombinant VV. Our laboratory typically uses coexpression of the *neo* gene as a selection scheme. This gene codes for neomycin phosphotransferase II, which confers resistance to G418 (a kanamycin analog). G418 (geneticin) inhibits both prokaryotic and eukaryotic protein synthesis so that untransformed cells or cells infected with wild-type VV eventually die, whereas cells infected with a recombinant virus expressing neomycin phosphotransferase inactivate G418 by phosphorylation and are able to form plaques (*see* **Note 11** for additional information on using G418). Recombinant virus can then be plaque-purified and screened by polymerase chain reaction (PCR). If coexpression of a selectable marker is not used, then plaque hybridization has been widely used to screen for recombinants.

3.3.1. Plaque Hybridization

1. Infect monolayers of TK– cells in 100-mm plates with harvested virus from **Subheading 3.2.3.**, **steps 6** or **7** by serial dilution in order to be able to isolate individual plaques.
2. Two hours after infection, remove inoculum and overlay the cells with cell infection medium supplemented with 25 μg/mL BrdU.
3. Incubate for 48 h at 37°C with 5% CO_2.
4. Aspirate off the media.
5. Transfer cell monolayer containing plaques to nitrocellulose filters by gently placing a circular filter over the cells. Blot the back of the filter evenly by using a Kimwipe soaked in blotting buffer.
6. Remove the filter and place in a new dish face-up on top of a PBS-M-soaked Whatman 9-cm filter.
7. Make a replica of the filter by pressing another nitrocellulose filter on top of the first using a soaked Kimwipe to apply pressure. Make an orientation mark or hole while the filters are together.
8. Place the original filter aside to air-dry. Place the duplicate on the PBS-M-soaked filter in a plate, seal with parafilm, and store at –70°C.

9. Denature the DNA on the first filter by placing it on a 0.5 *M* NaOH-soaked Whatman 3MM filter for 5 min. Blot on paper towels.
10. Repeat **step 9** three times.
11. Neutralize the filter by placing it on 1 *M* Tris-soaked Whatman paper. Repeat three times.
12. Wash the filter by soaking in blotting buffer for 5–10 min with shaking.
13. Air-dry the filter for 15 min, then place in an 80°C vacuum oven at 25 psi for 2 h.
14. Prehybridize the filter with sheared salmon sperm DNA.
15. Hybridize with nick-translated radiolabeled probe specific for the gene of interest.
16. Process using standard Southern analysis procedures.
17. The recombinants can be recovered from the duplicate filter by cutting the plaques out of the filter and placing in a sterile tube containing 200 μL PBS. Freeze-thaw the tubes three times and sonicate in a cup sonicator six times for 10 s each time to release the recombinant virus from the filter.
18. Recover virus by inoculating confluent BSC_{40} cells with the entire contents of the tube.

3.3.2. Recombinant Plaque Purification

To ensure that your recombinant virus does not contain contaminating wild-type or spontaneous TK-negative virus, it is recommended that the selected virus undergo plaque purification. Cells are infected at a low moi, overlaid with agarose to prevent the plaques from contaminating each other, picked via Pasteur pipet, and amplified and rescreened.

1. Grow confluent monolayers of TK– cells in 100-mm plates.
2. Inoculate with the crude recombinant virus contained in the lysate after transfection at a low moi (serial dilutions can be used to ensure that some plates will have only a few plaques) (*see* **Note 7**).
3. Allow the virus to adsorb for 30–60 min, then remove the inoculum and add a 1:1 mixture of prewarmed 2X plaque medium containing 50 μg/mL BrdU with an equal volume of 2.4% agarose (*see* **Note 12**). Each 100-mm plate needs approx 8 mL vol of this 1:1 mixture.
4. Incubate the plates at room temperature for 15 min to allow the agarose to solidify, then place in a 37°C incubator for 36–48 h.
5. Stain the monolayers by adding 5 mL neutral red/agarose stain on top of each agarose overlay in each plate. Incubate at room temperature for 15 min to allow agarose to solidify, then place in a 30°C incubator for 16–24 h (*see* **Note 13**).
6. Plaques should be visible to the naked eye at this point.
7. To harvest plaques, take a sterile Pasteur pipet, draw up a little PBS-M into the pipet, place directly over a plaque, and press down until the cell monolayer is reached. Apply gentle suction to draw up the agarose plug, being careful not to touch any other plaques in the process (*see* **Note 14**).
8. Blow the plug into a sterile tube containing 250 μL PBS-M. Vortex.
9. Use the entire contents of the tube (*see* **Note 15**) to inoculate fresh TK-negative cells to repeat plaque purification.

10. After two rounds of plaque purifications, begin to amplify the virus on confluent BSC_{40} cells.
11. Harvest cells when all the cells exhibit cytopathic effects and screen to ensure recombinants have the desired insert (e.g., *see* Chapters 9 and 10 for the PCR method to screen for recombinants).

VV continues to be a versatile tool for research and the expression of foreign gene products. Using the TK-cloning techniques, as well as many other cloning methods, allows for the development of recombinant poxviruses with potential uses in gene therapy, gene expression and regulation, and vaccine research. Vaccinia is particularly useful for these applications because of its cytoplasmic site of replication, ease of manipulation, and broad host range.

4. Notes

1. Besides WR, many other strains of VV (e.g., Copenhagen, IHD-J) have been used in the construction of recombinant VV.
2. VV early genes terminate transcription approx 50 bp downstream of a TTTTTNT (5TNT) sequence (***21***; *see* Chapter 11). This should be taken into consideration when designing the sequence of the foreign gene to be inserted. The termination signal is used early in infection, but not late.
3. Early gene expression may be desired for studies where the late cytopathic effects of VV might interfere with the assay. A natural vaccinia promoter may be desired to mimic wild-type expression levels. Late vaccinia promoters provide high-level genes of expression. Synthetic early/late promoters have been designed and put into useful plasmid transfer vectors ***(22)***.
4. Some examples of useful marker genes are the neomycin resistance gene, GUS reporter gene ***(23)***, β-galactosidase gene, or green fluorescent protein (GFP) gene (*see* Chapter 2).
5. There are a variety of commercially available competent *E. coli* cells that would work for this step, in addition to preparing them in the laboratory.
6. When passaging cells, it is best to use FCS at 10% (v/v), but infections are best carried out at a lower % FCS (2.5–5%).
7. The uninfected control plate is an important measure to ensure that there is no accidental contamination of any of the reagents with VV. The infected, but untransfected control plate is important to see the background level of spontaneous TK-negative virus present in the parental virus. Knowledge of such a background helps to determine how many plaques to pick during the first plaque purification (i.e., if little or no background TK-negative virus, then just a few plaques need to be picked). If there is a great quantity of background TK-negative virus, then many more plaques will have to be screened to find those with the insert.
8. The moi is determined by multiplying the number of cells on the plate by the desired moi, then dividing this number by the virus titer, which will provide the volume to add to the well in milliliters. This number can be multiplied by 1000 to determine the number of microliters to add. For example, if infecting 10 35-mm

dishes with an average of 1.2×10^6 cells/dish, with a virus at a titer of 1×10^8 pfu/mL, and an moi of 0.05 pfu/cell is desired, a total of 6 μL virus is necessary to infect all of the dishes.

9. Lipofectamine or other liposome agents may be used instead of DMRIE-C. Follow the procedures for the addition of any other reagent as indicated by the manufacturer.
10. As discussed in **Note 7**, such an amplification step also amplifies spontaneous TK-negative mutants, so further selection/screening is necessary.
11. If using a plasmid with the *neo* gene as a selectable marker, we have found that the cells need to be pretreated with G418 24-48 h before viral infection to achieve the maximum amount of inhibition of viral replication. G418 should be added to the media for a final concentration of 2 mg/mL.
12. Prepare 2.4% agarose in dH_2O and autoclave to sterilize. If put in use right away, allow it to cool by placing it at 37°C until ready. If it hardens prior to use, it can be melted in a microwave, then allowed to cool before using. It is critical that the agarose cool prior to adding it to cells, because hot agarose can kill the cells and the virus.
13. Some laboratories incubate the neutral red overlayed plates overnight at 37°C. We prefer to incubate at 30°C, because at such a temperature, it slows the growth of the uninfected cells so as not to start impinging on the large plaques that have formed over the preceding 48 h. Also, because neutral red can be toxic to the cells, we have found that the cells survive the staining. This technique allows us to consistently visualize nice round plaques in a sea of red-stained uninfected cells.
14. When picking plaques with a Pasteur pipet, it is often best to try and keep most of the contents in the tip of the pipet after aspiration and not draw it into the body of the pipet, which will make easier transfer of the agarose plug into a tube.
15. Some use only half of the plaque-picked lysate to infect cells to save a sample if a technical problem emerges during replaquing and growth steps.

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4

Transient and Inducible Expression of Vaccinia/T7 Recombinant Viruses

Mohamed Ragaa Mohamed and Edward G. Niles*

Summary

Recombinant DNA technology has made it possible to develop molecular cloning vectors that allow the expression of heterologous genes in a variety of animal viruses. This chapter discusses the use of vaccinia virus encoding bacteriophage T7 RNA polymerase as an expression vector system. A chosen gene is inserted into a plasmid vector designed to express genes under the control of the T7 promoter. Transient expression can then be achieved either by transfecting this plasmid into cells infected with the recombinant vaccinia virus expressing T7 RNA polymerase, vTF7-3 or by crossing this plasmid into the vaccinia virus genome and coinfecting cells with both viruses. Moreover, placement of *lacO* downstream of the vaccinia virus P11 late promoter regulating T7 RNA polymerase expression, and integration of *lac*I under vaccinia promoter control into the viral genome, vT7lacOI, yielded a recombinant virus capable of IPTG-inducible T7 promoter-controlled expression of foreign genes.

Key Words: T7; transient; inducible; expression; repressor; recombinant; vaccinia.

1. Introduction

Vaccinia virus has been developed as a vector for expression of heterogeneous genes ***(1–3)***. The availability of plasmid transfer vectors containing a vaccinia virus promoter, sites for insertion of foreign genes, and a variety of markers for selection and/or screening of recombinant plaques ***(4–6)***, has facilitated the use of this system. This expression system differs from others in that transcription occurs in the cytoplasm of the cell rather than in the nucleus. To enhance the expression of such genes, the incorporation of the highly effective phage T7 ***(7,8)***, T3 ***(9)***, or SP6 ***(10)***, RNA polymerases into the vaccinia virus expression system dramatically enhanced recombinant protein production. In its simplest, most versatile, and widely used format, cells are (1) infected

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with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase, noted for its high-transcriptase activity, stringent promoter specificity, and single subunit structure ***(11,12)***; and (2) transfected with one or multiple plasmids containing the target gene of interest preceded by a T7 promoter. Expression can be enhanced and made cap-independent by incorporation of a picornavirus untranslated leader sequence into the nascent mRNA ***(13)***. To enhance recombinant protein production, the T7 promoter-regulated gene of interest can be incorporated into the genome of a second recombinant virus, which is then used to coinfect cells with the virus expressing T7 RNA polymerase ***(7)*** or to infect cells that synthesize T7 RNA polymerase constitutively ***(14)***. However, missing from the vaccinia virus expression vectors is the capability of inducing specific gene expression on command. In the absence of any known naturally inducible vaccinia virus promoter, the importation of one from eukaryotic or prokaryotic sources was considered. Of the latter, the operator/repressor system of the *Escherichia coli* lactose operon ***(15)*** was transferred to a vaccinia virus expression vector ***(16,17)*** because it was well-characterized, exhibited both stringent repression and high inducibility, and had been adapted to mammalian cells ***(18–20)***. More recently, the regulatory components of the tetracycline (TET) operon have also been adopted for the construction of TET-inducible vaccinia virus recombinants ***(21)***.

However, for certain studies the cytopathic effects (CPE) of vaccinia virus may interfere with the analysis of the expression products. Additionally, stringent microbiological safety precautions must be taken, as vaccinia virus is a human pathogen. To overcome these limitations in using vaccinia virus with the T7 system, adoption of highly attenuated viruses is desirable. A novel vaccinia vector system was established on the basis of the host range restricted and highly attenuated modified vaccinia Ankara (MVA) strain of vaccinia virus ***(22–24)***. Derived by long-term serial passage in chicken embryo fibroblasts, MVA lost its capacity to grow productively in human and most other mammalian cells ***(25–27)***. Viral replication in human cells is blocked late in infection, preventing the assembly to mature infectious virions. Nevertheless, MVA is able to express viral and recombinant genes at high levels even in nonpermissive cells and can serve as an efficient and exceptionally safe expression vector ***(28)***.

2. Materials

2.1. T7 Transient Expression

2.1.1. Infection/Transfection Protocol

1. The plasmid vector pTM3 ***(29)*** is designed for expressing genes under control of the T7 promoter/EMCV UTR (**Fig. 1**). pTM3 also contains the *E. coli* guanine

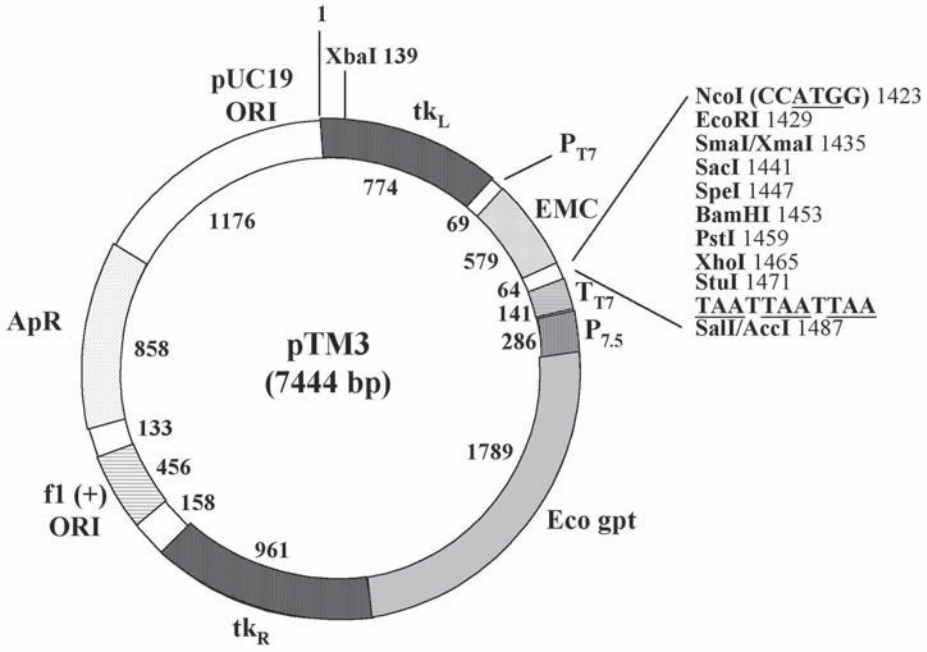

Fig. 1. The plasmid vector pTM3 ***(29)*** is designed to express genes under the control of the T7 promoter/EMCV UTR. The expression cassette is flanked by segments of the vaccinia virus TK gene-TK_L (left) and TK_R (right). pTM3 also contains the *E. coli* guanine phosphoribosyltransferase (Eco gpt) gene to permit MPA selection for recombinant vaccinia virus ***(6)***. P_{T7}, T7 promoter; T_{T7}, T7 terminator; EMC, encephalomyocarditis virus untranslated leader sequence; Ap^R, ampicillin resistant gene; ORI, origin of replication.

phosphoribosyl transferase (*gpt*) gene to permit mycophenolic acid (MPA) selection of recombinant vaccinia virus (***6***; *see* **Note 1**).

2. Exponentially growing African green monkey cells, BSC-40 (*see* **Note 2**).
3. Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (Hyclone).
4. Recombinant vaccinia virus, vTF7-3, expressing the bacteriophage T7 RNA polymerase gene ***(7)***.
5. Salmon sperm DNA.
6. 3 *M* sodium acetate, pH 7.0.
7. Absolute ethanol.
8. 2.5 *M* $CaCl_2$, prepare fresh (*see* **Note 3**).
9. 2X HEPES-buffered saline (HeBS), prepare fresh (*see* **Note 3**).
10. Phosphate-buffered saline (PBS).
11. Methionine-free DMEM.

12. [^{35}S]methionine.
13. 37°C, 5% CO_2 humidified incubator.
14. 100-mm tissue culture dishes.

2.1.2. Double Infection Protocol

1. Wild-type vaccinia virus.
2. Wild-type vaccinia virus DNA (*see* **Note 4**).
3. Human TK$^-$ 143 cells ***(30)***.
4. 5-Bromodeoxyuridine (BrdU) used at 25 μg/mL.
5. BSC-1 cells (*see* **Note 2**).
6. MPA, 10 mg/mL solution in water (*see* **Note 5**).
7. Xanthine, 10 mg/mL in water (*see* **Note 5**).
8. Hypoxanthine, 10 mg/mL in water (*see* **Note 5**).

2.2. Inducible Expression of Recombinant Viruses

2.2.1. Infection/Transfection Protocol

1. The recombinant vaccinia virus, vT7lacOI ***(31)***, expresses a version of the bacteriophage T7 RNA polymerase that is placed under the control of regulatory elements from the *E. coli* lac operon.
2. 100 m*M* IPTG.

2.2.2. Single Infection Protocol

1. Wild-type vaccinia virus DNA (*see* **Note 4**).

3. Methods

3.1. T7 Transient Expression

Expression can be obtained by either the infection/transfection or double infection protocols. With the latter, levels of protein made over a 24-h period can exceed 10% of the total cell protein ***(13)***. However, a recombinant virus that incorporates the gene of interest under the T7-promoter must be isolated.

3.1.1. Infection/Transfection Protocol

1. Insert the gene of interest at the multiple cloning region of pTM3 so that the *Nco*I site provides the translation initiation codon (*see* **Note 1**).
2. Purify the plasmid DNA twice using CsCl density gradient (*see* **Note 6**).
3. Split BSC-40 cells into 100-mm tissue culture dishes so they will be confluent on the day of transfection (*see* **Note 2**).
4. Infect the cells with the recombinant vaccinia virus, vTF7-3, expressing the bacteriophage T7 RNA polymerase at a multiplicity of infection (moi) of 10–20 pfu/cell (*see* **Note 7**). Leave the cells for 1 h at 37°C with occasional rocking of the plates to prevent drying.

5. Following the incubation, remove the virus inoculum and add 10 mL complete DMEM per 100-mm dish and continue the incubation at 37°C for another 2 h.
6. Precipitate the DNA to be transfected (10 µg plasmid DNA + 10 µg salmon sperm) with 1/10 vol of 3 *M* sodium acetate and 2.5 vol of absolute ethanol. Wash the precipitated DNA with 70% ethanol and air-dry by inverting the microcentrifuge tube on a fresh Kimwipe under the tissue culture hood.
7. Resuspend the DNA in 700 µL sterile H_2O and add 75 µL 2.5 *M* $CaCl_2$ (125 m*M* final). Add the DNA/$CaCl_2$ solution dropwise with a Pasteur pipet to a 750 µL 2X HeBS, vortex for a few seconds, and let it sit for 1 h at room temperature (*see* **Notes 3** and **8**).
8. Remove the medium from the infected cells and distribute the DNA/$CaCl_2$ precipitate evenly over the cells. Leave the DNA precipitate for 15 min at room temperature with periodic rocking, then add 10 mL complete DMEM, and continue the incubation at 37°C for another 4 h.
9. Remove the medium, wash cells with 10 mL of 1X PBS, then add 10 mL complete DMEM, and incubate overnight at 37°C.
10. There are multiple methods for analyzing the expression of the protein of interest. If antibodies are unavailable, pulse-label the cells for 1 h at 37°C with 65 µCi/mL [^{35}S]methionine in 2 mL methionine-free DMEM. After the labeling period, remove the isotope and wash the cells twice with PBS. Harvest the cells in the desired buffer for subsequent analysis.

3.1.2. Double Infection Protocol

To enhance recombinant protein production, the T7 promoter-regulated gene of interest can be incorporated into the genome of a second recombinant virus, which is then used to coinfect cells with the virus expressing T7 RNA polymerase *(7)*. For detailed protocols on the construction of the recombinant vaccinia virus using gpt and thymidine kinase (TK) selection, *see* Chapters 2 and 3, respectively. Briefly:

1. Infect the BSC-40 cells with wild-type vaccinia virus at a moi of 0.2 pfu/cell, at 37°C. Following incubation for 1 h at 37°C, transfect the infected cells with calcium phosphate-precipitated plasmid DNA (*see* **Notes 3**, **4**, and **8**) as described in **Subheading 3.1.1.**
2. After 4 h of incubation at 37°C, replace the medium with 10 mL fresh DMEM, and continue the incubation for 2–3 d at 37°C until they exhibit complete cytopathic effect. Scrape the infected cells and resuspend them in 300 µL PBS. Freeze and thaw the cells three times to release the virus.
3. Select the recombinant viruses formed by homologous recombination of the foreign gene into the TK locus by plaque assay on TK$^-$ 143 cell monolayers in the presence of 25 µg/mL BrdU; *see* **Note 9**. After multiple rounds of plaque purification, prepare virus stocks. The construct can be confirmed by isolating the DNA from the recombinant virus and analyzing the genome structure by polymerase chain reaction (PCR).

4. Coinfect a 100-mm dish of BSC-40 cells with 5 pfu/cell of each of the newly isolated recombinant vaccinia virus that harbors the foreign gene (under the control of the T7 RNA polymerase promoter) and vTF7-3 (expressing the T7 RNA polymerase) for 1 h at 37°C. Subsequently, add 10 mL fresh complete DMEM and continue incubation for another 8 h at 37°C.
5. Similar to **Subheading 3.1.1.**, **step 10**, if no antibodies to the expressed protein are available, proteins can be metabolically labeled during infection and analyzed for expression of the protein of interest. Briefly, pulse-label the cells for 1 h at 37°C with 65 μCi/mL [^{35}S]methionine in 2 mL methionine-free DMEM. After the labeling period, remove the isotope and wash the cells twice with PBS. Harvest the cells in the desired buffer for subsequent analysis.

3.2. Inducible Expression of Recombinant Viruses

Regulation of foreign gene expression from the recombinant vaccinia virus was achieved by transfer of the regulatory elements from the *E. coli* lactose operon into the genome of vaccinia virus ***(16)***.

3.2.1. Infection/Transfection Protocol

1. Insert the gene of interest at the multiple cloning region of pTM3 (*see* **Note 1**).
2. Purify the plasmid DNA twice using CsCl density gradient (*see* **Note 6**).
3. Infect a 100-mm dish of BSC-40 cells with the recombinant vaccinia virus vT7lacOI ***(31)***, expressing a regulated version of the bacteriophage T7 RNA polymerase at a moi of 20 pfu/cell. Incubate for 1 h at 37°C with occasional rocking of the plates.
4. Following the incubation, add 10 mL complete DMEM per 100-mm dish and continue the incubation at 37°C for another 2 h.
5. Precipitate the DNA to be transfected (10 μg plasmid DNA + 10 μg salmon sperm) with 1/10 vol of 3 *M* sodium acetate and 2.5 vol of absolute ethanol. Wash the precipitated DNA with 70% ethanol, and air-dry by inverting the microcentrifuge tube on a fresh Kimwipe under the tissue culture hood.
6. Resuspend the DNA in 700 μL sterile H_2O and add 75 μL 2.5 *M* $CaCl_2$ (125 m*M* final). Add the DNA/$CaCl_2$ solution dropwise with a Pasteur pipet to a 750 μL 2X HeBS, vortex for a few seconds, and let sit for 1 h at room temperature (*see* **Notes 3** and **8**).
7. Remove the medium from the infected cells and distribute the DNA/$CaCl_2$ precipitate evenly over the cells. Leave the DNA precipitate over the cells for 15 min at room temperature with periodic rocking, then add 10 mL complete DMEM, and continue the incubation at 37°C for another 4 h.
8. Remove the medium, wash cells with 10 mL 1X PBS, then add 10 mL complete DMEM containing 15 μ*M* IPTG, and leave overnight at 37°C.
9. To analyze expression of the protein of interest, pulse-label the cells for 1 h at 37°C with 65 μCi/mL [^{35}S]methionine in 2 mL methionine-free DMEM containing 15 μ*M* IPTG. After the labeling period, remove the isotope and wash the cells twice with PBS. Harvest the cells in the desired buffer for subsequent analysis.

3.2.2. Double Infection Protocol

1. To generate the recombinant virus, first infect the BSC-40 cells with wild-type vaccinia virus at a moi of 0.2/cell at 37°C. Following incubation for 1 h, transfect the infected cells with calcium phosphate-precipitated plasmid DNA (*see* **Notes 3**, **4**, and **8**) as described in **Subheading 3.1.1.**
2. After 4-h incubation at 37°C, replace the medium with 10 mL fresh DMEM and continue the incubation for 2–3 d at 37°C until they exhibit complete cytopathic effect. Scrap the infected cells and resuspend them in 300 µL PBS. Freeze and thaw the cells three times to release the virus.
3. Select the recombinant viruses formed by homologous recombination of the foreign gene into the TK locus by plaque assay on TK^- 143 cell monolayers in the presence of 25 µg/mL BrdU. Then isolate the DNA from the obtained TK^- viruses and analyze by PCR and agarose gel electrophoresis for the inserted sequences. For gpt selection, *see* **Note 9**.
4. Coinfect a 100-mm dish of BSC-40 cells with a moi of 5 pfu/cell of each of the newly isolated recombinant vaccinia virus that harbors the foreign gene (under the control of the T7 RNA polymerase promoter) and vT7lacOI (expressing a regulated version of the bacteriophage T7 RNA polymerase) for 1 h at 37°C with occasional rocking of the plates. Subsequently, remove the extra virus and add 10 mL fresh complete DMEM containing 15 µ*M* IPTG and continue incubation for another 8 h at 37°C.
5. Similar to **Subheading 3.1.1.**, **step 10**, if no antibodies to the expressed protein are available, proteins can be metabolically labeled during infection and analyzed for expression of the protein of interest. Briefly, with the protein of interest, pulse-label the cells for 1 h at 37°C with 65 µCi/mL [^{35}S]methionine in 2 mL methionine-free DMEM containing 15 µ*M* IPTG. After the labeling period, remove the isotope and wash the cells twice with PBS. Harvest the cells in the desired buffer for subsequent analysis.

4. Notes

1. An alternative to the pTM3 is pTM1 ***(29)***. However, pTM1 does not have the *E. coli* gpt gene that permits MPA selection for recombinant viruses. Therefore, when using pTM1, only the TK selection must be relied on for recombinant viruses (using cell line $HuTK^-$143B cells). Another alternative plasmid is pOS7 if there are no plans to generate a recombinant virus ***(29)***. This is a smaller plasmid that does not contain the tk or gpt sequences and thus can be used for infection/transfection protocols exclusively.
2. A 1:10 split of confluent cells takes approx 3–4 d to reach confluency at 37°C. Cells also tend to grow better and faster if split from a confluent dish rather than being split from a nonconfluent dish. Repeated splits at high dilutions (higher than 1:20) are not usually recommended because they exhaust the cells.
3. Because the optimum pH range for transfection is between 7.05 and 7.12 ***(32)***, another factor that affects the efficiency of transfection is the pH of the HeBS

buffer, which can change over time. Therefore, for the best results, the 2X HeBS buffer and 2.5 *M* $CaCl_2$ solution should be prepared fresh. Also, the pH of the medium should be carefully monitored. If the cultures become acidic, this dramatically reduces the transfection efficiency.

4. Although not absolutely required, adding 1–2 μg vaccinia virus DNA during transfection of the recombinant plasmid enhances the efficiency of recombination into the viral genome ***(33)***.
5. Stock solutions of MPA, xanthine, and hypoxanthine may need to be titrated with NaOH to go into the solution. Moreover, following the addition of MPA, xanthine, and hypoxanthine to the culture medium, readjust the pH of the medium with a few drops of 1 *M* HCl.
6. The efficiency of transfection is highly dependent on the quality and purity of plasmid DNA to be transfected; therefore, CsCl purification of the plasmid DNA is necessary.
7. Sample calculation. If you want to infect six wells of a six-well plate (1×10^6 cells/well × 6 wells = 6×10^6 cells). Stock of vTF7-3 is 5×10^8 pfu/mL. Thus, for a moi of 20 pfu/cell, 0.24 mL virus stock is needed (6×10^6 cells × 20 pfu/cell/5×10^8 pfu/mL).
8. We rely on the DNA precipitation method because it is one of the original methods and is easy, economical, and less problematic to use. However, any other transfection methods (e.g., lipofectamine, FuGENE, or any other commercially available products) should work fine, sometimes even with higher transfection efficiency. The problem with some of these reagents is that they may require transfection to be done in serum-free medium, which is a problem for cells that cannot tolerate the absence of serum for 2–24 h. Also, some of these reagents require the absence of any added antibacterial agents during transfection. Finally, some serum-free media formulations can inhibit cationic lipid-mediated transfection.
9. Alternatively, in the case of using a gpt-containing plasmid, recombinant viruses can be selected by gpt selection method (*see* Chapter 2). Briefly, BSC-1 cells are pretreated 1 d before infection with medium containing 25 μg/mL MPA, 250 μg/mL xanthine, and 15 μg/mL hypoxanthine (*see* **Note 5**). The pretreated cells are then infected with the transfection mix and, after an absorption period, overlayed with 1:1 mixture of 2X media containing 50 μg/mL MPA, 500 μg/mL xanthine, 30 μg/mL hypoxanthine, and low-melting point (LMP) agarose. Of note, while performing plaque purifications under semisolid overlay using BSC-1 cells, it is important to use LMP agarose in the overlay instead of the regular agar because BSC-1 cells seem to only survive with LMP agarose.

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5

Construction of Recombinant Vaccinia Viruses Using Leporipoxvirus-Catalyzed Recombination and Reactivation of Orthopoxvirus DNA

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Summary

Poxvirus DNA is not infectious because the initiation of the infective process requires proteins encapsidated along with the virus genome. However, infectious virus can be produced if purified poxvirus DNA is transfected into cells previously infected with another poxvirus. This process is termed *heterologous reactivation* if the infecting virus is different from the transfected virus. We describe a method in which the high-frequency recombination and replication reactions catalyzed by the Leporipoxvirus, Shope fibroma virus (SFV), can be coupled with SFV-promoted reactivation reactions to rapidly construct recombinant vaccinia viruses in high yields (25–100% recombinant progeny). The reactivated vaccinia viruses are easily purified free of the SFV helper virus by plating mixed populations of virus on cells that support only the growth of vaccinia virus. These heterologous reactivation reactions can be used to manipulate the structure of virus genomes and produce viruses that express recombinant proteins at high levels. We illustrate the method by polymerase chain reaction (PCR) cloning the gene encoding green fluorescent protein (GFP), then using double-strand break repair reactions to produce a recombinant virus that expresses high levels of GFP.

Key Words: Genetic recombination; green fluorescent protein (GFP); protein expression; reactivation; Shope fibroma virus (SFV); T7 expression vectors; vaccinia virus; virus vectors.

1. Introduction

An updated method ***(1)*** has been developed for reactivating transfected orthopoxvirus DNA using cells infected with Shope fibroma virus (SFV). This heterologous reactivation method takes advantage of the high-frequency recombination reactions promoted by SFV ***(2–4)***, to assemble recombinant vaccinia viruses. The procedure is quick and simple, yielding a sufficiently high

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proportion of recombinant viruses to avoid the necessity of performing ligation reactions ***(5,6)*** and/or using selection methods.

Purified poxvirus DNA is not infectious because, after viral entry and uncoating, starting the infection cycle requires transcription of early genes by virion-encapsidated RNA polymerases. This deficiency can be overcome if virus DNA is transfected into a cell already infected by a "helper" poxvirus, which can then provide all of the *trans*-acting factors needed to transcribe, replicate, and package the transfected genome ***(7)***. Of course, this scheme yields mixed viral progeny. But this problem can be overcome by performing heterologous reactivation reactions (i.e., using two different viruses) in a cell line permissive for both viruses, and then eliminating the helper virus by plating the mixture of viral progeny on cells that do not support its growth ***(6)***. In the method described here, BGMK cells, which support replication of both vaccinia virus and SFV, are used to generate recombinants, whereas BSC40 cells, which permit only vaccinia virus growth, are used to isolate the recombinant.

An attractive feature of this method is that replicating poxviruses require only limited end-sequence homology to promote recombination via double-stranded break repair reactions ***(4)***. This can be used to target polymerase chain reaction (PCR) amplicons into a restriction enzyme-cut vaccinia virus vector, using only the short (approx 18 nt) patches of sequence homology that can be easily incorporated into PCR primers. Used in conjunction with modified vaccinia virus expression vectors encoding T7 promoters ***(8)***, we show as an example how this method can be applied to rapidly clone and then overexpress green fluorescent protein (GFP).

2. Materials

1. Mammalian cells: BSC40 (ATCC, cat. no. CRL-2761), SIRC (ATCC, cat. no. CCL-60), BGMK (provided by G. McFadden, University of Western Ontario) (*see* **Note 1**).
2. Viruses: SFV (strain Kasza) and vaccinia virus (strain Western Reserve [WR]) were obtained from the ATCC. Recombinant vTF7.5 vaccinia virus was obtained from Dr. P. Traktman. Vaccinia strain XY_dBIDZ can be obtained from the authors.
3. Cell growth medium: Minimal essential medium (MEM) supplemented with 1X antibiotics and antimycotics, 2 m*M* glutamine, 0.1 m*M* nonessential amino acids, and 5% fetal bovine serum (FBS, purchased from Cansera).
4. Plaquing medium: Twice concentrated (2X) cell growth medium.
5. SIRC growth medium: MEM supplemented with 1X antibiotics and antimycotics, 2 m*M* glutamine, 0.1 m*M* nonessential amino acids, and 10% FCS.
6. 1 m*M* Tris: 1 m*M* Tris-HCl, pH 9; filter-sterilize.
7. 10 m*M* Tris: 10 m*M* Tris-HCl, pH 9; filter-sterilize.
8. Sucrose gradient solutions: 40%, 36%, 32%, 28%, and 24% sucrose (w/v) each made in 1 m*M* Tris; filter-sterilize.

9. Sucrose cushion: 36% (w/v) sucrose in 10 m*M* Tris.
10. Virion lysis buffer: 0.4 mg/mL proteinase K, 1.2% sodium dodecyl sulfate, 50 m*M* Tris-HCl, pH 8, 4 m*M* EDTA, pH 8, and 4 m*M* $CaCl_2$.
11. DNA suspension buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.
12. GFP plasmid: pEGFP-N1 (Clontech).
13. Oligonucleotide primers (*see* **Fig. 2B**).
14. Lipofectamine LF2000 (Invitrogen) and OptiMEM I (Gibco).
15. Phosphate-buffered saline (PBS): 1% NaCl, 0.025% KCl, 0.144% Na_2HPO_4, 0.025% KH_2PO_4, 0.01% $CaCl_2$, 0.01% $MgCl_2$.
16. Crystal violet solution: 0.5% crystal violet, 1% acetic acid, 0.7% formaldehyde, 14% ethanol.
17. Cell fixing solution: 2% formaldehyde and 0.2% glutaraldehyde in PBS.
18. X-gal solution: 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 2 m*M* $MgCl_2$, 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide in PBS.
19. 1.7% Noble agar (Difco) in water. Sterilize by autoclaving (*see* **Note 2**).
20. Nylon membrane (Bio-Rad).

3. Methods

To illustrate this method for producing recombinant vaccinia viruses, we will use as an example the construction of a virus expressing green fluorescent protein (GFP). The starting point for these studies is a recombinant vaccinia virus vector that we have designated XY_dBIDZ (**Fig. 1**). This virus encodes two unique restriction sites that, when cut with *Apa*I and I-*Sce*I, creates a double-stranded break flanked by non-homologous ends. This break would normally inactivate the virus DNA unless it can be repaired by a recombination event in the presence of a helper poxvirus. XY_dBIDZ was assembled using a series of steps, which is described elsewhere, including both traditional and reactivation-based recombinational methods. Furthermore, XY_dBIDZ includes a T7 promoter and an internal ribosome entry site derived from pTM3 ***(10,11)***, as well as a copy of the *Escherichia coli lacZ* gene under the independent regulation of a vaccinia virus P7.5 promoter. This "LacZ cassette" is incorporated into the virus downstream of the T7 promoter and is flanked by unique *Apa*I and *I-Sce*I sites. The whole expression construct replaces what, in vaccinia virus strain WR, would be the nonessential *Bgl*I-D restriction fragment. Deletion of *Bgl*I-D does not affect growth in culture, but might be expected to significantly attenuate the virus due to the deletion of two virokines, a host range function, and a serpin.

The overall strategy is to create a recombinational target by digesting XY_dBIDZ with *Apa*I and *I-Sce*I, then using coupled double-strand break repair and reactivation reactions to replace the excised LacZ cassette with a PCR-amplified copy of the GFP open reading frame (ORF). Recombinant viruses are formed in the presence of a helper virus and can later be identified

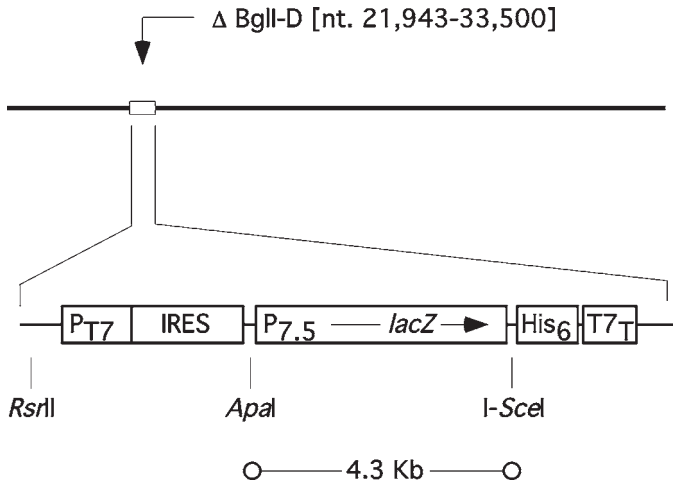

Fig. 1. Vaccinia virus gene expression vector XY_dBIDZ. The virus was assembled using a combination of homologous recombination and virus reactivation reactions. The expression cassette replaces an 11.6-kb *Bgl*I-D fragment of the vaccinia virus WR genome deleted at an earlier step in the construction of the vector. A T7 promoter (P_{T7}), internal ribosome entry site (IRES), and T7 terminator element ($T7_T$) regulates expression of any gene cloned between unique *Apa*I and I-*Sce*I sites. An *E. coli lac*Z gene, under the independent regulation of a vaccinia P7.5 promoter, provides a blue/white screen for recombinant viruses. A hexa-histidine tag can also be fused to the C-terminus of a recombinant protein if cloning is done in frame. (This tag was not used in the studies discussed in this chapter.) Part of this plasmid construct is derived from plasmid pTM3 ***(11)***, but another *Apa*I site encoded by that plasmid was deleted during the course of vector assembly.

by a *lacZ*–minus phenotype. The T7 promoter and internal ribosome entry site permit high-level expression of a GFP reporter protein in the presence of T7 polymerase.

3.1. Virus and Cell Culture

Vaccinia virus is routinely grown for 2–3 d in BSC40 cells in cell growth medium at 37°C in a 5% CO_2 atmosphere. SFV is grown for 5 d in SIRC cells in SIRC growth media at 37°C in a 5% CO_2 atmosphere. Vaccinia virus should be handled with care using BSL2 containment. SFV is not a known human pathogen, but it is still normally cultured under BSL2 conditions.

3.2. Preparation of Virus DNA from Purified Virions

Virus DNA is prepared from purified virions using a minor variant of a published procedure ***(9)***. There are two main steps in the process: purifying

virus particles from infected cell lysates using sucrose gradients, then isolating the virus DNA.

3.2.1. Preparation of Purified Vaccinia Virus from BSC40-Infected Cells

1. Prepare 15–20 150-mm plates of BSC40 cells and infect with a multiplicity of infection (moi) of 0.1 plaque-forming units (pfu) per cell.
2. Harvest the infected cells 2 d later by scraping the cells into the medium and then centrifuging the cell suspension for 5 min at 500*g* at 4°C. Discard the supernatant because most of the virus is cell associated (*see* **Note 3**).
3. Prepare six sucrose step gradients in SW41 tubes by carefully layering 3 mL of 40%, 2.2 mL of 36%, 2.2 mL of 32%, 2 mL of 28%, and 1 mL of 24% sucrose solutions into each tube. These are stored overnight at 4°C.
4. Resuspend the infected cell pellet in 8 mL of 10 m*M* Tris, and release the virus from the cells with a Dounce homogenizer. Do this in a biosafety hood as the procedure generates an aerosol.
5. Centrifuge the lysed cells for 10 min at 1100*g* at 4°C and collect the supernatant.
6. Resuspend the pellet with 4 mL 10 m*M* Tris, Dounce homogenize, and centrifuge again at 1100*g* at 4°C to extract more virus. Pool the two supernatants in sterile capped tubes.
7. Sonicate the supernatants in ice water (3 × 1 min) using a cup (*not* probe) sonicator filled with ice-water mixture.
8. Prepare two SW41 centrifuge tubes each containing a 6 mL sucrose cushion, and add the sonicated supernatant-containing virus to each tube.
9. Centrifuge 80 min at 33,000*g* at 4°C (*see* **Note 4**).
10. Discard the supernatant, resuspend the virus pellets from all of the tubes in a total of 4 mL 1 m*M* Tris, and sonicate again.
11. Overlay 1 mL of the sonicated virus onto each of the sucrose step gradients (**step 3**) using a total of four tubes at this point.
12. Centrifuge for 50 min at 26,000*g* at 4°C (*see* **Note 4**).
13. Collect and put aside the milky band of virus, located between the 30 and 40% sucrose layers, using a long Pasteur pipet.
14. Retrieve the pellets of aggregated virus from the bottom of these four tubes and resuspend in 1 mL 1 m*M* Tris.
15. Sonicate and "reband" using the remaining two sucrose gradients. Retrieve the bands of virus with a Pasteur pipet.
16. Pool the six bands of virus and add 2 vol 1 m*M* Tris.
17. Pellet the purified virus by centrifuging for 30 min at 33,000*g* at 4°C (*see* **Note 4**).
18. Discard the supernatant, then resuspend the pellet in 0.2 mL 1 m*M* Tris with sonication.

3.2.2. Extraction of Virus DNA

1. The virus suspension (0.2 mL) is mixed with 1.8 mL of virion lysis buffer, then incubated at 37°C for 4–16 h.
2. Virus DNA is gently extracted with 2 mL phenol followed by centrifugation at 15,000*g* for 10 min at room temperature, then with 2 mL phenol/chloroform (1:1),

and finally 2 mL chloroform. Use cut-off tips when transferring the DNA between tubes to minimize shear forces.
3. The DNA is ethanol-precipitated, rinsed with 70% ethanol, dried briefly, and resuspended in 0.25 mL sterile DNA suspension buffer or sterile water. The procedure should yield approx 100 μg virus DNA.

3.2.3. Restriction Enzyme Cleavage of XY_dBIDZ Virus DNA and PCR Amplification of a Recombinational Target (Gene Insert)

1. Cleave 10 μg virus DNA with 50 U *Apa*I in a 200-μL reaction overnight at 25°C, then add 25 U I-*Sce*I, and digest for 4 h at 37°C. Add another 25 U I-*Sce*I and incubate another 4 h at 37°C (*see* **Note 5**).
2. Extract the DNA with phenol/chloroform (1:1), then chloroform, and precipitate with 3 vol ethanol in the presence of 1/10 vol 3 *M* sodium acetate, pH 5. Recover the DNA by centrifugation (>15,000*g* for 2 min), wash with 70% ethanol and dry briefly.
3. Dissolve the DNA pellet in 50 μL DNA suspension buffer and determine the DNA concentration by spectrophotometry (*see* **Note 6**).
4. The GFP ORF is amplified by high-fidelity PCR using pEGFP-N1 as the template. The two PCR primers spanning the start and stop codons are described in **Fig. 2** (*see* **Note 7**). Typical thermocycler conditions are 94°C for 15 s, 50°C for 30 s, 72°C for 1 min, for 20–25 cycles.
5. The PCR products are gel-purified and recovered by electroelution, and the concentration is determined by spectrophotometry (*see* **Note 6**).

3.3. Infection and Transfection

1. One day prior to the planned infection/transfection, BGMK cells are split into six 60-mm dishes to be nearly confluent at the time of infection and transfection.
2. When a dish of cells has reached 95–100% confluency (*see* **Note 8**), five dishes are infected with SFV at an moi of approx 1 pfu/cell (approx 1×10^6 pfu) in 0.5 mL PBS/dish. The virus is left on the cells for 1 h at room temperature with periodic rocking.
3. The remaining plate is treated only with PBS and is used as a mock infection control.
4. After 1 h, the medium on the cells is replaced with 3 mL cell growth medium and returned to the 37°C incubator for another hour.
5. While the cells are incubating, a transfection mixture is prepared as shown in **Table 1**.
6. Distribute the DNA(s) into each numbered microtube and adjust to 0.5 mL with OptiMEM I and mix.
7. Prepare lipofection reagent in a second set of six microtubes by aliquoting 3 μL lipofectamine per 1 μg DNA (cut viral DNA + PCR product) (*see* **Note 9**) and adjust each volume to 0.5 mL with OptiMEM I. Mix well.
8. Combine the DNA into the lipofectamine, mix gently, and incubate at room temperature for 20 min.

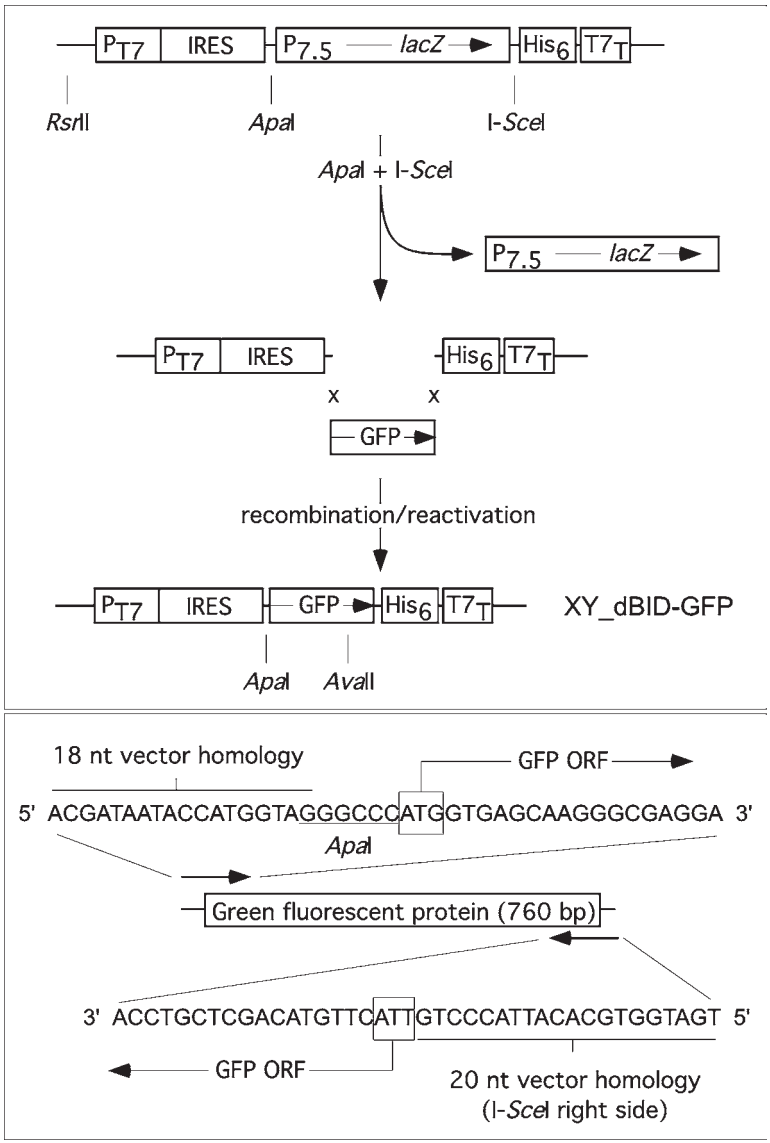

Fig. 2. Design and construction of a vaccinia virus expressing GFP. *Top panel*: The vaccinia virus expression vector (XY_dBIDZ) is digested with *Apa*I and I-*Sce*I, then the mixture of digestion products transfected, along with a PCR amplicon encoding GFP, into SFV-infected cells. Recombination and reactivation reactions generate recombinant vaccinia viruses encoding GFP (XY_dBID-GFP). The I-*Sce*I site is destroyed in the process. *Bottom panel*: The GFP ORF was cloned using the PCR and the two primers illustrated in the figure. The two PCR primers share 18 and 20 nt of homology with sequences flanking the *Apa*I and I-*Sce*I cut vaccinia vector. The leftmost primer encodes the GFP start codon (boxed), although a second, in frame, start codon is also provided by the virus vector. The rightmost primer encodes the GFP stop codon (boxed).

Table 1
Transfection of DNA into BGMK Cells[a]

Sample no.	1	2	3	4	5	6
SFV	+	+	+	+	+	–
XY_dBIDZ (RE-cut, μg)[b]	1	–	1	–	–	–
XY_dBIDZ (uncut, μg)	–	–	–	1	–	1
PCR amplicon (GFP, μg)[c]	–	0.16	0.16	–	–	–

[a]Reaction 3 contains the experimental sample; the other reactions serve as experimental controls.
[b]1 μg virus DNA is approx 8 fmol.
[c]A 40-fold molar excess of PCR-amplified DNA insert is used relative to the amount of virus DNA.

9. Add each transfection mixture to a dish of SFV-infected (or mock-infected) cells, mix by sliding the plate back and forth a few times, and return the dishes to the CO_2 incubator at 37°C for 4 h (*see* **Note 10**).
10. Replace the media with 5 mL of fresh cell growth medium and culture for 4 d.
11. After 3–4 d, scrape the SFV-infected/transfected BGMK cells into their growth medium and transfer the mixture (approx 5 mL) to a capped sterile tube. Virus particles are released by three consecutive freeze–thaw cycles (dry ice/37°C water bath).

3.4. Titration and Recovery of Reactivated Recombinant Vaccinia Virus

1. Split BSC40 cells into 60-mm dishes 1 d ahead of time, so that the cells are approx 80% confluent at the time of infection. About six plates are needed for each reactivated virus plus two for the control dishes. Eight additional plates are also needed for the plaque purification of recombinant virus, approx 40 in all (*see* **Note 11**).
2. Prepare 10-fold serial dilutions of these crude lysates (0.2 mL cell lysate plus 1.8 mL PBS).
3. Each dish of BSC40 cells is then infected in duplicate with 0.5 mL of three different dilutions of virus and cultured for 2–3 d (*see* **Notes 12** and **13**). Two dishes are also incubated with 0.5 mL 1/10 diluted media recovered from the mock infection control dish. Put aside the remaining eight dishes of BSC40 cells for recovery of the virus (**Subheading 3.5.**).
4. One hour later, replace the PBS dilution covering the cells with 5 mL fresh cell growth medium per plate.
5. After 2–3 d, when the plaques can be seen by eye, half of the infected cells are fixed and stained with 2 mL crystal violet solution to visualize the plaques formed by reactivated vaccinia virus. Count the number of plaques to determine the total virus titer (*see* **Note 14**).
6. Fix the second set of infected BSC40 cells for 5 min at room temperature with 2 mL cell fixing solution. Then add 2 mL X-gal solution and incubate at 37°C for at least 30 min. Count the blue plaques. This is the titer of *lac*Z-positive virus (*see* **Note 15**).

3.5. Recovery and Plaque Purification of Recombinant Virus

1. Take the eight remaining dishes of BSC40 cells that were prepared in **Subheading 3.4.**, and infect these cells with 0.5 mL diluted virus from lysates 3 and 4 (**Table 1**) from **Subheading 3.3.**, **step 11** (*see also* **Note 16**). Prepare two additional mock-infected controls. Let the virus absorb for 1 h, then replace the virus infection solution with 3 mL cell growth medium, and incubate for another 4–6 h at 37°C.
2. Remove the liquid medium and overlay the cells in each dish with 3 mL of an overlay containing a 1:1 mixture of 1.7% noble agar and 2X plaquing medium (*see* **Note 17**). Let the warmed mixture solidify at room temperature, then put dishes at 37°C for 2 d.
3. After the 2-d incubation, overlay the cells with 2 mL of the 1:1 mixture of 1.7% noble agar and 2X plaquing medium containing 300 μg/mL X-gal.
4. Incubate dishes another 24 h until blue plaques are seen in the plates derived from reaction 4 (**Table 1**). This positive control is important, because it confirms the β-galactosidase assay is working when no blue (parental) plaques are detected in reaction 3 (*see* **Note 18**).
5. Pick 5–10 white (*lacZ*-minus) plaques, generated by recombination in reaction 3 (**Table 1**), and suspend each plaque in 0.5 mL sterile PBS. Freeze–thaw the samples three times.
6. Plate 0.5 mL of the virus recovered from each plaque on 60-mm dishes of new BSC40 cells to increase the virus titer. Incubate the virus for 3 d, then scrape the cells into the culture medium. These virus stocks are then screened for recombinants (*see* **Note 19**).

3.6. Analysis of Viral DNA Structure by Southern Blot Analysis (see *Note 20)*

1. Infect a 60-mm dish of BSC40 cells with 0.1 mL of the stock of virus prepared in **Subheading 3.5.**, **step 6**.
2. Next day (approx 20-h post-infection), remove the media, and add 1 mL of virion lysis buffer.
3. Incubate at 37°C for 4 h. Prior to transferring lysed cells into a sterile tube, shear each sample by triturating the specimen 10–20 times using a sharp-tipped pipet (*see* **Note 21**).
4. Extract the DNA with phenol and chloroform, precipitate with ethanol, spin, dry, and dissolve the DNA in 0.1 mL H_2O.
5. Digest 50 μL DNA with *Ava*II overnight in a 0.1-mL reaction.
6. Separate the digested viral DNA using a 0.8% agarose gel, transfer to a nylon membrane, and hybridize to a probe prepared by random prime labeling the GFP-encoding PCR amplicon. **Figure 3** shows the resulting autoradiograph obtained after the membrane was exposed overnight to X-ray film (*see* **Notes 18** and **22**).

3.7. Expression of GFP from Recombinant Virus

1. Seed a sufficient number of small (22-mm-diameter) glass coverslips with BSC40 cells 1 d prior to infection.

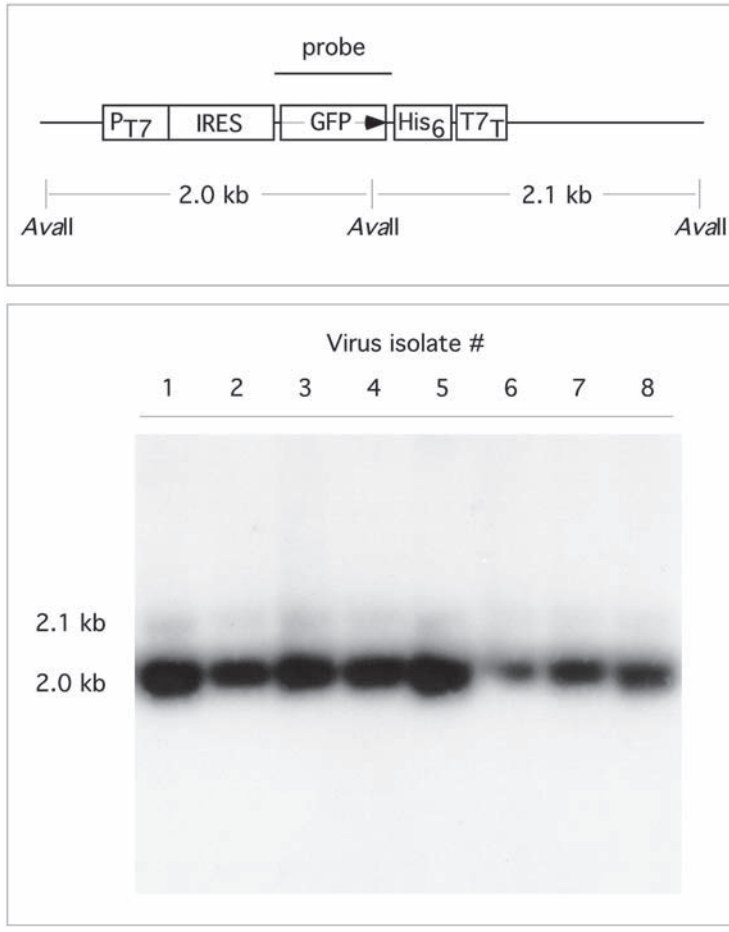

Fig. 3. Southern blot analysis of recombinant viruses encoding GFP. Putative recombinant viruses were digested with *Ava*II and subjected to Southern blot analysis using a radioactive probe spanning the GFP ORF. *Ava*II is predicted to produce two restriction fragments that are detectible with this probe, 2.0 and 2.1 kb in length (*top panel*). The 2-kb band is much darker than the 2.1-kb band (*bottom panel*), because the majority of the labeled probe hybridizes with the 2-kb fragment.

2. Coinfect the cells for 1 h with the newly isolated recombinant virus and a vaccinia virus expressing T7 RNA polymerase (e.g., vTF7.5, *see* **Note 23**). Infect with an moi of each virus at approx 2 pfu/cell.
3. Next day (approx 24 h postinfection), fix the cells for 30 min with 4% formaldehyde, wash twice with PBS, and mount in 80% glycerol. GFP expression is detected using a confocal fluorescence microscope and filter settings of 488 nm (excitation) and >500 nm (emission). **Figure 4** shows the expression pattern.

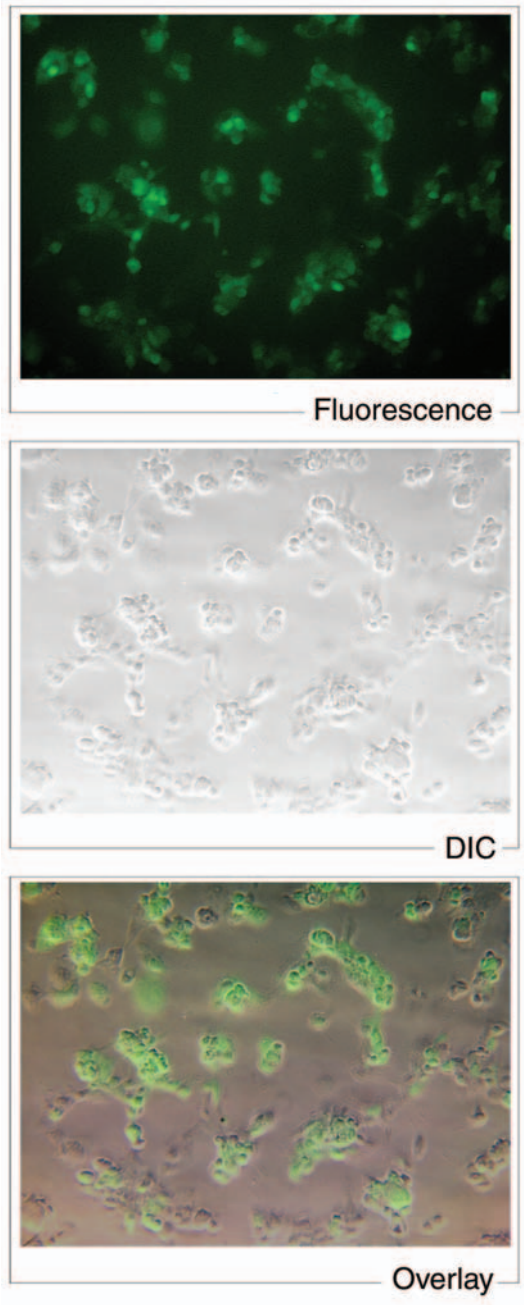

Fig. 4. Expression of recombinant GFP in BSC40 cells. BSC40 cells were coinfected with two recombinant vaccinia viruses, vTF7.5 and XY_dBID-GFP, and protein expression was detected 24-h post-infection by fluorescence microscopy. At an moi of 2 for each virus, about half of the cells express GFP. DIC, differential interference contrast.

4. Notes

1. Reactivation experiments employ BGMK and BSC40 cells. SIRC cells are used to propagate the SFV helper virus.
2. Noble agar is a higher quality agar used in cell culture.
3. The cell pellet can be stored frozen at this point if desired.
4. 33,000*g* is 17,000 rpm in SW41 rotor; 26,000*g* is 15,500 rpm in a SW41 rotor; 33,000*g* is 50,000 rpm in a 70Ti rotor.
5. I-*Sce*I is not very stable in either the *Apa*I reaction buffer (buffer 4) or in the I-*Sce*I reaction buffer provided by manufacturer (Biolabs). However, this enzyme is active in buffer 4, so we use this buffer and add two aliquots of I-*Sce*I to achieve complete DNA digestion. It is important that virus genomic DNA is cut to completion. This can be monitored using pulsed-field gel electrophoresis.
6. The concentration of DNA is determined by spectrophotometry: 50 μg/mL = 1 A_{260}.
7. The 5' ends of the primers encode additional sequences, which ensure that the amplicon's ends share 18–20 bp of homology with the flanking portions of the virus that are left behind after cutting XY_dBIDZ DNA with *Apa*I and I-*Sce*I (*see* **Fig. 2** for details).
8. The extent of confluency of the BGMK cells significantly affects the reactivation efficiency. This may be because greater confluency favors increased lipofectamine transfection efficiency and this, in turn, enhances virus yields.
9. The amount of DNA used to transfect BGMK cells also greatly influences the yield of virus from reactivation and recombination reactions. The optimum quantity of DNA is 2.0–2.3 μg per 60-mm dish of cells when using lipofectamine LF2000 under the recommended conditions. Adding more DNA has negative effects on cell viability. The ratio of targeted DNA to the inserted gene is also critical. A 40:1 molar ratio seems to maximize the recombination frequency.
10. Transfections are conducted in the presence of FBS, as it does not appear to affect the efficiency of transfection of BGMK cells.
11. Example of calculation of number of dishes needed to isolate recombinants: there are originally five infected/transfected dishes. Three dilutions per dish are necessary, plated in duplicate. A single dilution of the mock-infected extract is also plated in duplicate. Thus, (5 dishes × 3 dilutions/dish × 2 duplicate dishes) + 2 control dishes = 32 60-mm dishes.
12. Suggested dilutions are: 1/10–1/1000 dilutions of virus recovered from reactions 1–3, and 5 (**Table 1**), 1/1000–1/100,000 dilutions of reaction 4.
13. Do not plate too low a dilution of the reactivated virus, recovered from BGMK cells, on BSC40 cells. We typically plate at least a 1/100 dilution. A lower dilution of reactivated virus can be plated and still obtain vaccinia virus plaques, but the titers underestimate the virus yield calculated from plating greater dilutions of virus. We presume that dilution eliminates some form of interference caused by the SFV helper virus.
14. The reactivation efficiency (RE) can be calculated from the formula: RE = (plaques × 10 × dilution factor)/(viral DNA transfected μg).

Table 2
Titration of Reactivated Recombinant Virus

No.	Transfected DNA	SFV "helper"	Total virus (pfu/μg)	*lacZ*+ virus (pfu/μg)	Percent *lacZ*– virus
1	XY_dBIDZ (cut)	+	1.0×10^3	ND*	100
2	PCR-GFP	+	ND	ND	–
3	XY_dBIDZ (cut) + PCR-GFP	+	7.7×10^3	85	99
4	XY_dBIDZ (uncut)	+	7.1×10^4	6.8×10^4	~4
5	None	+	ND	ND	–
6	XY_dBIDZ (uncut)	–	ND	ND	–

*ND, not detected. No plaques were detected when 0.5 mL of a 1/10 diluted virus suspension was plated on BSC40 cells.

15. **Table 2** shows an example of the yields of *lacZ*-positive and *lacZ*-negative virus that can be expected to be obtained in such experiments. Virus from cells transfected with just the cleaved virus DNA are often recovered (**Table 1**, reaction 1), but usually much less than that obtained from cells transfected with both cleaved virus DNA and PCR amplicon (**Table 1**, reaction 3), and far fewer virus than are recovered from cells infected with the uncleaved virus DNA (**Table 1**, reaction 4). The greater the yield of *lacZ*-negative virus from cells transfected with both cleaved viral DNA and PCR amplicon (**Table 1**, reaction 3), relative to the number detected in reaction 1 (cleaved virus DNA alone), the greater the likelihood of obtaining recombinant virus encoding the expected insert. Most or all of the virus recovered from the reaction 3 should also produce white plaques due to the replacement of the LacZ cassette with the PCR amplicon. No virus should be isolated from the other control reactions (2, 5, and 6).
16. Dilute the virus recovered from reaction 3 (**Table 1**) 1/10-1/1000 and from reaction 4 (**Table 1**) 1/1000–1/100,000.
17. Solid overlay is made by melting 1.7% noble agar, then allowing it to cool to 42°C, and adding it to an equal volume of 37°C prewarmed 2X plaquing medium.
18. Recombinants typically comprise approx 50% (range 20–100%) of the *lacZ*-minus virus. Higher efficiencies can be obtained by increasing the length of homology from 20 to 50 bp.
19. We only do one round of plaque purifications, because the focus is high throughput. Once a potential recombinant is identified, it should be subjected to further rounds of purification.
20. Any suitable PCR reaction can also be used to detect recombinant virus (e.g., *see* Chapters 9 and 10). However, we prefer to use Southern blots because blots can more readily detect the unusual rearranged virus structures sometimes missed by PCR.
21. This is done to reduce the viscosity of the sample.

22. Occasionally, the recombination reactions do not precisely reconstruct the restriction sites that were built into the flanking the GFP integration sites (e.g., *Apa*I site; *see* **Fig. 2**). These sequence elements derived from the oligonucleotides used to PCR amplify the GFP or other amplicons, and we presume that they originate as primer synthesis errors. Such viruses produce anomalous bands on Southern blots.
23. The vaccinia virus 7.5K promoter (P7.5 or the 75K promoter ***[11]***) drives T7 RNA polymerase.

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6

Construction of cDNA Libraries in Vaccinia Virus

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Summary

Poxvirus expression vectors have gained widespread use for expression of foreign proteins and as delivery vehicles for vaccine antigens. We have developed a novel method using the poxvirus as a library vector for functional selection of specific cDNA. Poxviruses have several unique and useful properties as a library vector. Most importantly, because poxviruses are packaged into fully infectious particles in the cell cytoplasm, specific recombinants can be readily recovered even from a very small number of selected cells. Moreover, in contrast to libraries constructed in retrovirus or plasmid-based vectors, recombinant vaccinia virus can be efficiently recovered even from cells that have been induced to undergo apoptosis or cessation of cell growth. In the past, the major obstacle in this application to poxviruses has been the low frequency with which recombinants can be generated. The most commonly used method to construct recombinant poxvirus is homologous recombination. The frequency of recombinants derived in this manner is of the order of 0.1%, sufficient to recover a recombinant of a purified DNA clone in a transfer plasmid, but far too low to permit construction of a representative cDNA library. We have developed a method that generates nearly 100% recombinant vaccinia viruses at good titer. We have termed this method *trimolecular recombination*. cDNA libraries of as many as 10^7 or more independent viral recombinants can be constructed by trimolecular recombination. For the first time, large, diverse, and representative cDNA libraries can be screened in a vaccinia virus-based expression vector.

Key Words: cDNA library; fowlpox virus; trimolecular recombination; vaccinia virus.

1. Introduction

The ease of cloning and propagation in a variety of host cells has led to the extensive utilization of poxvirus vectors for foreign protein expression and as delivery vehicles for vaccine antigens ***(1)***. Generally, the target protein coding sequence is cloned under the control of a vaccinia promoter in a plasmid transfer vector. The promoter and insert are flanked by sequences homologous to a

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nonessential region in the poxvirus, often the thymidine kinase (*tk*) gene, so that the plasmid intermediate can be introduced into the viral genome by homologous recombination at that locus. The frequency of recombinants derived is of the order of 0.1%, enough to recover recombinants of a specific DNA clone, but not to permit construction of a large, representative cDNA library. Previously, we had attempted to generate diverse cDNA libraries in poxvirus employing a direct ligation method ***(2)***. Although this method did select for a higher frequency of recombinants, relatively low viral titers were generated. We describe here a new method to produce vaccinia virus recombinants at high frequency and good titer.

Poxvirus-based library vectors have several advantages relative to more common plasmid or retrovirus-based vectors. Poxvirus replicates and is packaged into fully infectious particles in the cell cytoplasm, and, as a result of its high infectivity, specific recombinants can be readily recovered even from very small numbers of selected cells, perhaps as few as a single cell. Additionally, unlike plasmid- or retrovirus-based vectors, recombinant genes in a poxvirus vector can be efficiently recovered even from cells that have ceased to divide or that have died as a result of expression of the selected recombinant gene. In the concluding section, we briefly describe examples of gene discovery applications we have developed using cDNA libraries constructed in vaccinia virus.

1.1. Trimolecular Recombination

The rationale for this strategy is that a high frequency of recombinants would be obtained if cells were transfected with defective vaccinia DNA that could be packaged into infectious particles only through recombination. One way to accomplish this is to cut the vaccinia DNA in the middle of the *tk* gene. Because there is no homology between the two *tk* gene fragments, the two vaccinia arms cannot be linked by homologous recombination except by bridging through the homologous tk sequences that flank the insert in a recombinant transfer plasmid. As naked vaccinia DNA is not itself infectious, production of infectious particles requires that transfection be carried out in cells infected with a helper virus. As previously described, fowlpox virus (FPV) does not productively infect mammalian cells, but does provide the necessary helper functions necessary for replication and packaging of mature wild-type or recombinant vaccinia virus particles ***(2–4)***.

A vaccinia virus vector, v7.5/tk, was constructed that incorporates the early/late 7.5k vaccinia promoter, as well as unique *Not*I and *Apa*I restriction sites downstream of the promoter into a recombinant vaccinia virus ***(2)***. Digestion of this virus with *Not*I and *Apa*I restriction endonucleases gives rise to two large fragments of approx 80 kb and approx 100 kb in size. Each of these arms include a nonoverlapping fragment of the *tk* gene for bridging by a transfer

plasmid. FPV-infected cells triply transfected with the two vaccinia arms and with DNA from a cDNA library constructed in a transfer plasmid leads to infectious vaccinia virus that is nearly 100% recombinant ***(5)***. This method is very reliable and has been used to construct vaccinia cDNA libraries from many different cell lines and normal tissue RNA.

2. Materials

1. Vaccinia virus DNA containing unique restriction sites in the tk gene (v7.5/tk; ***2***).
2. FPV strain HP1, provided to us by Dr. Bernard Moss (NIH, NIAID).
3. BSC1 cells: Maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), and glutamine.
4. Trypsin-EDTA (TE): 0.05% trypsin, 0.53 m*M* EDTA.
5. Hank's balanced salt solution (HBSS).
6. *Not*I and *Apa*I. (We generally use enzymes from Invitrogen, but other sources are acceptable.)
7. Lipofectamine and Opti-MEM I media (Invitrogen).
8. FPV infection media: M199 or DMEM base with 0.1% bovine serum albumin (BSA) and HEPES.
9. Polymerase chain reaction (PCR) machine and reagents.
10. QiaAMP DNA Blood Kit (Qiagen).
11. PCR primers MM428 (5'-GATATATTAAAGTCGAATAAAGTG) and MM430 (5'-GACATCACATAGTTTAGTTGC).

3. Methods

3.1. Transfer Plasmid

A cDNA library is constructed in a vaccinia transfer plasmid. Several vaccinia transfer plasmids have been described. We used a modified version of the p7.5/tk plasmid (**ref. *2*** and ES et al., unpublished data). Any transfer plasmid targeted to the vaccinia tk locus should work with this method. *See* **Note 1** for more information on preparation of the plasmid library.

3.2. Construction of cDNA Libraries in Vaccinia Virus

3.2.1. Cell and DNA Preparation

The first steps in this procedure are to seed the host cells for transfection and to prepare the v7.5/tk DNA arms by sequential digestion with *Apa*I and *Not*I.

1. Seed BSC1 cells 1:2 into 100-mm plates. Let cells adhere overnight. Cells should be 100% confluent by the next day (d 0). Generally two to four plates are transfected at one time.
2. Aliquot the required amount of vaccinia DNA from the stock tube into a 1.5-mL tube (*see* **Note 2**). Because these are large DNA fragments, *Use wide bore tips and never vortex vaccinia DNA*. Bring DNA to 1X concentration of Invitrogen

Table 1
Example of Initial *Apa*I Restriction Digest to Prepare DNA for Two Transfections

Reagent	Volume (μL)
Vaccinia DNA	12 (for 2 plates)
10X React 4 buffer (Invitrogen)	8
Molecular biology grade water	52
*Apa*I (10 U/μL)	8
Final	80

Table 2
Example of Second Restriction Digest with *Not*I to Prepare DNA for Two Transfections

Reagent	Volume (μL)
10X React 3 buffer (Invitrogen)	18
Molecular biology grade water	63
*Not*I (15 U/μL)	9
Final	90

React 4 Buffer (or buffer optimal for *Apa*I restriction digest). Add 1 μL *Apa*I enzyme for every 10 μL vol. **Table 1** provides an example digest of DNA for two 100-mm plates to transfect (with a stock of viral DNA at 0.5 μg/μL, use 6 μL vaccinia DNA/plate).

3. Incubate at 30°C for 3–4 h. Heat-inactivate at 65°C for 10 min, then place on ice.
4. In a fresh tube, prepare a mix that is 2X React 3 Buffer (Invitrogen) and contains 1 μL *Not*I for every 20 μL final reaction volume. **Table 2** provides an example calculation of such a reaction mix for one tube with 80 μL *Apa*I-digested vaccinia DNA. To the *Apa*I-digested vaccinia DNA, add a volume of this *Not*I mix that is equal to the volume of the *Apa*I reaction. That is, transfer 80 μL of the *Not*I mix outlined in **Table 2** into each tube of *Apa*I-digested DNA. (final volume = 160 μL).
5. Incubate at 37°C for 3–4 h. Heat-inactivate at 65°C for 10 min.
6. Store DNA at 4°C overnight.

3.2.2. Infection/Transfection

The vaccinia DNA arms are cotransfected along with recombinant transfer plasmid into FPV-infected BSC1 cells.

1. Infect BSC1 cells (prepared as described in **Subheading 3.2.1.**, **step 1**) with FPV at multiplicity of infection (moi) of 1.5 pfu FPV/cell (*see* **Note 3**). Transfer required amount of FPV to a 1.5-mL tube and add an equal volume of 1X TE.

Table 3
Preparation of Reagents for Lipofectamine-Mediated Transfection

Solution A	Solution B
800 μL Digested vaccinia DNA arms	48 μL Lipofectamine
12 μL Transfer plasmid library (50 ng/μL)	752 μL Opti-MEM I

Incubate in 37°C water bath for 10 min. Dilute virus into 6 mL FPV infection media (M199 or DMEM base with 0.1% BSA and HEPES) per plate. Aspirate media from BSC1 cells and add 6 mL FPV to each plate. Incubate at 37°C for approx 2 h (until ready to add transfection complexes).

2. Purify vaccinia DNA arms (prepared in **Subheading 3.2.1.**, **step 6**) with a Centricon 100 (C-100) column. Use two C-100 column/plate. Add 2 mL water to column. Remove 200 μL water from the column and add to the tube containing the digested vaccinia DNA (**Subheading 3.2.1.**, **step 6**). Add this water/DNA to the C-100 column. If one digestion reaction is going to be split into two C-100 columns, add 200 μL water from each column to the DNA, then add 200 μL diluted DNA to each C-100. Spin 15 min in an IEC Medispin or similar centrifuge. Invert tube and spin for 5 min. DNA volume should be approx 25–30 μL/column.
4. Bring vaccinia DNA to a volume of 800 μL/plate. Assume 25 μL DNA/C-100 column × 2 columns = 50 μL DNA. Add 750 μL Opti-MEM I media for one plate.
5. Prepare transfection mix of digested vaccinia DNA arms and approx 600 ng cDNA Library (in transfer plasmid) for transfection into FPV-infected BSC1 cells (**Subheading 3.2.2.**, **step 1**). As outlined in **Table 3**, in two 12 mm × 75 mm (4 mL) polypropylene tubes, mix the purified vaccinia DNA arms and approx 600 ng cDNA library (in transfer plasmid; solution A) and, in a second tube, mix 48 μL lipofectamine/plate in Opti-MEM I (solution B) following standard lipofectamine protocol (Invitrogen-BRL; *see* **Note 4**).
   a. Using wide bore tips, add solution B into solution A, mix gently.
   b. Incubate at room temperature for 15 min.
   c. Add an additional 2.4 mL Opti-MEM I to DNA/lipofectamine mixture.
6. Aspirate FPV infection inoculum from BSC1 cells (**Subheading 3.2.2.**, **step 1**). Wash 1X with 10 mL Opti-MEM I. Add 4 mL fresh Opti-MEM I to cells, followed by the DNA/lipofectamine mix (now at a volume of approx 4 mL) to cells. *Remember: Use wide bore tips and no vortexing!*
7. Incubate in 37°C CO_2 incubator for 4–5 h, then immediately proceed to the next section.

3.2.3. Plate Out Library

The transfected cells are harvested, mixed with uninfected filler cells, and plated into flasks. In addition, dilutions of each library are seeded into 96-well

plates to determine the efficiency of virus production. The cells are then incubated for 7 d to allow virus library production.

1. Four to five hours after transfection, harvest cells as follows:
   a. Aspirate off supernatant.
   b. Wash with 5 mL HBSS.
   c. Rinse with 1 mL TE.
   d. Add 1 mL fresh TE and incubate cells at 37°C until cells detach.
2. Increase to 50 mL final volume with DMEM 10% FBS (*see* **Note 5**).
3. Simultaneous with harvesting the FPV-infected/transfected cells, harvest fresh uninfected BSC1 cells from two 100-mm plates and put single-cell suspension in a final volume of 20 mL DMEM 10% FBS.
4. Use a portion of the infected/transfected cell suspension (**Subheading 3.2.3.**, **step 2**) to seed four 96-well plates for efficiency quality control as follows:
   a. Mix 1 mL infected/transfected cells (**Subheading 3.2.3.**, **step 2**) with 1 mL uninfected BSC1 cells (**Subheading 3.2.3.**, **step 3**). Increase to 20 mL vol with DMEM 10% FBS. Seed 200 µL into each well of a 96-well flat bottom plate.
   b. Mix 0.5 mL cells (**Subheading 3.2.3.**, **step 2**) with 1 mL uninfected BSC1 cells (**Subheading 3.2.3.**, **step 3**). Increase to 20 mL vol with DMEM 10% FBS. Seed 200 µL into each well of a 96-well flat bottom plate.
   c. Mix 0.25 mL cells (**Subheading 3.2.3.**, **step 2**) with 1 mL uninfected BSC1 cells (**Subheading 3.2.3.**, **step 3**). Increase to 20 mL vol with DMEM 10% FBS. Seed 200 µL into each well of a 96-well flat bottom plate.
   d. Mix 0.125 mL cells (**Subheading 3.2.3.**, **step 2**) with 1 mL uninfected BSC1 cells (**Subheading 3.2.3.**, **step 3**). Increase to 20 mL vol with DMEM 10% FBS. Seed 200 µL into each well of a 96-well flat bottom plate.
5. Add the remaining uninfected BSC1 cells from the two 100-mm plates (approx 16 mL) to the remaining infected/transfected cells (**Subheading 3.2.3.**, **step 2**) (approx 48 mL).
6. Increase volume to 210 mL final with DMEM 10% FBS.
7. Seed into seven T175s (30 mL/flask).
8. Incubate flasks and 96-well plates for 7 d at 37°C.

3.2.4. Harvest Library

1. Examine the 96-well plates from **Subheading 3.2.3.**, **step 4** to see how many wells contain virus. We usually examine 24–48 wells for each plate. The presence of one or more visible plaques indicates that a well contains the virus. Use the Poisson distribution to determine the number of independent virus-producing cells (*see* **Notes 6** and **7**). We have determined that under these conditions, most cells producing virus produce two to five different viral recombinants per cell (*see* **Note 7**). This should be determined for each library and batch of recombinants.

2. Harvest the cells from T175 flasks.
   a. Flasks should be covered with plaques.
   b. Harvest cells using TE as described in **Subheading 3.2.3.**, **step 1**. *Save all* supernatant, as some virus-containing cells will have detached by this point.
   c. Transfer cells and supernatant to 250-mL centrifuge bottle(s). Centrifuge at low speed (470*g*) for 5 min to pellet cells.
   d. Aspirate most of the supernatant, leaving approx 10 mL to resuspend pellet, and transfer to 15-mL centrifuge tube(s).
   e. Centrifuge again at low speed (470*g*) for 5 min.
   f. Aspirate supernatant. Resuspend cells in 3 mL DMEM 2.5% FBS.

3.2.5. Virus Library Titer and Quality Control

Follow standard procedures to titer and pick vaccinia plaques as described in Chapters 2 and 3 or **ref. *6***. Briefly:

1. Freeze–thaw the cell pellet, **Subheading 3.2.4.**, **step 2f**, in dry ice/isopropanol in a 37°C water bath three times.
2. Prepare 7–10-fold serial dilutions of viral stock starting at 10^{-2}.
3. Infect BSC1 in 12-well plates with 250 µL of each dilution in duplicate. Two days postinfection, stain with 0.1% crystal violet, which gives the titer of the library.
4. Analyze clones: infect in duplicate BSC1 cells in six-well plates with 500 µL of each dilution (**Subheading 3.2.5.**, **step 2**) and select for plaque analysis dilutions that yield 10–50 plaques/well (generally, dilution of 10^{-7} to 10^{-5}).
   a. Follow standard procedure to pick vaccinia plaques. Generally, 10 plaques are picked for each library.
   b. Transfer clones to 1.5-mL tubes containing 200 µL PBS. Mix briefly by vortexing.
   c. Purify DNA from 200 µL of each plaque using the QiaAMP blood DNA purification kit (Qiagen) following the manufacturer's recommendation.
   d. Use 5 µL purified DNA as template for a PCR reaction using vaccinia-specific primers that flank the insertion site in the *tk* gene. We use primers MM428 (5'-GATATATTAAAGTCGAATAAAGTG) and MM430 (5'-GAC ATCACATAGTTTAGTTGC). When used with empty v7.5/tk as the template, these primers will give a 310-bp PCR product.
5. Resolve PCR products on an agarose gel. PCR products should give a range of insert sizes comparable to the inserts in the plasmid library. There should be a minimum of less than 10% of clones that do not contain an insert.

3.3. Example Applications (See Note 8)

3.3.1. Selection of Fully Human Antibodies

We have constructed libraries of human immunoglobulin heavy- and light-chain genes in vaccinia virus from which fully human antibodies with a desired

antigen specificity have been selected. Combinatorial association of the separate heavy- and light-chain gene libraries generates a very large number of different antibodies. The ability to select fully functional bivalent antibodies from large random libraries in vitro overcomes some known limitations of antibody selection in normal or immunoglobulin transgenic mice. Most importantly, this strategy avoids limitations in the mouse antibody repertoire due to immune tolerance to highly conserved murine homologs of significant human proteins. Relative to phage display libraries, antibody expression in mammalian cells, as opposed to bacteria, facilitates folding and assembly of functional antibodies. This expression enhances the functional titer of the library, as well as the affinity of selected antibodies. It may also lead to the selection of antibodies better adapted to downstream manufacturing in mammalian production systems. This application is proprietary to Vaccinex and is described in a US patent application ***(7)***. A related application to select antibodies active in the cytoplasm of eukaryotic cells, so-called *intrabodies*, is described in another US patent ***(8)***.

3.3.2. Target Antigens of Cytotoxic T Cells

In addition to the increased efficiency with which low-frequency recombinants can be selected, an important advantage of the vaccinia virus library vector is that, unlike plasmid- or retrovirus-based vectors, recombinant genes in this vector can be efficiently recovered even from cells that have ceased to divide or that have died as a result of expression of the targeted gene. We have exploited this feature of the vector to select tumor genes encoding the target antigens of cytotoxic killer cells ***(5)***. This and related applications are more fully described in a US patent application ***(9)***.

3.3.3. Selection of Upstream Regulators of a Gene of Interest

The vaccinia virus library vector is a versatile tool also employed for functional selection of upstream genes that regulate a defined gene product. Strategies for selection of upstream regulators of a gene of interest at either the level of RNA transcription or protein expression have been devised. Assays employed in these strategies are dependent on specific biochemical or biological properties of the gene of interest. Generally, a library is constructed from specialized cells expressing high levels of the gene of interest and presumably all of its required regulators. This library is screened by infecting indicator cells that include, but fail to express, high levels of either the gene of interest or a reporter construct regulated by the isolated promoter of the gene of interest. Identifying an appropriate indicator cell is the most challenging part of this strategy. It is necessary to establish that the gene of interest, either naturally present or introduced by DNA transfection, can be expressed in the indicator

cells under some appropriate, but ill-defined conditions. In some cases, this follows from knowledge of the biology of the system.

Alternatively, it might be established by fusion of a high-expressor cell with an indicator cell carrying a marked version of the gene of interest. At the very least, we require that multiple independent transfectants of the indicator cell fail to express the gene of interest, whereas a marked version of the gene of interest transfected into high-expressor cells is expressed at high levels. This application is described more fully in US patent applications ***(10,11)***.

4. Notes

1. cDNA Library construction. The cDNA libraries are constructed in the plasmid transfer vector following standard molecular biology techniques. Following ligation, the library is transformed into bacteria and plated onto agar plates containing antibiotic. The library is allowed to grow overnight. On the next day, bacteria are harvested by scraping the plates with a cell scraper. Plasmid DNA is then extracted and used to make the vaccinia library.
2. Optimizing amount of vaccinia DNA to use. Vaccinia virus DNA is obtained from purified virus. More than enough DNA can be obtained from 2 L of infected HELA cells (1×10^6/mL) following the procedures outlined in Chapters 8 and 10 or **ref. *6***. Vaccinia DNA is then isolated from half of this purified virus. This vaccinia DNA is resuspended in a 200 µL final volume. The concentration of DNA should be approx 0.5 µg/µL. The quality of this DNA is verified by digesting 1 µL DNA with *Hin*dIII and resolving the fragments on an agarose gel. Distinct vaccinia DNA bands with a minimal smear of contaminating genomic DNA should be observed. We usually try using 6 µL (approx 3 µg) of this DNA to transfect one 100-mm plate of cells. If the DNA precipitates out of the solution when lipofectamine is added, then too much DNA was used. Repeat using 4 µL. If, on the other hand, the DNA concentration is low, try repeating with 8 µL DNA.
3. Preparation of FPV for infection. Thaw FPV stored at –80°C in a water bath. Example calculation for infecting at an moi 1.5 pfu/cell: Assuming 6×10^6 cells/plate × moi (1.5 pfu/cell) = 9×10^6 pfu/plate. Thus, if the stock of FPV = 1×10^8 pfu/mL, one needs 90 µL FPV stock/plate. Take 90 µL FPV, add 90 µL 1X TE, incubate at 37°C for 10 min, then dilute virus into 6 mL FPV infection media.
4. Choice of transfection reagents. There is a wide variety of transfection reagents available. Although we have not done an exhaustive analysis of these reagents, we have tested a number of them including: Lipofectamine (Invitrogen), Lipofectamine Plus (Invitrogen), Lipofectamine 2000 (Invitrogen), Superfect (Stratagene), Calcium Phosphate (Clontech), FuGENE 6 (Roche), and Gene Juice (Novagen). In our hands, Lipofectamine gives the best results.
5. Harvesting cells. It is important that the harvested infected (or uninfected) cells into a single-cell suspension. Thus, after trypsinization, it is often best to first add a small volume of media to the cells. After vigorous pipetting of this small vol-

Table 4
Sample Data for Poisson Distribution

No. of cells/well	No. of wells positive	F_0	μ	Frequency	Total no. of producing cells
1200 (= 1 mL)	28/36	0.22	1.5	1/797	7528
600 (= 0.5 mL)	29/36	0.20	1.6	1/372	16,000
300 (= 0.25 mL)	16/36	0.56	0.59	1/500	12,000
150 (= 0.125 mL)	9/36	0.75	0.29	1/521	11,500

Number of cells/well = the number of cells seeded into each well. That is, 6×10^6 cells diluted into 50 mL = 120,000 cells/mL. One milliliter is seeded into approx 100 wells which equals 1200 cells/well. Number of wells positive = the number of wells where plaques are visible. F_0 = Fraction of negative wells (1 – fraction of positive wells). For example, 16/36 wells are positive = 0.44. 1–0.44 = 0.56. μ = precursor frequency = -ln F_0. Frequency = number of cells/well/μ. Total number of producing cells = total number of transfected cells (example: 6×10^6 cells)/frequency. Total number of vaccinia clones produced = total number of producing cells × number of vaccinia clones produced/producing cell (*see* **Notes 6** and **7**).

ume (to break up cell clumps), more media can be added to bring the volume up to the recommended amount. Also, prewarmed media can also prevent cell clumping.

6. Poisson distribution. The Poisson distribution is used to calculate the diversity of the vaccinia libraries that are made. **Table 4** provides example data. From pool analysis (**Note 7**), we have observed that cells producing virus seem to produce multiple different viruses (two to five different vaccinia recombinants per producing cell). Therefore, if, in this example, we calculate that we have 12,000 cells producing virus and we have determined that each producing cell produces an average of three different vaccinia recombinants, then 12,000 × 3 = 36,000 vaccinia recombinants.
7. Pool analysis. We have observed that many wells that produce virus also produce numerous (two to five) different viruses, which was determined by harvesting wells from dilutions that gave less than 30% of a 96-well plate producing virus. DNA was extracted from these wells and analyzed by PCR using vector-specific primers (for empty v7.5/tk as the template, primers. MM428 (5'-GATATA TTAAAGTCGAATAAAGTG) and MM430 (5'-GACATCACATAGTTTAGTTGC) will give a 310-bp PCR product. This should be determined for each cDNA library that is constructed.
8. Availability of licenses for academic or commercial research. A license for commercial use of the protected technology by either academic institutions or more directly commercial entities is available in all fields of use except selection of human antibodies. Applications should be directed to Mr. Stan Holland, Vaccinex, Inc., 1895 Mt. Hope Avenue, Rochester, NY 14620.

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7

Construction and Isolation of Recombinant MVA

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Summary

Modified vaccinia virus Ankara (MVA) is a valuable tool for the expression of recombinant genes used for such purposes as the study of protein functions or characterization of cellular and humoral immune responses. A major advantage of MVA is its clear safety record, and it can be handled under biosafety level 1 conditions. Despite its replication deficiency in human and most mammalian cells, MVA provides high-level gene expression and has proven to be immunogenic when delivering heterologous antigens in animals and humans. This chapter provides state-of-the-art protocols for generation, plaque isolation, molecular characterization, as well as amplification and purification of MVA vector viruses to obtain recombinant viruses for further evaluation.

Key Words: Poxvirus; modified vaccinia virus Ankara (MVA); recombinant vaccine.

1. Introduction

Recombinant vaccinia viruses are very valuable research tools used in a broad range of applications in various fields, including cell biology, protein sciences, virology, immunology, or medicine. One appealing feature of the poxvirus double-stranded DNA genome is its capacity to accommodate large amounts of foreign DNA. Owing to their unique replication cycle, vaccinia vectors allow for high-level gene expression in the cytoplasm of mammalian cells. They are used to investigate protein functions, construct cDNA libraries, determine potential antigens for humoral- or cell-mediated immunity, and are being pursued as recombinant vaccines against infectious diseases and cancer ***(1–8)***. However, vaccinia virus can replicate in humans and has to be handled under biosafety level 2 laboratory conditions, and its imperfect safety record as a smallpox vaccine has been a concern for its use as a vector in clinical

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applications. Therefore, highly attenuated vaccinia viruses, such as modified vaccinia virus Ankara (MVA) or NYVAC, have been generated ***(9,10)***. These viruses share replication deficiency in human and most mammalian cells, allowing for handling under biosafety 1 conditions, but they have maintained the advantage of high-level gene expression. MVA has already demonstrated an excellent clinical safety record. It was used without any apparent incident as a smallpox vaccine in more than 120,000 humans, many of whom were considered a poor risk for the conventional smallpox vaccine ***(11,12)***. MVA vector vaccines induce significant levels of humoral and cellular immune responses to vaccine antigens and were found to be less affected by preexisting vaccinia virus-specific immunity when compared to replication-competent vaccinia virus vectors ***(13)***. Moreover, high doses of recombinant MVA vaccine are well-tolerated in immune-suppressed macaques ***(14)***, suggesting the safety of such vaccines even in potentially immunocompromised individuals.

Here, we describe an up-to-date methodology for the generation and characterization of recombinant MVA. The protocol includes different selection techniques for the isolation of cloned viruses and optimized procedures for virus amplification and titration.

2. Materials

2.1. Virus Strain

Vaccinia virus strain MVA (cloned isolate F6 ***[9]*** or II_{new} (***15***; *see* **Note 1**).

2.2. Cell Culture

1. Rabbit kidney (RK-13) cells (ATCC, CCL-37).
2. Baby hamster kidney (BHK-21) cells (ATCC, CCL-10).
3. HeLa cells (ATCC, CCL-2).
4. Primary chicken embryo fibroblasts (CEF), freshly prepared.
5. Cell growth medium: RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), 100 µg/mL streptomycin, 100 IU/mL penicillin, and 25 µg/mL amphothericin B (1% AB/AM).
6. Heat-inactivated fetal calf serum (FCS; Seromed, Biochrome KG, Berlin, Germany).
7. Antibiotics and antimycotics, such as streptomycin, penicillin, and amphothericin B (AB/AM) (Gibco BRL, Grand Island, NY).
8. Six-well tissue culture plates and T85 and T185 tissue culture flasks (Costar, Corning NY).

2.2.1. Preparation of Primary CEF

1. Ten 11-d-old eggs.
2. CEF growth medium: RPMI 1640 supplemented with 10% FCS and 1% AB/AM.
3. Trypsin: 1X trypsin-EDTA (Gibco BRL, Grand Island, NY).

4. Absolute ethanol (EtOH abs; laboratory-use grade, Merck KGaA, Darmstadt, Germany).
5. 50-mL Falcon tubes.
6. Sterile Petri dishes: 10-cm diameter (Costar, Corning, NY).
7. T185 tissue culture flasks.
8. Sterile instruments for dissection, e.g., scissors and forceps.
9. 10-mL syringes.
10. One sterile Erlenmeyer flask, two sterile beakers covered with two layers of gauze (wrapped in autoclave tape).

2.3. Virus Growth

2.3.1. Amplification of MVA

1. Confluent monolayers of CEF or BHK-21 cells grown on 60-mm^2 dishes and T85 and T185 tissue culture flasks.
2. Virus growth medium: RPMI supplemented with 2% FCS and 1% AB/AM.
3. Cell scraper.
4. 10 m*M* Tris-HCl, pH 9.0, autoclaved. Store at 4°C.

2.3.2. Purification of MVA

1. Cell scraper.
2. Cup sonicator and/or sonication needle (Sonopuls HD 200, Bandelin, Germany).
3. Dounce homogenizer: glass, tight-fitting, autoclaved.
4. 10 m*M* Tris-HCl, pH 9.0, autoclaved. Store at 4°C.
5. 1 m*M* Tris-HCl, pH 9.0, autoclaved. Store at 4°C.
6. 36% Sucrose (w/v) in 10 m*M* Tris-HCl, pH 9.0, sterile-filtered. Store at 4°C.

2.3.3. Titration of MVA Determining Amount of Infectious Units per mL (IU/mL)

1. Subconfluent monolayers of CEF or BHK-21 cells grown on six-well tissue culture plates (for growth conditions, *see* **Subheading 3.1.**).
2. Fixing solution: 1:1 mixture of acetone:methanol (Merck KGaA, Darmstadt, Germany, laboratory use grade). Store at 4°C.
3. Phosphate-buffered saline (PBS), pH 7.5.
4. Blocking buffer: PBS, pH 7.5, and 2% bovine serum albumin (BSA).
5. Primary antibody (1st Ab): polyclonal rabbit anti-vaccinia antibody (IgG fraction, Biogenesis Ltd, Poole, England, cat. no. 9503-2057) diluted 1:500–1:1000 in blocking buffer.
6. Secondary antibody (2nd Ab): horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (IgG [H+L]; Dianova, Hamburg, Germany, cat. no. 111-035-114) diluted 1:1000 in blocking buffer.
7. o-Dianisidine (Sigma, Deisenhofen, Germany); toxic.
8. Absolute EtOH.
9. Hydrogen superoxide >30% (H_2O_2 >30%; Sigma, Deisenhofen, Germany).

2.3.4. Titration of MVA Determining the Tissue Culture Infectious Dose 50 ($TCID_{50}$)

1. Subconfluent monolayers of CEF or BHK-21 cells grown on 96-well tissue culture plates (for growth conditions, *see* **Subheading 3.1.**).
2. For all additional materials, *see* **Subheading 2.3.3.**

2.4. Generation of Recombinant MVA

2.4.1. Molecular Cloning of Recombinant Gene Sequences

1. MVA targeting vector plasmids.
2. Restriction endonucleases.
3. DNA modifying enzymes, e.g., Klenow DNA polymerase, T4 DNA ligase.

2.4.2. Transfection of MVA-Infected Cells with Vector Plasmids

1. Subconfluent monolayers CEF or BHK-21 on six-well plates.
2. Recombinant MVA transfer vector plasmid, e.g., pIII dHR P7.5-target.
3. Serum-free RPMI: RPMI 1640 supplemented with 1% AB/AM. Store at 4°C.
4. FUGENE™ (Roche, Mannheim, Germany). Store at –20°C.

2.5. Isolation of Recombinant MVA (All Procedures)

1. Cup sonicator.
2. RPMI 1640 medium supplemented with 5% FCS and 1% AB/AM. Store at 4°C.
3. Subconfluent monolayers of RK-13 cells, primary CEF or BHK-21 grown on six-well plates in RPMI 1640 medium.
4. 1.5-mL Eppendorf vials.

2.5.1. Isolation of rMVA Screening for Transient β-Galactosidase Expression

1. X-gal solution: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Roche, Mannheim, Germany) 4% in dimethylformamide (DMFA; Sigma, Deisenhofen, Germany). Store at –20°C, light-sensitive, toxic.
2. 2X Plaquing medium: 2X RPMI 1640 supplemented with 4% FCS, 2% AB/AM. Store at 4°C.
3. 2% Low-melting-point agarose (LMP agarose; Life Technologies) in distilled water. Store at room temperature (RT).

2.5.2. Isolation of rMVA by Live Immune Detection of the Target Antigen

2.5.2.1. Concanavalin-A Coated Plates for Live Immunostaining

1. Concanavalin-A (Con-A; Sigma, Deisenhofen, Germany, cat. no. C-2010).
2. 10% Con-A in PBS (Con-A/PBS).

2.5.2.2. Immunostaining

See **Subheading 2.3.3.** with the following variations:

1. Confluent monolayers of CEF or BHK-21 cells on six-well tissue culture plates (for growth conditions *see* **Subheading 3.1.**).

2. 1st Ab: directed against target protein and diluted in blocking buffer.
3. 2nd Ab: horseradish peroxidase-conjugated antibody binding to 1st Ab and diluted in blocking buffer.

2.6. Characterization of Recombinant MVA Genomes by Polymerase Chain Reaction (PCR)

1. Confluent RK-13, BHK-21, or CEF cells grown on 6- or 12-well culture plates.
2. 10X TEN buffer, pH 7.4: 100 m*M* Tris-HCl, 10 m*M* EDTA, 1 *M* NaCl.
3. DNA grade proteinase K prepared as 1 mg/mL stock solution in 1 m*M* $CaCl_2$ (prot K; AGS GmbH, Heidelberg, Germany). Store at –20°C.
4. 20% Sodium dodecyl sulfate (SDS) in distilled water. DNase free; sterile-filtered.
5. Phenol-chloroform 1:1 mixture (Applied Biosystems, Foster City, CA). Store at 4°C.
6. 3 *M* Sodium acetate (NaAc) in distilled water (Merck KGaA, Darmstadt, Germany).
7. Absolute ethanol (EtOH abs) (Merck KGaA, Darmstadt, Germany).
8. 70% EtOH in distilled water.
9. Primer 1 (MVA-III-5') and primer 2 (MVA-III-3') dissolved in sterile distilled water to a final concentration of 5 pmol/µL, store at –20°C: MVA-III-5' (primer 1): 5'-GAATGCACATACATAAGTACCGGCATCTCTAGCAGT-3', MVA-III-3' (primer 2): 5'-CACCAGCGTCTACATGACGAGCTTCCGAGTTCC-3'.
10. Template DNA: viral genomic DNA prepared (as described in **Subheading 3.5.**) and plasmid DNA diluted to a final concentration of 100 ng/µL.
11. PCR master kit (Roche, Mannheim, Germany). Store at –20°C.

2.7. Growth of Virus Stocks from Single-Plaque Isolates

1. Confluent CEF or BHK-21 cells grown on 35-mm and 60-mm Petri dishes or in T85 and T185 tissue culture flasks.
2. Cell scraper.

2.8. Replication Deficiency Assay

1. Confluent HeLa and BHK or CEF cells, grown on six-well culture plates.
2. Cell scraper.

For additional materials, *see* **Subheading 2.3.3.**

3. Methods

3.1. Cell Culture

RK-13, BHK-21, and CEF cells are grown on six-well plates or T85 or T185 flasks in cell growth medium at 37°C and 5% CO_2.

3.1.1. Preparation of CEF (see *Notes 2* and *3*)

1. Prewarm 200 mL trypsin in a 37°C water bath.
2. Place eggs with blunt ends facing up to position the air space upward.
3. Wipe eggs with ethanol, crack eggshell with scissors, and cut it off, taking care not to damage the membrane.

4. Remove membrane and elevate the legs with forceps. Using a second pair of forceps to help support the embryo, transfer it into a Petri dish containing 10 mL serum-free RPMI.
5. Cut off the wings, legs, and head. Remove organs by holding the embryo with one set of forceps and scraping the belly with a second set of forceps.
6. Transfer remaining torso of the embryo to a second Petri dish containing 10 mL serum-free RPMI.
7. Homogenize the tissue by pressing five embryos at a time through a 10-mL syringe (*without* an attached needle) into an Erlenmeyer flask.
8. Add 100 mL trypsin (prewarmed to 37°C) and trypsinize the tissue by stirring for 10 min at RT using a magnetic stirrer.
9. Place cell suspension through gauze into a beaker, taking care not to transfer remaining embryo clumps.
10. Place remaining clumps in Erlenmeyer flask with 100 mL trypsin (prewarmed to 37°C). Stir for 10 min at RT.
11. Pass this trypsinized material through the gauze of a second beaker and pool filtrates in a 250-mL centrifuge bottle.
12. Spin for 10 min at 1800*g* and 4°C.
13. Discard supernatant and resuspend pellet by pipetting vigorously 10–15 times in 10 mL CEF growth medium, then add an additional 90 mL CEF growth medium to the cells.
14. Repellet cells by centrifuging for 10 min at 1800*g*.
15. Prepare 20 T185 culture flasks containing 40 mL CEF growth medium in each.
16. Resuspend cell pellet in 5 mL CEF growth medium, pipetting 10–15 times, then transfer to a 50-mL Falcon tube and fill to a final volume of 20 mL.
17. Add 1 mL cell suspension to each T185 flask.
18. Incubate for 3–4 d at 37°C until monolayers are confluent.
19. Split at ratio of 1:4 for six-well plates (2 mL/plate), 12-well plates (1 mL/plate), or T185 flasks.

3.2. Virus Growth (see Note 4)

3.2.1. Amplification of MVA

This protocol can be used either to amplify parental MVA or to grow stocks of recombinant MVA (rMVA). If titered MVA starting material is available for amplification, use a multiplicity of infection (moi) of 0.1 to 1 IU/cell for all infections. Either CEF or BHK-21 cells can be used for virus amplification, although growth in chicken cells yields higher virus output. Remove growth medium from cells before adding virus material to allow for efficient infection of cell monolayers (*see* **Notes 5** to **8**).

1. Infect cell monolayers of 10–40 T185 tissue culture flasks by inoculating each flask with 5 mL virus suspension (0.1–1 IU/cell).
2. Allow virus adsorption for 1 h at 37°C.

3. Gently rock flasks in 20-min intervals (*see* **Note 5**).
4. Add 30 mL virus growth medium per flask.
5. Incubate at 37°C for 2 d or until cytopathic effect (CPE) is obvious.
6. Remove approx 25 mL medium from each flask.
7. Scrape cells into remaining medium, and transfer to 50-mL centrifuge tubes.
8. Centrifuge 10 min at 1800*g* and 4°C.
9. Discard medium, resuspend, and combine cell pellets in 10 m*M* Tris. Use approx 1 mL per T185 culture flask.
10. Freeze-thaw virus material 3×, e.g., on dry ice and in a 37°C water bath. Vortex after each thaw.
11. Homogenize the material using a cup sonicator (*see* **Note 6**). Fill cup sonicator with ice-water (50% ice), place tube containing virus material in ice-water, and sonicate at maximal power for 1 min. Repeat 3×, taking care to avoid heating the sample by replenishing the ice in the cup. Store virus material at –80°C as crude material (*see* **Notes 7–9**) or until further purification (*see* **Subheading 3.2.2.**).

3.2.2. Purification of MVA

Crude stock preparations of MVA can be semipurified from cell debris and recombinant proteins by ultracentrifugation through a sucrose cushion.

1. Transfer virus material to a dounce homogenizer and dounce 5× on ice. Repeat for a total of five sets. Allow suspension to cool in between sets of strokes (*see* **Note 10**).
2. Transfer virus suspension to a 50-mL Falcon tube and centrifuge 5 min at 1800*g* and 4°C.
3. Collect supernatant in a 50-mL Falcon tube, resuspend pellet in 10 mL of 10 m*M* Tris, and repeat **steps 1–3**. Pool supernatants.
4. Prepare sterile 36% sucrose cushions by filling half a volume of an ultracentrifuge tube (e.g., SW 28) with sucrose. Overlay with equal volume of virus suspension.
5. Spin 60 min at 30,000*g* and 4°C.
6. Discard supernatant (cell debris and sucrose) and resuspend pelleted virus material in 1 m*M* Tris. Use approx 1 mL/10 cell culture flasks (*see* **Note 11**).
7. Store at –80°C.

3.2.3. Titration of MVA Determining Amount of IU/mL

To determine the infective titer of MVA stock preparations, foci of MVA-infected cells are visualized by specific immune-peroxidase staining of cells containing vaccinia viral antigens (*see* **Note 12**).

1. Thaw virus material and homogenize in cup sonicator as described in **Subheading 3.2.1.**, **step 11** (*see* **Note 13**).
2. Make 10-fold serial dilutions (ranging from 10^{-1} to 10^{-9}) of virus material in 3 mL RPMI/2% FCS/1% AB/AM.
3. Plate on CEF or BHK-21 six wells 1 mL virus suspension per well in duplicates; use 10^{-4} to 10^{-9} dilutions.

4. Incubate for 48 h at 37°C.
5. Remove medium from infected tissue culture plates. Fix and permeabilize cells with 1 mL fixing solution (1:1 mixture of acetone:methanol) per well for 2 min at RT.
6. Remove fixing solution and air-dry fixed monolayers.
7. Add 1 mL blocking buffer per well and incubate for 10 min (*see* **Note 14**).
8. Add 1 mL 1st Ab at 1:500–1:1000 in blocking buffer per well and incubate for 1 h at RT on a rocking device.
9. Remove 1st Ab and wash 3× with 1 mL PBS per well for 5 min each.
10. Add 1 mL 2nd antibody solution (2nd Ab at 1:1000 in blocking buffer per well) and incubate for 45–60 min at RT) on a rocking device.
11. Remove 2nd Ab and wash 3× with 1 mL PBS per well for 5 min each.
12. Prepare substrate solution in two steps as follows (recipe is enough for two six-well plates): make saturated dianisidine solution by adding a small amount of dianisidine (an amount that fills the tip of a small spatula) to 700 µL absolute EtOH. Mix by vortexing for 2 min. Centrifuge for 30 s at top speed at RT. Using supernatant only, prepare final substrate solution for peroxidase staining by adding 240 µL supernatant from the saturated dianisidine solution to 12 mL PBS in a 15-mL conical centrifuge tube. Mix by vortexing (*see* **Note 15**). Add 12 µL H_2O_2 (>30%), gently mix again, and use immediately.
13. Add 1 mL substrate solution per well and leave 15–30 min to clearly visualize stained viral foci.
14. To determine the titer, count stained foci in a suitable dilution. Count in both wells of the dilution and calculate the mean. To express titer as IU/mL, multiply the counted number of foci by the dilution. Wells with 20–100 viral foci generate the most accurate results.

3.2.4. Titration of MVA Determining the $TCID_{50}$

To titrate the infectivity of MVA stock preparations, foci of MVA-infected cells are visualized by specific immune-peroxidase staining of cells containing vaccinia viral antigen (*see* **Note 12**).

1. After thawing, homogenize MVA stock virus preparation by sonication as described in **Subheading 3.2.1.**, **step 11** (*see* **Note 13**).
2. Make 10-fold serial dilutions (ranging from 10^{-1} to 10^{-11}) of virus material in 1 mL RPMI/2% FCS/1% AB/AM.
3. Using 800 µL of each dilution, add 100 µL of each dilution in replicates of eight to subconfluent cell monolayers grown in 96-well plates using a multipipet and incubate at 37°C for 48 h.
4. Remove medium from infected tissue culture plates. Fix and permeabilize cells with 200 µL fixing solution per well for 2 min at RT.
5. Add 100 µL 1st Ab at 1:500–1:1000 in blocking buffer per well and incubate for 1 h at RT on a rocking device.

6. Remove 1st Ab and wash 3× with 200 µL PBS per well. For each washing step, allow to incubate with PBS for 10 min. Add 100 µL 2nd Ab solution at 1:1000 in blocking buffer per well and incubate for 45–60 min at RT on a rocking device.
7. Remove 2nd Ab and wash 3× with 200 µL PBS per well.
8. Prepare substrate solution as described in **Subheading 3.2.3.**, **step 12**.
9. Add 100 µL substrate solution per well and leave for 15–30 min to clearly see stained viral foci.
10. Monitor 96-well plate under a microscope and count all wells positive in which viral foci can be detected. Calculate titer according to the method of Kaerber ***(16)*** by determining the endpoint dilution that will infect 50% of the wells inoculated calculating in the following way (*see* **Note 16** for example of calculations):

$$\log_{10} 50\% \text{ endpoint dilution} = x - d/2 + (d\Sigma\, r/n)$$

x = highest dilution in which all eight wells (8/8) are counted positive
d = the $\log_{10}$ of the dilution factor ($d = 1$ when serial 10-fold dilutions are used)
r = number of positive wells per dilution
n = total number of wells per dilution ($n = 8$ when dilutions are plated out in replicates of eight)

3.3. Generation of Recombinant MVA (see *Fig. 1)*

3.3.1. Molecular Cloning of Recombinant Gene Sequences

Subclone recombinant genes into MVA plasmid vectors, such as those listed in **Table 1**. DNA fragments containing the coding sequence of the gene of interest, including authentic start (ATG) and stop (TAA/TAG/TGA) codons, are cloned into the multiple cloning site of the respective vector to generate the MVA transfer vector plasmid. The correct orientation of the recombinant gene is determined by the direction of promoter-specific transcription. Prepare stocks of transfer plasmid DNA (*see* **Note 17**) for generation of recombinant MVA (**Subheading 3.3.2.**).

3.3.2. Transfection of MVA-Infected Cells with Vector Plasmids

Recombinant plasmids are transfected into MVA-infected cells and homologous recombination between MVA and plasmid DNA generates a recombinant virus.

1. Grow CEF or BHK-21 cell monolayers to 80% confluence in six-well tissue culture plates. Use one well per transfection.
2. Discard medium and overlay cells with RPMI/2% FCS/1% AB/AM containing MVA at an moi of 0.01 (e.g., an inoculum of 1×10^4 IU MVA in 1 mL medium for one well with 1×10^6 cells). Incubate for 90 min at 37°C.
3. At 15–30 min postinfection start preparing FUGENE/plasmid DNA mix as described by the manufacturer in serum-free medium using 1.5 µg plasmid DNA.
4. Add FUGENE/plasmid DNA mix directly to the medium. Incubate for 48 h at 37°C.
5. Harvest cell monolayer with a cell scraper and transfer cells and medium into 1.5-mL microcentrifuge tubes. Store transfection harvest at –80°C to –20°C.

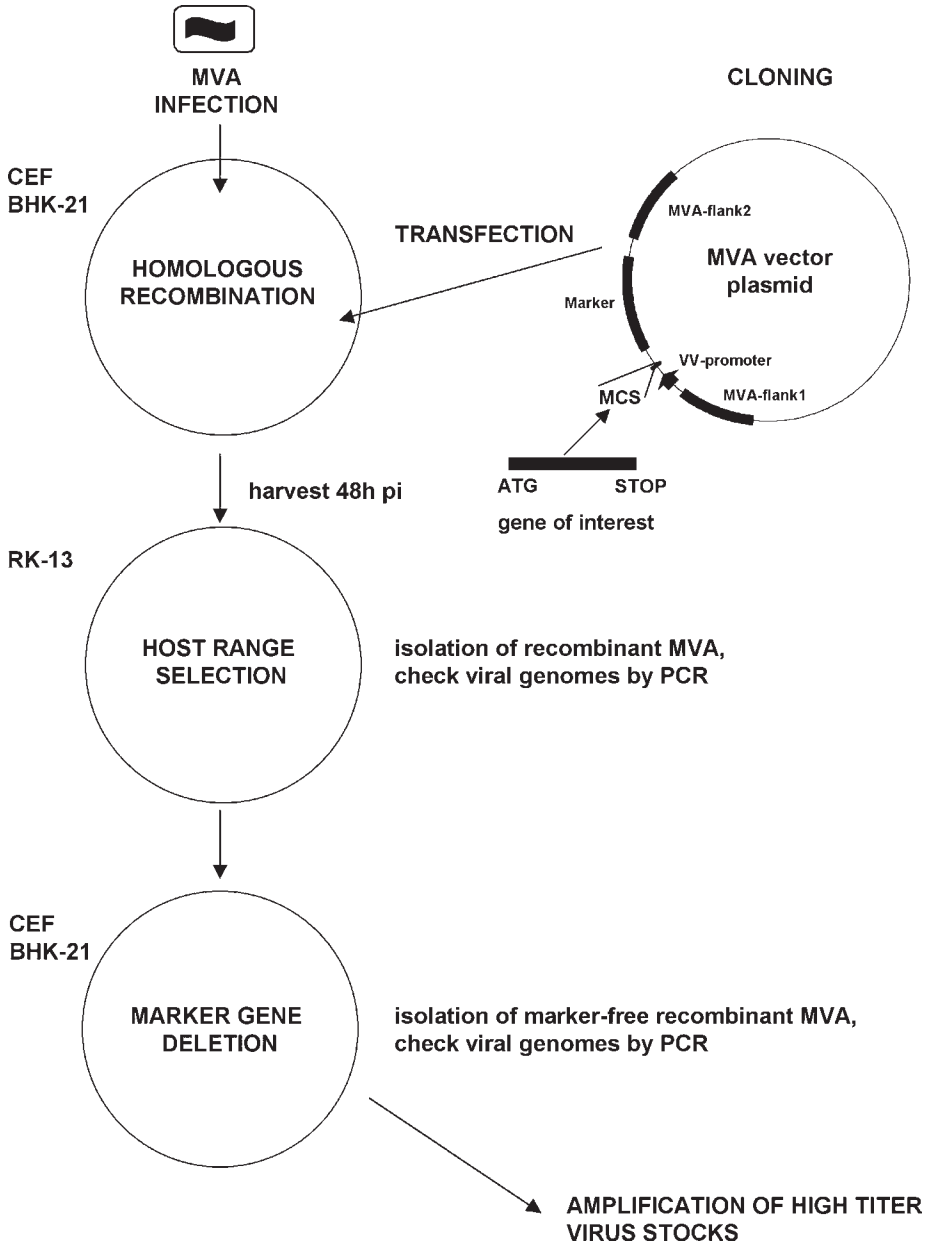

Fig. 1. Flow chart for the generation of recombinant MVA.

Table 1
MVA Transfer Vectors

Vector	Insertion site	Screening/selection	Reference
pIIIdHR-P7.5, pIIIdHR-sP	Del III	Transient K1L	***(15,17)***
pIIIdHR-PH5	Del III	Transient K1L	Unpublished
PVIdHR-PH5	Del VI	Transient K1L	Unpublished
pIILZ-P7.5	Del II	Stable β-galactosidase	***18***
pIILZdel-P7.5	Del II	Transient β-galactosidase	***20***
pLW7 (sP), pLW9 (PH5)	Del III	Immunostaining	***19***
pMC03 (sP)	Del III	β-glucuronidase	***21***
pIIIgptex-dsP	Del III	Immunostaining	***1***
pIIIgpt-dsP	Del III	Stable gpt	***1***

3.4. Isolation of rMVA (see *Note 18)*

3.4.1. Transient Host Range Selection

This technique relies on the inability of wild-type MVA to grow on RK-13 cells, whereas a recombinant expressing K1L can grow (**Fig. 1**). Vector plasmids contain the vaccinia virus *K1L* gene (along with the cloned foreign gene of interest) flanked by segments of MVA-DNA that direct integration into the viral genome precisely at the site of a naturally disrupted MVA gene sequence (e.g., deletion III; ***9***). rMVA expressing the recombinant antigen and expressing *K1L* coding sequences are isolated by consecutive rounds of plaque purification in RK-13 cell monolayers, selecting aggregates of infected RK-13 cells (*see* **Fig. 2**). The K1L expression cassette is designed to contain repetitive DNA sequences that allow for its deletion from the recombinant MVA genome when the virus is further plaque-purified under nonselective growth conditions (i.e., by additional rounds of plaque purification on CEF or BHK-21 cells).

1. Freeze–thaw transfection harvest 3× and homogenize in a cup sonicator similar to described in **Subheading 3.2.1.**, **step 11**.
2. Make four 10-fold serial dilutions (10^{-1} to 10^{-4}) of the virus suspension in virus growth medium by diluting 200 µL of the transfection harvest (or plaque pick) in 1.8 mL virus growth medium.
3. Remove growth medium from subconfluent RK-13 cell monolayers grown in six-well plates and infect with 1 mL diluted virus suspension per well. Incubate at 37°C for 48–72 h (*see* **Notes 19** and **20**).
4. Select typical cell aggregates of MVA/K1L-infected RK-13 cells under a microscope. Mark foci with a permanent marker on the bottom of the culture well.
5. Add 0.5 mL virus growth medium to sterile microcentrifuge tubes.

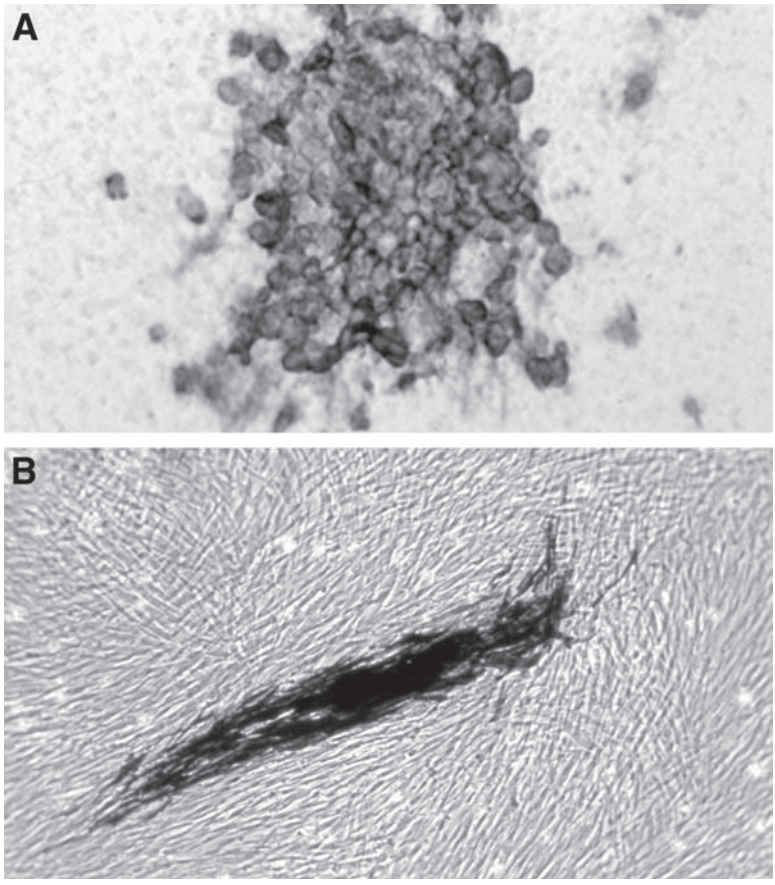

Fig. 2. Plaque morphology induced by MVA-K1L in RK-13 or CEF monolayers. **(A)** Visualization of a typical MVA-K1L-induced focus on RK-13 cells by specific immune-peroxidase staining of infected cells. **(B)** Detection of a typical MVA-LacZ induced plaque on CEF monolayers by X-gal staining.

6. Pick marked foci in a 20 μL vol by aspiration with an air-displacement pipet. Scrape and aspirate cells together with medium and transfer material to the tube containing 0.5 mL medium. Pick 5–15 foci using new tips each time and placing aspirates in separate tube.
7. Freeze–thaw, sonicate, and replate virus material obtained from plaque picks as described in **step 2** or store at –80°C.
8. Repeat as described in **steps 1–7** until clonally pure rMVA/K1L is obtained. This usually requires two to four rounds of plaque purification. Use PCR analysis of viral DNA to monitor for absence of wild-type MVA (*see* **Subheading 3.5.**).

9. After eliminating any contaminating wild-type MVA, proceed to plaque purification on CEF or BHK-21 cell monolayers, selecting for rMVA-specific foci. Repeat steps as described in **steps 1–7**. Use PCR analysis of viral DNA to monitor for absence of K1L selection cassette (**Subheading 3.5.**). This usually requires three to five rounds of plaque purification.
10. Amplify isolated virus (**Subheading 3.6.**) and analyze (**Subheading 3.5.**) the cloned rMVA.

3.4.2. Isolation of rMVA Screening for Transient β-Galactosidase Expression

rMVA expressing the recombinant antigen and transiently coexpressing β-galactosidase-coding sequences (rMVA/LZ) are cloned by consecutive rounds of plaque purification in CEF/BHK-21 cell monolayers stained with X-gal, selecting blue foci ***(19)***. To remove the reporter gene from rMVA, an additional round of plaque purification is carried out, screening for nonstained viral foci in the presence of X-gal (*see* **Note 21**).

1. Freeze–thaw transfection harvest 3× and homogenize in a cup sonicator similar to described in **Subheading 3.2.1.**, **step 11**.
2. Make four 10-fold serial dilutions (10^{-1} to 10^{-4}) of the virus suspension in viral growth medium by diluting 200 µL of the transfection harvest (or plaque pick) in 1.8 mL medium.
3. Remove growth medium from confluent cell monolayers grown in six-well plates and infect with 1 mL diluted virus suspension per well. Incubate at 37°C for 2 h.
4. Melt 2% LMP agarose and keep at 37°C until needed. Prewarm 2X plaquing medium and keep at 37°C until needed.
5. Two hours after infection of cell monolayers, mix equal amounts of 2% LMP agarose and 2X plaquing medium.
6. Remove inoculum from cells and overlay cell monolayers with 1 mL 2X plaquing medium/LMP agarose mixture. Allow agar to solidify at RT, and incubate for 48 h at 37°C.
7. Prepare second medium:agarose overlay containing X-gal by mixing equal amounts of 2% LMP agarose and 2X plaquing medium supplemented with 1/100 vol of X-gal solution. Add to each well 1 mL 2X medium/LMP agarose/X-gal mixture, allow to solidify at RT, and incubate for 4–12 h at 37°C.
8. Add 0.5 mL virus growth medium to sterile microcentrifuge tubes.
9. Pick blue foci of cells infected with recombinant MVA by inserting the tip of a sterile cotton-plugged Pasteur pipet through the agarose into the stained viral foci. Scrape and aspirate cells together with agarose plug into the Pasteur pipet tip, and transfer contents to the tube containing 0.5 mL medium by squeezing the rubber bulb on the Pasteur pipet. Pick 5–15 foci using separate sterile pipets and placing contents into separate tubes.
10. Freeze–thaw, sonicate, and replate virus material obtained from plaque picks as described in **steps 1–7** or store at –80°C.

11. Repeat steps as described in **steps 1–9** until clonally pure rMVA/LacZ is obtained. This usually requires 5–10 rounds of plaque purification. Use PCR analysis of viral DNA to monitor for absence of wild-type MVA (*see* **Subheading 3.5.** and **Note 21**).
12. Continue plaque purification in the presence of X-gal now selecting for *nonstaining* viral foci. Repeat steps as described in **steps 1–9** until all viral isolates fail to produce any blue foci in the presence of X-gal.
13. Amplify isolated virus (**Subheading 3.6.**) and analyze (**Subheading 3.5.**) the cloned rMVA.

3.4.3. Isolation of rMVA by Live Immune Detection of the Target Antigen

This is an alternate protocol that allows one to select recombinant MVA if an antibody that works by immunostaining specific for the recombinant gene product is available. The live immunostaining procedure enables specifically detecting cells infected with rMVA and producing the antigen of interest. In this approach, selective pressure is not used and works best for surface proteins (*see* **Note 22**). CEF or BHK-21 cells should be plated on culture wells pretreated with Concanavalin-A, which allows for tighter attachment of the monolayer to the plates during staining procedure (*see* **Note 23**).

3.4.3.1. Concanavalin-A Coated Plates for Live Immunostaining

1. Add 1 mL Con-A/PBS solution (10% Con-A in PBS) to each well of a six-well plate. Leave for 1 h at RT.
2. Rinse each well with 1 mL PBS and let plates dry in hood (sterile!).
3. Store in plastic bag at RT (plates will be stable for months).
4. When needed, plate cells into wells in the usual manner.

3.4.3.2. Immunostaining

1. Freeze–thaw transfection harvest 3× and homogenize in a cup sonicator similar to that described in **Subheading 3.2.1.**, **step 11**.
2. Make four 10-fold serial dilutions (10^{-1} to 10^{-4}) of the virus suspension in virus growth medium by diluting 200 µL of the transfection harvest (or plaque pick) in 1.8 mL medium.
3. Remove growth medium from subconfluent CEF or BHK-21 cell monolayers grown in six-well plates pretreated with Con-A and infect with 1 mL diluted virus suspension per well. Incubate at 37°C for 48–72 h. Use one cell monolayer as mock-infected control.
4. Dilute antibody in PBS/3% FCS. Optimum dilution has to be determined. Usually start with 1:500. Add 1 mL diluted antibody (1st Ab) per well and incubate for 1 h at RT rocking gently (*see* **Note 24**).
5. Remove 1st Ab and wash 2× with 2 mL PBS per well.
6. Dilute appropriate antispecies antibody conjugated to peroxidase (2nd Ab) 1:1000 in PBS/3% FCS.

7. Add 1 mL 2nd Ab dilution per well and incubate for 30–45 min at RT rocking gently.
8. Remove 2nd Ab and wash 2× with 2 mL PBS per well.
9. Prepare substrate solution as described in **Subheading 3.2.3.**, **step 11**.
10. Add 1 mL substrate solution per well. Incubate for up to 30 min at RT until foci of stained cell can be detected. Continue to monitor staining under a microscope.
11. Add 0.5 mL RPMI/2% FCS/1% AB/AM medium to sterile microcentrifuge tubes.
12. Pick stained foci in a 20 µL vol by aspiration with an air-displacement pipet. Scrape and aspirate cells together with supernatant and transfer material to the tube containing 0.5 mL medium. Pick 5–15 foci, each time using new tips and placing contents into a separate tube.
13. Freeze–thaw, sonicate, and replate virus material obtained from plaque picks as described in **steps 1–3** or store at –80°C.
14. Repeat as described in **steps 1–13** until clonally pure rMVA is obtained. Use PCR analysis of viral DNA to monitor for absence of wild-type MVA (**Subheading 3.5.**).
15. Amplify (**Subheading 3.6.**) and analyze (**Subheading 3.5.**) the cloned rMVA.

3.5. Characterization of Recombinant MVA Genomes by PCR

MVA-DNA is analyzed by PCR using oligonucleotide primers that are designed to amplify DNA fragments at the specific insertion site used within the MVA genome. Thus, genomes of rMVA and wild-type MVA can easily be identified and distinguished in DNA preparations from infected cell cultures. Elimination of wild-type MVA during plaque purification of rMVA can be monitored (*see* **Note 25**), and correct insertion of foreign DNA within the MVA genome can be ascertained. An example of a PCR analysis is described for using deletion III of MVA as an insertion site and transient K1L selection to generate recombinant viruses. Primers MVA-III-5' and MVA-III-3' anneal to template MVA-DNA sequences adjacent to insertion site III, and PCR will produce DNA fragments that are specific for wild-type MVA, for rMVA/K1L expressing the *K1L* marker gene, or for the final rMVA. Expected fragments will be:

Wild-type MVA: 0.7 kb, corresponding to empty deletion III
rMVA/K1L: 2.2 kb (Del III + K1L) + *x* kb (*x* = inserted gene sequence)
rMVA: 0.95 kb (rMVA/K1L-K1L) + *x* kb (*x* = inserted gene sequence)

The amplification product for wild-type MVA has a defined size of 0.7 kb, indicating that no foreign DNA is inserted into insertion site III. The expected molecular weight of the PCR product for rMVA/K1L can be calculated by adding the size of the recombinant insert to 2.2 kb (empty MVA and K1L marker cassette). DNA extracted from cells infected with wild-type MVA and plasmid DNA from pIIIdHR-P7.5-recombinant gene are used as control

templates (*see* **Note 26**). The size of the PCR fragment specific for the final recombinant virus rMVA results from the molecular weight of rMVA/K1L being reduced by 1.35 kb DNA corresponding to the desired loss of *K1L* reporter gene sequences. Further testing for the loss of K1L can also be performed (*see* **Note 27**).

1. Infect cell monolayer of one well in 6- or 12-well plate with 1 mL of 10^{-1} dilution of the virus suspension obtained from the last round of plaque purification, and incubate for 3 d at 37°C (*see* **Note 28**).
2. Discard medium, harvest cell monolayer in 400 µL distilled water and transfer into a 1.5-mL microcentrifuge tube.
3. Add 50 µL 10X TEN, pH 7.4, and freeze–thaw 3× (*see* **Note 29**).
4. Mix by vortexing, then microcentrifuge at 1800*g* for 5 min at RT to remove cellular debris.
5. Transfer supernatant into fresh 1.5-mL microcentrifuge tube. Add 50 µL prot K and 23 µL SDS 20%.
6. Vortex and incubate for 2 h at 56°C.
7. Extract DNA twice with phenol-chloroform by adding equal volumes of phenol-chloroform 1:1, mix and microcentrifuge at top speed for 5 min at RT. Pipet supernatant into new 1.5-mL microcentrifuge tube.
8. Add 1/10 vol of 3 *M* NaAc followed by 2 vol absolute EtOH. Mix gently and cool for 15 min at –80°C or for 30 min at –20°C. Centrifuge at top speed for 15 min at 4°C.
9. Aspirate off supernatant, wash DNA pellet with EtOH 70%, air-dry for at least 10 min (*see* **Note 30**). Resuspend dried pellet in 50 µL distilled water.
10. Prepare PCR reaction mix on ice by adding 39 µL distilled water, 5 µL primer 1, 5 µL primer 2, 1 µL template DNA (*see* **Note 31**) and 50 µL PCR master mix to obtain a total volume of 100 µL.
11. Perform PCR using the following conditions (*see* **Note 32**):

    Step 1: Denaturation at 94°C for 2 min.
    Step 2: Cycles 1–30: denaturation at 94°C for 30 s, annealing at 55°C for 40 s, elongation at 72°C for 3 min.
    Step 3: final elongation at 72°C for 7 min.
    Storage at 4°C.

12. Use 20 µL aliquot of each PCR reaction to perform agarose gel electrophoresis to visualize amplified DNA fragments and determine molecular weights in comparison to double-stranded DNA standards (e.g., 1-kb DNA ladder, Gibco BRL; *see* **Note 33**).

3.6. Growth of Virus Stocks from Single-Plaque Isolates (see Note 34)

1. Infect confluent CEF or BHK-21 cell monolayer grown in a approx 35-mm tissue culture dish, adding to the medium 250 µL virus suspension of isolated rMVA obtained from the last plaque purification. Incubate at 37°C for 2 d or until CPE is obvious.

2. Discard medium, harvest cell monolayer in 1 mL virus growth medium, transfer into 1.5-mL microcentrifuge tube, freeze–thaw, and sonicate as described in **Subheading 3.2.1.**, **step 11**. Either continue or store at –80°C to –20°C as first passage of rMVA.
3. Infect cell monolayer grown in approx 60-mm tissue culture dish by adding to 1 mL medium, 0.5 mL virus suspension obtained from first passage of rMVA. Allow virus to adsorb for 1 h at 37°C, add 2 mL virus growth medium and incubate at 37°C for 2 d or until CPE is obvious.
4. Scrape cells, transfer to 15-mL conical centrifuge tube, centrifuge 5 min at 1800*g*, discard medium, and resuspend cells in 2 mL virus growth medium, freeze–thaw, and sonicate as described in **Subheading 3.2.1.**, **step 11**. Either continue or store at –80°C to –20°C as second passage of rMVA.
5. Infect cell monolayer of one T85 tissue culture flask by adding 0.5 mL virus material from second passage of rMVA and 1.5 mL virus growth medium. Allow virus adsorption for 1 h at 37°C, rocking flask at 20-min intervals (*see* **Note 5**). Overlay with 10 mL virus growth medium, and incubate at 37°C for 2 d or until CPE is obvious.
6. Scrape cells, transfer to a 15-mL conical centrifuge tube, centrifuge 5 min at 1800*g*, discard medium, resuspend cells in 5 mL virus growth medium, freeze–thaw, and sonicate. Either continue or store at –80°C to –20°C as third passage of rMVA.
7. Infect cell monolayer in T185 tissue culture flask with 2 mL virus material from the third passage of rMVA. Allow virus adsorption for 1 h at 37°C, rocking flask at 20-min intervals, add 30 mL virus growth medium, and incubate at 37°C for 2 d or until CPE is obvious.
8. Scrape cells in medium, transfer to a 50-mL conical centrifuge tube, centrifuge 5 min at 1800*g*, discard medium, resuspend cells in 15 mL virus growth medium, freeze–thaw, sonicate, and store at –80°C to –20°C as fourth passage of rMVA (*see* **Note 35**).
9. Generate a high-titer seed virus stock following the procedure outlined in **Subheading 3.2.1.**

3.7. Replication Deficiency Assay

To monitor for preservation of safety (i.e., inability of recombinant virus to replicate in nonpermissive cell lines) of recombinant MVA after insertion of foreign genes, growth properties should be evaluated in human HeLa cells. Monolayers are infected with low moi of MVA and recombinant MVA for 72 h. A replication-competent vaccinia virus or MVA grown on permissive cells may serve as the control. Cells and supernatants are harvested at 0 h and 72 h postinfection, and infectivity is determined on BHK or CEF cells. (**Table 2** shows an example of such data for some recombinant viruses generated in our laboratory.)

Table 2
Replication Deficiency
of Recombinant MVA in Human HeLa Cells

Virus	t_{72}/t_0
MVA	0.46
rMVA-SIV-Tat	1.31
rMVA-SIV-gag/pol	1.00
rMVA-HCV-C/E1/E2	1.85
MVA on CEF	15,926.00

1. Infect confluent HeLa cells grown on six-well plates with 0.01 IU/cell of the respective virus in 1 mL medium. Infect two wells per time-point and virus.
2. Incubate at 37°C, 5% CO_2 for 1 h.
3. Remove inoculum. Wash twice with fresh medium.
4. Harvest 0 h time-points by scraping monolayers into the medium and transferring cells and supernatants into microfuge tubes. Store at –80°C.
5. Incubate remaining wells for 72 h at 37°C, 5% CO_2. Harvest wells (cells and supernatants) as described in **step 4**.
6. Titrate samples on confluent BHK or CEF six-well plates as described in **Subheading 3.2.3.**
7. Calculate the replication efficiency of each virus as a ratio t_{72}/t_0 using the mean titer for each time-point (four values: two samples for each time-point, titrated in duplicates).

4. Notes

4.1. Virus Stain and Preparation of CEF

1. F6 ***(9)*** is a cloned isolate of MVA originally obtained from seeding material of the 572nd CEF passage (02/22/1974) and currently available at the 582nd passage on CEF. II_{new} ***(15)*** is an engineered MVA virus, free of leftover K1L gene sequences, derived from F6 (582nd CEF passage).
2. Use 11-d-old embryonated eggs, 10-d-old are still small, and 12-d-old embryos may already develop feathers.
3. When the shell membrane is opened, check for viability of the embryo by monitoring the blood vessels on the chorioallantois membrane.

4.2. Virus Growth

4. For all steps, remember, when using frozen virus stocks, always sonicate 30 s in cup sonicator after thawing to disrupt clumps of virus.

4.3. Amplification of MVA

5. When infecting cell monolayers grown in larger tissue culture flasks (e.g., T185 flasks), avoid drying of the cell monolayer by rocking flask by hand at 20-min intervals.

6. To homogenize virus material most efficiently after amplification, we recommend the use of a sonication needle instead of the cup sonicator. Place the tube containing virus material in a small beaker with ice-water and plunge the sterilized sonication needle into virus suspension. Sonicate 4× for 15 s at maximal power. Take care to avoid heating of the sample.
7. Having obtained a rMVA stock virus, the following procedures are recommended: (1) Titer the virus stock on CEF or BHK-21 cell monolayers (*see* **Subheading 3.2.3.** or **3.2.4.**). (2) Check clonal purity and genomic stability of rMVA by PCR (*see* **Subheading 3.5.**) or Southern blot analysis of viral DNA. (3) Characterize synthesis of recombinant antigen by specific immunostaining of cell foci infected with virus expressing recombinant gene(s) (*see* **Subheading 3.2.3.**, but substitute 1st Ab with antigen-specific Ab) by immunoblot analysis of lysates from rMVA-infected cells or by immunoprecipitation of the target antigen made during rMVA infection following labeling with radioactive amino acids.
8. It is recommended to prepare a first seed virus stock as *primary* stock (material from approx 10 infected T185 culture flasks should be sufficient), which is used to amplify *working* stocks of rMVA. This is done to minimize multiple passages of the virus.
9. The crude material has typical titers of approx 3×10^9 IU per infected tissue culture flask.

4.4. Purification of MVA

10. If after amplification, virus material has been homogenized using a sonication needle, there is no need for douncing. Thus, the virus suspension can be transferred to a 50-mL Falcon tube, and centrifuge 5 min at 1800*g* and 4°C. Discard the pellet and continue purification with supernatant as described in **Subheading 3.2.2.**, **step 4**.
11. Normally, semipurified virus material pelleted through a sucrose cushion is fine for animal work. To obtain highly (i.e., band) purified viruses, material obtained from **Subheading 3.2.2.**, **step 6** can be centrifuged through a 25–40% sucrose gradient for 50 min at 28,000*g* and 4°C. Harvest virus band appearing at the lower half of the tube. To concentrate the virus in the band (and remove remaining sucrose), fill an ultracentrifuge tube with more than 3× vol 1 m*M* Tris, and pellet virus material at 38,000*g* for 1 h at 4°C. Resuspend the pellet in 1 m*M* Tris and store at –80°C. Usual titers for purified concentrated material are approx 1×10^{10} IU/mL. Band-purified material can be used as coating material for vaccinia virus-specific ELISAs.

4.5. Titration of MVA

12. We describe two different methods to determine titers of virus stocks. An advantage of the first method (amount of IU/mL, **Subheading 3.2.3.**) is the relative ease of using six-well rather than 96-well plates. Also, the handling for culture, plating of virus dilutions, and immunostaining is easier. On the other hand, determining the $TCID_{50}$ (**Subheading 3.2.4.**) of virus stocks may result in more repro-

ducible titers because a choice between infected or uninfected is independent of the number of plaques per well.

13. Before titration, virus material *must* be homogenized by sonication. Sonicate aliquots of maximal 1.5 mL virus suspension as described in **Subheading 3.2.1.**, **step 11**.
14. Alternatively, after the fixing of cell monolayers, incubation with blocking buffer can be done overnight at 4°C. In our experience, we actually do not require this blocking step and are able to proceed directly to adding the 1st Ab.
15. Owing to the toxicity of dianisidine and the need for such a tiny amount, we typically do not weigh it out, but just use an amount that fills the tip of a small spatula. To remove small clumps of o-dianisidine, filter the PBS/dianisidine mix through 0.2-μm filter into a new tube before adding the H_2O_2 >30%.

4.5.1. Determining $TCID_{50}$

16. Example for calculating $TCID_{50}$: If all eight wells are counted positive in dilution 10^{-7}, $x = 7$. If, five infected wells are found in dilution 10^{-8}, and the number of infected wells in dilution 10^{-9} (the highest dilution in which positive wells can be found) is 2, then the log 50% endpoint dilution would be: 7 – 1/2 + (8/8 + 5/8 + 2/8) = 7 – 0.5 + (1.875) = 7 + 1.375 = 8.375. Therefore, the endpoint dilution that will infect 50% of the wells inoculated is $10^{-8.375}$. The reciprocal of this number yields the titer in terms of infectious dose per unit volume. Because the inoculum added to an individual well was 0.1 mL, the titer of the virus suspension would therefore be: $10^{8.375}$ $TCID_{50}$/0.1 mL = $10^{9.375}$ $TCID_{50}$/mL.

4.6. Molecular Cloning of Recombinant Gene Sequences

17. For best transfection efficiencies, prepare clean, supercoiled DNA either by centrifugation through cesium chloride gradients or using plasmid purification kits (e.g., QIAGEN GmbH, Hilden, Germany).

4.7. Isolation of Recombinant MVA

18. For either selection technique, note the following. Because of its highly host-restricted nature, MVA does not produce the rapid cytopathic effect accompanied by destruction of the cell monolayer seen with standard strains of vaccinia (e.g., Western Reserve, Copenhagen) in mammalian cells. Plaque formation is only observed in CEF or BHK-21 cells or when providing K1L expression in RK-13 cells ***(15,17)***. Also, when picking rMVA plaques, preferably choose well-separated viral foci from wells infected with the highest dilutions, which will drastically reduce the number of plaque passages needed to isolate clonally pure rMVA.

4.7.1. Transient Host Range Selection

19. To distinguish rMVA-specific RK-13 aggregates, it may be helpful to use mock and wild-type MVA-infected control wells for comparison.

20. To allow for the most efficient plaque cloning, it may be helpful to perform plaque passages under agar (for overlay, follow **Subheading 3.4.2.**, **steps 4–6**).

4.7.2. Transient β-Galactosidase Screening

21. As the expression cassette containing the *lacZ* marker gene is designed to be efficiently deleted from the rMVA genome, nonstaining MVA foci may be observed during plaque purification even after all wild-type MVA has been successfully eliminated. To avoid needless plaque passages, it is important that the absence of parental MVA is confirmed by PCR analysis.

4.7.3. Screening by Live Immune Detection

22. For detection of intracellular target antigens, it may be helpful to freeze–thaw culture plates prior to staining. Incubate at –80°C without medium for 1–2 h.
23. The staining results will only be as good as the antibody. This procedure will be difficult if using weakly staining antibodies. The first round of picking for the recombinant is the most difficult, as recombinant foci may be small (one to four cells) due to the large amount of wild-type MVA present. In subsequent passages foci will be larger, more plentiful, and thus easier to pick.
24. No blocking step is done here, as speed is the most important factor at this point when working with live cells.

4.8. Characterization of Recombinant MVA DNA by PCR

25. To monitor the presence of wild-type MVA during plaque purification, viral DNA sufficient for PCR analysis is isolated from cell monolayers infected with the 10^{-1}-dilution of virus suspensions plated out for plaque passage.
26. Always use DNA of wild-type MVA and respective plasmid as control templates for PCR analysis.
27. Also check for deletion of K1L marker cassette from virus genomes by K1L-specific PCR using the following primers and 55°C annealing temperature: K1L-int-1: 5'-TGA TGA CAA GGG AAA CAC CGC-3', K1L-int-2: 5'-GTC GAC GTC AAT TAG TCG AGC-3'. If K1L is still present in viral genomes, this PCR yields a product of 290 bp. If K1L has successfully been recombined out, there will not be any PCR product. Always use controls (e.g., transfer plasmid and wild-type MVA) and perform PCR for specific insertion site in parallel to make sure that template DNA was present.
28. Virus material harvested from cell monolayers grown in 6-well/12-well plates and infected for 24 h with an moi of 10 IU/cell will yield a good amount of viral DNA for PCR/Southern blot analysis. If infectivity is too low, a second round of amplification may be necessary. Harvest amplification, freeze-thaw, sonicate, and replate on cell monolayers.
29. Avoid sonication of infected tissue culture material to be used for DNA extraction because unpackaged viral DNA will be destroyed and lost for analysis.
30. Carefully air-dry the pelleted DNA material to remove all EtOH.

31. As DNA preparations might contain variable quantities of viral DNA, the amount of template DNA used for PCR may be optimized.
32. PCR conditions (temperatures and number of cycles) may need to be optimized according to the size of the expected fragment to be amplified. Conditions as stated in the protocol have been used for amplification of up to 4 kb DNA inserted into the MVA genome.
33. If template DNA is derived from mixed virus populations on RK-13 cells containing both, rMVA/K1L, as well as wild-type MVA, PCR may amplify preferentially the fragment for wild-type MVA because of its smaller size, and rMVA/K1L may not be detectable. A signal for rMVA (having already lost the K1L gene) may also be detected, as the K1L marker cassette is designed to be efficiently deleted from recombinant genomes, and this process can occur in RK-13 cells.

4.9. Growth of Virus Stocks from Single-Plaque Isolates

34. The key to the amplification of a stock of virus from a single plaque is to follow a step-wise growth of virus on small, then larger amounts of cells. If one initially puts a low-titer growth on high cell numbers, there is a risk of losing the virus.
35. The titer of this material should be approx 10^9 IU. As a general rule, in growing up a seed stock material from one well-infected T185 culture flask can be used to infect 10 T185 flasks.

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8

Growing Poxviruses and Determining Virus Titer

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Summary

Poxviruses are cell-associated viruses that can be grown in adherent- or suspension-cell cultures or chorioallantoic membranes of embryonated hen's eggs. The main principle of isolating the virus is to mechanically lyse infected cells. The virus can then be semipurified by centrifugation through a sucrose cushion. Further purification can be achieved with a sucrose gradient. This chapter describes methods for the preparation of large-scale growth and purification of the virus.

Key Words: Poxviruses; vaccinia virus; virus purification; virus titration; DNA extraction.

1. Introduction

Poxviruses are the most complex and largest of all animal DNA viruses ***(1)***. The entire life cycle of poxviruses occurs in the host cell cytoplasm. They are brick-shaped viruses produced in the cell in viral factories. Generally, 100–10,000 virus particles are produced per cell. The virion can last at room temperature for several days, and lyophilized virions can maintain infectivity for several years. Vaccinia virus is the most studied member of the orthopoxvirus family, and a basic understanding in the viral life cycle is critical for understanding the scientific basis of its growth and purification ***(2)***.

Such basic factors include replication localized to the cell cytoplasm, aggregation of poxviral particles into inclusion particles ***(3)***, and the time taken for life cycle completion. Vaccinia virus and cowpox virus have a diverse host range, whereas other orthopoxviruses, such as modified vaccinia Ankara (MVA) and variola or monkeypox, are best grown in embryonated hen's eggs. This chapter focuses on growing wild-type poxviruses, obtaining virus titers, and isolating viral DNA from purified virions. Owing to the range of danger

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associated with the different poxviruses, it is essential to pay careful attention to the safety precautions when handling orthopoxviruses. Variola virus, the causative agent of smallpox, can only be handled in World Health Organization (WHO) designated biosafety level (BSL)-4 facilities. Monkeypox can be handled in a BSL-3 facility. Cowpox viruses and vaccinia virus should be worked within the BSL-2 laboratory (*see* **Note 1**).

2. Materials

2.1. Large-Scale Growth and Purification of Vaccinia Virus or Cowpox Virus in HeLa S3 Cells

1. HeLa S3 suspension culture cells (ATCC, # CCL2.2).
2. Various spinner bottle vented flasks, 1–10 L with Teflon stirrer (Bellco or Corning).
3. Cabinet for incubating the spinner bottles at 37°C.
4. Spinner medium (S-MEM): Joklik modified (minimal essential medium [MEM] for cell suspension calcium chloride-free) containing 1% penicillin, streptomycin, and fungizone, 10% highest grade fetal bovine serum (FBS; mycoplasma tested, complement-inactivated, endotoxin-free, virus-free, sterile, free of prions; *see* **Note 2**).
5. Crude sterile poxvirus stock with known virus titer (*see* **Note 3**).
6. Three types of refrigerated centrifuges: swinging bucket centrifuge (e.g., Sorvall RT6000 D); high-speed centrifuge (e.g., Haraeus or Beckman); ultracentrifuge (e.g., Sorvall Discovery 90; *see* **Note 4**).
7. Trypsin: Tissue culture grade filter-sterilized trypsin solution (0.25 mg/mL) stored frozen.
8. 10 m*M* Tris: 10 m*M* Tris-HCl, pH 9.0, filter-sterilized.
9. Glass Dounce homogenizer (Corning).
10. Sucrose solutions: Filter-sterilized sucrose solutions of 25%, 30%, 35%, 40%, and 45% (w/v) in 10 m*M* Tris-HCl, pH 9.0 (*see* **Note 5**).
11. Cup and probe sonicator.
12. Ear protection during sonication or soundproof assembly for housing sonicator.
13. Hemocytometer.
14. Trypan blue (0.4%): prepared in 0.85% saline and membrane-filtered.

2.2. Culturing of Poxviruses in Embryonated Hen's Eggs

1. Embryonated chicken eggs (fertilized 9–10 d previously).
2. Egg pricker (*see* **Note 6**).
3. Egg candler (*see* **Note 7**).
4. Physiological saline: 0.85% NaCl in sterile H_2O.
5. Tuberculin syringe: 1 mL tuberculin syringe with 29G needle.
6. 30 mL Universal screw-cap tubes.
7. Virus suspension mixture: 3:1 ratio of McIlvain's buffer: 0.1 *M* citric acid, 0.2 *M* Na_2HPO_4, adjust pH to 7.4 using either solution (i.e., if pH requires lowering, add more citric acid; if pH needs to be raised, add more Na_2HPO_4), stored at 4°C

with Arklone-X (1,1,2 trichloro-1,2,2 trifluoroethane; commercial dry-cleaning agent).
8. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 9.0.
9. Sucrose solution: 36% (w/v) made up in TE buffer.

2.3. Titering the Virus

1. BSC1 cells (ATCC, # CCL26).
2. Virus growth medium: MEM supplemented with 2% FBS.
3. Crystal violet solution: 0.1% in 20% ethanol.

2.4. Poxviral DNA Extraction

1. Lysis buffer: 50 m*M* Tris-HCl, pH 9.5, 700 m*M* NaCl, 10 m*M* EDTA, 1% sodium dodecyl sulfate (SDS), dH_2O to final required volume.
2. 50 mg/mL Proteinase K.
3. Phenol/chloroform: saturated phenol equilibrated with 50 m*M* Tris-HCl, pH 7.8:chloroform:isoamyl (25:24:1; *see* **Note 8**).
4. NaAc: 3 *M* sodium acetate, pH 7.0.
5. 95% Ethanol and 200 proof (100% ethanol).
6. 70% Ethanol.
7. UV and VIS spectrophotometer with quart cuvets (0.5–1-mL capacity).
8. Purified poxvirus preparation.
9. Microcentrifuge.
10. Deionized endotoxin-free sterile-distilled water with a conductive approx 18 mho.

3. Methods

3.1. Large-Scale Growth and Purification of Vaccinia Virus or Cowpox Virus in HeLa S3 Cells

A relatively easy method to grow large batches of vaccinia virus is to use HeLa suspension cells (*see* **Notes 9–11**) grown in spinner bottles. Maintenance of HeLa S3 over a long period requires very careful attention to temperature regulation, correct cell density, and spinning speed (*see* **Note 12**). Virus purification following growth in HeLa cells is carried out by first pelleting the virus through a sucrose cushion. Further viral purification by zonal sucrose gradient centrifugation is essential when undesirable cellular material is to be removed (*see* **Note 13**).

1. HeLa cells are maintained in S-MEM to a density of 5×10^6 cells/mL (*see* **Note 14**).
2. When ready to infect cells, count cells, then centrifuge in sterile bottles with tapering bottom at approx 2000 rpm (1500*g*) for 15 min at room temperature.
3. Discard supernatant and resuspend in S-MEM containing 2.5% FBS at a density approx 2×10^7 cell/mL.
4. Trypsinize the crude stock of virus that will be used to infect the HeLa cells by mixing equal volume of the stock virus with trypsin solution (*see* **Note 15**).

5. Infect HeLa cells with trypsinized crude virus stock at a multiplicity of infection (moi) of 1–5 pfu/cell and incubate for 1–2 h with constant stirring.
6. After this period of virus absorption onto cells, add medium to bring the cells back to their original density and stir for 48–72 h at 37°C.
7. At the end of the incubation period, chill the entire spinner bottle, then transfer the cell suspension to chilled centrifuge tubes. Lower the centrifuge temperature to 4°C and spin at 2000 rpm (1500*g*) at 4°C.
8. Discard the supernatant (*see* **Note 16**) and gently suspend the cell pellet in minimal amount of chilled 10 m*M* Tris (e.g., approx 10 mL) and transfer cell suspensions from each of the centrifuge tubes into a 15–20 mL Dounce homogenizer cooled on ice with the piston down.
9. Slowly raise the piston and gently push it down. Count each push downward as one complete cycle. Perform 25–50 such cycles, stopping after each set of five cycles (*see* **Note 17**).
10. Transfer the contents to a centrifuge tube and centrifuge at 1000 rpm (750*g*) to pellet out the cell nuclei and collect the nucleus-free cell lysate.
11. Sonicate the lysate (*see* **Note 18**).
12. In an ultracentrifuge tube (sterilized in an autoclave) appropriate for an ultracentrifuge SW41 rotor, add approx 2 mL of a 40% sucrose solution, carefully layer the sonicated lysate, and centrifuge at 35,000 rpm (approx 151,300*g*) for 2 h at 4°C.
13. At the end of centrifugation, carefully discard the supernatant. Resuspend the pellets in 200 µL of 10 m*M* Tris.
14. To further purify the virus, make a step gradient ranging from 25% to 40% by layering 2 mL of the highest concentration sucrose solution in the bottom, then 2 mL of the next highest and so on, leaving it overnight in a cold room, and using it the next morning (*see* **Note 19**).
15. The sucrose cushion-purified virus is carefully overlayed on the gradient and the tube is spun in a SW41 rotor at 25,000 rpm (approx 77,200*g*) for 1 h at 4°C.
16. The pellet is suspended in the 10 m*M* Tris, and the entire process can be repeated to obtain double-banded purified virus.
17. The virus should be titered (*see* **Subheading 3.3.**) and stored below –60°C in a freezer marked with biohazard stickers.

3.2. Culturing of Poxviruses in Embryonated Hen's Eggs

If there is no desire to grow and maintain spinner HeLa cells over a continuous period of time, we have been quite successful using embryonated hen's eggs to grow large amounts of virus. This method, which was developed prior to the advent of tissue culture, takes advantage of the egg's membrane that is rich in blood vessels and provides an environment that facilitates the growth and spread of the virus within the embryonated egg. The easily accessible membrane, known as the chorioallantoic membrane (CAM), is situated just below the shell. Virus grown on CAMs form raised circular pocks of varying size and

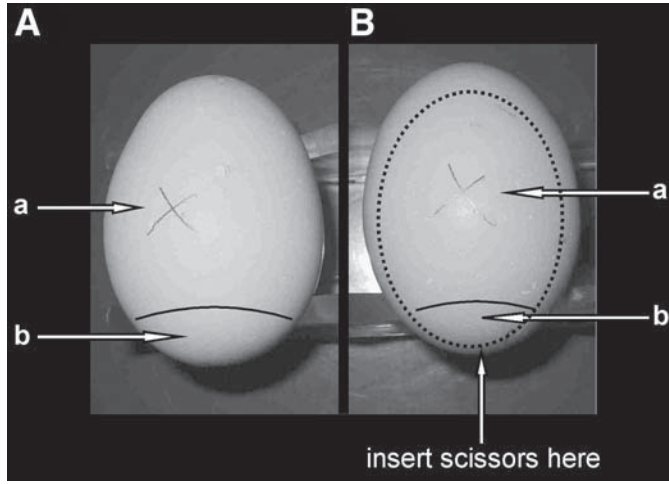

Fig. 1. Picture of an embryonated hen's egg. A. The dorsal **(A)** and air-sac **(B)** regions are indicated. B. Guideline for cutting of shell is indicated by broken lines when it is time to harvest virus.

color depending on the poxviral strain used (*see* **Note 20**). These pock lesions contain high titers of virus. Viral isolation and purification steps result in concentrated and clean viral suspensions. This method of virus culture was modified ***(3)*** from the one described by Joklik in 1962 ***(4)***.

3.2.1. Viral Inoculation

1. Clean the shell of 9–10-d-old embryonated chicken eggs with 70% ethanol to minimize bacterial contamination.
2. Examine each egg with the use of an egg candler. Note the air-sac region and blood vessels below the shell.
3. Make a pencil marking at the air sac and dorsal regions of each egg, respectively (*see* **Fig. 1A**).
4. Create a small opening at each marked region using a hole-puncher or egg pricker.
5. Place a small drop of melted candle wax above each dorsal opening, which will later be used to cover the opening to prevent bacterial contamination.
6. Apply a single drop of physiological saline onto each dorsal opening.
7. While examining eggs under the egg candler, use a pipet bulb to create a slight vacuum at the air-sac opening of each egg to drop or pull down the CAM area surrounding the dorsal opening. The dropped membrane area should be clearly visible in the light of the egg candler. If the membrane does not appear to drop, the air-sac opening may be too small. Enlarge the air-sac opening, and put an additional drop of physiological saline at the dorsal opening, followed by repeating bulb suction.

8. Incubate eggs at 37°C for 1.5 h to allow the dropped membranes to stabilize. (The dorsal opening remains uncovered for this stage.)
9. Using a tuberculin syringe, inoculate each dorsal opening with 100 μL poxviral suspension (*see* **Note 21**).
10. Cover the openings with candle wax to prevent contamination of inoculation site.
11. Incubate eggs at 37°C for 72 h (*see* **Note 22**).

3.2.2. Harvesting Virus from CAMs

1. Using a pair of sharp scissors inserted at the air-sac opening, cut along either side of the dropped CAM region, creating a "lid," which can be lifted to view the infected tissue (*see* **Fig. 1B**).
2. Cut away any membranous structures, attach the membrane to the chick embryo, and carefully remove the infected CAM region with forceps.
3. Rinse membranes in physiological saline to wash away any blood. Repeat rinse in fresh saline if necessary.
4. Transfer membranes to chilled thick-bottomed 30 mL Universal screw-cap tubes containing 1-cm of sterile glass beads.
5. Work on ice for remaining steps.
6. Add 1 mL virus suspension mixture per membrane (*see* **Note 23**).
7. Wrap Universal tubes in paper towels (as a precaution in the event of leakage). Shake for 2 min to break apart tissues and cells.
8. Centrifuge tubes at 800 rpm (600*g*) for 10 min at room temperature.
9. Transfer supernatants to clean chilled 30 mL Universal screw-cap tubes (no glass beads in these tubes). Leave on ice.
10. **Steps 6–9** are to be carried out three more times on pelleted material. No trypsinization or sonication steps are required.
11. Incubate pooled supernatants on ice for 1.5 h to allow for further sedimentation of the membrane debris.
12. Remove supernatant and centrifuge at 2000 rpm (1500*g*) for 10 min at room temperature.
13. Transfer supernatant to sterile 50-mL polyallomer centrifuge tubes.
14. Virus particles can be semipurified from this supernatant by pelleting through a sucrose cushion. Pipet 5 mL 36% sucrose at the base of ultracentrifuge tubes, then slowly pipet the supernatant-containing virus on top to form two distinct layers.
15. Centrifuge tubes at 11,000 rpm (5000*g*) for 1 h at 4°C.
16. Discard supernatant and resuspend pellets in 0.5 mL TE buffer overnight at 4°C (*see* **Notes 24** and **25**).
17. Store viral suspension at –20°C or –80°C

3.3. Virus Titration

The most commonly used laboratory strain of vaccinia virus (Western Reserve [WR] strain) forms large round plaques when grown in a confluent monolayer of cells under liquid overlay. Thus, titering of WR-based viruses

are quite easy. The first of these two titration methods is more rapid, but less accurate in comparison to the second method. However, it is appropriate to use for the comparison of viral titer between different stocks.

3.3.1. Method 1: Serial Dilutions from an Initial Dilution

1. Prepare six-well plates to be used when monolayers are at 100% confluency (*see* **Note 26**).
2. Remove media from each well of a six-well plate, then add 0.5 mL virus growth medium into each well.
3. Label each well of six-well plate to correspond to a specific virus dilution or control (e.g., 1:1000; 1:10,000, and 1:100,000).
4. Make 1:100 dilution of virus stock in virus growth medium (e.g., 10 μL virus stock in 1 mL; *see* **Note 27**).
5. Vortex to mix.
6. Transfer appropriate volume of virus dilution to a well-allocated first dilution step (i.e., 100 μL of 1:100 dilution stock in the well labeled 1:1000 dilution; 10 μL of the 1:100 dilution stock into the well labeled 1:10,000; 1 μL of the 1:100 dilution stock into the well labeled 1:100,000). Such titrations should be done in duplicate.
7. Continue dilution series by transferring a volume of diluted virus into the next well in the dilution series by using a clean pipet tip for each dilution step, leaving one well virus-free as a negative control.
8. Swirl media in plate gently.
9. Incubate at 37°C in a 5% CO_2 incubator for 2 h.
10. Add 1.5 mL virus growth medium into each well of six-well-plate, swirl briefly, and replace at 37°C in a 5% CO_2 incubator (*see* **Note 28**).
11. Incubate for 48–72 h, depending on stage of plaque formation as viewed under the microscope (*see* **Note 29**).
12. *See* **Subheading 3.3.3.** for staining.

3.3.2. Method 2

1. Prepare six-well plates to be used when monolayers are at 100% confluency (*see* **Note 26**).
2. Pipet 1 mL fresh virus infection medium into 10 sterile 15-mL disposable plastic tubes.
3. Label each tube with the appropriate dilutions required for selected dilution series (e.g., 1:100, 1:1000, and so on).
4. Transfer appropriate volume of virus stock to the first tube containing 1 mL virus infection medium (e.g., 10 μL virus suspension to 1 mL virus infection medium for a 1:100 dilution) (*see* **Note 27**).
5. Vortex tube briefly.
6. Transfer 100 μL from this first tube into the next tube using a new pipet tip (this is the 1:1000 dilution).

7. Continue dilution series using a clean pipet tip for each dilution step, leaving 1 mL aliquot virus-free as a negative control.
8. Label each well of six-well plate to correspond to a specific virus dilution (e.g., 1:100; 1:1000, and so on) made in the tubes (or control).
9. Remove media from each well of a six-well plate, then add 0.5 mL virus growth medium into each well.
10. Pour each dilution mix into a corresponding well (*see* **Note 30**).
11. Swirl gently and incubate at 37°C in a 5% CO_2 incubator for 2 h.
12. Add 1 mL virus growth medium into each well of six-well-plate, swirl briefly, and replace at 37°C in a 5% CO_2 incubator (*see* **Note 28**).
13. Incubate for 48–72 h, depending on the stage of plaque formation as viewed under the microscope (*see* **Note 29**).
14. *See* **Subheading 3.3.3.** for staining.

3.3.3. Staining

1. Aspirate off viral infection media from each well of the six-well plate.
2. Add 200–500 μL of crystal violet solution to each well of a six-well plate (add along the side of the well in order to not disrupt the cell layer).
3. Swirl gently and incubate for 1 h at room temperature.
4. Aspirate off the stained media.
5. Invert plate to allow dye to dry.
6. Plaques should become visible and easy to count. To calculate the viral titer, the number of plaques in each well are counted and multiplied by the virus dilution factor for that specific well. An average titer can be calculated following the determination of viral titer per well (*see* **Note 31** for an example).

3.4. Poxviral DNA Extraction

Viral DNA can be isolated from purified virions. Such DNA preparations are useful for polymerase chain reaction (PCR) experiments for the amplification of specific poxviral genes, restriction enzyme digestion, or sequencing of viral genomic DNA, as well as molecular cloning of poxviral genes.

1. In a 1.5-mL microcentrifuge tube, add 100 μL band-purified virus and 100 μL lysis buffer.
2. Invert tube gently to mix (solution should become clear; *see* **Notes 32** and **33**.
3. Incubate at room temperature for 10 min.
4. Add 0.05 vol (as according to original viral suspension volume) of proteinase K solution (*see* **Note 34**).
5. Mix gently (*see* **Note 32**) and incubate at 56°C for 1.5–2 h.
6. Begin to extract DNA by first adding equal volume of phenol:chloroform:isoamyl (25:24:1).
7. Invert to mix (*see* **Note 32**) and centrifuge at top speed (16,000*g*) for 10 min.
8. Remove top layer of solution and transfer to clean 1.5-mL centrifuge tube.
9. Repeat **steps 6** and **7** on the removed top layer.

10. Add 0.1 vol of ice-cold NaAc and 2.5 vol of ice-cold 95% or 100% ethanol.
11. Invert to mix (*see* **Note 32**) and incubate at –20°C overnight or –80°C for 1 h.
12. Pellet DNA by centrifuging at top speed for 20 min.
13. Discard supernatant (*see* **Note 35**).
14. Wash pellet with 200 µL ice-cold 70% ethanol.
15. Centrifuge at top speed for 10 min.
16. Discard supernatant (*see* **Note 35**).
17. Allow DNA pellet to dry; then, resuspend in 50 µL dH_2O or TE buffer, pH 7.5.
18. Measure absorbance at 260 nm in a quart cuvet by first blanking the instrument with 1 mL H_2O, then adding 1–10 µL of the DNA preparation and shaking prior to taking its reading (*see* **Note 36**).
19. Store at 4°C for short-term storage and –20°C/–80°C in aliquots for long-term storage.

4. Notes

1. *See* Chapter 1 and guidelines of the Centers for Disease Control (CDC): http://www.cdc.gov/mmwr/preview/mmwrhtml/00042032.htm and http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5010a1.htm.
2. This concentration facilitates rapid cell growth. 5% FBS may be used for more controlled cell growth. Spinner HeLa cells can also be maintained in 10% horse serum, which is a less expensive alternative to FBS.
3. A crude stock of virus is unpurified lysate from infected cells. For permissive cell lines infected with vaccinia virus, a titer of approx 10^8 pfu/mL virus can usually be obtained.
4. The swinging bucket centrifuge is used to harvest large volumes of infected cells. The high-speed centrifuge, which reaches a maximum speed of 10,000*g*, is for spinning down the dounce homogenate. The ultracentrifuge, which reaches speeds of more than 100,000*g*, is needed to pellet the virus through a sucrose cushion and for zonal sucrose gradient centrifugation.
5. For example, to make the sterile 25% sucrose solution, 25 g sucrose is weighed out in a graduated cylinder, and the volume is brought to 100 mL with 10 m*M* Tris. This solution is then filter-sterilized.
6. An egg candler should be available from egg or poultry farm suppliers.
7. An egg pricker is available at commercial retailers for the poaching of eggs.
8. Do not use phenol if pink. Use phenol only in a fume hood. Wear gloves when handling phenol.
9. Although HeLa cells can be obtained from ATCC, the author obtained his starting aliquot from Norman Cooper in the laboratory of Bernard Moss at the National Institutes of Health (NIH).
10. A notable exception for its inability to grow in HeLa cells is vaccinia virus strain MVA, which is grown in either chicken embryo fibroblasts (CEF) or BHK21 (*see* Chapter 7).
11. As an alternative to infecting and growing the virus in HeLa cells while in suspension, some laboratories plate spinner cells into flasks prior to infecting.

Some believe that the virus yields are improved when infecting monolayers. After 48 h of infection, most cells dislodge from the plastic and can therefore be easily harvested by combining all the cell suspensions and pelleting the cells by centrifugation. Obviously, the number of flasks can become quite large if growths of 1–4 L spinner HeLa are desired.

12. HeLa S3 are maintained at 37°C and spun at 1000 rpm. Watch to ensure that the stirring has not stopped so that clumping is prevented and to ensure proper aeration. Cells are split on a regular basis to keep the density no higher than 5×10^5 cells/mL. To avoid accidental viral contamination, it is best not to split the HeLa stock after virus use in the biosafety hood.
13. Semipurified or band-purified virus should be used for in vivo studies. Band-purified virus can be used when pure viral DNA is required.
14. Cell density is monitored by counting cells using a hemocytometer. For counting, equal volumes of cell suspension are mixed with trypan blue solution, and live cells are counted. If the proportion of dead cells is higher than 20%, then there could be a problem of contamination or overgrowth. Also, test for mycoplasma on a regular basis.
15. Viral factories and clumps of the virus are formed during the life cycle; therefore, a trypsinization or sonication step is absolutely essential to obtain free virus. If trypsin is undesirable because it may affect subsequent use, only sonication can be used.
16. It is necessary to be gentle to avoid lysing or shattering the cell nuclei.
17. During douncing, the douncer should be kept cooled on ice to prevent undo heating (and inactivation) of the virus. As the cells successfully break open, the suspension should become viscous and increasingly difficult to go through the douncing cycles.
18. These large growths are best sonicated with the use of a probe sonicator. *See* Chapter 1 for safety issues related to such a procedure. Smaller volumes can be sonicated in a cup sonicator.
19. Alternatively, a gradient maker can be used to make a 25–45% continuous sucrose gradient.
20. For example, cowpox virus (Brighton) forms hemorrhagic—appearing pocks, whereas vaccinia virus (e.g., Lister) forms white pocks ***(5)***. This reference also contains pictures of harvested membranes with pocks.
21. Not all poxviral strains grow equally well in embryonated hens eggs. Thus, it is advisable to make a dilution series of the poxviral stock to determine which viral titer produces the best pock confluency in the CAM. Very high titers may kill the membrane, whereas concentrations too low may result in poor pock confluency.
22. Attenuated strains of the virus may require an additional 24 h for good pock formation and higher viral titer production.
23. For example, for six membranes in one tube, add 3 mL McIlvain's buffer and 1 mL Arklone-X. These organic solvents help prevent aggregation of viral particles
24. The alkaline pH of the TE buffer helps prevent aggregation of viral particles in the solution.

25. We typically use 12–24 eggs at a time. Depending on the poxviral strain, one egg can produce a crude virus stock with a titer of up to 10^8 pfu/mL.
26. BSC-1 cells are the most commonly used cell lines for titering vaccinia virus. These cells form good monolayers and remain adherent after infection with vaccinia virus (WR strain).
27. Virus suspension should be vortexed and sonicated before titration to disrupt aggregated virus particles.
28. There are some strains of vaccinia virus (e.g., IHDJ) that release large amounts of extracellular enveloped virus (EEV). Such viruses do not form round plaques under liquid overlay; therefore, it is necessary to overlay them with semisolid overlay for accurate titering (*see* Chapter 15).
29. It is often best to stain cells infected with the WR strain at approx 36 h postinfection. Allowing infection to proceed longer under liquid overlay often results in the formation of satellite plaques from released EEV.
30. Making 1.2-mL dilutions, then transferring 1 mL from each dilution tube into a well may be preferred for greater accuracy.
31. Example titer: 100 plaques in a well infected with a 1:100,000 viral dilution indicates a viral titer of 1×10^7 pfu/mL viral suspension.
32. Do not vortex at any stage during the extraction procedure.
33. The virus is infectious until it is treated with lysis buffer. Thus, after the lysis buffer is added in the hood, it is safe to continue work outside the BSL-2 laboratory.
34. For example, 2 µL proteinase K solution per 100 µL virus suspension. Therefore, add 2 µL proteinase K solution to the 200 µL virus suspension/lysis buffer mixture.
35. Pipet off supernatants gently and consider putting in a clean tube to avoid accidental loss of the pellet.
36. At 260 nm, 1 OD corresponds to 50 µg/mL. For example, an OD_{260} reading of 0.05 for a 1:100 viral DNA dilution indicates a DNA concentration of ($50 \times 100 \times 0.05$) = 250 µg/mL. Alternatively, to quantitate minute quantities of DNA, use commercially available kits.

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9

Rapid Preparation of Vaccinia Virus DNA Template for Analysis and Cloning by PCR

Rachel L. Roper

Summary

This chapter describes the preparation of template DNA from poxvirus-infected cells, plaques, or crude virus stocks for polymerase chain reaction (PCR) amplification. The advantages of this technique are that it is rapid, inexpensive, and, most importantly, reliable, requiring only centrifugation, detergent, and protease treatment. The template preparation is suitable for PCR amplification for screening viruses, cloning, transfection, and DNA sequencing.

Key Words: DNA purification; PCR; virus preparation; virus screening; cloning; DNA sequencing; vaccinia; poxvirus.

1. Introduction

It is often desirable to screen for recombinant viruses or amplify genes for cloning from unpurified poxvirus (crude stock preparations, plaques, or infected cells) by polymerase chain reaction (PCR). This chapter describes simple, rapid, robust, inexpensive, and reproducible protocols for the preparation of template DNA from vaccinia virus-infected cells for PCR without the need for DNA purification. There are many chemical procedures and commercial columns available for DNA purification. However, some chemicals commonly used are hazardous, and the column kits are expensive. One of the main advantages of PCR is that it can amplify specific sequences from unpurified DNA. It has been estimated that each vaccinia virus-infected cell contains approx 10,000 copies of the poxvirus genome; thus, ample template DNA is present in a single cell ***(1)***. Yet, simply lysing infected cells does not consistently yield a product that can be used as a PCR template. The drawback is that cells contain known and unknown inhibitors of the PCR amplification. The protocols described here provide methods to remove, denature, or degrade cellular inhibitors of PCR in three simple steps. The first step is the removal of

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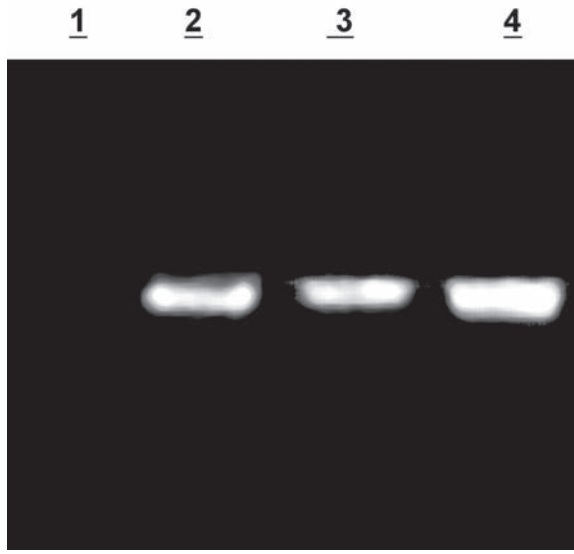

Fig. 1. Example of DNA amplification by protocol A. Crude virus stocks of rabbitpox (lane 2), cowpox (lane 3), and vaccinia virus (lane 4) were prepared from BSC1-infected cells, and the template was prepared for PCR using protocol A. PCR amplification was performed using *Taq* under standard reaction conditions, and the primers were conserved in all three viruses. Negative control (lane 1) contained no template DNA. PCR products were analyzed on agarose gel.

cellular debris after cell lysis. It is possible to successfully amplify DNA from centrifuged lysed infected supernatants, but it is not possible to amplify DNA from a whole crude preparation of virus-infected cells or cell pellets. Second, the addition of PCR-compatible detergents (e.g., NP-40 or IGEPAL), Tween-20, Triton X-100) is required ***(2)***. The detergents may act to release bound proteins from the template DNA, denature DNAses or proteins that bind dNTPs or cations, or disrupt the virus particles (although ample cytoplasmic DNA is likely present). Third, proteinase K is used to degrade inhibitory proteins. Finally, proteinase K must be heat-inactivated so that the *Taq* (or other DNA polymerases) is not degraded. PCR products generated by this method may be used for screening recombinant viruses and are compatible for use in DNA cloning, transfection assays, or DNA sequencing ***(3–8)***. This technique has been successfully employed with vaccinia, rabbitpox, cowpox, and ectromelia viruses ***(3–8)***, suggesting that it is widely applicable to poxviruses (*see* **Fig. 1**).

Two protocols are described: (1) template preparation for PCR amplification from a crude virus stock, the type commonly found in a poxvirus laboratory; and (2) describing template preparation directly from an infected tissue

culture well. The preparation of crude vaccinia virus stocks has been described in detail elsewhere (*see* **Note 1**). The second technique describes the direct aspiration of cells and medium (or agarose) from an infected cell area and has been used successfully on virus grown in cell culture for 8–72 h postinfection. Single plaques picked from under liquid medium or in agarose plugs have also been successfully PCR-amplified by this method. When virus is prepared from fresh infected cells rather than a crude stock, detergent and proteinase K are added first to disrupt the cell and release viral DNA. The centrifugation step is done after to remove the cellular debris. The advantage of this technique is that an infected well can be tested without terminating growth of the virus in the well.

2. Materials

Make sure all reagents are sterile, pure, and PCR-grade because this material will ultimately be used as a template in a PCR reaction.

1. Detergent solution: 0.9% NP40 or IGEPAL (Sigma), 0.9% Tween, 20 m*M* Tris-HCl, pH 8.3, 3 m*M* $MgCl_2$, 100 m*M* KCl, stored at –20°C.
2. Proteinase K at 5 mg/mL, stored at –20°C.
3. Water bath at 37°C or 45°C.
4. Heating block at 94°C.
5. Tris solution: 10 m*M* Tris-HCl, pH 9, filter-sterilized.

3. Methods

3.1. Protocol A: Amplification from a Crude Virus Preparation (Fig. 2)

Note that the endproduct is used as a PCR template. Autoclave all plastics and use sterile solutions and techniques to avoid contamination. Procedures are as follows:

1. Centrifuge the crude virus preparation in a microfuge on high speed at 14,000*g* for 10 s to pellet any cellular debris. The amount of crude virus preparation in the tube is not important if there is sufficient volume to remove 10 µL supernatant (*see* **Note 2**).
2. Remove 10 µL supernatant and dispense into a fresh microfuge tube.
3. Add 10 µL detergent solution: 2X PCR buffer containing 0.9% NP40 or IGEPAL and 0.9% Tween-20, stored at –20°C (*see* **Notes 3–5**).
4. Add 1.2 µL of a 5 mg/mL solution of proteinase K, stored at –20°C.
5. Incubate for 30 min to 1 h at 37°C or 30 min at 45°C (*see* **Note 6**).
6. Heat-inactivate the proteinase K for 10 min at 94°C (*see* **Note 7**).
7. Centrifuge in a microfuge for 5 s to collect the reaction mixture into the bottom of the tube.
8. Use 10 µL of this preparation in a 100-µL PCR reaction following standard PCR protocols (*see* **Notes 8** and **9**).

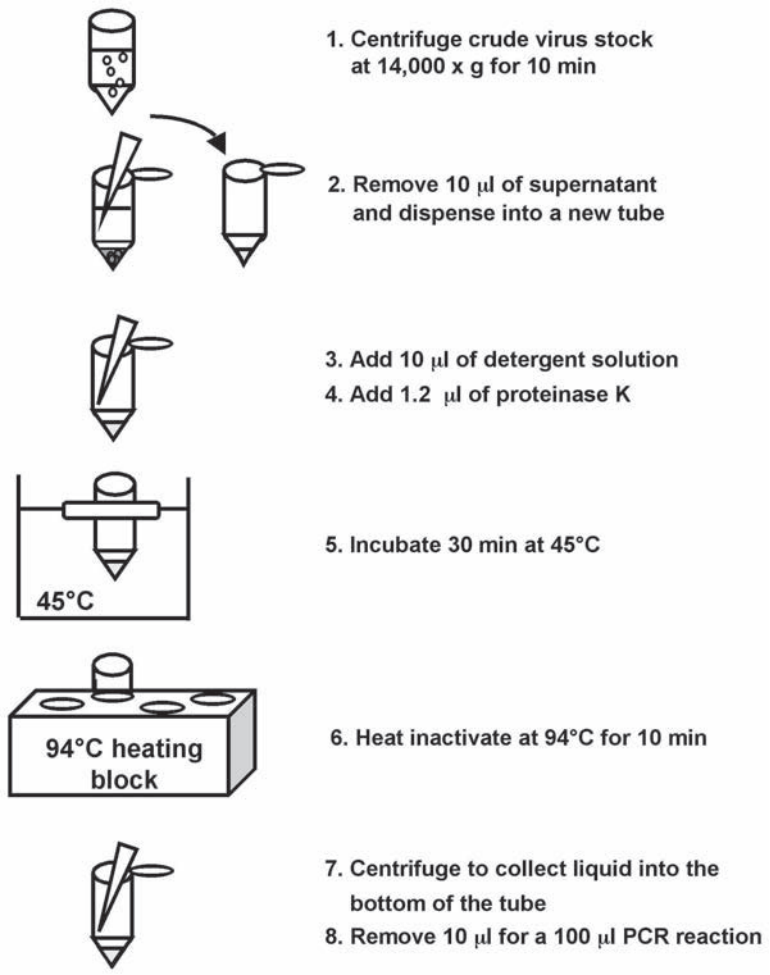

Fig. 2. Summary of protocol A. DNA template preparation from a crude virus stock in a 1.5-mL microfuge tube.

3.2. Protocol B: Amplification from Virus from an Infected Well or a Single Plaque

As noted, the endproduct will be used as a PCR template. Autoclave all plastics and use sterile solutions and techniques to avoid contamination.

1. Scrape a micropipet tip through an infected area of cells while aspirating 10 µL medium and cells. Dispense the 10 µL into a microfuge tube. Or pick a plaque in an agarose plug and put it into 30 µL media or Tris solution.
2. Vortex and remove 10 µL for the following steps, reserving the remaining sample for virus amplification.

3. Add 10 μL detergent solution: 2X PCR buffer containing 0.9% NP40/IGEPAL and 0.9% Tween-20, stored at –20°C (*see* **Notes 3–5**).
4. Add 1.2 μL 5 mg/mL solution of proteinase K, stored at –20°C.
5. Incubate for 30 min to 1 h at 37°C or 30 min at 45°C (*see* **Note 6**).
6. Heat-inactivate the proteinase K for 10 min at 94°C (*see* **Note 7**).
7. Centrifuge the microfuge tube on high at 14,000*g* for 10 s to remove cellular debris.
8. Pipet out 10 μL of this supernatant to use in a 100-μL PCR reaction following standard PCR protocols (*see* **Notes 8** and **9**).

4. Notes

1. Cells: This technique has been used on several mammalian cell lines, including HeLa, and BSC1 cell lines used commonly for vaccinia growth. Although numerous cell lines have not been screened, there has not been any cell line tested where this technique does not work.
2. For the purposes of this chapter, a crude virus stock is defined as virus-infected cells that have been concentrated approx 10 times relative to the volume in which the cells were grown during virus amplification (e.g., 20 mL culture grown in one T75 flask makes 2 mL crude stock, or one well of a six-well plate makes 100 μL crude virus preparation), and frozen and thawed three times to break open the cells. Ideally, every cell has been infected. The crude virus preparation may be resuspended in medium (e.g., minimum essential media), and the presence of 10% fetal bovine serum does not interfere with these protocols. Do not sonicate the crude virus preparation prior to performing this PCR-prep protocol because sonication breaks DNA.
3. Detergents: The detergent solution can be made with either NP-40 (now sometimes difficult to purchase) or IGEPAL (Sigma). If an alternate detergent is desired, a solution of 2% Triton X-100 in 2X PCR buffer (20 m*M* Tris-HCl, 3 m*M* $MgCl_2$, 100 m*M* KCl, pH 8.3) was found to be as effective as the combination of 0.9% NP40/IGEPAL and 0.9% Tween-20 in most, but not all, cases. Special care should be taken to avoid the introduction of sodium dodecyl sulfate (SDS), or other inhibitors of the PCR reaction or polymerases.
4. Commercial PCR buffers may be used to make up the detergent solution. For this protocol, the detergents are dissolved in 2X PCR buffer. Thus, when the detergent is mixed with the infected cell preparation, the final PCR concentration is 1X, as this will be directly introduced into the PCR reaction. The user should take this into account when preparing the PCR reaction to not add too much 10X PCR buffer.
5. For analyzing multiple reactions simultaneously, it is convenient to make a master mix of detergents and proteinase K in the proper proportions immediately prior to use, aliquot the mix into microfuge tubes, and then add virus samples. However, be careful with these techniques. The virus material being prepared provides the template in a PCR reaction. Contamination by any means should be strictly avoided.

6. The length of the protease digestion does not seem to be crucial. Times and temperatures known to work have been provided as guidelines.
7. Incubations: Normally, the detergent and proteinase K solution is incubated in a 45°C water bath, and the heat-inactivation step is incubated in a 94°C heating block. However, either heating method should suffice if the required temperature is maintained. Be careful not to allow water to contaminate the lip of the microfuge tube in a water bath, and ensure there is good contact between the microfuge tube and the walls of the heating block. Also, make certain that the 94°C heat-inactivation of proteinase K proceeds for the full 10 min. Lower incubation temperatures or shorter times may allow the remaining active proteinase K to digest the PCR polymerase enzyme.
8. Quantity of template preparation: 10 µL virus DNA template prepared by this method should be used in a 100-µL PCR reaction or 5 µL in a 50-µL PCR reaction. One microliter has also been found to be a sufficient template.
9. Samples prepared by this method work best when used for PCR within a few days (store at 4°C). To test primers and PCR reaction conditions, use primers on purified viral DNA or an appropriate plasmid as positive control templates. If there is no PCR product, try using less template mixture per PCR reaction. Often, the use of more template mixture increases inhibitory contaminants with a concomitant reduction in the PCR product. The most common problem experienced with this protocol is the omission of steps. If any step is omitted, the procedure will not work. Each step has been tested and is required for this protocol.

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10

Orthopoxvirus Diagnostics

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Summary

Biologic and antigenic properties are often useful for identifying and differentiating orthopoxviruses (OPV). However, polymerase chain reaction (PCR) amplification, with either restriction cleavage or sequencing of amplicons, has been gaining credibility as a more rapid, specific, sensitive, and often cost-saving technique for research and diagnostic laboratories. This chapter is consolidated using prior research papers from our laboratories with three different methods that should be suitable for the preparation of orthopoxvirus DNA from various sources (e.g., clinical specimens or cell cultures) and four different methods for PCR that should be useful for investigating orthopoxvirus species and strains.

Key Words: Orthopoxvirus; polymerase chain reaction (PCR); diagnostic; DNA preparation; hemagglutinin gene; A-type inclusion body gene; restriction fragment length polymorphism (RFLP); smallpox; variola virus; monkeypox virus; cowpox virus; vaccinia virus; camelpox virus; ectromelia virus; mousepox virus.

1. Introduction

The family *Poxviridae* is divided into two subfamilies: the *Entomopoxvirinae* of insects and the *Chordopoxvirinae* of vertebrates. The latter consists of eight genera and several unclassified viruses. The genus *Orthopoxvirus* comprises morphologically and antigenically closely related viruses, including the now eradicated variola (smallpox) virus and several pathogens of veterinary and zoonotic importance ***(1)***. Orthopoxviruses (OPVs) are allocated into 11 species, eight Eurasian-African species (variola, monkeypox, vaccinia, cowpox, camelpox, ectromelia, taterapox, and Uasin Gishu disease virus) and three North American species (raccoon poxvirus, volepox virus, and skunkpox virus). DNA maps and several other molecular biologic features, including full genome sequencing, have been determined to various extents (***2***; *see also* Chapter

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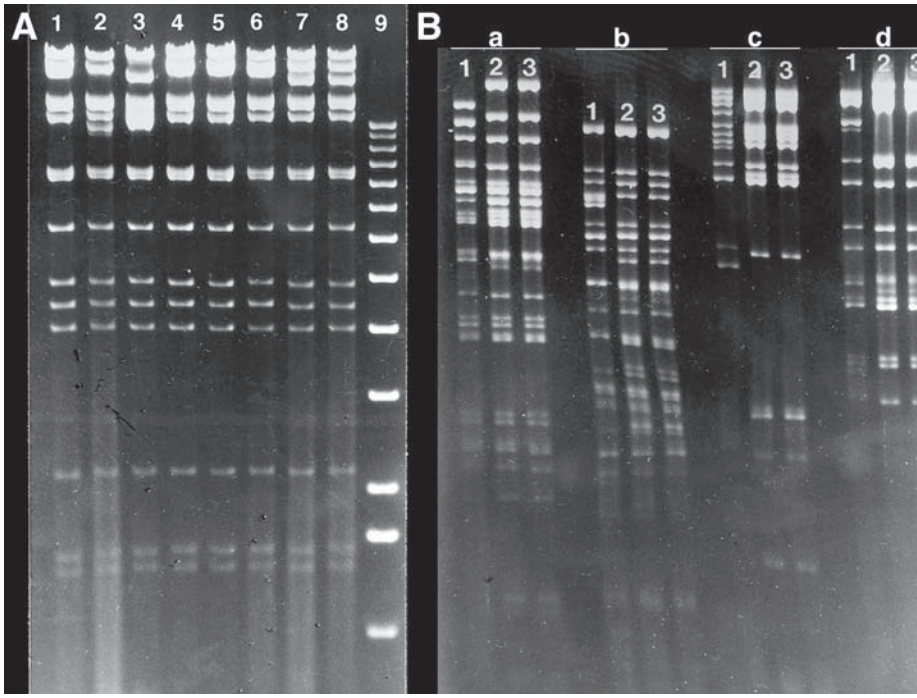

Fig. 1. Electropherogram of different vaccinia viruses and a human orthopoxvirus isolate. **(A)** Genome DNAs were digested with *Hin*dIII. Lanes (1) buffalopox virus BP-1, (2) vaccinia virus Elstree, (3) vaccinia virus M1, (4) vaccinia virus IHD-J, (5) human orthopoxvirus isolate OPV 85, (6) vaccinia virus Levaditi, (7) vaccinia virus Western Reserve, (8) rabbitpox virus Utrecht, (9) 1-kb ladder weight marker. **(B)** Electropherograms of (1) vaccinia virus Levaditi, (2) human orthopoxvirus isolate OPV 85, and (3) vaccinia virus IHD-J. Genome DNAs were digested with (a) *Bam*HI, (b) *Eco*RI, (c) *Sal*I, and (d) *Xho*I.

23 and **Note 1**). Identification and differentiation have been achieved by different methods, including restriction endonuclease cleavage site mapping ***(3–5)***, which has provided definitive information for virus classification in support of immunologic and biologic criteria. Recently, polymerase chain reaction (PCR) methods have been applied to rapidly identify and subtype available OPVs (***6–10***; *see also* **Note 2**).

This chapter encompasses prior research papers from our laboratories ***(3–11)***: three procedures found useful for preparing OPV DNA (*see* **Subheadings 3.1.–3.3.**) and four suitable protocols that use gel electrophoresis for PCR identification and differentiation of OPVs (**Subheadings 3.4.–3.7.**). Viral DNA extracted according to the first method (**Subheading 3.1.**) is more useful for

large-scale DNA preparations, generally for sequencing and restriction enzyme fragment length polymorphism (RFLP) analysis. An example of the latter is given in **Fig. 1A**, which depicts an electropherogram of *Hin*dIII-digested genomic DNA of various vaccinia viruses (VACV), and an OPV (OPV 85) isolated from a lesion on the hand of a laboratory worker who handled various VACV strains. To identify the virus strain that caused the infection, we compared the *Hin*dIII RFLP pattern of OPV 85 with those of different VACVs in the laboratory. Although the patterns resemble each other considerably, there are differences caused by size variation of the second and third largest digest fragments. Identical patterns of OPV 85 can be observed for VACV strains Levaditi and IHD-J (lanes 4–6, respectively). Therefore, further RFLP analyses were conducted using these strains and *Bam*HI, *Eco*RI, *Sal*I, and *Xho*I restrictases (**Fig. 1B**). In each case, identical RFLP patterns were observed for OPV 85 and VACV IHD-J, thus indicating the infection was with the IHD-J strain. The results demonstrate the usefulness and level of precision that RFLP analyses provide for strain or virus isolate identification.

DNA prepared by all three procedures described in this chapter is suitable for PCR analysis. However, DNA can be prepared relatively quickly from infected chick embryo chorioallantoic membranes (*see* Chapter 8) or various clinical samples (e.g., crusted scab, dried vesicle fluid of lesions; *see* **Note 3**) by using the steps described in **Subheading 3.3.** or from infected cell cultures by using the steps in **Subheading 3.2.** The four PCR protocols presented here should enable relatively simple and accurate detection and differentiation of OPVs, as judged from a limited number of analyses with field samples to date (*see* **Notes 4** and **5**).

2. Materials

2.1. Large-Scale Preparation of Poxvirus DNA from Cytoplasm of Infected Tissue Culture Cells

1. TSE buffer: 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 5 m*M* Na_2EDTA.
2. TKE buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* KCl, 5 m*M* Na_2EDTA.
3. 10% Triton X-100. Filter through 0.45-μm nitrocellulose membrane.
4. 14.3 *M* 2-mercaptoethanol.
5. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* Na_2EDTA.
6. 54% (w/w) sucrose.
7. 10 mg/mL proteinase-K, stored in aliquots at –20°C.
8. 10% sodium dodecyl sulfate (SDS; genetic technology grade).
9. 5 *M* NaCl.
10. Phenol:chloroform:isoamyl (25:24:1) alcohol mixture (genetic technology grade phenol is washed three times with TE buffer, then combined with chloroform and isoamyl alcohol).

11. Chloroform:isoamyl (24:1) alcohol mixture.
12. BPB dye: 0.25% bromphenol blue in 40% (w/v) sucrose.
13. SeaKem-GTG agarose (FMC BioProducts, Rockland, ME).
14. TAE buffer: 40 m*M* Tris-HCl, 5 m*M* sodium acetate, 1 m*M* Na_2EDTA, pH 8.0, prepared from 50X concentrate. (Prior to use, for 1X buffer, add ethidium bromide solution [3 mg/mL] to 0.5 μg/mL; wear gloves.)
15. 1-kb Ladder DNA marker (Gibco-BRL, Gaithersburg, MD).
16. Restriction endonucleases (store at –20°C) and appropriate 10X reaction buffers (Boehringer-Mannheim, Germany).

2.2. Small-Scale Preparation of Poxvirus DNA by Lysis of OPV-Infected Cells for Use in PCR

1. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* Na_2EDTA, 0.5% Tween-20. (Prior to use, add proteinase K to a final concentration of 50 μg/mL.)

2.3. Preparation of Poxvirus DNA from Clinical Samples (See Note 3)

1. Microfuge tube pestle (Kontes, Inc., Vineland, NJ).
2. Lysis solution: 50 m*M* Tris-HCl, pH 8.0, 100 m*M* Na_2EDTA, 100 m*M* NaCl, 1% SDS.
3. 20 mg/mL Proteinase-K, stored in aliquots at –20°C.
4. Phenol:chloroform:isoamyl (25:24:1) alcohol mixture and chloroform:isoamyl (24:1) alcohol mixture (same as in **Subheading 2.1.**).
5. 100% ethanol.
6. 70% ethanol.

2.4. Detection and Differentiation of OPV by ATI-PCR Assay

1. DNA prepared as described above.
2. Primers: ATI-up-1, 5'-AATACAAGGAGGATCT-3', and ATI-low-1, 5'-CTT AACTTTTTCTTTCTC-3'.
3. GeneAmp PCR Reagent Kit (Perkin-Elmer, Norwalk, CT).
4. NuSieve-GTG and SeaKem-GTG agarose (FMC BioProducts).
5. *Bgl*II and *Xba*I (store at –20°C) and appropriate 10X reaction buffers (Boehringer-Mannheim).

2.5. Detection and Differentiation of OPV by Using Hemagglutinin-PCR Assay (HA-PCR)

1. DNA prepared as described above.
2. Eurasian-African OPV-HA consensus primers EACP1, 5'-ATGACACGA TTGCCAATAC-3', and EACP2, 5'-CTAGACTTTGTTTTCTG-3'.
3. North American OPV-HA consensus primers NACP1, 5'-ACGATGTCG TATACTTTGAT-3', and NACP2, 5'-GAAACAACTCCAAATATCTC-3'.
4. GeneAmp PCR Reagent Kit (Perkin-Elmer).
5. NuSieve GTG and SeaKem GTG agarose (FMC BioProducts).
6. *Rsa*I and *Taq*I, store at –20°C (Boehringer-Mannheim).

2.6. Detection and Differentiation of OPV by Using Species-Specific PCR

1. DNA prepared as described above.
2. Thermocycler (e.g., Perkin-Elmer, model 9600).
3. 25 m*M* $MgCl_2$.
4. GeneAmp PCR Reagent Kit (Perkin-Elmer).
5. NuSieve GTG and SeaKem GTG agarose (FMC BioProducts).
6. Variola-specific primers VAR1, 5'-TAAATCATTGACTGCTAA-3', and VAR2, 5'-GTA-GATGGTTCATTATCATTGTG-3'.
7. Vaccinia-specific primers VAC1, 5'-ATGCAACTCTATCATGTAA-3', and VAC2, 5'-CATAATCTACTTTATCAGTG-3'.
8. Camelpox-specific primers CML1, 5'-GCCGGTACTTATGTATGTGT-3', and CML2, 5'-GATCTTCTTCTTTATCAGTG-3'.
9. Monkeypox-specific primers MPV1, 5'-CTGATAATGTAGAAGAC-3', and MPV2, 5'-TTGTATTTACGTGGGTG-3'.
10. Ectromelia-specific primers ECT1, 5'-CATACAGTCACAGACACTGTTG-3', and ECT2, 5'-GATGCTTTCTACAGTTGTTGGTA-3'.
11. Cowpox-specific primers CPV1, 5'ATGACACGATTGCCAATACTTC-3', and CPV2, 5'-CTTACTGTAGTGTATGAGACAGC-3'.
12. Taterapox-specific primers GBL1, 5'-CGTCGGTATTCGAAATCGCGAA-3', and GBL2, 5'-GTTTTGTATTTACGTGAACGG-3'.
13. Raccoon poxvirus-specific primers RCN1, 5'-GATGATACGCAATATAATGT-3', and RCN2, 5'-TCTACCGTTGTTGGTATCGAG-3'.
14. Skunkpox virus-specific primer pair SKP1, 5'-AGTTCTGCTAATATCGCTAG-3', and SKP2, 5'-AGTGGTTGTGGGAGCAGTGG-3'.
15. Volepox virus-specific primers VPX1, 5'-CCATCACCAGAAGTAGTTGCAG-3', andVPX2, 5'-ATATGTGCTCCATATGAACT-3'.

2.7. Detection of Monkeypox Virus Using PCR

1. DNA prepared as described above.
2. Primers: Gabon-1, 5'-GAGAGAATCTCTTGATAT-3'; and Gabon-2, 5'-ATT CTAGATTGTAATC-3'.
3. GeneAmp PCR Reagent Kit (Perkin-Elmer).
4. SeaKem-GTG agarose (FMC BioProducts).
5. *Bgl*II (store at –20°C) and appropriate 10X reaction buffer (Boehringer-Mannheim).

3. Methods

3.1. Large-Scale Preparation of Poxvirus DNA from Cytoplasm of Infected Tissue Culture Cells

1. Prepare 10 150-cm^2 culture flasks cell containing monolayers of monkey kidney or other suitable cell line grown to 95% confluence (*see* **Note 6**).
2. Infect flasks using a virus input multiplicity of infection (moi) of 0.1–0.01 pfu/cell, and incubate at 37°C until severe cytopathic effect is evident (usually 3 d).

3. After chilling cultures at 4°C for 2–16 h, scrape infected cells from the tissue culture flask.
4. Transfer cell suspension to conical 50-mL centrifuge tubes. Collect cells by centrifugation for 10 min at 500*g* at room temperature.
5. Suspend cells in 100 mL TSE buffer, and centrifuge into a pellet as in **step 4**.
6. Suspend the pellet in 18 mL TKE buffer at 4°C for 10 min by gently vortexing the mixture for 15 s every 5 min.
7. Add 2 mL 10% Triton X-100, mix gently. Add 50 μL 2-mercaptoethanol, then mix gently and chill mixture on ice.
8. Vortex the chilled mixture slowly after 5 min and again at 10 min. Observe cells for cytoplasmic lysis by use of a microscope.
9. Remove nuclei by centrifugation for 10 min at 1500*g* at 4°C.
10. Transfer the supernatant (cytoplasmic extract) to a fresh polycarbonate tube.
11. Concentrate viral cores by centrifugation for 30 min at 20,000*g* at 4°C.
12. Aspirate the supernatant and suspend the pelleted viral cores in 0.8 mL cold TE buffer by use of a syringe with a 20-gauge needle attached.
13. Add in order by gentle mixing: 1.4 mL 54% sucrose, 15 μL 2-mercaptoethanol, and 50 μL of 10 mg/mL proteinase-K. Incubate mixture on ice for 15 min.
14. Place tubes at 37°C, add 250 μL 10% SDS, mix gently, and incubate at 37°C overnight or at 50°C for 2–4 h.
15. Add 0.4 mL 5 *M* NaCl and mix gently.
16. Extract DNA from the digested lysate three times with an equal volume of phenol:chloroform:isoamyl alcohol mixture, using gentle manual shaking for several minutes or motor-driven rocker to avoid shearing the DNA. Separate the extraction phases at 8000*g* for 1 min, and remove phenol (bottom) phase using a Pasteur pipet.
17. Remove final phenol phase completely, and gently extract the aqueous phase twice using an equal volume of chloroform:isoamyl alcohol mixture.
18. Transfer aqueous phase to a small wetted dialysis tubing. Dialyze TE buffer overnight with at least two buffer changes. Transfer dialyzed DNA solution to a microfuge tube and add a few drops of chloroform to preserve DNA solution at 4°C.
19. Estimate the DNA concentration by measuring the optical density at 260 nm (1 OD_{260} = 45 μg DNA). DNA prepared by this method is suitable for restriction enzyme analysis or PCR assay.
20. Prepare a restriction digest reaction using approx 2 μg DNA in a total volume of 20 μL. Digest for 3 h to overnight at 37°C. Add 2 μL BPB dye solution prior to separating DNA fragments using a submerged 0.6% agarose gel in TAE buffer. Visualize gel stained with ethidium bromide and photograph.

3.2. Small-Scale Preparation of Poxvirus DNA by Lysis of Infected Tissue Culture Cells for Use in PCR (See Note 7)

1. Infect a 25-cm^2 cell monolayer (monkey kidney or other suitable cell line) grown to 95% confluence with a virus moi of 0.1–0.01 pfu/cell and incubate at 37°C until severe cytopathic effect is evident (approx 3 d).

2. Harvest infected cells from the surface by scraping into the medium.
3. Remove 100 µL of this infected cell suspension and mix with an equal volume of lysis buffer.
4. Incubate at 56°C for 1 h.
5. Inactivate the proteinase K in the lysate by incubating at 100°C for 15–20 min and chill on ice.
6. Use 1–2 µL of this preparation for each PCR assay.

*3.3. Preparation of Poxvirus DNA from Clinical Samples (***See *Note 3)***

1. Suspend a small aliquot of crusted scabs in 90 µL lysis solution, add 10 µL proteinase K, and digest for 10 min at 37°C.
2. Disrupt the scab with a microfuge tube pestle.
3. Add another 350 µL lysis solution and 50 µL of 20 mg/mL proteinase K, mix gently, and then incubate for 2 h at 37°C.
4. Extract the lysed suspension twice with an equal volume of phenol:chloroform: isoamyl alcohol mixture. Separate the extraction phases at 8000*g* for 1 min.
5. Aspirate the phenol (bottom) phase completely and extract the aqueous phase twice with equal volumes of chloroform:isoamyl alcohol mixture.
6. Transfer aqueous phase to a new tube and add 2 vol cold absolute ethanol.
7. Place tube at –70°C for approx 30 min to precipitate DNA.
8. Collect the DNA precipitate by centrifugation at 15,000*g* for 5 min at 4°C.
9. Aspirate the supernatant completely and wash the pellet with 70% ethanol by centrifugation (8000*g* for 1 min).
10. Air-dry DNA for a few minutes at room temperature and dissolve the DNA pellet in 10 µL H_2O.

3.4. Detection and Differentiation of OPV by ATI-PCR Assay

The ATI-PCR assay ***(6)*** was accomplished by the selection of two oligonucleotides that correspond to sequences within the gene that encodes the acidophilic or A-type inclusion protein (ATI) of the cowpox virus. The ATI protein is truncated in cells infected with the variola, vaccinia, monkeypox, or camelpox virus. The primer pair described is based on sequences flanking a region that exhibits various deletions in a comparison of corresponding sequences determined for vaccinia, ectromelia, monkeypox, camelpox, and variola viruses. Thus, depending on which OPV species is being examined, the ATI-PCR method provides a DNA fragment of distinct size (**Table 1**). The steps described here, combined with *Xba*I digest electrophoresis of the resultant fragment, has enabled correct species assignment of 73 different virus isolates already known to belong to eight different OPV species (*see* **Table 1** and **Note 8**).

1. Prepare template DNA from virions, chemical samples, or infected cells as outlined in **Subheadings 3.1.–3.3.**

Table 1
Fragment Sizes of Amplicons from 76 Different OPV DNAs Amplified by PCR Using Primer Pair ATI-up-1 and ATI-low-1 and Sizes of *Xba*I Amplicon-Digest Fragments

Species	Amplicon size (bp)	*Xba*I digest fragments (bp)	No. of specimens tested
Eurasian-African			
Variola	1582	1269, 144, 100, 69	6
Cowpox	1673 or 1601	645, 515, 344, 100, 69 or	10
		645, 344, 299, 216, 100, 69 or	4
		645, 443, 344, 100, 69	8
Vaccinia	1615	907, 323, 216, 100, 69	4
Monkeypox	1517 or 1067	834, 306, 144, 100, 69, 64 or	8
		384, 306, 144, 100, 69, 64	4
Ectromelia	1220	575, 341, 153, 151	9
Camelpox	879	494, 216, 100, 69	20
North American			
Raccoon poxvirus	None		1
Volepox virus	510	356, 154	1
Skunkpox virus	530	530	1

2. In a 0.5-μL microcentrifuge tube, add the following for a total reaction volume of 100 μL: 2 μL of each dNTP, 10 μL 10X PCR buffer, 60 ng each of primer pair ATI-up-1 and ATI-low-1, and appropriate volume H_2O. Then, add 50 ng template DNA and 2.5 U *Taq* polymerase.
3. Place sample into a thermocycler. Heat mixture for 6 min at 94°C and cycle 24–29 times for 1 min at 94°C, 1 min at 45°C, and 2.5 min at 72°C. Heat for 10 min at 72°C and cool reaction at 4°C until analysis.
4. For analysis, mix 10 μL reaction mixture with 1 μL BPB dye solution and load onto 1% SeaKem-GTG submerged agarose gel in TAE buffer. Load a parallel lane with DNA-marker ladder. Separate the reaction products at 100 V. Visualize DNA and photograph.
5. To enhance the resolution of fragment size differences, add 5 U *Xba*I to 20 μL PCR mixture and incubate for 2 h at 37°C. Perform electrophoresis in a 3% NuSieve-GTG agarose-1% SeaKem-GTG submerged agarose gel. Visualize and photograph as mentioned previously (*see* **Note 8**).

3.5. Detection and Differentiation of OPV by Using HA-PCR Assay

The PCR assay for sequences encoding the hemagglutinin (HA) protein (HA-PCR) targets the open-reading frame (ORF) encoding for the HA glycoprotein *(7)*. This protein is a major component of the HA antigen, an infected

cell-membrane complex that distinguishes OPVs from all other poxvirus genera. A consensus-sequence primer pair was devised to amplify an HA-DNA fragment from genome DNA of known North American OPVs (raccoon poxvirus, skunkpox virus, and volepox virus) that are approx 75% identical in sequence to Eurasian-African OPVs. A second pair of consensus primers was devised to amplify an HA-DNA fragment from the genome DNA of Eurasian-African OPVs (variola, vaccinia, cowpox, monkeypox, camelpox, ectromelia, and taterapox viruses). Because no virus or DNA has been available for Uasin Gishu disease virus, which was reported in the 1970s ***(2)***, this was not included in our analysis. Using HA-PCR, *Rsa*I digest and electrophoresis of the HA-PCR product from the North American OPVs enhances resolution of fragment differences. *Taq*I digestion enables identification and differentiation of the seven Eurasian-African OPVs. To date, 83 isolates have been verified (**Table 2**). Of interest, use of primers with no mismatches had further enabled precise distinction of individual virus DNAs. In this regard, Ropp and colleagues ***(7)*** showed amplification and discernment of individual virus species by using deliberately cross-contaminated viral DNA samples (*see* **Note 9**).

1. Prepare template DNA as outlined in **Subheadings 3.1.–3.3.**
2. Set up the reaction mixture as in **Subheading 3.4.**, **step 2**, except use 60 ng of each primer NACP1 and NACP2 or EACP1 and EACP2.
3. Place sample in a thermocycler. Heat mixture for 6 min at 94°C and cycle 24 times for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Heat for 10 min at 72°C and cool mixture at 4°C until analysis.
4. Mix 15 µL reaction mixture with 1.5 µL BPB dye solution and load into a submerged gel (3% NuSieve-GTG agarose-1% SeaKem-GTG agarose). Load a parallel lane with DNA marker ladder. Separate fragments by electrophoresis in TAE buffer. Visualize DNA and photograph.
5. To enhance the resolution of fragment size differences, restrictase-treat a portion of the HA amplicons of North American OPVs by using 5 U *Rsa*I, and for Eurasian-African OPVs, use *Taq*I in a 30-µL reaction volume (incubate 2 h at 37°C for *Rsa*I or at 65°C for *Taq*I). Perform electrophoresis in 3% NuSieve-GTG agarose-1% SeaKem-GTG agarose gel (include DNA size marker). Visualize and photograph (*see* **Note 8**).

3.6. Detection and Differentiation of OPV by Species-Specific PCR

This PCR assay also targets the *HA* gene; however, in this assay, a small region within the HA ORF is amplified, and the fragment size designates the species ***(7)***. For this method, a set of primers and modified PCR conditions had to be devised from a comparison of multiple aligned HA base sequences for 50 different OPV isolates and by various titrations to establish suitable PCR reagent concentrations (*see* **Note 10**).

Table 2
Fragment Sizes of Amplicons of 83 OPV DNAs Amplified by PCR Using Primer Pair NACP1 + 2 or EACP1 + 2, and Sizes of PCR Products After *Taq*I or *Rsa*I Restriction Cleavage

Species	Amplicon (bp) NACP1 + 2	Amplicon (bp) EACP1 + 2	Restrictase digest fragments (bp)	No. of specimens tested
Eurasian-African			*Taq*I	
Variola	None	942	536, 406	33
Vaccinia	None	948	451, 295, 105, 97	5
Camelpox	None	960	474, 331, 90, 75	4
Monkeypox	None	942	451, 220, 105, 91, 75	24
Ectromelia	None	846	343, 220, 111, 97, 75	3
Cowpox	None	942	303, 289, 115, 96, 91	9
Taterapox	None	960	342, 331, 97, 80, 75, 35	1
North American			*Rsa*I	
Raccoon poxvirus	652	None	194, 192, 153, 113	2
Volepox virus	580	None	467, 113	1
Skunkpox virus	658	None	264, 202, 113, 71, 8	1

Table 3
OPV-Specific Primer Pairs, dNTPs, and $MgCl_2$ for Detection and Differentiation of OPV by Species-Specific PCR (Subheading 3.6.)

Primer pair	m*M* dNTP	m*M* $MgCl_2$	ng each primer
MPV1 + 2	800	2.5	500
RCN1 + 2	800	2.5	500
VPX1 + 2	800	2.5	500
ECT1 + 2	800	1.5	500
VAR1 + 2	25	1.5	500
SKP1 + 2	25	1.5	100
GBL1 + 2	8	1.5	500
CPV1 + 2	8	1.5	250
CML1 + 2	8	1.5	25
VAC1 + 2	8	1.5	25

1. Into each of 10 separate microfuge tubes, add 10 µL 10X reaction buffer and appropriate amount of dNTPs, $MgCl_2$, and the respective OPV-specific primer pair as outlined in **Table 3**.
2. Add 50 ng template DNA and 2.5 U *Taq* polymerase (100 µL total volume).
3. Place sample in a thermocycler. Heat for 6 min at 94°C and cycle 24 times for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Heat for 10 min at 72°C and cool mixture to 4°C until analysis.
4. Mix 15 µL of the reaction products with 1.5 µL BPB dye solution and load onto 3% NuSieve-GTG agarose-1% SeaKem-GTG agarose submerged gel. In one lane, load 1-kb DNA ladder as size marker. Perform electrophoresis in TAE buffer. Visualize and photograph (*see* **Note 8**).

3.7. Detection of Monkeypox Virus by PCR

The PCR assay described here also targets sequences of the *ATI* gene, but is used to specifically identify monkeypox virus. It had been shown that an 8-bp deletion located within this gene is responsible for a frame shift and, consequently, for a premature stop when compared with those for other OPV *ATI* genes ***(9)***. This unique alteration enabled design of a primer pair that flanked that deletion and amplified a 601-bp fragment that specifically identified all 19 monkeypox virus strains examined. DNA of other OPVs was not amplified. Specificity of the amplicons is confirmed by cleavage with *Bgl*II, which produced three subfragments of expected sizes (*see* **Note 11**).

1. Prepare template DNA as outlined in **Subheadings 3.1.–3.3.**
2. Set up the reaction mixture as in **Subheading 3.4.**, **step 2**, except 60 ng each of primers Gabon-1 and Gabon-2.

3. Place sample into an automatic thermocycler. Heat for 6 min at 94°C and cycle 24 times for 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. Heat for 10 min at 72°C, then cool and store at 4°C until analysis.
4. Mix 15 μL reaction mixture with 1.5 μL BPB dye solution, and load onto submerged gel (1% SeaKem-GTG agarose). Load a parallel lane with marker DNA. Separate by electrophoresis in TAE buffer. Visualize DNA and photograph.
5. To confirm specificity of the amplicon, add 5 U *Rsa*I to 20 μL of the resulting PCR mixture and incubate 2 h at 37°C. Perform electrophoresis in 1% SeaKem-GTG agarose gel (include DNA size marker). Visualize and photograph (*see* **Note 8**).

4. Notes

1. We also refer the reader to the Internet site, www.poxvirus.org, which catalogs poxvirus genomes and constantly updates this information.
2. It is suggested that in addition to direct sequencing of the products from the four PCR methods described in this chapter, serologic and biologic tests, such as electron microscopy, virus growth morphology on chorioallantoic membranes of 12-d-old hens' eggs, and antigenic analysis ***(2,4,5)***, should be considered for reference identification of virus isolates suspected to represent new OPV species or strains.
3. Various clinical and research laboratory issues, such as sample handling, collection, triage, and other diagnostic test methods for the identification and differentiation of poxviruses can be found in **ref. *12***. Information is also available on the Centers for Disease Control (CDC) website: http://www.bt.cdc.gov/agent/smallpox/lab-testing/index.asp.
4. Knight and colleagues ***(8)*** described a PCR method where alastrim variola minor virus, which causes mild smallpox, can be distinguished from other variola viruses because of a distinctive sequence insertion within the right-end region of alastrim virus. However, Meyer et al. ***(13)*** used the protocol to investigate a collection of German cowpox viruses, and the alastrim-specific PCR product was amplified in 6 of 15 virus isolates tested. The amplicon size corresponded to the size of alastrim variola minor strains, and subsequent sequencing showed a sequence homology of approx 97%. The observation in certain cowpox virus isolates of what was thought to be an alastrim-unique sequence, and the fact that other variola viruses have part of the sequence, appears to support the view that variola virus derives from a cowpox virus-like progenitor. An alternative possibility is that somehow recombination events favoring sequence exchange from alastrim to cowpox occurred during alastrim introductions into Europe by infected travelers from the Americas.
5. In a recent article, Espy and colleagues ***(14)*** report the specific detection of variola virus DNA by real-time PCR using a LightCycler (Roche, Inc.). Mismatches in the so-called FRET probes used in this method enabled discrimination of variola virus from other OPVs by DNA melting curve analysis. However, the authors did not include DNA of camelpox virus in their assay. Preliminary studies (H. Meyer, unpublished data) indicate that the melting temperature for authentic

camelpox virus DNA is rather close to that described for variola virus and, therefore, most likely will crossreact with the probes. PCR using the LightCycler and other real-time instruments is rapid and may provide first-line support for the physician who suspects on clinical grounds that a patient has smallpox. Further validation using a large number of different OPV specimens, including variola virus DNA, provides information on the extent of efficacy, reproducibility, accuracy, and reliability of real-time PCR.

6. HeLa cells are also used either in flasks or in suspension for large-scale virus preparations (*see* Chapters 8 and 15).
7. *See also* Chapters 9 and 10 for another description of a method for PCR analysis of a small virus growth for PCR analysis useful when screening for recombinant viruses.
8. In applying the present protocols, we suggest that PCR be done first by using consensus primers (i.e., the ATI or HA primer pair; **Subheadings 3.4.** or **3.5.**). This procedure enables differentiation of species by RFLP profile differences of the PCR products. The primers that we developed appear to work well for a variety of purified virus DNA preparations, and they have been successfully used to examine several clinical samples (**Tables 1** and **2**). Investigation of various monkeypox virus strains by ATI-PCR has shown different sized amplicons of 1517-bp and 1067-bp, respectively. This difference is owing to a 450-bp deletion in strains originating from Central Africa, mainly the Democratic Republic of Congo, where monkeypox virus is somewhat endemic ***(2,11)***. This deletion does not appear in strains from Western African rainforest countries, where the disease occurs very sporadically. The results confirm earlier observations that monkeypox viruses can be subgrouped by RFLP ***(3,10)***. The size of the amplicons obtained for cowpox viruses accounts for either 1673 bp or 1701 bp, and this is caused by a 72-bp deletion ***(13)***. It should also be noted that the ATI primer pair fails to amplify DNA of the highly attenuated VACV strain MVA and strain Copenhagen because of extensive deletions in the ATI ORF.
9. Ropp et al. ***(7)*** also describe an alternative method of amplifying the HA ORF of different OPVs, using a set of primer pairs of more exact sequence specificity compared with the consensus primers described here. Although more experimentation is needed, the RFLP patterns observed when we used the set of primers with no mismatches were rather comparable to patterns observed when we used the consensus primers.
10. We currently suggest that the results obtained with this method should be interpreted with caution because the assay has not been rigorously evaluated as extensively as the HA- or ATI-PCR methods (**Subheadings 3.4.** and **3.5.**). However, work to improve the concept of this method is in progress. The specificity of the primers relies on the precise use of the PCR conditions described.
11. The monkeypox virus assay enables specific amplification of monkeypox virus only and has been successfully applied to several specimens from suspected cases of human monkeypox ***(11)***. The disease is associated with generalized rash, clinically undistinguishable from smallpox, caused by the variola virus, which is pres-

ently the major bioterror threat infectious agent because smallpox is eradicated. By applying the monkeypox virus-specific protocol, infection with variola virus can be ruled out.

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11

An In Vitro Transcription System for Studying Vaccinia Virus Early Genes

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Summary

Transcription of the vaccinia virus early genes occurs within the confines of the virion core structure. Therefore, isolated virions are a particularly rich source of proteins that function in early mRNA biosynthesis. Methods are described here for the extraction of purified vaccinia virions to yield protein mixtures with high transcriptional activity on viral early gene templates, responding specifically to both transcriptional initiation and termination signals in the DNA.

Key Words: RNA polymerase; transcription factors; early genes; in vitro transcription.

1. Introduction

Vaccinia virus genes are activated at the level of transcriptional initiation at early, intermediate, or late promoters, where approximately half of the vaccinia virus genes belong to the early class. Viral genes driven by early promoters include encoding factors required for DNA replication, such as DNA polymerase and genes encoding factors, such as cytokines and proteins, which counter host defenses. Early genes possess a complete transcription unit, that is, they are driven by a transcription promoter located just 5' of the open-reading frame and signaled to terminate transcription by a sequence near the 3' end of open-reading frames. Early promoters require approx 30 nt upstream of transcriptional start sites ***(1)*** and can often be identified visually on the nontemplate strand of the DNA as a G residue near nt –20 relative to the start site, flanked on both sides by a 8-nt A-T-rich clusters. The termination signal is the simple sequence TTTTTNT, where N is any nucleotide, also on the nontemplate strand of DNA ***(2)***. Termination of transcription occurs with some heterogeneity at 30–50 nt downstream of the signal (*see* **Note 1**).

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Transcription of vaccinia early genes is accomplished by a fairly well-characterized group of virus-encoded proteins. Transcription initiation appears to require only the viral RNA polymerase and early transcription factor ***(3)***. RNA polymerase must possess the 95-kDa subunit for activity on early gene templates ***(4)***. The termination of transcription requires the viral-capping enzyme ***(5)*** and nucleoside phosphohydrolase I ***(6,7)***. The A18R protein has been implicated in the elongation of transcription ***(8)***.

Although multiple proteins are required to reconstitute transcription of early genes in vitro, reactions can be studied using extracts derived from cells or virus particles. Whole-cell extracts from HeLa cells infected with vaccinia support transcription on early gene templates (*see* Chapter 12). However, whole-cell extracts are problematic in that both deoxyribonucleases and ribonucleases released by cell lysis interfere with mRNA product accumulation. All of the proteins required for early gene transcription are packaged within the virus particle to support transcription without the requirement of host functions. Virion extracts are essentially devoid of nucleases; thus, they are a preferred source of early gene transcription activity. This chapter describes methods for the extraction and assay of early gene transcription activity from purified virions.

2. Materials

2.1. Infection of Cells and Purification of Virus

2.1.1. Equipment

1. Roller bottles: 700 cm^2 (Bellco Glassware Co., Vineland, NJ).
2. Ultracentrifuge rotor: SW28 rotor (Beckman Coulter, Palo Alto, CA).

2.1.2. Cell Lines and Virus

1. HeLa S3 cells (American Type Culture Collection # CCL-2.2).
2. Virus used is the Western Reserve (WR) strain, obtained from Bernard Moss (National Institutes of Health [NIH], NIAID).
3. Culture medium: modified essential medium supplemented with 10% calf serum.
4. Infection medium: modified essential medium supplemented with 5% calf serum.

2.1.3. Reagents and Solutions

2.5 mg/mL Trypsin (Life Technologies, Rockville, MD).

2.2. Extraction of Vaccinia Cores and Removal of Viral DNA

2.2.1. Equipment

1. Centrifuge tubes rated to 12,000*g*.
2. Large Dounce homogenizer.
3. 10-cc syringe fitted with 22-gauge needle.
4. Small chromatography column with 10 mL or greater capacity.

2.2.2. Reagent and Solutions

1. Membrane-stripping solution: 50 m*M* Tris-HCl, pH 8.0, 50 m*M* dithiothreitol (DTT), 05% Nonidet P40 (BDH Chemicals, Poole, UK).
2. Buffer A: 0.25 *M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 10 m*M* DTT, 0.1 m*M* EDTA.
3. 10% (w/v) sodium deoxycholate.
4. DEAE-cellulose (e.g., Whatman DE52) equilibrated in buffer A.
5. Protein assay kit (Bio-Rad Laboratories, Richmond, CA).
6. Glycerol.
7. Dialysis buffer: 50 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA, 1 m*M* DTT, 0.01% Nonidet P40, 10% glycerol.

2.3. Transcription of Early Gene Templates

2.3.1. Equipment

1. Water bath set to 30°C.
2. Table-top centrifuge equipped to hold 1.5-mL Eppendorf tubes.
3. Vertical electrophoresis apparatus and power supply.
4. Vacuum gel dryer.

2.3.2. Reagents and Solutions

1. Suitable template DNA (0.1–0.5 pmol/reaction).
2. 10X Transcription buffer: 500 m*M* Tris-HCl, pH 8.0, 40 m*M* $MgCl_2$, 10 m*M* DTT.
3. 10X Nucleotide mix: 10 m*M* ATP, 10 m*M* CTP, 1 m*M* UTP (and 10 m*M* GTP if not using G-less cassette template).
4. ^{32}P-α-UTP.
5. Transcription stop solution: 8 *M* urea, 1% sodium dodecyl sulfate, 10 m*M* EDTA, 0.25 mg/mL tRNA.
6. Phenol/chloroform mix (1/1 v/v).
7. 7.5 *M* ammonium acetate.
8. Absolute ethanol.
9. Formamide dye mix: 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol in deionize formamide.
10. Urea-polyacrylamide gels.

3. Methods

3.1. Infection of Cells and Purification of Virus

We routinely grow vaccinia on HeLa S-3 cells. Because these cells can be propagated in monolayer or suspension culture, either format can be used. We have determined, however, that the yield of virus is consistently higher when cells are infected on monolayers. To minimize the amount of culture medium required, cells are grown in roller bottles (*see* **Note 2**). We routinely use the WR strain of vaccinia. To increase infectivity of stocks used to infect roller bottles, we treat the virus with trypsin. Virus is released from infected

cells by hypotonic shock and Dounce homogenization and purified by sucrose gradient centrifugation. The basic protocol used here closely resembles that described in Chapters 8 and 15. Only the adaptations to large-scale virus production are described here.

1. Each 700 cm^2 roller bottle is seeded with 2×10^8 cells in 175 mL culture medium and allowed to attach overnight at a roller speed of 500 revolutions/min.
2. Virus (approx 5 plaque forming units [pfu] per cell) is treated with 0.25 mg/mL trypsin for 30 min at 37°C prior to infection (*see* **Note 3**).
3. Virus is diluted to 20 mL per roller bottle with infection medium and applied to roller bottles previously drained of culture medium.
4. After a 1-h attachment period, an additional 30 mL culture medium is added to each roller bottle.
5. After 48 h, cells are no longer attached to the bottle and are harvested by centrifugation and lysed by Dounce homogenization in a hypotonic buffer solution.
6. Nuclei are removed by sedimentation at 2000*g* for 5 min and the supernatant retained.
7. Virus is purified by pelleting through a sucrose cushion, followed by band purification in a sucrose gradient, and finally pelleted as described in Chapters 8 and 15.
8. Virus pellets are resuspended in 10 m*M* Tris-HCl, pH 9.0 (approx 1 mL per initial roller bottle), and virus is quantitated by absorbance at 260 nm (A_{260}) (*see* **Note 4**). A 1000-fold dilution of virus is usually adequate for absorbance measurements.

3.2. Extraction of Vaccinia Cores and Removal of Viral DNA

The vaccinia virion can be unsheathed with combinations of mild detergent and reducing reagent. Nonidet P40 extraction will strip the viral membrane from the core particle to yield a core capable of active transcription of all early viral genes. Further extraction with deoxycholate solublizes much of the core particle to release its contents (*see* **Note 5**). This procedure yields active enzymes for essentially all of the multitude of activities previously identified in the vaccinia core. The viral DNA can be removed by passage over an anion exchange resin in the presence of 0.25 *M* NaCl to dissociate protein from DNA while the DNA is trapped on the resin. The following description is suitable for 300–1000 A_{260} U of virus that will yield high activity for in vitro transcription reactions.

1. Purified vaccinia virions isolated in **Subheading 3.1.**, **step 8** are collected by centrifugation at 12,000*g* for 15 min.
2. The virus pellet is resuspended in 5 mL membrane-stripping solution. The pellet is moderately compact and will require repeated pipetting up and down to resuspend fully.
3. Incubate the suspension for 1 h at 37°C.

4. Collect the viral cores by centrifugation at 12,000*g* for 15 min.
5. Resuspend the core pellet in 2 mL buffer A.
6. Add sodium deoxycholate to 0.4% and mix thoroughly.
7. Incubate on ice, mixing occasionally for 1 h. The mixture should acquire a thick viscosity. If the solution does not become viscous, add an additional 0.2% sodium deoxycholate and continue to incubate on ice for an additional 30 min (*see* **Note 5**).
8. To reduce the viscosity of the extract, pass it through a 22-gauge needle, being careful to minimize foaming (*see* **Note 6**).
9. Remove insoluble material by sedimentation at 15,000*g* for 30 min.
10. Apply the supernatant to a 2 mL DEAE-cellulose column previously equilibrated with buffer A.
11. Collect fractions of the flow-through material (0.5-mL fractions are suggested), eluting with buffer A.
12. Test fractions for protein content with the protein assay according to the manufacturer's suggestions.
13. Add glycerol to peak fractions to a final concentration of 10% (*see* **Note 7**).
14. Divide fractions into aliquots useful for a single experiment (*see* **Note 8**).
15. Store at –70°C or lower temperature.

3.3. Transcription of Early Gene Templates

If preparation of total early mRNA is desired, cores are stripped of the viral membrane with Nonidet P40 and resuspended in a buffered solution that contains nucleoside triphosphates and $MgCl_2$. Nascent RNA is released from the cores, which can be removed by centrifugation.

If transcription on defined early gene templates is desired, soluble core extract is incubated with template DNA in the presence of nucleoside triphosphates and $MgCl_2$. Proteins are removed by organic extraction, and nucleic acid is precipitated with ethanol for subsequent analysis by denaturing polyacrylamide gel electrophoresis. The choice of template DNA to be used is an important consideration. Initiation of transcription by most RNA polymerases occurs fortuitously on cryptic initiation sites in bacterial plasmids, and the vaccinia early transcription system is no exception. Hence, a DNA fragment with the essential transcription elements resident on the DNA must be purified away from plasmid sequences. Early promoters require approx 30 nt upstream of the transcriptional start site. For run-off transcription reactions, DNA is cleaved with a restriction endonuclease downstream of the start site. If termination of transcription is to be observed, the DNA should be cleaved at a distance downstream of the termination signal (i.e., >50 nt downstream of the TTTTTNT signal) sufficient to discriminate between terminated RNA vs read-through transcripts.

A useful approach to avoid purification of DNA fragments as template is the use of a "nucleotide omission" template in which one of the four nucle-

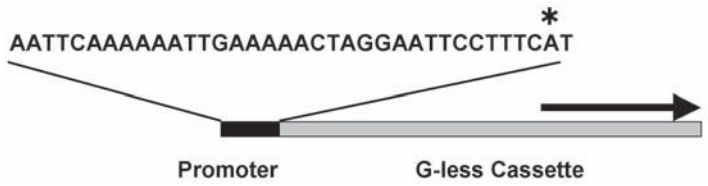

Fig. 1. Diagram of the pSB24 early transcription template. The synthetic promoter drives transcription across the 400-nt G-less cassette in the direction of the arrow. The sequence of the promoter is shown above, with the start site for transcription indicated with an asterisk.

otides is not included in the template downstream of the transcriptional start site. For example, the popular pSB24 template was designed to have a powerful synthetic early promoter to drive the transcription of a 400-nt G-less cassette (**Fig. 1**). For this template, GTP is omitted from the transcription reaction, and in fact, the chain terminating nucleotide 2'-O-methyl-GTP can be used as a replacement. Any fortuitous initiation of transcription within the bacterial sequences in the plasmid will quickly terminate as soon as a G residue is required in the transcript. This plasmid construct was not designed to observe bona fide termination of transcription. To observe transcriptional termination, a plasmid, such as G21(TER29)A78 ***(6)***, should be used.

1. Immediately prior to setting up transcription reaction, virion extract is thawed on ice.
2. Reaction solutions are assembled by mixing approx 0.5 pmol template DNA, 5 µL 10X transcription buffer, 5 µL 10X nucleotide solution, 0.5 mCi ^{32}P-UTP, and H_2O to a final volume of 50 µL, minus the volume of virion extract to be added. If desired, 2'-O-methylGTP is added to a final concentration of 10 µ*M*. Virion extract is added last to initiate the reaction (*see* **Notes 9** and **10**).
3. Reaction tubes are transferred to a 30°C water bath for 1 h.
4. Fifty microliters Transcription stop solution is added to each reaction, and the mixture is extracted once with an equal volume of phenol/chloroform.
5. The aqueous phase of the extraction is transferred to a new tube and 50 µL 7.5 *M* ammonium acetate and 300 µL ethanol are added to the tube.
6. Precipitates are allowed to form for at least 30 min in a –20°C freezer.
7. Tubes are centrifuged in an Eppendorf-style centrifuge on full speed for 10 min. The supernatants are discarded into radiological waste.
8. Pellets are washed by addition of 0.5 mL 70% ethanol and centrifuged for an additional 5 min. Supernatants are discarded.
9. Pellets are dried under vacuum and resuspended in 5 µL formamide dye mix.
10. Prior to electrophoresis, RNA is heated to 65°C for 5 min and cooled to room temperature.

11. Electrophoresis is on an urea-polyacrylamide gel. For the pSB24 template, the transcript is approx 400 nt in length. To observe this product, we use a 6% acrylamide gel and run the gel until the xylene cyanol dye reaches the bottom. End-labeled DNA serves as useful size markers.
12. The gel is dried under vacuum and either exposed to X-ray film or analyzed with a phosphorimager.

4. Notes

1. Knowing about the TTTTTNT termination signal is important when constructing recombinant vaccinia viruses expressing foreign proteins driven by an early promoter. If the foreign gene sequence serendipitously contains this T5NT sequence near the 5' end of the open-reading frame, it will lead to expression of truncated products at early times during infection. If possible, one should therefore perform site-directed mutagenesis to alter a base (without altering the amino acid) to disrupt the T5NT sequence.
2. Virus production is most efficient when cells are grown in a monolayer. Stationary cultures produce the highest yield. Roller bottles are the more cost-effective alternative, although somewhat less efficient. We routinely recover approx 100 A_{260} U of virus per 700 cm^2 roller bottle.
3. Virus is treated with trypsin prior to infection to increase its infectivity.
4. Absorbance 260 (A_{260}): an A_{260} unit is defined as absorbance/mL of virus solution. A 1000-fold dilution of virus is usually adequate for obtaining an absorbance measurement of less than 1.0.
5. In the extraction of virus, do not allow the final concentration of sodium deoxycholate to exceed 0.8%, as this leads to serious reduction in transcription activity of the extract.
6. Do *not* use sonication to reduce the viscosity of the extract. Sonication has been reported to destroy the activity of the vaccinia RNA polymerase ***(9)***.
7. Failure to add glycerol to extracts prior to freezing inactivates the RNA polymerase.
8. Repeated freezing and thawing of virus extract results in significantly decreased transcription activity.
9. If optimal transcription activity is desired, it is recommended that an extract titration experiment be performed. Transcription activity is dependent on the extract:template ratio. Addition of too much extract can result in diminution of the RNA product.
10. Consideration must be given to the NaCl concentration in the transcription reaction that is contributed by the extract solution. The concentration of NaCl in the transcription reaction should not exceed 75 m*M*, or inhibition of transcription will be observed. If addition of greater amounts of extracts is anticipated, addition of more glycerol to the extract is not needed, and the extract should be dialyzed for several hours against the Dialysis buffer prior to freezing.

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12

An In Vitro Transcription System for Studying Vaccinia Virus Late Genes

Cynthia F. Wright

Summary

This chapter describes a protocol that allows accurate in vitro transcription of vaccinia virus late genes. In this method, extracts are made from vaccinia virus-infected cells and used as enzyme sources to produce mRNAs from plasmid templates containing late gene promoter sequences.

Key Words: In vitro transcription; vaccinia virus; postreplicative gene expression; transcription factor; G-less cassette.

1. Introduction

The development of in vitro extracts that accurately transcribe appropriate DNA templates has contributed substantially to determining the identity and role of proteins involved in mRNA biogenesis for both cellular and viral systems. This chapter describes a system that has been developed for the transcription of vaccinia virus late genes in vitro, where extracts are prepared from vaccinia virus-infected HeLa cells. These extracts are also capable of transcribing both early and intermediate-gene templates ***(1–3)***. Early gene-specific transcription extracts can be made by solubilizing purified virion particles as described in Chapter 11, and intermediate gene-specific extracts can be made by infecting cells in the presence of DNA synthesis inhibitors, hydroxyurea, or cytosine arabinoside ***(3–5)***. For in vitro transcription reactions, the extract from infected cells is used as the enzyme source, and a plasmid containing a vaccinia virus late promoter sequence is used as the template. In this system, radiolabeled nucleotides are incorporated into the message, and the formation of an appropriately sized labeled RNA transcript is monitored by polyacrylamide gel electrophoresis, followed by autoradiography. Fine mapping of the

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5' ends of the transcripts made using this system has revealed that the messages made in vitro are the same as those made in vivo, including the addition of the 5' poly(A) leader characteristic of vaccinia virus late messages ***(6)***. Biochemical fractionation of these extracts has revealed that the late-transcription system is complex and requires numerous virally encoded proteins, viral RNA polymerase, and host cell factors ***(2,7–11)***.

2. Materials

2.1. Vectors

1. pCFW9 ***(2)*** or pCFW10: Available on request from C. Wright at wrightcf@musc.edu.

2.2. Whole-Cell Extract Preparation

1. HeLa S3 cells (ATCC, #CCL-2.2).
2. Modified eagle's medium (MEM) for spinner culture (Gibco Invitrogen Corp., Grand Island, NY, cat. no. 11380-037).
3. Spinner culture media: modified MEM containing 5% horse serum, 100 U/mL penicillin,100 mg/mL streptomycin sulfate, 25 m*M* HEPES, and 2 m*M* glutamine.
4. Cell resuspension buffer: 0.01 *M* Tris-HCl, pH 8.0, 0.001 *M* EDTA, and 0.005 *M* dithiothreitol (DTT; add immediately before use) chilled to 4°C.
5. Extraction buffer: 0.05 *M* Tris-HCl, pH 8.0, 0.01 *M* $MgCl_2$, 0.002 *M* DTT, 25% sucrose, and 50% glycerol, chilled to 4°C.
6. Saturated $(NH_4)_2SO_4$ solution.
7. Solid $(NH_4)_2SO_4$.
8. 1 *M* NaOH.
9. Buffer A: 0.1 *M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA, 0.01% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO), 10% glycerol, and 2 m*M* DTT, chilled to 4°C.
10. Prepared dialysis tubing (12,000–14,000 molecular weight cutoff [MWCO], Invitrogen Life Technologies, Carlsbad, CA, cat. no. 15961-022).

2.3. Transcription Reactions

1. Polyvinyl alcohol, average molecular weight 30,000–70,000 (Sigma-Aldrich, make a 24% stock solution and store at 4°C up to several months).
2. 100 m*M* ATP, CTP, and UTP stock solutions (Amersham Biosciences, Piscataway, NJ).
3. 3'-O-methylGTP (Amersham Biosciences).
4. [α-^{32}P]UTP or [α-^{32}P]CTP, 3000 Ci/mmol, 10 mCi/mL (Amersham Biosciences). Caution—radioactive material.
5. Transcription reaction mix: 8% polyvinyl alcohol, 1 μg vector DNA, 0.14 m*M* EDTA, 1 m*M* ATP, 100 μ*M* CTP, 20 μ*M* UTP, 5 μCi of [α-^{32}P]UTP, and 2 m*M* $MgCl_2$.
6. Stop buffer: 80 m*M* EDTA, 100 m*M* NaCl, and 1% sodium dodecyl sulfate (SDS). Immediately before use, add 10 μg yeast tRNA per 50 μL stop buffer.

7. tRNA, from Baker's yeast (Sigma-Aldrich). Make a stock at 10 mg/mL and store at –20°C.
8. Phenol/chloroform/isoamyl alcohol (25:24:1), store at 4°C.
9. Ribonuclease (RNase) T1.
10. 7.5 *M* Ammonium acetate.
11. Absolute ethanol.
12. Gel running dye: 95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. Store at –20°C.
13. 1X TBE buffer: 0.089 *M* Tris-borate, 0.089 *M* boric acid, and 0.002 *M* EDTA.

3. Methods

The methods described below outline (1) the construction of the plasmids used as templates, (2) preparation of extracts from vaccinia virus-infected HeLa cells, and (3) the protocol for the in vitro transcription reaction.

3.1. Vector for Transcription

Plasmids used as templates for transcription are based on the pC_2AT G-less cassette vector of Sawadogo and Roeder ***(12)***. This vector contains a synthetic 380-bp DNA sequence that lacks cytidine residues on one strand so that when a promoter is cloned into it, transcripts without guanosine residues are produced. This allows transcription reactions to be conducted in the absence of exogenously added GTP, which has been found to reduce the level of background transcription with crude cell extracts. Plasmids, pCFW9 and pCFW10, contain the late promoter from the vaccinia virus F17R gene cloned into the G-less cassette vector. **Figure 1** shows the sequence of the promoter and G-less cassette junction of these plasmids.

3.2. Whole-Cell Extract Preparation

Extracts are prepared from HeLa S3 spinner cultures infected with vaccinia virus. The cells are processed essentially as described by Manley and Gefter ***(13)***.

1. Crude virus stocks are grown and titered as described in Chapter 8.
2. HeLa S3 cells are maintained in spinner culture media at densities ranging from 2 to 5 × 10^5 cells/mL (*see* **Note 1**).
3. To prepare cells for infection, cells are centrifuged and resuspended in a small sealed flask at 1/10 the original volume with fresh media.
4. Infect with virus at a multiplicity of infection (moi) of 10.
5. After 30-min incubation with stirring at 37°C, increase the volume back to the original volume with fresh media (*see* **Note 2**).
6. Allow infection to proceed overnight (approx 15 h) with stirring at 37°C.
7. After incubation, cells are harvested by centrifugation in 600-mL bottles in a Beckman JS 5.2 rotor at 2000 rpm (approx 700*g*) for 10 min (*see* **Note 3**).

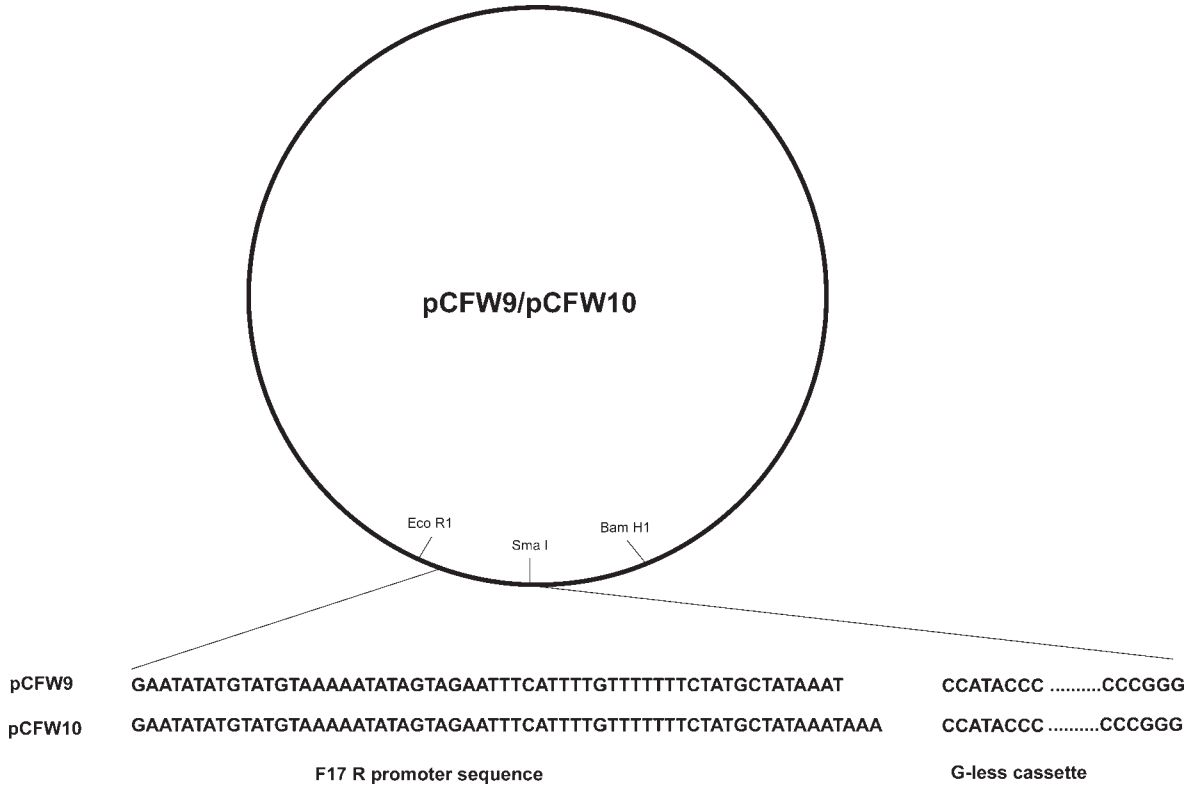

Fig. 1. Schematic drawing of pCFW9 and pCFW10 late promoter-containing plasmids used as transcription templates. Plasmids were adapted from the pC_2AT vector of Sawadogo and Roeder ***(12)***, which is based on pUC13. To construct these vectors, pC_2AT was digested with Sst1, the overhanging ends were blunted using T4 DNA polymerase, and double-stranded blunt-ended oligonucleotides (sequences for pCFW9 and pCFW10, respectively, are shown) containing the F17R promoter were ligated in.

8. Resuspend the cell pellet in 1/10 the original volume of fresh media lacking serum.
9. Repellet the infected cells by transferring the infected cell suspension to 50-mL polypropylene tubes and recentrifuging.
10. Discard the supernatant and record the packed cell pellet volume (*see* **Note 4**).
11. Resuspend the cell pellet with gentle pipetting in a volume of cell resuspension buffer that is four times the volume of the pellet recorded in **step 10**.
12. Allow the cells to swell on ice for 20 min.
13. Transfer the cells to a Dounce homogenizer on ice and lyse the cells with 20 strokes of a "B" pestle, keeping the homogenizer on ice (*see* **Note 5**).
14. Transfer the homogenized lysate to a prechilled beaker and add a volume of extraction buffer that is four times the volume of the pellet recorded in **step 10**.
15. Stir gently at 4°C.
16. Add dropwise a volume of saturated $(NH_4)_2SO_4$ that is equal to the volume of the pellet recorded in step 10 and stir the lysate gently at 4°C for 20 min.
17. Transfer the extract (which is very viscous) into polycarbonate bottles for a Beckman 70.1 Ti rotor.
18. Spin the bottles in a prechilled rotor at 60,000 rpm (approx 265,000*g*) at 4°C for 2 h.
19. Remove the supernatant into a clean beaker and record this volume (*see* **Note 4**).
20. Precipitate proteins by addition of solid $(NH_4)_2SO_4$ at 0.33 g/mL suspension with stirring at 4°C.
21. After the $(NH_4)_2SO_4$ is dissolved, add 0.01 mL 1 *M* NaOH per 10 g of $(NH_4)_2SO_4$ and stir the suspension for 30 min at 4°C.
22. Pellet the precipitate by centrifugation in a Beckman 70.1 Ti rotor at 13,000 rpm (approx 12,400*g*) for 20 min at 4°C.
23. Resuspend the pellet in chilled buffer A using a volume that is 1/10 the volume recorded in **step 19**.
24. Dialyze this suspension in a cold room or cold box against two changes of 100 vol of buffer A for 4–15 h each.
25. During this dialysis, a portion of the proteins will precipitate out, so the final dialysate must be centrifuged at 10,000*g* for 10 min at 4°C in an SS-34 rotor to clarify the suspension.
26. The final clarified supernatant is snap-frozen on dry ice and stored in aliquots at –80°C (*see* **Note 6**).

3.3. Transcription Reactions

Transcription reactions are done in microcentrifuge tubes (*see* **Note 7**) in a final volume of 50 µL.

1. Pipet 25–30 µL transcription reaction mix into a microcentrifuge tube (*see* **Notes 8** and **9**).
2. Add a combination of protein extract (isolated in **Subheading 3.2.**, **step 26**) and buffer A equivalent to 20–25 µL and incubate at 30°C for 30 min.
3. Stop the reactions by adding 50 µL stop buffer.

4. Add 50 µL phenol/chloroform/isoamyl alcohol (25:24:1) to extract the aqueous solution one time (*see* **Note 10**). Usually approx 90 µL of aqueous phase can be recovered.
5. Add 45 µL 7.5 *M* ammonium acetate and 330 µL ethanol and incubate on ice for 10 min (*see* **Note 11**).
6. Centrifuge tubes at full speed in a microcentrifuge for 15 min.
7. Wash the RNA pellet with 80% ethanol and briefly air-dry.
8. Resuspend the pellet in 6 µL gel running dye and heat at 65°C for 5 min.
9. Apply all of the resuspended reaction to a 4% polyacrylamide gel containing 8 *M* urea using 1X TBE as the running buffer (*see* **Note 12**).
10. For templates containing a transcript that is 380 nt in length, run the gel until the xylene cyanol dye is at the bottom of the gel.
11. After running the gel, adhere it to a piece of Whatman 3 MM chromatography paper and dry on a gel dryer, if desired.
12. Expose the gel to X-ray film and develop the film.
13. Robust reactions will result in signals of 380 nt that are visible after an exposure of several hours to overnight at –70°C using an intensifying screen.

4. Notes

1. Cells double in less than 24 h. Thus, either an equal volume of fresh media must be added daily (which results in culture expansion), or half the volume of the cell suspension should be discarded daily and an equivalent amount of fresh media added. Any amount of cells can be grown, but for ease of subsequent manipulation, at least 1 L of cells is recommended.
2. Attempting to reuse the media that the cells were originally grown in has resulted in poor-quality extracts.
3. At this point, it is important to note that the entire culture is now contaminated with the virus, and the supernatant and culture flask must be decontaminated prior to disposal and washing.
4. The volume of reagents added in subsequent steps is based on the volume recorded.
5. Based on analogy to cellular systems, whole-cell extracts were initially used to study vaccinia virus transcription in vitro. However, because vaccinia virus replicates in the cytoplasm of infected cells, cytoplasmic extracts can also transcribe viral genes. Cytoplasmic extracts can be made by taking the lysate after Dounce homogenization (**Subheading 3.2.**, **step 13**) and centrifuging it in a 50-mL tube in a Beckman JS 5.2 rotor at 2000 rpm (approx 700*g*) for 10 min to pellet out the cell nuclei. The resulting supernatant is then processed exactly the same way as described for the whole-cell extract and is easier to manipulate because of its reduced viscosity. The only potential disadvantage of using cytoplasmic extracts is that the isolation of cellular proteins found to stimulate transcription and are predominantly nuclear may not be optimal. Extracts from lysolecithin-permeabilized infected cells have also been used successfully as enzyme sources to transcribe early, intermediate, and late genes ***(3)***.

6. The protein concentration of the clarified extract is usually 10 mg/mL. Usually, 5 µL (50 µg) of this extract is adequate to support robust transcription in vitro; therefore, aliquots of 100 µL are sufficient for many transcription reactions. The extracts are stable for several freeze–thaw cycles without noticeable loss of activity.
7. Because RNA is easily hydrolyzed by RNases present in the environment, it is important to use clean plastic tubes and pipets, as well as to wear gloves when performing the reactions.
8. Either pCFW9 or pCFW10 may be used, and both supercoiled templates and templates linearized by a restriction digestion will work. In practice, we make enough of the transcription reaction mix for all reactions that contain DNA template, nucleotides, polyvinyl alcohol, $MgCl_2$, and EDTA, and add 25–30 µL of this to 25–20 µL of the protein mixture.
9. [α-^{32}P]UTP is the preferred nucleotide label for crude cell extracts that contain tRNA and the tRNA cytidylyltransferase activity. Upon fractionation and purification of the system [α-^{32}P]CTP is the preferred labeled nucleotide because the transcript contains twice as many cytosine as adenosine or uracil residues. If labeled CTP is used, the amount of unlabeled UTP should be increased to 100 µ*M* and unlabeled CTP decreased to 10 µ*M*. Note that this system uses radioactive nucleotides that must be procured and handled according to the radiation safety procedures of the relevant institute. These reactions may also be supplemented with 0.1 m*M* 3'-O-methylGTP and RNase T1 (15 U) to terminate and hydrolyze, respectively, read-through transcripts and other labeled products that might be generated by contaminating GTP and nucleic acids presented in crude extracts or nucleotide mixes.
10. Because the polyvinyl alcohol is viscous and creates a broad interface between the phenol and aqueous layers, it is important to spin the tube after adding the phenol-chloroform mixture at full speed in a microcentrifuge for at least 10 min.
11. RNA is stable in ethanol and can be stored for longer periods of time at –20°C.
12. We use the minigel format that includes gel plates that are 8 × 10 cm in size and 1.5 mm thick spacers.

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13

Studying Vaccinia Virus RNA Processing In Vitro

Paul D. Gershon

Summary

The study and use of vaccinia virus-derived RNA-modifying proteins has made a significant contribution to molecular biology. Here, the purification and assay of two such proteins, comprising the vaccinia poly(A) polymerase/cap-specific mRNA 2'-O-methyltransferase, is described.

Key Words: Cap; poly(A); mRNA; protein purification.

1. Introduction

Viral gene-regulatory systems, including those of vaccinia (the prototypic poxvirus), have contributed significantly to the elucidation of general mechanisms for mRNA synthesis and modification. For example, studies of vaccinia mRNA and RNA-modifying enzymes contributed to the elucidation of the mRNA 5' cap structure ***(1)***, demonstration of mRNA-associated poly(A) ***(2)***, the characterization of enzymatic activities contributing to cap formation ***(3)***, and the isolation and characterization of a poly(A)-polymerizing enzyme ***(4)***.

Vaccinia transcripts are not subject to splicing, though mature vaccinia mRNAs have eukaryotic features, including a standard mRNA 5' cap structure that consists of a 7-methylguanosine connected via a 5' to 5' triphosphate bridge to the first transcribed nucleotide (cap 0; ***1***). Cap 0 may be modified to the corresponding cap I structure by 2'-O-methylation of the first transcribed nucleotide. A fraction of the mRNA population may also be 2'-O-methylated at the second transcribed nucleotide ***(5)***. If the latter contains an adenine base, this is frequently also methylated at position 6 of its ring, leading to a doubly-methylated nucleotide (m^6A^m; ***6***). Vaccinia mRNA 3' ends possess poly(A) tails with a median length of approx 100 nt ***(7)***.

The cap and poly(A) tail are added to vaccinia transcripts by four virus-encoded proteins. Thus, the mRNA 5'-triphosphate of the nascent-transcribed

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mRNA is converted to a cap 0 structure through the action of the heterodimeric vaccinia-capping enzyme ***(3,8,9)***. The poly(A) tail is added by the viral poly(A) polymerase (PAP, also referred to as VP55, encoded by the E1L open-reading frame [ORF]), and a PAP-associated vaccinia-encoded protein, VP39 (encoded by the J3R ORF) functions at the mRNA 3' end in poly(A) tail elongation, as a PAP processivity factor ***(10)***. VP39 also acts at the mRNA 5' end as an RNA methyltransferase—catalyzing the cap-specific 2'-O-methylation of the first transcribed nucleotide of the mRNA ***(11–13)***. VP39 is one of only a handful of poxvirus proteins whose three-dimensional structure has been elucidated ***(14–18)***. The VP39 crystal structure provides a wealth of data on the general mechanism of mRNA cap recognition by proteins and a basis for the investigation of VP39's mechanism as a PAP processivity factor ***(19–21)***.

Various aspects of vaccinia proteins VP55 and VP39 have been previously reviewed ***(10,22–25)***. The vaccinia PAP subunits are commercially unavailable, but can be expressed and purified on a noncommercial basis. This is relatively straightforward, and protocols applicable to the expression and purification of VP55, VP39, and the VP55–VP39 heterodimer are given here (**Subheading 3.**), along with protocols for assaying their enzymatic activities. Although comparable protocols have already been outlined ***(24,25)***, here they are updated, given in more practical detail, and described in a step-by-step protocol format. In addition to these protocols, a myriad of protein–substrate interaction and enzymatic kinetic assays have been developed over the years. Although these assays are beyond the scope of this chapter, they can be found elsewhere ***(17,19–21,24–30)***. Practical uses of VP55 in RNA 3'-end labeling with a single, labeled chain-terminating nucleotide, assays of poly(A) tail length by RNase A digestion of the 3'-end labeled RNA, and addition of short poly(N) tracts to RNA 3' ends have also been described ***(25)***.

2. Materials

1. Vaccinia virus strain Western Reserve (WR; ATCC, #VR1354).
2. HeLa S-3 cells (ATCC, #CCL-2.2).
3. Virion lysis buffer: 0.1 *M* Tris-HCl, pH 8.5, 0.25 *M* NaCl, 0.01 *M* dithiothreitol (DTT), and 0.2% sodium deoxycholate.
4. DEAE-cellulose (DE-52, Whatman) column (3 × 1.5 cm).
5. DEAE-cellulose or DEAE-Bio-Gel column (5 × 1 cm).
6. Single-stranded DNA-agarose (Invitrogen-Life Technologies) column (1.5 × 20 cm).
7. Heparin-agarose (Gibco BRL) column (10 × 13 and 8 × 60 mm).
8. Carboxymethyl-Sephadex (Amersham-Pharmacia) column (1 × 15-cm).
9. Phosphocellulose (P11, Whatman) column.
10. Cibacron blue (F3GA)-agarose (Affigel blue; Bio-Rad) column.
11. Poly(A)-Sepharose (Pharmacia) column.
12. Reduced glutathione (GSH)-Sepharose beads (Pharmacia).

13. Heparin-agarose column (HiTrap-Heparin, Amersham-Pharmacia).
14. Buffer A: 0.15 *M* Tris-HCl, pH 8.4, 1 m*M* DTT, 0.1 m*M* EDTA, 0.1% Triton X-100, 10% glycerol, 0.25 *M* NaCl.
15. Buffer B: 0.05 *M* Tris-HCl, pH 8.0, 1 m*M* DTT, 0.1 m*M* EDTA, 0.01% Nonidet P-40, 10% glycerol.
16. Buffer C: 50 m*M* Tris-HCl, pH 8.0, 0.02 m*M* EDTA, 1 m*M* DTT, 20% glycerol.
17. Buffer D: 50 m*M* sodium acetate, pH 6.0, 1 m*M* DTT, 1 m*M* EDTA, 0.1% Triton X-100, 10% glycerol, 0.2 *M* NaCl.
18. Buffer E: 0.05 *M* Tris-HCl, pH 8.0, 15% glycerol, 0.1 m*M* EDTA, 2 m*M* DTT.
19. Buffer F: 10–20 m*M* HEPES-NaOH, pH 7.5–8.0, 10% glycerol, 1 m*M* 2-mercaptoethanol, 0.1% NP40.
20. Buffer G: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* 2-mercaptoethanol, 10% glycerol.
21. Buffer H: 20 m*M* HEPES, pH 7.6, 0.5 m*M* EDTA, 0.5 m*M* DTT, 10% glycerol.
22. Buffer I: 50 m*M* Tris-HCl, pH 8.4, 1 m*M* DTT, 1 m*M* EDTA, 0.1% Triton X-100, 10% glycerol, 0.05 *M* NaCl.
23. Dilution buffer: 1 m*M* DTT, 0.1 m*M* EDTA, 0.1% Triton X-100, 10% glycerol.
24. Swelling buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 5 m*M* DTT, 63 μg/mL PMSF, 1.25 μg/mL pepstatin.
25. 10X TD buffer: 0.5 *M* Tris-HCl, pH 9.0, 50 m*M* DTT.
26. TCA/pyrophosphate solution: 10% trichloroacetic acid (TCA) + 1% pyrophosphate.
27. 10X cap-specific methyltransferase reaction buffer: 250 m*M* HEPES-NaOH, pH 7.5, 10 m*M* DTT.
28. Recombinant vaccinia virus vTF7-3 (which contains a copy of the T7 RNA polymerase gene; ***31,32***).
29. Recombinant vaccinia virus vPG172a (which contains an additional copy of the VP55 gene under control of a phage T7 promoter; ***33***).
30. Recombinant vaccinia virus vPG175 (which contains an additional copy of the VP39 gene under control of a phage T7 promoter; ***34***).
31. Anti-VP55 N-terminal peptide antibody: generation described in **ref. *35***.
32. Anti-VP39 N-terminal peptide antibody: generation described in **ref. *35***.
33. The GST-VP39 expression plasmid (pPG177), constructed by cloning the VP39 coding region between the *Nco*I and *Sal*I sites of plasmid pGEX-KG ***(36,37)***.
34. High Five insect cells (Invitrogen).

3. Methods

3.1. Purification of the VP55–VP39 Heterodimer and VP39 Monomer from Purified Virions

The vaccinia VP55–VP39 heterodimer and VP39 monomer are both present within the virion. Until the early 1990s, the majority of studies with these enzymes employed material isolated from purified vaccinia virions. Protocols for the maintenance of HeLa S-3 cell suspension cultures, preparation of vaccinia (WR strain) virions from infected HeLa cells, and their purification by sucrose density gradient centrifugation are covered in Chapters 8 and 15, as

well as in **refs. *38–40***. The extraction and purification of PAP and 2'-O-methyltransferase from whole virions were initially described in **refs. *4*** and ***12***. Since VP39 is present in molar excess over VP55 within the virion (***35***; unpublished data), monomeric VP39 is typically found in addition to the heterodimer in the soluble fractions of virion extracts ***(35)***.

1. Incubate 100 mg of twice gradient-purified virions (*see* Chapters 8 and 15) in 12 mL virion lysis buffer for 30 min at 0°C (*see* **Note 1**).
2. Remove insoluble proteins by centrifugation in an SW41 rotor at 35,000 rpm (approx 151,000*g*) at 4°C for 30 min and collect the supernatant.
3. Shear the released DNA by forcing the supernatant two times through a 0.5-in. long, 26-gauge hypodermic needle.
4. Add glycerol, Triton X-100 detergent, and EDTA to final concentrations of 10%, 0.1%, and 0.1 m*M*, respectively.
5. Remove any residual nucleic acid by applying the resulting material to a DEAE-cellulose column (DE-52; 3 × 1.5-cm) equilibrated with buffer A at 20 mL/h. (The DNA remains bound to the column.)
6. Collect the flow-through (containing vaccinia enzymes) and dilute it with two equal volumes of dilution buffer (*see* **Note 2**).
7. Apply this material to a DEAE-bio-gel or 5 × 1-cm DEAE-cellulose column that has been pre-equilibrated with buffer B containing 0.08 *M* NaCl at 20 mL/h. Unbound (flow-through) fractions contain VP39 monomer and the VP55–VP39 heterodimer (*see* **Note 3**).
8. Apply flow-through fractions to a single-stranded DNA-agarose column (15 × 200-mm) equilibrated in buffer B containing 0.08 *M* NaCl.
9. Elute bound proteins using a 500-mL linear gradient of 0.08–0.6 *M* NaCl in buffer B. NaCl-gradient fractions from this column should contain in order of ascending fraction numbers, the capping enzyme, VP39 monomer, and VP55–VP39 heterodimer (*see* **Note 4**).
10. Monomeric VP39 can be further purified by application of gradient fractions from **step 9** to a 10 × 13-mm heparin-agarose column equilibrated with buffer C.
11. VP39 can be eluted from the column with a 9.6-mL linear gradient of 0.2–0.65 *M* KCl in buffer C (***35***; *see* **Note 4**).
12. In some preparations, highly purified VP55–VP39 heterodimer can be obtained directly from the single-stranded DNA-agarose column gradient fractions (**step 9**). In others, an additional purification step may be required.
13. To affect the further purification, single-stranded DNA-agarose column gradient fractions containing the heterodimer (i.e., PAP activity; *see* **Note 4**) are pooled and applied to a 8 × 60-mm heparin-agarose column equilibrated in buffer C containing 0.05 *M* NaCl ***(42)***.
14. Elute this column with a 40-mL linear gradient of 0.05–0.6 *M* NaCl in buffer C (***35***; *see* **Note 5**).
15. The resulting gradient fractions can be assayed for PAP activity or by silver staining (*see* **Notes 4**, **6**, and **7**).

3.1.1. Purification of the VP39 Monomer: Alternative Method

Owing to difficulties separating VP39 monomer from closely eluting proteins, alternative purification schemes for virion-derived monomeric VP39 can be performed ***(12)***.

1. Flow-through fractions from the DEAE-cellulose column described in **Subheading 3.1.1.**, **step 7** are pooled and dialyzed against at least 10 vol of buffer D.
2. Apply dialyzed material to a 1 × 15-cm carboxymethyl-Sephadex column preequilibrated in buffer D.
3. Elute with a 200-mL vol of linear gradient of 0.2–0.6 *M* NaCl in buffer D and collect gradient fractions and assay for VP39 (*see* **Notes 8** and **9**).

3.2. Overexpression of VP55 and VP39 in Vaccinia-Infected HeLa Cells and Purification from HeLa Cell Cytoplasmic Extract

Recombinant vaccinia viruses have been constructed for the individual overexpression of VP55 and VP39 in HeLa cells using the vaccinia-T7 system ***(31,32)*** and used as described below.

3.2.1. Overexpression and Purification of VP55

1. Spinner HeLa cells (5×10^9) are coinfected with recombinant vaccinia virus vPG172a ***(33)*** along with the recombinant vaccinia virus vTF7-3 ***(31,32)***, each virus at a multiplicity of infection (moi) of 10 pfu/cell using standard infection protocols as described in Chapter 8 and **ref. *38***.
2. After 18-h infection, cells are harvested, and the cell pellet is suspended in 80 mL swelling buffer.
3. The cell suspension is then Dounce-homogenized on ice with 10–20 sharp strokes.
4. Nuclei are then pelleted by centrifugation at 1500*g* for 10 min.
5. The supernatant is subjected to an additional clarification step (120,000*g* for 2 h), and the supernatant from this step is adjusted to 15% glycerol and 250 m*M* NaCl.
6. Residual nucleic acid is removed by application to a 40-mL DEAE-cellulose column (DE-52) equilibrated in buffer E containing 0.25 *M* NaCl.
7. Collect and pool the flow-through, which contains the protein.
8. Dilute the pooled flow-through (which is in buffer E containing 0.25 *M* NaCl) to 0.1 *M* NaCl.
9. Apply this material to an 80-mL phosphocellulose column.
10. After extensive washing with buffer E containing 0.2 *M* NaCl, the phosphocellulose column is eluted with a 500-mL linear gradient of 0.2–0.5 *M* NaCl in buffer E.
11. VP55-containing gradient fractions can be identified by PAP assay (**Subheading 3.5.**) or by immunoblotting.
12. Active gradient fractions from the phosphocellulose column are pooled, diluted to 0.1 *M* NaCl with buffer E, and applied to a 5-mL heparin-agarose column preequilibrated in buffer E.

13. After washing the heparin-agarose column with buffer E containing 0.15 *M* NaCl, protein is eluted with a 150-mL linear gradient of 0.15–0.6 *M* NaCl in buffer E.
14. VP55-containing gradient fractions from the heparin-agarose column are pooled and diluted to 0.1 *M* NaCl with buffer E.
15. This material is then applied to a 3.5-mL Cibacron blue (F3GA)-agarose column.
16. This column is then washed with buffer E containing 0.15 *M* NaCl.
17. Protein is eluted with a 150-mL linear gradient of 0.15–1 *M* NaCl in buffer E.
18. VP55-containing gradient fractions from the Cibacron blue column are pooled and diluted to 0.02 *M* NaCl with buffer E.
19. Pooled fractions are applied to a 4-mL poly(A)-Sepharose column.
20. This column is washed with buffer E containing 0.02 *M* NaCl.
21. Proteins are eluted with a 100-mL linear gradient of 0.02–0.2 *M* NaCl in buffer E.
22. The resulting VP55 is essentially free of detectable contaminants by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.2. Overexpression and Purification of VP39

1. Spinner HeLa cells (5×10^9) are coinfected with recombinant vaccinia virus vPG175 ***(34)***, along with the recombinant vaccinia virus vTF7-3 ***(31,32)***, each virus at a moi of 10 pfu/cell using standard infection protocols as described in Chapter 8 and **ref. *38***.
2. Similar to what has just been described for VP55 (**Subheading 3.2.1.**), follow **steps 2–9**.
3. After extensive washing of the loaded phosphocellulose column with buffer E containing 0.2 *M* NaCl, the protein is eluted with a 500-mL linear gradient of 0.2–0.55 *M* NaCl in buffer E.
4. VP39-containing gradient fractions can be identified by immunoblotting.
5. VP39-containing gradient fractions from the phosphocellulose column are pooled, diluted to 0.2 *M* NaCl with buffer E, and applied to a 12-mL Cibacron blue (F3GA)-agarose column.
6. This column is washed with buffer E containing 0.4 *M* NaCl.
7. Proteins are eluted with a 240-mL linear gradient of 0.4–1.4 *M* NaCl in buffer E.
8. VP39-containing gradient fractions can be identified by immunoblotting (*see* **Note 10**).
9. Active fractions from the Cibacron blue-agarose column are pooled, diluted to 0.1 *M* NaCl with buffer E, and applied to a 2-mL heparin-agarose column. The column is then washed with buffer E containing 0.1 *M* NaCl.
10. Protein is eluted with a 60-mL linear gradient of 0.1–0.5 *M* NaCl in buffer E.
11. VP39-containing gradient fractions are identified by various means (*see* **Note 11**).
12. VP39-containing gradient fractions from the heparin-agarose column are pooled and diluted to 0.05 *M* NaCl with buffer E, then applied to a 3-mL poly(A)-Sepharose column. This column is then washed with buffer E containing 0.05 *M* NaCl.
13. Protein is eluted with an 84-mL linear gradient of 0.05–0.3 *M* NaCl in buffer E.
14. The resulting VP39 should be essentially free of detectable contaminants by silver-stained SDS-PAGE.

3.3. E. coli Overexpression and Purification of VP55 and VP39

Attempts have been made to express both 6His- and glutathione-*S*-transferase (GST)-tagged versions of VP55 in *Escherichia coli*. However, under a variety of expression and extraction conditions with various fused tags and *E. coli* strains, low concentrations of recombinant protein are recovered in the soluble fraction. Although VP55 recovered from the soluble protein extract is active, significant purification is necessary to fractionate *E. coli*-derived polymerizing activities, and extensive precipitation of *E. coli*-expressed VP55 can occur during purification and concentration. Owing to the above difficulties, *E. coli* expression of VP55 is not described here.

VP39 has been successfully expressed to high levels in *E. coli* as an N-terminally tagged GST fusion protein from plasmid pPG177 ***(36,37)*** and purified to apparent homogeneity ***(15,24–26,36)***. In one study ***(15)***, this procedure yielded 160 mg of a point mutant of VP39 in a highly purified form for successful crystallography from a 14 L fermentation culture.

1. *E. coli* strain HB101 is transformed with plasmid pPG177.
2. Superbroth containing 0.1 mg/mL ampicillin is inoculated with colonies of the transformed *E. coli*, and cultures are grown to the stationary phase at 37°C.
3. Isopropyl 0.4–0.5 m*M* 1-thio-β-D-galactopyranoside is added, followed by a further 2–4 h incubation at 37°C (*see* **Note 12**).
4. Cells are pelleted, and the pellets are resuspended in 2–5 mL/g wet weight of *E. coli* pellet in buffer F containing 0.1 *M* NaCl, 0.1 mg/mL PMSF, and 2 μg/mL pepstatin.
5. After sonication (*see* **Note 13**), the broken cell suspension can be supplemented with $MgCl_2$ (to 1 m*M* final concentration) and DNAse I (Boehringer) and incubated for 15-min incubation at room temperature.
6. The lysate is then ultracentrifuged in an SW41 rotor at 20,000 rpm (49,400*g*) for 20 min at 4°C
7. The supernatant is removed and saved (*see* **Note 14**).
8. Reduced GSH-Sepharose beads from which the manufacturer's storage buffer has been drained are mixed with a 15–20-fold volume excess of supernatant from **step 7**.
9. Incubate the mixture at room temperature for 20 min with continuous inversion.
10. Wash beads three or four times with a 5–20-fold volume excess of buffer F containing 0.1 *M* NaCl.
11. Bead-bound GST-VP39 can be eluted using buffer F containing 60 m*M* NaCl plus 10 m*M* reduced GSH that has been adjusted to pH 7.5 with NaOH.

3.3.1. Alternative Protocol for Eluting Bead-Bound GST-VP39

The described elution procedure (**Subheading 3.3.**, **step 11**) can be substituted with thrombin treatment in order to specifically release VP39 from the bead-bound GST moiety ***(26)***.

1. Beads adsorbed with GST-VP39 (from **Subheading 3.3.**, **step 10**) are supplemented with 1–2 vol of buffer F containing 0.1 *M* NaCl, plus either 1/10 vol of 10X thrombin cleavage buffer (Novagen) or 2.5 m*M* $CaCl_2$.
2. 1.5–10 U Thrombin protease (Novagen) is then added per milliliter of packed beads.
3. Beads are then incubated at room temperature for 30–240 min (with rotation). This is sometimes followed by overnight incubation at 4°C (*see* **Note 15**).
4. VP39-containing supernatants are decanted from the settled beads, and the beads are then washed three to five times with at least 1.5 vol buffer F NaCl containing 0.1 *M* to collect the released VP39.
5. Supernatant and wash fractions are pooled.
6. To purify to apparent homogeneity, pooled VP39-containing fractions are diluted with an equal volume of water and applied to a heparin-agarose column (HiTrap-Heparin; approx 1 mL of resin per approx 15 g of *E. coli* wet weight in **Subheading 3.3.**, **step 4**) pre-equilibrated in buffer G.
7. After washing the column extensively in buffer G, protein is eluted with a linear NaCl gradient (0–500 m*M*) in buffer G, collecting gradient fractions (*see* **Note 16**).

3.4. Baculovirus Overexpression and Purification of VP55 and VP39

Baculoviruses have been isolated that express VP55 and VP39. Because baculovirus has shown no discernible advantage over *E. coli* for VP39 expression, the baculovirus-expressing GST-VP55 only is described.

3.4.1. GST-VP55

1. A recombinant baculovirus was generated using the transfer plasmid (pPG220-10; *see* **Note 17**) following transfection protocols from Invitrogen Inc. After multiple rounds of plaque purification, a virus stock was generated by three stages of amplification. Virus titers were determined according to protocols from Invitrogen Inc.
2. High Five insect cells were cultured in either serum-free medium or Grace's medium containing 10% fetal bovine serum (Invitrogen Inc.) according to protocols from Invitrogen Inc.
3. Titered virus is added to cells at a moi of 5–10 pfu/cell, and the infected cells were incubated at 27°C over a 2.5–3.5-d period for protein expression ***(19)***.
4. Infected cells were then pelleted and lysed (*see* **Note 18**), and recombinant GST-VP55 is purified as previously described for *E. coli* expressed GST-VP39 (**Subheading 3.3.**, **steps 4–11**, **Subheading 3.3.1.**; ***19***).

3.5. VP55 and VP55-VP39 PAP Activity Assays

Unlike other enzymes, the vaccinia PAP is unusual in being able to rapidly and processively add long poly(A) tails to RNA substrates without requiring the presence of specific RNA sequences (*see* **Note 19**).

3.5.1. Assay by Incorporation of Labeled Nucleotide into TCA-Precipitable Material

The incorporation of ^{32}P-AMP from [α-^{32}P]ATP into TCA-precipitable RNA by PAP has been described in **ref. *4*** and can be implemented as follows:

1. Assay mixtures contain 1/10 vol of 10X TD buffer supplemented with 1 m*M* ATP containing [α-^{32}P]ATP (5 mCi/mmol final), and poly(C) (0.1 m*M* in nucleotide final concentration, dissolved in RNase-free water) in a final volume of 0.1 mL (*see* **Note 20**).
2. After 30-min incubation at 37°C, assays are terminated by the addition of 5 µL 1 mg/mL yeast tRNA (Gibco-Life Technologies) followed immediately by 0.3 mL TCA/pyrophosphate solution.
3. Samples are then placed on ice for 60 min.
4. The samples are then centrifuged in a microcentrifuge for approx 5 min at full speed.
5. Precipitates are resuspended in TCA/pyrophosphate solution and applied to GF/C (glass microfiber) filter discs (Whatman) under vacuum.
6. The discs are washed four times (under vacuum) with approx 5 mL 10% TCA, then once with 5 mL ethanol:water (19:1).
7. The discs are allowed to air-dry and filter-bound ^{32}P is quantitated by scintillation counting (*see* **Note 21**).

3.5.2. Assay Using End-labeled Primer/Electrophoretic Readout

An electrophoretic time-course PAP assay has been described and subsequently refined ***(33,34,42,43)***, in which a discrete 5' end-labeled RNA primer is employed, and polyadenylylation products are analyzed by PAGE. As described in **ref. *42***, reaction conditions for this assay were similar to those initially described for [α-^{32}P] AMP incorporation into TCA-precipitable RNA (***4,24,25***; **Subheading 3.5.1.**), except for a lowering of the $MnCl_2$ concentration to 0.6 m*M*, the omission of labeled ATP, use of small (10 µL) reaction volumes, and use of the discrete RNA primer U_{10} (after 5'-end labeling of the latter using [γ-^{32}P]ATP plus T4 polynucleotide kinase followed by gel-purification of the labeled primer). With further slight modifications, the assay has been implemented yet more recently (e.g., **refs. *29,33,34,43***), in combination with a variety of RNA and RNA/DNA chimeric primers (synthesized either chemically or enzymatically) mostly in the 25–40-nt size range. These recent modifications include (in most instances), the substitution of $MnCl_2$ entirely with $MgCl_2$ (*see* **Note 22**), assaying at room temperature instead of 37°C to slow the reaction slightly and ease the rapid sampling of early time-points, gel purification of the unlabeled primer stocks, and use of labeled primer directly from the labeling reaction (after heat-killing the polynucleotide kinase used for labeling) without further gel purification. Protocols for assaying either monomeric

VP55, mixtures of separately purified recombinant VP55 and VP39, or the purified virion VP55–VP39 heterodimer are as follows:

1. Purified chemically synthesized primers (*see* **Note 23**) are labeled directly with [γ-^{32}P]ATP using polynucleotide kinase with any standard manufacturer's protocol (*see* **Note 24**).
2. 10 µL polyadenylylation reactions are typically set up containing 1 µL 10X TD buffer, 0.6 µL 1 *M* NaCl (*see* **Note 25**), 1 µL 10 m*M* ATP, and 1 µL kinase mix (from which 1 m*M* (final) Mg^{2+} and primer is derived). 2 µL resulting reaction mixture can be transferred to 2 µL deionized formamide as a "time-zero" sample.
3. Working at room temperature, the assay is initiated by adding VP55 (e.g., 1 µL 250 n*M* preparation; *see* **Note 20**) to the remainder of the reaction mixture. (Typically, primer is present in the assay at more than threefold molar excess over the enzyme.)
4. 2-µL Aliquots of the resulting mixture are withdrawn at various intervals and mixed with equal volumes of deionized formamide (*see* **Note 26**).
5. After all time-points have been taken, products are analyzed by electrophoresis in polyacrylamide gels containing 7 *M* urea and TBE buffer (*see* **Note 27**), followed by autoradiography or phosphor-plate imaging.

3.6. Cap-Specific (Nucleoside-2'-O-)-Methyltransferase Assays of VP39

The cap-specific (nucleoside-2'-O-)-methyltransferase assay ***(12,13,36)*** requires, as a substrate, RNA terminated at the 5' end with cap 0 (*see* **Subheading 1.**). Previously, Brome mosaic virus (BMV) RNA was employed as the capped RNA substrate. Although BMV RNA is commercially available (Promega), package sizes are small and expensive. However, it can be prepared in larger quantities with appropriate greenhouse facilities and has been provided in the past as a gift to those who first reported the assay ***(12)*** and also to this author. Alternatively, specific mRNA species requiring cap 2'-O-methylation may be synthesized initially with cap 0 by in vitro transcription of a phage RNA polymerase promoter-driven template in the presence of dinucleotide cap analog ***(14,27,45)*** or in the absence of dinucleotide cap analog via posttranscriptional treatment with the vaccinia-capping enzyme ***(40)***.

3.6.1. Enzyme Activity Assays

1. 100-µL reactions typically contain 1/10 vol of 10X cap-specific methyltransferase reaction buffer, 1 µ*M* adenosyl [^{3}H-methyl]methionine (e.g., approx 1 µL of approx 5–15 Ci/mmol, approx 0.55 mCi/mL adenosyl [^{3}H-methyl]-methionine, Perkin Elmer/NEN Life Sciences), 5 µg BMV RNA, and VP39 or fractions containing VP39 (*see* **Notes 20** and **28**).
2. After 30-min incubation at 37°C, VP39 is inactivated by incubating samples at 100°C for 2 min.

3. To quantitate the radioactively labeled RNA product, samples are deposited onto DEAE filters (DE-81, Whatman), which are subsequently washed under vacuum four times with 5 mL vol of 25 m*M* ammonium formate, followed by a wash with 5 mL water, then two washes with 5 mL vol of ethanol:ether (1:1).
4. After air-drying under ambient conditions, filters are subjected to scintillation counting.

3.6.2. Quantitative Conversion of Cap 0- to Cap I-Terminated RNA

Cap I-terminated RNA may be required, e.g., for mRNA expression studies or studies of influenza virus transcriptional priming ***(46,47)***. To subject a chosen RNA species to the cap 0 - cap I conversion reaction, the reaction conditions given in **Subheading 3.6.1.** can be used with the following modifications/considerations:

1. BMV RNA should be replaced with the chosen RNA.
2. The ^{3}H-AdoMet should be replaced with unlabeled AdoMet at a concentration of 2–500 μ*M*.
3. Owing to VP39's low rate of chemistry/turnover, conversion levels can be maximized by including VP39 in the reaction in molar amounts approaching equimolarity with the RNA substrate. The larger quantities of VP39 required for this can most easily be achieved using *E. coli*-expressed VP39 generated as described in **Subheading 3.**
4. VP39 can effect cap 0–cap I conversion in capping enzyme buffer, which is similar to the cap-specific methyltransferase reaction buffer described in **Subheading 3.6.1.** except for the additional presence of $MgCl_2$ (unpublished data).
5. By employing mixtures of VP39 with the vaccinia-capping enzyme, it should be possible to synthesize cap I-terminated RNA directly from the triphosphate-terminated counterpart in a one-tube reaction.

4. Notes

1. Enzymes from purified whole virions are released in the presence of sodium deoxycholate, DTT, and salt at the indicated concentrations ***(41)***.
2. Addition of this buffer to fractions lowers the Tris-HCl and NaCl concentrations to 0.05 and 0.08 *M*, respectively, while maintaining the concentrations of other buffer components.
3. The NaCl can be substituted with KCl ***(35)***.
4. Heterodimer-containing fractions can be assayed for PAP activity (**Subheading 3.5.**), and the active fractions pooled. VP39 monomer can be detected by either immunoblotting (35) or assaying column fractions for cap-specific (nucleoside-2'-O-)-methyltransferase activity (**Subheading 3.6.**).
5. As an alternative to heparin-agarose for the final purification step of the heterodimer, a 1 mL Mono S HR5/5 column can be used ***(35)***. First, dialyze pooled single-stranded DNA-agarose column fractions against buffer H containing 0.05 *M* NaCl. The Mono S column is eluted with a 40-mL linear gradient of

0.05–1 *M* NaCl in buffer H, collecting 0.5-mL fractions. The two subunits do not appear to separate significantly during Mono S column chromatography performed in this manner.

6. Heparin-agarose column gradient fractions containing the VP55–VP39 heterodimer should be essentially free of other proteins as judged by SDS-PAGE. As a result of this purity, instead of assaying for PAP activity (**Subheading 3.5.**), heparin-agarose column fractions can also be analyzed for the presence of the complex by silver-stained SDS-PAGE.
7. Although even greater purity of the VP55–VP39 heterodimer can be achieved by running a NaCl gradient that is up to 8 times shallower, this may lead to some disassociation of the heterodimer subunits (unpublished). This is presumably the result of slightly different salt concentrations required for elution of the individual PAP subunits from heparin-agarose ***(33)***, coupled with a possibly destabilizing effect of NaCl upon dimerization (unpublished).
8. Column fractions can be assayed for cap-specific 2'-O-methyltransferase activity (**Subheading 3.6.**). Alternatively, monomeric VP39 is usually obvious in stained gels given its known size of 39 KDa ***(13)***.
9. Minor contaminants can be removed by dialysis of pooled peak fractions into buffer I, application to an ADP-agarose column ***(12)***, and elution with a linear gradient of 0.05–0.5 *M* NaCl in buffer I. SDS-PAGE of gradient fractions containing 2'-O-methyltransferase activity typically shows a single 39-kDa protein band. Of note, the original purification protocol ***(12)*** suggested the occasional use of one additional column, namely, poly(U)-Sepharose, as a final purification step, but the enzyme appeared pure prior to this step.
10. If purified VP55 ***(33)*** is available, one can also assay for adenylyltransferase-stimulatory activity in the presence of highly purified VP55 as described in **Subheading 3.5.** and illustrated in **Fig. 1** of **ref. *34***.
11. VP39-containing fractions are identified by immunoblot, the adenylyltransferase-stimulatory assay, or by silver-stained SDS-PAGE.
12. Some mutants of VP39 express to higher levels in *E. coli* at 27°C than at 37°C.
13. Sonication:10–25× 5-s bursts, full power, 50% duty cycle, 550 Sonic Dismembrator, Fisher Scientific.
14. The pellet may be resonicated in a second aliquot of buffer F containing 0.1 mg/mL PMSF and 2 μg/mL pepstatin, and **steps 5** and **6** of **Subheading 3.3.** are repeated. The two supernatants are then pooled.
15. Larger amounts of thrombin can be employed to shorten the incubation times. Extended thrombin cleavage times, perhaps in combination with protein freeze-thawing, may lead to truncation of the recombinant protein at a protease-hypersensitive site located 26–36 amino acids from the natural VP39 C-terminus. To help circumvent this, small amounts of PMSF can be added at the end of the thrombin cleavage step.
16. A 100–200-column volume gradient aids in obtaining protein at very high purity. Peak fractions may contain highly purified VP39 at concentrations of several mg/mL.

17. Baculovirus transfer plasmid pPG220-10 was generated as follows. (a) The GST ORF from plasmid pPG177 was amplified by polymerase chain reaction (PCR) as a *Hin*dIII-*Pst*I fragment, and cloned between the *Hin*dIII and *Pst*I sites of plasmid pBlueBac III (Invitrogen) using standard cloning techniques to give plasmid pSTONE2. (b) The VP55 gene was amplified from vaccinia genomic DNA as a *Hin*dIII fragment with an *Nco*I site at the VP55 gene initiation codon, and cloned in plasmid pBluescript SK^+ (Stratagene) to give plasmid pPG219a. (c) The *Nco*I–*Hin*dIII fragment of pPG219a was cloned in *Nco*I–*Hin*dIII cleaved pSTONE 2 to give plasmid pPG220-10, the transfer vector used for generating recombinant baculovirus-expressing GST-VP55.
18. Baculovirus-infected cells are lysed only with five 15-s bursts of sonication.
19. Conditions for in vitro PAP reaction vary depending on the importance of complete substrate utilization and target tail length. Reaction variables include: (i) whether the VP55 subunit is alone or if both subunits are present; (ii) time and temperature; (iii) whether Mn^{2+} is present as well as/in place of Mg^{2+}; (iv) concentration of monovalent cation; (v) relative concentrations of PAP, RNA, and ATP; and (vi) the presence/absence of ATP analogs.
20. The volume of column fraction necessary to achieve activity is entirely empirical. But for a specific column, the same volume of each fraction should be used.
21. The sensitivity of this assay may be increased by excluding unlabeled ATP from the reaction, increasing incubation times to 60 min, and increasing the PAP concentration by decreasing reaction volumes to 10–20 μL ***(35)***. The use of poly(A) or oligo(A) primers ***(35)*** may also improve sensitivity. Under the modified conditions, TCA-insoluble radioactivity is directly proportional to enzyme concentration over a range of PAP concentrations ***(35)***.
22. In reactions where $MnCl_2$ is required, $MnCl_2{\cdot}4H_2O$ is dissolved immediately before use, and $MgCl_2$, Tris-HCl, and DTT in both the assay mixtures and polynucleotide kinase-labeling reactions are substituted with $MnCl_2$, HEPES-NaOH, and 2-mercaptoethanol, respectively ***(48)***, to retard the chemical conversion/oxidation of the manganese to VP55-inhibitory products.
23. Approximately 30–100-μg aliquots of RNA or RNA/DNA chimeric primers synthesized by solid-phase chemistry are purified by denaturing PAGE, detecting products by ultraviolet (uv) shadowing and band excision from the gel using standard methods ***(44)***. The primer is typically eluted from the crushed gel slice by overnight incubation in approx 0.4 mL 0.1% SDS, 1 m*M* EDTA, followed by ethanol precipitation of the primer from the supernatant (separated from crushed polyacrylamide with the help of a gel-loader pipet tip) in the presence of glycogen carrier. The precipitated primer is redissolved in approx 5–20 μL RNAse-free water. The concentration of primer in such preparations is typically 10–50 μ*M*.
24. A typical 10-μL labeling reaction might contain 10–20 pmol (0.5–1 μL; **Note 23**) of purified primer, 1 μL manufacturer's 10X buffer (typically 0.7 *M* Tris-HCl, pH 7.6, 100 m*M* $MgCl_2$, 50 m*M* DTT), 1 μL [γ-^{32}P]ATP (3000 mCi/mmol, 10 mCi/mL) and 5 Richardson units of polynucleotide kinase (typically 0.5 μL). Thirty-minute incubation at 37°C is followed by 15-min incubation at 70°C.

25. Inclusion of NaCl prevents nonspecific interaction of VP55 and primer (*see* **Fig. 8C** of **ref. *34***).
26. Formamide stops the reaction very rapidly.
27. To achieve single-nucleotide PAGE resolution of polyadenylylation products, an 8% polyacrylamide sequencing-length/thickness gel can be run. Samples are loaded in alternate wells of a shark's tooth comb with the intervening wells being loaded with formamide alone. Dyes are not present in the formamide that is mixed with samples, but bromophenol blue and xylene cyanol are instead present in a dye track loaded at one side of the gel.
28. Final NaCl concentration should remain below approx 50 m*M **(11)***.

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14

Methods for Analysis of Poxvirus DNA Replication

Paula Traktman* and Kathleen Boyle

Summary

Cytoplasmic replication of poxviruses dictates the encoding of most, if not all, of the *trans*-acting factors required for faithful genome duplication. Several of these proteins have been identified through genetic and biochemical evaluation, including the catalytic DNA polymerase (E9), an essential and stoichiometric component of the processive polymerase (A20), a single-strand DNA-binding protein (I3), a type I topoisomerase (H6), the uracil DNA glycosylase (D4), a nucleic acid-independent nucleoside triphosphatase (D5), a serine/threonine protein kinase (B1), and a Holliday Junction resolvase (A22). All of these factors work in concert to faithfully duplicate the viral genome. Although a replication origin has not been defined for the poxviruses, *cis*-acting sequences found within the telomeric 200 bp have been implicated as necessary and sufficient for minichromosome replication. Replication occurs within cytoplasmic foci from approx 3 to 12 h postinfection. This chapter includes several methodologies to assay and quantitate replication in vivo, visualize replication foci microscopically, and test the integrity of central replication enzymes in vitro.

Key Words: Vaccinia virus; DNA replication; dot-blot hybridization; immunofluorescence; concatemer resolution; minichromosome replication; processive DNA polymerase.

1. Introduction

Cytoplasmic replication of poxviruses requires the encoding of most, if not all, the *trans*-acting factors necessary for faithful genome duplication ***(1,2)***. The current working model of poxvirus DNA replication is diagrammed in **Fig. 1**. DNA replication is considered to initiate from a nick (i) at either of the hairpin termini, exposing a 3'-OH group. This free 3' terminus serves as a site for the processive viral polymerase complex to perform strand-displacement primer extension (ii). The nascent strand and template are self-complementary and can isomerize into the form shown (iii). Extension of the nascent strand

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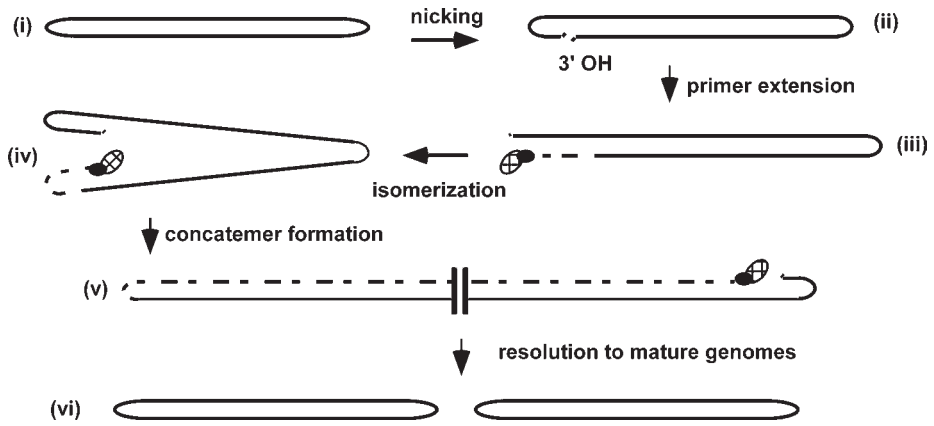

Fig. 1. Schematic diagram of the current model for poxvirus DNA replication. The 200 kB ds-DNA genome is nicked, exposing a free 3'-OH group that serves as a site for the polymerase complex to initiate strand-displacement primer extension. The nascent DNA (dashed line) and replicated template can then isomerize, and the processive polymerase complex is able to continue synthesis along the length of the genome to generate tail–tail concatemeric dimers that are ultimately resolved into mature monomeric genomes.

occurs by copying the remainder of the template genome, moving through the hairpin structure and along the other template strand (iv). The resultant DNA structures are tail/tail concatemers (v) that are resolved into mature monomeric genomes (vi). Several protocols have been developed to analyze DNA replication both in vivo and in vitro. Monitoring ^{3}H-thymidine incorporation can assess the rate of DNA synthesis, while the steady-state levels of viral DNA accumulation are accurately quantitated by dot-blot Southern hybridization. To visualize the subcellular sites of DNA replication in vivo, viral DNA can be labeled by bromodeoxyuridine (BrdU) incorporation and subsequently detected by immunofluorescence microscopy using an anti-BrdU antibody. To determine if the maturation and processing of replication intermediates has occurred, the genome resolution assay and pulse field gel electrophoresis can be used. To investigate the *cis*-acting regulatory elements (sequence and/or structure) involved in vaccinia DNA replication, the minichromosome replication assay can be used. Finally, assessment of viral polymerase activities can be monitored in vitro by quantitating the ability of cytoplasmic extracts or purified protein(s) to incorporate radiolabeled nucleotides into a nicked double-stranded template or to convert a primed/single-stranded M13 template to a fully replicated RFII product (replicative form II; double-stranded nicked circular DNA product).

2. Materials

1. Tissue culture media: Dulbecco's modified Eagle's medium (DMEM).
2. Vaccinia virus: Western Reserve (WR) strain (wild-type [wt] and/or temperature sensitive [ts] strains; *see* **Note 1**).
3. Cell lines: BSC40 American Type Culture Collection (ATCC # CRL-2761) and L 929 (ATCC # CCL-1).
4. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 2 m*M* KCl, 10 m*M* Na_2HPO_4, 1 m*M* KH_2PO_4, pH 7.4.
5. Glass beads for DNA purification.
6. TE buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.
7. End-over-end rotator.
8. 1X TAE: 40 m*M* Tris acetate, 1 m*M* EDTA.
9. Kodak BioMax MS or MR scientific imaging film (Eastman Kodak Co, Rochester, NY).
10. Kodak BioMax MS intensifying screen.
11. GF/C glass fiber filters (Whatman Inc., Maidstone, England).
12. Zeta probe membrane (Bio-Rad, Hercules, CA).
13. 20X SSC: 3 *M* NaCl, 0.3 *M* Na_3 citrate (pH 7.0).
14. Hybridization solution: 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 6X SSC, 5X Denhardt's solution, 100 μg/mL boiled salmon sperm DNA.
15. Phosphorimager system.

2.1. ^{3}H-Thymidine Incorporation

1. Methyl-^{3}H-thymidine.
2. NP-40 lysis buffer: 150 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.8, 1.5 m*M* $MgCl_2$, 0.65% NP-40.
3. Ready protein liquid scintillation cocktail (Beckman Coulter, Fullerton, CA).

2.2. Dot-Blot Southern Hybridization

1. Dot-blot loading buffer: 10X SSC, 1 *M* NH_4Ac.
2. Dot-blot apparatus (Bio-Rad).
3. DNA denaturing solution: 0.5 *M* NaOH, 1.5 *M* NaCl.
4. *Hin*dIIID and *Hin*dIIIE fragments of the vaccinia virus genome.

2.3. BrdU Incorporation and Immunofluorescence

1. 5-Bromo-2'-deoxyuridine: 2.5 mg/mL stock solution.
2. Anti-BrdU antibody (Developmental Studies Hybridoma Bank, University of Iowa).
3. 16% paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA).
5. Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Eugene, OR).
6. Slow Fade equilibration buffer system (Molecular Probes).

2.4. Genome Resolution

1. Isatin-β-thiosemicarbazone (IBT; Pfaltz and Bauer, Waterbury, CT).
2. Cell suspension solution: 20 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 10 m*M* EDTA.

2.5. Pulsed-Field Gel Electrophoresis

1. CHEF suspension buffer: CHEF genomic DNA plug kit (Bio-Rad).
2. Clear-cut agarose (Bio-Rad).
3. Proteinase K reaction mixture (Bio-Rad).
4. Wash buffer (Bio-Rad).
5. CHEF-DRII apparatus (Bio-Rad).
6. Immobilon-Ny+ nylon membrane (Millipore, Billerica, MA).

2.6. Minichromosome Replication Assay

1. pBluescript II KS (Stratagene, La Jolla, CA).
2. 26-mer oligonucleotide: 5'-TCGACGGTACCGCTTTTGCGGTACCG-3'.
3. pUC19 (New England Biolabs, Beverly, MA).
4. pHS (3; *see* **Note 2**).
5. Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA).
6. Hypotonic lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* KCl, 5 m*M* EDTA.
7. Minichromosome preparation reaction mixture: hairpin and stuffer DNA, 1 m*M* $MgCl_2$, 5 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 0.1 m*M* DTT, 10 m*M* rATP, 4000 U T4 DNA ligase, and 10 U each of *Sal*I and *Xho*I in final volume of 50 μL.
8. Cytoplasmic lysate buffer: cell lysate supplemented with 90 μg/mL proteinase K, 0.6% SDS, and 176 m*M* NaCl.

2.7. Detection of Processive Polymerase Activity

1. 24-mer oligonucleotide: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'.
2. ssM13mp18 (New England Biolabs).
3. *Escherichia coli* single-strand binding (SSB) protein (Stratagene).
4. Single-primed M13 replication reaction mix: 25 μL containing 10 m*M* Tris-HCl, pH 7.5, 40 mg/mL bovine serum albumin (BSA), 4% glycerol, 0.1 m*M* EDTA, 5 m*M* DTT, 8 m*M* $MgCl_2$, 25 fmol primed M13 template, 750 ng *E. coli* SSB, 60 μ*M* each of dCTP and dGTP, and viral proteins.
5. Stop solution: 1% SDS/40 m*M* EDTA.
6. RFII-TBE: 50 m*M* Trizma base, 50 m*M* boric acid, 1 m*M* EDTA.

2.8. Incorporation of dNTPs into a Nicked ds-DNA Template

1. Salmon sperm type III DNA (Sigma, St. Louis, MO).
2. SS DNA buffer: 10 m*M* Tris-HCl, pH 7.2, 5 m*M* $MgCl_2$.
3. Incorporation reaction mixtures: 100 μL containing 50 m*M* phosphate buffer, pH 7.4, 0.5 mg/mL BSA, 5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.3 mg/mL activated salmon sperm DNA, 100 μ*M* each of dGTP, dATP, dCTP, and 10 μ*M* [^{32}P]TTP (1500 cpm/pmol).

3. Methods

3.1. ^{3}H-Thymidine Incorporation in Infected Cells

To measure the rate of viral DNA synthesis, one can monitor the incorporation of radiolabeled thymidine into the cytoplasmic pools of DNA ***(4,5)***. However, owing to changes in nucleotide pools and feedback inhibition of the viral thymidine kinase, this method can only accurately gauge the levels of viral DNA accumulation up to approx 5-h postinfection (hpi). An additional caveat to this method is that it can only be used with thymidine kinase-positive viruses. Nevertheless, it is the most accurate way to analyze the early stages of DNA synthesis and to monitor the short-term and/or rapid impact of changes in incubation temperatures or added inhibitors on viral DNA synthesis (*see* **Note 3**).

3.1.1. Infection and Radiolabeling

1. Confluent 35-mm dishes of BSC40 cells are infected at multiplicity of infection (moi) of 5 pfu/cell and maintained at either 37°C (*wt*) or 31.5°C/40°C (*ts* mutants; *see* **Note 4**).
2. At the indicated time-points (e.g., 1.5, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 8 hpi), rinse cells in serum-free DMEM and incubate with DMEM containing 10 μCi/mL methyl-^{3}H-thymidine (83.7 Ci/mmol).
3. After 30-min labeling, chill cells by placing dishes on ice, remove the media, and wash the cell monolayer gently with cold PBS.
4. Remove the PBS wash and add 500 μL ice-cold NP-40 lysis buffer to the cells (*see* **Note 5**).
5. After 1–2 min, gently remove all of the fluid to an Eppendorf tube without disrupting the adhered material.

3.1.2. Quantitating ^{3}H-Thymidine Incorporation

1. To the DNA-containing cytoplasmic lysate, add an equal volume of cold 20% (v/v) trichloroacetic acid (TCA). Mix well by vortexing and incubate on ice for ≥30 min.
2. Prewet GF/C filters in 10% TCA and load them into a vacuum filtration apparatus (*see* **Notes 6** and **31**).
3. Vortex each sample right before applying it to the manifold. Wash the filters well by filling the manifold chamber two times with ice-cold 10% TCA and draining under vacuum.
4. Perform a final wash with 95% ethanol to dry the filters.
5. Transfer filters to scintillation vials and allow to air-dry.
6. Cover filters with Ready Protein liquid scintillation cocktail; cap the vials and shake well to allow the scintillant to come into contact with the sample (*see* **Note 7**).
7. Quantitate the cpm present on each filter, and therefore incorporated into each culture, in a β-scintillation counter (*see* **Note 8**). Data should be acquired in duplicate and then graphed.

3.2. Dot-Blot Southern Hybridization for Viral DNA Quantitation

To assess the steady-state levels of viral DNA, the accumulation of viral DNA sequences can be most accurately achieved using dot-blot Southern hybridization ***(5,6)***. Although levels of viral DNA are not strongly detected until approx 4–5 hpi, accurate data points can be obtained up to 24 hpi (*see* **Note 9**).

3.2.1. Infection and Harvesting

1. Confluent 35-mm dishes of cells are infected at the desired moi (2–15 pfu/cell) and maintained at either 37°C (*wt*) or 31.5°C/40°C (*ts* mutants).
2. At the indicated time-points (e.g., 3, 6, 9, 12, and 24 hpi), cells are chilled by placing dishes on ice. An ethanol-flamed rubber policeman is used to scrape the plate so that the cells become detached from the dish and suspended in the culture medium.
3. The cells are recovered from the harvested medium by low-speed centrifugation (800*g* for 5 min at 4°C) and washed once with cold PBS.
4. After a second centrifugation, the pellet is resuspended in 500 µL dot-blot loading buffer and subjected to three cycles of freeze-thawing to disrupt the cells.

3.2.2. Spotting of Viral DNA onto Membranes and Detection

1. Assemble the dot-blot apparatus with a hydrated Zeta probe membrane and connect to a vacuum aspirator (vacuum off).
2. To equilibrate the membrane, 50 µL dot-blot loading buffer is added to each well with a multichannel pipettor. Vacuum is applied to draw the wash through and then turned off.
3. Vortex each sample immediately prior to removing 25 µL for addition to the appropriate well (*see* **Note 10**).
4. Apply vacuum slowly to allow each sample to drain thoroughly. Turn vacuum off.
5. Denature DNA *in situ* by adding 50 µL DNA denaturing solution (aspirator off) with a multichannel pipettor and incubate for 10 min. Apply vacuum slowly to allow each well to drain thoroughly. Turn vacuum off.
6. Neutralize by performing two washes (5-min incubation each) with 50 µL 10X SSC. In each case, apply solution with vacuum off, let stand 5 min, and then drain.
7. Remove membrane from apparatus and allow to air-dry.
8. Prepare a ^{32}P-radiolabeled probe by nick translation or random priming using standard protocols ***(12)***. Use 100 ng of the *Hin*dIIID and *Hin*dIIIE vaccinia virus genome fragments to obtain good coverage of the viral genome (*see* **Note 11**).
9. Perform a ≥1 h prehybridization of the membrane in hybridization buffer at 42°C (*see* **Note 12**).
10. For hybridization, prepare fresh hybridization buffer to which you add 1 × 10^6 cpm/mL probe DNA that has been boiled together with carrier DNA (i.e., salmon sperm). Incubate at 42°C for 12–18 h.
11. Wash the membrane as follows: 2X SSC at room temperature (3 × 15 min), followed by 0.2X SSC/0.1% SDS at 55°C (2 × 30 min).

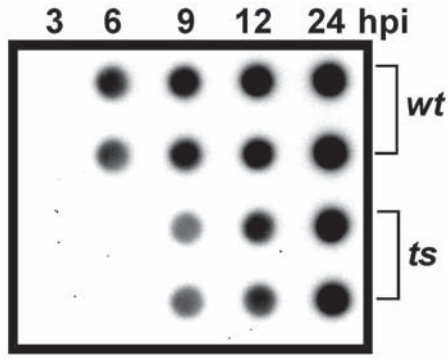

Fig. 2. Accumulation of viral DNA analyzed by dot-blot Southern hybridization. Cell lysates were prepared at 3, 6, 9, 12, and 24 hpi at 40°C with *wt* virus or a replication-impaired *ts* mutant. Lysates were spotted onto a Zeta probe membrane, and viral DNA was visualized after hybridization with radiolabeled probe, representing fragments of the vaccinia genome. The autoradiograph clearly shows that the accumulation of viral DNA in cells infected with the *ts* mutant is significantly delayed when compared to that seen with the *wt* virus.

12. Air-dry the membrane and visualize the radiolabeled dots by autoradiography on Kodak MS film (–80°C with intensifying screen for approx 3 h); quantitate using a phosphorimager system. *See* **Fig. 2** for example result of such a procedure.

3.3. Incorporation of BrdU to Visualize Viral DNA by Indirect Immunofluorescence Microscopy

To observe the subcellular sites of viral DNA synthesis, immunofluorescence microscopy can be used ***(8)***. This method allows the visualization of both the size of the viral DNA factory as well as the intensity of staining (*see* **Note 13**).

3.3.1. Infection

1. Seed cells on 35-mm dishes (*see* **Note 14**) and when confluent, infect with *wt* virus (or any thymidine kinase-positive derivative) at an moi of 5 pfu/cell.
2. After the 30-min adsorption period, the inoculum is removed and cells are refed with fresh medium containing 25 μg BrdU/mL.

3.3.2. Immunofluorescence Analysis

1. At 7 hpi, place dish on ice, remove medium, and rinse cells gently with cold PBS.
2. Fix cells with 4% PFA in PBS for 15 min on ice.
3. Gently wash cells two times with cold PBS.
4. Permeabilize the cells by adding 0.1% Triton X-100/PBS for 5 min on ice.

5. Rinse once (gently) with cold PBS.
6. Incubate with 5 μg/mL anti-BrdU antibody in PBS (1.5 mL/35-mm dish) for 1 h at room temperature.
7. Rinse five times (gently) with cold PBS.
8. Incubate with 1:400 dilution (in PBS) Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody for 1 h room temperature.
9. Rinse five times (gently) with cold PBS.
10. Equilibrate with 1 drop Slow Fade pre-equilibration buffer and incubate for 5 min at room temperature.
11. Aspirate any excess liquid and mount a circular coverslip using one drop of the Slow Fade light component A. Store dishes in the dark at 4°C.
12. Examine dishes on an upright or inverted microscope set up for immunofluorescence analysis and photograph.

3.4. Genome Resolution Assay

As described in **Subheading 1.**, and diagrammed in **Fig. 1**, tail–tail genomic concatemers that form during viral DNA replication are resolved by the vaccinia Holliday Junction resolvase, A22R ***(9)***. The following protocol can be used to detect the concatemer junction fragment, providing evidence for the accumulation of concatemers that have not undergone resolution to mature genomes ***(9,10)***.

3.4.1. Infection and Harvesting Viral DNA

1. Confluent 35-mm dishes of BSC40 cells are infected with *wt* virus at an moi of 2 pfu/cell and maintained at 37°C (*see* **Note 15**).
2. At 18 hpi, cells are chilled on ice, scraped with an ethanol-flamed rubber policeman, collected by centrifugation (800*g* for 5 min at 4°C), and rinsed with cold PBS (*see* **Note 16**).
3. Resuspend cell pellet in 50 μL cell suspension solution and incubate on ice for 10 min.
4. Adjust the sample to a final volume of 300 μL with more cell suspension solution, such that the final volume also contains 0.6% SDS and 0.7 mg/mL proteinase K, and incubate at 37°C for 6 h on an end-over-end rotator.
5. Purify viral DNA by organic extraction and ethanol precipitation.
6. Resuspend the DNA pellet in 50 μL TE and shear the nuclear DNA by passing through a 25-gauge needle five times.

3.4.2. DNA Digestion and Southern Blot Analysis

1. Digest 1 μg DNA with *Bst*EII and resolve on a 20-cm 1% agarose gel and run in 1X TAE at 25 V for 16 h (*see* **Note 17**).
2. Rinse the gel well in dH_2O, soak in 0.25 *M* HCl for 10–15 min at room temperature, and rinse again with dH_2O three to five times.
3. To prepare the gel for transfer, soak in 0.4 *N* NaOH for approx 30 min (with multiple solution changes).

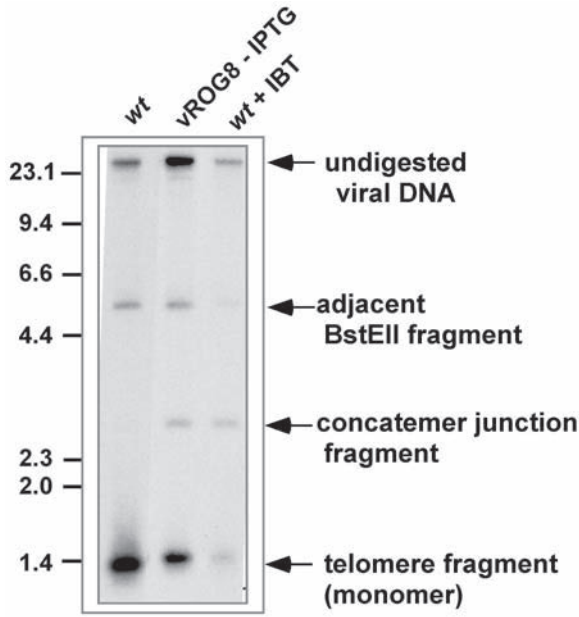

Fig. 3. Resolution of replicated genomic concatemeric structures into mature genomes. In cells infected with the *wt* virus in the presence of IBT, or cells infected with vROG8 in the absence of IPTG, there is an accumulation of concatemeric DNA intermediates that yield a 2.6-kb junction fragment in this *Bst*EII digest. In control *wt* infections, resolution occurs efficiently and only monomeric genomes, which yield a 1.3-kb *Bst*EII fragment, are seen. DNA molecular markers on noted on the left in kilobase.

4. Transfer the DNA (by capillary action) to Zeta probe (*see* **Note 18**) using 0.4 *M* NaOH for 8 h.
5. To prepare the probe, release a 1.2-kb *Pvu*II/*Eco*RI fragment from pSV9 ***(11)***. ^{32}P-radiolabel the purified fragment by nick translation or random priming ***(12)***.
6. Perform a ≥1-h prehybridization of the membrane in hybridization buffer at 42°C (*see* **Note 12**).
7. For hybridization, prepare fresh hybridization buffer to which is added 1×10^6 cpm/mL of probe DNA that has been boiled together with the carrier (i.e., salmon sperm) DNA. Incubate at 42°C for 12–18 h.
8. Wash the membrane as follows: 2X SSC at room-temperature (3 × 15 min), followed by 0.2X SSC/0.1% SDS at 55°C (2 × 30 min).
9. Air-dry the membrane and visualize the DNA by autoradiography on Kodak MR film (4–18-h exposure at –80°C with intensifying screen) or on a phosphorimager system. *See* **Fig. 3** for the example result of such a procedure.

3.5. Analysis of Viral DNA by Pulse-Field Gel Electrophoresis

To analyze the accumulation of larger genomic structures during DNA replication, viral DNA can be harvested and analyzed by pulse-field gel electrophoresis (PFGE). This technique allows the detection of intact monomeric genomes, multimers, or larger branched structures that appear to accumulate during some mutant infections ***(9,13)***.

3.5.1. Infection and Harvesting

1. Confluent 35-mm dishes of BSC40 cells are infected at an moi of 5 pfu/cell.
2. At the indicated time-points (e.g., 2, 4, 6, 10, 12, and 24 hpi), dishes are chilled on ice and cells are released into the media with an ethanol-flamed rubber policeman.
3. Cells are pelleted by centrifugation (800*g* for 5 min at 4°C), washed once with cold PBS, and resuspended at a concentration of 10^7 cells/mL in CHEF suspension buffer.

3.5.2. Preparation of Samples, PFGE, and Southern Blot Analysis

1. The cell pellets are warmed to 50°C, mixed with clear-cut agarose to a final concentration of 0.75%, and formed into 100-µL agarose plugs.
2. The agarose plugs are incubated with the proteinase K reaction mixture at 50°C for 24 h, then equilibrated in wash buffer.
3. One-third of the agarose plug is loaded into a 1% agarose gel and subjected to PFGE in 0.5X TBE using a CHEF-DRII apparatus.
4. PFGE is performed at 5.8 V with a switching-time gradient of 50–90 s for 22 h at 14°C (*see* **Note 19**). The agarose gel is stained with 0.5 µg ethidium bromide/mL and ultraviolet (UV)-irradiated on a transilluminator for 10 min, followed by soaking in 0.25 *M* HCl for 30 min to nick the DNA.
5. DNA is transferred to an immobilon-Ny+ nylon membrane and detected by Southern blot hybridization ***(7)*** using radiolabeled vaccinia virus genome fragments (*see* **Subheading 3.2.2.**, **step 8**).

3.6. Minichromosome Replication Assay

The minichromosome assay was established to study the *cis*-acting sequences that enable templates to be efficiently replicated by the viral replication machinery. Minichromosomes are generated by flanking a central plasmid sequence ("stuffer") with sequences derived from the telomeres of the viral genome, which include the extrahelical base-containing hairpin termini ***(3)***. The minichromosomes are introduced into infected cells by lipofection, and replication is quantitated by determining the levels of *Dpn*I-resistant DNA present within cytoplasmic extracts harvested at 24 hpi.

3.6.1. Preparation of Minichromosomes

1. Prepare the stuffer by digesting approx 5 µg pBluescript II KS^{-sal} (*see* **Notes 20** and **21**) with *Xho*I.

2. Purify the plasmid DNA by organic extraction and ethanol precipitation and resuspend in TE buffer, aiming for a concentration of approx 200 ng/μL (approx 100 fmol/μL).
3. Prepare the hairpin termini by digesting 7–8 μg pHS (***3***; *see* **Notes 2** and **20**) with *Sal*I. Resolve the DNA on a 1% agarose gel cast and run in 1X TAE.
4. Excise the 414-nt fragment from the gel, purify on glass beads, and elute into 10 μL TE buffer by incubation at 37°C for 2 h (with gentle rotation if possible; *see* **Note 2**). Quantitate by resolving an aliquot on a 1% agarose/1X TAE gel followed by ethidium bromide staining/UV illumination.
5. Heat the purified fragment at 95°C for 5 min and then snap-cool by rapidly placing on ice for 5 min to convert the palindromic duplex into two hairpin isoforms that contain the authentic set of extrahelical bases (***14***,***15***; *see* **Note 22**).
6. Generate the minichromosomes by ligating the hairpins to plasmid stuffer at a 3:1 molar ratio (1200 fmol hairpins/400 fmol stuffer) in the presence of DNA ligase, *Sal*I and *Xho*I. Minichromosome preparation is optimal when performed at 16°C for 24 h in a final volume of 50 μL minichromosome preparation reaction mixture (*see* **Notes 23** and **24**).
7. Heat-inactivate the reaction at 68°C for 10 min.
8. Resolve entire reaction on a 1% agarose/1X TAE gel, excise the band corresponding to the minichromosomes, and purify using glass beads.
9. Resuspend purified minichromosomes in approx 20 μL sterile TE; quantitate by examining an aliquot on a 1% agarose/1X TAE gel followed by ethidium bromide staining/UV illumination.

3.6.2. Replication Assay

1. Confluent 60-mm dishes of L929 cells are infected with *wt* virus at an moi of 3 pfu/cell.
2. After 30-min adsorption, cells are transfected with 15 fmol of the minichromosome templates using lipofectamine plus reagent.
3. At 6 hpi, the medium is removed and cells are fed with fresh DMEM supplemented with 5% fetal bovine serum (FBS).
4. Cells are released from the dish at 24 hpi by scraping with an ethanol-flamed rubber policeman, collected by centrifugation (800*g* for 10 min at 4°C), and washed with cold PBS (*see* **Note 25**). The cell pellet is resuspended in 135 μL hypotonic lysis buffer and incubated on ice for 5 min.
5. Triton X-100 is added to 1% and β-mercaptoethanol to 40 m*M*, and incubation is allowed to proceed for 10 min on ice.
6. Nuclei are removed by centrifugation at 700*g* for 10 min at 4°C.
7. The supernatant fluid, representing a cytoplasmic lysate, is then supplemented with cytoplasmic lysate buffer and incubated at 37°C for 2 h on an end-over-end rotator.
8. DNA is purified by organic extraction and ethanol precipitation and resuspended in 20 μL TE.

3.6.3. Analysis of the Replication Assay

1. 10 μL (one-half) of each DNA sample is digested with *Dpn*I for 2 h at 37°C.
2. The undigested and digested DNA samples are resolved on a 20-cm 1% agarose/1X TAE gel for 2 h at 200 V (*see* **Note 26**).
3. The gel is soaked in 0.25 *M* HCl (2 × 15 min) and the DNA is then transferred to a Zeta probe (*see* **Note 18**) using 0.4 *N* NaOH for 16 h.
4. To prepare the probe (the *E. coli* β-lactamase gene), pUC19 DNA is digested with *Ssp*I and *Afl*III to release a 1.7-kb fragment, which can be purified and ^{32}P-radiolabeled by nick translation or random priming ***(12)***.
5. Perform a 1–4-h prehybridization of the membrane in hybridization buffer at 42°C (*see* **Note 12**).
6. For hybridization, prepare fresh hybridization buffer to which 1×10^6 CPM/mL of probe DNA that has been boiled together with the carrier (i.e., salmon sperm) DNA is added. Incubate at 42°C for 12–18 h.
7. Wash the membrane as follows: 2X SSC at room temperature (3 × 15 min), followed by 0.2X SSC/0.1% SDS at 55°C (2 × 30 min).
8. Air-dry the membrane and visualize by autoradiography on Kodak MS film (16 h exposure at –80°C with intensifying screen).

3.7. Single-Primed M13 Replication Assay

This assay measures the ability of purified viral proteins or cytoplasmic lysates to convert a primed single-stranded M13 template to the duplex form in a manner that requires processive DNA synthesis ***(16–18)***. It provides a measure of the stability and activity of components of the processive polymerase complex.

3.7.1. Preparation of Primer/Template

The 24-mer oligonucleotide primer is annealed to the ssM13mp18 template at a 20:1 molar ratio (3.21 μ*M* primer/160 n*M* M13; ***17***). The reaction is set up in a screw-cap tube, placed in a boiling water bath for 5 min, and allowed to cool to room temperature for 1–2 h (*see* **Note 27**).

3.7.2. Reaction Conditions

1. Reaction mixtures (25 μL) contain single-primed M13 replication reaction mix as well as the protein(s) or lysate of choice. For cytoplasmic lysates, 2 μg protein is used per reaction (*see* **Note 28**).
2. Reactions are incubated for 3 min in a 30°C water bath to allow replication complexes to assemble at the primer template junction.
3. Replication is then allowed to proceed by the addition of 60 μ*M* dATP and 20 μ*M* [α^{32}P]TTP (5 μCi/nmol); incubations are continued for an additional 1–30 min at 30°C.
4. If synthesis is processive, the RFII form should first be seen after 5-min incubation, with levels increasing as the incubation continues.
5. Reactions are terminated by addition of equal volume of stop solution.

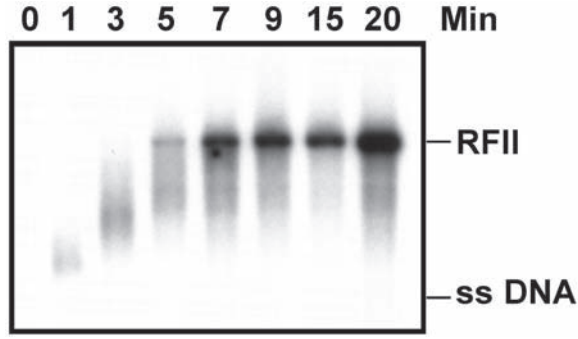

Fig. 4. Processive polymerase activity detected using a primed M13 template. In this time-course experiment, the protein mixture assayed can complete RFII formation within 5-min incubation; the levels of product increase thereafter.

3.7.3. Visualization of RFII Formation

1. A 16-μL aliquot of the above reaction is resolved on a 20-cm 0.8% agarose gel containing 0.125 μg ethidium bromide/mL, cast and run in 1X RFII-TBE.
2. Electrophoresis is performed for 16–18 h at 15 mA (*see* **Note 29**).
3. The gel is then photographed, dried on a gel drier without heat, and exposed to film for autoradiography (30-min exposure at –80°C with intensifying screen). *See* **Fig. 4** for the example result of such a procedure.

3.8. Incorporation of dNTPs into an Artificially Nicked ds-DNA Template

This assay provides the most basic quantitation of the DNA polymerase activity present in cytoplasmic extracts or purified protein preparations ***(19)***.

3.8.1. Preparation of Template

1. 50 mg salmon sperm (SS) type III DNA is dissolved in 100 mL SS DNA buffer and incubated at 4°C for 72 h.
2. After organic extraction, the DNA is recovered by ethanol precipitation and resuspended in TE at a concentration of 5 mg/mL.

3.8.2. Incorporation of Radiolabeled Nucleotide into the Nicked Template

1. 100 μL incorporation reaction mixtures are initiated by the addition of the polymerase or extract and incubated for 30 min at 37°C (*see* **Note 30**).
2. Reactions are quenched on ice by the addition of an equal volume of ice-cold 20% TCA/0.2 *M* sodium pyrophosphate, mixed well, and kept on ice for at least 10 min.
3. Samples are applied to GF/C glass fiber filters (prewet in 10% TCA) using a vacuum filtration apparatus (*see* **Notes 6** and **31**).

4. The filters are washed well two times under vacuum by the application of ice-cold 10% TCA (fill the manifold cup each time). Filters are then washed once with 95% ethanol to facilitate their drying.
5. Filters are transferred to scintillation vials and allowed to air-dry (*see* **Note 8**). The bound radioactive material is measured by Çerenkov counting (*see* **Note 32**).

4. Notes

1. Growth of wild-type vaccinia virus (*wt*) in tissue culture is carried out at 37°C. Temperature-sensitive mutants (*ts* mutants) are grown at either 31.5°C (permissive) or 40°C (nonpermissive).
2. The pHS plasmid contains a 414-bp insert comprising two copies of viral telomeric sequences in a tail-to-tail, palindromic arrangement; this insert was prepared from an intracellular concatemeric junction fragment ***(3)***. When purifying the 414-bp insert on glass beads, elution temperatures of higher than 37°C cannot be used, because the AT-rich palindromic duplex will melt and the resultant single-stranded DNA will bind irreversibly to the glass beads.
3. Determining the rate of viral DNA synthesis as measured by ^{3}H-thymidine incorporation has several caveats. (1) As thymidine incorporation declines after approx 5 hpi due to a feedback inhibition of the viral thymidine kinase, the most accurate time-points at which to assess DNA synthesis with this assay are between 1 and 5 hpi. (2) The assay has to be performed with a thymidine kinase-positive virus. (3) In terms of the assay itself, it is import to use GF/C glass filters, and the ready protein scintillation cocktail.
4. Include an uninfected culture as a control.
5. This treatment disrupts the plasma membrane, but leaves the nuclei intact and adherent to the tissue culture dish.
6. It is useful to include a blank filter as a control for the background radioactivity that may be contaminating the manifold.
7. It is useful to include a vial with scintillant alone as a control.
8. Static from gloves can lead to erroneous readings; thus, remove gloves while loading the vials into the scintillation counter.
9. In addition to providing a temporal profile of DNA accumulation, a drug of interest can also be added, or cultures infected with a *ts* virus can be shifted to a higher temperature after the onset of replication (e.g., 4 hpi) to monitor a direct effect on ongoing synthesis.
10. For accurate quantitation of viral DNA accumulation, each sample should be spotted in duplicate. Vortexing each time an aliquot of the sample is removed improves the reproducibility of the duplicates. If applying samples in duplicates, vortex sample again before removing and loading the second aliquot to dot-blot apparatus.
11. Using the *Hin*dIIID and *Hin*dIIIE vaccinia virus genome fragments represents approx 31 kb of the vaccinia virus genome.
12. Use standard hybridization procedures, such as “Seal-a-meal” pouches in a water bath or hybridization bottles in a rotating hybridization oven.

13. Along with revealing the subcellular compartmentalization of viral DNA replication, immunofluorescence analysis can be used to detect colocalization of replication proteins and/or viral DNA ***(8)***.
14. Cells can be cultured in tissue culture dishes of various sizes or in Lab-Tek (Nalg Nunc) chamber culture slides. Infection with a thymidine kinase-positive virus is required for incorporation of BrdU, thus allowing detection with the BrdU antibody.
15. As a control for concatemer accumulation, cells can be infected with *wt* virus (moi of 2 pfu/cell) in the presence of 60 m*M* IBT. IBT is an antipoxviral drug that causes an abortive infection as a result of mRNA breakdown and, hence, diminished expression of late proteins. As an additional control for the accumulation of concatemeric DNA, infections can be performed with recombinant virus vROG8 ***(20)*** in the absence of IPTG at an moi of 15 pfu/cell.
16. To provide a temporal view of concatemer accumulation, cultures can be harvested as early as 6 hpi.
17. Electrophoresis can also be performed at 100–110 V for 3–4 h, although resolution is not optimal.
18. Besides the Zeta probe, any filter suitable for Southern blot analysis can be used.
19. Several critical parameters should be kept in mind when optimizing the PFGE technique. These parameters include voltage (typically 5–10 V/cm), temperature, recirculation of buffer to prevent uneven heating of the gel while running, buffer choice (0.5X TBE is best because of the minimal amount of current it carries), use of PFGE-agarose, and pulse times.
20. All plasmid preparations used to prepare the stuffer and termini of the minichromosomes must be purified with the alkaline lysis protocol. Minichromosomes prepared in this way are intact and remain stable upon transfection. If plasmids are prepared with Qiagen kits (Valencia, CA), the minichromosomes are rapidly degraded upon transfection.
21. To generate the plasmid used as the source of the stuffer DNA (pBSIIKS^{-Sal}), pBluescript II KS DNA was digested with *Sal*I, the recessed 3' ends were filled in with the Klenow fragment of the *E. coli* polymerase I in the presence of dNTPs, and the DNA was religated and introduced into *E. coli*.
22. These hairpin isoforms have been referred to as "flip" and "flop" in the literature (e.g., **refs. *14,15***).
23. The ligation reaction used to assemble the minichromosomes includes both *Sal*I and *Xho*I to cleave any stuffer–stuffer or hairpin–hairpin multimers that form and thereby drive the reaction to generate hairpin–stuffer–hairpin minichromosomes.
24. Control minichromosomes are prepared in a parallel manner, with the exception that the viral hairpin termini are replaced in the ligation mixture with a 26-mer hairpin oligonucleotide.
25. Although quantitation of minichromosome replication is optimal at 24 hpi, samples can be harvested at early time-points (e.g., <1–4 h postlipofection) to confirm the intracellular delivery and stability of the minichromosomes.
26. An aliquot of the minichromosome preparation used for transfection and/or the purified plasmid stuffer sequence should be loaded in a control lane to serve as a size marker.

27. The annealed primer/template can be stored at –20°C.
28. The RFII assay is performed under conditions (8 m*M* $MgCl_2$) in which the purified DNA polymerase (E9 protein) is distributive and cannot direct the formation of RFII product under conditions of template excess. Thus, this assay measures the productive association of the polymerase with factor(s) conferring processivity. To assay pure polymerase alone, 1850 fmol of E9 protein added to the standard reaction mixture generates RFII in a distributive manner in a 30-min reaction ***(18)***. Alternatively, the $MgCl_2$ can be reduced to 1 m*M*; under these conditions, 37 fmol of E9 completes RFII formation in 5 min ***(17)***.
29. In terms of visualizing assay products, electrophoresis at 75 mA for 2–3 h allows the detection of fully replicated RFII products, but better separation of the RFII product from the single-strand template is obtained if electrophoresis is performed at slower amperage for a longer period of time.
30. Include a control sample lacking polymerase or extract.
31. Use of GF/C glass filters is optimal.
32. Çerenkov counting: filters placed in vials are counted in a scintillation counter in the absence of any scintillation fluid.

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15

Studying the Binding and Entry of the Intracellular and Extracellular Enveloped Forms of Vaccinia Virus

Mansun Law and Geoffrey L. Smith*

Summary

This chapter describes the methods for the study of binding and entry of the two different forms of vaccinia virus (VV)—the intracellular mature virus (IMV) and extracellular enveloped virus (EEV)—using immunofluorescent staining and confocal microscopy. After binding to or penetration of the cells, IMV, EEV, and virus cores are distinguished by different antibodies. Bound virus or penetrated cores are visualized and recorded by confocal microscopy and can be accurately counted. Although specific antibodies to IMV, EEV, and virus cores are required, this method is highly quantitative and also allows the recognition of virus aggregates, which would not be possible using other techniques, such as flow cytometry and radiolabeling of virus particles. Furthermore, this method bypasses the need for EEV purification that may damage the EEV membrane and release an IMV particle.

Key Words: Vaccinia virus; intracellular mature virus; extracellular enveloped virus; binding; entry; confocal microscopy.

1. Introduction

The study of vaccinia virus (VV) binding and entry is complicated by the fact that two different infectious virions, e.g., intracellular mature virus (IMV) and extracellular enveloped virus (EEV), are produced during the virus replication cycle (*see* **Fig. 1A**). IMV is robust and suitable for transmission between hosts, whereas EEV and the morphologically indistinguishable cell-associated enveloped virus (CEV) are responsible for spreading infection within the host (reviewed in **ref. *1***). CEV and EEV are different from IMV both structurally and antigenically (*see* **Fig. 1B**). Because the outermost membrane of EEV is fragile and readily ruptured by various physical conditions, such as ultra-

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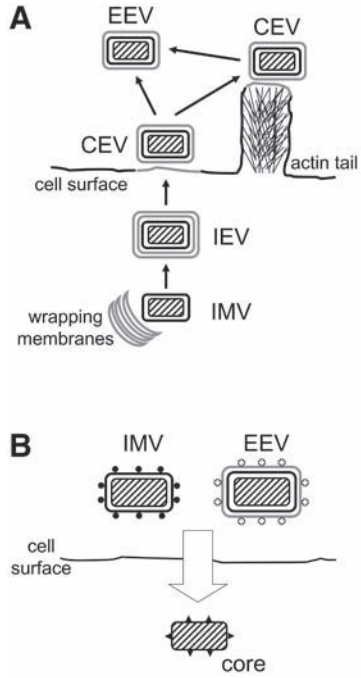

Fig. 1. Different forms of VV. **(A)** Morphogenesis of VV. IMV is the first infectious virion produced during an infection. A fraction of IMV is wrapped by the host membranes to form intracellular enveloped virus (IEV). IEV moves to the cell surface using microtubules, and fusion of the IEV outer membrane with the plasma membrane produces a CEV on the cell surface. A CEV may either induce an actin tail to facilitate dissemination into a neighboring cell or is released from the cell surface to form EEV for long-range spread. Adapted from **ref.** ***1***. **(B)** Schematic illustration of IMV, EEV, and the virus core to emphasize their antigenic difference. The IMV and EEV shown are attached on the cell surface, whereas the virus core has penetrated into the cytosol.

sonication, hypotonicity, freezing and thawing, and low pH, the purification of EEV reduces the level of intact EEV that is resistant to IMV-neutralizing antibody ***(2–5)***. To study the binding and entry of VV and other poxviruses, it is necessary to first define which form of virus is being studied (*see* **Note 1**). If EEV is used, care should be taken to minimize damage to the EEV membrane.

Many techniques have been exploited for the study of poxvirus binding and entry, such as plaque formation ***(6,7)***, electron microscopy (EM; ***8–12***), flow cytometry ***(13)***, and confocal microscopy ***(5,12,14)***. For these assays, viruses labeled with radioisotopes ***(12,15,16)***, biotin ***(13)***, or fluorescent lipids ***(17)*** have been used. Alternatively, recombinant viruses expressing reporter genes

(luciferase or β-galactosidase) have been used to detect cells that have been infected ***(18–24)***. However, virus labeling may not be suitable for EEV because additional steps in virus purification and labeling can disrupt the EEV membrane. Recently, green fluorescent protein (GFP)-labeled poxviruses were generated, although their use for binding and entry have not been demonstrated ***(25–27)***. The pros and cons of the above techniques are summarized in **Table 1**.

Here, methods based on the study of VV-binding and entry using unlabeled virus and confocal microscopy are provided in detail ***(5,12,14,28,29)***.

2. Materials

1. VV strain Western Reserve (WR) or mutants thereof.
2. BHK-21, BS-C-1, and RK_{13} cells for growing and titrating virus infectivity.
3. Cells of interest for studying virus-binding and entry.
4. BS-C-1 and RK_{13} cell growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin (pen-strep), L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS).
5. BHK-21 cell growth medium: Glasgow-modified Eagle's medium (GMEM) supplemented with pen-strep, L-glutamine, tryptose-phosphate broth, and 10% FBS.
6. Virus growth medium: DMEM supplemented with pen-strep, L-glutamine, and 2% FBS.
7. Phosphate-buffered saline (PBS).
8. Tissue culture flasks (T175) and 6-well plates.
9. 13-mm or 22-mm glass coverslips, autoclaved, for growing of adherent cells.
10. Carboxymethylcellulose (CMC), sodium salt, low viscosity, 3% (w/v) dissolved in hot water and autoclaved.
11. 2X DMEM supplemented with 2X pen-strep, L-glutamine and FBS as in virus growth medium.
12. Crystal violet solution: prepare a stock solution of 1% crystal violet (w/v) in absolute ethanol and dilute to 0.1% crystal violet in 15% (v/v) ethanol.
13. Tight-fitting Dounce homogenizer (10- or 15-mL capacity).
14. 10 m*M* Tris-HCl, pH 9.0, sterile-filtered.
15. 40% (w/v) sucrose in 10 m*M* Tris-HCl, pH 9.0, sterile-filtered. This is used as a stock solution for making up other lower dilutions (24%, 28%, 32%, and 36%).
16. Ultracentrifuge with a SW28 swing-out bucket rotor (Beckman) or equivalent.
17. Clear centrifuge tube to fit the rotor above, e.g., Ultra-Clear centrifuge tube size 25 × 89 mm from Beckman for SW28 rotor.
18. Cup-horn ultrasonicator.
19. 4% (v/v) paraformaldehyde solution. Prepare fresh by diluting 16% paraformaldehyde stock solution into 1:1 using distilled water, then further dilute to 4% using equal volume of 0.5 *M* HEPES buffer, pH 7.4.
20. 20 m*M* glycine in PBS.
21. 0.1% (w/v) saponin in PBS.
22. IMV-neutralizing monoclonal antibody (MAb), e.g., mouse MAb 2D5 (anti-L1R; ***3***) or mouse MAb 5B4/2F2 (anti-A27L; ***30***).

Table 1
Techniques Used for the Study of Poxvirus Binding and/or Entry

Method	Pros	Cons
Infectivity assay	Simple and requires no additional equipment.	Relies on measurement of productive infection; therefore, data may not correlate directly with virus-binding or entry. Not useful for cells resistant to infection.
Electron microscopy	IMV and EEV can be distinguished. Ultrafine structures, such as membranes and fusion events, can be visualized.	Time consuming, impractical for large number of samples, possible subjective selection of images, difficult to quantify, and prone to artefacts.
Flow cytometry	Fast, easy, and useful in assays with many samples. Results are expressed as the mean of total binding events.	Only been shown to work using biotin-labeled virus and may not be sensitive enough for detecting virus using VV-specific antibody. Do not recognize virus aggregates. Purification and biotinylation of virus may damage EEV membrane. May not be useful for entry study.
Confocal microscopy	Unlabeled virus used directly with minimal treatment, particularly useful for the study of EEV. Bound and entered virus particles can be quantified. Nonspecific background, such as virus aggregates, can be eliminated based on particle shape and size.	Dependent on the availability of good antibodies. Time consuming to acquire and analyze data.

Radioisotope labeling	Useful in assays with many samples. Results are expressed by the mean of total-binding events.	Use of radioactive substance. Do not recognize virus aggregates. Require purification of the virus that may damage EEV. May not be useful for entry study.
Labeling viral membrane with fluorescent lipids	Real-time measurement of viral membrane fusion using spectrofluorometer.	Requires purification of virus before viral membrane labeling that may disrupt the EEV membrane.
GFP-labeled virus	GFP signal is strong and specific. Detection of GFP-labeled virus is fast and straightforward. GFP may be linked to different components of the virus particle for the study of different stages of virus infection.	Requires construction of recombinant virus. GFP may alter the function of the fused viral protein and therefore affect the binding/entry properties of the virus.

23. Primary antibody against IMV, e.g., mouse MAb 1.1 (anti-D8L; ***31***) (*see* **Note 2**).
24. Primary antibody against EEV, e.g., rat MAb 19C2 (anti-B5R; ***32***) or rat MAb 15B6 (anti-F13L; ***32***).
25. Primary antibody against virus core, e.g., rabbit anticore antibody (***33***; *see* **Note 3**).
26. Fluorescent dye-labeled secondary antibodies, e.g., FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rat IgG, TRITC-conjugated anti-rabbit IgG.
27. Microscope slides and coverslips.
28. Mounting medium, e.g., Mowiol or from a commercial source. The preparation of Mowiol mounting medium has been described in **ref. *34***. Incubate 2.4 g Mowiol 4-88 (Calbiochem) with 3 g glycerol for 1 h, then add 6 mL water, and mix the solution for another 2 h. The solution is mixed with 12 mL 0.2 *M* Tris-HCl, pH 8.5, and incubated at 50°C for 10 min. The resulting solution is centrifuged to remove any undissolved materials. If desired, add 1 μg/μL 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) to the supernatant for DNA visualization. Aliquot the completed mounting medium and store at –20°C until use.
29. Confocal microscope with appropriate filters, e.g., Zeiss Laser Scanning Microscope 510.

3. Methods

The methods described below outline (1) the preparation and quantitation of IMV and EEV, (2) virus binding assays, (3) virus entry assays, and (4) confocal microscopy and data analysis.

3.1. Preparation of IMV and EEV

3.1.1. Production of Fresh EEV Using BHK-21 Cell System

EEV represents less than 1% of total infectivity produced after infection with many VV strains ***(35)***, and purification of EEV introduces damage to the EEV membrane. Therefore, it is important to produce EEV in a cell system that makes high levels of EEV with minimal IMV contamination. Previously, VV strain International Health Department (IHD)-J was reported to produce 3–4-fold more EEV from RK_{13} cells than HeLa and SIRC cells ***(36)***, and the IHD-J strain made 10–500-fold more EEV than many other strains including WR (50-fold) using RK_{13} cells ***(35)***. Therefore, the IHD-J strain is convenient to study EEV binding and entry. However, because many VV mutants have been generated from the WR strain, another system is introduced here that produces EEV of WR virus up to 1×10^7 plaque forming units (pfu)/mL with approx 80% intact EEV (G. Rodger, M. Law, G. Carter, and G. L. Smith, unpublished data). For other VV strains or mutants that make less EEV, other cell systems or methods to concentrate EEV may be necessary. The quality of EEV produced in any culture system with minimal contamination with IMV and maximal EEV membrane integrity should be considered in addition to the total yield of extracellular virus (*see* **Subheading 3.1.3.**).

1. Grow BHK-21 cells in T175 flasks to confluency in a 5% CO_2 incubator.
2. Wash cells once with PBS to remove cell debris and growth medium.
3. Infect cells with VV WR at 3 pfu/cell (approx 1.5×10^8 pfu/flask) for 2 h in 5 mL virus growth medium with occasional agitation.
4. Wash cells twice with PBS to remove unbound virus (mainly IMV), overlay cells with 10 mL virus growth medium, and incubate for 24 h.
5. Harvest supernatant and remove detached cells by centrifugation at 400*g* for 15 min at 4°C. The infected cells left in the flasks can be scraped into 10 m*M* Tris-HCl, pH 9.0, and stored at –70°C prior to extraction of IMV.
6. Keep the fresh EEV on ice until use and titer virus infectivity (*see* **Subheading 3.1.3.**).

3.1.2. Purification of IMV by Sucrose Density Gradient Velocity Sedimentation

IMV are released by cell lysis that may be induced by hypotonic shock, freezing and thawing, or Dounce homogenization. Because of this, IMV has to be purified away from cellular debris before use. Owing to their structural differences, IMV and EEV sediment at 1.27 and 1.23 g/mL, respectively, in cesium chloride gradient equilibrium sedimentation ***(36,37)***. However, these virions can also be purified in a cheaper way using sucrose gradient velocity sedimentation ***(38)***. To produce high titer of IMV, RK_{13} cells are used.

1. Infect RK_{13} cells with WR virus at 0.1 pfu/cell for 48 h or until full cytopathic effect (CPE) develops. Alternatively, IMV can be extracted from the frozen infected cells in **Subheading 3.1.1.** (go directly to **step 5**).
2. Tap the flasks once or twice to dislodge infected cells. The majority of infected cells should come off; otherwise, leave the flasks for longer incubation.
3. Pellet cells by centrifugation at 400*g* for 10 min at 4°C.
4. Resuspend cell pellet in 10 mL 10 m*M* Tris-HCl, pH 9.0, and leave on ice for 10 min.
5. Dounce-homogenize the cells with 15–20 strokes while keeping the homogenizer in an ice bath.
6. Spin the homogenate at 750*g* for 10 min to pellet nuclei.
7. Collect the supernatant and resuspend the pellet in 10 m*M* Tris-HCl, pH 9.0, rehomogenize and spin as above. Pool the supernatants.
8. Layer the pooled supernatant onto 14 mL 36% (w/v) sucrose cushion in Ultra-Clear centrifuge tubes. Fill up and balance the tubes to 2–3 mm below the top with 10 m*M* Tris-HCl, pH 9.0, and spin at 25,000*g* for 80 min at 4°C in a Beckman SW 28 swing-out bucket rotor (*see* **Note 4**).
9. During the centrifugation, prepare 24–40% (w/v) sucrose gradients by carefully overlaying 7 mL 40%, then 36%, 32%, 28%, and 24% (w/v) sucrose solutions on top of each other into centrifuge tubes. Leave the tubes for 2.5 h or seal the tubes and carefully layer the tubes horizontally for 1 h to facilitate diffusion. Alternatively, the continuous gradients can be prepared directly using a gradient former.

10. After centrifugation through the sucrose cushion, discard the supernatant and resuspend the pellet that contains both virus and cell debris in 2 mL 10 m*M* Tris-HCl, pH 9.0. Sonicate the pellet to disperse any aggregates using a cup-horn ultrasonicator.
11. Overlay the virus suspension onto the sucrose gradients, top-up with 10 m*M* Tris-HCl, pH 9.0, and balance the tubes as in **step 8**. Spin the tubes at 19,000*g* for 50 min at 4°C. Virus from a maximum of five T175 flasks should be loaded onto each gradient.
12. Carefully remove the virus band using a fine pipet (*see* **Note 5**). If necessary, the pellet can be resuspended in 10 m*M* Tris-HCl, pH 9.0, sonicated, and loaded onto another gradient to recover more IMV.
13. Dilute the virus band at least threefold with 10 m*M* Tris-HCl, pH 9.0, and pellet the virus at 30,000*g* for 60 min at 4°C.
14. Resuspend the pellet in a small volume (0.5–1 mL) of 10 m*M* Tris-HCl, pH 9.0, and sonicate gently to disperse any aggregates. Keep aliquots of virus at –70°C.
15. Determine virus infectivity by plaque assay (*see* **Subheading 3.1.3.**) and virus particle concentration by measuring optical density (OD) at 260 nm (1 OD unit = 64 μg virus/mL = 1.3×10^{10} virus particles/mL).

3.1.3. Titration of IMV and EEV

Although different cell types may be used for binding and entry assays, it is useful for virus infectivity to be standardized using a reference cell line. BS-C-1 cells are used in this laboratory because they are relatively large cells with good cell–cell contact inhibition and remain attached to tissue culture plastic for a long period (>10 d in low-serum concentration; ***39***). Clear plaques can be obtained not only for wild-type virus, but also for slow growing strains/mutants that require longer incubation periods before the plaques can be visualized.

IMV can be titrated easily following a standard procedure (*see* **Chapter 8**). EEV infectivity can only be determined when the infectivity of contaminating IMV and broken EEV are subtracted. This can be done with a standard plaque assay with the inclusion of an IMV-neutralizing MAb.

1. Prepare confluent monolayers of BS-C-1 cells on six-well plates.
2. Make dilutions of IMV and EEV in virus growth medium to obtain approx 200 pfu/well for statistically reliable counts (*see* **Note 6**).
3. For EEV, add IMV-neutralizing MAb to the diluted virus and incubate at 37°C for 1 h. The final MAb concentration should be enough to inhibit more than 95% infectivity of purified IMV under similar experimental conditions.
4. Wash cell monolayers once with PBS.
5. Inoculate 0.5 mL virus dilutions into triplicate wells and incubate for 1.5 h with regular agitation to prevent cell monolayer from drying.
6. Aspirate the virus inoculum and wash cells once with PBS.
7. Overlay each well with 2 mL 1.5% CMC solution (mix 1:1 [v/v] of 3% CMC solution and 2X DMEM) and incubate for 40 h for wild-type virus (*see* **Note 7**).

8. Aspirate overlay completely and cover each well with 1 mL 0.1% crystal violet solution in 15% ethanol. Rock the plates several times and leave the plates for 30 min to stain.
9. Remove the stain and rinse the plates gently with tap water. Count plaques when plates are dried.
10. Calculate the IMV and EEV infectivity in pfu/mL. The quality of the EEV preparation is expressed as % EEV (titer with IMV-neutralizing MAb/titer without IMV-neutralizing MAb × 100%). The cell system described previously (*see* **Subheading 3.1.1.**) normally produces approximately 80% EEV infectivity.

3.2. Virus-Binding Assay

Even when using purified IMV and freshly prepared EEV, low levels of the other virus form may be present and, therefore, MAbs specific to IMV and EEV are useful reagents to distinguish and quantify these particles by indirect immunofluorescent staining and confocal microscopy. If necessary, IMV and EEV can be detected at the same time by double immunofluorescent staining ***(5)***. Another advantage of this technique is that the presence of virus aggregates can be observed ***(29)***. An example of applying this technique in a virus binding study is shown in **Fig. 2**.

1. Grow a confluent monolayer of cells on autoclaved glass coverslips.
2. Dilute IMV in ice-cold virus growth medium or other appropriate buffer. If frozen virus is used, sonicate the virus after thawing and before dilution. For EEV, use undiluted fresh EEV preparation.
3. Cool cells on an ice platform and wash cells once with ice-cold PBS.
4. Allow virus binding to cells in the minimum volume sufficient to cover the cells (approx 50 µL/cm^2 of cells) and incubate with occasional agitation for the desired time at 4°C to prevent virus entry. A greater incubation volume may be required for EEV as EEV may be at low concentrations (normally <10^7 pfu/mL) in fresh preparation.
5. Remove unbound virus by washing cells twice with cold PBS.
6. Fix cells with 4% paraformaldehyde in 250 m*M* HEPES for 10 min at 4°C, then for 20 min at room temperature. The remaining steps can be performed at room temperature.
7. Aspirate the fixative, wash the cells once with PBS, and then quench fixation with 20 m*M* glycine in PBS for 5 min.
8. If the antigens to be detected are not exposed on the virus (e.g., F13L of EEV), the membrane is permeabilized and blocked at the same time using 0.1% saponin (w/v) in PBS with 10% FBS for 20 min. Otherwise, proceed to next step.
9. Block nonspecific-binding sites on samples with PBS containing 10% FBS (blocking buffer) for 20 min.
10. Prepare appropriate dilution of primary antibodies specific for IMV or EEV in blocking buffer (*see* **Note 8**). If IMV and EEV are to be detected simultaneously, the IMV- and EEV-specific primary antibodies should be of different species or

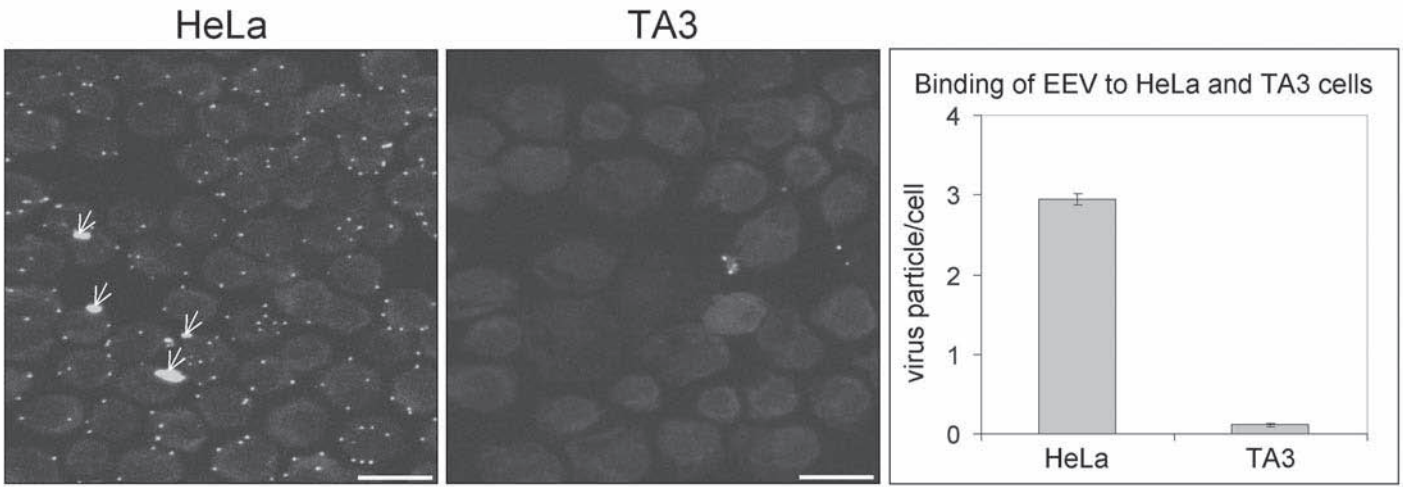

Fig. 2. Binding of EEV to HeLa and TA3 cells. Previously TA3 cells were shown not to bind IMV ***(20)***, but their ability to bind EEV is addressed here. TA3 cells were grown in suspension culture, and HeLa cells were detached by incubation in PBS containing 5 m*M* EDTA for 20 min at 37°C. The suspension cells (2×10^6) were bound with fresh EEV (1 mL, 1×10^7 pfu/mL, 70% EEV) on ice for 2 h. Bound virus particles were detected using rat MAb 19C2 (anti-B5R) and FITC-conjugated donkey anti-rat IgG as described in **Subheading 3.2.** Optical sections of the cells were collected using a confocal microscope (*see* **Subheading 3.4.**), and the images shown are a projection of the optical sections. Arrows indicate virus aggregates that were excluded in the counting of virus particles. Virus aggregates would not have been distinguished from individual particles by other methods, such as flow cytometry and radioisotope labeling. Bound virus particles of each image were counted, and the average of virus particle/cell ± standard deviation ($n = 6$) is shown. Total numbers of HeLa and TA3 cells counted were 197 and 182, respectively. Bar = 20 μm. Similar to IMV, the results showed that EEV does not bind TA3 cells efficiently.

isotypes. For instance, use mouse MAb 1.1 for IMV and rat MAb 19C2 for EEV. In this situation, use the same antibody concentrations as for single-color staining.

11. Pipet 50 μL antibody onto a parafilm sheet for each 22-mm coverslip.
12. Gently lift up the coverslips with a pair of fine forceps and absorb excess liquid with a piece of tissue. Place the coverslips onto the antibody with the cells facing down and incubate for 45 min at 37°C in a humidified chamber.
13. Wash cells three times by placing the coverslips onto 100 μL PBS and incubate for 5 min. Between each wash, absorb excess liquid with tissue and pass coverslips over large-quantity PBS in a container.
14. Prepare appropriate dilution of fluorescent dye-labeled secondary antibodies specific for the primary antibodies and incubate and wash the samples as in **steps 11–13** (*see* **Note 9**).
15. Pass the coverslips over distilled water after the final wash in PBS before mounting.
16. Mount the coverslips onto glass slides with commercial-mounting product or Mowiol-mounting medium and leave slides in the dark for the mounting medium to dry.
17. Store slides at 4°C and analyze results within 2 weeks (*see* **Subheading 3.4.**).
18. For binding assays with suspension cells, *see* **Note 10**.

3.3. Virus Entry Assay

Poxvirus core antigens are masked by the IMV membrane and are only exposed after virus entry or after the IMV membrane is disrupted using a reducing agent, such as dithiothreitol (DTT), and/or a detergent, such as NP-40 ***(14,33,40)***. Interestingly, a rabbit polyclonal anticore antibody only labels cores that have entered cells, but not cores within IMV or EEV that have been fixed and permeabilized by paraformaldehyde and saponin ***(14)*** or Triton X-100 (***12***; *see* **Note 3**). Therefore, the entry of VV can be followed using antibody specific for core antigens, but *see* **Note 3** (*see* **Fig. 3**).

1. Bind IMV or EEV to cells following **steps 1–5** of **Subheading 3.2.** Because IMV is present in fresh EEV preparation, EEV is pretreated with IMV-neutralizing MAb at 37°C for 1 h before use.
2. After virus-binding and washing, cells are covered in virus growth medium or an appropriate buffer. IMV-neutralizing MAb is added to the medium when EEV is used.
3. Incubate the coverslips at the specific temperature and for the desired length of time according to experimental design. Meanwhile, keep one sample at 4°C as a control for background core staining.
4. To stop virus entry, wash cells once with cold PBS, then fix and quench, permeabilize, and block samples as in **steps 6–8** of **Subheading 3.2.**
5. At this point, the samples are ready for staining with anticore antibody as in **steps 10–16** of **Subheading 3.2.** (*see* **Note 11**).

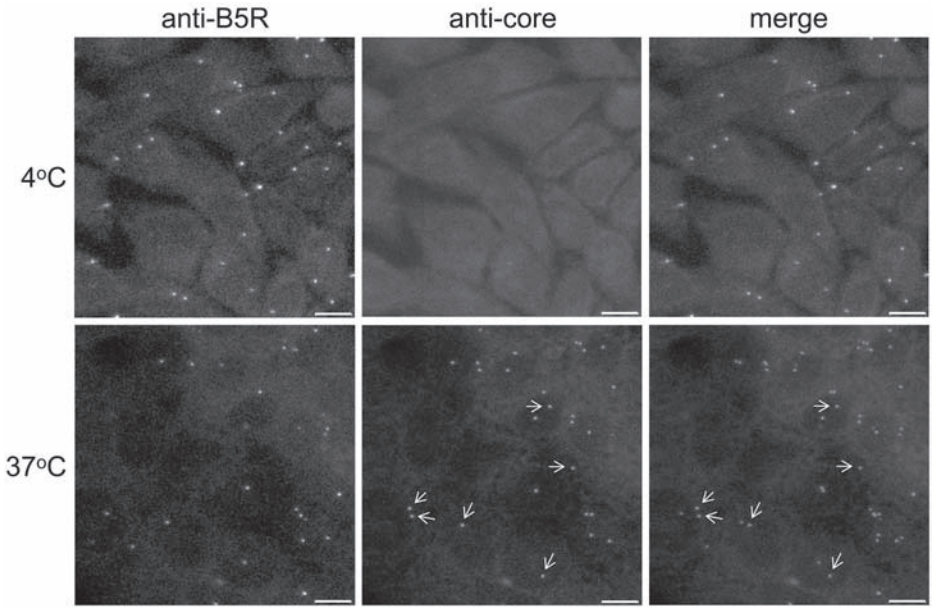

Fig. 3. Entry of EEV. HeLa cells grown on 13-mm glass coverslips were incubated with 0.5 mL fresh EEV (same EEV preparation as in **Fig. 2**) at 4°C for 2 h. IMV-neutralizing MAb was added to inhibit the entry of IMV. Unbound virus particles were removed, and the cells were either fixed immediately after binding or after 1 h at 37°C to permit virus entry (*see* **Subheading 3.3.**). Bound virus particles and cores in the cytoplasm were detected using anti-B5R (MAb 19C2) and rabbit anticore antibody, followed by FITC-conjugated donkey anti-rat IgG and TRITC-conjugated donkey anti-rabbit IgG, respectively. Images shown are the projections of *z*-series optical sections of the samples. Arrows highlight some of the cores that are clearly distinct from bound EEV. Bar = 10 μm. The results showed that virus entry is temperature-dependent, and virus cores are intracellular and do not colocalize with EEV bound onto the cell surface.

3.4. Confocal Microscopy and Data Analysis

The following section outlines some considerations for the examination of the samples used for the binding and entry assays. Images should be acquired for the purpose of virus particle quantification rather than high-quality image presentation. For the general operation of a confocal microscope, refer to the manufacturer's manual.

1. Examine the slides using an oil-immersion objective lens (e.g., ×40) that will normally give an image of 30–40 cells in a single scan.

2. Select a view randomly and take a bright-field image to record the number of cells.
3. Select an appropriate laser and filter set and adjust the various parameters for optimal signal-to-noise ratio of the images. As signals of antibody-labeled virus particles can be relatively weak, set the detection gain of the detector to maximum, and increase the pinhole size until virus particles are seen clearly.
4. Scan the image by multiple average scan (e.g., 4 for Zeiss LSM510 confocal microscope) to reduce nonspecific noise.
5. Section optically through the whole cells perpendicular to the *z*-axis at approx 0.5 μm steps to cover the entire cell surface.
6. Acquire six or seven images per sample to collect data from approx 200 cells.
7. Reconstruct images by projecting all *z*-series optical sections. The total number of virions bound on the surface of cells or the total number of cores inside the cells can be determined either manually or with the aid of imaging software that has a pixel-counting function, e.g., "Esivision" Soft Imaging Software (SIS) image analysis package (Soft Imaging Software, GmbH, Germany). Another advantage of using software is that the size and intensity of each particle signal can also be determined, which is useful for distinguishing single-virus particles from aggregates.
8. The number of particles counted per cell is then calculated. If necessary, the significance of the results can be addressed statistically by the student's *t*-test.

4. Notes

1. There have been two reports regarding the identification of poxvirus receptors ***(23,41)***, where neither study specified which form of virus was binding to these receptors, despite IMV and EEV being different both structurally and antigenically ***(1)***. It is worth noting that the claims for identification of specific protein receptors have been refuted ***(13,42)***.
2. The optimal working concentration of the primary and secondary antibodies should be determined in preliminary experiments. VV-infected cells can be used as a positive control for the primary and secondary antibodies but a 2–5-fold higher concentration may be required for detecting virus particles instead of virus antigens in infected cells.
3. This rabbit polyclonal anticore antibody had been shown to label only virus cores that have entered cells or have been extracted chemically, but not cores within fixed and permeabilized IMV and EEV particles ***(12,14)***. It was suggested that fixation crosslinked IMV membrane proteins to form a lattice around the core, thus preventing staining by the antibody ***(14)***. For other anticore antibodies, it is necessary to check that the antibody only stains intracellular cores, but not virus particles that have been fixed and permeabilized.
4. If a different rotor is used, layer the supernatant onto an equal volume of 36% (w/v) sucrose cushion and centrifuge at 25,000*g* for 80 min at 4°C.
5. VV is a biological category II microorganism, and it is recommended not to use needles to puncture centrifuge tubes for harvesting the virus.

6. A T175 flask of confluent BHK-21 cells normally produces approx 10^8–10^9 pfu of IMV (after sucrose gradient purification) and approx 5×10^6 pfu/mL fresh EEV of VV WR virus.
7. CMC overlay is a semisolid overlay that is more convenient to use than agarose. An overlay prevents formation of secondary plaques, and this is important for accurate plaque counting, particularly if the plate is left for more than 36 h. Also, an overlay is necessary for vaccinia virus strains or mutants that make more EEV.
8. Mouse MAb 1.1 (purified IgG, 10 μg/mL; ***31***) specific for the IMV surface antigen D8L is used in this laboratory for detecting IMV particles ***(5)***. Other good IMV-specific MAbs, such as MAb 2D5 specific for L1R ***(3)*** and MAb 5B4/2F2 specific for A27L ***(30)***, can also be used, especially if D8L is not a suitable antigen in some mutants. For EEV, rat MAbs against B5R (MAb 19C2, tissue culture supernatant, diluted 1/8; ***32***) and F13L (MAb 15B6, tissue culture supernatant, diluted 1/5; ***32***) generally give good staining results. F13L is located underneath the EEV outer membrane and permeabilization of the membrane is required when MAb 15B6 is used. Conversely, permeabilization is unnecessary for MAb 19C2 because the antigenic epitope on B5R is exposed on EEV surface.
9. To prevent false labeling of the other virus form in double immunofluorescent staining, use secondary antibodies that have been preadsorbed against the counterpart species. Secondary antibodies conjugated with two different fluorescent dyes, e.g., FITC and TRITC, are employed to visualize and differentiate the corresponding primary antibodies.
10. For suspension cells, virus-binding can be done in a small volume, e.g., 100 μL, or greater volume for fresh EEV with a minimum of 1×10^6 cells. The cells are pelleted by low-speed centrifugation after virus-binding, washing, antibody labeling, and so on, according to the procedure in **Subheading 3.2.** Before mounting onto microscopic slides, the cells are washed once with water. If cytospin centrifuge is available, the cells (approx 1×10^5 dependent on cell size) can be spun onto glass slides and covered with mounting medium (approx 20 μL) and glass coverslips. Otherwise, the cell pellet is dislodged by gently tapping the tubes several times before adding the mounting medium. The mounting medium (approx 30 μL) is mixed carefully with the cells without introducing air bubbles, and the mixture is then transferred onto a glass microscope slide and covered with a glass coverslip.
11. If virus particles bound on the cell surface and cores penetrating the cytosol are to be detected simultaneously, e.g., in the study of the relative ratio of virus particles that can bind, but not enter, two primary antibodies can be used. Follow the instruction listed in **Note 9** for double immunofluorescent staining.

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16

Pox, Dyes, and Videotape

Making Movies of GFP-Labeled Vaccinia Virus

Brian M. Ward

Summary

The large size of poxvirus virions (approx 250 × 350 nm) makes them ideal candidates for microscopic studies. Recombinant vaccinia viruses that express a viral envelope-specific, green fluorescent protein (GFP) chimera produce enveloped virions that fluoresce green. This fluorescent labeling allows the live, real-time study of viral egress using a variety of microscopic techniques. The methods presented here describe how to image the movement of intracellular enveloped virions that are labeled with green fluorescent protein using time-lapse laser scanning confocal microscopy. Details are also provided for analyzing the images obtained and converting them into QuickTime movies suitable for presentation.

Key Words: Vaccinia virus; B5R; green fluorescent protein (GFP); intracellular enveloped virus; intracellular movement; confocal microscopy; time-lapse microscopy; NIH image; QuickTime.

1. Introduction

Cloning of the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* in 1992 started a revolution in the field of biology ***(1)***. From simple protein trafficking and localization to the construction of complex biosensors, there are few areas in the life sciences that have not been affected by this revolution. The most widely used version is the red-shifted enhanced GFP, which fluoresces brightly at 507 nm when excited with light at a wavelength of 488 nm. This fluorescence is easily visualized using a standard fluorescein isothiocyanate (FITC) filter set that can be found on most fluorescent microscopes. Alternatively, GFP is spectrally ideal for visualization using laser-scanning confocal microscopy because its excitation peak nearly perfectly matches the emission wavelength of the argon laser commonly found on these systems.

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The versatility of GFP as a reporter protein is a result of its ability to fluoresce without the addition of extra substrates or cofactors, making it possible to visualize the fluorescence in live cells. Appending GFP to proteins or signal sequences targets it to specific organelles and fluorescently labels them so that their intracellular dynamics may be monitored live using fluorescent microscopy. Recently, this idea was applied to the study of viruses when it was demonstrated that a recombinant vaccinia virus expressing a chimeric GFP targeted to the viral envelope produces envelope virions that fluoresce. Subsequently, several studies have used recombinant vaccinia viruses that express GFP chimeras to study viral egress ***(2–6)***.

Poxvirus morphogenesis is a complex process due, in part, to the formation of different infectious forms. Morphogenesis occurs in the cytoplasm and gives rise to two forms of virions: a nonenveloped, but membrane-bound form called intracellular mature virions (IMV; ***7–10***) and an enveloped form with two distinct types—intracellular-enveloped virions (IEV) and extracellular-enveloped virions (EEV). IEV are a subset of IMV that have received an extra double membrane derived from the *trans*-Golgi network (TGN) or endosomal cisternae. IEV lose one of the newly acquired membranes after fusion with the plasma membrane, releasing the EEV form of the virus from the cell ***(11–16)***. To investigate IEV morphogenesis, a recombinant vaccinia virus was constructed that expressed the enveloped specific protein B5R fused to GFP (B5R-GFP; **Fig. 1**) in place of the normal B5R protein ***(3)*** using standard protocols outlined in this book for the construction of recombinant poxviruses. Because the B5R protein is specifically targeted to the wrapping membrane of the enveloped virus, the B5R-GFP chimera fluorescently labels IEV for visualization by fluorescent microscopy. Careful characterization of the recombinant virus expressing B5R-GFP (vB5R-GFP) determined that expression of the chimera was similar to B5R and that there was no effect on plaque size. Fluorescent microscopy of cells infected with vB5R-GFP demonstrated that B5R-GFP localized to the juxta-nuclear region (the site of wrapping) and also at the cell periphery (**Fig. 1C**), as had previously been reported for B5R ***(3,17–19)***. Furthermore, individual virions (arrowheads, **Fig. 1C**) could be visualized, indicating that the fluorescent signal was incorporated into enveloped virus,

Fig. 1. *(opposite page)* B5R-GFP. **(A)** Schematic representation of B5R-GFP chimera showing the lumenal domain (LD), transmembrane domain (TMD), and cytoplasmic tail domain (CD) in relation to GFP. **(B)** Nucleotide and corresponding amino acid sequence at the fusion junction of B5R (underlined) and GFP. **(C)** Subcellular localization of B5R-GFP chimera. Arrow points to the site of wrapping in the juxtanuclear region of the cell. Arrowheads point to virion-sized particles. Concave arrowheads point to the collection of B5R-GFP signal in the vertices of the cell.

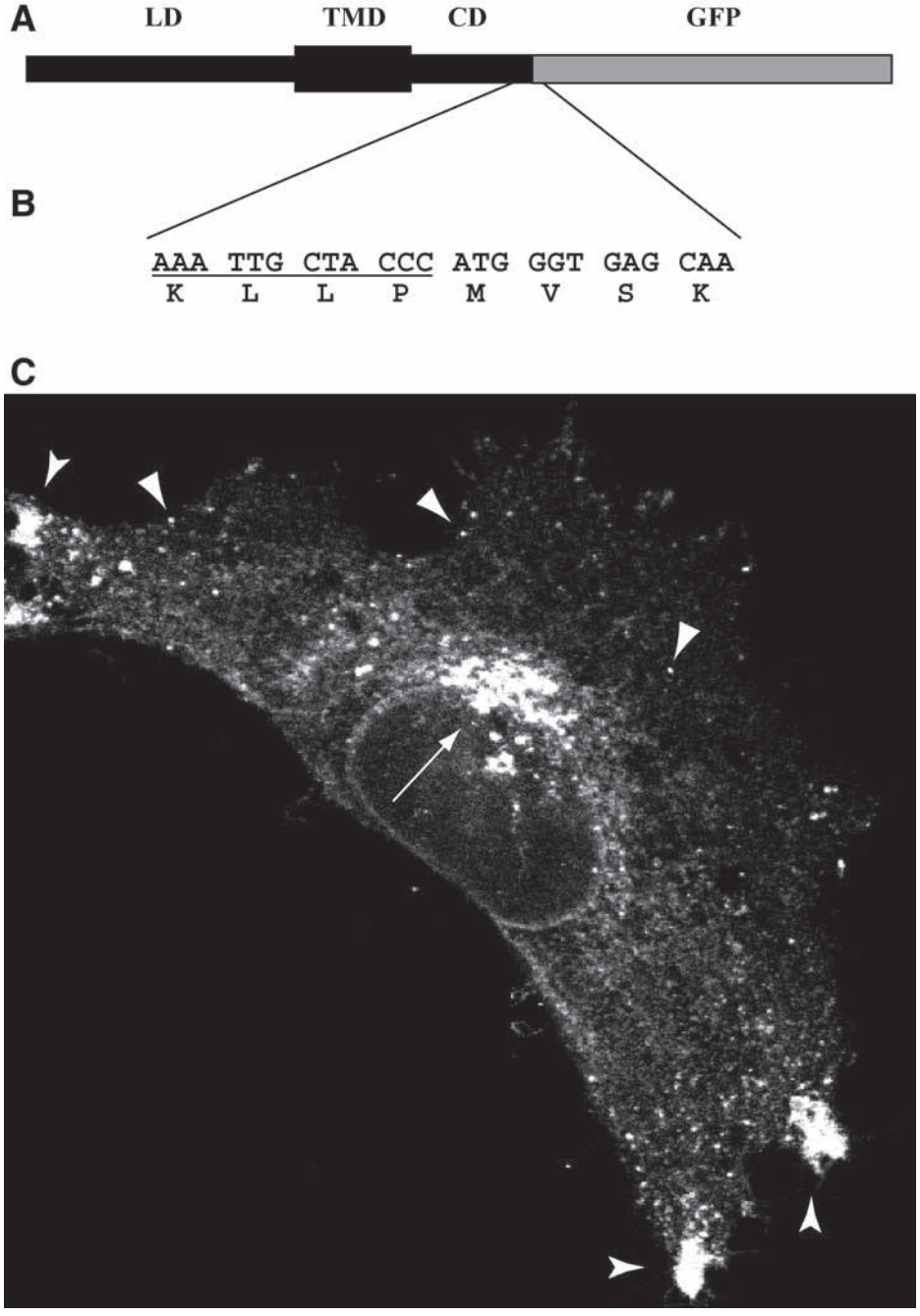

Fig. 1.

which was confirmed by immunoelectron microscopy using GFP-specific antibodies.

Subsequent studies using GFP-labeled virions by this laboratory and others have demonstrated that IEV move from the site of wrapping to the cell periphery using microtubules ***(2–6)***. These studies have challenged the long-held belief that intracellular movement of enveloped vaccinia virus is achieved by rapid actin polymerization. The methods presented here describe how to prepare cells and infect them with vB5R-GFP. After infection, details are provided for the visualization of B5R-GFP-labeled virions in the infected cells using live time-lapse laser-scanning confocal microscopy. Once images are obtained, instructions are given for analyzing virion movement and creating a QuickTime movie from the time-lapse series.

2. Materials

2.1. Virus and Cells

1. Crude lysate stock (with known titer) of recombinant vaccinia virus expressing B5R-GFP in place of the normal copy of B5R (*see* **Note 1**). Store in 0.1-mL aliquots at –70°C indefinitely until used.
2. DMEM-10 and DMEM-2.5: Dulbecco's modified Eagle's medium (DMEM; Quality Biological) containing 0.03% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and either 10% fetal bovine serum (FBS; DMEM-10) or 2.5% FBS (DMEM-2.5).
3. HeLa cell monolayer maintained in DMEM-10.
4. Video media: DMEM, containing 25 m*M* HEPES, that does not contain phenol red (Biowhittaker). Adjust the pH to 7.4 and filter-sterilize. Add glutamine, penicillin, and streptomycin to sterilized media at concentrations described above. Add FBS to 2.5%. Protect from light and store up to several months at 4°C.
5. Cell passage trypsin: 0.25% trypsin, 0.02% EDTA in Hank's balanced salt solution (Quality Biological).
6. Viral infectivity trypsin: 0.25 mg/mL 2X crystallized, salt-free, trypsin (Worthington) in water. Filter-sterilize and store indefinitely at –20°C.
7. Material and equipment for routine tissue culture, including plasticware, laminar-flow hood. CO_2 incubator and centrifuge.

2.2. Microscopy and Image Analysis

1. Bio-Rad MicroRadiance laser-scanning confocal system with an argon laser-running LaserSharp2000 v4.1 software attached to a Zeiss Axiovert 135 inverted microscope equipped with an oil emersion plan-apochromat 63×, 1.4 NA objective. The system should be setup for both epifluorescence and transmitted light microscopy. Ideally, the microscope should be mounted on an air table (*see* **Note 2**).
2. ΔT culture dish system (Bioptechs Inc.), including:

a. Delta T culture dishes with 0.17-mm thick glass.
b. Zeiss stage adapter.
c. Culture dish controller.
d. Objective heater/sensor.
e. Objective heater/sensor controller.
f. Hinged perfusion support needles.
g. Supply tubing (Tygon) 1/16-in. inner diameter.
h. Microperfusion pump.

3. 100% Ethanol.
4. Sterile water.
5. NIH Image version 1.61 software: available for free at http://rsb.info.nih-gov/nih-image/ and a Power Mac G4 computer (*see* **Note 3**).
6. QuickTime player software: available for free at http://www.apple.com/quicktime/download/.

3. Methods

3.1. Cell Culture

HeLa cell monolayers (*see* **Note 4**) are grown in a 75-cm^2 tissue culture flask and passaged upon reaching confluency. Two days prior to microscopy, confluent monolayer cells are trypsinized, seeded into a ΔT culture dish, and allowed to adhere overnight.

1. Aspirate off growth media from the 75-cm^2 tissue culture flask and rinse cell monolayer once with 2 mL cell passage trypsin preheated to 37°C.
2. Add 2 mL preheated cell passage trypsin and rock flask to evenly distribute over monolayer.
3. Incubate cells in trypsin at 37°C for 5 min.
4. Add 10 mL DMEM-10 to inactivate trypsin and pipet up and down to break up clumps of cells.
5. Pipet cell suspension into a 15-mL sterile conical tube and centrifuge for 5 min at 300*g*.
6. Aspirate off media and resuspend cell pellet in 10 mL DMEM-10 by pipetting up and down.
7. Add 100 μL resuspended cells (approx 1×10^5 cells) into a ΔT culture dish (*see* **Note 5**).
8. Add 1 mL DMEM-10 to the dish and gently rotate to evenly distribute the cells being careful not to touch or scratch the glass coverslip that forms the bottom of the dish (*see* **Note 6**).
9. Incubate the dish overnight at 37°C in a humidified, 5% CO_2 incubator.

3.2. Viral Infection

On the evening before microscopy, HeLa cells in the ΔT dish are infected with trypsinized vB5R-GFP at a low multiplicity of infection (moi). Infected cells are incubated overnight and imaged the next morning.

1. Thaw stock of vaccinia virus and mix with an equal volume of viral infectivity trypsin. Vortex and incubate in a 37°C water bath for 30 min. Vortex every 5–10 min.
2. Dilute trypsinized virus in DMEM-2.5 to achieve a titer of 1×10^4 plaque-forming units (PFU)/mL. (Aim for a low moi of approx 0.01 to 0.05 pfu/cell.)
3. Check ΔT dish to ensure cells have seeded properly.
4. Remove media from dish and infect cells by adding 0.2 mL diluted virus.
5. Bring volume in dish to 0.5 mL with DMEM-2.5 and place back in CO_2 incubator.
6. Gently rock the dish every 20 min for 1 h to uniformly spread inoculum and keep cells moist.
7. Remove media, add 1 mL DMEM-2.5, and incubate overnight at 37°C in a humidified, 5% CO_2 incubator.

3.3. Microscopy

On the morning after infection, cells are equilibrated in video media while the confocal microscope is set up. Infected cells are then visualized and images are collected using the laser-scanning confocal microscope.

3.3.1. Preparation of Cells

1. Warm video media to 37°C.
2. Check infectivity of cells using a fluorescent microscope equipped with a standard FITC filter set. Infected cells should appear as green fluorescent foci in the monolayer (*see* **Note 7**).
3. Remove media from dish and replace with 0.7-mL video media. Place dish back into 37°C incubator until the microscope is set up.

3.3.2. Preparation of Confocal Microscope and Stage

1. Turn on the ultraviolet (UV) lamp.
2. Turn on the computer that controls the confocal system.
3. Turn on the main switch to the μRadiance control unit and start the argon laser.
4. Turn on the computer that controls the confocal microscope and start the LaserSharp2000 software.
5. Attach the objective heater/sensor to the 63× oil immersion lens and turn on the objective heater/sensor controller. Set the temperature to 37°C (*see* **Note 8**)
6. Set up the perfusion system by attaching the tubing to the microperfusion pump and hinged perfusion support needles, taking care to note the intake and waste tube.
7. Connect the hinged perfusion support needles with a short piece of tubing and rinse the entire tubing system with 3 mL 100% ethanol, followed by 3 mL sterile distilled water and finally 3 mL video media.
8. Retrieve ΔT culture dish containing infected cells in video media. Before mounting the dish on the stage adapter, apply a drop of immersion oil on the temperature probe of the adapter and on the 63× objective.
9. Mount the ΔT culture dish onto the stage adapter and place the perfusion support needles into the holes provided on the stage. Adjust needles so that the ends sit

inside the dish. Rapidly perfuse media into the dish until the level reaches the needles and media is seen in the waste tubing indicating that it is being removed. Set the perfusion pump to a rate of 0.1 mL/min (*see* **Notes 9** and **10**).

10. Turn on the culture dish controller and set the temperature to 37°C.
11. Bring the objective up so that the oil makes contact with the bottom of the dish, and let the entire system equilibrate for 5–10 min.

3.3.3. Image Acquisition

1. Open a new project in the LaserSharp software by selecting the New Experiment option under the File menu.
2. If not already set, make the following settings in the Control Panel Toolbox window:
   a. 63× oil objective.
   b. 512 × 512 box size.
   c. 0.30 Argon laser power (*see* **Note 11**).
   d. 166 Lines/s scan speed.
   e. Direct collection filter.
3. Select the Optics button from the Control Panel ToolBox window and make the following settings:
   a. 488 Excitation filter.
   b. HQ500LP emission filter.
   c. 100% detector 1.
4. Using epifluorescence, find a fluorescent cell and center it in the field of view. A typical infected cell is shown in **Fig. 1C**.
5. Start acquiring images by selecting the LiveScan option under the Acquire menu. While images are being acquired, adjust the focus, iris size, gain, and offset as needed to obtain a clear bright signal.
6. Center a cell in the middle of the image display window using the set of arrow buttons in the Pan section of the Control Panel ToolBox window. Also, adjust the zoom so that the cell fills the pane. Stop scanning. Document the pixel size and box size (*see* **Note 12**).
7. Click the automatic acquisition (z-series) button in the Control Panel ToolBox window and select the time series tab.
8. Check the enable box. Set the cycle number to 100 and the cycle time to 6 s and select Start. The software should collect an image every 6 s for 10 min (*see* **Notes 13** and **14**).
9. After the automatic acquisition is complete, preview the collected time-lapse series by either dragging the scroll bar at the bottom of the window or clicking the Back-Forth button at the top of the window. A representative set of images depicting virion movement is shown in **Fig. 2** (*see* **Note 15**).
10. Save the entire stack of images to the disk as a single file by selecting the Save Experiment option under the File menu (*see* **Note 16**).
11. Continue collecting several time-lapse series on several different infected cells by repeating **steps 4–11**.

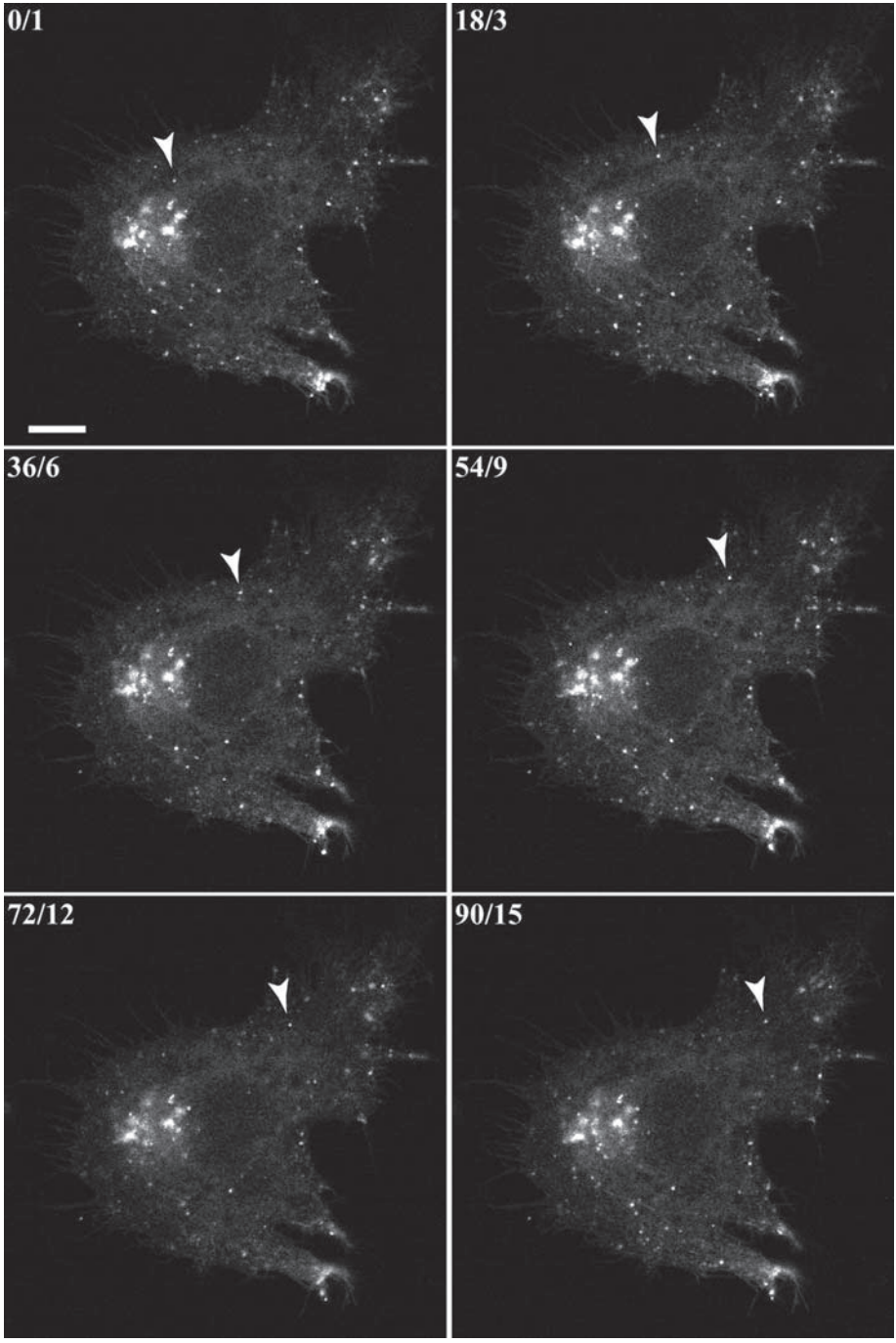

Fig. 2.

3.4. Image Analysis and Processing

3.4.1. Analysis of Movement

Image files are transferred from the computer controlling the confocal microscope onto a Power Macintosh computer that is running NIH Image software for analysis of virion movement (*see* **Note 17**).

1. Open NIH Image software.
2. Select the Load Macros option under the Special menu.
3. Navigate to the Macros folder in the NIH Image folder and open the file titled Input/Output Macros.
4. A new set of options should now appear under the Special menu. Select the Import Biorad MRC 600 Z Series… command. Navigate to the folder containing the time series collected above and highlight one of the time-lapse files; it should have the general format of "(name)_raw01.pic," then click the Open button. Do not change the other settings. Click OK to start with image 1.
5. Click the window containing the image to bring it to the front, and press the command (⌘) and equals (=) keys simultaneously to start cycling through the individual frames in sequential order. Change the speed of frame display (frame rate) by pressing the number keys (higher numbers result in faster frame rates and lower numbers result in slower frame rates). Alternatively, frames may be advanced forward or backward one frame at a time by pressing the period (.) or comma (,) key, respectively.
6. Find a virion moving in the sequence of images and use the comma key to move backward through the stack to find the starting point of its movement.
7. Select Set Scale… from the Analyze menu and make sure it is set to Pixels.
8. Select the line tool from the Tools window and place the crosshairs over the fluorescent virion. Advance the stack one frame using the period key (*see* **Note 18**).
9. While holding down the mouse button, move the crosshairs to the new position of the virion. This should draw a line from the previous virion position to the new position. Before unclicking the mouse button, note the length of the line displayed in the Info window. This number represents the number of pixels traveled. Multiply this number by the pixel size denoted in **Subheading 3.3.2.**, **step 18** to get the number of micrometers moved. Continue measuring the distance moved for as many frames as the virion is visible. Be sure to also note frames with no movement, as this affects the overall speed of the virion.
10. To calculate the speed of movement in micrometers per second, sum the total number of micrometers moved and divide this by the elapsed time (number of frames in which movement was measured, including frames of no movement,

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Fig. 2. Visualization of real-time IEV intracellular movement using time-lapse laser-scanning confocal microscopy. Elapsed time (seconds)/frame number is shown in upper left. Bar = 10 μm. The entire time-lapse video may be viewed at http://www.urmc.rochester.edu/gebs/faculty/Brian_Ward.htm.

Table 1
Frame-by-Frame Measurements of Virion Movement

Frame no.	Elapsed time (s)[a]	Distance moved (pixels)	Distance moved (μm)[b]
1	6	9.26	1.39
2	12	18.65	2.80
3	18	23.58	3.54
4	24	12.65	1.90
5	30	21.92	3.29
6	36	3.91	0.59
7	42	16.03	2.40
8	48	27.66	4.15
9	54	6.50	0.98
10	60	4.61	0.69
11	66	6.05	0.91
12	72	6.54	0.98
13	78	3.02	0.45
14	84	21.82	3.27
15	90	0.00	0.00
Total		182.2	27.33
Speed (μm/s)			0.30

[a]Cumulative.
[b]One pixel = 0.15 μm.

multiplied by the cycle time between each frames). An example of measurements for an individual virion, their conversion into micrometers and a calculation of average speed is shown in **Table 1** for the movement of the virion depicted in **Fig. 2**.

3.4.2. Data Presentation

Individual images from the time-lapse series demonstrating movement may be made into montages like the one shown in **Fig. 2** by copying the individual slices from NIH Image and pasting them directly into a publication quality imaging software program, such as Adobe Photoshop. Alternatively, entire time-lapse sequences may be converted to QuickTime movies to use as supplemental material for publication or to show virion movement during presentations (*see* **Note 15**).

1. Open a time-lapse sequence following **steps 1–5** from **Subheading 3.4.1.**
2. Click the window containing the image to bring it to the front, and press the command (⌘) and equals (=) keys simultaneously to start the software displaying the frames in sequential order. Frames may also be advanced forward or backward one frame at a time by pressing the period (.) or comma (,) key, respectively.

3. Use the number keys on the keyboard to slow down or speed up the number of frames shown per second until they are displayed at a desirable rate. Frames should be shown rapidly enough that the animation is visible, but not so fast that it is difficult to follow. Once the frames are being displayed at a desirable rate, note the number in the Info window next to fps, which is the frame rate in frames per second.
4. Select the Save As… option under the File menu, rename the file, check QuickTime, and Save. In the next window, select Graphic for the compression, change Color to Grayscale, and set the Quality to Best. In the Motion section, enter the frame rate noted above for the frames per second and click OK. Key frames may be set if desired, but it is not necessary. The program will save the entire sequence as a QuickTime movie with the frames advancing at the frame rate specified.
5. Open the movie using QuickTime Player to preview the movie to be sure the frame rate is appropriate. If the frame rate is too fast or slow, repeat **step 4** using either a faster or slower frame rate (*see* **Note 19**).

4. Notes

1. The protocols described utilize the recombinant vaccinia virus vB5R-GFP expressing a B5R-GFP chimera in place of the normal B5R protein. It should be noted that F13L-GFP and A36R-GFP, both enveloped specific protein-GFP chimeras, have been described in the literature ***(4,20)*** to label IEV and are therefore also suitable for the procedures described in this chapter.
2. The laser-scanning confocal microscope described here is the system used by the author. In principle, any confocal system that has a time-lapse acquisition function and a temperature control stage and is able to resolve GFP-labeled vaccinia virions should suffice. Alternatively, the author has used digital-video fluorescent microscopy to document virion movement, but finds the confocal system described here to be the most convenient method for these types of studies.
3. The author uses a PowerMac G4 450 MHz single processor with 512MB RAM and a 25 gigabyte hard drive running OS 9.2.2. It should be noted that slower computers may be adequate for processing images, although at a much slower pace.
4. HeLa cells are preferred by the author over the more commonly used BSC1 cells because they remain adherent and relatively flat when infected, making them easier to image. RK13 cells have also been uses with satisfactory results.
5. Cells are purposefully seeded at subconfluent numbers causing them to spread out, become flatter, and thus easier to image. The author has also passed cells early in the morning and infected later in the evening (approx 10 h later) for imaging the next day.
6. ΔT culture dishes are packaged with a protective cardboard insert. To protect the glass bottom, incubate the dish on top of the cardboard insert that has been placed in the inverted lid of a pipet tip box.

7. Depending on the intensity of the fluorescent bulb and the quality of the lenses on the microscope used, the reader may or may not be able to visualize the fluorescence without the use of an oil immersion lens. Care should be taken when viewing the cells, as the fluorescence may fade with prolonged exposure to intense excitation light.
8. The oil immersion lens makes physical contact via oil with the bottom of the dish. This connection allows the transfer of heat from the dish to the lens, effectively lowering the overall temperature in the dish. Heating the objective helps to maintain a constant temperature in the dish. To reduce stress and possible damage caused by drastic temperature shifts, the author stores the 63× objective in a 37°C incubator when not in use.
9. Perfusion of fresh media during the experiment is required to prevent the dish from drying out due to evaporation. A flow rate of 0.1 mL/min is fast enough to prevent complete drying, but slow enough to not drastically change the temperature in the dish, which would cause the focal plane to shift. Initially, the pump setting that corresponds to a flow rate of 0.1 mL/min needs to be determined empirically. Choose a setting on the pump and let the pump fill a graduated cylinder with water for 20 min. Adjust the pump setting as required and repeat until a rate of 0.1 mL/min is achieved.
10. The medium is not warmed before addition to the dish because, at this slow of a flow rate, most of the heat is lost in the tubing before the medium reaches the dish.
11. To avoid any toxic effects of the laser and GFP photobleaching, the lowest laser-power setting possible should be used that still allows imaging of the fluorescence. Although 0.30 is the setting used on our system, depending on the strength of the laser and the quality of the optics, higher (or lower) settings may be required when using other systems.
12. The pixel size is calculated based on the values entered in the objective lens setup window of the software. It is important that the correct objective is chosen and that the values for the objective being used are accurately entered for reliable measurements to be made later.
13. It is important that the cycle time is precisely 6 s. Start timing at the beginning of a scan and stop at the beginning of the next scan—they should be exactly 6 s apart. Some slower scanning systems may not be able to scan fast enough to accommodate a 6-s cycle time. In this case, extend the cycle time so that the system is able to complete a scan in the set time. Some systems may be able to scan quicker, in this case, the temporal resolution may be increased by using cycle times shorter than 6 s. Alternatively, the area scanned may be decreased, which leads to shorter scan times.
14. Using this particular system and settings, usually 10–20 min is the longest time a single-focal plane can be maintained. For other systems, the number of scans may be increased or decreased depending on how long the imaged cell stays in focus. Eliminating drafts around the scope and maintaining room temperature close to that of the experiment should help stabilize the focal plane.

15. Movies of infected cells imaged using this technique may be viewed at http://www.niaid.nih.gov/dir/labs/lvd/movies.htm and http://www.urmc.rochester.edu/gebs/faculty/Brian_Ward.htm.
16. Image files can be quite large depending on the number of scans and channels imaged (e.g., approx 250 Mb per time lapse sequence so a full day can be approx 2–4 Gigs). Before collecting the time-lapse series, it is important to ensure there is sufficient hard disk space to accommodate the large files.
17. There is no need to convert the image files; NIH image is able to open the PC files on a Macintosh computer.
18. It may be easier to follow virion movement more accurately and draw lines on a magnified image. To magnify the image, choose the magnify tool in the tools window and click on the image to magnify. To restore the image to the normal size, double click on the magnify tool in the tools window.
19. QuickTime movies may be inserted directly into PowerPoint presentations by selecting the Movies and Sound command under the Insert menu.

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17

Interaction Analysis of Viral Cytokine-Binding Proteins Using Surface Plasmon Resonance

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Summary

Surface plasmon resonance (SPR) biosensors have become an increasingly popular technology for characterizing the protein–protein interactions of virus–host interactions. Various studies have exploited the versatility of SPR to probe the interaction between virus and host components, including constituents of virus particles and host cellular receptors, as well as interactions between viral proteins and host immune molecules. This chapter describes basic procedures for employing SPR to study the interaction between poxvirus proteins and host immune-signaling proteins. We also identify how this methodology may be adapted toward other applications relevant in the study of poxvirus–host interactions.

Key Words: Poxvirus; surface plasmon resonance; Biacore; binding kinetics; virulence factors.

1. Introduction

Surface plasmon resonance (SPR) biosensors have become a common technology for characterizing the protein–protein interactions of virus–host interactions. A multitude of analyses have exploited SPR versatility to explore the interaction between virus and host components, including constituents of virus particles and host cellular receptors, along with interactions between viral proteins and host immune molecules. Basic protocols are defined for using SPR to study the interaction between poxvirus proteins and host immune-signaling proteins. Also described is the methodology that may be adapted for utilization in other applications in the study of poxvirus–host interactions.

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1.1. SPR Applications: Poxvirus–Host Interactions

There are numerous applications of SPR technology (**Table 1**). Simple experiments that qualitatively question whether binding occurs can be set up with relative ease, but require special attention to incorporate proper controls. Detailed kinetic analysis involves careful planning in all aspects of experimental design and should ideally involve well-characterized and pure samples. Screening methods may also be developed to enable the identification of ligands for orphan-binding proteins or receptors. Moreover, structural information may be obtained when combined with, for example, the analysis of site-directed mutants in epitope mapping experiments or when screening a panel of molecules of known structure. Finally, thermodynamic, concentration, and solution affinity analyses, as well as more complex ligand fishing experiments, have also been developed for SPR instruments.

Poxviruses possess extensive inventories of gene products whose function is to dampen the host inflammatory response and/or modulate the host microenvironment. These viral proteins include a number of secreted binding proteins, termed "viroceptors," that are defined as virus-encoded receptor-like proteins generally secreted from virus-infected cells and act to competitively inhibit inflammatory cytokines or chemokines from binding their cognate host receptors (**Table 2**; ***1,2***). Poxviruses also produce cytokine, chemokine, or growth factor mimics, termed "virokines," which are capable of engaging host cell-surface receptors as either agonists or antagonists and in this manner can modulate a variety of host systems that benefits poxvirus survival (**Table 2**).

SPR biosensors, such as Biacore, have become useful tools for characterizing the interactions of poxvirus viroceptors and virokines with host-signaling molecules or host cellular receptors, respectively (**Table 2**). Such studies have yielded a better understanding of how these viral proteins function to modulate the host immune response by not only understanding the binding kinetics that govern the interactions, but also, in some cases, the specificity and stoichiometry of binding. Notwithstanding, SPR analysis can also be adapted to examine other poxvirus–host interactions, including those that involve proteins, oligonucleotides, lipids, or even whole virions and virion components. As a model application for using SPR, we focus here on the use of SPR for the kinetic- and affinity-binding analysis of viroceptors with host-signaling molecules and are directed at users familiar with the basic usage of Biacore instrumentation.

1.2. Principles of SPR and Biacore Instrumentation

To date, Biacore has released a series of SPR-based instruments that provide various levels of sensitivity, automation, and utility (**Table 3**). These instruments permit the real-time analysis of the interaction between two

Table 1
Applications of SPR Technology

Application	Notes
Kinetic analysis (k_{on}, and $k_{off,}$ K_d)	Determines kinetic and equilibrium binding parameters: association (k_{on} or k_{ass}), dissociation constant (k_{off} or k_{diss}) and equilibrium dissociation constant (K_d). Analysis should use low-density surfaces and high flow rates.
Equilibrium analysis (K_d)	Determines K_d. Analysis can be performed using higher density surfaces and slow flow rates.
Specificity/epitope mapping	Kinetic or equilibrium analysis of mutants of analyte and/or ligand or panel of molecules of known structure.
Stoichiometry	Uses observed binding at saturation during kinetic or equilibrium analysis. Requires knowing the molecular masses of the analyte and ligand and the level of immobilized protein. Should be validated using other methods.
Determination of assembly of complex	Sequential injection of analyte over surfaces to build complexes or parallel injection of two or more analytes over surface to resolve complex binding mechanisms.
Competition analysis	Involves preincubation of analyte with increasing concentrations of competing ligand before injection. Determines inhibitory constant (K_i). Analysis can use higher density surfaces and slower flow rates.
Concentration analysis	To determine the active concentration of protein in a solution. Using equilibrium binding analysis of unknown sample against a standard curve of binding at equilibrium of known concentrations. Can also use initial rate of binding to determine concentration (partial or full mass transport methods).
Thermodynamics	Performing kinetic and/or equilibrium analysis at various temperatures to extract thermodynamic properties of interaction. Complementary to isothermal titration calorimetry and can be performed when the amounts of sample are limiting.
Analyte screening/ligand fishing/ small molecule screening	Involves screening many analytes for binding to orphan receptors, find novel binding partners or screen for binding inhibitors.
SPR-mass spectrometry	Involves using SPR as a front-end screen for novel binding partners and identifying binding partner by mass spectrometry. Uses either on-chip or off-chip approaches.

Table 2
Poxvirus Proteins Analyzed by SPR

Virus/protein	Function	Host ligand or receptor	Reference
Myxoma virus			
M-T1	Chemokine binding protein	Glycosaminoglycans	***(10)***
MGF	Myxoma growth factor	EGF receptor	***(11)***
Vaccinia virus			
B29R (VV-35kDa)	Chemokine binding protein	CC-chemokines	***(12,13)***
VGF	Vaccinia growth factor	EGF receptor	***(11)***
A41L	Unknown	Unknown	***(14)***
IL-18BP	Inhibits IL-18	IL-18	***(15)***
Profilin	Unknown	Polyphosphoinositides, actin	***(16)***
CrmD	TNF receptor homolog	TNF	***(17)***
B8R	IFN-γ receptor homolog	IFN-γ	***(18)***
VP-39	Cap-dependent 2'-O-Methyltransferase/ poly(A) polymerase processivity factor	Uncapped and 5'-cap 0-terminated oligo(A)	***(19,20)***
Cowpox virus			
p35	Chemokine binding protein	CC-chemokines	***(8,15)***
CrmD	TNF receptor homolog	Human and rat TNF	***(21)***
Molluscum contagiosum virus			
MC054L	IL-18 binding protein	IL-18	***(7,22)***
Ectromelia virus			
p13	IL-18 binding protein	IL-18	***(15,23)***
Orf virus			
ORFV-CBP	Chemokine binding protein	C and CC-chemokine inhibitor	***(24)***
Tanapoxvirus			
2L	TNF binding protein	Human TNF	***(25)***

Table 3
Biacore Instruments

Instrument	Features
BiacoreX	Semiautomatic
	Two flow cells in series
	Flow rates: 1–100 μL
	Sensitivity = > 200 Dalton
	Temperature range: 4–37°C
	Manual sample recovery
Biacore 1000	Automatic
	Four flow cells—single channel
	Flow rates: 1–100 μL
	Sensitivity = > 1000 Dalton
	Temperature range: 20–37°C
	2- × 96-well-plate holder
Biacore 2000	Automatic
	Four flow cells—multichannel (in series)
	Flow rates: 1–100 μL.
	Sensitivity = > 200 Dalton
	Temperature range: 4–40°C
	2- × 96-well-plate holder
	Automatic sample recovery
Biacore 3000	Automatic
	Four flow cells—multichannel (in series)
	Flow rates: 1–100 mL
	Sensitivity = > 200 Dalton
	Temperature range: 4–40°C
	2- × 96-well-plate holder
	Automatic sample recovery at high concentration
Biacore S51	Automatic
	Three addressable spots
	Flow rates: 2–95 μL
	Sensitivity = >100 Dalton
	96- or 384-well microplate
	Temperature range: 4–45°C

macromolecules without requiring labeling. Detecting a binding event using SPR involves either covalently or noncovalently immobilizing one of the reactant macromolecules (sometimes referred as the ligand) onto a chip surface and monitoring its interaction with a mobile-phase reactant, often referred to as the analyte. Immobilization is typically performed on a chip surface com-

Table 4
Biacore Sensor Chips

Chip	Features	Applications
CM5	Carboxymethyl dextran matrix	General purpose chip (amine, thiol, aldehyde coupling)
SA	Carboxymethyl dextran matrix with coupled streptavidin	Biotin-tag capture
NTA	Carboxymethyl dextran matrix with coupled nitriloacetic acid (NTA)	His-tag capture
HPA	Hydrophobic surface	Construct lipid monolayer for analysis of integral membrane proteins or for analysis of analyte interactions with lipids.
B1	Low-charge carboxymethylated dextran matrix	For reduced nonspecific binding and lower capacity surfaces.
F1	Short dextran matrix	Large analytes (e.g., viral particles).
C1	Flat carboxymethyl without dextran	No dextran. Ideal when dextran is a cause of nonspecific binding.
J1	Gold surface with no modifications	User-defined surface
L1	Lipophilic dextran	Liposome capture for analysis of integral membrane proteins.

posed of a carboxymethylated dextran matrix (**Table 4**) to which the ligand or the ligand's capture agent can be chemically coupled.

SPR biosensors detect a binding event by exploiting the optical phenomenon of SPR to measure the refractive index change very close to a chip surface that occurs from the accumulation of the analyte on the surface (**Fig. 1A**). When an analyte is injected into the flow cell containing the immobilized ligand, the change in the refractive index as a result of the binding of the analyte to the immobilized ligand is monitored in real time. The refractive index change is measured in arbitrary resonance units (RUs) and is approximately proportional to the protein mass accumulated on the surface (1 RU = 1 pg mm^{-2}). SPR instruments therefore allow the accurate and very sensitive monitoring of the association and dissociation phases of a binding event, which is plotted in a sensorgram, where the mass of protein binding is plotted on the vertical axis (in RUs) with respect to time on the horizontal axis (**Fig. 1B**).

The SPR detection method has many advantages, including that it dispenses the need to use labeled reactants, which might otherwise interfere with binding recognition. The presence of multiple flow cells in Biacore instruments enables the user to not only monitor the binding response of the active flow cell (i.e., contain-

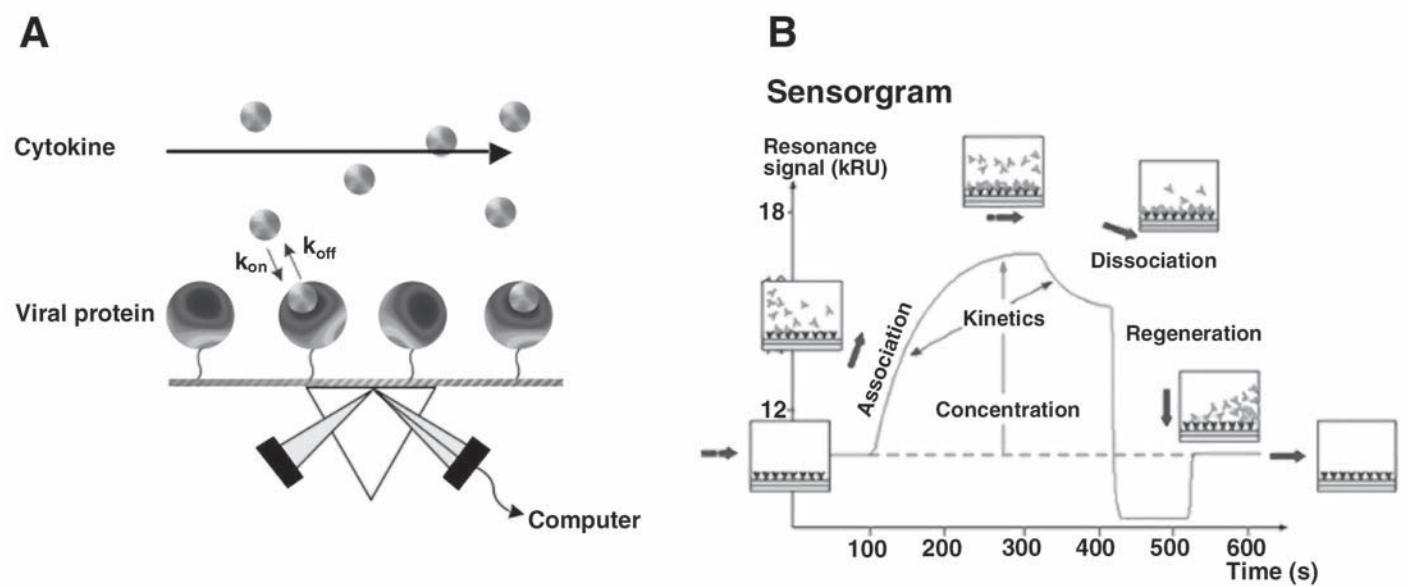

Fig. 1. Schematic of the Biacore SPR detector unit and sensorgram. **(A)** Representation of the detection unit and flow cell. Plane polarized monochromatic light is reflected off a prism under conditions of total internal reflection. At a particular angle, a loss of energy to the gold creates oscillating waves in the gold termed "surface plasmons." Owing to the loss of energy to this electron dense surface, there is a reduction in the intensity in the reflected light. As the refractive index of the medium influences the angle of the dip in reflected light intensity, any change in the refractive index, caused either by a change in the medium composition or the binding of a biomolecule close to the surface, is detected as a change in the angle of the reflected intensity or RU. Flowing an analyte (host cytokine) into a flow channel containing an immobilized protein (viral protein) may be detected and plotted on a computer as a sensorgram. **(B)** Sensorgrams are plotted as binding (in RU) vs time. Binding occurs upon injection of the analyte (shown at time 100 s). After a 200-s injection, only running buffer is injected over the surface to observe the dissociation of the analyte from the surface. Following the dissociation phase, regeneration buffer is injected over the surface to remove the analyte from the surface. Reproduced with permission of Biacore Inc.

ing the immobilized protein of interest), but also permits the inclusion of a flow cell with either no protein or an immobilized mock protein to control for background responses, including nonspecific binding and bulk refractive shifts due to minor differences in analyte and running buffer composition. The subtraction of the background binding sensorgram from the total binding sensorgram using Biacore software yields a specific-binding sensorgram.

One of the primary benefits of SPR analysis is that it permits the monitoring of both the association and dissociation phases in real time, allowing for detailed kinetic analysis yielding quantitative information regarding the interaction's binding parameters. Equilibrium binding analysis is also a standard procedure that monitors binding curves at each analyte concentration at equilibrium. Such analysis is suitable for characterizing the equilibrium constants when kinetic rates are too fast or slow to resolve and can also be used as an internal control to test the validity of the equilibrium constants derived from kinetic studies. Moreover, because the mass of analyte at saturating concentrations can be detected bound to the immobilized protein, the stoichiometry of the interaction can be deduced, provided the mass of the protein on the surface and the molecular masses of the proteins of interest are known. Therefore, sensorgrams generated during SPR experiments are rich with information about the interactions between biological macromolecules.

1.3. Kinetic Analysis

Kinetic analysis using global analysis software to fit datasets to a simple-binding model places great demand on obtaining high-quality data ***(3,4)***. To generate high-quality data, both analyte and immobilized protein must be sufficiently pure to minimize nonspecific or spurious interactions. Moreover, low-surface densities of the immobilized reactant (i.e., binding at saturation, R_{max}, should be <50 RU) should be routinely used to minimize artifacts, such as mass transport limitations, aggregation, and steric hindrance because of crowding. Moreover, high-analyte flow rates (>50 mL/min) should be used to increase transport to the surface and prevent rebinding. Another potential pitfall may arise from the use of amine coupling to directly immobilize the ligand. Although relatively easy to perform, the random nature of this method increases the possibility of obstructing the binding site on the immobilized protein. To prevent such occlusion of the binding site, alternative immobilization strategies should be considered, including immobilization with a capture tag on the surface protein (e.g., histidine tag), a capture antibody, or thiol coupling using a unique or engineered-free cysteine.

A consequence of using low-density chip surfaces is the reduced signal-to-noise ratio, which exposes random noise of the detector. Moreover, low-density chips also reveal instrument noise, and such sensorgram deviations can be

problematic in properly fitting datasets. Fortunately, kinetic data are unaffected by random noise, and systematic instrument noise can be corrected not only by referencing for nonspecific binding (i.e., using a control surface), but also by referencing against a blank injection (buffer only) in a process referred to as "double-referencing" ***(3,4)***. This process entails that specific binding sensorgrams suspected of having unwanted system noise should be subjected to an additional subtraction using a sensorgram generated from a buffer-only injection.

Finally, analysis of kinetic data is now routinely performed by simultaneously fitting the association and dissociation profiles of sensorgrams generated from a series of concentrations to determine the rate constants. Although such global analysis requires the burden of generating high-quality data, the advantages of this analysis not only provides a test of the system's validity, but also allows for the discrimination between binding mechanisms. Global analysis can be performed using either the BIAevaluation software provided by Biacore, the free software SPRevolution (http://www.bri.nrc.ca/csrg/equipf.htm#biacore), or CLAMP (***5***; http://www.hci.utah.edu/cores/biacore/).

2. Materials

2.1. Equipment

1. Biacore instrument (Biacore AB, Uppsala; **Table 3**).
2. SPR evaluation software: BIAevaluation (Biacore AB, Uppsala) and SPRevolution or CLAMP.
3. Biacore CM5 sensor chip (**Table 4**; *see* **Note 1**).
4. Degassing apparatus.
5. 0.2-μm disposable filters.

2.2. Reagents and Buffers

1. 0.1 *M N*-hydroxysuccinimide (NHS).
2. 0.4 *M N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (EDC).
3. Coupling buffers: 10 m*M* formate, pH 3.0, 10 m*M* acetate, pH 4.0–5.5, or 5 m*M* maleate, pH 5.5–6.0 (*see* **Note 2**).
4. Blocking and deactivation reagent: 1 *M* ethanolamine, pH 8.5.
5. Running buffer (HBS-EP): 20 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 3.4 m*M* EDTA, and 0.005% surfactant P-20 (*see* **Note 3**).
6. Regeneration buffer: 10 m*M* glycine/HCl, pH 2.0–3.0, but this depends on type of interaction (*see* **Table 5** for other options).
7. Ligand: purified soluble viral protein (*see* **Note 4**) in a buffer that is completely absent of primary amines (e.g., avoid Tris).
8. Analyte: purified host protein (depending on application) at a relatively high concentration (if possible) and preferably dialyzed or buffer exchanged into the running buffer (*see* **Note 4**). For ligand fishing or screening applications, crude mixtures may be used for analysis (*see* **Note 5**).

Table 5
Regeneration Solutions*

Bond strength	Acidic	Basic	Hydrophobic	Ionic
Weak	pH > 2.5 formic acid/HCl, 10 m*M* glycine-HCl	pH < 9 10 m*M* HEPES/NaOH	pH < 9 50% Ethylene glycol	1 *M* NaCl
Intermediate	pH 2–2.5 formic acid, HCl, H_3PO_4, 10 m*M* glycine/HCl	pH 9–10 NaOH 10 m*M* glycine/NaOH	pH 9–10 50% Ethylene glycol	2 *M* $MgCl_2$
Strong	pH < 2 formic acid, HCl, H_3PO_4, 10 m*M* glycine/HCl	pH > 10 NaOH	pH > 10 25–50% Ethylene glycol	4 *M* $MgCl_2$ 6 *M* Guanidine hydrochloride

*Reproduced from Getting Started BiacoreX handbook with permission of Biacore Inc.

3. Methods

3.1. Immobilization

A variety of chips are available from Biacore, and the choice of chip to use depends on the samples being analyzed and the intended application (**Table 4**). For standard protein–protein analysis, the most commonly employed chips are the CM5 chip, the low-charge CM4 chip, or the ready-made streptavidin SA chip (*see* **Note 6**). Whereas the SA chip assumes the immobilized protein is biotinylated (*see* **Note 7**), the CM5 chip and CM4 chip can be used with a variety of coupling chemistries, including amine coupling, thiol coupling, and aldehyde coupling. Here, we focus on the factors that might be considered when performing amine coupling, although such considerations should be applicable when considering other covalent-coupling strategies.

Deciding which protein to immobilize depends on various factors, including the size, charge, and stability of the proteins of interest. In many instances, the smaller of the two reactants are immobilized to ensure the binding signal is maximized, although more recent studies with small molecules has demonstrated that the sensitivity of Biacore instrumentation is amenable to the detection of molecules as small as 200 Dalton under optimized conditions ***(6)***. Other variables include the number and location of primary amine groups in the proteins of interest, which may determine whether amine coupling is appropriate. For instance, amine coupling should be avoided if it is known that lysine residues encompass the binding site (*see* **Note 8**). The stability of the immobilized protein should also be considered, as an unstable protein may be unsuitable for a prolonged study requiring multiple regenerations. Finally, if a protein possesses two or more binding sites (e.g., an antibody or an Fc-fusion of a viroceptor), it should be used as the immobilized proteins because avidity effects impact the fitting to a simple-binding model.

The basic procedure of amine coupling involves two steps. The first step is to determine the preconcentration buffer conditions, and the second step is the actual coupling using EDC/NHS to activate the carboxyl groups on the carboxymethylated dextran matrix to create reactive succinimidyl ester groups for coupling the protein. Preconcentration is necessary to concentrate the protein to a high level onto the dextran matrix by lowering the pH and ionic strength of the buffer. The purpose of this is to encourage enough of the protein to interact electrostatically with the carboxylated dextran matrix surface for efficient coupling. Therefore, test injections of the protein to be immobilized is required to determine the optimal buffer composition, typically below the isoelectric point (pI) of the protein (*see* **Note 2**) and the protein concentration required for effective binding of the protein to the surface. Once conditions have been optimized, the actual coupling can be performed, which involves

activating the surface, flowing the ligand over the activated surface, preferably in a controlled manner, and then blocking the active sites with ethanolamine.

3.1.1. Preparing the Instrument

1. Turn on the instrument and computer and run Biacore Control Software according to the Biacore manual. Allow the instrument temperature to stabilize.
2. Warm HBS-EP running buffer to room temperature. Filter using a 0.2-μm filter and degas (*see* **Note 3**).
3. Remove unused CM5 chip from refrigerator and warm to room temperature.
4. Dock chip according to Biacore instrument manual.

3.1.2. Determining Preconcentration Buffer and Protein Concentration Conditions

1. Dilute the protein to be immobilized at various concentrations (e.g., 10–100 μg/mL) in a volume of 100 μL of various test buffers (e.g., 10 m*M* formate, pH 3.0, 10 m*M* acetate, pH 4.0–5.5, or 5 m*M* maleate pH 5.5–6.0). To ensure a sufficiently positive charge on the protein, the pH of the injection solution should ideally be at least one pH unit below the pI of the protein (*see* **Note 2**).
2. Run sensorgram at a flow rate of 5 μL/min using a single-flow cell that is eventually used for the actual coupling, but reserve flow cell 1 to serve as the control surface.
3. Inject 50–80 μL of each sample and monitor the binding response (RUs) that each concentration of protein generates. An ideal response should result in a steady increase in binding, achieving maximum levels of 5000–15,000 RUs during one injection.
4. Following each injection, the bound protein should rapidly dissociate from the surface, such that the RUs should drop back to baseline levels. If residual binding remains, inject three quick pulses of regeneration buffer or until signal is back to baseline.
5. If the highest concentration of protein fails to bind or binds too slowly to the surface, increase the concentration of protein (within limits of solubility) and/or alter the pH of the protein's buffer. However, if the pH is too low, the amino groups become protonated and coupling efficiency is diminished. Once the optimal conditions are identified, proceed with the coupling protocol.

3.1.3. Amine Coupling

Before coupling, it is useful to determine the approximate level of protein that is needed to be immobilized to observe an adequate binding signal. For kinetic analysis studies, it is desirable to have low levels of immobilized protein on the surface to minimize artifacts, such as mass transport limitations, crowding, or aggregation, which may affect kinetic constants. Therefore, aim to use the lowest possible saturable-binding level ($R_{max} < 50$ RU) allowed based on the molecular masses of the proteins being studied. Conversely, SPR bind-

ing equilibrium studies do not depend on kinetics and therefore do not necessarily require low levels of immobilized protein. Similarly, screening or fishing studies may benefit from higher levels of immobilized protein to maximize the sensitivity of detection (R_{max} ~ 500–1000 RU). Regardless of the intended application, one should use the stoichiometry equation (*see* **Note 9**) to estimate the level of immobilized protein required to achieve the desired R_{max} level before beginning any immobilization procedure.

1. Prepare the protein to be immobilized under the optimized buffer conditions as determined in **Subheading 3.1.2.**
2. Run sensorgram at a flow rate of 5 μL/min over the same flow cell (i.e., flow cell 2) used in the preconcentration determination procedure. Wait for the signal to stabilize. Just prior to beginning the coupling procedure, mix equal parts (1:1) of 0.4 *M* EDC and 0.1 *M* NHS together (40 μL of each should be sufficient).
3. To activate the carboxymethylated dextran matrix, inject 35 μL EDC/NHS mixture containing the immobilized protein over flow cell 2.
4. Using the manual injection mode, inject 50 μL protein to be immobilized (in optimized buffer) over flow cell 2.
5. Monitor the amount (RUs) of protein binding to the surface. Because coupling is inefficient, the observed amount of protein at the surface does not reflect the true amount that is coupled. Therefore, to judge the approximate amount of protein coupled to the surface, stop the injection intermittently and observe the change in the baseline RU level relative to the baseline prior to injection. This reflects the amount of protein that has been coupled to the surface.
6. To achieve an adequate level of coupled protein based on the theoretical stoichiometry (*see* **Note 9**), continue the injection of protein over the surface until the desired amount of protein is coupled to the surface. It should be noted that coupling efficiency is greatly reduced after 7 min.
7. After the protein has been coupled to the surface, inject 35 μL ethanolamine at 5 μL/min over the surface to block unreacted ester groups. Inject the flow cells with three 5-μL pulses (at a flow rate of 100 μL/min) of regeneration buffer to remove any inefficiently coupled protein.
8. If a control protein is available (*see* **Note 10**), immobilization of the control protein onto the reference surface can be performed by repeating **steps 1–7** on flow cell 1 using fresh EDC/NHS. If no control protein is available, the reference cell should at least be activated with EDC/NHS and blocked with ethanolamine.

3.2. Interaction Analysis

3.2.1. Binding Kinetics

As Biacore instrument models vary in their level of automation, note that the following methods are performed using either the semiautomation features of the BiacoreX or the full automation of the Biacore 1000, 2000, or 3000. Please refer to Biacore manuals for details.

1. Prepare serial dilutions of the analyte using the running buffer as the diluent to minimize the effect of bulk refractive index changes (*see* **Note 11**). The final concentration range should ideally span two orders of magnitude on either side of the K_d and include at least four to six concentrations. Also, prepare a vial of running buffer for the blank buffer injections to control for system deviations.
2. Run sensorgram and set the flow rate preferably greater than 50 μL/min. Before performing injections, ensure the signal has been stabilized.
3. Inject 100 μL of each concentration of the analyte over both the control surface and the active surface. (Flow cells are in series on the BiacoreX, 2000, and 3000 instruments, and users should use the in-line subtraction of sensorgrams generated from flow cell 1 from flow cell 2; Biacore 1000 users must perform these injections separately over the two surfaces. For BiacoreX users, use the bubble technique detailed in the Biacore manual to prevent analyte dilution or mixing with running buffer [*see* **Note 12**]. Use the KINJECT command on Biacore 1000–3000 instruments.) To monitor the dissociation phase of binding, incorporate a delay of at least 120 s or until the binding has decreased to at least one half-life. The delay time ultimately depends on the rate of dissociation. For accurate determination of the dissociation rate, rebinding effects should be minimized (*see* **Note 13**).
4. After the end of the dissociation phase, inject three short pulses of 10 μL regeneration buffer to remove bound protein from the ligand on the surface. After each injection of a given concentration sample, include a 100 μL blank running buffer injection and incorporate a delay time following the injection that corresponds to the delay used in the injection of the analyte in **step 3**. The sensorgram from this injection can be used for a second referencing to subtract out system noise (*see* **Subheading 3.2.2.**) and as an additional control to ensure that there is no carryover from the injection of the analyte. Repeat **step 4** if there is evidence of carryover (*see* **Note 14**). Blank buffer injections can also be performed before injection of the analyte.
5. Perform replicates to ensure the activity of the chip is intact following regeneration and to demonstrate reproducibility. If activity of the protein is compromised with the regeneration buffer, experiment with other milder regeneration solutions. Randomize and repeat sample injections to ensure a robust dataset.

3.2.2. Data Analysis of Sensorgrams to Determine Kinetic Parameters

Data analysis has been greatly improved with global analysis, provided sensorgrams have been generated under optimal conditions. Although users are urged to experiment with the different software available (*see* **Subheading 1.3.**), Biacore's current version of BIAevaluation (version 4.0.1) is sufficient for most routine analyses of sensorgram data.

1. To analyze sensorgram data, open curves generated from **Subheading 3.2.1.** in the BIAevaluation software.

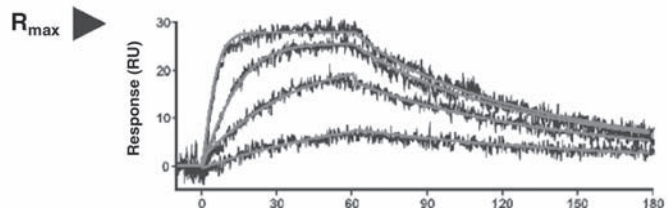

Fig. 2. Kinetic analysis of an immobilized Fc-fusion of the vaccinia virus chemokine-binding protein interacting with a CC-chemokine. A Fc-fusion of the vaccinia virus (VV)-35-kDa chemokine-binding protein was injected at a slow flow rate and captured onto a CM5 chip that had been preimmobilized with amine-coupled protein A. Once the VV-35kDa-Fc protein was loaded onto the protein A surface of flow channel 2, MCP-1 was injected over the surface of flow channels 1 and 2. Sensorgram curves from flow channel 1 was automatically subtracted from the sensorgram from flow channel 2. Sensorgram is showing the specific-binding profiles of various concentrations of (0.93, 2.8, 8.3, and 25 nM; from bottom to top) of chemokine binding to the VV-35-kDa-Fc chemokine-binding protein surface. Data were globally fitted to a 1:1 mass transport model using BIAevaluation 3.0 software (light gray lines). Reproduced with permission of the Journal of Leukocyte Biology ***(9)***.

2. Select each curve and open in graph mode. Zero the curves on the *y*-axis and align the start of the curves (i.e., the beginning of the injection) on the *x*-axis. If the control surface (flow cell 1) sensorgram has not already been subtracted during the experimental run, subtract the sensorgram generated from flow cell 1 (nonspecific binding) from flow cell 2 (total binding) to generate a sensorgram of specific binding.
3. To correct for system noise, subtract blank buffer sensorgrams that have been zeroed on the *x* and *y* axes from specific-binding curves.
4. Perform a simultaneous fit on all data curves (**Fig. 2**). Assuming a 1:1 model ($A + B \Leftrightarrow AB$), select a 1:1 Langmuir-binding model. If the rate constants are greater than $10^6\ M^{-1}s^{-1}$, then the kinetic data may be mass-transport-limited. In this case, select a 1:1 mass transport model that incorporates an additional variable to account for mass transport to the surface.
5. If the data deviate from a 1:1 model, a thorough reassessment of the experimental conditions should be performed (**Table 6**). If experimental artifacts can be confidently ruled out and there is strong experimental evidence that the interaction is known to occur with an alternate binding mechanism, other binding models may be used to fit the data. However, it is highly recommended that other biophysical techniques be used to validate the binding mechanism (e.g., analytical ultracentrifugation, isothermal titration calorimetry, mass spectrometry, NMR).

Table 6
Improving Data Quality for Kinetic Analysis

Problem	Possible cause	Details of cause	Solution
Heterogeneous binding kinetics	Heterogeneous surface	Immobilization by amine coupling may result in binding site occlusion.	Use alternate couple methods including antibody capture, affinity capture methods or thiol coupling using a unique free cysteine.
		Ligand is impure.	Further purify sample.
	Heterogeneous analyte	Analyte is impure.	Further purify sample.
	Protein aggregation	Protein aggregates preventing the discrimination of simple binding kinetics	For analyte, determine solution oligomerization state by analytical centrifugation. Change buffer conditions.
	Nonspecific binding	Interaction of analyte with surface due to high charge or nonspecific binding to dextran matrix.	Use reference cell with appropriate immobilized control protein. Use low-charge dextran matrix chip (Biacore CM4 or C1 chip) Include BSA in sample buffer or soluble carboxymethylated dextran in analyte solution.
	Sample carry-over	Ineffective washing of microfluidics cartridge. Monitored by incorporating blank injections before and after sample injections.	Incorporate wash steps before and after sample injections.
No binding	Steric hindrance	Crowded surface.	Use lower surface densities.
		Affinity tag is interfering with binding site.	Alter location of the tag.

Mass transport limitations	Analyte rate of binding is in excess of its diffusion rate.	Localized depletion of analyte at surface results in distorted association kinetics. Rebinding occurs due to high association rate and inadequate mass transport away from surface.	Utilize lower surface densities and fast flow rates (e.g., > 30 μL/min). Account for mass transport using mass transport model during data analysis. Inject competing ligand during dissociation.
Complex binding mechanism	Multivalent interactions	Avidity effects may arise when multivalent protein is used as the analyte.	The monovalent protein should be used as the analyte and the multivalent reactant should be immobilized.
		Binding mechanism is confirmed to deviate from a simple 1:1 binding model using other biophysical techniques.	Use more complex binding models to describe data.
Drifting baseline	Immobilized ligand is not stable.	Use of noncovalent capture of ligand results in dissociable interaction. Amine coupled protein is poorly coupled.	Use stronger interaction to couple (e.g., biotin/streptavidin coupling) or covalent coupling (e.g., thiol coupling). For poorly coupled amine proteins, inject EDC/NHS over unstable surface but perform controls to ensure binding is not compromised. Use drifting baseline model to fit the data.

3.3. Assessing K_d from Binding Equilibrium Experiments

Equilibrium-binding experiments are a simpler variation of the methods utilized in performing binding kinetics (**Subheading 3.2.1.**). Rather than focusing on the association and dissociation profiles, this analysis is primarily concerned with observing the binding of the analyte at equilibrium at several concentrations and therefore requires that each curve be allowed to proceed until the rate of change in binding RUs is zero. Moreover, this analysis does not depend on flow rate; thus, slow or fast flow rates may be used, provided the binding profiles reach equilibrium at all concentrations. This analysis can also tolerate higher levels of immobilized protein on the surface, although too high of a density may cause crowding effects to arise.

1. Prepare chip surface as in **Subheading 3.1.** Prepare serial dilutions as in **Subheading 3.2.1.**, **step 1**.
2. Perform sequential injections (*see* **Note 15**) of analyte at various concentrations and record the binding level (in RU) at equilibrium (i.e., when the change in binding RUs vs time = 0).
3. The K_d of the interaction can be determined by graphing the binding at equilibrium (in RU) vs time and fitting the data to the single site binding equation $B_{eq} = (B_{max} \times C)/(K_d + C)$, where B_{eq} is the response bound at equilibrium, B_{max} is binding at saturation, and C is the concentration of analyte.

3.4. Equilibrium Competition Analysis

Competition analysis can be treated as a variation on equilibrium-binding analysis (**Subheading 3.3.**). The assay involves injecting a particular concentration of the analyte over the ligand surface and inhibiting the analyte's ability to bind to the surface using several concentrations of binding competitor. It is advisable to perform an equilibrium-binding analysis (**Subheading 3.3.**) of the interaction before proceeding with a competition analysis, as knowing the K_d of the interaction can be useful for assessing the amount of analyte required to achieve equilibrium binding within a reasonable period of time. It is also absolutely essential to ensure that the competitor does not bind the immobilized protein. Therefore, injection of the competitor alone over the active and control surfaces should be performed prior to commencing the study.

Prepare a dilution series of the competitor (0 to 1000-fold dilution) and mix each concentration of competitor with a single concentration of analyte (preferably at the K_d). Sequentially, inject the ligand/competitor mixture over the ligand and control surfaces from the highest concentration of competitor to the lowest and then to zero concentration inhibitor (the last injection yielding the maximum level of binding). Record the binding level at equilibrium for each injection. Plot the specific-binding response (in RU) over inhibitor concentration. To determine the inhibitory concentration (IC_{50}) of the interaction,

use the simple equation $B_{eq} = B_{bound}/[1 + (C/IC_{50})^P]$, where B_{eq} is the response bound at equilibrium, B_{bound} is the response bound at equilibrium in absence of competitor, C is the concentration of competitor, and IC_{50} is the concentration at which 50% binding is observed, and P is the Hill coefficient.

3.5. Determining the Interaction Stoichiometry

Sensorgrams generated during either kinetic or equilibrium analysis (**Subheading 3.2.** or **3.3.**) can be used to determine the stoichiometry of the interaction, provided that the experimental conditions satisfy the requirements of the stoichiometry equation (*see* **Note 9**). To determine the binding stoichiometry, R_{max} must be determined experimentally by injecting increasing concentrations of analyte until the observed binding RUs at equilibrium no longer increases (*see* **Note 15**). The RU level where binding saturates is the R_{max} value. If the molecular masses of the analyte and ligand are known, the R_{max} value and the amount (in RUs) of protein on the surface can be inserted into the stoichiometry equation to solve for valency (*see* **Note 9**).

Challenges to this approach include the necessity to achieve saturation of all binding sites, which may result in the potential oligomerization of some analytes at high concentrations. As the equation assumes that the immobilized protein is 100% active, this method nearly absolutely requires that the protein be immobilized via a directed capture method that orients the molecule's binding site out of the matrix (*see* **Notes 7** and **8**). Although the use of a noncovalent capture of a tagged ligand may be used, it is preferable that the surface be sufficiently stable, such that baseline drift is minimal to be able to resolve the saturated binding at equilibrium. While directed biotin tags are ideal (*see* **Note 7**) because the dissociation rate of biotin and streptavidin is almost negligible, other noncovalent capture tags may have variable success depending on the context of the protein they are incorporated into. Finally, it is important that the oligomeric state of the proteins alone being characterized are known, as the deduced stoichiometry hinges on this knowledge.

3.6. Analyte Screening to Identifying Cytokine-Binding Partners for Orphan Viral-Binding Proteins by SPR

The high sensitivity of Biacore makes it an ideal instrument to identify novel binding partners for orphan viral proteins. One of the methods that we recently used successfully involved screening a panel of known cytokines and chemokines against a novel viral protein using SPR (Seet, McFadden, and Fleming, unpublished data) (*see* **Note 16**). After immobilizing the viral protein onto a flow cell of a Biacore chip at high density (*see* **Note 17**), purified cytokines or chemokines were injected over the control and active surfaces at relatively high concentration (50–200 n*M*) in hopes of identifying binding profiles indicative of specific interactions. Any specific binding event that occurred

was then further characterized using Biacore by performing more detailed kinetic- or equilibrium-binding analysis (**Subheadings 3.2.** or **3.3.**) to assess whether the affinity of the interaction is biologically relevant. If the binding partner is a cytokine with a known receptor, a recombinant form of the receptor can be immobilized onto a Biacore chip surface, and a competition analysis can be performed to assess whether the viral protein inhibits the interaction of the cytokine with its receptor (**Subheading 3.2.**). Thus, Biacore can provide valuable information that can be further tested and confirmed in biological systems.

3.7. Conclusion

The infection of a virus with its host entails a multitude of interacting biomolecules that collectively impart biological and biochemical specificity to the process of infection. As such, SPR biosensors have the potential to aid in understanding many aspects of poxvirus biology at the molecular interface with their hosts. The ability to acquire kinetic, equilibrium, stoichiometric, and thermodynamic information, and its ability to examine many types of biomolecules, including viral particles, proteins, lipids, and carbohydrates, makes SPR analysis a highly valuable tool for the study of poxviruses. Future studies using SPR to study poxviruses will no doubt exploit its versatility to explore unique aspects of poxvirus–host interactions.

4. Notes

1. Biacore chips should be stored at 4°C in a 50-mL Falcon tube with only a small drop of distilled water.
2. It is important that the coupling buffer containing primary amines (such as, Tris) be avoided. Lowering the pH of the analyte solution during coupling aids in concentrating the analyte to the negatively charged surface. Although pH 4.0 is generally acceptable for most purposes; the pH of the coupling buffer can be raised or lowered and may depend on the pI of the protein. The running buffers listed in **Subheading 2.2.** are all used experimentally to find out which works best (*see* **Subheading 3.1.2., step 1**).
3. SPR analysis is amenable to numerous running buffers and can be varied depending on the application and sample being used. A range of pH, ionic strength, and detergents may be used during analysis, although to prevent nonspecific binding, NaCl levels should be kept above 100 m*M*, and pH levels should be maintained above 7. Ready-made buffer (HBS-EP) that is filtered and degassed may be purchased from Biacore. However, if preparing buffer in-house, it is highly advisable to prepare fresh running buffer each day. Buffer should be filtered using a 0.2-μm filter and degassed under vacuum.
4. Proteins used for kinetic analysis should be purified to homogeneity and assessed for purity (preferably more than 95%) using silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To avoid bulk shifts,

the analyte buffer should be matched to the running buffer. If the stock analyte solution is at a relatively high concentration, effects of bulk shifts may be minimized through the protein dilution in the running buffer. Otherwise, buffer exchange or dialysis may be required to prevent unwanted bulk shifts.

5. Applications involving the use of crude supernatants or lysates requires more attention to reduce nonspecific binding. Some measures that can reduce nonspecific binding include raising the ionic strength of the analyte solution and/or including soluble carboxymethyl dextran in the analyte solution.
6. As a cost-saving measure, purified soluble streptavidin can be amine-coupled to a CM5 chip, rather than using a prepared SA chip purchased from Biacore.
7. Xiang and Moss *(7)* have used a method for immobilizing the molluscum contagiosum interleukin-18 (IL-18) binding protein homolog onto an SA chip. They engineered a flexible linker (GGGGSGGGGS), followed by a biotinylation sequence (GLNDIFEAQKIEWHE) and a six-histidine tag onto the viral IL-18-binding protein. The expressed protein was purified by incubating the medium containing the secreted viral protein with Ni-nitrilotriacetic acid resin (Qiagen) to purify the histidine-tagged protein. After washing, the resin was incubated with the *Escherichia coli* biotin holoenzyme synthetase, BirA (Avidity, Colorado). The resin was washed and packed into a column and the biotinylated viral protein was eluted with 300 m*M* imidazole in phosphate-buffered saline. The biotinylated protein was immobilized onto a Biacore SA chip and analyzed for its interaction with human and mouse IL-18 *(7)*.
8. Random immobilization of proteins to surfaces by amine coupling may result in the inadvertent occlusion or obstruction of the binding site. Therefore, alternate methods to immobilize the target protein may include the use of thiol-coupling that exploits either a unique free-thiol group or an engineered-free cysteine. Moreover, a capture tag (i.e., six-histidine tag) or affinity capture antibody (toward an epitope tag or to the native polypeptide itself) can be used to orient the molecule to ensure the binding site is unobstructed.
9. Ideally, low-surface densities (less than 50 RU) should be used in kinetic analysis to minimize mass transport limitations and aggregation at the surface. The stoichiometry equation is useful when determining how much should be immobilized onto the chip surface or when deducing the stoichiometry of the interaction. The equation to be used is $R_{max} = (MW_{analyte}/MW_{ligand}) \times (RU_{immobilized}) \times$ (stoichiometry), where R_{max} is the level of RU at saturation, the $MW_{analyte}/MW_{ligand}$ is the molecular mass ratio between the analyte and the ligand, and $RU_{immobilized}$ is the amount of RUs of ligand immobilized to the chip surface. Because the equation assumes 100% active protein on the surface, when calculating the amount of protein to immobilize onto the surface during amine coupling, it may be necessary to overestimate the amount of immobilized protein required to achieve a desired R_{max} value, as the random nature of amine-coupling immobilization may block anywhere from 10% to 50% of the binding sites. If more than 50% of theoretical sites are blocked after immobilization, it is recommended to use an alternate immobilization strategy.

10. As a true control, the immobilized protein on the control surface should match the protein on the active surface in terms of molecular mass, charge, and hydrophobicity. The ideal control protein is a recombinant site-directed mutant of the active protein that does not bind the analyte.
11. Because SPR monitors the refractive index, any minor differences between the running buffer and analyte buffer appear as large shifts in RUs at the beginning and end of the injections. Although the control reference surface serves to limit this effect by subtracting it out from the active flow surface sensorgram, residual effects in the form of spikes are likely to still be apparent. Thus, great care should be taken to always match the running buffer and analyte buffer for cleaner data by diluting the analyte with the running buffer. If the analyte stock concentration is relatively low, and the stock buffer has a substantial refractive index difference from that of the running buffer, buffer exchange or dialyze the analyte into the running buffer to completely match the buffers. Perform subsequent serial dilutions into the running buffer.
12. On the manual instrument (BiacoreX), all micropipet injections of sample into the instrument for the collection of experimental data should incorporate a 5–10-μL bubbles before and after sample analyte to prevent sample mixing and dilution with running buffer that would otherwise lower the effective concentration of the sample being injected. This technique is described in the BiacoreX manual.
13. Accurate assessment of the dissociation rate constant can be affected by rebinding the analyte to the surface, particularly when the association rate is very fast, which may result in a slower observed rate constant. To minimize rebinding, high-flow rates are recommended. Moreover, injection of a soluble form of the ligand (immobilized protein) over the surface should be performed following the association phase to compete for the rebinding to the surface. Whereas this can function as a remedy for rebinding, such a technique should be used as a routine test for rebinding when performing kinetic analysis for the first time using uncharacterized proteins.
14. Carryover occurs when sample analyte is insufficiently cleared from the surface after regeneration or when the analyte has high nonspecific binding to the surfaces of the microfluidics cartridge, resulting in either reduced-binding capacity on the surfaces and/or may result in inaccurate-binding analysis. It can be monitored by performing replicates and control injections to observe reduced-binding capacity, poor reproducibility, and spurious binding events during blank buffer injections.
15. Sequential injections can be performed by separate injections of a given concentration of analyte followed by a regeneration step or can be performed by sequential injections without regeneration. In the latter case, injections must be performed with the lowest concentration first until equilibrium is attained, then without regeneration the next higher concentration can be injected immediately until equilibrium is attained. This can be repeated until the final injection of the highest analyte concentration.
16. A variation on this theme has been used by Smith et al. ***(8)*** to identify a binding partner for the cowpox virus CC-chemokine-binding protein. The viral protein

was immobilized onto a Biacore chip and then exposed to conditioned supernatants from a cell line. Once a binding activity was identified by SPR, the positively identified supernatants were passed over an affinity column containing the immobilized viral protein. The binding partner was eluted and sequenced.

17. Because the molecular mass of the protein being screened is unknown, higher density surfaces should be used to ensure enough protein is on the chip surface to permit a binding signal large enough to be detected.

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18

Monitoring of Human Immunological Responses to Vaccinia Virus

Richard Harrop, Matthew G. Ryan, Hana Golding, Irina Redchenko, and Miles W. Carroll*

Summary

For the last 30 yr, interest in vaccinia virus immune monitoring has focused on the use of the vaccinia virus as a recombinant vaccine vector and the potential detrimental effect of antivector immunity on subsequent vaccination with a recombinant vaccinia virus. However, interest in this area has intensified after the publication of reports suggesting that smallpox may be a major pathogen selected for bioterrorist activities. Owing to the unacceptably high incidence of complications induced by previous effective smallpox vaccine strains, alternative safer strains (e.g., modified vaccinia Ankara [MVA]) are being assessed for their antigenicity in clinical trials. The exact immune effector mechanism responsible for vaccine-induced protection to smallpox infection has not been fully elucidated, although it is believed that neutralizing antibody plays a major role. This chapter describes a simple enzyme-linked immunosorbent assay (ELISA) to quantify vaccinia virus antibody titer. Additionally, to define serum-neutralizing activity, both a classical plaque reduction assay and a high-throughput 96-well plate method based on reduction of recombinant vaccinia virus expressed β-galactosidase is described. Furthermore, details are given for a T-cell proliferation assay, primarily for monitoring T-helper CD4 activity and an enzyme-linked immunospot (ELISPOT) assay for CD8 analysis. The use of reliable immunological assays is vital in assessing the potential efficacy of new vaccines to protect against smallpox infection.

Key Words: Human immune assays; vaccinia virus; MVA; ELISA; neutralization; proliferation; ELISPOT.

1. Introduction

After recent reports suggesting that smallpox may be a major pathogen selected for bioterrorist activities, detailed analyses of the immunological responses induced following vaccinia virus immunization has intensified.

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The full impact of releasing variola major—the causative agent of smallpox—on the current population is unknown. Vaccination against smallpox was stopped in 1971 in the United States, leaving many adults with a history of distant immunization and the youth without any protection. The exact immune effector mechanism responsible for vaccine-induced protection to smallpox infection has not been fully elucidated. Reports of complications following vaccinia virus infection in recipients with either humoral or cell-mediated deficiencies ***(1)*** suggest that both arms of the immune response are required for efficient protection. More recent reports in animal models suggest a far greater importance of the humoral response ***(2)***. However, long-term immunological memory may still rely on the induction of effective T-cell responses.

Despite the effectiveness of vaccinia virus vaccine strains (e.g., Wyeth's DryVax), there is still considerable concern regarding the relatively high level of vaccine complications associated with their use ***(1)***. It is estimated that up to 20% of the present population would not be given DryVax because of predisposing factors that could result in serious adverse events. The potentially safer modified vaccinia Ankara (MVA) strain has been proposed as an alternative vaccine candidate, although its ability to induce protection against smallpox has never been tested in humans. To this end, the safety and efficacy of DryVax, MVA, and other candidate smallpox vaccines need to be determined in both nonhuman primate and human clinical trials. The use of validated high-throughput immunological assays is important in assessing the effectiveness of new vaccines to protect against smallpox infection. Such high-throughput assays have been developed to quantify neutralizing antibody titers by utilizing reporter gene expression ***(3,4)***.

Since the description of the potential utility of vaccinia virus as a recombinant vaccine vector, many thousands of people have been exposed to this virus as part of infectious disease and cancer immunotherapy vaccine protocols. Immunological parameters analyzed within such clinical trial settings have often focused on the anti-vector antibody response and the induction of antibodies capable of neutralizing vaccinia infection (especially in homologous prime-boost protocols). It is argued that high levels of neutralizing antibodies can impact the magnitude of the immunological response induced to the target antigen delivered by the recombinant vector. Indeed, the presence of pre-existing vaccinia-specific antibodies was thought to have a profound negative effect on the efficacy of vaccination with a recombinant virus; although recent data suggest that this may not be the case (Redchenko, I. and Carroll, M., unpublished data).

We have utilized MVA as a vaccine vector to deliver a tumor-associated antigen to late-stage cancer patients enrolled into a clinical trial. Throughout the trial, humoral and cellular immune responses mounted by patients to both the tumor antigen and MVA were monitored. Antibodies specific for MVA

and those capable of inhibiting viral infection of susceptible cells were measured by enzyme-linked immunosorbent assay (ELISA) and plaque reduction assay, respectively. Additionally, the cellular responses mounted to MVA were monitored by proliferation assay and enzyme-linked immunospot (ELISPOT). Such analyses have enabled assessment of the effects of vaccinia virus immunity on the ability to induce immune responses to the gene product delivered by recombinant MVA. We also describe a high-throughput vaccinia virus neutralization assay based on the reduction of recombinant vaccinia virus-expressed β-galactosidase, which is applicable for the immune analysis of large-scale smallpox vaccine clinical trials.

2. Materials

2.1. ELISA

1. Vaccinia virus (double sucrose-cushioned; *see* Chapters 8 and 15).
2. Carbonate coating buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3. Add 800 mL H_2O and adjust pH to 9.6.
3. Phosphate-buffered saline (PBS; Sigma).
4. Wash buffer: PBS + 0.1% Tween-20.
5. Blocking buffer: PBS + 5% fetal bovine serum (FBS).
6. 96-Well ELISA plates (e.g., Immulon 4-HBX, Thermo Labsystems).
7. Human sera, stored at –20°C.
8. Secondary α-human immunoglobulin horseradish peroxidase (HRP)-conjugated antibody (e.g., DAKO).
9. Colorimetric substrate (e.g., Sigma FAST OPD Peroxidase substrate tablets).
10. Stop solution: 3 *M* HCl or 3 *M* H_2SO_4.
11. Microplate reader (e.g., Dynex MRX).

2.2. Plaque Reduction Assay

1. Primary chicken embryo fibroblasts (CEF; Veterinary Laboratories Agency, Weybridge, Surrey), VERO (ATCC, # CCL-81), and BSC-1 (ECACC, # 85011422) cells.
2. CEF growth medium: Minimal essential medium (MEM) supplemented with 2 m*M* glutamine, 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin.
3. Human sera (test and control).
4. Sucrose cushion-purified MVA expressing LacZ (MVA-LacZ) or DryVax vaccinia virus.
5. TEAE cellulose resin, e.g., Sigma T-9658.
6. Dilution buffer: PBS and 2% FBS.
7. Virus growth medium: MEM supplemented with 2 m*M* glutamine, 2% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin.
8. Fixing solution: 4% neutral-buffered formalin in PBS.
9. X-gal stock solution: 2% X-gal (Alexis) in dimethylformamide.
10. X-gal stain: 4 m*M* potassium ferricyanide, 4 m*M* potassium ferrocyanide, 2 m*M* magnesium chloride, 0.04% X-gal in PBS.

2.3. High-Throughput Neutralization Assay

1. Dilution medium: EMEM, 10% FBS; heat-inactivated.
2. HeLa cells (ATCC, # CCL-2).
3. Trypsin solution: prepared by a 1:10 dilution of a 10X trypsin stock (2.5% in Hank's balanced salt solution [Mediatech]) in calcium- and magnesium-free PBS.
4. Cell lysis buffer: 4% solution of IGEPAL detergent (Sigma-Aldrich, cat. no. CA630) in dilution medium.
5. Immulon 2 plates (Thermo Labsystems).
6. β-Galactosidase enzyme (1500 U/mL β-gal; Roche Diagnostics, Corp).
7. 15 U/mL β-Gal working solution: B-gal in 50% glycerol solution in PBS containing 400 μg/mL bovine serum albumin (BSA). Aliquots are stored at –20°C.
8. Z buffer (Sigma-Aldrich): 98.55 mL 0.1 *M* sodium phosphate, 1 mL 1 *M* KCl, 0.1 mL 1 *M* $MgSO_4$, 0.35 mL β-mercaptoethanol.
9. Chlorophenol red-β-D-galactopyranoside monosodium salt (CPRG) substrate stock (Roche Diagnostics): 4 mg/mL 0.1 *M* sodium phosphate, pH 7.0, prepared fresh for each assay.
10. Stop solution: 1 *M* Na_2CO_3.

2.4. Proliferation Assay

1. Human peripheral blood mononuclear cells (PBMCs).
2. Ficoll gradient (Accuspin columns; Sigma).
3. PBMC culture media: RPMI-1640, 10% FBS, 2 m*M* glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin.
4. Cell freezing medium (Sigma, cat. no. C-6164).
5. Antigens: ultraviolet (UV)-irradiated MVA.
6. Mitogen: 10 μg/mL PHA working stock.
7. 96-Well tissue culture plates (Falcon Microtest 96).
8. 1 mCi/mL ^{3}H-thymidine (Amersham Pharmacia).
9. Cell harvester (Packard FilterMate Cell Harvester).
10. Scintillant (MicroScint-20, Packard).
11. Glass fibre filters (Packard).
12. Scintillation/Micro-Beta counter (Topcount, Packard).

2.5. ELISPOT

1. 96-Well ELISPOT plates (Multiscreen IP, Millipore).
2. Wash buffer: PBS + 0.1% Tween-20.
3. Antigens: PHA and MVA, untreated.
4. IFNγ ELISPOT Kit (MABTECH, which includes antibodies 1-D1K and 7-B6-1 [1 mg/mL]).
5. Alkaline phosphatase (AP) substrate (Bio-Rad; 5 mL is required per plate): 4.8 mL sterile-distilled water, 200 μL AP color development buffer. Mix well, then add 50 μL AP color reagent A and 50 μL AP color reagent B.
6. ELISPOT plate reader (AID).

7. $CD4^+$ T-cell depletion kit (Dynabeads M-450 CD4; Dynal).
8. Dilution buffer: PBS + 2% FBS.
9. Cell culture medium: RPMI-1640, 10% FBS, 2 m*M* glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin.
10. Magnetic separation device.

3. Methods

The methods described here detail the measurement of vaccinia virus-specific antibodies (**Subheading 3.1.**), antibodies capable of neutralizing vaccinia virus infection of target cells (**Subheading 3.2.**) and vaccinia-specific T-cell responses (**Subheadings 3.3.** and **3.4.**). With the exception of the high-throughput neutralization assay (**Subheading 3.2.3.**), each procedure uses double sucrose cushion-purified virus to decrease the potential problem of nonspecific responses against proteins derived from cells in which the virus was propagated. All assays described are for the analysis of human immune responses, but can be readily adapted to monitor murine responses to vaccinia virus.

3.1. Measurement of Antivaccinia Antibody Responses by ELISA

As stated previously, vaccinia virus-specific antibodies are thought to play a major role in protection against smallpox infection. ELISA is a flexible and relatively high-throughput technique that facilitates the quantitation of soluble antibody molecules in serum. The antigen of interest (vaccinia virus) is coated onto the surface of a 96-well plate and subsequently incubated with the test sample (serum). Any antibodies specific for the antigen will bind and, after extensive wash steps, can be detected using an enzyme-conjugated secondary antibody.

To accurately determine the MVA-specific antibody response, it is important to use double sucrose cushion-purified virus ***(5)*** to reduce the potential of detecting nonspecific anticellular antibody responses (*see* **Note 1**). Additionally, as the particle-to-infectivity ratio can differ considerably between different virus preparations, it is advisable to perform a checkerboard ELISA to optimize antigen and antibody concentrations for each different viral stock. Wherever possible, the same viral stock should be used to analyze responses in a single test group. **Figure 1** illustrates a checkerboard ELISA in which both the primary test sera and the antigen have been titrated. At an MVA concentration of more than 1.25×10^6 pfu/well, the optical density (OD) reading reaches a plateau for all concentrations of this high-titer human serum. This indicates that the antigen is in excess and that the quantity of MVA-specific antibody in the serum (at these dilutions) is limiting.

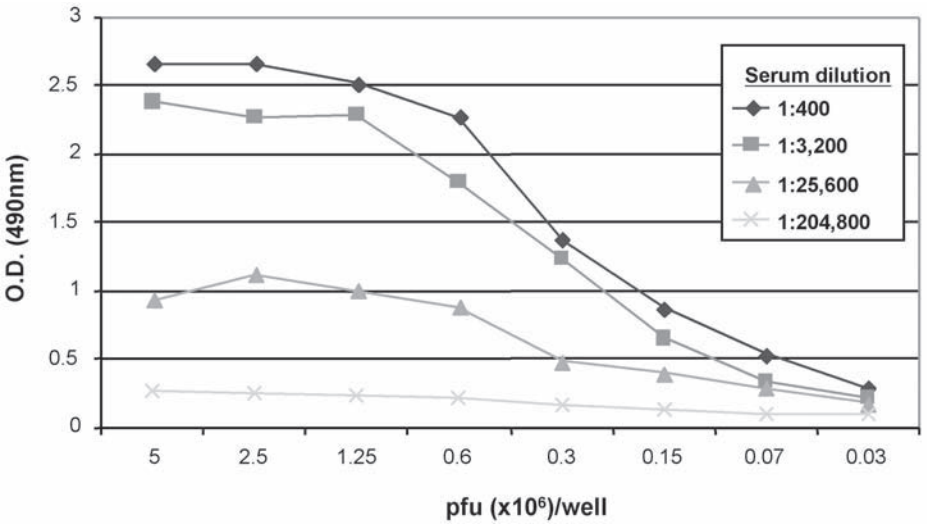

Fig. 1. MVA checkerboard ELISA. Double sucrose cushion-purified virus is diluted serially across the plate from 5×10^6 to 0.03×10^6 pfu/well. Test sera is diluted down the plate. For clarity, only four serum dilutions (1:400, 1:3,200, 1:25,600, and 1:204,800) are shown. The test serum was taken from a donor immunized with a recombinant MVA vector.

3.1.1. Preparation of ELISA Plates Coated with Antigen

1. Dilute MVA to 5×10^7 pfu/mL (*see* **Note 2**) in carbonate coating buffer (*see* **Note 3**).
2. Add 100 µL of this diluted antigen to each well of the 96-well plate (i.e., 5×10^6 pfu/well).
3. Incubate overnight at 4°C in a humid environment.
4. Aspirate solution and wash each well briefly with wash buffer.
5. Add 200 µL blocking buffer to each well (*see* **Note 4**).
6. Incubate for 1 h at room temperature in a humid environment.

3.1.2. Dilution and Addition of Control and Test Sera (see ***Note 5***)

1. Thaw aliquots of negative control (*see* **Note 6**) and test serum. If available, a positive control should also be included (*see* **Note 7**).
2. Dilute sera to the same concentration in wash buffer (*see* **Note 8**). Place the diluted sera on ice.
3. Add 200 µL of the diluted serum samples (test and negative and positive control sera) to appropriate wells; assay at least in duplicate.
4. Add 100 µL wash solution to all remaining wells.
5. Perform doubling dilutions across the plate by taking 100 µL from column 1 and pipetting it into the wells of column 2. Mix and continue across the plate as required. *See* **Fig. 2** for an example of a plate template.
6. Incubate at room temperature for 2 h in a humid environment.

	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
	1	2	3	4	5	6	7	8	9	10	11	12
A	-ve						Test 1					
B	-ve						Test 1					
C	-ve						Test 1					
D	-ve						Test 1					
E	+ve						Test 2					
F	+ve						Test 2					
G	+ve						Test 2					
H	+ve						Test 2					

Fig. 2. Example plate template for the determination of antigen-specific antibody titer. To columns 1 and 7 of a precoated plate, 200 µL negative control (–ve), positive control (+ve), or test serum (all diluted to 1:1000) are added. To all remaining wells, 100 µL wash buffer is added. Serial dilutions are then made across half the plate using a multichannel pipettor by removing 100 µL from column 1 and dispensing into column 2, and so on.

3.1.3. Detection of Bound Antibody

1. Dilute the secondary antihuman Ig antibody 1:1000 in wash buffer (*see* **Note 9**). Place on ice.
2. Wash plates three times with wash buffer.
3. Add 100 µL diluted secondary antibody to each well.
4. Incubate for 2 h at room temperature in a humid environment.
5. Wash plate five times with wash buffer.
6. Detect bound antibody by using an OPD substrate system. For each plate, dissolve one tablet OPD and one tablet urea-H_2O_2 in 20 mL H_2O (*see* **Note 10**).
7. When completely dissolved, add 200 µL/well (*see* **Note 10**).
8. Allow color to develop at room temperature. Color development should be timed and the reaction stopped while the negative control sample is still relatively clear and the OD of the test sera lies within the dynamic range of the plate reader (i.e., all within 20 min).
9. Stop the reaction by addition of 50 µL stop solution.
10. Read the absorbance values at 490 nm using a microplate reader.
11. Plot mean absorbance value against serum dilution for each sample. *See* **Fig. 3** for an example; *see also* **Note 11**.

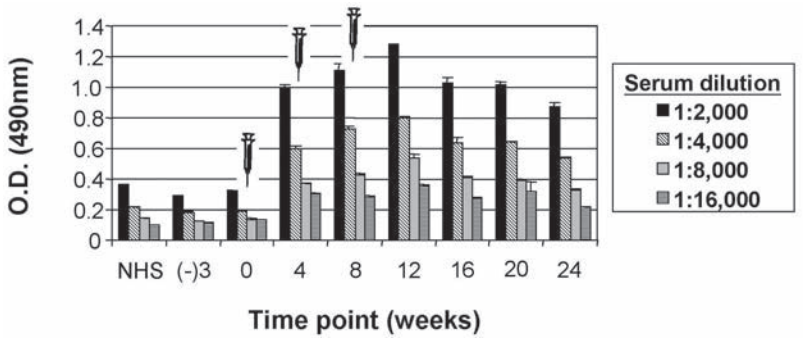

Fig. 3. Anti-MVA antibody response measured by ELISA in an individual immunized with a recombinant MVA vector at wk 0, 4, and 8. Postimmunization samples are compared to two internal control preimmunization serum samples (–3 and 0 wk), as well as to the negative control (a pool of human serum recovered from five known vaccinia naïve donors [NHS]). Serial dilutions from 1:2,000 to 1:16,000 are illustrated. For clarity, the positive control sample is not shown.

3.2. Measurement of Vaccinia Virus-Neutralizing Antibodies

Although antiviral antibody levels can be monitored by ELISA, this assay provides no functional information on the ability of the antibodies to inhibit viral infection of target cells. Plaque reduction assay (neutralization assay) can provide such functional information. The presence of a high vaccinia virus-neutralizing antibody titer is believed to be one of the major mechanisms that can help confer protection against smallpox infection.

The plaque reduction assay is a relatively simple technique that provides a quantitative measurement of antibodies capable of vaccinia virus neutralization ***(6,7)***. As our clinical trials have utilized recombinant MVA as the viral vector, we primarily use MVA in our assays. However, as described below, the assay can be easily modified for use with replication-competent vaccinia viruses (e.g., Wyeth's DryVax strain and Western Reserve [WR]; *see* **Note 12**). As MVA does not form obvious plaques, a recombinant MVA expressing the *Escherichia coli* LacZ marker gene ***(8)*** is used to enable infected foci visualization by employing the X-gal substrate. Essentially, a known titer of MVA-LacZ is mixed with test serum prior to incubation on a permissive cell line, such as primary CEF. The BHK21 cell line is an adequate alternative to CEFs ***(9)***. Any reduction in the expected number of plaques when compared to virus treated with control serum is likely to be caused by the binding of antibodies to viral entry proteins that abrogates infection of CEF cells (*see* **Subheading 3.2.2.** for detailed methodology). Although the plaque reduction assay is relatively simple and requires no specialized equipment, it is time-consuming, and

high-throughput assays based on LacZ (*see* **Subheading 3.2.3.**) and green fluorescent protein (GFP) expression have been developed ***(3,4)***.

There are two major forms of vaccinia virus: intracellular mature virus (IMV), which remains within the cells and is released after cell lysis; and extracellular-enveloped virus (EEV), which is released from the intact infected cell. Different antibodies are able to neutralize the two virus forms because the envelope of IMV and EEV are made up of different viral proteins ***(10)***. The virus used in our assay is primarily IMV and is the standard virus source used historically in vaccinia virus neutralization assays. Assays that measure EEV-neutralizing antibodies have also been developed ***(11)***.

3.2.1. Removal of Heparin from Sera to Prevent Heparin Interference with the Measurement of Neutralizing Antibody Titers

When a fresh blood sample is collected from a donor, it is frequently collected in the anticoagulant heparin (40 U/mL). Several publications have demonstrated that heparin interferes with vaccinia infectivity ***(12)***. We found that the accurate quantification of neutralizing antibody levels in the plaque reduction method was markedly compromised by the presence of heparin, especially at high-serum concentrations (*see* **Fig. 4**). This is especially true for MVA on CEF cells. DryVax is less affected by the presence of heparin. It is likely that heparin exerts this inhibitory effect by binding to the heparin sulfate receptor shown to play a role in viral cell entry ***(12)***. To facilitate the accurate measurement of neutralizing antibody titers in human sera collected in heparin, we developed a method for removing or blocking the heparin effect on MVA infectivity. (This method is not required where blood is recovered without the use of heparin.) Heparin antagonists tested included protamine sulfate, polybrene, and poly-D-lysine, but their narrow concentration window for optimal heparin blocking made reproducible results between samples difficult. The anion exchanger TEAE (triethylaminoethyl) cellulose ***(13)*** was eventually selected for its superior ability to bind and remove heparin. The primary advantage of using the readily extractable TEAE resin to sequester heparin is that it can be removed from the serum sample prior to the measurement of neutralizing antibodies.

1. Remove control and test sera from storage at –20°C (*see* **Note 13**).
2. Heat inactivate the complement component of both control and test sera by incubating samples at 56°C for 30 min.
3. Add TEAE cellulose resin to a final concentration of 20 mg/mL to the heat-inactivated sera.
4. Mix the sera–TEAE suspension by gentle inversion using a rotary mixer for 10 min at room temperature.

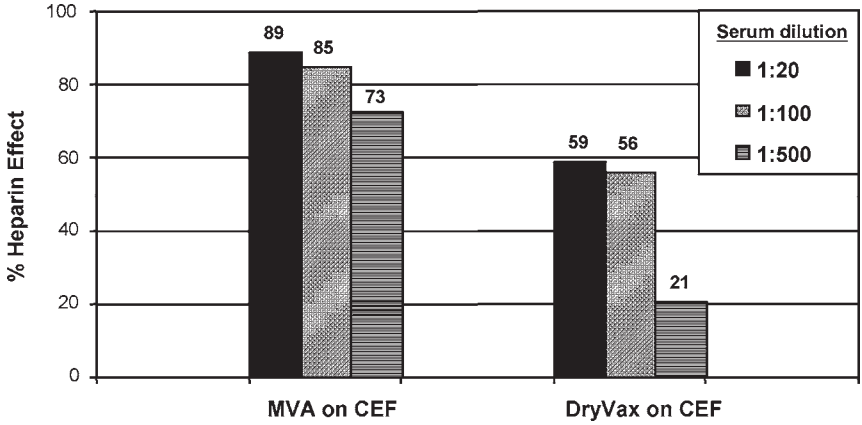

Fig. 4. Inhibitory effect of soluble heparin on MVA and DryVax viral infection of CEF cells. This figure illustrates the percentage reduction in MVA or DryVax plaque-forming units (pfu) attributable to the presence of heparin in the test sera. The percentage of heparin effect is determined for each virus using serum from a vaccinia naive donor (at three different dilutions of 1:20, 1:100, and 1:500) and by comparing the number of pfu produced by each virus in the absence (TEAE-treated serum) or presence (untreated serum) of heparin. When compared to DryVax infection of CEF cells, a similar degree of inhibition was also observed following DryVax infection of VERO cells, suggesting that this phenomenon is not cell specific (data not shown). % Heparin effect = [((average no. of pfu in the absence of heparin) – (average no. of pfu in the presence of heparin))/(Average no. of pfu produced in the absence of heparin)] × 100%

5. Sediment the TEAE resin–heparin complex from the serum by centrifuging at 2000*g* for 10 min at 4°C.
6. Aspirate and collect the clarified serum supernatant, taking care not to disturb the pelleted TEAE resin.
7. Dilute both control and test sera to the same concentration with the addition of ice-cold dilution buffer. Place the diluted serum on ice.
8. Inclusion of a positive-control serum sample (if available) of known neutralizing titer can be included to check the validity of the assay (*see* **Note 5**).

3.2.2. Standard Plaque Reduction Assay

3.2.2.1. Preparation of MVA-LacZ Working Stocks and CEF Cells

1. Based on the viral titer of the MVA-LacZ master stock, dilute the virus in PBS and dispense into cryovials at a concentration of 1×10^5 pfu/mL.
2. Store at –80°C until required. These aliquots constitute the MVA-LacZ working stock for each assay (*see* **Note 14**).
3. CEF cells are seeded into six-well plates at a concentration of 5×10^5 cells/well in CEF growth medium and incubated at 37°C, 5% CO_2 for 18–24 h prior to assay initiation.

3.2.2.2. Addition of MVA Virus to Control and Test Sera

1. Dilute the MVA-LacZ virus working stock in dilution buffer to yield 200 pfu/mL.
2. Add 1 mL MVA-LacZ (containing 200 pfu) to an equal volume of diluted serum (*see* **Note 15**).
3. Incubate the virus plasma mixture for 1 h at 37°C.

3.2.2.3. Infecting CEF Cells with Serum-Treated MVA Virus and Determining Virus Titer

1. The virus–plasma mixture is dispensed into duplicate wells of a six-well plate (1 mL/well) containing a confluent monolayer of CEF cells (prepared the day before as described in **Subheading 3.2.2.1.**).
2. Incubate for 1 h at 37°C.
3. After 1 h adsorption, the virus plasma mixture is replaced with virus growth medium and the plates are incubated overnight at 37°C 5% CO_2.
4. At 24 h postinfection, remove medium from the six-well plate and add 1 mL fixing solution to each well.
5. Incubate at room temperature for 5 min.
6. Remove the fixing solution and carefully wash the cells with 2 mL PBS.
7. Add 1 mL X-gal stain and incubate the cells 4 h to overnight at 37°C (*see* **Note 16**).
8. Remove the X-gal substrate from the six-well plate.
9. Rinse each well twice with 2 mL PBS and discard wash.
10. Invert rinsed plates and place on light box to quantify the numbers of stained foci.
11. For counting purposes, a cluster of ≥10 positive blue cells constitutes a single focus.
12. The neutralizing titer of the serum is defined as the highest dilution that yields ≥50% reduction in the number of plaques. The percentage neutralization is calculated as (no. of pfu with immune serum/no. of pfu with naïve serum) × 100. An experimental example is illustrated in **Fig. 5**.

3.2.3. High Throughput β-Gal-Based Vaccinia Virus Neutralization Assay

The classic vaccinia neutralization assays are all based on plaque reduction-neutralizing tests (PRNT). They are slow (4–7 d), require a relatively large volume of supplies (because they are conducted in 6- or 12-well plates), and they are also somewhat subjective- and operator-dependent as the read-out is visual ***(6,14,15)***. Therefore, the PRNT assays are considered difficult to standardize, validate, and transfer between laboratories. Furthermore, owing to the low throughput, only limited numbers of samples can be tested in each assay, making it impractical for evaluating hundreds of samples from clinical trials. A reporter gene-based assay with an automated readout provides an alternative to the classical PRNT assays. Because it does not require several rounds of viral replication, it can be read within 24 h or less. We reasoned that an enzyme-encoding gene would provide augmented sensitivity when compared with other reporter genes, as each enzyme molecule converts multiple substrate molecules

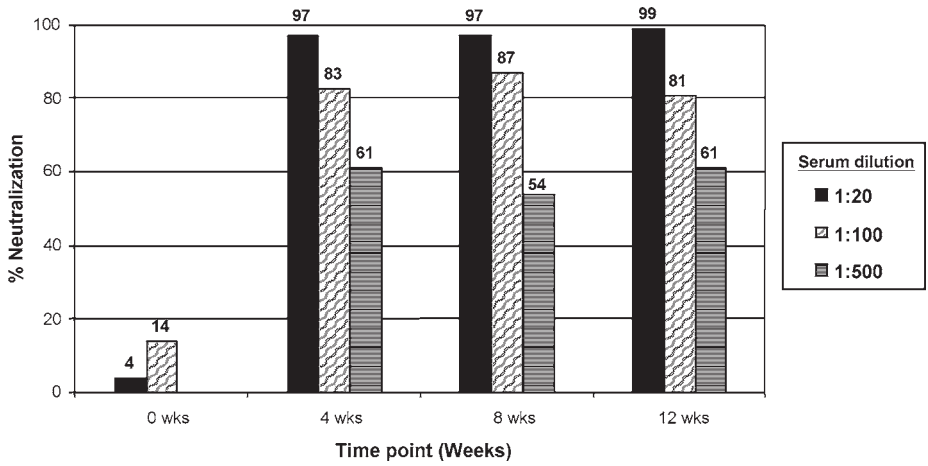

Fig. 5. MVA neutralization assay. This figure illustrates the ability of serum (pretreated to remove heparin) recovered from a single donor to neutralize MVA infection of CEF cells. The donor was vaccinated with a recombinant MVA vector at 0, 4, and 8 wk. Serum dilutions of 1:20, 1:100, and 1:500 are illustrated. Because the reduction in pfu is more than 50% at a dilution of 1:500 at 4, 8, and 12-wk time-points, the neutralizing antibody titer is at least ≥1:500.

within the time-frame of the assay. We demonstrated that the new assay is rapid (24 h), of equal sensitivity to PRNT assays, reproducible, and objective. The new assay is a high-throughput assay and amenable to validation. The assay is currently used by several laboratories for preclinical and clinical studies of new smallpox vaccines, establishing the potency of new vaccinia immunoglobulin (VIG) products, and screening monoclonal antibodies and entry inhibitors ***(3)***.

3.2.3.1. Assay Set-Up

This assay is based on the expression of a reporter gene encoding the bacterial β-gal enzyme under the control of a vaccinia virus-synthetic early/late promoter ***(16)***. In each assay, WR-based recombinant vaccinia virus expressing β-gal (vSC65) is incubated with test sera or with positive and negative controls serially diluted in dilution medium. A control plate contains limited virus titration (0.125–0.03 pfu/cell) (*see* **Note 17**). In most assays, virus is used at 0.06 pfu/cell.

1. 96-Well round-bottom plates are seeded with 1×10^5 HeLa cells per well (in 50 μL/well).
2. Prior to setting up the appropriate virus and antibody dilutions, the β-gal-expressing virus is prepared by mixing equal volumes of the crude virus stock and trypsin solution in a microtube and incubating at 37°C for 30 min (*see* **Note 18**).

3. Virus/antibodies mixtures are incubated in microtubes at 37°C for 1 h (*see* **Note 19**).
4. After the 1 h incubation, 50 µL of this mixture is then dispensed into wells of 96-well round-bottom plates containing the HeLa cells.
5. Incubate virus titrations and virus neutralization plates for an additional 16 h at 37°C in a humidified CO_2 incubator.
6. Add 100 µL cell lysis buffer of to each well and incubate for 30 min at 37°C.
7. At this point, the plates may be frozen at –70°C (up to 1 mo). Prior to the enzymatic assay, plates are thawed completely and kept at 4°C.

3.2.3.2. Measurement of β-Gal Activity (*see* **Note 20**)

1. Each β-gal enzymatic assay includes generation of a standard curve using a recombinant β-gal. To generate a standard curve, the β-gal working solution (15 U/mL) is diluted 1:100 in Z buffer/PBS at 1:4 ratio to 0.15 U/mL (150 mU/mL).
2. This diluted solution is then followed by a serial twofold dilutions in Z buffer/PBS (4:1 ratio; *see* **Note 21**).
3. Supernatants from cell lysates prepared in **Subheading 3.2.3.1.**, **step 7** are diluted by taking 20 µL cell lysate supernatant and transferring to a well containing 180 µL PBS (1:10 dilution). Mix by multiple pipetting.
4. Transfer 10 µL of this dilution to an adjacent well containing 90 µL Z buffer (final dilution 1:100).
5. Twenty microliters of fresh CPRG substrate is added to all wells containing the β-gal standard curve and to wells containing experimental samples diluted in Z buffer.
6. Plates are incubated in the dark at room temperature for 30 min, and the enzymatic reaction is stopped by the addition of 50 µL stop solution to each well.
7. OD is determined at 575 nm by an ELISA reader.
8. OD readings are transferred to Microsoft Excel for analysis. The equation ($y = bx + a$) of the linear portion of the standard curve and initial dilution factor (1:100) are used to convert OD values of each sample to β-gal activity in mU/mL, and the mean ± SD for each group of replicates are calculated. y = % of β-gal activity in the virus only control; x = log dilutions of specimen.

3.2.3.3. Calculation of Neutralization Titers and 50% Inhibitory Dilution Values

The β-gal activity in the virus-only plate determines the 100% activity level (uninhibited control), and the β-gal activity for each experimental group is compared to this value and expressed as percentage of control. Optimally, control β-gal activity should range between 300 and 900 mU/mL. Microsoft Excel is used to plot the percentage of control values for the serial dilutions of a given antibody preparation vs log dilutions. The equation of each curve is used to calculate the 50% inhibitory dilution (ID_{50}) for each test article. In the case of VIG and VIGIV, the protein concentrations are known (5–10% solutions are equal to 50–100 mg/mL) so the 50% inhibitory dilutions can be converted to ID_{50} in mg/mL. The Food and Drug Administration (FDA) VIG standard

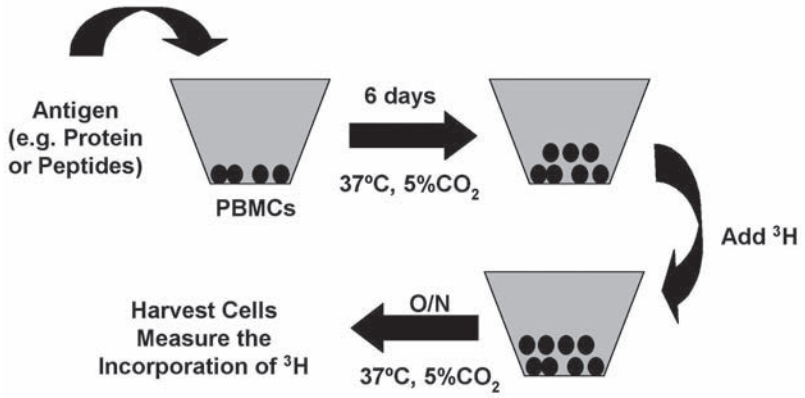

Fig. 6. Flow diagram illustrating the steps required to perform a proliferation assay. PBMCs isolated from whole blood are added to wells of a 96-well plate. Antigen is added and cocultured with PBMCs for 6 d. Antigen-specific T cells will respond by proliferating, which is monitored by the incorporation of radiolabel (^{3}H-thymidine) into cellular DNA.

(50 mg/mL) should give an ID_{50} between 1:200 and 1:3000 dilution (i.e., 17–25 μg/mL).

3.3. Measurement of MVA-Specific Cellular Responses by Proliferation Assay

Measurement of proliferative responses of human lymphocytes is a fundamental technique for the assessment of reactivity to various antigenic stimuli (illustrated schematically in **Fig. 6**). Incorporation of a radiolabel (^{3}H-thymidine) into cellular DNA is a common method to assess a proliferative T-cell response to an antigen. This assay continues to be used extensively, because clonal expansion of a specific T-cell population is the desired outcome of any vaccination protocol. The assay is usually used as a surrogate for a class II (CD4)-restricted responses, as the incubation of antigen-presenting cells with soluble antigen requires protein uptake, and most likely, preferential processing of that antigen in the class II pathway. Results from proliferation assays are often reported as a stimulation index (SI) that is defined as:

SI = Incorporation of ^{3}H-thymidine by PBMCs cultured with test antigen/ incorporation of ^{3}H-thymidine by PBMCs cultured with medium alone

An SI more than 2 is considered to be a positive result. An increase in SI to a specific antigen following immunization is indicative of a positive immune response induced by the immunogen.

In all cases, medium alone serves as a negative (no stimulation) control and a polyclonal stimulator (e.g., phytohemagglutinin [PHA]) is used as a positive

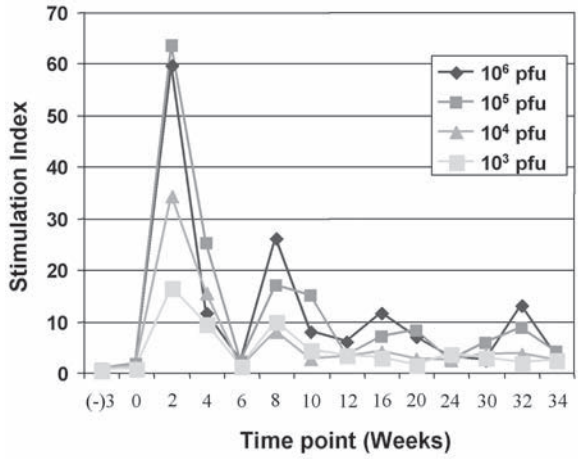

Fig. 7. Analysis of the MVA-specific proliferative responses of PBMCs recovered from an individual who was vaccinated with a recombinant MVA vector at 0, 4, 8, and 28 wk. The UV-inactivated MVA antigen was at a range of concentrations of 10^6 to 10^3 pfu/well. Stimulation index is defined in **Subheading 3.3.**

control. Wherever possible, the optimal concentration of the test antigen should be determined (usually in the range of 1–10 μg/mL for protein antigens). Where responses to vaccinia virus are being monitored, it is important to use a purified viral preparation. Simple cell lysate preparations risk potentially confusing results due to the presence of either inhibitory or stimulatory proteins derived from the cell line used to propagate the virus. **Figure 7** shows an example proliferative response of PBMCs from a donor immunized with a recombinant MVA vector throughout a vaccination time-course.

3.3.1. Preparation of PBMCs

1. PBMCs should be prepared from fresh blood by standard protocols (*see* **Note 22**).
2. To prepare PBMCs, fresh heparinized (or other suitable anticoagulant) blood should be diluted with an equal volume of sterile PBS.
3. Layer the diluted blood carefully onto a Ficoll gradient.
4. Centrifuge the samples at 400*g* for 20 min at room temperature (no brake).
5. Carefully remove the cellular interface (containing the PBMC fraction) from each tube.
6. Wash PBMCs twice with PBS and once with PBMC growth medium.
7. Count the cell number and use fresh or freeze immediately in cell-freezing medium and store under liquid nitrogen until required (*see* **Note 23**).

3.3.2. Addition of Antigen and Cells

1. All test (e.g., UV-inactivated MVA; *see* **Note 24**) and control (e.g., PHA) antigens should be diluted to the appropriate concentrations in PBMC growth

medium and sufficient stocks frozen to enable multiple repeats of the assay (*see* **Note 25**).
2. Resuspend PBMCs to a concentration of 1×10^6/mL in PBMC growth medium and add 100 µL diluted PBMCs (1×10^5) to appropriate wells of a 96-well tissue culture plate.
3. Add 100 µL antigen or mitogen (PHA), each diluted in PBMC growth medium, to appropriate wells (each antigen/mitogen should be assayed at least in triplicate).
4. Incubate plates at 37°C, 5% CO_2 for 6 d.

3.3.3. Addition of ^{3}H-Thymidine and Measurement of the Incorporation of ^{3}H-Thymidine into Cellular DNA

1. Dilute stock ^{3}H-thymidine (1 mCi/mL) 1:20 in PBMC growth medium to a final concentration of 50 µCi/mL (2 mL is required per plate; *see* **Note 26**).
2. Add 20 µL ^{3}H-thymidine to each well of the microtiter plate (approx 1 µCi/well).
3. Return the plate to a humidified incubator at 37°C, 5% CO_2 for an additional 18 h overnight.
4. Harvest the cultures on to a glass fiber filter using a cell harvester (*see* **Note 27**) by first washing the filter with water, then adding the harvested cell lysates to the filter.
5. Place the dried filter mat into an appropriate carrier plate, add 15 µL scintillation fluid, and count in a micro-β-counter.

3.4. Measurement of MVA-Specific Cellular Responses by Interferon-γ (IFNγ) ELISPOT

ELISPOT is a highly sensitive assay that detects cytokines released from individual cells following antigenic stimulation of PBMCs in vitro (illustrated schematically in **Fig. 8**). The cytokine most frequently measured is IFNγ that can be secreted by $CD4^+$ (Th1) and $CD8^+$ (cytotoxic) T cells. Depending on the starting cells (whole PBMCs or those depleted of CD4 cells) and the antigen used for stimulation (whole protein, live or inactivated/killed virus, or peptides), the assay can be used to measure IFNγ secretion by either $CD4^+$ and/or $CD8^+$ T cells. In a healthy donor, the percentage of most antigen-specific T cells is usually below the detection limit of 1:100,000 PBMCs. However, during a viral infection or following vaccination, the percentage of antigen-specific T cells present in the periphery may increase dramatically. Thus, an increase in specific T-cell precursor frequency following immunization or infection would be indicative of a positive immune response induced by the vaccine/pathogen.

3.4.1. Preparation of ELISPOT Plates

1. Dilute the coating (capture) antibody (e.g., 1-D1K) to 15 µg/mL in sterile PBS.
2. Add 100 µL to each well of the 96-well ELISPOT plate.
3. Incubate overnight at 4°C in a humid environment.
4. Flick off 1-D1K antibody from the plate and wash each well six times with 100 µL RPMI (flick off each wash).

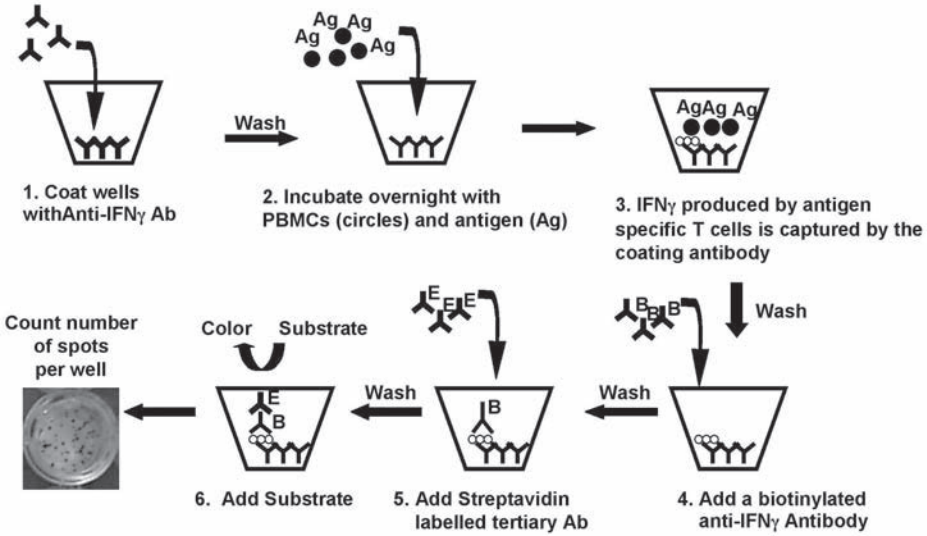

Fig. 8. Schematic representation of the ELISPOT assay. Wells of an ELISPOT plate are coated with the appropriate capture antibody (e.g., anti-IFNγ). Cells (black dots) and antigen (Ag) are subsequently cocultured in precoated wells at 37°C, 5% CO_2 for 16–20 h. If cells specific for the test antigen respond by secreting IFNγ (open circles), this is captured on the plate by the bound antibody (Ab). This "footprint" can be subsequently detected and is seen as a "spot" on the well. Each spot represents one antigen-specific cell.

5. Block nonspecific binding by the addition of 100 μL PBMC growth medium to each well.
6. Incubate at room temperature for 30–60 min.

3.4.2. Addition of Cells and Antigen

1. Count PBMCs and dilute to a concentration of 5×10^6 cells/mL in PBMC growth medium. If PBMCs are to be depleted of $CD4^+$ T cells, follow the protocol provided in **Subheading 3.4.4.**
2. Add 100 μL PBMCs to appropriate wells of the washed and blocked ELISPOT plate (5×10^5 cells/well; *see* **Note 28**).
3. Dilute appropriate positive controls (PHA to 10 μg/mL) and antigens (MVA to 10^7 pfu/mL). Where possible, use a range of antigen dilutions, e.g., MVA concentrations ranging from 10^7 to 10^4 pfu/mL (*see* **Note 29**). Untreated MVA should be used rather than UV-irradiated (*see* **Note 30**).
4. Add 100 μL-positive PHA and negative PBMC growth medium controls and antigen to appropriate wells; each should be assayed at least in triplicate.
5. Incubate for 16–20 h at 37°C, 5% CO_2. Do not move the plate during this incubation period.

3.4.3. Detection of IFNγ-Producing Cells

1. Remove ELISPOT plates from the incubator and aspirate off liquid from each well in the appropriate biosafety cabinet.
2. Wash each well six times with 100 µL wash buffer. Flick off contents each time.
3. Dilute biotinylated monoclonal antibody 7-B6-1 1:1000 from 1 mg/mL to 1 µg/mL in PBS.
4. Add 100 µL diluted antibody to each well.
5. Incubate at room temperature for 2–4 h.
6. Wash each well six times with 100 µL wash buffer. Flick off contents each time.
7. Dilute streptavidin–alkaline phosphatase secondary antibody 1:1000 in PBS and add 100 µL diluted antibody to each well.
8. Incubate at room temperature for 1–2 h.
9. Wash each well six times with 100 µL wash buffer. Flick off contents each time.
10. During the last wash step, prepare the alkaline phosphatase substrate (*see* **Subheading 2.5.**, **item 5**).
11. Flick off last wash from the plate and add 50 µL substrate to each well.
12. Leave for 15 min to 1 h or until spots have developed.
13. Stop the reaction by washing thoroughly with tap water. Air-dry the plate.
14. Read the number of spots per well using a dissecting microscope or, ideally, a dedicated ELISPOT plate reader.

3.4.4. Depletion of $CD4^+$ T Cells

Prior to use in cellular assays (e.g., ELISPOT), total PBMCs can be depleted of $CD4^+$ T cells to facilitate analysis of the CD8 arm of the immune response to specific antigens. Various methods are available to enrich, purify, or deplete different cellular subsets. One of the most rapid and simple techniques to deplete $CD4^+$ T cells is based on immunomagnetic cell isolation. The CD4-depleted PBMCs can be subsequently assayed by ELISPOT and compared with total PBMCs to demonstrate the component of the vaccinia virus-specific T-cell response that is mediated by $CD8^+$ T cells (*see* **Note 31**). **Figure 9** illustrates an ELISPOT assay used to investigate the MVA-specific $CD8^+$ T-cell response induced in a cancer patient following vaccination with a recombinant MVA vector at wk 0.

1. Wash the desired number of Dynabeads in dilution buffer according to the manufacturer's instructions.
2. Resuspend PBMC at 1×10^7 cells/mL in dilution buffer and incubate at 2–8°C.
3. Add the cells to prewashed Dynabeads.
4. Incubate for 30 min at 2–8°C on an apparatus that provides both gentle tilting and rotation.
5. Isolate the rosetted $CD4^+$ T cells by placing the tube into an appropriate magnet for 2–3 min.
6. Transfer CD4-depleted supernatant into a fresh tube, count the cells, and resuspend in PBMC growth medium at 2×10^6 cells/mL.

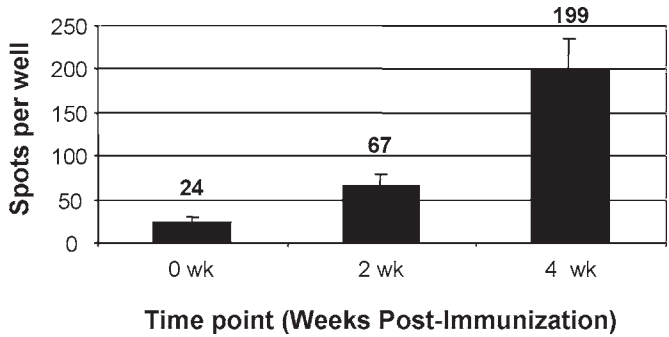

Fig. 9. ELISPOT analysis of the MVA-specific CD8 response of PBMC recovered from a colorectal cancer patient vaccinated with 1×10^8 pfu of a recombinant MVA vector at wk 0. This figure illustrates the numbers of spots (IFNγ-secreting cells) per 2×10^5 CD4-depleted PBMCs.

7. To measure $CD8^+$ T-cell-mediated vaccinia virus-specific response by ELISPOT, add 100 µL CD4-depleted PBMCs to appropriate wells of the washed and blocked ELISPOT plate (2×10^5 cells/well).
8. Proceed with the ELISPOT as detailed in **Subheading 3.4.2.**

4. Notes

4.1. ELISA

1. With the use of double-band purified virus for the assay, one will only detect antigens present in IMV and not EEV-specific envelope proteins. *See* **Subheading 3.2.** and Chapter 15 for further discussion of IMV and EEV.
2. Optimized for each viral preparation by performing a checkerboard ELISA as discussed in **Subheading 3.1.** and illustrated in **Fig. 1**.
3. Depending on laboratory containment level, it may be advisable to UV-irradiate the virus prior to setting up ELISA plates ("in-plate" fixation of the virus with formalin is an alternative). UV inactivation of MVA can be achieved using a DNA crosslinker. The MVA stock should be added to wells of a 96-well plate (100 µL/well) and placed in a DNA crosslinker for 5 min at 900,000 µJ/cm^2. Following irradiation, the inactivated virus can be pipetted out, diluted to an appropriate concentration (e.g., 10^8 pfu/mL in sterile PBS), aliquoted into sterile vials, and stored at –80°C until required. A plaque assay should be subsequently performed to ensure complete inactivation of the virus.
4. This blocking step helps prevent nonspecific binding of antibodies to the well.
5. Wherever possible, key assays used in the analysis of immunological responses to vaccinia virus should be fully validated and "fit for purpose." Assays should be accurate, specific, sensitive, and reproducible. Additionally, the analytical samples should be suitable for the specific assay. Care should be given to appropriate internal negative and positive controls for each assay (e.g., sera from known

vaccinia naïve and experienced donors as negative and positive controls, respectively, for ELISA and neutralization assays). Such controls can be used to set acceptable limits by which the assay can be accepted or discarded.

6. The negative control serum (normal human sera [NHS]) used should be made from a pool of at least five known vaccinia naïve donors.
7. If available, a positive-control serum sample of known titer should be included, which can be used to assess assay reliability.
8. Starting dilutions have to be determined empirically and may need to be altered from subject to subject.
9. See manufacturer's datasheet for recommended working concentrations of anti-human conjugate. Ten milliliters is required per plate.
10. The substrate should be prepared just prior to washing the plates. Because the OPD substrate is light-sensitive, dissolve in the dark or cover flask with foil. Ensure that the OPD substrate tablets are completely dissolved. If a small amount of particulate matter remains, strong false-positive color reactions can occur in wells.
11. We have defined MVA-specific antibody titers as the highest dilution that gives ≥2 fold higher OD than the NHS negative control at the same dilution.

4.2. Neutralization Assay

12. VERO and BSC1 cells are used for neutralization assays with DryVax™ and WR.
13. Volumes required will depend on assay size and the neutralizing titer of test sera.
14. To minimize the experimental variation between each neutralization assay, a master bank of diluted MVA virus should be prepared and used for all experiments where side-by-side comparisons are important.
15. Serum dilutions may vary between individuals, but we found 1:20, 1:100, and 1:500 to be a good working range.
16. As mentioned in **Note 12**, if DryVax™ or WR are used in the neutralization assays, these viruses form visible round plaques on cell monolayers. Thus, at 48 h postinfection, remove medium from each six-well plate and add 1 mL crystal violet stain (0.15% crystal violet, 8% formaldehyde, 5% ethanol) and incubate the cells at room temperature for 5 min. Aspirate off the crystal violet stain and let plates air-dry in flow cabinet. Plaques can be counted using a light box.
17. Each assay should include a titration of vSC56 at a multiplicity of infection (moi) of 0.125, 0.06, and 0.03 pfu/cell. This span of moi is to ensure linearity of viral dilutions and obtain control values for the assay. Most assays are run at an moi of 0.06 or 0.125 pfu/cell. β-gal values in the absence of inhibitors should be between 400 and 800 mU/mL.
18. Virus used in this assay are crude extracts released by three freeze–thaw cycles of infected HeLa cells. To break up virus clumps, the virus stock is treated with an equal volume of trypsin solution for 30 min at 37°C. The virus stock is then titered on BSC1 cells. Although virus clumps can be broken up by sonication, we have found that the trypsin treatment was more reliable and gave more reproducible virus titrations.

19. We typically perform four to six replicates per antibody dilution. We use FDA vaccinia immune globulin standard reference as a positive neutralization control. Pooled sera from vaccinia-naïve individuals, preimmune animal sera, and serial dilutions of 5% BSA solution are used as negative controls. We also include in each assay a series of virus titrations that do not contain any antibodies.
20. The enzymatic reactions are conducted in 96-well Immulon 2 plates.
21. Each assay plate contains β-gal standard at concentrations ranging from 18.75 down to 0.3 mU/mL (in duplicates).

4.3. Proliferation Assay

22. Alternatively, frozen vials of PBMCs should be thawed and allowed to recover following overnight culture in PBMC culture medium.
23. Yields of PBMCs/mL whole blood vary dramatically from donor to donor. However, in general, yields of 10^6 PBMCs/mL blood can be expected. PBMCs are frozen at a concentration 10^7 cells/mL in cell-freezing medium.
24. MVA should be UV-inactivated (*see* **Note 3**) prior to use in proliferation assays. If left untreated, viral gene products will result in the shutdown of host cell protein synthesis and ultimately cause cell death.
25. Ideally, sufficient single-use aliquots of antigen should be frozen to enable the experiment to be repeated at least once. However, where multiple time-points are being assayed, the quantity of antigen stocks should be determined by the researcher.
26. Adhere to local safety rules for the handling and disposal of ^{3}H-thymidine.
27. Ideally, use a cell harvester that is compatible with 96-well plates and can simultaneously aspirate as solutions are added.

4.4. ELISPOT

28. Whenever possible, key assays used in the analysis of immunological responses to vaccinia virus should be fully validated and "fit for purpose." Assays should be accurate, specific, sensitive, and reproducible. Additionally, the analytical samples should be suitable for the specific assay. For example, there are reports that PBMCs that are not processed and frozen within 24 h can show greatly reduced responses in ELISPOT *(17)*. Attention should be given to appropriate internal negative and positive controls for each assay. Such controls can be used to set acceptable limits by which the assay can be accepted or discarded.
29. We have tested MVA dilutions ranging from 10^7 to 10^5 pfu/mL in IFNγ ELISPOT assays and found little difference in the number of IFNγ producing cells detected. However, this should be determined empirically and may vary when whole PBMCs or those depleted of $CD4^+$ T cells are used.
30. For use in ELISPOT, untreated virus usually gives a stronger response (greater number of spots) than UV-inactivated. Presumably, following infection of antigen-presenting cells, viral antigens are presented primarily via the class I-restricted pathway and, to a lesser degree, by the class II pathway, thus mainly stimulating MVA-specific $CD8^+$ T cells. The use of UV-inactivated virus in this assay would result in responses mediated primarily by $CD4^+$ T cells.

31. To compare the MVA-specific cellular immune responses in $CD4^+$ and $CD8^+$ T cells, we compared the number of IFN-γ-producing cells in total PBMC vs CD4-depleted PBMC in response to untreated MVA. We demonstrated that the number of IFN-γ-producing cells was twofold higher in depleted CD4 when compared to untreated PBMCs.

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19

Vaccinia Virus as a Tool for Immunologic Studies

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Summary

Studies that involve antigen processing and presentation often require *de novo* biosynthesis of the antigen both in vitro and in vivo. Additionally, biosynthesis of the antigen or engineered variants within the antigen-presenting cells is usually simpler than providing purified recombinant proteins from bacteria, yeast, or insect cells. For these purposes, recombinant vaccinia virus-based expression has several advantages over other expression systems employed in the field. Insertion of large pieces of recombinant DNA into the vaccinia virus genome, easy recombination and selection of vaccinia viruses, and the ability of these viruses to infect a variety of cells are some key aspects that have made this system popular. Although their efficacy is proven in studies of major histocompatibility complex (MHC) class I-restricted antigen processing and presentation, it is challenging to use them in MHC class II-restricted antigen processing and presentation owing to many reasons specified in this chapter. This chapter aims to describe the commonly used procedures in this field that employ vaccinia virus systems, particularly troubleshooting common problems encountered during experiments.

Key Words: Recombinant vaccinia virus systems; MHC class I and class II pathways; antigen processing and presentation; in vitro and in vivo assays; procedures and troubleshooting.

1. Introduction

Vaccinia viruses have been used by laboratories to study processing of antigens and presentation on major histocompatibility complex (MHC) class I and class II molecules (e.g., **refs. *1–5***). The advantages of using a vaccinia expression system are: (1) recombinant viruses are easily made and produce high amounts of proteins under the control of various vaccinia promoters ***(6–9)***; (2) inserts of up to 25 kb can be placed into the vaccinia genome; (3) vaccinia virus has a very wide tropism, allowing for many different cell types to be used in in vitro experiments; (4) this wide tropism also allows for in vivo experiments

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in many animal models, from mouse to monkeys; and (5) vaccinia is a highly stable virus that can be stored at –80°C for an indefinite period of time. The vaccinia virus system has been demonstrated to be particularly useful in studying variations in antigenic structure, stability, cellular location, and expression levels ***(10)***. One limitation of this system stems from the cytopathic nature of the virus; however, this can be overcome by one of two ways. First, a less cytopathic version of the virus, such as modified vaccinia Ankara (MVA), can be used ***(11)***. Second, one can block the late phase of the viral life cycle with the drug cytosine β-D-arabinofuranoside (Ara-C), thereby preventing DNA replication and expression of late genes. This chapter describes the basic methodologies employed to study antigen processing and presentation through the use of recombinant vaccinia viruses. It should be emphasized that these techniques may have to be optimized for a particular set of experiments.

2. Materials

2.1. General

1. Tissue culture equipment: laminar flow hood, plates, flasks, incubators, and so on.
2. Cell culture media: Dulbecco's modified Eagle media (DMEM), RPMI 1640.
3. Fetal calf serum (FCS): both high-quality (e.g., Hyclone) and general use.
4. Assay media: RPMI 1640 media containing 5.5×10^{-5} *M* 2-mercaptoethanol (Gibco BRL), 10% high-quality FCS serum, 10 μg/mL gentamicin (Sigma).
5. Balanced salt solution (BSS)/bovine serum albumin (BSA): 0.15 *M* NaCl, 5 m*M* KCl, 1.22 m*M* $MgSO_4$, 1.3 m*M* K_2HPO_4, 0.74 m*M* KH_2PO_4, (mix K_2HPO_4 and KH_2PO_4 and adjust pH to 7.2), 10 m*M* HEPES, pH 7.2, 2.5 m*M* $CaCl_2$, 0.1% w/v BSA (Sigma; *see* **Note 1**).
6. Cup Sonicator (Branson Sonifier 450; *see* **Note 2**).
7. Tissue grinders (homogenizers, e.g., VWR, Thomas).
8. 70-μm Sterile cell strainers (Falcon).
9. Dissecting tools: scissors, forceps.
10. Microscope.

2.2. Chromium Release Assays, Priming Assays, and Limiting Dilution Analysis

1. Inbred mouse strains.
2. 0.5-mL insulin syringes.
3. MHC-matched target cells.
4. Alternative method of T-cell restimulation (other than vaccinia; *see* **Note 3**).
5. Recombinant mouse interleukin-2 (IL-2; Roche).
6. β2 Microglobulin (Scripps, San Diego, CA).
7. 96-well round-bottom plates.
8. ^{51}Cr as sodium chromate in a sodium chloride solution (Amersham).
9. Liquid scintillation counter or gamma counter.
10. 96-well plates for liquid scintillation counter.

11. Plastic tubes (for samples if using a gamma counter).
12. Sealing tape for plate (Fisher).
13. Scintillation fluid.
14. 15-mL conical tubes and rack.
15. Table-top centrifuge.
16. Infection medium: DMEM, 5% FCS.
17. Assay media: *See* **Subheading 2.1.**
18. Multichannel pipet.
19. Triton X-100 (Fisher).
20. Rotator for 15-mL conical tubes.
21. Gamma counter or beta plate reader.

2.3. Enzyme-Linked Immunospot (ELISPOT) Assay

1. ELISPOT plates, i.e., 96-well nitrocellulose-backed microtiter plates (Millipore).
2. Cytokine-specific primary antibody: purified rat anti-mouse interferon-γ (IFN-γ; Pharmingen).
3. Labeled secondary cytokine-specific antibody: biotinylated rat anti-mouse IFN-γ (Pharmingen).
4. Detecting antibody or protein: horseradish peroxidase avidin D (Vector).
5. Sigma Fast 3,3'-diaminobenzidine tablet set (DAB peroxidase substrate; Sigma).
6. Coating buffer: 0.015 *M* sodium borate, 0.15 *M* NaCl, pH 8.5.
7. Dilution buffer: phosphate-buffered saline (PBS) plus 1% BSA.
8. Wash buffer: PBS plus 0.25% Tween-20 (Sigma).
9. Blocking buffer: PBS plus 5% BSA.
10. ACK buffer: 0.15 *M* NH_4Cl, 1 m*M* $KHCO_3$, 0.1 m*M* Na_2EDTA, pH to 7.2–7.4 using 1 *N* HCl; filter-sterilize through a 0.2-μm filter and store at room temperature.
11. Assay media: *see* **Subheading 2.1.**
12. Cytokine-secreting cells.
13. Human recombinant IL-2 (National Institutes of Health [NIH] AIDS Research and Reagents Program).
14. Developing substrate: DAB peroxidase substrate (*see* **Note 4**).
15. Light microscope for counting spots or, alternatively, a digital camera and spot-counting software.

2.4. Tetramer Analysis

1. Tetramer: streptavidin-linked and fluorochrome-conjugated, phycoerythrin (PE), or allophycocyanin (APC; *see* **Note 5**).
2. ACK buffer: *see* **Subheading 2.3.**
3. Cell strainers.
4. 96-well U-bottom plate or FACS tubes.
5. Fc Block (Pharmingen).
6. FACS buffer: PBS, 1% FCS, 0.02% sodium azide.
7. Anti-CD8 antibody (Pharmingen).
8. 2% Paraformaldehyde (Electron Microscopy Sciences).
9. Flow cytometer.

2.5. Intracellular Cytokine Staining (ICC)

1. Phorbol myristate acetate (PMA; ICN).
2. Ionomycin (Sigma).
3. Brefeldin A (BFA; Sigma).
4. Alternate form of restimulation (i.e., peptide).
5. Fluorochrome-conjugated anticytokine antibody (e.g., anti-IFN-γ).
6. Isotype-matched negative control antibody.
7. Fluorochrome-conjugated anti-CD8 antibody.
8. Staining buffer: PBS, 3% heat-inactivated FCS, 0.09% sodium azide.
9. FACS buffer without azide: PBS, 1% FCS (*see* **Note 6**).
10. Cytofix/cytoperm solution (Pharmingen).
11. Perm/wash solution (Pharmingen).
12. Flow cytometer.

2.6. LacZ Assay

1. Tissue culture needs: Flat- and round-bottom 96-well plates, conical tubes, pipets, multi-channel pipets, and so forth).
2. APC and T-cell hybridomas.
3. Recombinant vaccinia viruses and synthetic peptides.
4. Potassium ferrocyanide and potassium ferricyanide.
5. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).
6. Fixative solution: 2% formaldehyde and 0.2% glutaraldehyde in water.
7. Substrate solution: 5 m*M* potassium ferrocyanide (100 μL from 0.5 *M* stock), 5 m*M* potassium ferricyanide (100 μL from 0.5 *M* stock, light-sensitive compound), 2 m*M* $MgCl_2$ (20 μL from 1 *M* $MgCl_2$), 1 mg/mL X-gal (1 mL from 10 mg/mL stock made in dimethylsulfoxide [DMSO]); bring up to 10 mL in PBS.
7. 4-Methyl umbelliferyl β-D-galactoside (MUG): 33 mg/mL MUG stock solution is made in DMSO and stored frozen at –20°C.
8. Fluorescent substrate solution: 100 m*M* 2-mercaptoethanol, 9 m*M* $MgCl_2$, 0.125% NP40, 3 m*M* MUG.
9. Stop solution: 300 m*M* glycine and 15 m*M* EDTA in water.
10. Plate reader, such as Victor 2 Wallach reader.

3. Methods

3.1. In Vitro Class I-Restricted Chromium Release Assay (See Notes 7 and 8)

Cytotoxic T-lymphocyte (CTL) assays are employed to measure specific immune responses. To do these experiments, MHC-matched (for the epitope of interest) cells are infected with the recombinant vaccinia viruses expressing the protein under study. These infected cells serve as target cells subsequently loaded with an indicator to measure CTL-specific cell lysis. A population of CTL specific for the epitope of interest can be generated by priming mice and restimulating CTLs in vitro or by using CTL cell lines. A limitation

of this assay is the time required from the beginning of viral infection of targets to the harvesting of culture supernatants. If performed efficiently, this assay will take approx 10–11 h. Time considerations must be observed because longer incubation times may result in greater spontaneous lysis and variable results owing to the cytopathic nature of vaccinia at the late stages of its life cycle.

1. Six to seven days prior to the day of the chromium release assay, CTLs should be harvested from mice of interest and the epitope-specific CTL population expanded by in vitro restimulation for 6–7 d. (For more detail on how to do this, *see* **Subheading 3.2.1.**)
2. Typically, 5×10^5 MHC-matched target cells are infected with 5–10 plaque-forming units (pfu)/cell of the recombinant vaccinia expressing the target protein of interest (vac; *see* **Note 9**). In a 15-mL conical tube, add the appropriate amount of virus to BSS/BSA for a final volume of 200 µL. Then, to the vac-containing tubes, add 200 µL suspension containing target cells in BSS/BSA. Set targets at a concentration of 2.5×10^6 cells/mL (*see* **Note 10**).
3. Place cells into a rotator at 37°C for 1 h. Make sure that the rotator is set in a position so that the tubes are nearly horizontal to prevent the cells from pelleting at the bottom. After 1 h, add 2 mL infection medium to the tubes and continue to incubate for an additional 3 h.
4. At any time during the infection, consider adding BFA at 5 µg/mL (*see* **Notes 11** and **12**).
5. Set up the effector population in 96-well plates. CTLs are plated in triplicate and four dilutions are set up for a total of four effector-to-target ratios (E:T) for each target to be tested (*see* **Note 13**). Using a multichannel pipettor, add 150 µL effector cells to the top row of the plates, and from these, remove 50 µL cell suspension and add it to the next row, which will already contain 100 µL assay media. Repeat this step for the two more rows, always starting from the row just above that into which the cells are added. This will result in 1:3 dilutions of the effector cells. Discard the 50 µL from the last row. Place the plate in a 37°C incubator at 5% CO_2 to await the addition of target cells (*see* **Note 14**).
6. Set up another plate to assess spontaneous and maximal ^{51}Cr release by target cells. For each target cell, add 100 µL assay media to five wells and 100 µL 1% Triton X-100 (in PBS) solution to another five wells. Place plate(s) into a 37°C incubator at 5% CO_2 to await the addition of target cells (*see* **Note 14**).
7. After the infection of the target cells is complete (4 h total, **step 2**), pellet the infected cells by centrifugation at 1000*g* for 5 min. Remove as much of the media as possible (*see* **Note 15**).
8. Target cells (*see* **Note 16**) will be labeled in a total volume of 25 µL containing 50 µCi/5×10^5 cells of ^{51}Cr diluted in infection medium. The cells can be resuspended in the ^{51}Cr by pipetting up and down with a p200 pipet. Place tubes back in the rotator for 1 h.
9. After labeling, pellet cells as previously done and wash two times with 3 mL cold PBS for each wash. It would be useful to remove the first supernatant (containing

the high-activity ^{51}Cr) by pipetting with a p200 and placing it into a shielded container. The wash supernatants contain relatively low amounts of radioactivity and may therefore be decanted into a low-activity waste receptacle (according to institutional guidelines; *see* **Note 14**).
10. Resuspend the cells with the appropriate amount of assay medium. For example, when using only one effector population, 2.7 mL assay medium is added to each target cell population, which allows for the addition of 100 µL target cell suspension to the wells containing effector cells and the wells containing medium and Triton X-100 (*see* **Note 17**).
11. Incubate cells in 37°C incubator at 5% CO_2 for 4 h.
12. Carefully remove plates after 4-h incubation not to disturb the cell pellet at the bottom of each well. Transfer 50 µL supernatant to a new 96-well plate (specific for gamma counter) that already contains 50 µL scintillation fluid (*see* **Note 18**).
13. Calculate percentage lysis by: percentage lysis = [(experimental–spontaneous)/(maximal–spontaneous)] × 100. The spontaneous lysis value is obtained by counting counts per minute (cpm) from the target cells added to media in the absence of CTL, and the maximal lysis value is obtained from target cells added to Triton X-100.

3.2. In Vivo MHC Class I-Restricted Assays

Using vaccinia priming as a starting point, it is relatively easy to determine CD8+ T-cell responses in vivo. The number of epitope-specific T cells generated can be determined by a number of different assays. Activated, epitope-specific CD8+ T cells can be quantitated using LDA ***(12,13)***, IFN-γ ELISPOT ***(14)***, staining with MHC-I/peptide tetramers ***(15,16)***, and intracellular cytokine staining ***(16)***.

3.2.1. Priming Assay

1. Dilute recombinant vaccinia viruses into BSS/BSA at a final volume of 200 µL. Usually vaccinia from 10^5 to 10^7 pfu is sufficient (*see* **Note 19**).
2. Prime mice intraperitoneally (ip) or intravenously (iv) using insulin syringes fitted with a 28-gauge needle (*see* **Notes 20** and **21**).
3. After a minimum of 2-wk infection, mice are sacrificed and the spleens are removed.
4. The spleen is homogenized using a tissue grinder in 7 mL assay medium and the resulting cells are pelleted by spinning at 1000*g* for 5 min. Do all subsequent steps on ice.
5. Lyse red blood cells by adding 1 mL ACK buffer and incubate on ice for exactly 1 min. After 1 min, immediately add 30 mL PBS.
6. Pellet cells at 1000*g* for 5 min at 4°C. Wash 1X with PBS.
7. Resuspend cells in assay medium and pour through a cell strainer back into assay medium.
8. Count cells and set at appropriate concentration (usually 10^7 cells/mL).

9. In vitro restimulation is likely required, as primary ex vivo CTL activity is not typically robust.
10. To restimulate, using a second method of epitope presentation is required, which serves to avoid expansion of vaccinia-specific responses that could mask responses to the desired epitope (*see* **Note 22**). A protocol for restimulation using peptide follows.
11. Count splenocytes. If comparing different spleen populations, keep the total number of cells the same between populations.
12. For each priming condition, two-thirds of the total spleen cells is used to generate epitope-specific CTL, and one-third is used to assess vaccinia-specific priming.
13. Naïve spleen cells from uninfected mice are used as the restimulators for vac-specific responses and is added at a 1:3 ratio to responders. For vac-specific responders, infect the appropriate number of restimulators with 3–5 pfu of wild-type vaccinia virus in a total volume of 400 µL BSS/BSA by rotating the cells for 1 h at 37°C.
14. After incubation, pellet cells by washing once with BSS/BSA, resuspend in the appropriate volume of assay media, and add to responder cells. Keep the vac-specific CTLs in a T-25 flask in a final volume of 12 mL.
15. For the epitope-specific CTL populations, pulse 10^{-8} to 10^{-7} *M* peptide and 33 µg/mL β2-microglobulin onto irradiated MHC-matched cells, and rotate at 37°C for 1 h. Wash peptide-loaded cells once with BSS/BSA and add them to the CTL cultures at a ratio of 10:1 (CTL: restimulators; *see* **Note 23**).
16. Restimulation is for 6–7 d. Generally, the best CTL activity is on d 6. D 7 is slightly worse, and after d 7, there is a sharp drop-off of CTL activity (*see* **Note 24**).
17. CTL activity can be assessed via chromium release assay (*see* **Subheading 3.1.**).

3.2.2. Limiting Dilution Assay (LDA)

LDA can be used to estimate the proportion of specific T cells in a mixture that can respond to an activation stimulus. Other assays that measure the activation of T cells do not provide information regarding the actual number of responding cells. However, LDA has its limitations, underestimating the true frequency of antigen-specific precursors by 5–100-fold ***(16–18)***. But LDA data about kinetics, duration, and the relationship between responses are equivalent to that from assays that employ MHC tetramers and intracellular cytokine staining ***(18)***. LDA can assess the cytolytic capacity of CTL precursors (CTLp) whose frequency may be at the lower limit of detection for flow cytometric techniques. A typical LDA assay is performed as follows.

1. Prime mice and prepare spleen cells as described in **Subheading 3.2.1.**
2. Infect APC with recombinant vaccinia viruses (*see* **Note 25**) or pulse with synthetic peptides and irradiate them.
3. Make 4–8 dilutions (24 or more replicates each) of the responder populations and plate them out in 96-well V-bottom plates (100 µL/well; *see* **Note 26**).

4. Spin and wash APC and resuspend in assay medium at a density of 2.5–5 × 10^6/mL with 2X IL-2 (usually 10–40 U/mL, but this should be determined experimentally), and add to plates with responders in a total of 200 μL/well.
5. After 6–7 d, wells can be tested for cytotoxic activity against virus-infected or peptide-pulsed cells. Each well will be split two ways to assay for epitope-specific (or vaccinia-specific) lysis and background lysis against unpulsed (or uninfected) targets. Label MHC-matched peptide-pulsed or MVA-infected targets with ^{51}Cr (100 μCi/10^6 cells in 50 μL) and plate 10^4 targets/well.
6. Transfer effector cells to a 96-well round-bottom plate.
7. Wash labeled cells as described earlier and plate them into wells containing the appropriate populations of effectors (*see* **Note 27**). Incubate plates at 37°C for 7–8 h to allow detectable lysis even by a low number of effectors from the microtiter restimulation.
8. Complete CTL assay as described in **Subheading 3.1.** and calculate percentage lysis for each well.
9. Positive wells are identified as those that cause lysis of the appropriate targets (virus-infected or peptide-pulsed) three standard deviations above the mean lysis of the control targets (unpulsed or uninfected) for each group. Precursor frequencies can be calculated by linear regression analysis using the cut-off of 37% of negative wells to give an estimate of frequencies or the X^2 method described by Taswell ***(19)***.

3.2.3. ELISPOT Assay

An advantage of ELISPOT assays over some of the above mentioned in vivo assays is that the precursor frequency of T cells can actually be quite low, and ELISPOT can be used to quantitate smaller populations of CTL ***(16)***. Tetramer assays and intracellular cytokine staining (ICS) may require somewhat larger CTL precursor populations. It should be noted that ELISPOT assay works best for CTL populations taken from a mouse at least 14-d postvaccinia priming. CTLs collected prior to this time tend to produce a high number of nonspecific IFN-γ spots for reasons that are not entirely understood.

1. Prime mice and prepare spleen cells as described in **Subheading 3.2.1.**
2. On the day before harvesting the spleens, coat the wells of the 96-well ELISPOT plate with the anticytokine antibody (usually 10–40 μg/mL in freshly prepared coating buffer). Use 50 μL coating solution in each well. Wrap the plate in parafilm and incubate at room temperature for 2 h (*see* **Note 28**).
3. After 2 h, remove the coating buffer and wash the plates three times with wash buffer. Use 200 μL wash buffer in each well (*see* **Note 29**).
4. Block wells overnight at 4°C with 200 μL blocking buffer per well. Wrap plates in parafilm to avoid evaporation.
5. On the following day, discard blocking buffer. Wash plates three times with wash buffer, 1X with PBS (*see* **Note 30**), and allow to dry completely. Add 100 μL assay media to cells and place into a 37°C incubator at 5% CO_2.

6. Remove spleens, homogenize as described in **Subheading 3.2.1.**, **step 4**, and pellet the cells by centrifugation at 1000*g* for 5 min. Keep cells on ice for this and subsequent steps.
7. Lyse red blood cells by adding 1 mL ACK buffer and leaving on ice for 1 min. Immediately after 1 min, add 30 mL sterile PBS. Pellet cells and resuspend in 5 mL assay medium and count cells.
8. Harvest MHC-matched stimulator cells from naïve mice. These cells can be infected with a recombinant vaccinia virus expressing the required epitope (*see* **Note 31**) or pulsed with peptide (*see* **Note 32**). Whether peptide pulsing or vaccinia infecting, do this at 37°C for 1 h.
9. Consider irradiating stimulator cells (*see* **Note 33**).
10. Wash and resuspend stimulator cells in assay medium containing 40 U IL-2. The final IL-2 concentration should be 20 U/mL after the addition of the spleen cells. A good starting point is 5×10^6 cells/mL (2.5×10^5 cells/50 μL). Remove assay medium from plates in the incubator and add 50 μL cell suspension into each well (*see* **Note 34**).
11. Set the concentration of the spleen cells for the appropriate dilutions. For epitope-specific responses, 5×10^6/mL (2.5×10^5/50 μL) is a good starting concentration, and, for vaccinia-specific responses, 5×10^4/50 μL is a good starting point. At least two fivefold dilutions should be done for each spleen population. Add 50 μL to wells that already contain stimulator cells.
12. Incubate cells at 37°C for 18–24 h.
13. After incubation wash cells nine times with wash solution and dry.
14. Add secondary biotinylated cytokine-specific antibody (4 μg/mL in 50 μL) and incubate at room temperature for 2 h.
15. Wash plate six times with wash solution and dry.
16. Add horseradish peroxidase (HRP)–avidin (use 10 μg/mL in 50 μL) and incubate at room temperature for 2 h.
17. Wash plate five times with wash solution and once with water; allow to dry.
18. Add developing substrate. Once spots have developed, wash plates with water to stop the reaction and allow plates to dry.
19. Count spots and determine CTL frequencies (*see* **Note 35**).

3.2.4. Tetramer Staining

Tetramer analysis is a very convenient method to determine the number of epitope-specific CTLs that arise after an animal is primed with a specific virus. Although it should be mentioned, in some cases, not all tetramer-positive cells appear to be functional ***(20,21)***. Therefore, it is worth measuring cytolytic ability, as well as cytokine secretion of the tetramer-positive population, to achieve a full understanding of these cells.

1. Prime mice and prepare spleen cells as described in **Subheading 3.2.1.** and dilute cells to an appropriate concentration (usually 10^7 cells/mL).
2. Plate 10^6 cells/well of a 96-well V-bottom plate. Spin plate (as above) and remove supernatants.

3. Resuspend cells in 50 μL/well of Fc block (*see* **Note 36**).
4. Incubate for 15 min either on ice or at 4°C.
5. Wash cells one time with FACS buffer.
6. After washing, resuspend pellet in 50 μL PBS containing diluted tetramer (*see* **Note 37**). Do all subsequent steps by keeping cells in the dark.
7. Incubate either at room temperature or on ice for 30 min (*see* **Note 38**).
8. Wash cells one time with FACS buffer.
9. Add other surface-marker antibodies diluted in FACS buffer (*see* **Note 39**).
10. Incubate on ice for 30 min.
11. Wash one time and resuspend in 200 μL 2% paraformaldehyde (PFA).
12. Run samples through a flow cytometer.

3.2.5. Intracellular Cytokine Staining (ICS)

For intracellular cytokine staining, an excellent protocol is available on the Pharmingen website (www.pharmingen.com) and is summarized for use with vaccinia systems here.

1. Prime mice and prepare spleen cells as described in **Subheading 3.2.1.** and dilute cells to an appropriate concentration (usually 10^7 cells/mL).
2. Samples should be set up for stimulation to have a set of cells for experimental, positive, and negative controls (*see* **Note 40**).
3. The experimental set of cells should be pulsed with 10^{-10} to 10^{-6} *M* of appropriate peptide for 4–6 h at 37°C (*see* **Note 41**). For the positive control, stimulate with 50 ng/mL PMA and 700 ng/mL ionomycin for 4 h at 37°C. Lastly, for the negative control, use an irrelevant peptide or perform a mock stimulation without any peptide present. 10 μg/mL BFA must be added to each set of conditions for the last 3-h stimulation.
4. Count cells and place 10^6 cells/well in a 96-well round-bottom plate.
5. Once all sets of cells have been stimulated, staining for a particular cytokine can proceed.
6. Cells should be incubated in a total of 50 μL staining buffer with a predetermined optimal concentration of a fluorochrome-conjugated cell-surface monoclonal antibody, such as anti-CD8.
7. Staining should take place for 20–30 min at 4°C, keeping the samples in the dark.
8. After the incubation is complete, wash the cells two times with FACS buffer without azide, pellet cells by centrifugation at approx 1000*g*, and remove supernatant.
9. Fix and permeabilize cells by thoroughly resuspending them in 100 μL cytofix/cytoperm solution and incubating on ice for 10–20 min in the dark.
10. Wash cells two times with perm/wash solution, pellet, and remove supernatant.
11. Resuspend cells in the perm/wash solution containing either the anticytokine antibody or the isotype-matched antibody (negative control) at the proper concentration.
12. Incubate for 30 min on ice in the dark.
13. After incubation wash cells two times with perm/wash solution.

14. Wash cells one time with FACS buffer without azide.
15. Fix cells with 2% PFA. Add 100 μL/well.
16. Analyze by flow cytometry.

3.3. MHC Class II-Restricted T-Cell Responses

Analysis of MHC class II-restricted T-cell responses that employ recombinant vaccinia viruses is often more challenging than MHC class I-restricted T-cell responses for the following reasons. (1) MHC class II-restricted responses are generally studied using traditional methods such as in vitro proliferation assays and quantitation of IL-2 released by activated T cells or T-cell hybridomas. As these assays require lengthy in vitro culture periods (usually 2–5 d), the use of cytopathic vaccinia viruses in such assays leads to major complications. Such problems can be alleviated if vaccinia viruses are rendered replication-incompetent, which can be achieved by psoralen-ultraviolet (UV) treatment of vaccinia viruses ***(22,23)***. Alternatively, less cytopathic MVA ***(11)*** can be used in these assays. We have not tested these possibilities in our laboratory, but prefer to employ assays of shorter duration to evaluate MHC class II-restricted responses. In vitro ^{51}Cr-release assays can be performed if MHC class II-restricted cytotoxic T-cell clones are available. Perhaps the most convenient method is to utilize T-cell hybridomas that express β-galactosidase following antigen-driven stimulation, a method devised by Shastri and coworkers ***(24)***. (2) Additionally, it may be essential to use purified vaccinia viruses while assaying MHC class II-restricted responses. Crude preparations of vaccinia viruses may contain contaminating exogenous antigens that complicate the interpretation of experimental results. Vaccinia viruses can be purified using sucrose density gradients, which use standard methods as described in Chapters 8 and 15 and **ref. *25***.

Owing to their cytopathic nature and their probable effect on integrity of intracellular structures following the infection of target cells, vaccinia viruses had been considered unsuitable for targeting antigens to different compartments within the APC. However, we and others have successfully targeted antigens to distinct compartments within the APC and used them in MHC class I- and class II-restricted assays ***(10,26–28)***. Furthermore, assessing the components required for the presentation of an epitope is an important area in MHC class II-mediated antigen processing and presentation. Nonclassical APC that do not normally express components, such as H2-M and invariant chain essential for MHC class II-restricted presentation, can be infected with recombinant vaccinia viruses expressing such molecules, and their requirement for the presentation of epitopes can be assessed. We have coinfected fibroblasts with recombinant vaccinia viruses expressing α and β chains of MHC and found that such cells are able to present antigens to T-cell hybridomas (unpublished

results from our laboratory). Recently, cells expressing Cd1d molecules from vaccinia viruses have been shown to present lipid antigens ***(29)***. Thus, vaccinia viruses may be used to express components of antigen processing and presentation, which may be much more efficient than transient transfections, as vaccinia viruses infect a wide variety of cells, and the expression of recombinant proteins can be quickly assessed.

Unlike priming mice with vaccinia viruses to study CD8 responses, we prefer not to use vaccinia viruses to study $CD4^+$ T cell responses because of several limitations just described. However, several groups have used modified immunization protocols to generate in vivo $CD4^+$ T-cell responses. Subcutaneous or footpad immunizations with vaccinia viruses emulsified in Freund's adjuvant has been reported to generate efficient $CD4^+$ responses ***(26)***. Antigens fused with an endosome-sorting signal from the LAMP protein ***(26)*** and invariant chain ***(27)*** have been shown to prime $CD4^+$ T cells efficiently in vivo following immunization with recombinant vaccinia viruses. Hence, it may be necessary to modify antigens and/or employ modified priming protocols that may prove useful in generating successful in vivo $CD4^+$ T-cell responses.

3.3.1. Cytotoxic T-Cell Assays

Primary $CD4^+$ T-cell cultures generally have a lower cytolytic capacity than $CD8^+$ T cells as a result of reduced perforin expression, as well as lower precursor frequency of Fas ligand-expressing cells ***(30)***. To have measurable cytolysis, it is essential to use target cells that abundantly express Fas. Although longer assay periods may allow for the upregulation of Fas, again, the cytopathic nature of vaccinia viruses may pose problems. (As Fas expression is necessary for CTL-mediated killing, it may be useful to sort high Fas-expressing cells by flow cytometry and employing them as targets in CTL assays ***(30)***. Thus, assays are done in a similar manner as MHC class I-restricted CTL assays, with the exception that coincubation of effector and target cells is longer to allow Fas-mediated killing, but it is sufficiently shorter than conventional MHC class II-restricted assays, which avoids the nonspecific lysis of target cells by vaccinia viruses. MHC class II-restricted CTL assays are performed as follows:

1. Target cells at 2×10^7 cells/mL density are infected with vaccinia virus (5 pfu/cell) for 1 h at 37°C in BSS/BSA in 15-mL conical tubes.
2. Spin down the cells, resuspend in 5 mL assay medium, and incubate for an additional 4 h at 37°C.
3. Load cells with ^{51}Cr as described in **Subheading 3.1.**
4. Mix 1×10^4 radiolabeled cells and effector cells at desired E:T ratios in 96-well round-bottom plates.
5. Incubate overnight at 37°C and harvest supernatant. The percentage of specific lysis is calculated as described in **Subheading 3.1.**, **step 13**.

3.3.2. LacZ-Inducible T-Cell Hybridomas

T-cell hybridomas that express β-galactosidase following antigen-driven stimulation are possibly the most convenient system to assess MHC class I- and class II-restricted responses. Splenocytes or T-cell clones are fused with the BWZ.36 cell line stably transfected with the nuclear factor-activated T cells (NFAT) enhancer element-lacZ construct. Thus, β-galactosidase is specifically produced in response to stimulation by antigen/MHC class II complexes ***(24)***. However, recombinant vaccinia viruses with the standard β-galactosidase selection markers cannot be used in conjunction with lacZ-inducible T-cell hybridomas because of the high levels of background from vaccinia-infected APC. Ara-C, a compound that inhibits vaccinia DNA synthesis, can be used to block β-galactosidase expression if the β-galactosidase is driven by a late-vac promoter. However, we obtain highly variable results using this compound; thus, we recommend the generation of recombinant vaccinia viruses using alternate selection markers, such as β-glucuronidase, which does not cleave β-galactosidase substrates ***(11,31)***.

Although these T-cell hybridomas are easy to use, for unknown reasons, they are highly unstable. Some are more stable than others, perhaps due to the nature of the T-cell clones used in the fusion procedure. Recloning several times by limiting dilution may be necessary before stable and reliable T-cell hybridomas can be developed. Once a stable line is established, cells can be maintained in the assay medium without any selection pressure.

There are two widely used methods for detecting and measuring T-cell hybridoma activation using insoluble and soluble substrates for β-galactosidase. Assays are set up as described below.

1. Infect APC with recombinant vaccinia viruses at 5 pfu/cell in BSS/BSA for 1 h at 37°C.
2. Wash cells once with assay medium and plate 1×10^5 cells in 100 μL assay medium in a 96-well flat-bottom plate.
3. Add 1×10^5 T-cell hybridoma in 100 μL assay medium (*see* **Note 42**).
4. Incubate plate at 37°C overnight.
5. Wash cells once with PBS.
6. Add substrate using protocols from either **Subheading 3.3.2.1.** or **Subheading 3.3.2.2.**

3.3.2.1. Detection of Activated T-Cell Hybridomas by Microscopic Enumeration

Activated T-cell hybridomas can be treated with an insoluble substrate [fluorescein di-β-galactoside (FDG) or X-gal] that is then used as an indicator substrate by β-galactosidase expressed in activated cells. As a result, activated T-cell hybridomas turn blue, which can be quantitated microscopically.

1. After overnight incubation of T-cell hybridomas with APC, wash cells once with PBS.
2. Fix cells with 50 µL ice-cold fixative solution for 5 min at 4°C.
3. Wash cells once with PBS.
4. Add 50 µL substrate solution.
5. Incubate the plate overnight at 37°C and quantitate blue cells microscopically (*see* **Note 43**).

3.3.2.2. Quantitation of Activated T-Cell Hybridomas Using Fluorescent Substrates

Quantitation of activated T-cell hybridomas using fluorescent substrates is preferred over the previous method of microscopic enumeration because it is quick, simple, and avoids potential bias in counting. Various substrates can be used to measure T-cell hybridoma activation. We use MUG in our efforts to quantitate T-cell hybridoma activation (*see* **Note 44**).

1. Instead of fixing the cells as in the previous method, cells are washed once with PBS and lysed with 100 µL fluorescent substrate solution and incubated at 37°C for 4 h.
2. Stop enzymatic reaction by adding 50 µL stop solution.
3. Transfer 50 µL to a round-bottom plate and measure fluorescence emission using a plate reader (360 nm excitation; 460 nm emission; *see* **Note 45**).

4. Notes

1. PBS + 0.1% BSA may be substituted for BSS/BSA.
2. Because of safety concerns, when sonicating vaccinia viruses, a cup sonicator is safer because a probe sonicator will aerosolize the virus.
3. If there is not an efficient alternate method for restimulation, try to restimulate with vaccinia, but generally this produces a high vac-specific response, which masks any epitope-specific response being assayed.
4. Some substrates, such as DAB, do not dissolve completely; therefore, the solution should be filtered through a 0.22-µm filter to avoid background spots on the nitrocellulose.
5. Currently, tetramers can be obtained from the NIAID Tetramer Facility, Emory University Vaccine Center at Yerkes, or purchased from Beckman Coulter Inc.
6. FACS buffer for ICS (**Subheading 3.2.5.**) does not contain sodium azide, as it is toxic to the cells.
7. Over the years, our laboratory has tested several nonradioactive methods for measuring cytolysis. Thus far, no method has performed as well as the chromium release assay.
8. Although chromium release assays have been exploited by many laboratories to explore questions in antigen processing and presentation, other assays can also be employed to this end. One such assay is the use of T-cell hybridomas transfected with *lacZ*, which offers an alternative read-out to chromium release. The *lacZ*-transfected T-cell hybridomas engineered in the Shastri laboratory

(Berkeley, CA; ***24***) are a good example of such cells, and the use of this system is described in **Subheading 3.3.2.** *LacZ*-based assays for class I-restricted responses are identical to those described for class II assays.

9. If MHC-matched target cells are not readily available, one can coinfect with a vac expressing the appropriate MHC element. If infecting with more than one recombinant vaccinia, try to limit the total moi to 15 pfu/cell.
10. For convenience, it may be beneficial to set up the tubes containing vaccinia in BSS/BSA the night before the assay, then keep the tubes at 4°C until the next morning, at which time target cells can be added to begin the infection.
11. Vaccinia infection of targets can also be used to study aspects of protein processing and presentation following infection with a recombinant vaccinia virus. Using this method, assessment can be made of the impact of targeting a protein of interest to different cellular compartments, making mutations within a protein of interest, and by altering the vaccinia promoter strength driving the foreign gene, effects of the protein expression level on antigen processing and presentation can be examined. Additionally, the assay allows the investigation of the pathways and proteases required for the production of particular antigens. BFA is added to the cells to inhibit transport of MHC class I/peptide complexes to the cell surface ***(32)***. Using BFA is an easy way to gauge the efficiency of presentation in cases where maximal presentation of epitopes is reached at relatively early time-points in the course of vaccinia infection. For rapidly processed epitopes, considerable differences in presentation efficiency may be masked without the addition of BFA simply because the rate of presentation of all epitopes is so rapid as to reach maximal levels by the time CTL are added to the targets. The appropriate time to add BFA needs to be determined experimentally. In our experience, 110-min postinfection is a good starting point. In cases where agents should be added to the target cells potentially harmful to T cells (e.g., protease inhibitors), addition of BFA following ^{51}Cr labeling and washing can obviate the necessity to have the inhibitor present during coincubation with T cells. Lastly, another area in which use of BFA may be useful is in ensuring equal infection of target cells. Use of vac-specific CTL to confirm equal infection is complicated by the magnitude of the anti-vac response, which often results in similar killing even at different E:T ratios. Addition of BFA at various times postinfection may limit presentation, such that a titration of specific lysis is observed with different E:T ratios.
12. BFA loses its potency over a period of weeks while stored at –80°C.
13. While we do four E:T ratios, some laboratories do as many as eight.
14. When target cells are being added to the 96-well plates, it is best to add cells to the TX-100-containing wells last to avoid any splashing of the detergent. Even an imperceptible amount of TX-100 on the tip of a pipet yields high lysis in the next wells plated out. When setting up the plates that include the TX-100 samples, it is helpful to keep the TX-100 wells separated from the assay media-containing wells. We do this by establishing a two-row gap between our spontaneous and maximal lysis groups.

15. The total volume in which the cells are resuspended for ^{51}Cr-labeling has an impact on the efficiency of labeling. Therefore, it is important to keep the volume equal from target to target. Carefully suctioning all of the liquid from the last wash (just prior to addition of ^{51}Cr) is critical for achieving equivalent volumes. It is often necessary to suction each tube a second time to allow PBS on the walls of the tube to flow to the bottom.
16. It is possible to count target cells before plating out to adjust them to the proper cell density. This is likely unnecessary if all the tubes have been handled similarly. In addition, the total amount of chromium released (lysis by TX-100) indicates whether the cells per well for each target is equivalent. The calculation of E:T ratio is actually arbitrary when using bulk populations of CTL from the spleen because the number of true epitope-specific cells is unknown. One exception is with the comparison of infected to uninfected targets; in this case, cell loss during washes may occur to differing degrees due to early cytopathic effects of vac infection. If so, it is necessary to count target cells before plating out.
17. At this point, a multidosing pipet can be used, but be sure to rinse the pipet with PBS between additions of targets to avoid crosscontamination. Do not use water to rinse tips, as this results in cell lysis.
18. Some scintillation fluids kill vaccinia virus, but check the scintillation fluid being used. In our laboratory, we use Optiphase (Wallac), which has shown to be effective in killing the vaccinia used in the assays. Be sure to wash the multichannel pipet tips with water between effector dilutions to not cross-contaminate samples. Seal the plates with tape and shake at a low speed for 20 min after mixing read plates in counter. If using a conventional gamma counter, the protocol is modified such that 100 μL supernatant should be transferred to the appropriate tubes and chased with 100 μL 10% bleach for counting.
19. To evaluate relevant differences in priming capacities of recombinant vaccinia viruses, do a series of dilutions for each virus.
20. Mice can be primed either (ip) or (iv). For chromium release assays, ip priming is usually sufficient. But for assays where priming efficiency must be equivalent between mice (i.e., in vivo assays), we generally obtain more consistent results when the mice are primed iv through the tail vein.
21. It is beneficial to use the smallest volume possible for injection and also a small-gauge needle. Insulin syringes work well for this purpose. These requirements help to reduce any reflux of the inoculum at the injection site. Additionally, primed mice should be caged separately from unprimed mice. This helps prevent crosscontamination that may result from virus leakage or shedding (although the latter will be minimal with vaccinia).
22. One method is the use of a synthetic peptide pulsed onto irradiated MHC-matched-presenting cells. Cells can be irradiated with 10,000 cGy in a cesium source. Another method is the addition of 1 μg/mL peptide directly to harvested splenocyte cultures for 6 d. Alternatively, express the antigen via a heterologous viral system (e.g., influenza or adenovirus) to infect and present antigen.

23. The optimal concentration of peptide-pulsed onto the restimulator cells needs to be determined. Peptide-pulsed restimulators can be substituted with transfected cells (after irradiation) at a ratio of 1:25 (transfectant:CTL).
24. If it is desirable to keep CTL populations longer than 1 wk, the addition of 20–40 U/mL IL-2 may allow the CTL to survive another 6–7 d. This has not generally proven to produce high-activity CTL, but may be helpful in some instances.
25. The CPE of vaccinia virus may affect the restimulation of low numbers of CTLp in microtiter wells. To avoid these issues, MVA can be used ***(11,33)***.
26. V-bottom plates are preferable to round-bottom plates in LDA, as cell-to-cell contact between the APC and T cells is better in V-bottom plates.
27. Remember to include appropriate controls for measuring spontaneous and total lysis.
28. All antibody concentrations should be optimized for the particular antibody being used.
29. Some protocols recommend completely drying nitrocellulose by removing the backs of the plates and allowing time for the wells to dry completely between washes. We have found this to be unnecessary and can be omitted. However, we do completely dry the wells before the addition of cells, antibodies, or detecting reagents.
30. Be sure to remove all Tween-20.
31. If using vaccinia virus to infect stimulator cells, include a wild-type vaccinia virus to determine any cytokine secretion by the CTL that is vaccinia-specific. This method is useful because it allows the assessment of the priming level for each animal used in the assay. Corrections for uneven priming can be made once the primary data have been obtained ***(34)***. For infections, 5–10 pfu/cell is sufficient.
32. Optimal concentrations for peptide pulsing should be determined, but range from 10^{-11} to 10^{-7}.
33. For some cell types, it might be useful to irradiate the stimulator cells prior to peptide pulsing. The irradiation step inhibits growth of the stimulator cells while being cocultured with the CTL population. We have determined that for assays using L cells as stimulators, the irradiation step can be skipped.
34. The optimal density of stimulators per well must be determined experimentally for each system.
35. This process can be automated. There are complete ELISPOT-counting setups that are commercially available. The AID ELISPOT reader system from Autoimmun DiagnostikaGmbH (Germany) and AELVIS GmbH (Germany) soft- and hardware for ELISPOT analysis are some examples. AELVIS GmbH (Germany) offers an ELISPOT analysis service worldwide for complete analysis of ELISOPT plates.
36. In place of Pharmingen Fc Block reagent, one can use 2.4G2A hybridoma supernatant (contains MAbs against Fcγ receptor) and normal goat serum at a 2.5:1 ratio.
37. The optimal concentration of tetramer must be determined for each preparation of tetramer used.

38. Incubation temperature must be determined experimentally for a particular assay. Also, as a reminder, sodium azide cannot be used if incubating at room temperature.
39. Antibody concentration must be optimized. One should remember to at least include an anti-CD8.
40. For these experiments, several in vitro methods for stimulating cytokine production have been described. Common methods include the use of phorbol esters (PMA) and calcium ionophores. As for a negative control, activated cells should be stained with an irrelevant isotype-matched antibody. Also of note, it has been reported that cell activation with PMA alone causes a transient loss of CD4 expression from the surface of mouse T cells ***(35)***. Additionally, cell activation with PMA and a calcium ionophore together can cause a greater and more sustained decrease in CD4 and, to a lesser extent, CD8 from the surface of mouse thymocytes and mouse and human peripheral T lymphocytes.
41. The optimal concentration must be determined for each peptide used.
42. Although most of the T-cell hybridomas that we have tested respond well at the indicated APC and T-cell hybridoma ratios, it is useful to determine appropriate ratios for individual T-cell hybridomas. Depending on the density of specific peptide/MHC complexes presented by the APC and T-cell receptor affinity for the complex, T-cell hybridoma responses may attain saturation quickly. Hence, it is advisable to double-dilute APC and assess T-cell hybridoma activation to obtain graded responses.
43. Microscopic examination and quantitation of activated T-cell hybridomas can be time-consuming and labor-intensive. We have automated this process using a microscope attached to a computer, which is aided with software to spot and count cells.
44. Fluorescent substrates are light-sensitive. Thus, it is necessary to protect assay plates from direct light. Also samples should be kept devoid of bubbles, as bubbles may interfere with the assay reading. Brief centrifugation of assay plates prior to reading is recommended.
45. Other substrates, such as 0.15 m*M* chlorophenol red β-galactopyranoside (CPRG) or 5 m*M* O-nitrophenol-β-galactopyranoside (ONPG) or 1 m*M* FDG can also be used with absorption wavelengths at 595, 415, or 495 n*M*, respectively, and 635 n*M* as the reference wavelength. MUG and CPRG seem to be the most sensitive substrates in these assays.

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Mouse Models for Studying Orthopoxvirus Respiratory Infections

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Summary

Concern regarding the use of variola and monkeypox viruses as bioterrorist agents has led to an increased study of orthopoxviruses to understand the molecular and cellular basis of pathogenesis and develop safe and effective antivirals and vaccines against smallpox. Crucial to these efforts is the availability of animal models, which are inexpensive, genetically homogeneous, and recapitulate the human disease. The popular small-animal orthopoxvirus models employ the inbred mouse as the host, the respiratory tract as the site of virus inoculation, and orthopoxviruses—vaccinia, cowpox, and ectromelia viruses—as surrogates for variola virus. Ectromelia virus is likely the best surrogate for variola virus in a mouse model, as it is infectious at very low doses of virus, and the mousepox disease is associated with high mortality in the susceptible A, BALB/c, and DBA/2 stains of mice, but causes an unapparent infection in the C57BL/6 mouse strain. This chapter describes an ectromelia virus respiratory infection model in the mouse.

Key Words: Mouse models; intranasal; intratracheal; aerosol; mousepox; ectromelia virus.

1. Introduction

Although four orthopoxviruses have been shown to cause disease in humans, only vaccinia virus (VACV), cowpox virus (CPXV), and monkeypox virus still cause human infections ***(1)***. With the global eradication of the smallpox disease in 1979, the causative agent, variola virus, no longer circulates in human populations. However, because the respiratory tract is a major route of infection for the monkeypox and variola virus, there is concern that variola virus could be reintroduced through bioterrorism and/or biowarfare ***(2,3)***.

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Human monkeypox and smallpox have been modeled using various combinations of orthopoxviruses, hosts, and routes of inoculation. Rabbitpox is a disease model that employs a respiratory route infection of rabbits with rabbitpox virus (RPV; *see* **Note 1**). Mice have been extensively studied using VACV (*see* **Note 2**) and CPXV (*see* **Note 3**) challenge by various routes of inoculation. Ectromelia virus (ECTV) is a natural orthopoxvirus pathogen of mice (*see* **Note 4**). Although this chapter exclusively describes the use of ECTV infection in mice, similar approaches could be used with other orthopoxviruses in mice, rats, and rabbits. Using ECTV, we describe three major approaches for infecting the respiratory tract. The choice of technique is dependent on the question being asked. If the upper respiratory tract is the region of interest, then an intranasal infection is the method of choice; yet, the lower respiratory tract may also become infected. If the goal is to mimic a deep-lung infection, then a small-particle aerosol should be used. Intratracheal infections are utilized only when a portion of the lung is to be infected or if the aim is to introduce large doses of the infecting agent into the lung.

ECTV can infect all strains of laboratory mice and can cause either a severe disease (characterized by severe illness and/or sudden death) or silent infections (no clinical signs, but virus is shed by infected mice). Thus, to prevent a disastrous outbreak in the mouse facility, special precautions when handling ECTV-infected mice are prudent. Bedding contaminated with infectious virus serves as a source of infection to other mice; thus, use of particulate bedding, such as beta chips, or the use of excess amounts of bedding must be avoided. Poxviruses are relatively resistant to heat or drying and many chemical disinfectants. Poxviruses are sensitive to moist heat (autoclaving) and chlorine-based disinfectants (*see* **Note 5**). The following precautions should be taken. Inoculated animals should be housed in microisolators with "care-fresh" bedding in a designated cubicle. All personnel should wear eye protection and a disposable mask, gloves, and gown when working with inoculated animals or potentially contaminated equipment. Dispose of the mask, gloves, and laboratory coat in a biohazard container maintained within the room. Animal and cage manipulations should only take place under the laminar flow hood. The work surface of the hood should be wiped with disinfectant (*see* **Note 5**) between each cage change and manipulation. To further diminish the possible transfer of virus, the exterior of the gloves should be kept moist with disinfectant at all times. After the last cage manipulation, all exposed hood surfaces should be sprayed with disinfectant and left wet for at least 10 min prior to final wipe. Cages and solid equipment should be disinfected (sprayed or dipped) with a virucidal agent or autoclaved prior to transport to the sanitation area.

2. Materials

2.1. Aerosol Route

1. Nose-only inhalation exposure system (NOIES, also known as a Jaeger-NYU NOIES; CH Technologies, cat. no. 201-666-2335; www.toxics.com).
2. Vacuum pump (Gast Manufacturing, IDEX Corporation, cat. no. DOA-V191-AA).
3. Recirculating pump (Fluid Metering Inc., cat. no. QG50/QG50MB).
4. Magnehelic pressure gauge +/– 2 in (Dwyer, cat. no. 2304).
5. Two clamp stands.
6. HEPA filter (Millipore Opticap Filter, cat. no. KTGR04NP3) with a magnehelic pressure gauge +/– 15 in (Dwyer, cat. no. 2330).
7. Flow meter (Dwyer, cat. no. VA22436).
8. Tubing and fittings to connect all components (Fisher Tygon and polyurethane tubing).
9. High-vacuum grease (Dow Corning), 0.5-in. Teflon tape (Fisher, cat. no. 14831300A).
10. Ectromelia virus pelletted through a sucrose cushion (resulting titer approx 1×10^9 plaque-forming units [pfu]/mL).
11. Media for diluting the viral dose and for impinger (Fisher, cat. no. BW12-604F, Dulbecco's modified Eagle's medium [DMEM]).
12. Reusable animal holders (CH Technologies) with fitted latex probe covers (Carolina Medical).
13. No. 6 neoprene rubber stoppers (Fisher, cat. no. 14-141G).
14. 10% bleach (Chlorox).
15. Sterile H_2O.
16. 70% ethanol (Aaper Alcohol and Chemical Co).
17. CV-1 cells (green monkey kidney epithelial cells; ATCC, # CCL-70).
18. CV-1 growth medium: DMEM supplemented with 10% fetal calf serum (FCS; Hyclone SH30066), 1X penicillin/streptomycin (pen/strep) (Fisher, cat. no. BW17603E), 2 m*M* L-glutamine (Fisher, cat. no. 17605E), and 1X nonessential amino acids (Fisher, cat. no. 13114E).
19. 24-well tissue culture-treated plates (Fisher, cat. no. 07-200-84).
20. Phosphate-buffered saline (PBS) without calcium or magnesium (Fisher, cat. no. 17516F) supplemented with 1% FCS.
21. Carboxymethylcellulose (CMC) overlay media: DMEM supplemented with 5% FCS, 1X pen/strep, 2 m*M* L-glutamine, 0.1% CMC (Sigma, cat. no. C4888; *see* **Note 6**).
22. Crystal violet stain solution: 0.13% crystal violet (Fisher, cat. no. C58125) in 11.1% formaldehyde (*see* **Note 7**).
23. Glass-dosing jar (CH Technologies).
24. Glass impinger (Ace glass, cat. no. 7540).
25. Envirocide® (Viro Research, cat. no. 30024).
26. Lint-free absorbent wipes.
27. Tools for assembly, including Allen wrenches, small adjustable crescent wrenches, and razor blades.
28. Tubing cutters (CH Technologies).

2.2. Localization of Foci of Infection

1. Tissue freezing mixture: 1:1 mixture of PBS (Bio Whittaker, cat. no. 17-516F) and tissue freezing medium (TFM; Fisher, cat. no. 15-183-13).
2. Luer-Lok 3-mL syringe (VWR, cat. no. BD309585).
3. 19-gauge 1.5-in. needle (Fisher, cat. no. 14-826-52).
4. Dissecting scissors (Fisher, cat. no. 13-804-6).
5. Specimen forceps (VWR, cat. no. 25719-044, 25729-387).
6. Sterile surgical blades (VWR, cat. no. BD371110).
7. Scalpel handle (VWR, cat. no. 25856-023).
8. Dissecting hemostat (VWR, cat. no. 25601-060).
9. Liquid nitrogen.
10. 2-Methylbutane (2MB; Sigma, cat. no. 27,034-2).
11. Cryostat chucks (Leica, cat. no. CM1900).
12. Sample bags (VWR, cat. no. 56766-016).
13. Dry ice.
14. Cryostat (Leica, cat. no. CM1900).
15. Disposable microtome blades (VWR, cat. no. 25608-964).
16. 4% paraformaldehyde (Sigma, cat. no. P-6148) in PBS.
17. X-gal stock: 4% X-gal (Roche, cat. no. 745740) in *N*,*N*-dimethylforamide (Sigma, cat. no. D-8654).
18. X-gal buffer: 5 m*M* potassium ferricyanide (Sigma, cat. no. P-8131), 5 m*M* potassium ferricyanide (Sigma, cat. no. P-9387), 2 m*M* $MgCl_2$ (Sigma, cat. no. M-1028). Add PBS to 100 mL.
19. X-gal staining solution: 800 μL X-gal buffer + 20 μL X-gal stock.
20. Staining jar (VWR, cat. no. 25457-200).
21. Six complete staining dishes (Wheaton, cat. no. 900200).
22. Nuclear Fast Red (Newcomer Supply, cat. no. 1255A).
23. 95% ethanol (Aaper Alcohol and Chemical Co.).
24. 100% ethanol (Aaper Alcohol and Chemical Co.).
25. Xylene (Fisher, cat. no. X3P-1Gal).
26. Superfrost Plus slides (Fisher, cat. no. 12-550-15).
27. Slide tray (Newcomer Supply, cat. no. 6841A).
28. Coverslip (VWR, cat. no. 48404-452).
29. Cytoseal mounting medium (VWR, cat. no. 48212-154).
30. MarkerII/Superfrost (Fisher, cat. no. 14-905-30).
31. Clear nail polish (generic drugstore brand).

2.3. Intranasal and Intratracheal Routes

1. 100 mg/mL ketamine HCl (Sigma, K2753).
2. 10 mg/mL xylazine (Sigma, X1126).
3. Anaesthetic solution: final 90 mg/kg ketamine/final 10 mg/kg xylazine.
4. Saint Louis University mouse/rat intubation platform (cat. no. 1225).
5. Isotonic saline: 0.9% NaCl at room temperature.

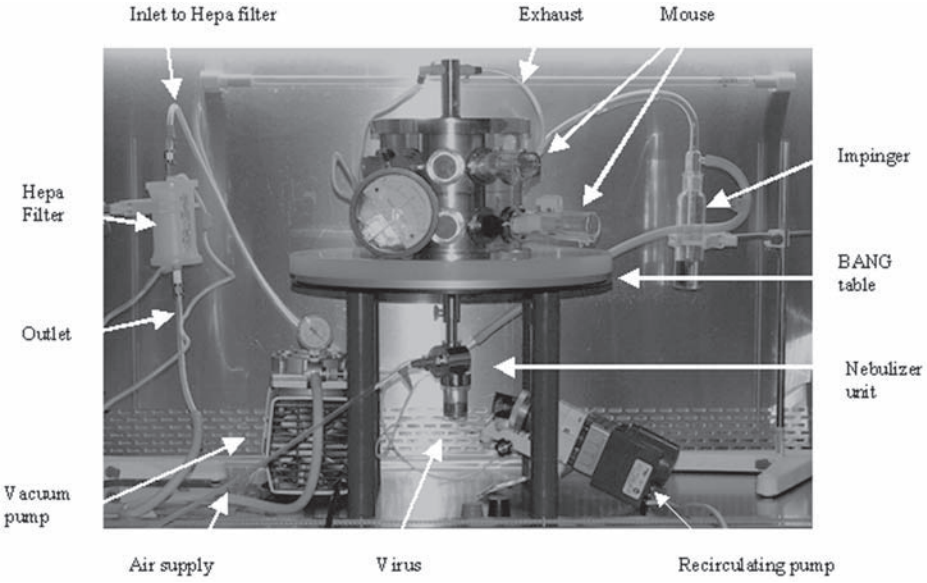

Fig. 1. NOIES.

6. Series IA-1B intratracheal aerosolizer (PennCentury, Philadelphia, PA).
7. Hamilton gas-tight syringe (Hamilton, cat. no. 81220).
8. Micropipetter, range 1–5 μL.
9. Virus suspension in filtered buffer.
10. Sterile barrier tips.

3. Methods

3.1. Aerosol Route: NOIES Assembly and Operation

The NOIES is composed of a single-jet BioAerosol Nebulizing Generator (BANG) and a series of 12-port manifolds designed to deliver an aerosol exposure dose simultaneously to multiple animals (*see* **Fig. 1** and **Note 8**). The virus suspension in the dose jar is pumped through the outlet probe via tubing to the nebulizing chamber (*see* **Fig. 2**). Here, the liquid feed meets an air stream, which enters the nebulizer through a fine-bore channel. The collision of the liquid and air stream yields an aerosol. The aerosol is driven by air pressure and pulled by a vacuum into the nebulizing outlet column, where larger droplets recondense and return to the dose jar, and droplets 0.7–2.5 μm in diameter enter the aerosol exposure chamber. The exposure chamber is made from modular segments, each sufficient for exposing 12 mice (*see* **Fig. 3** and **Note 9**).

The aerosol is ported from the central aerosol chamber to individual exposure stations where the animal inhales a portion of the delivered aerosol

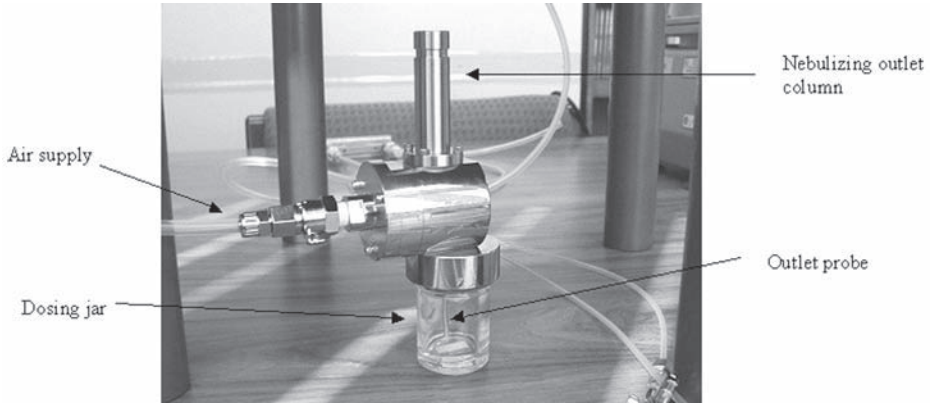

Fig. 2. The nebulizing unit.

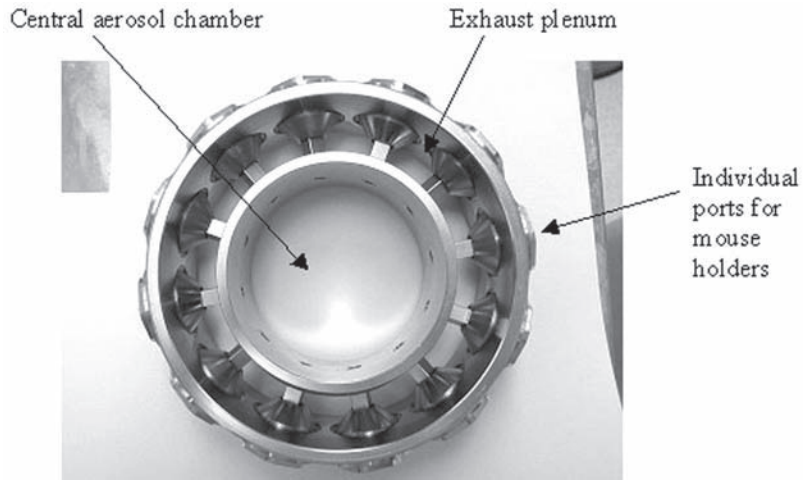

Fig. 3. A 12-port modular unit of the aerosol chamber.

(*see* **Fig. 4**). Exhaled gases and the noninhaled aerosol exit the individual exposure stations via an exhaust plenum through a rotating sealed T-connector. The left side of the connector leads to a magnehelic gauge, which measures the pressure differential between the exhaust plenum and the ambient air, whereas the right side connects to an impinger containing a diluent that traps residual virus in the aerosol stream. The impinger outlet is connected to an inline HEPA filter and exhausts through a vacuum pump, which pulls the aerosol through the system at approx 1.5 L/min when the input air pressure is set at 18 psi and a 5-L flow restrictor is in place. The pump exhausts into a

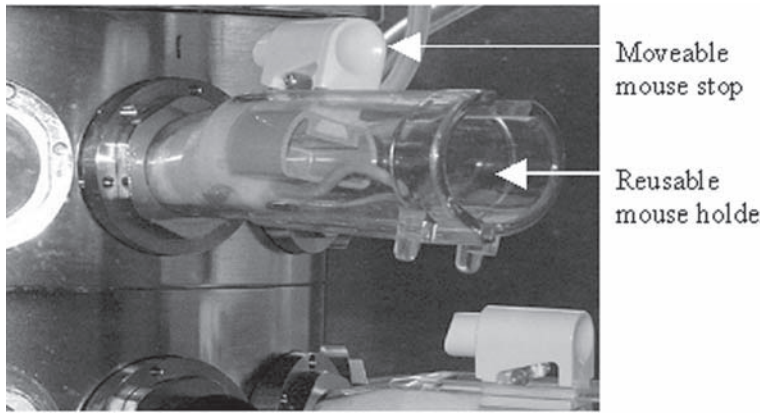

Fig. 4. Mouse holder containing a mouse.

class 2 biosafety cabinet hard-ducted through a HEPA filter to the outside environment.

1. Assemble the NOIES following the manufacturer's instructions (*see* **Note 10**).
2. Dilute the input virus in DMEM using a minimum volume of 5 mL for every 15-min aerosol generation (*see* **Notes 11**, **12**, and **13**).
3. The liquid feed containing the virus is pumped up to approx 1 cm within the tubing preceding the nebulizing chamber, and the pump is turned off. The vacuum pump is engaged. The mice are placed in mouse holders with their noses snugly fitted into the open conical end of the mouse tube holders (*see* **Note 14**). The movable stop is placed against the back of the mouse to prevent withdrawal of the nose from the exposure chamber (*see* **Fig. 4**). The open-tube ends are covered with fitted latex probe covers. The air tank outlet pressure is set at 20 psi, and the air flow is started at approx 1.5 L/min. The virus suspension is then fed into the nebulizer at a rate of 0.5 mL/min.
4. After the designated time (15 min), the virus feed to the nebulizer is stopped, while the 1.5 L/min air flow is maintained for an additional 5 min to move through any residual aerosol left in the aerosolizing chamber and mouse-dosing plenum. The airflow is then stopped.
5. With the vacuum pump still on, the mouse holders are removed and replaced with rubber stoppers to seal the system. The latex covers are removed and the mice are returned to their cages. With removal of the last mouse holder, the vacuum pump may be turned off.
6. The NOIES is then disassembled. The impinger jar is sealed with sterile aluminum foil and placed on ice for titration of "trapped" virus. The HEPA filter and magnehelic are wiped with Envirocide® and stored in a biohazard bag that is kept on a small laboratory cart that is adjacent to the hood. The vacuum pump is also wiped with Envirocide® and stored in a biohazard bag on the laboratory cart.

7. The viral suspension is collected by unscrewing the dose jar from the NOIES. The jar is held under the liquid outlet probe, and the liquid remaining in the tubing is pumped back into the dose jar. The viral dose jar is sealed with sterile aluminum foil and held on ice for titration of virus and measurement of infectivity of the remaining virus suspension.
8. In a separate dosing jar, add 15 mL 10% bleach. The bleach is recirculated through the tubing at 4 mL/min for 5 min. The bleach is discarded and the jar is rinsed with sterile H_2O. Fifteen milliliters of sterile H_2O is added to the dose jar and recirculated through the tubing at 4 mL/min for 5 min. The water is discarded. Fifteen milliliters of 70% ethanol is added to the dose jar and recirculated at 4 mL/min for 5 min. The ethanol is discarded. This step cleans, disinfects, and sterilizes the junction between the recirculation pump and the NOIES tubing only.
9. The rubber stoppers are removed from the aerosol chamber segments and placed in a small wire autoclavable basket along with small metal fittings from the nebulizer. The basket is placed in a biohazard bag. The outside of the bag is wiped with Envirocide®, and this bag is placed into a second bag outside of the class 2 biosafety cabinet. The lid on the top segment of the aerosol chamber is removed and wiped with Envirocide®. Each individual segment is separated, wiped with Envirocide®, and placed in a biohazard bag in the class 2 biosafety cabinet. The bag is wiped with Envirocide® and placed into a second bag outside of the class 2 biosafety cabinet.
10. The ring stand and NOIES table is wiped with Envirocide® and stored in biohazard bags on the cart. All remaining elements of the NOIES are wiped with Envirocide® and removed from the class 2 biosafety cabinet. Once the class 2 biosafety cabinet is emptied of all equipment, interior surfaces are wiped with Envirocide®. All NOIES metal fittings, stainless-steel components, and tubing are autoclaved for 25 min on a wrapped setting.
11. The autoclaved stainless-steel components of the NOIES are cleaned with 70% ethanol. The nebulizer is disassembled and soaked in distilled H_2O. The components are carefully dried and reassembled. The nebulizer is autoclaved again for 25 min on a wrapped setting.
12. While the NOIES components are in the autoclave, the input virus and impinger fluid are titrated using CV-1 monolayers (50–90% confluent) in a 24-well-plate format. The volumes of virus suspension and impinger fluid are recorded to permit calculation of the presented virus dose (*see* **Note 15**).

3.2. Localization of Foci of Infection

To determine the number and location of the lung foci of virus infection, mice infected as described in **Subheading 3.1.** with an ECTV recombinant expressing *lac*Z (*see* **Note 16**) are sacrificed with an overdose of CO_2 at different times postinfection (*see* **Note 17**).

1. The chest cavity is opened with dissecting scissors to expose the trachea and lung.

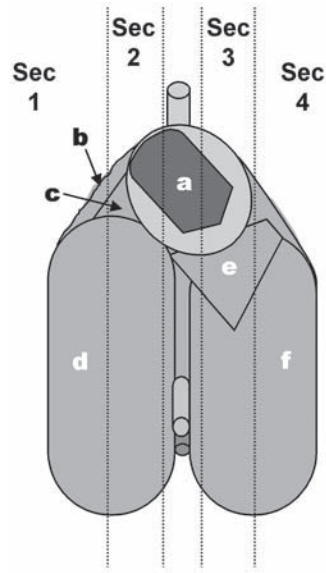

Fig. 5. Schematic view of the ventral surface of mouse lung and heart. Adapted from Popseko et al. ***(38)***. Letters: a: heart; b–e: right lung. b, cranial lobe; c, medial lobe; d, caudal lobe; e, accessory lobe; f, left lung. Vertical lines represent the four sections (Sec) of the lungs harvested from a mouse. Note that the accessory lobe (e) is reflected to the left and is sectioned along with the left lung.

2. Using a Luer-Lok syringe with needle, TFM is infused into the lungs via the trachea until all lobes are inflated.
3. To prevent rapid deflation, the trachea is clamped with a hemostat (*see* **Note 18**).
4. The entire lung is removed from the thoracic cavity by gently cutting underneath the trachea (*see* **Note 19**).
5. Using a scalpel and forceps, section the lung as illustrated in **Fig. 5**.
6. In anticipation of freezing the lung tissue, 2MB is placed in a container that has been carefully lowered into a thermos containing liquid nitrogen (*see* **Note 20**).
7. A clean cryostat chuck is labeled to identify the specimen and orientation (*see* **Note 21**).
8. The cryostat chuck is layered with TFM and, using long forceps, is immersed in 2MB until frozen (*see* **Note 22**).
9. All four lung sections (*see* **Fig. 5**) are rapidly transferred to the frozen cryostat chuck prepared in **step 8** with sections 1 and 4 positioned cut side-up and sections 2 and 3 placed bronchial side-up (*see* **Note 23**).
10. The sections are quickly covered with a complete layer of TFM.
11. The cryostat chuck is kept upright and immersed in 2MB using long forceps until frozen.

12. The cryostat chuck is placed in a labeled sample bag and the bag is stored on dry ice until transferred to a –70°C freezer for storage until processing for cryosections.
13. In anticipation of sectioning, adjust the cryostat temperature to –20°C. Prior to cutting sections, place the chuck in the cryostat for 15–20 min to allow the tissue to reach cryostat temperature.
14. Attach the chuck to the assembly and tighten the bolt to hold it firmly.
15. Adjust the thickness of the cut using the micrometer dial (*see* **Note 24**).
16. For each cut section gently smooth out any wrinkles with a brush kept inside the chamber, then place on a slide, and hold it there for a few seconds to allow the section to "melt" onto the surface.
17. Keep the slide outside of the cryostat chamber until ready to pick up another section. Fit as many sections on each slide as possible.
18. Place the filled slide on a rack in the cryostat chamber until the block is completely trimmed.
19. When finished, remove tissue waste from the cryostat chamber and lock the cutting handle. Return the chuck and tissue to –70ºC for storage.
20. The slides are stored in a sealed box overnight at –20ºC or directly processed for X-gal staining.
21. The slides are incubated at room temperature for no longer than 30 min prior to placement in a staining jar and fixed in fresh 4% paraformaldehyde for 15 min at 4ºC.
22. The slides are rinsed twice in PBS for 10 min each at 4ºC, then incubated overnight at 37ºC in X-gal staining solution ***(4)***.
23. The slides are rinsed three times in PBS for 10 min or until solution is no longer yellow (*see* **Note 25**).
24. The slides are rinsed in deionized water and placed in a staining jar containing nuclear fast red for 8 min. The slides are again rinsed with deionized water and dehydrated through sequential immersions in graded alcohol and xylene reagents (*see* **Note 26**).
25. A coverslip is mounted over the sections using cytoseal.
26. Following drying at room temperature, clear nail polish is applied around the edge of the coverslip, which minimizes drying out of the slide.
27. Examine by light microscopy. Using this protocol, a 40-μm frozen section of an A/J mouse lung infected by aerosol with ECTV, sacrificed 3 d postinfection, and stained with X-gal is presented in **Fig. 6**.

3.3. Intranasal and Intratracheal Routes

If the upper respiratory tract is the region of interest, then an intranasal infection will be the method of choice, although the lower respiratory tract may also become infected. Intratracheal infections are utilized when only a portion of the lung is to be infected or the aim is to introduce large doses of the infecting agent into the lung.

1. Mice are anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg ketamine/10 mg/kg xylazine.

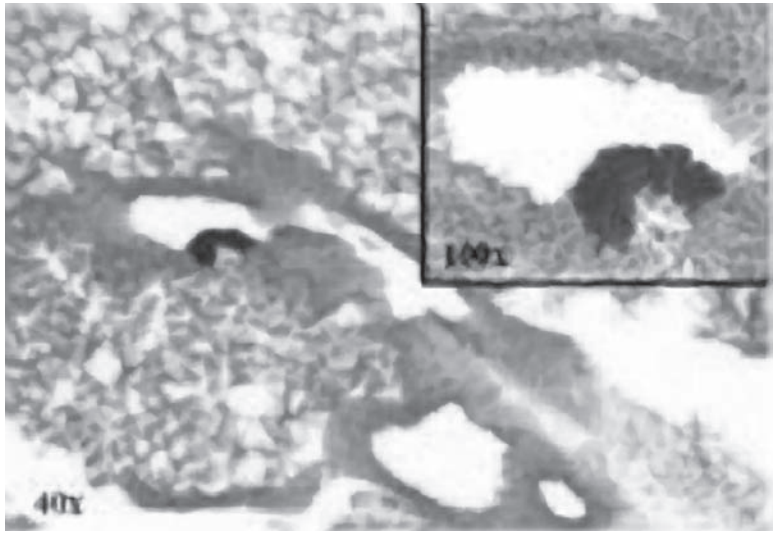

Fig. 6. ECTV focus of infection in the lung. 40-μm Frozen section of an A/J mouse lung infected by aerosol with ECTV, sacrificed 3-d postinfection, and stained with X-gal.

Fig. 7. Platform and mouse position for intranasal and intratracheal administration of virus.

2. The anesthetized animal is laid on its back on the intubation platform at a 45° angle. The mouse is held in place by side supports and a strap under the roof of the mouth (**Fig. 7**).
3. For an intranasal inoculation, 3–5 μL in a sterile barrier tip is dispensed into one of the nares followed by a 10-min incubation to allow for complete aspiration of

the drop. A second dose is then applied to the other nare followed by an additional 10-min inspiration time. The animal is then returned to its cage and maintained under a heat-lamp until fully recovered.

4. For an intratracheal inoculation, a modified otoscope is used to visualize the vocal chords. A Hamilton syringe fitted with a series IA-1B intratracheal aerosolizer is inserted down the throat to the carina. A maximum of 20 μL solution is dispensed, taking no longer than 30 s. The mouse is returned to a cage with 100% O_2 atmosphere until recovery is ensured (i.e., resumption of mobility).

4. Notes

1. Two strains of RPV have been identified. The Rockefeller virus strain was isolated from a spontaneous outbreak of severe disease in rabbits by Greene ***(5–8)***. The Utrecht strain of RPV was isolated in a similar manner by Jansen ***(9)***. Both virus strains were associated with high levels of mortality, although experimental findings suggested the Utrecht strain was possibly more virulent. Although VACV and RPV cause distinctly different diseases in domestic rabbits, the viruses are closely related, genetically belonging to the same species as determined by pocking virus isolates on the chorioallantoic membrane of the chick embryo, intradermal inoculation into rabbit skin, and crossprotection studies in rabbits. More recently, *Hin*dIII, *Sma*I, and *Xho*I restriction endonuclease analysis patterns of the genomes of multiple VACV strains and Utrecht RPV strain have confirmed this conclusion ***(10,11)***. Both VACV and RPV require little more than one infectious particle to initiate an infection in rabbits by the respiratory route. However, here the similarity ends. VACV failed to produce a fatal disease or even a severe infection with doses as high as 1.3×10^4 pfu ***(12)***. Aerosol infection with RPV, on the other hand, produced nearly uniform fatalities with the lowest dose administered (15 pfu; six deaths of seven animals inoculated). Epidemiological studies suggested that RPV transmission is mediated through aerosols. Experimental respiratory infections of rabbits with RPV have been studied in great detail. The clinical, virological, pathological, and epidemiological findings of this disease are reviewed elsewhere ***(12–14)***.
2. The origins of VACV remain a mystery. It has no known natural reservoir, and is not a strain of CPXV, but is a distinct species. VACV is highly infectious, but is of low virulence for a wide variety of mouse strains. With the exception of immunocompromised mice, it is next to impossible to observe mortality following percutaneous or footpad inoculation of VACV even at doses as high as 10^7 pfu ***(15)***. Following infection, the virus replicated efficiently at the site of application, but was rarely detected in the draining lymph node and more rarely in the spleen or liver. Similarly, intravenous and intraperitoneal doses of VACV in excess of 10^7 pfu are necessary to observe mortality ***(16,17)***. The nonphysiological intracranial route of inoculation is the most sensitive of all routes tested, and LD_{50} values can be achieved in the area of 10 pfu, but are dependent on the strains of VACV and strain of mice used ***(18,19)***. The intranasal route of VACV inoculation is the most often studied of all the infection routes, and LD_{50} values

have been reported, which range from 3.3×10^5 to greater than 6×10^8 pfu, also dependent on the strain of VACV and strains of mice ***(16,20,21)***.

3. CPXV was named as a result of its association with the pustular lesions on the teats of cows and the hands of milkers, although repeated studies have shown that bovine cowpox is not, and never has been, a common disease. The natural reservoir in nature is not the cow, rather it is likely to be rodents (voles, wood mice, and/or rats). The pathogenesis of CPXV has been thoroughly studied following intradermal inoculation of guinea pigs and rabbits by Downie in the 1930s ***(22)***. In the late 1960s, Subrahmanyan and Mims carried out less detailed studies with the Brighton CPXV strain in various strains of mice ***(23,24)***. Outbred mice inoculated with CPXV by intravenous, intranasal instillation, and intracranial routes yielded LD_{50} values of approx 10^7, 10^7, and 10^4, respectively. Bray et al. ***(25)*** reported that 4-wk-old female mice inoculated by tail scarification with 10^6 pfu of the Brighton strain of cowpox had a more generalized infection with 40% associated mortality. Huggins and his colleagues have used a lethal aerosol and intranasal CPXV challenge model to test a experimental orthopoxvirus therapeutic, cidofovir [[HPMPC]] (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine) ***(25,26)***. The disease pattern can be very dependent on the age of the mice.
4. ECTV is the causative agent of mousepox, a naturally occurring acute exanthematous disease found in mouse colonies in Europe, Japan, and China ***(19)***. The natural reservoir for ECTV is unknown, but one report provides evidence that wild mice may be involved. Laboratory studies have shown ECTV to have a very narrow host range, infecting only certain mouse species ***(19)***. The Moscow, Hampstead, and NIH79 strains are the most thoroughly studied. The Moscow strain is the most virulent and infectious for the mouse ***(27–30)***. Studies in the last five decades from a succession of investigators have resulted in a detailed description of the virologic and pathologic disease course in genetically susceptible and resistant inbred and outbred mice. This work has led to the identification and characterization of the important cell-mediated and innate responses for recovery from infection and the discovery of rmp-1, rmp-2, rmp-3, and rmp-4 loci, which govern resistance to severe mousepox ***(31–33)***. All mice are highly susceptible to infection with ECTV by a peripheral route of infection, such as the footpad. However, mouse strains vary considerably with regard to the severity of disease. The C57BL/6 and AKR strains are resistant to severe disease, whereas the A, BALB/c, DBA, and C3H strains are susceptible. Also, the disease-resistance patterns are very dependent on route of inoculation as demonstrated by the studies of Schell in the early 1960s ***(34)***. Because no other orthopoxvirus has been studied in its natural host to this level of detail, we believe the ECTV mouse aerosol infection is one of the best models for human monkeypox and smallpox.
5. Disinfectants include freshly made 10% Chlorox, Spor-klenz, or Expor solutions.
6. One gram of CMC is added to a 1-L bottle that is autoclaved for 15 min. Sterile 950 mL DMEM is added to the bottle along with a sterile 2-in. magnetic stir bar. The bottle is placed on a stirrer plate at room temperature for 24–48 h. Once the CMC is dissolved, the other components are added aseptically.

7. Crystal violet solution is made by dissolving 1.3 g crystal violet (Fisher, cat. no. C58125) in 50 mL ethanol with stirring for 1 h. Then, add 300 mL 37% formaldehyde (Fisher, cat. no. F79) and fill to 1 L with distilled H_2O and stir overnight.
8. BANG was designed and engineered by Robert Gussmon for CH Technologies. With newly purchased systems, a field engineer from CH Technologies sets the system up and instruct the user in its operation.
9. The maximum number of mice that can be dosed at one time is dependent on the number of segments that are stacked to make the aerosol chamber and exhaust plenum. Exposure units of up to six segments have been used to dose 72 animals at a time. Each segment has the capacity to hold up to 12 mouse holders. Although the NOIES has been used with four segments for a total capacity of 48 mice, we do not use more than three segments due to the time required for putting the mice into, and removing them from, the holders.
10. Remember to lubricate all rubber gaskets and impinger glass junction with high-pressure vacuum grease. Line all metal- and glass-screw fittings with Teflon tape, including the dosing jar. The pressure differential between the NOIES aerosol chamber and interior of the class 2 biosafety cabinet must be checked prior to the generation of an aerosol, which should be at least –0.1 in. of water differential pressure with respect to the pressure within the biosafety safety cabinet. Also, the pressure differential across the HEPA filter must be measured to verify that the filter is functioning (between 5 and 10 in. of pressure differential is normal). Sentinel mice kept in the class 2 biosafety cabinet during the NOIES operation have never been infected, suggesting that the NOIES is effective in containing the aerosolized virus.
11. We use the Moscow strain of ECTV as our challenge virus. Using the NOIES with our standard conditions (18 psi tank pressure, 1.5 L/min air flow, 0.5 mL/min liquid feed, 1×10^7 pfu/mL virus concentration in liquid feed, 15-min exposure) results in a presented dose of 5.6×10^4 pfu (*see* **Note 12**). We have obtained reproducible infectivity levels in the impinger fluid (approx 6.6×10^4 pfu) and obtained consistent mean day-of-death values with little variance (A/J mice; experiment 1: 7.3 ± 0.5 d; experiment 2: 7.5 ± 0.5 d; and experiment 3: 6.5 ± 0.4 d). We found that virus concentrations of less than 10^4 pfu/mL of liquid feed did not result in reproducible detection of virus infectivity in impinger fluid, even though mice became infected. In our studies, susceptible mouse strains, A/J and BALB/c, have an average day of death of 6–10-d postinfection depending on the presented dose of virus.
12. The presented dose of virus that mice are exposed to is calculated by multiplying the concentration of virus in the aerosol (C_A) in pfu/L by the total volume (V_m) of air respired by the mouse (of given weight in grams) over the exposure time (t) using Guyton's formula, $V_m = 2.1 \times (\text{weight})^{0.75}$ ***(35)***. The C_A can be calculated in two ways. The first approach uses the initial virus concentration in the liquid feed (C_A = concentration of virus in liquid feed, pfu/mL × total quantity of liquid feed nebulized, mL ÷ total volume of aerosol, L. The second method calculates C_A using the virus infectivity present in the impinger liquid, C_A = concentration of

virus in impinger fluid, pfu/mL × total volume of impinger fluid, mL ÷ rate of air flow through impinger fluid, L/min × time, min). An assumption of the second approach is that all residual infectivity is captured in the impinger fluid. This should be tested experimentally. Because the virus can undergo multiple aerosolization and condensation steps prior to becoming a small enough particle to enter the aerosol chamber, it is important to measure virus stability under NOIES operating conditions. In our experience, ECTV in the liquid feed is stable for at least 30 min in an operating NOIES.

13. Before challenging mice in the NOIES, it is important to standardize parameters that can affect the properties of the aerosolized dose (primary air flow, secondary air flow, virus concentration in liquid feed, rate of liquid feed, time of exposure, stability of virus infectivity in liquid feed, and so on). Using virus infectivity levels in the impinger fluid as an assay, these parameters should be examined in pilot experiments (without mice). On completion of optimization experiments, pilot mouse experiments can then be attempted. Several successful small mouse experiments should be completed prior to attempting a large-scale mouse experiment.
14. Mice 10–12 wk of age (15–20 g) are used, as mice of this size are unable to turn around in the mouse holders and avoid the aerosol. Mice must be held securely in the mouse holder for consistent results.
15. To titrate the input virus, a series of 10-fold dilutions in PBS/1% FCS from 10^{-1} to 10^{-6} is carried out. For impinger fluid, 10^{-0} and 10^{-1} dilutions are made. Aspirate all but approx 0.1 mL of the growth media covering the CV-1 cells (to prevent the cells from drying). Plate 0.1 mL of each dilution on a monolayer. Incubate the plate at 37°C in a tissue culture incubator, rocking the plate every 15 min. After 30–60 min, overlay the monolayers with 1 mL CMC overlay media per well. (The CMC overlay restricts secondary plaque formation and makes counting plaques easier and more accurate). Incubate approx 4 d or until discrete plaques are visible under the microscope. Add 0.25 mL crystal violet stain solution directly to each well (i.e., without removing the overlay). Vigorously rock plate over the sink to mix the stain–fix solution. After 30 min at room temperature, invert the plate in a running water bath and shake vigorously until all the CMC overlay and residual stain is washed off of the stained monolayer. Dry by inverting the plate on a lined rack overnight at room temperature or in a hot-room. Count plaques in the different dilution wells and calculate the titer of the given dilution = (no. of plaques) × (1/dilution) × (10) pfu/mL.
16. To enhance ECTV utility as a challenge virus in antiviral or vaccine studies, we have recombined a *lacZ* reporter gene into the naturally fragmented and nonfunctional CHO hr locus, which does not modify the pathogenesis pattern of the virus (N. Chen and R. M. L. Buller, unpublished data). The SV40 tumor nuclear-localizing signal was fused in frame with the *lacZ* gene, and the chimeric gene was expressed from a consensus strong early VACV promoter ***(36)***.
17. 24-h Postinfection is the earliest time-point we have sacrificed and tried to locate where the virus is.

18. Suture can be used to tie off the trachea to prevent deflation after infusion. Also, *see* **ref. *37***.
19. When removing the lung, the heart can remain attached, but is not included with the lung sections. Also, the trachea and esophagus are not included.
20. The liquid nitrogen is contained in a thermoflask and the 2MB container is held on a stand with a support ring. The 2MB must be cold, but without the formation of ice crystals.
21. It is helpful to have an assistant prepare the cryostat chuck while the lung is harvested. Specimens that are cut repeatedly over a period of time are left on the cryostat chuck. To conserve resources and expand options, our machine shop fabricated similar cryostat chucks made from aluminum. It is important the cryostat chucks be made of metal to permit better temperature control of the specimen.
22. The TFM will turn white and hard on the cryostat chuck.
23. Mounting the lung sections as described will allow the investigator to determine the distribution of infected cells progressively from the hilum to the lung periphery within the air passages. Thus, it is possible to determine the density of infected cells within the bronchi, bronchioles, respiratory bronchioles, alveolar ducts, and alveoli and deduce from that data which sized passage is most often the site of the initial infection event.
24. Although frozen sections are most often cut at 5–8 μm, we cut at approx 40 μm to permit the processing of the complete lung in a reasonable number of sections.
25. Slides can be left in PBS overnight at 4ºC.
26. Ten times each in 95% and 100% ethanol, xylene, and xylene.

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21

Viral Glycoprotein-Mediated Cell Fusion Assays Using Vaccinia Virus Vectors

Katharine N. Bossart and Christopher C. Broder*

Summary

The vaccinia virus-based expression of viral envelope glycoprotein genes—derived from enveloped viruses that infect their respective host cells through a pH-independent mechanism of membrane fusion—has been a powerful tool in helping to characterize these important attachment and fusion proteins. The cellular expression of these viral envelope glycoproteins has allowed for the measurement of membrane fusion events using cell–cell fusion or syncytia formation. This method has been enhanced by the addition of a reporter-gene system to the vaccinia virus-based cell–cell fusion assay. This improvement has provided a high-throughput and quantitative aspect to this assay, which can serve as a surrogate for virus entry and is therefore ideally suited in the characterization of numerous enveloped viruses, including biological safety level-4 (BSL-4) agents. This chapter will detail the methods of the vaccinia virus-based reporter-gene fusion assay and how it may be used to characterize the fusion mediated by the BSL-4-classified Hendra and Nipah viruses.

Key Words: Paramyxovirus; Hendra virus; Nipah virus; envelope glycoprotein; quantitation membrane fusion; syncytia; vaccinia virus; β-galactosidase; quantitative; virus; entry; infection; emerging viruses.

1. Introduction

Among the many applications of foreign gene expression that recombinant vaccinia virus techniques have been employed (for recent reviews, *see* **refs. *1–4***), the in vitro production of envelope glycoproteins from other viruses has served as a powerful platform for the characterization of these important structural components. Recombinant vaccinia viruses have been instrumental

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in several unique approaches related to the study of those viral envelope glycoproteins that function with a pH-independent fusion mechanism using cell–cell fusion or syncytia formation assays. Indeed, one of the earliest examples of this technique was the construction of the first vaccinia virus recombinant encoding the envelope glycoprotein gene from human immunodeficiency virus type-1 (HIV-1), then known as human T-cell leukemia virus type-III, and the subsequent demonstration of syncytia formation in a CD4-dependent manner ***(5,6)***. This syncytia formation assay using HIV-1 envelope glycoprotein encoded by recombinant vaccinia virus vectors has been wildly employed in the study of HIV-1 envelope-mediated membrane fusion (reviewed in **ref. *7***). In subsequent studies, the vaccinia virus-based cell fusion approach was coupled with a bacteriophage T7 promoter, reporter-gene-based system. This improved assay was critical in the characterization of the requirements for HIV-1 envelope-mediated membrane fusion ***(8–10)*** and in the identification of the first HIV-1 coreceptor ***(11)***. Here, in addition to the viral envelope glycoprotein(s) and viral receptors being expressed on effector and target cell populations, respectively, one cell population also expresses bacteriophage T7 RNA polymerase and the other a T7 promoter-driven *Escherichia coli lacZ* cassette that are both introduced by separate recombinant vaccinia viruses ***(9,12–14)***. Thus, cell fusion results in the specific production of β-galactosidase (β-gal) that can be quantified (**Fig. 1**). The subsequent detection of β-gal synthesis using colorometric substrates has a high level of sensitivity. This combined with very low levels of background substrate cleavage activity makes this assay very useful for detecting and measuring rare fusion events and/or subtle differences in the fusion permissivity between envelope glycoprotein-expressing effector cells with different target cell types. Frequently, these subtle differences cannot be detected using simple syncytia formation assay techniques.

Envelope glycoproteins derived from various viruses that employ a pH-independent mechanism of fusion for virion entry have been studied using this reporter-gene assay ***(15–22)***. Many of these examples are with the envelope glycoproteins derived from members of the *Paramyxoviridae* family, which are negative-sense RNA-enveloped viruses and encompass a variety of important human and animal pathogens (reviewed in **ref. *23***). Unlike HIV-1 and other retroviruses, paramyxoviruses contain two principal membrane-anchored envelope glycoproteins. One glycoprotein is associated with virion attachment to the host cell and, depending on the particular virus, has been designated as either the hemagglutinin-neuraminidase glycoprotein (HN), the hemagglutinin glycoprotein (H), or the G glycoprotein that has neither hemagglutinating nor neuraminidase activities (reviewed in **ref. *24***). The other glycoprotein is the fusion glycoprotein (F), which is directly involved in facilitating the fusion of the viral and host cell membranes (reviewed in **ref. *23***).

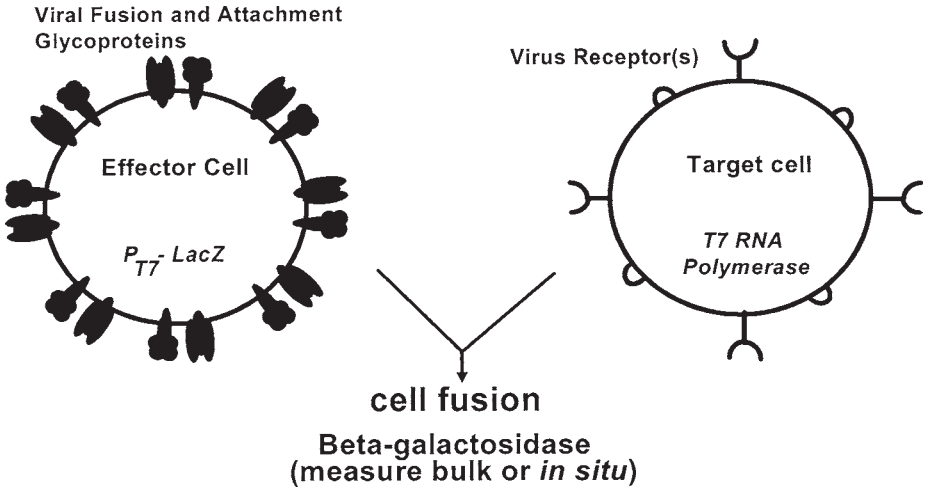

Fig. 1. Vaccinia virus-based reporter gene activation assay for viral envelope glycoprotein-mediated cell fusion. In addition to the viral envelope glycoproteins being examined, an effector cell population is prepared where these cells are infected with a second recombinant vaccinia virus encoding bacteriophage T7 promoter-driven *E. coli. lacZ* cassette. The target cell population being examined is then prepared by infection with another recombinant vaccinia virus that encodes a T7 RNA polymerase ***(9,12–14)***. If the target cell population is permissive for cell fusion (i.e., displays functional receptors for the viral envelope glycoprotein presented on the effector cells), then cell–cell fusion ensues and results in the specific production of β-gal, which may be quantified. Cell–cell fusion reactions are conducted with various cell mixtures in 96-well plate format, and, typically, the ratio of target and effector cells is 1:1 (2×10^5 total cells/well, 0.2-mL total volume). For quantitative analyses, the cell mixtures are lysed at 2.5–3 h postmixing and aliquots of the lysates are assayed for β-gal at ambient temperature with a chromogenic substrate. Fusion results are calculated and expressed as rates of β-gal activity (change in optical density at 570 nm/min × 1000).

Because the recombinant vaccinia virus-based cell fusion system can be performed using only the envelope glycoproteins of the virus of interest, it is an ideal platform for safely studying highly pathogenic viral agents. Recently, two new paramyxoviruses—Nipah virus (NiV) and Hendra virus (HeV)—have been identified, which are closely related members of a new genus, *Henipavirus*, within the *Paramyxoviridae* family. HeV was identified first from cases of severe respiratory disease that fatally affected both horses and humans ***(25)***. Subsequent to the appearance of that virus, an outbreak of severe febrile encephalitis associated with human death occurred in Malaysia, and later studies identified a Hendra-like virus (now known as NiV) from that episode ***(26–28)***. Among the paramyxoviruses these viruses are unusual in their ability to

infect and cause potentially fatal disease in many host species, including humans. Thus, they are classified as zoonotic biological safety level-4 (BSL-4) agents. We recently applied the vaccinia virus-based reporter-gene assay for cell fusion to the study of these new BSL-4 pathogens ***(29,30)***. The purpose of this chapter is to give a detailed overview of the methods employed in performing the vaccinia virus-based cell fusion assay. We describe the steps of this assay using the NiV and HeV envelope glycoproteins as examples.

2. Materials

2.1. Cell Culture

1. Cell lines: HeLa cells (ATCC, #CCL 2), HuTK$^-$143B (TK$^-$) (ATCC, #CRL 8303), and U373-MG (provided by Adam P. Geballe, Fred Hutchinson Cancer Research Center, Seattle, WA; ***31***).
2. DMEM-10: Dulbecco's modified Eagle's medium (DMEM; Quality Biologicals, Gaithersburg, MD) supplemented with 2 m*M* L-glutamine and 10% cosmic calf serum (CCS; HyClone, Logan, UT).
3. DMEM-2.5: DMEM, 2 m*M* L-glutamine, 2.5% CCS.
4. EMEM-10: Eagle's minimal essential medium (EMEM; Quality Biologicals) supplemented with 2 m*M* L-glutamine and 10% CCS.
5. EMEM-2.5: EMEM, 2 m*M* L-glutamine, 2.5% CCS.
6. Trypsin–EDTA solution: 0.25% trypsin–0.02% EDTA (Quality Biologicals).
7. Plasticware: 25-cm^2, 75-cm^2, and 150-cm^2 tissue culture flasks (Falcon, BD Laboratories). 50-mL polypropylene conical centrifuge tubes and 96-well flat-bottom plates (Falcon, BD Laboratories).

2.2. Recombinant Vaccinia Viruses and Expression Plasmids

1. Recombinant vaccinia viruses: vCB21R (vaccinia virus-encoding *E. coli lacZ* gene linked to the T7 promoter; ***11***), vTF7.3 (vaccinia virus-encoding T7 RNA polymerase; ***32***), vKB-1 (vaccinia virus-encoding HeV F), vKB-2 (vaccinia virus-encoding HeV G), vKB-6 (vaccinia virus-encoding NiV G), and vKB-7 (vaccinia virus-encoding NiV F).
2. Expression plasmid constructs are prepared using vaccinia virus promoter-driven expression vector pMC02 ***(33)***: pKB-1 (HeV F in pMC02), pKB-2 (HeV G in pMC02), pKB-6 (NiV G in pMC02), and pKB-7 (NiV F in pMC02).

2.3. Reporter-Gene Assay Specialized Reagents and Equipment

1. *N*-(1-(2,3-Dioleoyloxy)propyl]*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) liposomal transfection reagent (Roche Diagnostics Corp., Indianapolis, IN); store at 4°C.
2. 40 m*M* HEPES, pH 7.4, store at 4°C.
3. 100X AraC: 4 mg/mL cytosine arabinoside in water, filter-sterilized (*see* **Note 1**).
4. Nonidet P-40 stock solution (Calbiochem): 20% (v/v) in H_2O; store at 4°C.

5. CPRG substrate: chlorophenol red-D-galactopyranoside (PRG; Roche Diagnostics Corp.); light-sensitive, store at –80°C.
6. 10X Z buffer: 0.6 *M* Na_2HPO_4, 0.4 *M* NaH_2PO_4, 0.1 *M* KCl, 0.01 *M* $MgSO_4$, 0.5 *M* β-mercaptoethanol; store at –20°C.
7. 10X CPRG stock: 250 mg CPRG substrate dissolved in 5.14 mL 10X Z buffer (*see* **Note 2**).
8. Microplate absorbance reader (MRX; Dynatec Laboratories) equipped with a 570-nm filter or similar 96-well absorbance detector.

3. Methods

The methods described below outline (1) protein expression in effector and target cells, (2) preparing effector and target cell populations for the fusion assay, (3) quantitative analysis of β-gal synthesized during the fusion assay, and (4) adaptations of the quantitative fusion assay for diagnostic purposes. This reporter-gene assay for measuring viral-mediated membrane fusion is extremely sensitive and is not dependent on the extensive cell–cell fusion events usually required for the visualization of syncytia or giant-cell formation. It is also a quantitative and fairly rapid assay. Typically, the cell–cell fusion reactions are conducted only 2.5–3 h at 37ºC. The reader is encouraged to review the data and descriptions that detail the assessments of the sensitivity and quantitative abilities of the assay elsewhere ***(12,34)***.

3.1. Protein Expression in Effector and Target Cell Populations

Effector cells express both the envelope glycoproteins of interest and bacteriophage T7 RNA polymerase (*see* **Notes 3** and **4**). Target cells express the receptor needed for viral envelope glycoprotein-mediated membrane fusion and contain the *E. coli lacZ* gene linked to the bacteriophage T7 promoter (*see* **Note 5**). The T7 RNA polymerase is introduced into the effector cells by recombinant vaccinia virus vTF7.3 ***(35)*** and the *E. coli lacZ* gene cassette, whose expression is dependent on T7 RNA polymerase, is introduced into the target cells by recombinant vaccinia virus vCB21R ***(10)***. The vTF7.3 and vCB21R viruses may be switched in the two cell populations if needed. **Figure 1** provides an overall scheme of the fusion assay.

Typically, for adherent cells, the effector and target cell types used are seeded into appropriately sized cell culture flasks at a density whereby an overnight incubation yields cultures near confluency the following day (*see* **Note 6**). The plasmid transfection and vaccinia virus infection protocols for preparing effector and target cell populations are very similar to each other. For the experiments included in this chapter, all vaccinia virus infections were carried out using a multiplicity of infection (moi) of 10 plaque-forming units (pfu) per cell, which ensures that all cells are infected with vaccinia virus. All cell lines used as examples in the chapter are adherent. Suspension cell lines may

also be employed (*see* **Note 7**). The protocol described here employs HeLa as the viral envelope glycoprotein expressing effector cell type introduced by vaccinia virus infection or plasmid DNA transfection of cell monolayers. The viral envelope glycoproteins from NiV and HeV expressed by vaccinia virus and presented here as examples of the fusion assay are shown in **Fig. 2**.

3.1.1. Effector Cell Preparation: Viral Envelope Glycoprotein Expression Using Plasmid DNA

1. Detach HeLa cells with the trypsin–EDTA solution, quench with DMEM-10, harvest the cells by standard centrifugation and resuspension procedures, and count. Add 1.8×10^6 cells per 25-cm^2 flask in 10 mL DMEM-10 and incubate overnight at 37°C under a humidified 5% CO_2 atmosphere. This yields approx 3×10^6 cells/flask the following day (*see* **Note 8**).
2. Prepare 2.5 μg plasmid DNA in 30 μL water with 20 μL 40 m*M* HEPES in a 12 × 75-mm polystyrene tube (*see* **Note 9**).
3. Premix 19 μL water, 12 μL DOTAP, and 19 μL 40 m*M* HEPES, then add to each DNA/HEPES mixture from the previous step. Do not pipet up and down to mix; instead, gently tap the tube to mix (*see* **Note 10**).
4. Incubate 30 min at room temperature.
5. Add 0.9 mL DMEM-2.5 to each transfection tube (*see* **Note 11**).
6. Aspirate the DMEM-10 from the 25-cm^2 flask of HeLa cells and replace with the DNA/HEPES/DOTAP/medium transfection mixtures from **step 5** (*see* **Note 12**).
7. Incubate 4 h at 37°C in a humidified tissue culture incubator.
8. Thaw the vTF7.3 and sonicate for 30 s in a cup sonicator filled with an ice-water slurry. Maintain virus stock on ice.
9. For each transfected 25-cm^2 flask of HeLa cells, add 3×10^7 pfu of vTF7.3 in a total volume of 1 mL DMEM-2.5 and vortex (*see* **Note 13**).
10. Aspirate the DNA transfection mixture from the 25-cm^2 flask of HeLa cells and replace with the 1 mL inoculum of vTF7.3 from **step 9**.
11. Incubate 2 h at 37°C in a humidified tissue culture incubator, gently rocking the flask every 20 min to avoid drying of any monolayer areas. Proceed to **Subheading 3.2.**

3.1.2. Effector Cell Preparation: Viral Envelope Glycoprotein Expression Using Recombinant Vaccinia Virus

1. Detach HeLa cells with the trypsin–EDTA solution as described in **Subheading 3.1.1.**, **step 1**.
2. Thaw the appropriate envelope glycoprotein-encoding recombinant vaccinia viruses. The examples used here are: vKB-1 (HeV F), vKB-2 (HeV G), vKB-6 (NiV G), vKB-7 (NiV F), and vTF7.3. Sonicate for 30 s in a cup sonicator filled with an ice-water slurry. Maintain virus stocks on ice.

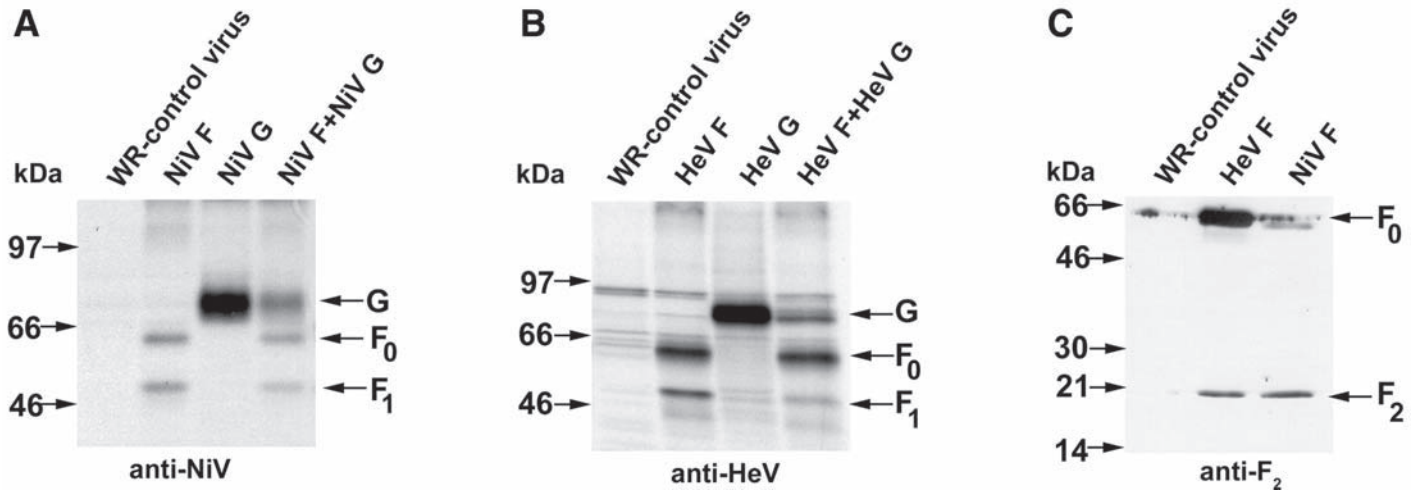

Fig. 2. Expression of recombinant NiV F and G glycoproteins. The NiV F and G glycoprotein open-reading frames were subcloned into a vaccinia virus promoter-driven expression vector pMC02 ***(33)*** and recombinant viruses were made ***(29,30)***. HeLa cells were infected with HeV or NiV F or G encoding viruses and incubated 16 h at 37°C. Beginning at 6-h postinfection, the cells were either labeled overnight with [^{35}S]methionine/cysteine for immunoprecipitation or cultured in medium alone for Western blotting. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit anti-NiV or rabbit anti-HeV antiserum followed by protein G-sepharose. Western blot was performed using rabbit polyclonal antiserum against a synthetic F_2 peptide ***(30)***. The metabolically labeled proteins were resolved by 10% SDS-PAGE under reducing conditions and detected by fluorography; lysates for Western blot were resolved by 10% SDS-PAGE under reducing conditions and detected by chemiluminescence. **(A)** Immunoprecipitation; **(B)** immunoprecipitation; **(C)** Western blot. Reproduced from Bossart et al. ***(29)***, with permission.

3. Add 3.5×10^7 pfu of the appropriate recombinant vaccinia virus or viruses and 3.5×10^7 pfu of vTF7.3 to 1 mL DMEM-2.5. Vortex (*see* **Notes 13** and **14**).
4. Aspirate DMEM-10 from each 25-cm^2 flask of HeLa cells being employed and replace with the 1 mL virus inoculum from the previous step.
5. Incubate 2 h at 37°C in a humidified tissue culture incubator, gently rocking the flask every 20 min to avoid drying of any monolayer areas. Proceed to **Subheading 3.2.**

3.1.3. Target Cell Population Preparation

The experimental examples presented in this chapter employ target cells capable of endogenously producing the necessary viral receptor for envelope glycoprotein-mediated fusion. Although no specific examples are presented in this chapter, the methods for the introduction of recognized and cloned viral receptors into a nonpermissive target cell type can be done exactly as described for expression of the viral envelope in **Subheadings 3.1.1.** and **3.1.2.** (*see* **Note 15**).

1. As specific examples for the fusion assay as it is applied to the NiV and HeV system, detach the U373-MG and TK^- cell lines with the trypsin–EDTA solution, quench with media containing 10% CCS, harvest the cells by standard centrifugation and resuspension procedures and count. Seed 5×10^6 U373-MG cells in a 150-cm^2 flask in 40 mL DMEM-10, and 5×10^6 TK^- cells to a 150-cm^2 flask in 40 mL EMEM-10. Incubate overnight, which yields approx 1.0×10^7 cells/flask the following day.
2. Thaw *E. coli lacZ* gene-T7 promoter-encoding vaccinia virus recombinant vCB21R and sonicate for 30 s in a cup sonicator filled with an ice-water slurry. Maintain virus stocks on ice.
3. Add 1.0×10^8 pfu of vCB21R to 5 mL DMEM-2.5 (for U373-MG), and 1.0×10^8 pfu of vCB21R to 5 mL EMEM-2.5 (for TK^-). Vortex.
4. Aspirate media from 150-cm^2 flask of U373-MG and TK^- cells and replace with 5 mL virus mixtures from previous step (*see* **Note 13**).
5. Incubate 2 h at 37ºC in a humidified tissue culture incubator, gently rocking the flask every 20 min. Proceed to **Subheading 3.2.**

3.2. Reporter Gene Fusion Assay

After DNA transfection or vaccinia virus infection procedures for preparation of the effector and target cell populations are carried out, the cells are recovered from the culture flasks and prepared as suspension cultures as outlined below.

3.2.1. Preparation of the Effector and Target Cell Populations

1. Remove the virus inoculum and wash the monolayer with 1 mL trypsin-EDTA solution to a 25-cm^2 flask or 5 mL to a 150-cm^2 flask. Briefly rock flask to cover entire surface area and immediately remove.

2. Add another fresh 1 mL trypsin–EDTA solution to a 25-cm^2 flask or 5 mL to a 150-cm^2 flask. Rock flask to cover entire surface area and incubate until cells have detached from the flask.
3. Quench the with 10 mL DMEM-10 for a 25-cm^2 flask or 25 mL to a 150-cm^2 flask. Mix by pipet and transfer the cells to a sterile 50-mL polypropylene conical tube (*see* **Note 16**).
4. Pellet the cells by centrifugation at 500*g* for 10 min.
5. Wash twice with 20 mL DMEM-10 by centrifugation at 500*g* for 10 min (*see* **Note 17**).
6. Count the cell populations and resuspend the cells at a density of 2×10^5cells/mL in DMEM-10 in the 50-mL conical tubes (*see* **Note 18**). Do not exceed 30 mL/tube.
7. Position tubes at a near horizontal angle to allow adequate gas exchange with the medium and avoid having the cells settle into a pellet at the bottom of the tube. Incubate all the cell populations at 31°C (*see* **Note 19**) under a humidified 5% CO_2 atmosphere. Typically, an overnight incubation is performed (12–15 h) for convenience, but the cell populations may be used in the fusion assay following a 4–6-h incubation period to ensure maximal recombinant protein expression.

3.2.2. Fusion Assay Set-Up

1. Wash all cell populations twice with DMEM-10 by centrifugation and resuspend at a density of 1×10^6 cells/mL (*see* **Note 20**).
2. Add 100X AraC to all cell population at 40 μg/mL (1X) (*see* **Note 21**).
3. Prepare two blank wells (mock wells) in a tissue culture 96-well flat-bottom plate by adding 200 μL DMEM-10 containing 1X AraC.
4. Prepare two wells containing target cells only by adding 100 μL target cell suspension to each of the two wells and add an additional 100 μL DMEM-10 containing 1X AraC to each well.
5. Prepare two wells containing effector cells only by adding 100 μL of each effector cell population to each of two wells and add an additional 100 μL DMEM-10 containing 1X AraC to each well.
6. Using a multichannel pipet and disposable plastic cell culture trough to aid in the simultaneous addition and mixing of cell populations, add 100 μL of each effector cell population to each of the two wells for every target cell type being tested.
7. When all the effector cell containing wells have been prepared, add 100 μL of each target cell population to the wells containing the 100 μL suspension of effector cells using a multichannel pipet and mix by pipetting up and down three times. Change the pipet tips for each row of wells. The final cell–cell mixtures being assessed for fusion contain 2.0×10^5 cells in 200 μL.
8. Incubate the 96-well plate for 2.5–3 h at 37°C in a humidified tissue culture incubator.
9. The remaining effector and target cell populations can be used to set up a duplicate 96-well plate to examine syncytia formation or lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to examine envelope and/or receptor protein expression in a similar fashion as depicted in **Fig. 2**. An example of the syncytia formation is shown in **Fig. 3**.

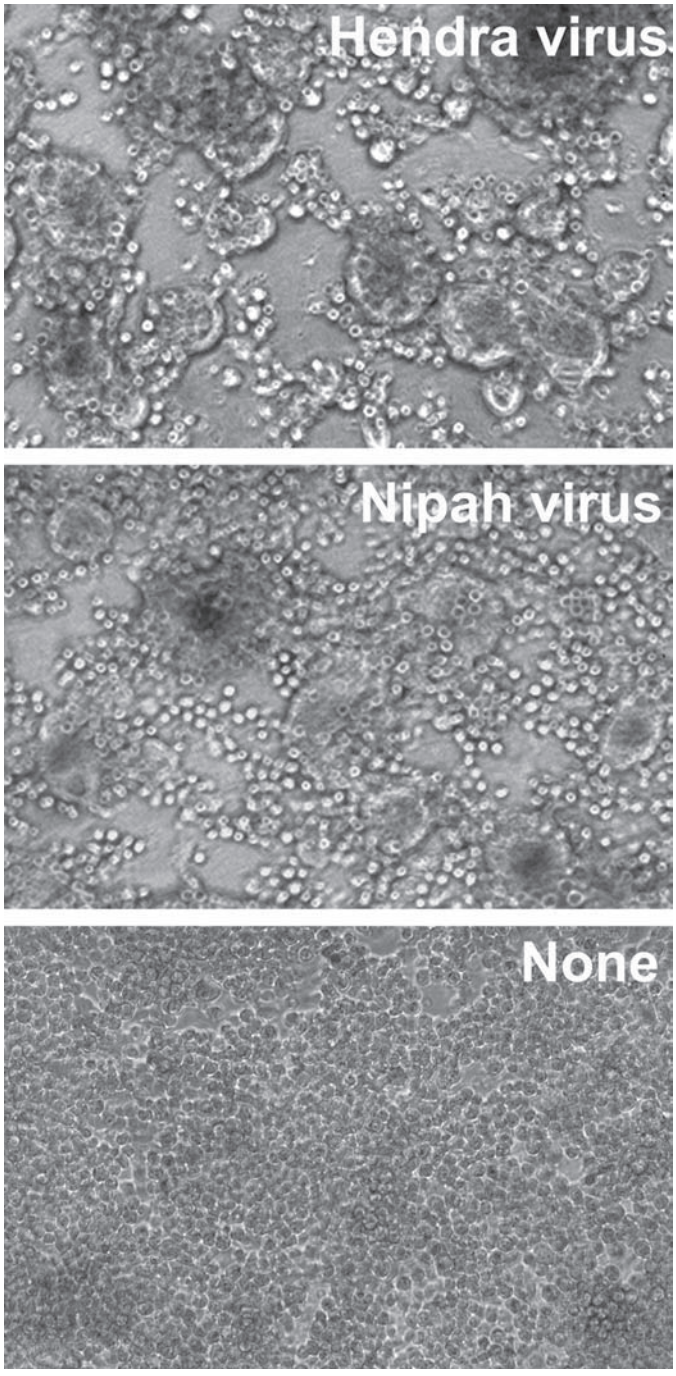

Fig. 3.

10. Following the fusion period, add 10 µL 10% NP-40 solution to each well of the 96-well plate using a multichannel pipet and mix. Avoid bubble formation by not pipetting up the entire sample (*see* **Note 22**).
11. Seal the plate with parafilm and place the plastic lid on the plate.
12. Freeze and store at –80°C until ready to proceed to the β-gal assay.

3.3. Quantitative Measurement of β-Gal Synthesized During the Fusion Assay

3.3.1. β-Gal Assay

1. Remove the 96-well plate from –80°C and thaw at room temperature.
2. Prepare 10X CPRG stock (*see* **Note 2**).
3. Turn on the 96-well absorbance reader with a 570-nm filter. If possible, program the instrument to read all wells while subtracting the absorbance of one of the blank wells (prepared without any cells) from the experimental wells.
4. Dilute the required amount of the 10X CPRG stock 1:5 with water to make a 2X CPRG stock solution (*see* **Note 23**).
5. Using a multichannel pipet, mix the contents of each well of the 96-well fusion assay plate while avoiding bubble formation by not drawing up the entire contents of any well.
6. Using a multichannel pipet, transfer 50 µL of the contents of each well of the 96-well fusion assay plate to a new 96-well plate in the same configuration.
7. Using a multichannel pipet, add 50 µL 2X CPRG stock solution to each well, pipetting up and down once, and replacing the tips for each subsequent row of wells. This procedure should be done as rapidly as possible while avoiding bubble formation.
8. Set a timer to count forward.
9. Take an initial reading of the 96-well plate and start the timer.
10. Read the plate at approx 5-min intervals and record the exact minute and second reading on each print-out of the 96-well plate absorbance values measured. Record three to four measurements (*see* **Note 24**).

3.3.2. Determining the Levels of β-Gal Activity by Calculating the Rates of Substrate Cleavage

Although several measurements have been taken, only two absorbance values and the time between those values are required. The rate that is calculated

Fig. 3. *(previous page)* Syncytia formation mediated by homotypic NiV and HeV envelope combinations. HeLa cells were infected with vaccinia recombinants encoding either the HeV F and G glycoproteins, the NiV F and G glycoproteins, or none, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). The effector cells (1×10^5) were mixed with the target U373 cells (1×10^5) in duplicate wells of a 96-well plate and incubated at 37°C. After 30 h, photographs were taken at ×400 magnification.

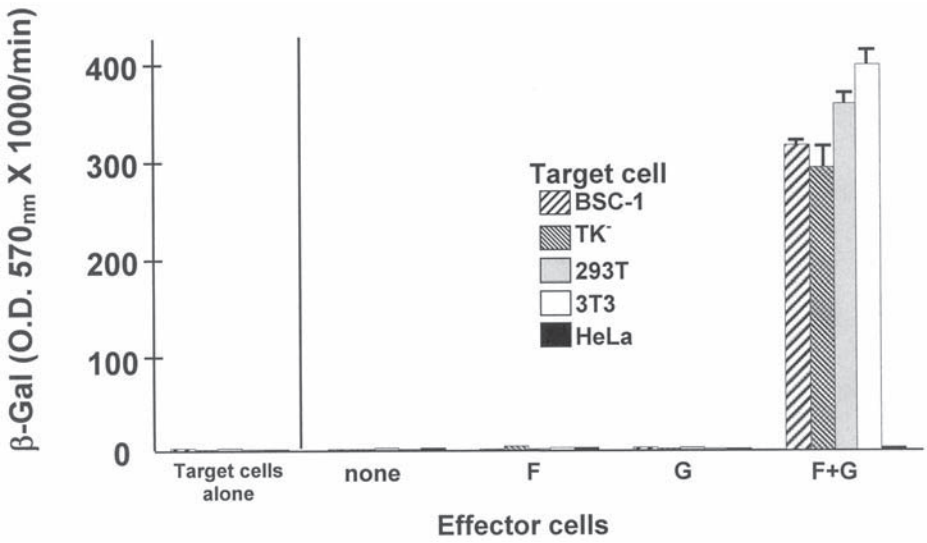

Fig. 4. Quantitation of HeV-mediated cell fusion. HeLa cells were infected with vaccinia recombinants encoding the HeV F, G, both F and G glycoproteins, or none, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E. coli LacZ*-encoding reporter vaccinia virus vCB21R. Effector cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 h at 37°C, cells were lysed and β-gal activity was quantitated. The level of background β-gal activity in target cell populations alone is indicated. The β-gal activity from target cells mixed with HeLa effector cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding HeV glycoproteins is indicated as none. Reproduced from Bossart et al. ***(30)***, with permission from Elsevier Science.

should be nearly identical between any two readings and is therefore not affected by the actual numerical absorbance value recorded. The rates of substrate cleavage are determined as follows:

$$\text{Rate} = (\text{OD}_{\text{time 2}} - \text{OD}_{\text{time 1}})/\text{time interval (min)} \times 1000$$

Because of the time that may elapse between the addition and mixing of substrate to each row of wells, as well as the layout of the replicates in the 96-well plate, a rate calculation should be determined for each individual well before making averages and range calculations of replicate wells from the fusion assay. Typically, the rate values are also determined for each cell population alone (effector and target), and these are presented as negative controls for fusion. Examples of the rates of β-gal activity determined from a fusion assay with different effector cell populations is shown in **Fig. 4**. Examples of

the rates of β-gal activity determined from a fusion assay with several alternate target cell populations is shown in **Fig. 5**.

3.4. Adaptations of the Reporter Gene Fusion Assay

Owing to the assay's high sensitivity, low background, 96-well plate high throughput, and quantitative capability, it is an ideal tool for measuring reagents capable of inhibiting the viral-mediated membrane fusion step as a rapid surrogate assay for virus entry. Some of these applications include the evaluation of neutralizing antibody responses elicited in naturally or experimentally infected animals and humans and in the screening of novel drugs or small molecules as inhibitors of virus-mediated membrane fusion. Furthermore, it is ideal for studying the entry mechanism of viruses that are otherwise restricted to the highest level of biocontainment (BSL-4). The final section of this chapter describes the application of the reporter-gene assay to measure the specific inhibitory activity of antibodies and peptides on the cell-fusion process of the BSL-4 agents, HeV and NiV.

Typically, antiviral antibodies, which block virus entry or viral-mediated membrane fusion, exert their effects by blocking the viral glycoproteins required for receptor recognition and/or the fusion process itself. Recent advances in understanding the mechanisms involved in the fusion of the membrane of enveloped viruses with their host cell membrane support a model involving the formation of a trimer-of-hairpins structure of the fusion glycoprotein whose oligomeric coiled-coil formation is mediated by the two α-helical heptad repeat domains of the fusion protein (i.e., HIV-1 gp41 or paramyxovirus F; reviewed in **refs. *36–39***). Peptides derived from either of the α-helical heptad repeat regions of enveloped viral fusion proteins have previously been shown to be potent inhibitors of the fusion process for many viruses when present during the fusion process ***(40–46)***.

3.4.1. Fusion Inhibition Assay

As multiple concentrations of test reagents are typically used to determine dose responses, it is important to prepare a large number of both target and effector cell populations.

1. Prepare effector and target cells as described in **Subheadings 3.1.** and **3.2.**, but at **step 6** in **Subheading 3.2.1.**, adjust the cell densities to 2×10^6 cells/mL.
2. During the centrifugation and washing steps of the effector and target cell populations, prepare dilutions of antiviral peptides or antiviral antisera in 100-μL vol using DMEM-10 containing 1X AraC, and determine the total number of concentrations that will be tested. The examples here are using these peptides: HeV FC2, NiV FC1, and ScHeV FC2, as well as rabbit polyclonal anti-NiV antisera (*see* **Notes 25** and **26**).

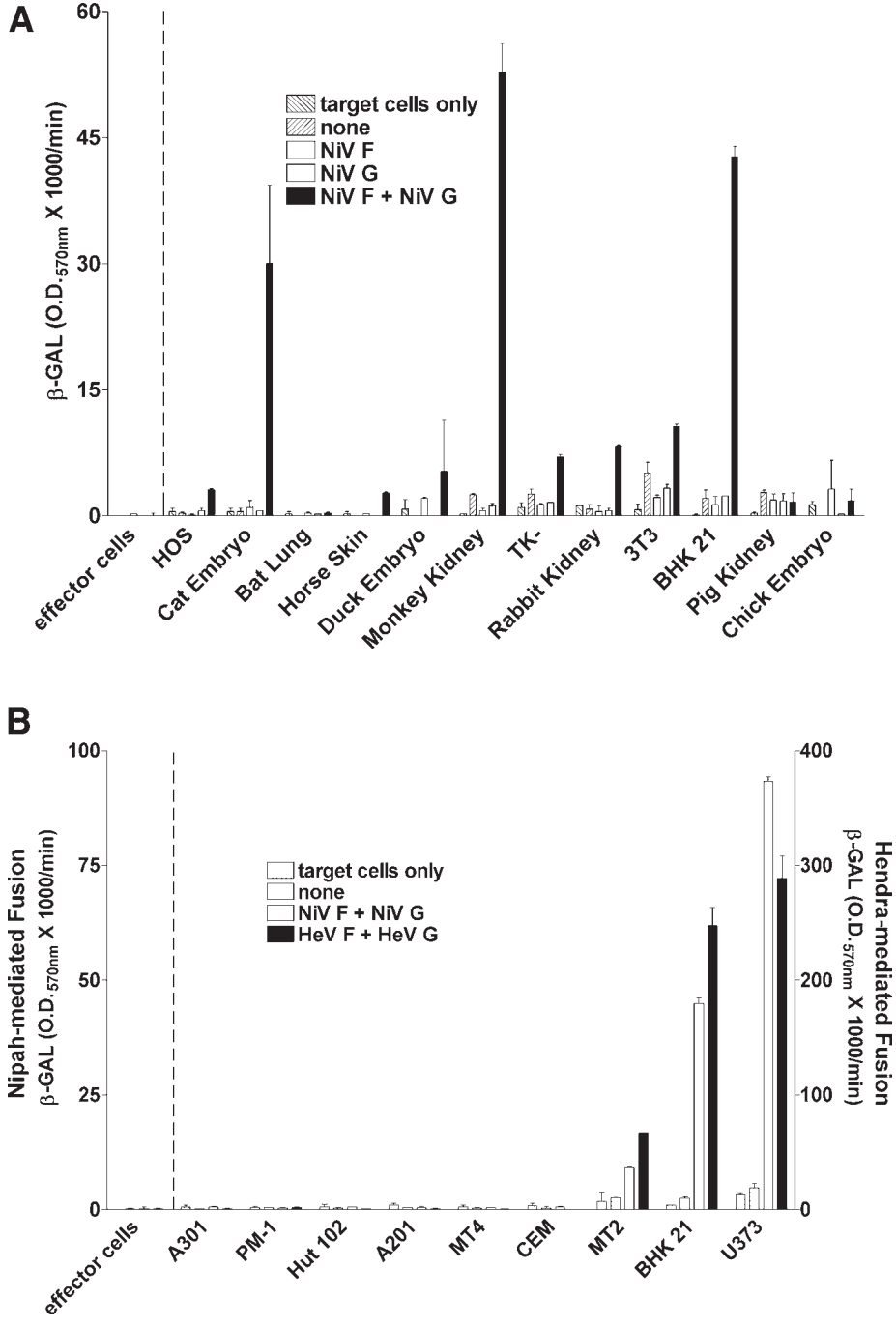
A
β-GAL (O.D.570nm X 1000/min)
60
45
30
15
0
target cells only
none
NiV F
NiV G
NiV F + NiV G
effector cells
HOS
Cat Embryo
Bat Lung
Horse Skin
Duck Embryo
Monkey Kidney
TK-
Rabbit Kidney
3T3
BHK 21
Pig Kidney
Chick Embryo
B
Nipah-mediated Fusion
β-GAL (O.D.570nm X 1000/min)
100
75
50
25
0
target cells only
none
NiV F + NiV G
HeV F + HeV G
Hendra-mediated Fusion
β-GAL (O.D.570nm X 1000/min)
400
300
200
100
0
effector cells
A301
PM-1
Hut 102
A201
MT4
CEM
MT2
BHK 21
U373

Fig. 5.

3. Prepare two blank wells (mock wells) in a tissue culture 96-well flat-bottom plate by adding 200 μL DMEM-10 containing 1X AraC.
4. Prepare two wells containing only target cells by adding 50 μL of the target cell suspension to each of two wells. Add an additional 150 μL DMEM-10 containing 1X AraC to each well.
5. Prepare two wells containing only effector cells by adding 50 μL of each effector cell population to be tested to the 96-well flat-bottom plate in duplicate. Add an additional 150 μL DMEM-10 containing 1X AraC to these wells.
6. For the remaining fusion reactions, add 50 μL effector cells to the number of wells needed to test all dilutions of the antisera or inhibitors in duplicate. For example, if testing 10 peptide dilutions, plate 20 wells of effector cells for HeV FC2, 20 wells of effector cells for NiV FC1, and 20 wells of effector cells for ScHeV FC2. A similar approach would be taken for setting up a series of antisera dilutions. At least two wells are also prepared that contain no inhibitors or antisera.
7. Add 100 μL of each peptide or antisera dilution to effector cells from **step 6** in duplicate. Add 100 μL DMEM-10 containing 1X AraC to the control wells receiving no inhibitors or antisera. The total volume in each well should be 150 μL (*see* **Note 26**).
8. Add 50 μL target cells to all remaining effector cell-containing wells from **step 7**.
9. Mix all wells with a multichannel pipet and incubate 2.5–3 h at 37°C in a humidified tissue culture incubator.
10. Following the fusion period, add 10 μL 10% NP-40 solution to each well of the 96-well plate using a multichannel pipet and mix. Avoid bubble formation by not pipetting up the entire sample.
11. Seal the plate with parafilm and place the plastic lid on the plate.
12. Freeze and store at –80°C until ready to proceed to the β-gal assay.

Fig. 5. *(previous page)* Quantitation of NiV envelope glycoprotein-mediated cell fusion. HeLa cells were infected with vaccinia recombinants encoding NiV F, G, both NiV F and G, neither (none), or both HeV F and G, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E. coli LacZ*-encoding reporter vaccinia virus vCB21R. The NiV or HeV glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (1×10^5) in duplicate wells of a 96-well plate. After 3 h at 37°C, cells were lysed and β-gal activity was quantitated. The level of background β-gal activity in target cell populations alone is indicated as target cells only, whereas the β-gal activity from target cells mixed with HeLa effector cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding NiV or HeV glycoproteins is indicated as none. The level of background β-gal activity in effector cell populations alone labeled as effector cells on the *x*-axis. **(A)** Species tropism of NiV-mediated cell fusion. **(B)** NiV-mediated cell fusion with human T-cell and neuroblastoma cell lines as compared to HeV-mediated fusion. Reproduced from Bossart et al. ***(29)***, with permission.

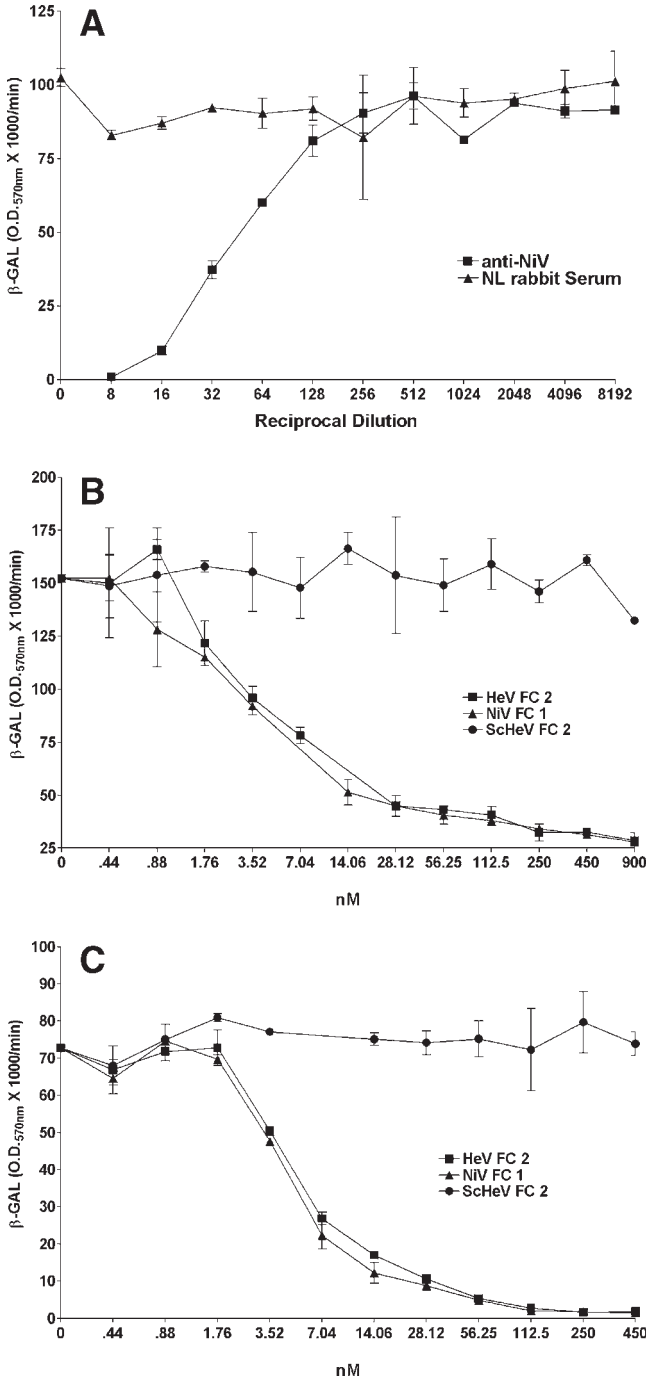
A
β-GAL (O.D.570nm X 1000/min)
125
100
75
50
25
0
anti-NiV
NL rabbit Serum
0
8
16
32
64
128
256
512
1024
2048
4096
8192
Reciprocal Dilution
B
β-GAL (O.D.570nm X 1000/min)
200
175
150
125
100
75
50
25
HeV FC 2
NiV FC 1
ScHeV FC 2
0
.44
.88
1.76
3.52
7.04
14.06
28.12
56.25
112.5
250
450
900
nM
C
β-GAL (O.D.570nm X 1000/min)
100
90
80
70
60
50
40
30
20
10
0
HeV FC 2
NiV FC 1
ScHeV FC 2
0
.44
.88
1.76
3.52
7.04
14.06
28.12
56.25
112.5
250
450
nM

Fig. 6.

13. Follow steps outlined in **Subheading 3.3.** to quantitate the fusion activity by measuring the levels of β-gal synthesized.

The results presented in this section employ U373-MG as the target cells in fusion inhibition assays owing to their capability for the highest levels of cell fusion with both HeV and NiV envelope glycoprotein expressing effector cells. An example of results from these fusion inhibition assays is shown in **Fig. 6**.

4. Notes

1. A stock solution may be prepared ahead of time and stored for 1 mo at –20°C.
2. Preparation of 10X CPRG stock: remove the 10X Z buffer from –20°C and thaw in 37°C water bath; vortex occasionally. Remove one vial (250 mg) of the CPRG substrate from –80°C and add 5.14 mL 10X Z buffer and dissolve completely. Any remaining 10X stock can be stored at –20ºC, wrapped in foil.
3. Ideally, a receptor-negative cell line should be used for effector cells expressing envelope glycoprotein expression.
4. Effectors: The envelope glycoprotein may be linked to either a vaccinia virus promoter or the T7 RNA polymerase promoter. In either case, expression is driven by coinfection with vTF7.3. Alternatively, T7 RNA polymerase may be produced using vTF1-1 (P11 natural late vaccinia virus promoter; ***35***), or any vaccinia virus strain or recombinant may be employed if the viral envelope glycoprotein gene of interest is linked to a vaccinia virus promoter. Choices for the appropriate promoter type for gene expression depends on a number of factors, including desired level of expression ***(47)***, permissiveness of the cell type for the various stages of a vaccinia virus infection, and the level of background activity that the fusion assay can tolerate. For example, whereas the vaccinia virus infects most cell lines, the expression from various vaccinia virus promoters may be hindered in some cell types, especially some primary cells (*see* Chapter 22 for a strategy to overcome such a block).
5. Target cells: The expression of the receptor protein must be high. Thus, use either a vaccinia virus promoter or a T7 promoter. If the T7 promoter is chosen, vTF7.3

Fig. 6. *(previous page)* Fusion inhibition assay. HeLa cells were infected with vaccinia recombinants encoding both NiV F and G glycoproteins or both HeV F and G glycoproteins, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Human U373 cells were infected with the *E. coli LacZ*-encoding reporter vaccinia virus vCB21R (target cells). Peptides or rabbit polyclonal anti-NiV serum were diluted and added to the effector cells (1×10^5) in a 96-well plate. U373 target cells were then added (1×10^5). Each peptide and serum concentration was performed in duplicate in 96-well plate format. After 3 h at 37°C, cells were lysed and β-gal activity was quantitated. **(A)** Inhibition of HeV-mediated fusion by C-terminal synthetic F peptides. **(B)** Inhibition of NiV-mediated fusion by C-terminal synthetic F peptides. **(C)** Inhibition of NiV-mediated fusion by anti-NiV antiserum. Reproduced from Bossart et al. ***(29)***, with permission.

can be used in target cells instead of effector cells. If this is done, then effectors must be prepared using the reporter virus vCB21R. Choices of promoters discussed in **Note 5** also apply here.

6. The necessary number of effector and target cells dictates the flask size necessary for each cell type. For example, if many different target cells are tested, more effector cells should be used. If different envelope combinations are tested, then a larger number of target cells is needed. Generally, a 25-cm^2 flask yields approx 3.5×10^6 cells, a 75-cm^2 flask yields approx 7×10^6 cells, and a 150-cm^2 flask yields approx 10×10^6 cells. Each cell type used varies slightly, and cell numbers must be optimized accordingly. When using different cell lines, plate the cells in the base medium that the cells normally grow in.
7. Cells that grow as suspension cultures (e.g., T-cell lines) may also be used in the fusion assay. Here, the infection of suspension cells with vaccinia virus is performed by first harvesting and counting the cells to be infected by centrifugation, then preparing a suspension of the required number of cells at a density of 1×10^7 cells/ml using the appropriate culture medium containing 2.5% serum in a conical (usually 15-mL sterile disposable) polypropylene tube. The required amount of vaccinia virus (at an moi of 10) is added to the cell suspension, the cells are gently vortexed to mix, and the virus infection is allowed to proceed for 1–2 h at 37ºC in a humidified tissue culture incubator while swirling the cells every 20 min. Following the infection period, 10 mL of the appropriate cell culture medium is added, the cells are transferred to a 50-mL disposable polypropylene tube, and the remaining procedures are as detailed in **Subheading 3.2.1.**, **step 7**.
8. If envelope glycoproteins or cellular receptors are introduced into effector and target cells, respectively, using transfection, plate cells to be 80% confluent after overnight incubation.
9. The 40 m*M* HEPES must have a pH value of 7.4. Small variations in pH decrease the transfection efficiency dramatically.
10. Any alternate transfection reagent or procedure in place of the DOTAP example in this chapter may be employed, along with the corresponding protocols. When different flask sizes are used, the amount of reagents at each step is adjusted accordingly.
11. For all effector and target cell population preparations made by plasmid DNA transfection, use the appropriate base medium for the cell line being used, but adjust the final serum concentration to 2.5%.
12. For both target and effector cell transfections, remember to include a mock transfection as a negative control.
13. All vaccinia virus infections were carried out using a moi of 10 pfu/cell, which insures that all cells are infected with vaccinia virus. Vortexing leads to volume loss, so it is helpful to prepare more than needed. For example, if doing three 25-cm^2 flask effector cell infections after transfection, prepare an inoculum of 40 moi of vTF7.3 in 4 mL, vortex, and then add 1 mL/25-cm^2 flask.
14. If introducing envelopes into effector cells with recombinant vaccinia viruses, it is important to remember to include controls. For example, infect with vTF7.3

alone and no recombinant vaccinia virus as a negative control. An infection with 10 moi of wild-type vaccinia strain Western Reserve (WR; ATCC, #VR1354) can be substituted instead of the recombinant virus to keep the overall moi constant. When performing coinfections, it is important to expose the cells to both viruses simultaneously, e.g., if doing three 25-cm^2 flask target cell infections with recombinant vaccinia expressed receptors, prepare 40 moi. vTF7.3 and 40 moi recombinant virus in 4 mL, vortex, and then add 1 mL/25-cm^2 flask.

15. If introducing receptors into target cells with recombinant vaccinia viruses, it is important to infect with vCB21R alone and no recombinant vaccinia virus as a negative control. Also, an infection with 10 moi of WR can be substituted instead of the recombinant virus to keep the overall moi constant.
16. Avoid the use of polystyrene tubes, which may allow cells to readhere to the inside of the tube.
17. Spray aspirator with 70% ethanol in between aspirating different virus infection groups to minimize crosscontamination of viruses during the washing procedure.
18. Polypropylene tubes are preferred over polystyrene during the overnight incubation stage to minimize cell adherence to plastic. Because low pH induces fusion of vaccinia-infected cells, it is critical to avoid acidification during the overnight incubation. For this reason, the cells are suspended at a cell density below 3×10^5 cells/mL.
19. Overnight incubation is carried out at 31°C to reduce vaccinia virus cytopathic effect (e.g., less trypan blue cells). The lower incubation temperature might be slowing down vaccinia-mediated cell death. This results in better fusion and higher reporter signals.
20. Use DMEM-10 for all effector and target cells from this point on. Count cells after the last wash prior to centrifugation. If the cells clump where they are difficult to count, try resuspending them in 1 mL using a 1-mL pipet man and then increasing the volume as necessary to obtain an accurate count.
21. AraC is included in the assay from this point forward to reduce the background level of any new β-gal expression because of the potential superinfection of target cells (infected with the vCB21R reporter virus) by the T7-polymerase encoding vaccinia recombinant after the two cell populations are mixed. Such unwanted infection could result in β-gal activity that does not represent cell fusion. The inclusion of AraC, which inhibits vaccinia virus DNA replication, prevents expression of proteins driven by intermediate and late promoters. The T7-polymerase protein expressed by vTF7.3 ***(32)*** is under an early/late promoter; thus, it has only a marginal benefit in the presence of AraC. A more pronounced benefit in background reduction may be seen when using vTF1.1 ***(35)***, which employs a vaccinia virus late promoter and, consequently, expression of new T7-polymerase can be inhibited with AraC. Alternate nonionic detergents in place of NP-40 can be used to lyse the cells.
22. Alternate nonionic detergents aside from NP-40 can be used to lyse the cells.
23. Precalculate the amount of 2X CPRG (50 μL/well) needed based on the number of wells to be assayed. Remove the volume of 10X CPRG necessary to make 2X

CPRG and transfer to a new tube. Freeze the remainder of the 10X CPRG stock at –20°C, wrapped in foil.

24. Time-points may need to be adjusted based on the level of the β-gal present in any one fusion assay. 50 μL Cell lysates can be mixed with 2X CPRG again in a new 96-well plate and assayed again if necessary. It is important to make at least three readings prior to the absorbance, increasing to a level that exceeds the plate reader's measuring ability. The amount of cell lysate may also be varied (lowered) using the appropriate diluent to maintain the same concentrations of substrate and detergent in a total volume of 0.1 mL/well.
25. The fusion inhibiting peptides used were derived from the α-helical heptad repeats of either HeV F (HeV FC2) or NiV F (NiV FC1). A scrambled version of the HeV F-derived peptide was used as a negative control (ScHeV FC2). Anti-NiV polyclonal rabbit serum and serum from a naive rabbit were used in the presented example and was set up in the same manner as the peptide inhibitor dilutions added at **step 7**. In this example, the peptides and antisera have been described elsewhere ***(29,30)***.
26. When measuring the inhibitory activities of virus-specific antisera, it is often informative to measure the same dilution series in parallel using naive or normal antisera. Additionally, it is usually better to mix the antiviral antisera and controls with the effector cell population rather than the target cell populations.

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22

Use of Dual Recombinant Vaccinia Virus Vectors to Assay Viral Glycoprotein-Mediated Fusion with Transfection-Resistant Primary Cell Targets

Yanjie Yi, Anjali Singh, Joanne Cutilli, and Ronald G. Collman*

Summary

Fusion mediated by the human immunodeficiency virus type-1 (HIV-1) envelope (Env) glycoprotein and the cellular CD4/chemokine receptor complex is the first step in entry and is often analyzed in cell–cell fusion assays that require Env expression by recombinant vaccinia viruses and/or target cell transfection. Primary lymphocytes and macrophages are the principal targets for HIV-1 in vivo, but are poor substrates for transfection, and constructing recombinant vaccinia viruses expressing every novel or mutant *env* gene is laborious. This chapter describes a fusion assay using two recombinant vaccinia viruses that express distinct RNA polymerases suitable for transfection-resistant targets, such as primary human lymphocytes and macrophages. It also uses *env* genes contained in plasmid vectors, eliminating the need to construct recombinant vaccinia viruses to analyze each construct. Effector 293T cells are cotransfected with SP6-driven reporter gene and T7-driven *env* plasmids, then infected with recombinant vaccinia virus expressing T7 polymerase. Primary lymphocyte or macrophage targets are infected with recombinant vaccinia virus expressing SP6 polymerase. Fusion mediated by effector cell Env and endogenous CD4/coreceptors in target lymphocytes or macrophages enables SP6 polymerase-mediated reporter gene transactivation. This approach provides an efficient tool to study fusion mediated by multiple-cloned primary isolate or mutant HIV-1 *env* genes with the primary target cell types relevant to infection in vivo.

Key Words: HIV-1; envelope; fusion; CD4; CCR5; CXCR4; chemokine receptor; lymphocyte; macrophage; SP6 polymerase; T7 polymerase; vaccinia virus.

1. Introduction

Receptor-mediated membrane fusion is a critical step for enveloped virus entry into target cells. Cell–cell fusion assays have proven to be a valuable

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technique in the study of human immunodeficiency virus (HIV) and Simian immunodeficiency virus (SIV) entry, tropism, and coreceptor utilization ***(1,2)***. Both the discovery that HIV-1 requires the chemokine receptors CCR5 or CXCR4 in addition to CD4 for entry and the identification of chemokine receptor selectivity as the basis for target cell tropism were done in cell–cell fusion assays using heterologous systems, whereby target cells are transfected with a reporter plasmid activated upon fusion with *env* expressing effector cells ***(3–5)***.

Primary lymphocytes and macrophages represent the major physiologically relevant target cells for HIV-1 in vivo. Although chemokine receptor utilization in heterologous systems often predicts use in primary cells, important exceptions exist. For example, it was initially discovered that CCR5 is used as an entry coreceptor by macrophage (M)-tropic prototype isolates that infect primary macrophages and lymphocytes, but not cell lines (R5 strains), CXCR4 by T-cell line (T)-tropic strains that infect primary lymphocytes and lymphoid cell lines (X4 strains), and both chemokine receptors by dual-tropic prototypes that infect all three types of cells (R5X4 strains). These observations led to the expectation that macrophages would express CD4 and CCR5, T-cell lines would express CD4 and CXCR4, and lymphocytes would express CD4 and both coreceptors. However, it was later recognized that macrophages express both CXCR4 and CCR5, and that some X4 strains can use CXCR4 on macrophages, as well as on lymphocytes and cell lines (dual-tropic X4), whereas other isolates use CXCR4 on lymphocytes and cell lines but not on macrophages (T-tropic X4 strains; ***6–9***). Thus, fusion and coreceptor use in primary target cells relevant to infection in vivo is not always predicted by studies in other systems.

Several variations of cell–cell fusion assays have been described, including the protocol in Chapter 21 and **ref. *10***. One common approach uses effector cells infected with recombinant vaccinia viruses expressing *env* and transfected with a reporter gene plasmid under control of the T7 promoter. Target cells are then transfected with CD4/coreceptor plasmids and infected with recombinant vaccinia virus expressing the T7 polymerase. If fusion occurs, cytoplasmic mixing enables T7 polymerase expression in the target cell to transactivate the reporter plasmid in the Env-expressing effector cells. Although this approach is suitable for transfection-resistant targets, such as primary lymphocytes and macrophages that express endogenous fusion receptors ***(2)***, it requires the construction of a recombinant vaccinia virus for each *env* to be tested. An alternative approach involves transfection of effector cells with *env* under control of the T7 promoter and infection with recombinant T7 polymerase-expressing vaccinia virus, which drives *env* expression ***(10)***. Target cells are then trans-

fected with a reporter gene construct under control of the T7 promoter. However, this method requires efficient transfection of target cells; thus, it is not suitable for primary human macrophage or lymphocyte targets.

The approach for analyzing fusogenicity and specificity of HIV-1 described here is based on dual-recombinant vaccinia viruses expressing distinct RNA polymerases and a luciferase reporter system that does not require target cell transfection. This assay also employs HIV-1 *env* genes cloned into plasmid vectors and therefore does not require constructing individual recombinant vaccinia viruses for each *env* ***(11)***. This assay provides a quantitative, sensitive, rapid, and efficient tool to study fusion mediated by multiple primary isolates, chimeric or mutant HIV-1 Env glycoproteins with physiologically relevant primary lymphocyte and macrophage targets.

2. Materials

2.1. Reagents and Supplies

1. Heparin and Ficol-Hypaque (Pharmacia, Piscattaway, NJ) for separation of peripheral blood mononuclear cells (PBMC).
2. Tissue culture flasks coated with 2% endotoxin-free gelatin (Sigma, St. Louis, MO; ***12***).
3. Peripheral blood lymphocyte (PBL) medium: RPMI supplemented with 10% fetal bovine serum (FBS), 600 μg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.
4. Monocyte-derived macrophage (MDM) medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 10% horse serum, 600 μg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.
5. 293T medium: DMEM supplemented with 10% FBS, 600 μg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.
6. MDM wash solution: 10 m*M* EDTA in PBS mixed 1:1 with DMEM containing 20% horse serum.
7. 5 μg/mL phytohemagglutinin (PHA) for PBL activation.
8. Rifampicin (Sigma): 100 mg/mL stock solution in dimethylsulfoxide (DMSO), stored at –20°C (*see* **Note 1**).
9. Cytosine arabinoside (Ara-C) (Sigma): 10 μ*M* stock solution in H_2O stored at –20°C (*see* **Note 2**).
10. FuGENE 6 transfection reagent (Roche Inc., Indianapolis, IN).
11. Lysis buffer: 0.5% Triton X-100 in PBS.
12. Luminescence analysis: luciferase substrate (Promega, Madison, WI); luciferase activity is read using 96-well luminometry plates (Corning Labs, Corning, NY) and a plate luminometer. We use an MLX Microtiter Plate Luminometer, Thermo LabSystems (Franklin, MA).
13. Other supplies: flasks, plates, pipets, screw-cap tubes, microfuge tubes, pipet tips, and 37°C and 32°C CO_2 incubators.

2.2. Recombinant Vaccinia Viruses Expressing Distinct RNA Polymerase

1. Recombinant vaccinia virus vP11T7gene1 (also known as vTF1.1) expresses the T7 RNA polymerase under control of a vaccinia virus strong late promoter (***13***; *see* **Note 3**).
2. Recombinant vaccinia virus $vSIMB_{E/L}$ expresses the SP6 RNA polymerase under control of a synthetic vaccinia virus early/late promoter (***14***; *see* **Note 4**).

2.3. Plasmids Containing *HIV-1* env Genes Under Control of the T7 Promoter (See Note 5)

1. T-tropic X4 prototype IIIB cloned into pSP73 (Promega, Madison, WI).
2. M-tropic R5 prototype JRFL cloned into pCDNA3 (Invitrogen, Carlsbad, CA).
3. Dual-tropic R5X4 prototype 89.6 cloned into pCR3.1 (Invitrogen).

2.4. Reporter Gene Clones

1. SP6-luc reporter plasmid (Promega) contains the luciferase gene under control of the SP6 promoter.

2.5. Effector and Target Cells

1. 293T cells (ATCC, #SD-3515; *see* **Note 6**).
2. Primary human lymphocytes and macrophages: PBL and MDM are derived from PBMC obtained by venipuncture from healthy volunteers (*see* **Subheading 3.1.**).

3. Methods

To assess Env-mediated fusion with lymphocytes, the nonadherent PBL targets are aliquoted and added to individual wells in 24-well plates of Env-expressing effector cells. To assess fusion with macrophages, the Env-expressing effector cells are detached, aliquoted, and added to individual wells of adherent target MDM. *See* **Fig. 1** for scheme of the fusion assay.

3.1. Preparation of Primary Macrophages and Lymphocytes

1. In advance of obtaining whole blood donation, gelatin-coated flasks are prepared by adding 10 mL 2% endotoxin-free gelatin to a T-75 flask, incubating for 2 h in a dry 37°C incubator, aspirating excess gelatin, and returning them to the incubator (caps loose) to dry for at least 2 d (*see* **Note 7**).
2. Heparinized whole blood obtained from normal volunteers (*see* **Note 8**) is mixed with an equal volume of PBS.
3. Of this mixture, 20 mL is layered on 15 mL Ficoll-Hypaque and centrifuged at 4°C for 40 min at 2000*g*.
4. The PBMC layer is collected without further washing, diluted to 2×10^6 cells/mL in serum-free DMEM, and 20 mL is placed in a gelatin-coated T-75 flask (*see* **Note 9**).

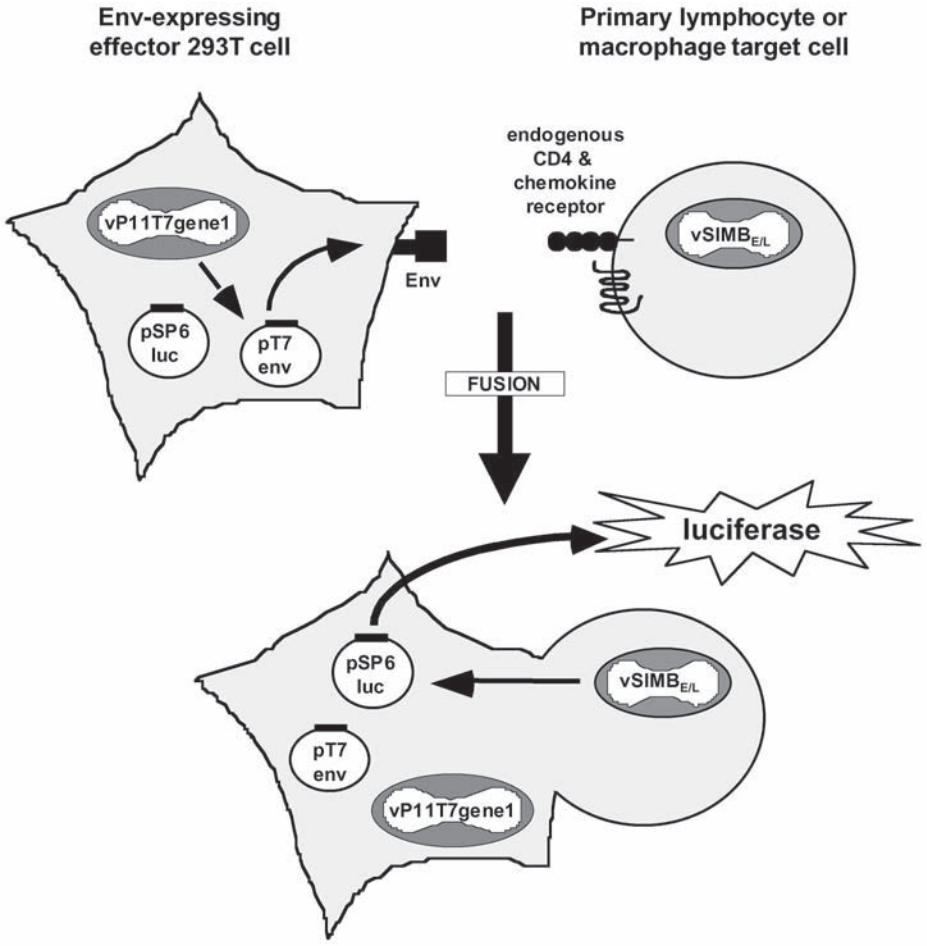

Fig. 1. Scheme of fusion assay. Env is expressed in effector cells by infection with T7 polymerase-expressing recombinant vaccinia virus vP11T7gene1 and transfection with T7-driven *env* gene plasmids. Effector cells are also cotransfected with a plasmid encoding the luciferase reporter gene under control of the SP6 RNA polymerase promoter. Target PBL or MDM are infected with a recombinant vaccinia virus vSIMB$_{E/L}$, which expresses the SP6 RNA polymerase. Mixing of target and effector cells enables fusion to take place if the expressed *env* utilizes coreceptors present on the lymphocytes or macrophages, leading to SP6-driven luciferase expression.

5. After incubation for 1 h at 37°C, the nonadherent cells, which represent mainly PBL, are collected, washed twice with serum-free RPMI, and suspended at 1.5 × 10^6 cells/mL in PBL medium.
6. PBLs are stimulated for 3 d with 5 μg/mL PHA, at which point they may be used (*see* **Note 10**).

7. To obtain MDM, the gelatin-adherent cells from **step 5** (≥96% monocytes) are washed four times with serum-free DMEM and once with 10 mL MDM wash solution.
8. Adherent cells are then detached by 15-min incubation with 10 mL MDM wash solution, washed in serum-free DMEM, and suspended at 5×10^5 cells/mL in MDM medium.
9. Cells are then seeded at 2×10^5 cells/well in 48-well plates and maintained at 37°C in 5% CO_2. Monocytes are maintained in culture for 5–7 d to allow differentiation into macrophages.
10. Prior to use, MDM cultures are washed with PBS to remove any residual nonadherent cells and fed with fresh MDM medium (*see* **Notes 11** and **12**).

3.2. PBL Fusion Assay

3.2.1. Preparation of 293T Effector Cells for PBL Fusion Assay

1. On the day prior to the fusion assay, plate 293T cells in 24-well plates at 2×10^5 cells/well to achieve 80% confluency the next day (*see* **Note 13**).
2. The next day, 293T cells in 24-well plates are then infected for 30 min with vP11T7gene1 at a multiplicity of infection (moi) of 10 plaque-forming units (pfu) per cell.
3. Cells are then washed, replenished with 1 mL fresh medium, and cotransfected with *env* and reporter gene plasmids.
4. For transfection, 100 μL serum-free DMEM is placed in a sterile tube and 6 μL FuGENE 6 reagent is added directly into this medium and mixed gently by tapping (do not vortex; *see* **Note 14**).
5. Then, add 10 μL DNA mixture containing plasmids that encode the SP6-driven reporter gene (1.5 μg) and T7-driven HIV-1 *env* (1 μg). Gently tap and incubate for a minimum of 15 min at room temperature.
6. The DNA/FuGENE 6 mixture is added to 293T cell wells and incubated for 4 h at 37°C.
7. Then, an additional 0.5 mL 293T medium is then added and supplemented with rifampicin at 100 μg/mL final concentration. Effector cells are then incubated at 32°C overnight (*see* **Notes 1** and **15**).

3.2.2. Preparation of PBL Target Cells

1. Two days prior to fusion assay, prepare PBL as described in **Subheading 3.1.**
2. PBLs are suspended at 1.5×10^6 cells/mL and infected for 30 min with $vSIMB_{E/L}$ at a moi of 5 pfu/cell. Cells are then washed once with PBL medium, resuspended in fresh PBL medium supplemented with 100 μg/mL rifampicin, and incubated overnight at 32°C (*see* **Notes 1** and **15**).

3.2.3. PBL Fusion Assay

1. Following overnight incubation of 293T effector cells and PBL target cells, medium is removed from the 293T effector cells and replenished with 0.5 mL fresh 293T medium containing rifampicin and 0.1 μ*M* Ara-C (*see* **Note 2**).

2. Target PBL are washed three times with serum-free RPMI and resuspended at 7.5 × 10^6 cells/mL in PBL medium containing 100 μg/mL rifampicin and 0.1 μ*M* Ara-C.
3. PBL are agitated to generate a uniform cell suspension, and 200 μL containing 1.5 × 10^6 cells are added to each well of *env*-expressing effector 293T cells.
4. Target PBL/effector 293T cell cocultures are then incubated at 37°C for 6 h to allow for fusion and reporter gene transactivation (*see* **Note 16**).
5. After 6-h coculture, the medium containing most of the PBL suspension is collected and transferred to a 15-mL centrifuge tube. Remaining cells are detached with 0.5 m*M* EDTA followed by several washes and added to the suspension cells.
6. Cells are pelleted, liquid is removed by careful aspiration, and cells are lysed by incubation for 5 min at room temperature in 100 μL lysis buffer.
7. Proceed to **Subheading 3.4.** for luciferase read-out.

3.3. MDM Fusion Assay

3.3.1. Preparation of 293T Effector Cells for MDM Fusion Assay

1. On the day prior to the fusion assay, plate 293T cells in 12-well plates at 4 × 10^5 cells/well to achieve 80% confluency the next day (*see* **Notes 13** and **17**).
2. 293T cells in 12-well plates are then infected for 30 min with vP11T7gene1 at a moi of 10 pfu/cell.
3. Cells are then washed, replenished with 1.5 mL fresh DMEM, and cotransfected with *env* and reporter gene plasmids.
4. For transfection, 200 μL serum-free DMEM is placed in a sterile tube and 12 μL FuGENE 6 reagent is added directly into this medium and mixed gently by tapping (do not vortex; *see* **Note 14**).
5. Then add 10 μL DNA mixture containing plasmids encoding the 3 μg SP6-driven reporter gene and 2 μg T7-driven HIV-1 *env*. Gently tap and incubate for a minimum of 15 min at room temperature.
6. The DNA/FuGENE 6 mixture is added to 293T cell wells and incubated for 4 h at 37°C.
7. Then, an additional 0.5 mL 293T medium is then added and supplemented with rifampicin at 100 μg/mL final concentration. Effector cells are then incubated at 32°C overnight (*see* **Notes 1** and **15**).

3.3.2. Preparation of MDM Target Cells

1. Six days prior to fusion assay, prepare MDM as described in **Subheading 3.1.**
2. MDM in 48-well plates are infected for 1 h with $vSIMB_{E/L}$ at an moi of 5 pfu/cell. Cells are then washed once with PBL medium, resuspended in fresh PBL medium supplemented with 100 μg/mL rifampicin, and incubated overnight at 32°C (*see* **Notes 15** and **18**).

3.3.3. MDM Fusion Assay

1. Following overnight incubation of 293T effector cells and MDM target cells, target MDM are washed three times with serum-free DMEM and replenished with MDM medium containing 100 μg/mL rifampicin and 0.1 μ*M* Ara-C.

2. Effector 293T cells (approx 5×10^5 cells/well) are washed with PBS, detached with 0.5 m*M* EDTA in PBS, and then washed two times with PBS and once with 293T medium.
3. The effector 293T cells are then suspended in 400 μL 293T medium containing 100 μg/mL rifampicin and 0.1 μ*M* Ara-C, and 100 μL is added to each well of target MDM in 48-well plates.
4. Target MDM/effector 293T cell cocultures are then incubated at 37°C for 6 h to allow for fusion and reporter gene transactivation (*see* **Note 16**).
5. The medium is then removed and 100 μL lysis buffer is added to each well and incubated for 10 min at room temperature (*see* **Note 19**).
6. Proceed to **Subheading 3.4.** for luciferase read-out.

3.4. Luciferase Read-Out

1. Cellular lysates are transferred to a microfuge tube, cleared of debris by pelleting, and 50 μL of clarified lysate is transferred to the well of a 96-well luminometry plate.
2. An equal volume of luciferase substrate is then added.
3. Luciferase activity measured by plate luminometry (*see* **Notes 20–22** and **Figs. 2** and **3** for examples of results of PBL and MDM fusion assays, respectively).

Fig. 2. *(opposite page)* Fusion with primary PBL mediated by Envs from the prototype macrophage-tropic R5 HIV-1 isolate JRFL, T-cell line-tropic X4 isolate IIIB, and dual-tropic R5X4 isolate 89.6. PBL from a CCR5 wild-type donor is shown on the left, whereas PBL lacking the CCR5 chemokine receptor from a donor homozygous for the nonfunctional ccr5Δ32 deletion allele are shown on the right. The specificity of fusion is confirmed by blocking CD4 using a monoclonal antibody (MAb 19; 5 μg/mL; ***6***) or a small-molecular-weight CXCR4 antagonist (AMD3100; 10 μg/mL; ***20***). Effector cells transfected with empty vector (no Env) served as a negative control. The CXCR4 antagonist and CD4 blocking MAb were added to target PBL for 1 h prior to and during incubation with effector 293T cells. The data show that all three strains require CD4 for fusion with PBL. CCR5 on PBL is used for fusion by both JRFL and 89.6, whereas PBL CXCR4 is used by IIIB and 89.6.

Fig. 3. *(opposite page)* Fusion with primary MDM mediated by Envs from the prototype macrophage-tropic R5 HIV-1 isolate JRFL, T-cell line-tropic X4 isolate IIIB, and dual-tropic R5X4 isolate 89.6. The left two panels show MDM from CCR5 wild-type donors, whereas the right panel shows MDM that lack CCR5 from a donor homozygous for the ccr5Δ32 allele. The CXCR4 antagonist AMD3100 (10 μg/mL) and CD4 blocking MAb 19 (5 μg/mL) were added to MDM 1 h prior to and during incubation with effector 293T cells. The data show that JRFL uses macrophage CCR5 for fusion, 89.6 uses both CCR5 and CXCR4 for fusion, but IIIB fuses poorly with macrophages despite that it uses CXCR4 on lymphocytes for fusion, as shown in **Fig. 2**.

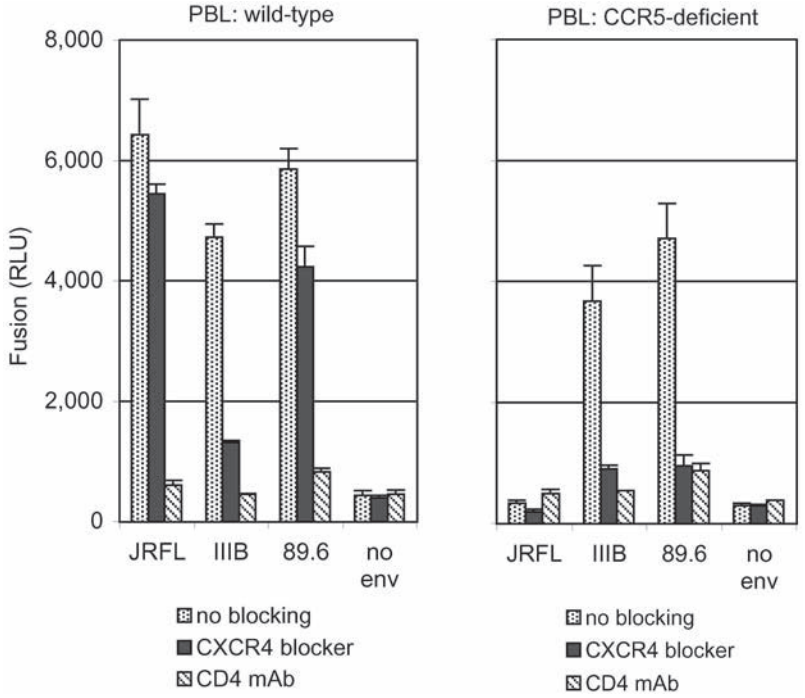
PBL: wild-type
PBL: CCR5-deficient
8,000
6,000
4,000
2,000
0
Fusion (RLU)
JRFL
IIIB
89.6
no env
no blocking
CXCR4 blocker
CD4 mAb

Fig. 2.

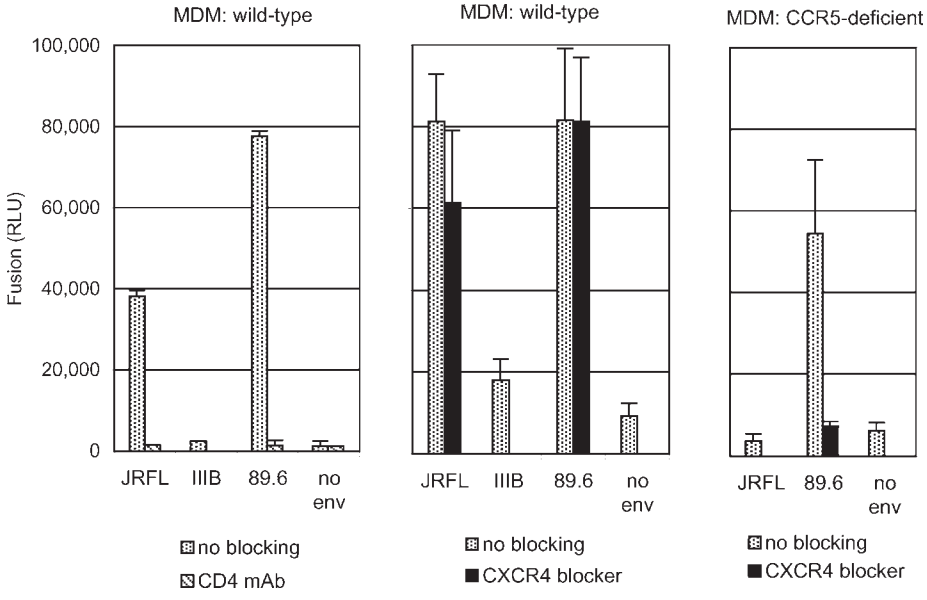
MDM: wild-type
MDM: wild-type
MDM: CCR5-deficient
100,000
80,000
60,000
40,000
20,000
0
Fusion (RLU)
JRFL
IIIB
89.6
no env
no blocking
CD4 mAb
CXCR4 blocker

Fig. 3.

3.5. Controls

It is critical to set up appropriate controls by which the specificity of fusion can be determined. In cell line-based fusion assays where both Env in effector cells and CD4/coreceptors in target cells are introduced by transfection, both Env-negative effectors and receptor-negative targets can be used as negative controls. Similarly, in this assay, effector cells that do not contain Env plasmid (transfected with empty vector) should be used. However, because endogenous CD4 and chemokine receptor expression is responsible for MDM or PBL fusion, blocking CD4 or the chemokine receptor should be included to support the specificity of fusion. CD4 may be blocked by preincubation with anti-CD4 monoclonal antibodies, such as OKT4A (Ortho Biotech, Bridgewater, NJ), Leu3a (Becton Dickinson, Franklin Lakes, NJ), or other blocking antibodies. If blocking CD4 does not block the luciferase activity, it indicates that the activity measured is likely nonspecific, rather than Env-mediated fusion (*see* **Notes 21** and **23**).

4. Notes

1. Rifampicin is used in the assays because it inhibits vaccinia virus assembly, thereby minimizing cytopathic effects that increase background.
2. Ara-C inhibits vaccinia virus DNA replication and transcription from vaccinia late promoters. It is used in the assays to reduce the background.
3. Recombinant vaccinia virus-expressed T7 RNA polymerase enables high-level *env* expression and, because transcription takes place in cytoplasm, they do not depend on expression of the HIV-1 auxiliary protein Rev for *env* mRNA transport. Use of plasmid-encoded *envs* obviates the need to generate a recombinant vaccinia virus for every viral glycoprotein tested and is therefore ideal for rapid analysis of *env* mutants or primary isolate *env* cloned by PCR.
4. Use of a recombinant vaccinia virus, such as $vSIMB_{E/L}$, that utilizes an early promoter for expression in target macrophages is critical, as the vaccinia virus late promoter is not active in primary human macrophages ***(15)***.
5. Essentially, any T7 promoter-containing plasmid vector can be used, including those designed for direct cloning of PCR products (e.g., pCR-blunt; Invitrogen). Many vectors, such as pcDNA3 and pCR3.1, also contain the CMV promoter, but nuclear export of HIV-1 *env* RNA requires coexpression of the Rev protein ***(16)***, so CMV-driven nuclear transcription would not contribute to Env expression, and only cytoplasmic T7-driven expression would lead to fusion.
6. Effector cells for Env expression should be highly transfectable and show minimal cytopathic effect with vaccinia infection. Moreover, the cells must highly express and appropriate glycosylate and cleave the *env* appropriately. We use 293T cells for this purpose.
7. Gelatin-coated flasks may be stored (tightly capped) for up to 2 wk prior to use ***(12)***.

8. Blood donors. Approximately 1% of the European and North American white population lack CCR5 expression owing to homozygosity for a nonfunctional 32-bp CCR5 deletion mutation (ccr5Δ32) and are nonpermissive for R5 HIV-1 strains ***(17)***. If prototype R5 M-tropic Envs fail to fuse with MDM or PBL from a particular donor, the CCR5 genotype can be easily checked by polymerase chain reaction (PCR) for the presence or absence of the Δ32 allele ***(17)***.
9. From a 60-mL blood donation, we typically get approx 6×10^6 MDM and approx 3×10^7 PBL.
10. Other methods of PBL separation or even whole PBMC may also be used. If a more stringently defined population of primary lymphocyte targets is needed, then various methods of T-cell or CD4 cell purification may be performed.
11. If desired, MDM medium may be supplemented with 100 U/mL M-CSF to enhance maturation.
12. Other methods of monocyte purification, such as elutriation, are equally suitable.
13. One confluent T-75 flask of 293T cells yields approx 2×10^7 cells. Effector cell density is critical. Do not use cells more than 80% confluent at the day of transfection.
14. FuGENE 6 transfection should be done in strict accordance with the manufacturer's instructions. Do not allow FuGENE 6 reagent to come in contact with plastic surfaces other than the pipet tip. Do not try to aliquot the FuGENE 6 reagent. FuGENE 6 should be diluted in serum-free medium before mixing with DNA. It is not necessary to remove the FuGENE 6/DNA mixture as the FuGENE 6 has very low toxicity in the cells.
15. Incubation at 32°C is carried out to reduce vaccinia virus cytopathic effect, as well as to minimize new vaccinia virus production.
16. The combination of viral inhibitors, overnight incubation at 32°C, and extensive washing prior to mixing helps reduce background luciferase activity that might result from infection of effector cells by residual or newly synthesized SP6 polymerase-expressing vaccinia virus.
17. Prepare the number of effector wells needed by calculating that each well can be used with three to four wells of target MDM.
18. As mentioned in **Note 1**, rifampicin inhibits virus assembly. However, although mature MDM can be infected by vaccinia virus and express early viral proteins, there is a block in DNA replication ***(15)***, resulting in no new progeny virus. Thus, the addition of rifampicin to vaccinia-infected MDM is likely unnecessary, but we add it to keep the reagents in the assay consistent.
19. All cells should be adherent at this point; thus, do not worry about cells in the medium.
20. The absolute level of fusion seen in different experiments varies, likely owing to different coreceptor and/or CD4 expression levels on different donors' primary cells ***(18)***. Generally, the levels of luciferase expression generated in PBL fusion assays are lower than those seen in MDM tested in parallel, which may be due to the result of the less efficient contact between suspension PBL targets and 293T effector cells.

21. The most common difficulty encountered in fusion experiments using primary cells is high background. Many factors may contribute to nonspecific reporter gene expression. Most commonly, background luciferase is a result of residual vaccinia virus or leaking of vaccinia virus in target cells, allowing direct infection and transactivation of effector cells. Thorough washing at each step and the inclusion of rifampicin and Ara-C are therefore critical. Negative controls must be included, such as effector cells infected with T7 polymerase-expressing vaccinia virus and transfected with reporter gene plus empty vector lacking *env*. This control should be considered as the background. Positive controls should include prototype Envs known to use the coreceptor of interest on the target cell being tested.
22. Another possible scenario is low or absent signal. The most likely problem is poor effector cell transfection efficiency. The quality of plasmid DNA is critical and should be confirmed by agarose gel electrophoresis. Preparation by the Qiagen method (Qiagen, Valencia, CA) works well in our experience. As mentioned in **Note 14**, FuGENE 6 protocols should be followed precisely, and inadvertent adsorption of the reagent by exposure to plastic should be avoided. Avoid using excessively small tubes for FuGENE 6 dilution and mixing with DNA, as this may lead to inadequate mixing and inefficient transfection. Vaccinia virus is rarely the problem, but if low signal persists, the virus should be retitered to ensure that the infection is actually at the desired moi. Mycoplasma contamination of effector cells should also be considered.
23. Blocking CD4 to confirm fusion specificity is described in **Subheading 3.5.** CXCR4 or CCR5 may also be blocked, but they are more difficult to target than CD4. Although CXCR4 can be blocked in some contexts using MAb 12G5 (***19***; Pharmingen, San Diego, CA) or the chemokine SDF-1α (available from Peprotech, Rocky Hill, NJ), we have not found these particularly efficient at blocking MDM and PBL fusion. Rather, we find the small molecule antagonist 5 μg/mL AMD3100; ***(20)*** is quite efficient, although this is not commercially available. The anti-CCR5 monoclonal antibody 2D7 (Pharmingen) and chemokines MIP-1α, MIP-1β, and RANTES (Peprotech) may block CCR5 coreceptor function in some contexts, but we have not found these to be useful in blocking cell–cell fusion. Rather, we use cells derived from donors who naturally lack CCR5 expression owing to homozygosity for the ccr5Δ32 deletion allele ***(6,7,17)***. Only approx 1% of the European white population carry this genotype. Small molecule CCR5 antagonists have been described, but either they have been unsuccessful in blocking fusion in our studies ***(21)*** or have yet to be available to us to test for PBL or MDM fusion inhibition ***(22)***.

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23

Poxvirus Bioinformatics

Chris Upton

Summary

Biochemical and functional analysis of poxvirus genomes, genes, and proteins has entered a new era with the recent sequencing of more than 30 poxvirus genomes. The management and analysis of this volume of sequence data in an efficient and effective manner requires specialized computer software. This chapter describes a number of bioinformatics techniques useful for analyzing poxvirus genomes. Some of the software discussed here have been developed by members of the Poxvirus Bioinformatics Resource Center (PBRC; funded by National Institutes of Health [NIH]) specifically for use with poxvirus genomes. These programs or, more accurately, suites of programs have many functions dedicated to poxvirus genome characterization. Significantly, this software has been designed with ease of use at a single location as the major goal.

Key Words: Poxvirus; vaccinia virus; smallpox; bioinformatics; genomics; dotplot; multiple alignment; POCs; VOCs; VGO; BLAST; NAP; saturated BLAST.

1. Introduction

Since the first complete poxvirus genome was announced in 1990 ***(1)***, there has been an explosion in the accumulation of DNA sequence data; more than 30 poxvirus genomes have been reported (*see* **Note 1**). Additionally, there is a series of variola virus strains sequenced by the Centers for Disease Control (CDC), Atlanta, GA (J. Esposito, personal communication). Organizing and manipulating the data contained in genomes that can be more than 200 kb, and the bioinformatic analysis of these genes and proteins, is not trivial and is often very time-consuming. The correct choice of computer software can make the process much more efficient and permits researchers to ask a variety of otherwise intractable questions. All the software described here is free to academic research institutes and most runs on Windows, Macintosh, and LINUX operating systems or via a WWW interface. In these examples, the author has chosen software that is relatively straightforward to use; this review focuses on

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software available at the Poxvirus Bioinformatics Resource Center (PBRC; http://www.poxvirus.org/) because it is a comprehensive site with updated and corrected genome information. We have designed the Viral Genome Organizer (VGO) and Poxvirus Orthologous Clusters (POCs) programs specifically for use with poxvirus genomes, but they can be used for other viruses, including intron-containing viruses, such as herpesviruses, adenoviruses, and retroviruses [to reflect this general utility, POCs is being renamed VOCs (Virus Orthologous Clusters)]. The VGO software has been designed to handle multiple genomes and also much larger DNA sequences, such as those found in bacterial genomes.

The goal of this chapter is to introduce readers to numerous techniques for characterizing poxvirus genomes, including: (1) comparison of entire poxvirus genomes; (2) comparison of poxvirus genes and proteins within orthologous families; (3) similarity searching of poxvirus proteins against databases for functional prediction; and (4) analysis of poxvirus proteins for motifs and domains for function prediction. Additionally, this chapter examines techniques to work with complete genomes, simplifying and speeding up procedures, as one of the more difficult problems the researcher faces is how to organize the large amount of data in more than 30 poxvirus genomes. Although it is impossible to go into great detail for all the software mentioned, help files and manuals are available and detailed instructions are provided on how to access the software used through the PBRC site (*see* **Note 2**).

2. Materials

1. The user must have access to a reasonably up-to-date computer (*see* **Note 3**) and operating system, such as a G4 Macintosh with OS X (*see* **Note 4**), Pentium IV with Windows 2000, or either type of machine running a LINUX operating system, e.g., Red Hat 7.2.
2. We have preferred the client–server format (*see* **Note 3**), where a copy of the software is automatically loaded onto the user's computer. This is preferred because of the greater flexibility and functionality it allows in the client software (*see* **Note 5**), and it also overcomes the problem of supporting a large variety of WWW browsers.
3. Key software:
   a. *ARTEMIS*: a JAVA (*see* **Note 6**) program for helping with DNA sequence annotation ***(2)***. It runs on multiple platforms and is available from http://www.sanger.ac.uk/Software/Artemis/.
   b. *DOTTER*: runs on UNIX and Windows systems ***(3)***. We have developed a JAVA client–server version of DOTTER (JDOTTER; unpublished) that interfaces with a database containing result files from all poxvirus genomes plotted against each other (PBRC website or http://athena.bioc.uvic.ca/pbr/dotter). JDOTTER can also produce dotplots for protein sequences and multiple DNA or protein sequences.

c. *LAJ*: local-alignment JAVA ***(4)***. Although the program can be installed on most computers and used locally (http://globin.cse.psu.edu/dist/laj/laj_readme.html/). PBRC provides a WWW interface to the poxvirus genomes aligned by LAJ to simplify its use.
d. *NAP*: a global nucleotide to amino acid alignment program ***(5)***. This program runs on UNIX machines, but we have recently developed a JAVA client–server version of this program and incorporated it into POCs and VGO, as well as having developed a stand-alone client that is available at http://athena.bioc.uvic.ca/pbr/nap/.
e. *POCs*: SQL database (*see* **Note 7**) of information derived from all complete annotated poxvirus genomes. A powerful, but easy-to-use interface has been built to query the database. It can be accessed via the PBRC website or directly at http://athena.bioc.uvic.ca/pbr/pocs/.
f. *Saturated BLAST* runs on UNIX operating systems using an X-Windows interface. Computer accounts that permit access to a PBRC machine running this software are available through the PBRC.
g. *VGO*: software for visualizing complete poxvirus genomes. The program allows access to protein–DNA sequences from a genome and preprocessed result files from a variety of analyses. It can be accessed via the PBRC website or directly at http://athena.bioc.uvic.ca/pbr/vgo/.

3. Methods

3.1. Connecting to the PBRC Website

The PBRC website is reached by pointing an Internet Browser to http://www.poxvirus.org. It is well worth spending time browsing this site, but most of the software discussed here can be found as follows:

1. Click on the *Analysis* link (in the main header menu).
2. Click on the *Genome Analysis* link.
3. Click on the *POCs, VGO, LAJ*, or *JDOTTER* links.
4. Follow links to *Launch* button.
5. Some programs require JAVA-Web Start to be installed on the local computer, which is built into the Macintosh OS X operating system, but does not run on previous versions of the Macintosh OS (*see* **Note 4**). On other operating systems, you will be alerted if you need to install JAVA-Web Start (*see* **Note 8**).
6. Follow the automatic links to the SUN computer web site and install the software (*see* **Note 8**).

3.2. Organizing Poxvirus Genome Data

Although a 200–300 kb genome is small by current sequencing capacities, analysis of the information contained within a single poxvirus genome, regardless of the 30 such genomes, requires specialized bioinformatics tools. Among the programs we have developed for the characterization of poxvirus genomes,

two described here have the specific goals of (1) providing a dedicated database to organize and store poxvirus sequence data and (2) giving users intuitive interfaces to this database and other comprehensive analysis programs, eliminating the need to access multiple websites. These two programs are described first because several other sections of this review refer to the analyses that they can perform.

3.3. Poxvirus Orthologous Clusters

POCs ***(6,7)*** is a JAVA client–server application that accesses a large, up-to-date, MySQL database containing all complete and fully annotated poxvirus genomes. The database is administered by the author's laboratory and is available to all researchers via the PBRC website or directly at http://athena.bioc.uvic.ca/pbr/POCs/pocs.html/. One of the key features of POCs is that the poxvirus genomes are automatically processed to place genes into orthologous gene families based on BLASTP (protein query search against a protein database) scores that are then assessed by a human database curator. The POCs database also stores complete corrected GenBank files, individual gene, protein and promoter region sequences, promoter designation (if known), annotations, molecular weights, isoelectric points, nucleotide and amino acid (aa) frequencies, codon usages, and BLASTP scores. Many of the poxvirus GenBank sequence files contain errors in annotations and DNA sequences; we update our database as soon as we become aware of any corrections to these files. Therefore, POCs is the most current source of poxvirus genomic information. Currently (*see* **Note 1**), the database contains 30 complete poxvirus genomes with 6625 predicted genes or gene fragments. These predicted genes have been grouped into 792 families (requiring a representative from two or more different viruses) that have been given names and functions when known. Among the applications integrated into POCs are: PSI-BLAST (position-specific iterative BLAST), BLASTP, BLASTX (DNA query translated and searched against a protein database), TBLASTN [protein query search against a DNA database (translated to six reading frames on the fly; ***8***)]; LAJ (local alignment JAVA; ***9***); Base-By-Base (unpublished); JDOTTER ***(3)***; and NAP (for comparing a protein against a DNA sequence; ***5***). The BLAST searches are run against the sequences in the POCs database, which simplifies the results by restricting them to poxvirus sequences. Because the POCs database is relatively small, the searches are run interactively and are very fast. However, from within POCs, users can link to a separate database that the author's group maintains of BLAST search results of each protein when compared to the National Center for Biotechnology Information Nonredundant (NCBI NR) protein and expressed sequence tag (EST) DNA database. These

Table 1
Examples of Queries that Can Be Performed with POCs

Window	Query
SQ	Find genes with names that match A1*
SQ	Find genes with DNA sequence containing a given sequence of characters
SQ	Find proteins with 4.5 < pI < 6.5
SQ	Find proteins with 25,000 < MW < 30,000 Dalton
SQ	Find proteins with *serine* content greater than *13%*
SQ	Find genes with *leu + ile + val + ala > 40%*
GFA	Find the gene family containing EV gene X
GFA	Find a specific gene family or group of gene families by annotation
GFA	Find genes matching a query protein with a BLAST score of more than 50
GFA	Find gene families that contain a gene from all orthopoxviruses
GFA	Find gene families present in myxoma virus, but absent from SFV

SQ, Sequence Query; GFA, Gene Family Analyzer.

search results are updated at least monthly. This allows a user to browse through BLAST search results very rapidly without waiting for the searches to actually run.

Each time a user opens the POCs program, JAVA-Web Start automatically checks the version of the client on the user's machine and downloads an updated version if required. POCs, like VGO, was specifically designed to be used by molecular biologists. The POCs interface makes it simple for researchers to make a variety of otherwise complex SQL database queries easily and rapidly. Two windows are used to navigate through the POCs program: the Sequence Query window and Gene Family Analyzer window. The Sequence Query window allows users to search the database for genes based on such parameters as gene name, size, pI, NCBI protein ID, as well as nucleotide and amino acid sequences and constraints. The Gene Family Analyzer window allows users to search the database based on family name, ID, numbers of genes per family, and specific annotations. **Table 1** shows some example queries that can easily be made within POCs. Once a database query has been selected, the results of the query can be seen as a Gene Count or Family Count (*see* **Note 9**), in which case only the number of hits in the database is displayed, or the list of genes and families can be viewed in a new window. The count function is used to check the query and prevent the accidental download of large numbers of genes, which would waste time. For an additional safeguard, POCs asks users to confirm that they want to display more than 1000 genes. The Gene and Family Results Tables can be sorted, and the results can be graphed in various formats. The genes and families of genes are also available for display or fur-

ther analysis using the aforementioned tools. It is quite simple to select a gene family, then view all members of that family. Their protein, DNA, or promoter region sequences can be selected and put into a multiple alignment, or particular sequences can be used to search the POCs database using any of the BLAST programs.

For users wishing to set up their own database, there is also an administrative client program that offers additional functions over the client program to allow for database management. These functions include: adding a genome and all its genes into the database from a given GenBank format file; deleting or modifying genomes and/or genes; assigning genes to gene families based on user-assigned BLASTP *E*-values; and editing, deleting, or modifying user notes.

3.3.1. Example 1: Generate a Multiple Alignment of Poxvirus Uracil DNA Glycosylase Proteins

1. To select the gene family, open POCs as described in **Subheading 3.1.**
2. Click on *Gene Family Analyzer* tab at the top of the window.
3. If you know the gene name, in the *Gene in Family Query* section, select the checkbox at the left and select virus *VV_Cop* from the pull down menu and gene *VV_Cop-D4R* (uracil DNA glycosylase).
4. If you do not know the specific gene name, scroll down to the *Gene Families* section, select the checkbox (select only *Gene Families*), and select *Uracil-DNA glycosylase* from the alphabetized *gene family names* menu.
5. To make an alignment, click on *Family View* button, select the gene family by clicking on the family in the table, click on the *Align* button at the bottom of the window, and select alignment program of your choice.
6. Or, if you want to make an alignment of a subset of the family, click on the *Gene View* button, select the proteins to be aligned by clicking on the rows of the table using shift or control (Macintosh apple) key to select several genes. From *Alignment* menu, choose *Protein Sequence Alignment* and the type of alignment (use T-coffee for more distantly related proteins).
7. The alignment is returned in a Base-By-Base Lite window (*see* **Notes 10** and **11**).

3.3.2. Example 2: Find Gene Families Present in Myxoma Virus but Absent from SFV

1. Open POCs as described in **Subheading 3.1.** or go to the *Select* menu and *Clear All* to clear any previous search parameters.
2. Click on *Gene Family Analyzer* tab at the top of the window.
3. Scroll down to the *Family Query* section, select checkbox for *"Select gene families that…"*
4. Select *Contain* radio button, select *MYX from* menu, and click *Add Criteria* button below this.
5. Select *AND* operator radio button at the right side and click *Add Operator* button.

6. Select *Do NOT Contain* radio button, select *SFV from* menu, and click *Add Criteria* button. Your query will show in the window.
7. Click on *Family Count* or *Family View* button (*see* **Note 9**). The search requires only a few seconds (*see* **Note 12**).

3.4. The Viral Genome Organizer

VGO ***(10)*** was originally based on Genotator ***(11)***, an annotation workbench designed for the analysis of eukaryotic genomic sequences, but has been extensively redesigned and now uses a JAVA interface instead of an X-Windows/Perltk interface and connects to a MySQL database (e.g., POCs) instead of using flat files. VGO provides a simple-to-use graphical interface to complete poxvirus genomes. It manages large amounts of information, including the complete genome sequence and all gene and protein sequences. Also, it can display: (1) coding regions designated in the genome GenBank file; (2) computer-predicted open-reading frames (ORF) of any user-selected size; (3) all start and stop codons; (4) search results for restriction sites or any other subsequence defined in a regular expression (e.g., TTTTTNT); (5) graphs of nucleotide composition; and (6) results from a user-defined input file (*see* **Note 13**). VGO also permits the user to quickly display gene and protein sequences, as well as the DNA sequence of any selected region of the genome. These sequences are all available in windows that allow the user to copy the sequences for pasting into other analysis programs.

An important feature of VGO is that it shows a graphical representation of most of the numerical data in the POCs database (e.g., amino acid composition of all proteins in a genome or the level of conservation of the genes among all poxvirus genomes; **Fig. 1**). VGO can be used to access the same prerun BLASTP and PSI-BLAST searches against the NCBI databases that were described previously. The program also provides an interface to MVIEW ***(12)***, which generates colored multiple alignments of these BLAST results. Such alignments are extremely useful for identifying weak similarities between protein sequences (*see* below).

VGO can also display multiple genomes at once, limited only by screen "real-estate," so that related genomes can be more easily compared. The *autohighlight related genes* feature highlights orthologs of any selected gene in the other viral genomes being displayed. When comparing multiple genomes, it is convenient to label the genes by their POCs family number because orthologous genes in the multiple viruses then appear with the same number (although these family numbers may appear unfamiliar). For example, this solves the problem that the DNA ligase gene is called A50R, J4R, K4R, 163R, 148, 171, and 192 in a series of orthopoxviruses, but they are all in the same POCs family, #1159.

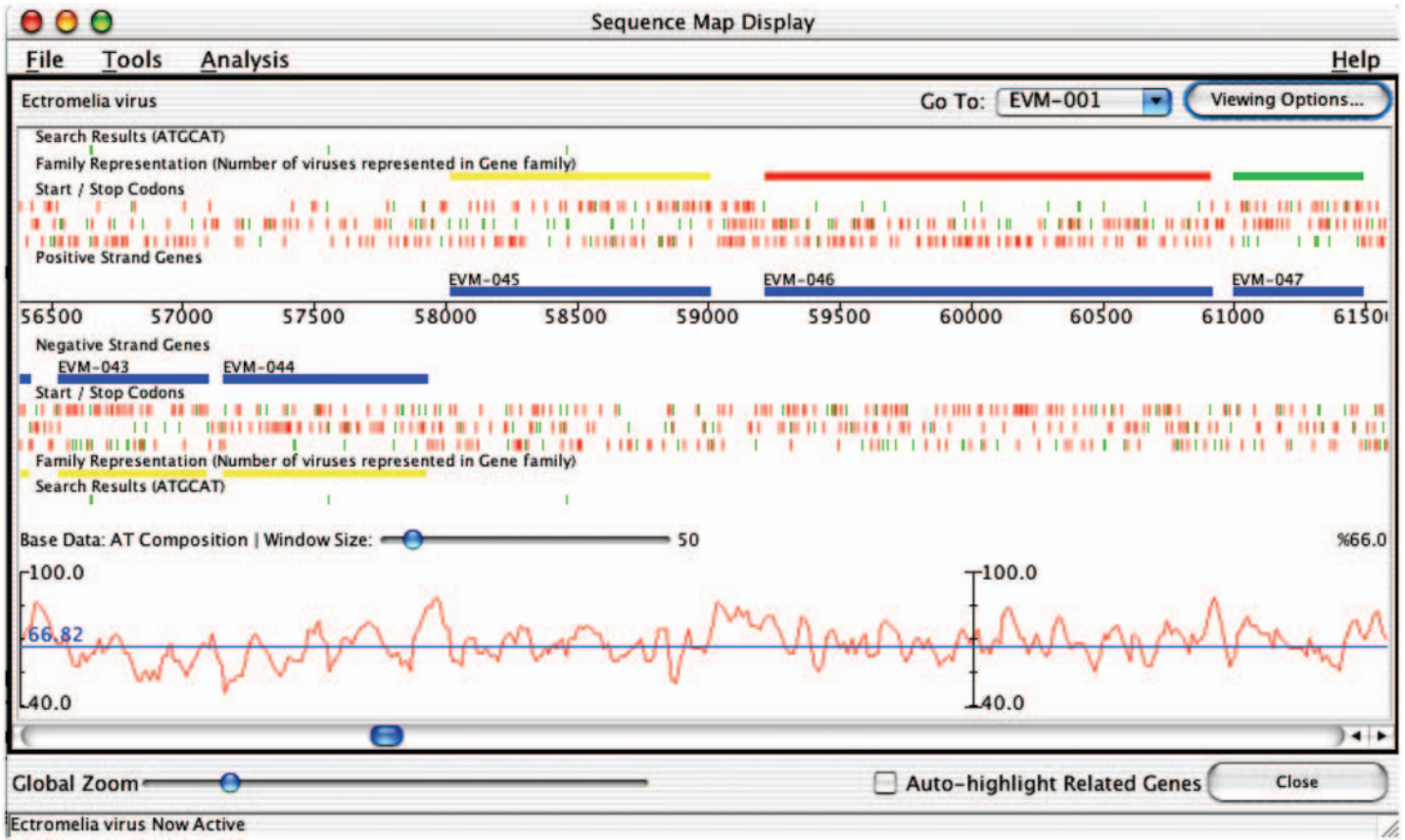
Sequence Map Display
File
Tools
Analysis
Help
Ectromelia virus
Go To:
EVM-001
Viewing Options...
Search Results (ATGCAT)
Family Representation (Number of viruses represented in Gene family)
Start / Stop Codons
Positive Strand Genes
EVM-045
EVM-046
EVM-047
56500
57000
57500
58000
58500
59000
59500
60000
60500
61000
Negative Strand Genes
EVM-043
EVM-044
Start / Stop Codons
Family Representation (Number of viruses represented in Gene family)
Search Results (ATGCAT)
Base Data: AT Composition | Window Size:
50
%66.0
100.0
66.82
40.0
100.0
40.0
Global Zoom
Auto-highlight Related Genes
Close
Ectromelia virus Now Active

Fig. 1.

3.4.1. Example 1

Determine if a large (1-kb) noncoding region in Ectromelia virus (EV) is actually fragmented genes present in cowpox virus (*see* **Note 14**).

1. Open VGO as described in **Subheading 3.1.**
2. From *File* menu, choose *Open* and select both *Ectromelia virus* and *Cowpox virus* (*see* **Note 15**); click OK.
3. From the *Organism List* (pink bars in VGO window), click on the two viruses for analysis (*see* **Note 15**).
4. From *View* menu, choose *Sequence Map.*
5. If stop/start codons are not visible by default, use the *Viewing Options* button to show them for both viruses. Stretch the windows vertically to see all frames for the two viruses (*see* **Note 16**).
6. In the EV window, use the *Global Zoom* scroll bar to zoom until genes can be viewed as a reasonable size and gene names appear (*see* **Notes 16** and **17**).
7. In the EV window, use the *Go To* menu to move to gene 028.
8. Select the *Auto-highlight Related Genes* box at the bottom of the window.
9. Click on the EV gene 028 to select it (color changes to light blue and the area is shaded).
10. Scroll along the cowpox genome until the orthologous-highlighted gene (light blue) appears. (In this example, this turns out to be gene 051.)
11. Manually align the two genes in the CPV and EV windows.
12. Click on EV gene 029, and VGO shows that this is equivalent to cowpox 053. (This suggests that the EV 1-kb region between 028 and 029 may be homologous to the cowpox 052 gene.) Test this as follows.
13. Open NAP from the VGO *Tools* menu.
14. Double click on the cowpox 053 gene to display the protein sequence and copy it into the *Protein Sequence* section of the *NAP* window using standard *copy/paste* commands of your operating system.
15. Open *Genome Subsequence* from the *VGO View* menu. A new window appears.
16. Use your cursor to drag a box over the region of the EV genome between gene 028 and 029. (This fills in the correct coordinates into the *Genome Subsequence* window.)
17. Click the *Display* button in the *Subsequence Grabber* window and copy the DNA sequence into the *DNA Sequence* section of the *NAP* window.

Fig. 1. *(previous page)* The sequence map display of a portion of EV (Moscow strain) genome in VGO. Moving up (top strand) and down (bottom strand) from the nucleotide scale close to the center of the window are: (1) coding regions (blue) annotated in the GenBank file; (2) start/stop codons for three frames (red—stop, green—start); (3) family representation, bars are color-coded to represent how many genomes contain an ortholog (red bar for gene 046 indicates that all poxviruses contain this gene); and (4) location of ATGCAT subsequences within genome. The line graph at the bottom of the window displays AT content along the genome.

18. Click the *Submit* button.
19. Scroll through the NAP alignment of the DNA and protein sequences. Aligned positions are shown as dots between them and gaps are indicated with dashes (*see* **Note 18**).

3.5. Comparison of Poxvirus DNA Sequences

An indispensable tool for the comparison of large DNA sequences is the dotplot. Each nucleotide or small window of nucleotides of one sequence is compared to every nucleotide of another. For analysis of poxvirus genomes, the software must be able to handle DNA sequences of in excess of 300 kb. We have found that DOTTER ***(3)*** is an extremely effective tool because after the plot is performed, it permits the user to change parameters in real time while viewing the plot. It is also possible to zoom into particular regions of the dotplot by pointing the cursor over the region, which results in recalculation of the plot in that small region. **Figure 2** shows a dotplot comparison of the genomes of the variola virus (Bangladesh strain) and monkeypox virus (Zaire strain). The regions of high similarity, as well as gaps in the alignment, are immediately obvious. The greyramp tool (**Fig. 2**; inset) is used to rapidly change scoring parameters without the need for recalculation of the complete dotplot. An alignment tool is also available (not shown), which displays a continuously scrollable window that shows the alignment of the two DNA sequences at any point in the plot chosen by the user. Dotplots are very useful for detecting direct and inverted repeats. Part of the viral inverted terminal repeats are visible in the top right of dotplot in **Fig. 2**. Users of dotplots should be aware that the resolution of the plot is low (related to the number of nucleotides/screen pixel) when large sequences are compared; therefore, to get a good sense of fine-sequence similarity from a dotplot, it is necessary to zoom in. We have created a new interface for DOTTER (JDOTTER) that permits it to be used as a graphical display in the POC and VGO packages with gene or protein sequences or genome segments selected by the user.

Local Alignment Java (LAJ; http://globin.cse.psu.edu/) produces a dotplot-like graph, but positions of local alignments are the datapoints that are marked in the window. These local alignments are calculated by a version of the BLAST algorithm ***(8)*** and may be short or long, reflecting the degree of similarity between the two sequences being compared. A LAJ plot of the left ends (approx 25 kb) of the variola virus (Bangladesh strain) and monkeypox virus (Zaire strain) genomes is shown in **Fig. 3**. Underneath the main plot window, there are the following sections: (1) thick bars representing the ORFs and DNA sequence (mousing-over these bars displays the name of the ORFs at the top of the window and clicking a bar opens the corresponding NCBI sequence www page); (2) a percent identity plot (PIP), where the scale of the PIP is 50–100%;

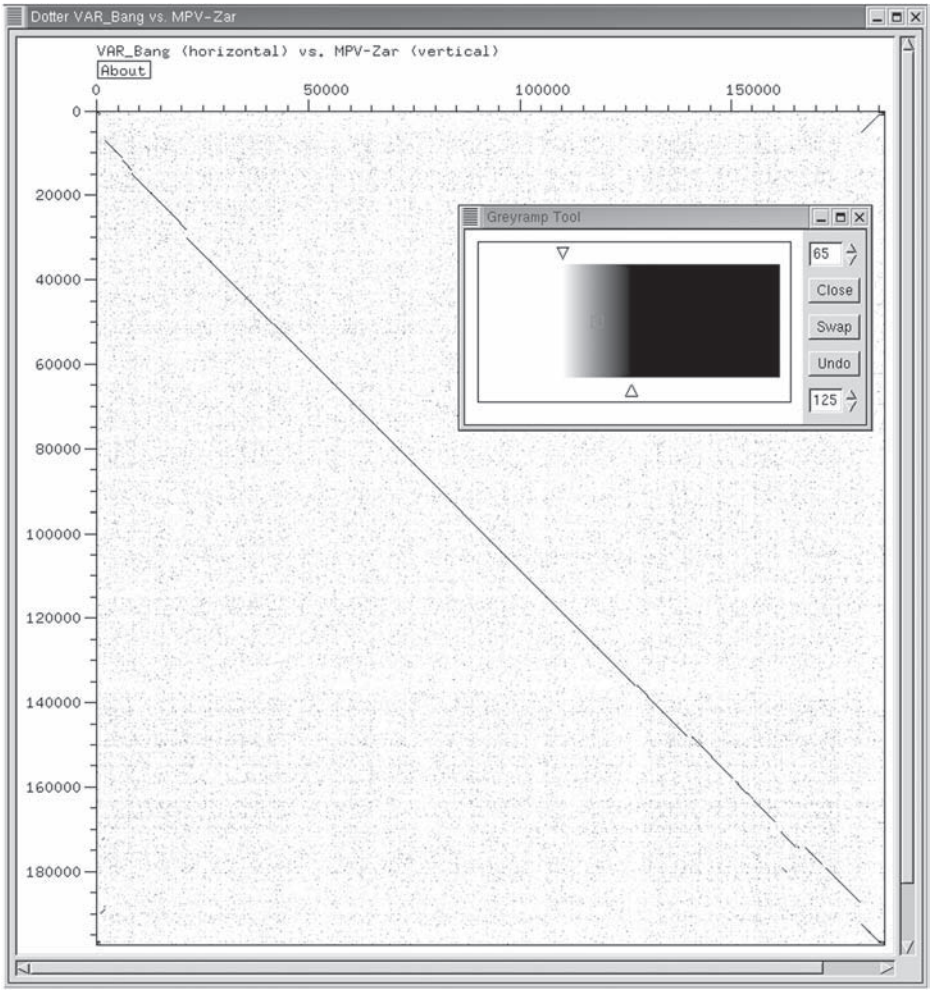

Fig. 2. Dotplot of complete genomes (DNA) of variola virus (Bangladesh strain) vs monkeypox virus (Zaire strain). Inset shows the *Greyramp Tool* for changing scoring parameters.

(3) DNA sequence alignment from a particular local alignment selected in the top widow. When using LAJ, it is important to use all of the information provided by the three windows, because unlike DOTTER output, the main window does not provide information on the degree of similarity, only the size and position of the local alignments. The similarity information is provided by the PIP. An advantage of LAJ and the PIP is that it is possible to have an appreciation of the similarity of two large DNA sequences without zooming in.

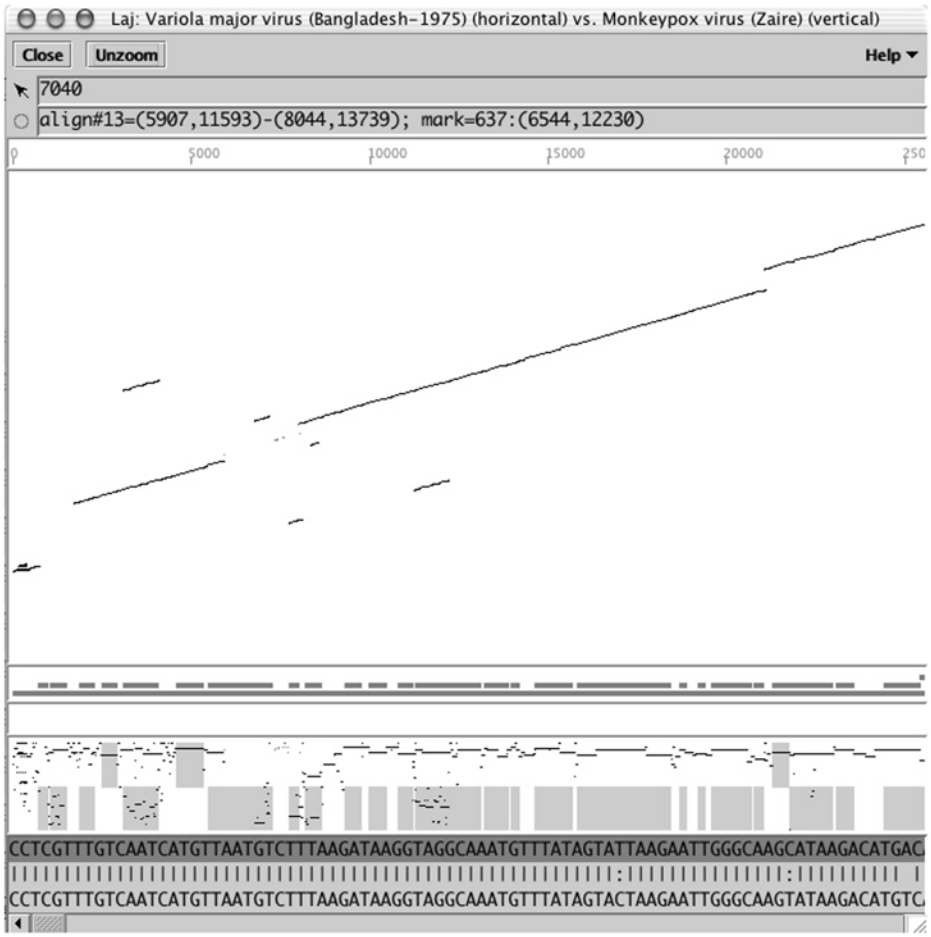

Fig. 3. LAJ plot of the left ends (approx 25 kb) of the variola virus (Bangladesh strain) vs monkeypox virus (Zaire strain) genomes. Under the main plot window, there are: (1) thick bars representing ORFs and DNA sequence; (2) a PIP, the scale of the PIP is 50–100% (Thus, most of the two sequences are more than 90% identical as shown by the horizontal lines across the top of the PIP); and (3) DNA sequence alignment from a particular local alignment selected in the top window.

The LAJ program (http://globin.cse.psu.edu/dist/laj/laj_readme.html/) can be installed on most computers and used locally; however, this is not an easy process. Therefore, PBRC has generated LAJ plot data of all the pairwise combinations of complete poxvirus genomes and has made these available through a simple Web interface (http://www.poxvirus.org/laj.asp). Users simply have to select the two sequences they wish to compare from menus, the data and the

JAVA program (also called an applet) load automatically, and the plot is displayed on the user's local computer. LAJ is also available through a menu in POCs.

Yet another useful way to display similarities between poxvirus genomes is with Synteny plots. These are scatter diagrams of protein orthologs shared between pairs of virus genomes. **Figure 4** shows the result of a comparison of variola virus (Bangladesh strain) and monkeypox virus (Zaire strain) performed using software with a Web interface at the PBRC (*see* **Note 19**). The program permits a stringency parameter to be set to determine a percentage similarity cut-off for display of protein pairs.

3.6. Annotation of Poxvirus Genomes

Having determined the DNA sequence of a poxvirus genome, there remains the equally challenging problem of annotating the genome. The level of difficulty is inversely proportional to the similarity of the genome to other previously properly annotated sequenced genomes. Although poxvirus genes do not contain the multiple introns that complicate the prediction of many eukaryotic genes, one of the most difficult problems (which is exacerbated in GC-rich genomes because of the low frequency of stop-codons in noncoding sequence) is to correctly designate an ORF as a gene.

Many genes are simple to annotate because they are highly conserved in numerous poxviruses. However, small ORFs within a newly sequenced genome with no orthologs are especially problematic. We have found that ARTEMIS ***(2)***, which has a graphical interface, is a very useful tool in helping to annotate genomes. ARTEMIS displays a variety of information about the sequence, including: ORFs, start–stop codons, six-frame translations, and nucleotide composition graphs. It also provides links to BLAST search programs, as well as features to semiautomate the naming of ORFs. There are several criteria that can be applied to the ORFs to help with the designation, which include: ORF size, overlap with other genes, presence of promoter like elements, isoelectric point, amino acid composition ***(13)***, and codon usage. For vaccinia virus and other AT-rich poxviruses, the coding strand of genes is positively correlated with purine content (unpublished data). Vaccinia virus (Copenhagen strain) is a useful test system because the genome was initially annotated with 200 genes and subsequently reannotated with 64 more ORFs, most of which substantially overlap with larger genes conserved in other genomes. These extra ORFs were named "X-ORF-Y," where X represents the *Hin*dIII genome fragment, and Y represents the rank of the ORF from left to right. Very few of these ORFs have been shown to be functional genes. **Figure 5** illustrates that ranking all genes and ORFs from vaccinia virus (Copenhagen strain) by purine content, which separates these two sets quite well. Combining amino acid content of

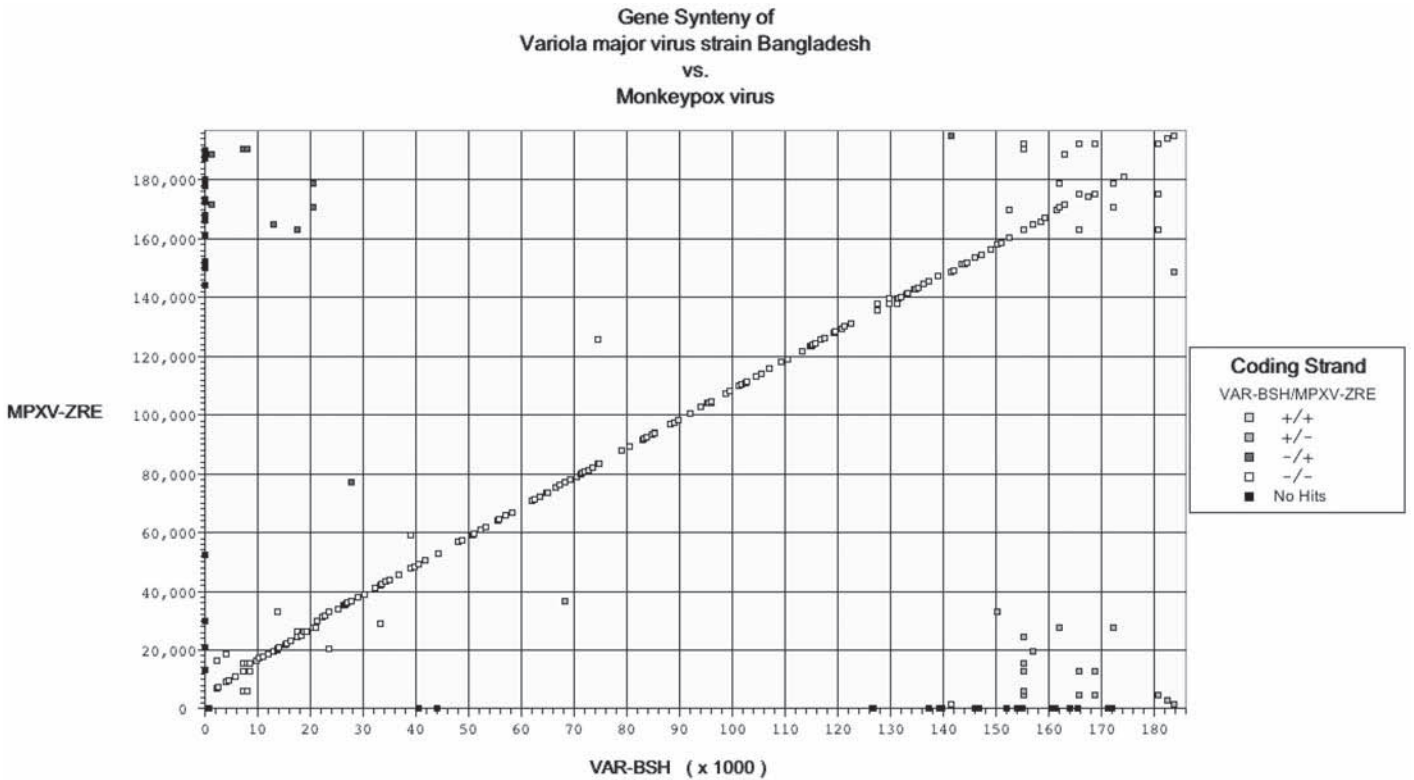

Fig. 4. Gene Synteny plot (low stringency) of variola virus (Bangladesh strain) vs monkeypox virus (Zaire strain). Proteins can be compared at high or low stringency. Mousing-over particular spots displays gene identities. (Original figure is displayed in color.)

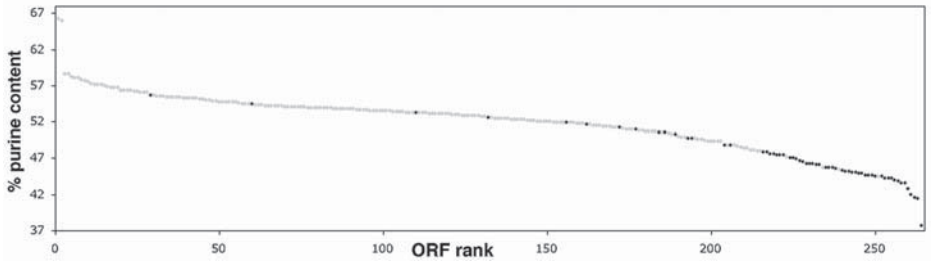

Fig. 5. All vaccinia virus (Copenhagen strain) ORFs as annotated in the GenBank file were ranked by purine content before plotting. Gray dots represent "normal" genes; black dots represent ORFs that overlap larger genes (named X-ORF-X, and so on in the GenBank file).

predicted proteins with the purine content of the ORF is even more effective in discriminating between groups of ORFs likely or unlikely to encode a protein (unpublished data). Clearly, there are exceptions to this predictive strategy, but the goal of this analysis is to flag ORFs that need more careful scrutiny by the genome annotator.

Another useful tool for the characterization of new genomes is NAP ***(5)***, a global nucleotide vs amino acid alignment program, available from within POCs and VGO (*see* VGO example 1, **Subheading 3.4.1.**). The utility of this program lies in the fact that it not only generates alignments between protein and DNA sequences, but that it produces a gapped-global alignment. Therefore, it becomes very easy to locate and evaluate potential sequencing errors that may break genes into fragments by the introduction of frame-shifting mutations or stop codons. We have developed an easy to use JAVA client–server interface to the NAP program and have integrated this software into POCs and VGO; it is also available as a stand-alone at http://athena.bioc.uvic.ca/pbr/nap/.

3.7. Searching for Distantly Related Proteins

One of the most common questions in bioinformatics is "What is my protein (or DNA) sequence similar to?" Similarity searches in which a protein or DNA sequence is searched against a database of all known sequences are most often performed with one of the BLAST programs ***(8)***. There are various search algorithm strategies, and an important design factor is how they balance search sensitivity and search speed. Another key difference is if the program performs a local or global alignment, as the latter may be more sensitive, but takes much more computer time. For "routine" database searches, BLASTP is sufficient to find database matches at more than 30% identity. However, a more sensitive

database search program is Position Specific Iterative BLAST (PSI-BLAST), which automatically constructs a new position-specific scoring matrix (PSSM) using a multiple alignment of the highest scoring database matches used in the next round of an iterative series of BLAST searches. Thus, on each round of searching, the program uses a modified scoring matrix that reflects the most conserved residues in sequences already identified as similar to the query. For example, the author has found that PSI-BLAST is superior to BLASTP in the identification of members of the poxvirus uracil DNA glycosylase family, which share very little similarity with other uracil DNA glycosylases ***(14)***. Each month, the VGO program runs BLASTP and PSI-BLAST searches of all poxvirus proteins in the POCs database against an updated NCBI NR protein database; both VGO and the POCs software programs can be used to access the results of these searches.

Saturated BLAST ***(15)*** is also useful for searching for weak similarities between proteins. It is similar to PSI-BLAST in that it uses an iterative process, but Saturated BLAST adopts an intermediate sequence search (ISS) method rather than modifying the scoring matrix. ISS is a strategy for recognizing distant homologs using transitive sequences. The hypothesis is that when the similarity between two remotely homologous sequences cannot be detected by normal sequence comparison, if there is an intermediate sequence with significant alignment scores to both of them, their similarity can still be established. Although ISS and its extension, multiple ISS (which applies more than one intermediate step) has been proven to be sensitive, performing such searches manually is tedious and time-consuming. The advantage of the Saturated BLAST software is that it has a graphical user interface that enables the user to perform the iterated multiple intermediate sequence searches efficiently and in a somewhat automated fashion (http://bioinformatics.ljcrf.edu/xblast/).

It is also often insightful to view BLASTP result files as a pseudo-multiple alignment. For example, the similarity between the poxvirus IFN-γ binding proteins (MYX-L 007R) and the mammalian (IFN-γ receptors is very low ***(16)***; 23%) as shown in the pairwise alignment in **Fig. 6**. At this level of similarity between two proteins, it is very difficult to decide whether an alignment is significant. However, by examining the multiple sequence alignments (**Fig. 6**),

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Fig. 6. *(opposite page)* **(A)** BLASTP alignment of myxoma virus IFN-γ binding protein with human IFN-γ receptor. The *E* value is 0.001, and there are only 23% identical residues. **(B)** An MVIEW alignment of BLASTP results after database search with myxoma virus IFN-γ binding protein. Proteins from (1) myxoma virus; (2) swinepox; (3) sheeppox; (4) vaccinia virus; (5) rat; and (6) human. Residues conserved throughout this diverse group of proteins strongly suggest that they are all related. (Original alignment is colored.)


A
Query: 19 ITSYKFESVNFDSKIEWTGDGLYNISLKNYGIKTWQTMYTNVPEGTYDISAFPKNDFVSF 78
ITSY V + ++ + +K Y + W TN+ +I S
Sbjct: 37 ITSYDLNPVVHWKHQNVSQAAVFTVQVKMYP-EYWTDACTNIAHHYCNIYKHISYPDSSA 95
Query: 79 WVKF-------EQGDYKVEEYCTGLCVEVKIGPPTVTLTEYDDHINLYIEHPYATRGSKK 131
W + E + EE+ +C + K+GPP + + +D + ++I HP +
Sbjct: 96 WARVKAKVGQRESAYAQSEEFI--MCRKGKVGPPGLDIGRKEDQLIVHIFHPKVNVSQE- 152
Query: 132 IPIYKRGDMCDI--YLLYTANFTFGDSEEPVTYDIDDYDCTSTGCSIDFATT---EKVCV 186
++ G+ C Y ++ ++ G+ + + DC+ T C ++ + + CV
Sbjct: 153 -TMFGDGNTCYTFDYTVFVKHYRSGEILH-TEHSVLKEDCSETLCELNISVSTLNSNYCV 210
Query: 187 TAQGATEGFLEKITPWSSEVCL 208
+ G + F + T S + C+
Sbjct: 211 SVVGKS-SFWQVNTETSKDACI 231
B
1 WQDDGYTYNVSIKPYTTATWINVCEWASSSCNVSLALQYDLDVVSWARLTRVGKYTEYSLEPTCAVARFSPP
2 WDNNVISYDVELMQYSHDEWRTVCTNSLGYCNLTNS-DIDNDDETWVRFKYENKTseHNIGRVCEIVQITSP
3 whDNSSSYKVMIMTYSSGEWKQACNYTTQqcNVSPFINDNTDDF-WIKFISIDNKEsfTFKPICESVVITTP
4 WTGDGL-YNISLKNYGIKTwtNVPE---GTYDISAFPKNDF-VSFWVKFEQGDYKVEEYCTGLCVEVKIGPP
5 WKHQNVsfTVQVKMY-PEYWTDAcnIAHHYCNIYKHISYP-DSSAWARvaKVGqyAQSEEFIMCRKGKVGPP
6 WepQVPVFTVEVKNykNSEWIDACINISHhcNISDHVG-DPSNSLWVRvaRVGqyAKSEEFAVCRDGKIGPP
1 EVQLVRTGTSVEVLVRHPVVYLRGQEVSVYGHSFCDYDFGYKTIFLFSKNKRAEYVVPGRYCDNVEC
2 IVNMTRDGSIILLDIHHPMTY--DNQYYIYNntLCGFEFIYEATFIIN-DTIIPYSIDNQYCDDVHC
3 SVSIKRQDVNTVIAINHPLAKDNKKNSPIYNNdyCNIVFKYILSITFGDSNTIKYEVDEQFCDKKRC
4 TVTLTEYDDHINLYIEHPYATRGSKKIPIYKrdMCDICLLYTANFTFGDSKevPYDIDDYDCTSTGC
5 GLDIGRKEDQLIVHIFHPKVNV--SQETMFGDGNTCYTFDYT---VFVKHYRseHSVLKEDCSETLC
6 KLDIRKEEKQIMIDIFHPSVFVNGDEQEV------DYdiRVYNVYVRMNGSEIQYKILTQkcDEIQC

Fig. 6.

it becomes quite apparent that the few amino acids conserved between the myxoma virus and human sequences are also conserved in other poxvirus and mammalian orthologs. From within VGO, one can convert BLAST results pairwise alignments into a multiple alignment with MVIEW ***(12)***.

3.8. Motif Searches

A protein motif can be defined as a pattern of amino acids characteristic of a functional/structural unit or protein domain of a series of proteins. A protein domain is often defined as an "independently folding structural unit." One of the most commonly used motif databases is PROSITE (***17***; www.expasy.ch/prosite/). In November 2003, PROSITE contained more than 1665 different entries. Motif searches are frequently useful in identifying domains within a protein when no other large areas of similarity exist with other proteins.

For example, [KR]-[LIV]-[LIVC]-[LIVM]-x-G-[QI]-D-P-Y is the PROSITE (PS00130) motif for uracil DNA glycosylases. It is common to all uracil DNA glycosylases present in the SWISS-PROT database and generates no false-positive hits. This motif is written as a regular expression, a format that allows mismatches and variability in spacing between residues in the motif. Software at PBRC permits searching of a database of poxvirus proteins for specific PROSITE motifs (link: DATA:GENES) and software at www.expasy.ch/prosite/ can be used to search: (1) a protein sequence against all motifs or (2) a PROSITE or user-created motif against all protein sequences. The POCs database also allows users to search for protein or DNA motifs. For a protein motif search, simply select *Protein Sequence—MATCH* and type in the regular expression for the motif. An advantage of performing the search through POCs is the speed and the fact that only poxvirus sequences are searched. However, it should be noted that the PROSITE (PS00130) motif only matches 18 of the 21 uracil DNA glycosylases present in POCs. This is because PROSITE is out of date, and the motif needs to be relaxed so that it includes the genes from lumpy skin disease virus, sheeppox virus, and melanoplus sanguinipes entomopoxvirus. Thus, the modified motif [KLNR]-[LIV]-[LIVC]-[LIVM]-x-G-[QIY]-[RD]-[SP]-[YF] finds all 21 uracil DNA glycosylases in POCs and 73 proteins in SWISS-PROT, only one of which is not annotated as a uracil DNA glycosylase. As yet, it is unknown if the sheeppox virus and melanoplus sanguinipes entomopoxvirus proteins are functional uracil DNA glycosylases.

The ProfileScan Server (http://hits.isb-sib.ch/cgi-bin/PFSCAN) searches user-supplied protein sequences for Pfam (Protein Families database of alignments and Hidden Markov Model [HMMs]) and PROSITE motifs. The Pfam website (http://www.sanger.ac.uk/Software/Pfam/index.shtml) lists 63 families of poxvirus proteins and is an excellent source of information, although the output can be rather overwhelming and difficult to interpret.

InterPro (http://www.ebi.ac.uk/interpro/) is another comprehensive assembly of motif and sequence databases that is, in turn, connected to a variety of other databases. It can be useful for looking at conserved motifs or domains in protein families, but for the most part, it is overkill for simple motif searches.

3.9. Multiple Sequence Alignments

The generation of multiple sequence alignments (MSAs) is a common technique to try to determine what amino acids are key to the structure or function of a group of proteins. Residues that are most conserved are likely to have important roles in the function of these proteins. These conserved residues are also used to define the motif(s) characteristic of protein families. Multiple alignments are also the basis of all phylogenetic analyses. To generate the best multiple alignments, it is common to first align the sequences with a computer program, such as CLUSTALW ***(18)***, and then edit the computer-generated alignment by hand. There are many parameters associated with the programs, and the most important are related to the insertion of gaps in the alignments and the choice of comparison matrix. T-COFFEE ***(19)*** and DIALIGN ***(20,21)*** are two programs that have been reported to produce better alignments than CLUSTALW with sequences with low (less than 30% identity) similarity. One of the more time-consuming steps is the final polishing of the alignment, which requires the use of a sequence alignment editor. Most of the alignment programs do not provide this feature. Yet another hurdle is the production of a publication quality figure, if required. Many commercial packages can do all three steps in a fairly seamless process, but suffer from the problems in importing all the sequences in to the program.

There are two software solutions to these problems available from the PBRC website. First, we have integrated into both POCs and VGO the CLUSTALW and T-COFFEE alignment programs together with Base-By-Base, which is our own JAVA MSA editor. The result is that it is now very simple to select a protein family or any series of proteins from POCs and use them as input for alignment, then have results automatically fed into Base-By-Base. Once in Base-By-Base, they can be viewed as colored alignments and edited as required. Gene DNA sequences, as well as the regions upstream of each gene, can also be aligned using this method. Base-By-Base is also able to import sequences from the user's local computer and can summarize differences between complete genome alignments in graphic or tabular formats. The second solution is the use of PepTool (http://www.biotools.com/), a commercial program produced by BioTools, which has been licensed by PBRC for use through the ATHENA server (http://athena.bioc.uvic.ca/pbr/biotools/). PepTool is a comprehensive package of protein analysis programs, and, in collaboration with BioTools, we have prepared all the protein sequences available in the

POCs database in PepTool format and organized them by virus to simplify loading into the program. We have also taken all the protein families from POCs and formatted each as PepTool MSA to be opened directly in the MSA tool of PepTool. Unfortunately, to make this program available to all PBRC users, it is necessary to run it on the PBRC LINUX server, which requires connections to be made through a X-windows interface. X-windows emulators are available for all platforms (e.g., VNC; http://www.uk.research.att.com/vnc/index.html), and this connection does not take away from the normal graphical interface of PepTool that can generate publication quality output.

3.10. Future Work

It is difficult to predict where the explosion in genomic and proteomic information will take us. However, it is obvious that computers and bioinformatics are here to stay. The molecular biological databases are not only continuing to grow in size, but are also proliferating in number. The Infobiogen catalog of biological databases lists 511 (http://www.infobiogen.fr/services/dbcat/), and they are becoming populated with sequences that are more and more closely related. Therefore, it is a safe assumption that we will be able to find the answers to more detailed questions. A good example of this is the accumulation of complete sequences of very closely related poxvirus genomes. We are now able to move from comparing vaccinia and variola genomes to comparing many different strains of a single virus, such as variola (CDC; personal communication) or monkeypox (PBRC). With this comes the need to develop novel software. Currently, the POCs database is constructed to answer questions like "which genes are present in variola, but absent from vaccinia." We are now developing strategies to systematically examine differences between very closely related strains of viruses at the single nucleotide level. (Base-By-Base is the first version of this software package.)

Another problem that must be addressed is that the databases are growing on a daily basis, and new poxvirus genomes are being determined on a monthly basis. What is needed is a notification system to provide users with updates of relevant new information in the databases. Numerous journals can email titles of new publications to users based on key word information. NCBI provides users with a storage area or cupboard ("Cubby") to aid in searching for new publications with a series of search parameters. However, reporting new hits in biological sequence databases is a far more complex problem because of the volume of data in both the public protein databases and the poxvirus database, along with the difficulties arising from small changes to the accession numbers or sequences themselves in the NCBI databases. PBRC hopes to develop such a reporting system.

Finally, as more protein structures are solved for poxvirus proteins, the availability of good bioinformatics tools to interface between structural data and the large volume of sequence data will maximize the benefit of high-resolution structures and should enhance our understanding of the poxvirus life cycle and development of therapeutics.

4. Notes

1. This review was submitted in December 2002 and updated in proof, January 2004.
2. PBRC (http://www.poxvirus.org/).
3. It is not necessary to have the "latest and greatest" personal computer, as most of the software described in this chapter is available in either (1) a simple WWW form (e.g., BLAST at NCBI) interface that sends requests to a remote computer and displays the results in a WWW browser; or (2) a client–server format in which the local client program is far more sophisticated than a WWW interface and only connects to the remote server to download information from a database or to off-load computationally intensive calculations.
4. Mac OS 9 does not have the required JAVA capabilities and therefore cannot be used.
5. Much of the PBRC software is written in JAVA to make it available for multiple computer-operating systems. The combination of JAVA and the client–server format has another advantage in that the program on the UNIX server performing the central processing unit (cpu)-intensive numerical calculations can be written in C or another language, and only the interface needs to be in JAVA, which may be slower.
6. JAVA is a multiplatform, platform-independent, object-oriented programming language.
7. SQL stands for sequence query language database. (Note that the word sequence does not refer to DNA or protein sequence.)
8. Downloading JAVA-Web Start is straightforward and takes only a few minutes even for a novice. When JAVA-Web Start downloads one of the JAVA clients of POCs, VGO, or JDotter to the user's local machine for the first time, it displays a warning window by default that informs the user that permitting this program to be installed on their computer is potentially dangerous. This is because the software is like most other programs on your computer and can write to your hard drive. The default warning message also includes information about the origin of the software and developers to allow the user to determine if the software comes from a trustworthy site. We recommend that you accept the software. The PBRC site is secure behind a firewall to prevent potential hackers from tampering with these files. The PBRC groups use this software daily in exactly the same way as external users; thus, we would quickly detect any problems with the software.
9. The *Family Count* button gives the number of different gene families represented in the result of a query. The *Gene Count* button gives the total number of genes in the result. Therefore, these two numbers are usually different as there are often many genes per family.

10. Base-By-Base permits editing of the MSAs, which is often required.
11. If searching for a vaccinia gene in the *Gene Family Analyzer* returns no sequences, check that the virus selector has not been used to restrict the search to another virus or that no other search criteria were inadvertently selected. The *Clear All* feature of the *Select* menu can be used to remove all queries.
12. Use the shift key to select everything between two rows in a table; use the command (apple key on a Macintosh) or the control key on other systems to select a series of nonadjacent genes.
13. An example of the results from a user-defined input file is a simple-format text file defining position, length, and color of boxes to be drawn on the VGO sequence map. We frequently use it to graphically display results of otherwise obtuse text files generated by promoter prediction programs.
14. Between EV genes 028 and 029, there is approx 1 kb with no large ORF present in this region. It is unusual to have noncoding regions in poxvirus genomes; therefore, it would be useful to determine how this region in EV compares to the cowpox genome. The hypothesis to be tested is that this region of EV is a fragmented gene equivalent to a complete gene in cowpox.
15. Use the control or command (Macintosh apple) key for multiple selections.
16. Scrolling is faster with fewer items displayed on the screen. To maximize speed, do not display start/stop codons unless needed and zoom in before scrolling.
17. The virus genes are shown in dark blue above (transcribed to the right) and below (transcribed to the left) the scale bar.
18. The NAP results clearly demonstrate that the two regions of the genomes are homologous, but that a small number of nucleotide deletions cause a series of frame-shifts in the EV gene that destroy its functionality. Aligned positions are shown as dots between them and gaps are indicated with dashes. This is can also be seen by looking at the start/stop codons in VGO.
19. From the PBRC homepage, use the following links: ANALYSIS, ORTHOLOG COMPARISON and GRAPHICAL ORTHOLOG SYNTENY COMPARISONS.

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24

Preparation and Use of Molluscum Contagiosum Virus from Human Tissue Biopsy Specimens

Nadja V. Melquiot and Joachim J. Bugert*

Summary

Molluscum contagiosum virus (MCV) lesions are limited to the human epidermis and can persist for months, showing only weak signs of inflammation. Because a culture system has not yet been created to replicate MCV, this chapter describes how the virus can be isolated from patient specimens. From this material, we describe how MCV is purified and used for infection studies, electron microscopy, viral DNA extraction, and analyses of early mRNA synthesized by in vitro transcription of permeabilized virions. The complete MCV-1 genome has been sequenced, and a redundant MCV genome fragment library of MCV type 1 (available from American Type Culture Collection [ATCC]) is useful for the cloning and study of individual MCV genes.

Key Words: Molluscum contagiosum virus (MCV); MCV virion particles; human tissue biopsy; central umbilication; electron microscopy; in vitro transcription assay; cell culture; MCV genome fragment library; MCV transcriptional signals.

1. Introduction

After the eradication of variola virus, the only strictly human pathogenic poxvirus that remains is the molluscum contagiosum virus (MCV), the sole member of the genus *Molluscipoxvirus*. MCV causes benign growths of the skin mainly in children and immunosuppressed individuals. The lesions are limited to the epidermis and can persist for months, displaying only weak symptoms of inflammation. Once inflammation occurs, the viral lesion is quickly eliminated ***(1)***. MCV lesions have been histopathologically classified as acanthomas—benign hyperproliferative processes confined to the epidermis.

MCV does not produce infectious progeny in cell culture ***(2)*** and can best be isolated from the central umbilication of noninflamed lesions larger

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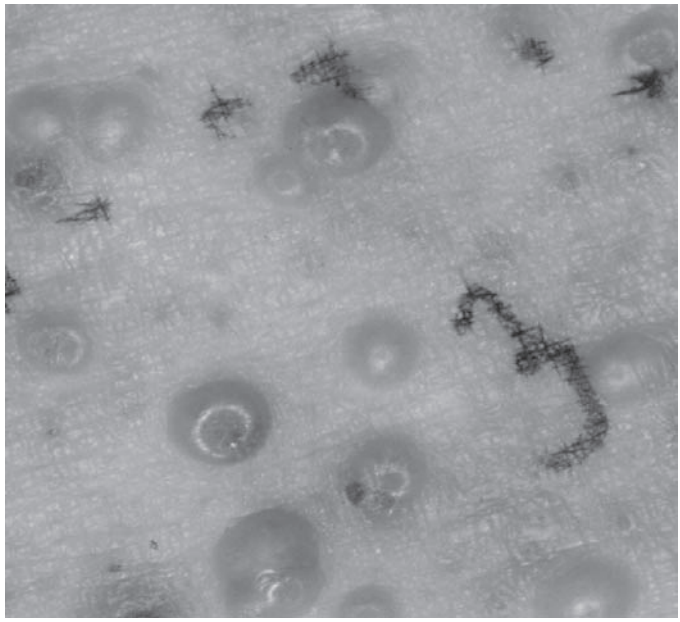

Fig. 1. MCV lesions in an immunocompromised host. Individual lesions are between 0.5 and 3-mm diameter. All lesions are umbilicated; four lesions are irritated and inflamed by scratching. (Taken from Turner and Bugert, NIH, Dermatology Service and NIAID, 1995.)

than 3-mm diameter (**Figs. 1** and **2**). The waxy plug that can be removed from the lesions is composed of approx 90% virus particles and 10% lipid-rich cellular debris. Very clean MCV preparations of infectious virus for infection studies, electron microscopy, and viral DNA extraction can be obtained by ultracentrifugation of this material through a sucrose cushion or a sucrose gradient. MCV lesions that are inflamed or smaller than 3-mm diameter without an identifiable central plug can be removed from the skin by curettage with a sharp spoon instrument, and the material can be used for isolation of infected cells and viral mRNA ***(3)***. Early and very small amounts of intermediate and late MCV mRNA can be isolated from MCV-infected human primary fibroblast cultures ***(2)***. More abundant amounts of MCV early mRNA can be synthesized by permeabilized virions in an in vitro transcription assay optimized for MCV ***(4)***.

MCV DNA has a 60% average GC content and a number of terminal and inverted repeats that can occasionally complicate resolution in manual and automated sequencing protocols. The complete MCV genomic sequence has been determined ***(5,6)***, and an updated MCV gene feature table is available

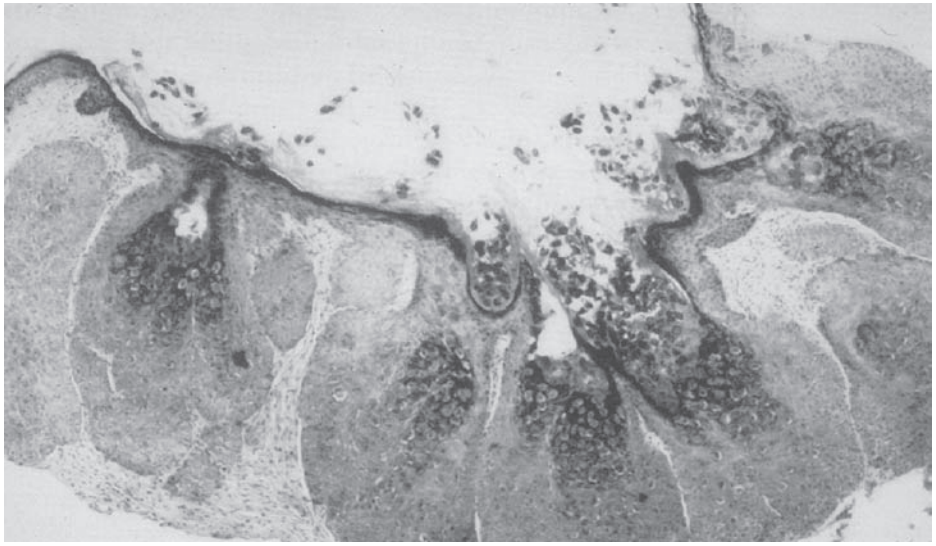

Fig. 2. Microscopic section through a typical MCV lesion. Normal epidermis to the extreme left and right. In between, there is enlarged epidermis with MCV-infected lobuli in various stages of opening up into an umbilication in center top. Released MCV-infected cell debris in the lumen can also be seen. (Taken from Bugert, University of Heidelberg, Department of Medical Virology, 1993.)

(***7***; *see* **Note 1**). For the cloning of individual MCV genes, either viral DNA isolated from MCV particles or a redundant MCV genome fragment library of MCV type 1 (available from the American Type Culture Collection [ATCC]) can be used. Vaccinia virus-infected cells transfected with MCV genes can express the MCV protein because the MCV transcriptional signals (i.e., promoters) are recognized by other poxviruses ***(8)***.

2. Materials

2.1. Isolation and Quantitation of MCV Particles from Lesion Plugs

1. 1 m*M* Tris: 1 m*M* Tris-HCl, pH 8.
2. PolybeadSelect® particles (Polysciences, cat. no. 21401): certified size standards, 0.324 μm, 2% solids (2.1×10^{12} particles/mL).
3. Ammonium molybdate solution: 2% aqueous solution.

2.2. MCV Viral DNA Preparation

1. Chloroform-isoamylalcohol (24:1).
2. Saturated phenol, pH 8: 1 kg phenol melted at 65°C, then mixed with 300 mL 1X TNE, pH 8.3. After the phases have separated, 0.5 g 8-hydroxychinolin is added to the organic phase.

3. 20X sodium citrate buffer (SSC): 0.15 *M* NaCl, 0.015 *M* Na_3 citrate, pH 7.2.
4. 10X TNE Stock buffer: 0.05 *M* Tris-HCl, pH 7.5, 0.001 *M* EDTA, 0.1 *M* NaCl.
5. Lysis buffer: 1X TNE with 1% sodium dodecyl sulfate (SDS) and 100 μg/mL Proteinase K.

2.3. MCV Early Viral mRNA In Vitro Transcription

1. Shand buffer: in vitro transcription buffer (final concentrations in 200-μL reaction): 1.0 *M* Tris-HCl, pH 8.5, 50 m*M* $MgCl_2$, 0.25% 2-mercaptoethanol, 0.05% Nonidet P40, 4.0 m*M* ATP, 2.0 m*M* CTP, UTP, and GTP, 7.5 m*M* phosphoenolpyruvate, 4.0 μg pyruvate kinase, 1.0 μL RNAsin (40 U/μL, Boehringer, Mannheim, Germany).
2. [α-^{32}P]UTP: specific activity 1 to 3 × 10^3 Ci/mmol.
3. 10% trichloroacetic acid.

2.4. MCV Infection Studies

1. MRC-5 cells (ATCC, # CCL-171).
2. HaCaT keratinocytes (DKFZ, Germany, obtained from Prof. Dr. Norbert Fusenig).
3. Growth medium: Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 m*M* glutamine, and 5% fetal calf serum (FCS; *see* **Note 2**).

2.5. Total MCV-Infected Cell RNA Preparation

RNeasy Mini Kit (Fa. Qiagen GmbH, Hilden, cat. no. 74104).

2.6. MCV Enzyme-Linked Immunosorbent Assay (ELISA)

1. MDB buffer: 20 m*M* Tris-HCl, pH 8, 1 m*M* EDTA, 100 m*M* NaCl, 0.5% NP40, 1 μL/mL proteinase inhibitor cocktail (Sigma).
2. ELISA blocking buffer: 1% skim milk powder in phosphate-buffered saline (PBS).
3. ELISA wash buffer: 0.05% Tween-20 in PBS.

3. Methods

3.1. Protocols Using MCV Particles from Lesion Plugs

MCV viral particles can best be isolated from the central umbilication of noninflamed lesions larger than 3-mm diameter (**Figs. 1** and **2**). The waxy plug, removable from the central indentation of individual lesions, is composed of approx 90% virus particles and 10% lipid-rich cellular debris.

3.1.1. Isolation of MCV Particles from MCV Lesion Material

1. Sanitize the area of the lesion with 70% ethanol swabs.
2. Use sterile forceps to apply light pressure from two sides of the lesion.
3. Remove extruded plug of cellular debris and virus with sterile forceps (*see* **Note 3**).

4. Deposit plug into a sterile plastic tube.
5. Aspirate the plug through a 20-gauge needle in 1 mL 1 m*M* Tris (*see* **Notes 4** and **5**).
6. Ultracentrifuge this material at 31,000*g* for 1.5 h at 4°C in a SW50 rotor through a 5 mL 36% sucrose cushion (*see* **Note 6**).
7. To purify the pelleted virus with a sucrose gradient generate a 20–60% sucrose gradient (*see* Chapter 8 and **Note 7**). Ultracentrifuge at 100,000*g* for 1 h at 4°C. MCV bands are between 30% and 40% sucrose.
8. Store purified MCV infectious viral particles in 1 m*M* Tris at –70°C (*see* **Notes 8** and **9**).

3.1.2. Quantitation of MCV Particle Concentration

Because MCV does not grow in tissue culture, the number of infectious particles cannot be biologically determined by plaque formation. Therefore, for quantitation of the MCV particle yield, electron microscopy is done on a mixture of virus and a known quantity of latex particles of standard size (324-nm diameter; *see* **Notes 5**, **10**, and **11**).

1. Mix 5 µL PolybeadSelect® particles with 5 µL viral particle suspension and put on parafilm.
2. Mount copper grid on mixture for electron microscopy.
3. Dry at room temperature, then wash twice with H_2O.
4. Put copper grid on droplet (approx 10 µL) of ammonium molybdate solution for 10 min (*see* **Note 12**).
5. Wash twice with H_2O and let dry for electron microscopy.
6. Count the number of viral particles per number of latex particles in 10 individual grid-sections and determine mean relation. Calculate viral particle concentration (*see* **Notes 9** and **13**).

3.1.3. Preparation of MCV Genomic DNA from MCV Particles

Purified DNA from MCV can be used for multiple purposes, including DNA fingerprinting ***(9)***, polymerase chain reaction (PCR) amplification of MCV-specific DNA sequences (*see* **Note 14**), cloning of MCV restriction or PCR fragments, and DNA nucleotide sequence analysis ***(10)***. DNA restriction analysis has been used to identify MCV types. Restriction enzymes *Bam*HI, *Cla*I, *Eco*RI, and *Hin*dIII lead to typical DNA restriction fragment patterns that, when compared to published patterns, identify the MCV type (*see* **Note 15**).

1. Use MCV lesional debris (**Subheading 3.1.1.**, **step 3**), or for best results, purified MCV particles (**Subheading 3.1.1.**, **step 7**) from one to as many lesions as possible.
2. Incubate 1 mL debris, purified virus, or lesional material with a volume equivalent of 1 mL in 10 mL lysis buffer overnight at 37°C.
3. Add 25 mL saturated phenol and centrifuge at 2016*g*, room temperature for 10 min.

4. Remove aqueous supernatant and reextract with 25 mL saturated phenol and centrifuge at 2016*g*, room temperature for 10 min.
5. Remove aqueous supernatant and add 25 mL chloroform/isoamylalcohol, and centrifuge at 3000 rpm at room temperature for 10 min. Repeat once.
6. Remove aqueous supernatant (approx 9 mL) and add 1 mL saturated NaCl.
7. Then, ethanol-precipitate with 2.5 vol of absolute ethanol for 2 h at –20°C.
8. Centrifuge at 8064*g* at –20°C for 30 min.
9. Air-dry pellet and resuspended in 1.5 mL 0.1 × SSC (*see* **Note 16**).

3.1.4. MCV Early Viral mRNA Transcription In Vitro

MCV early mRNA alone can be synthesized by permeabilized virions in an in vitro transcription assay optimized for MCV ***(4)***. The in vitro transcription assay is based on a protocol described by Shand et al. ***(11)*** and has been modified for the production of mRNA suitable for reverse transcriptase-PCR (RT-PCR). Supernatant can be used for hybridization and RT-PCR experiments. The outcome of the assay depends on the quality of the virus preparation and the agent type used for resolution of disulfide bonds (*see* **Note 17**).

1. Incubate 50 µL virus suspension (approx 1 OD_{260}, [*see* **Note 10**] or 5×10^9 MVA particles) with Shand buffer (200-µL final reaction volume) at 35°C for 120 min (*see* **Notes 17** and **18**).
2. For MCV early mRNA transcription kinetics, take 20 µL aliquots at different time-points (e.g., 0, 60, 120, and 180 min) and immediately put on ice.
3. For purification of MCV mRNA out of 200 µL total reaction volume or from 20-µL aliquots (add 180 µL Shand buffer), use Qiagen lysis buffer RLT, RNeasy Shredder® and RNeasy-Mini columns® following manufacturer's instructions. Then, continue with RT-PCR protocols.

3.2. MCV Infection of Human Fibroblast Cell Cultures

MCV does not produce viral progeny in cell culture. However, in human diploid fibroblasts and HaCaT keratinocytes, MCV transcribes early mRNA and small amounts of intermediate and late mRNA. Human diploid fibroblasts are currently tested with different transgenes and treated with antibodies, e.g., anti-β-interferon, to minimize apoptosis induced in MCV-infected cell cultures. Such experiments have the ultimate goal to overcome the lack of MCV replication in cell culture.

1. Prepare 12-well plates of human MRC-5 fibroblasts or HaCaT keratinocytes in growth medium.
2. Inoculate with 5×10^6 particles (*see* **Note 19**) in 300 µL/well of DMEM without additives.
3. Adsorb for 45 min at 37°C.
4. Wash cells twice with PBS.

5. Add 1 mL growth medium
6. Observe typical cytopathic effect (CPE) with cell rounding beginning at approx 13-h postinfection (*see* **Fig. 3** and **Note 20**).

3.3. Preparation of Total MCV-Infected Cell RNA

1. Use MCV lesions smaller than 3-mm diameter without an identifiable central plug and remove by curettage (*see* **Fig. 1** and **Note 21**).
2. Freeze instantly in liquid nitrogen or use immediately.
3. Put biopsy (or MCV-infected MRC-5 cells) directly into GTC-containing buffer (e.g., RLT lysis buffer of RNEasy mini kit) containing fresh 2-mercaptoethanol (1%).
4. Isolate total RNA using RNeasy Shredder and Mini columns following manufacturer's instructions (*see* **Notes 21** and **22**).

3.4. MCV ELISA

3.4.1. Preparation of MCV Protein Lysate and Coated ELISA Plates

MCV virion proteins are prepared from purified MCV virus particles using a mild detergent, which has been described by Konya and coworkers ***(12)***. A panel of control sera should be tested on each plate.

1. Lyse 1 mL 1 OD virus suspension (or 10^{11} MCV particles) with 1 mL MDB for a total volume of 2 mL on ice for 30 min.
2. Determine protein concentration using the Bradford method.
3. Coat 96-well flat-bottom ELISA plates with 5 mg/mL MCV virion protein. Store at 4°C for up to 4 wk.

3.4.2. Running a MCV ELISA

1. Incubate 96-well ELISA plates with ELISA blocking buffer for 1 h at 37°C.
2. Dilute test or control sera 1:40 in ELISA wash buffer. (Run duplicates.)
3. Add to ELISA plate and incubate for 18 h at 47°C.
4. Wash five times for 5 min in ELISA wash buffer.
5. Add peroxidase-conjugated goat anti-human IgG (1:4000 in ELISA blocking buffer) for l h at 37°C.
6. Wash five times for 5 min in ELISA wash buffer.
7. Add peroxidase substrate solution at room temperature for 30 min.
8. Read absorbances at 690 nm and express as OD units (mean of the test wells subtracted by the mean of the blank wells).

4. Notes

1. The partial MCV type 2 genomic sequence (approx 60 kb) has been determined (Lohmüller, C., Doctoral dissertation). The sequenced MCV-2 DNA displayed between 78% and 99% DNA homology and between 45% and 98% amino acid homology to sequences of MCV-1.
2. Inactivate FCS for 2 h at 56°C. Keep at 4°C.

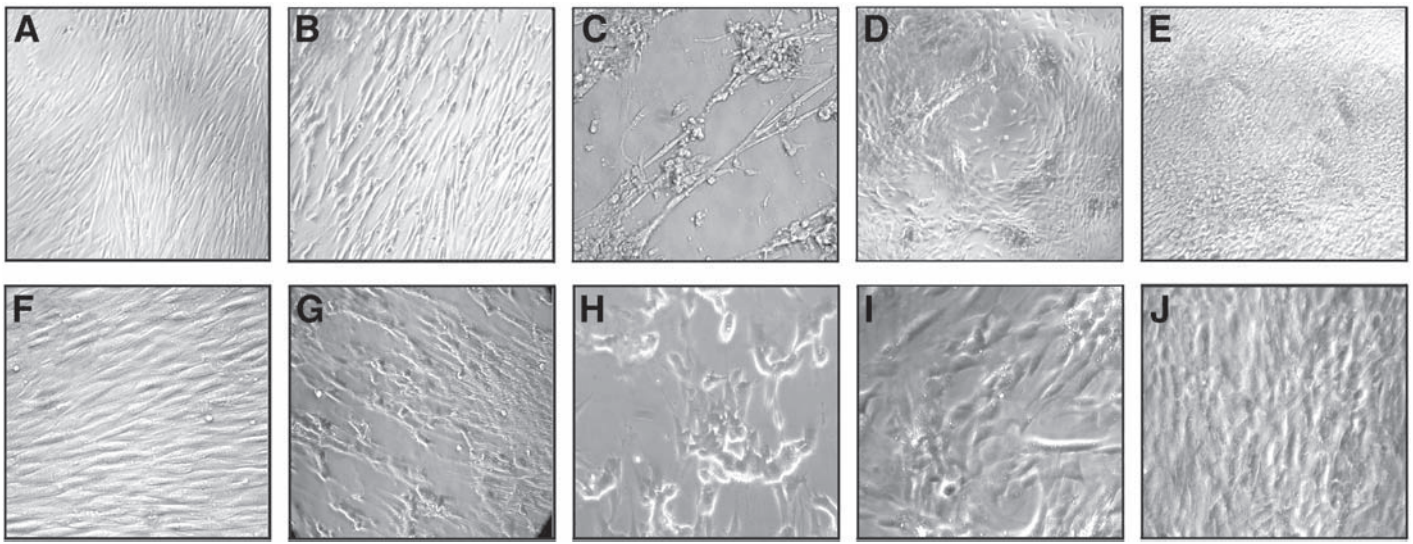

Fig. 3. Time-course of MCV infection of MRC-5 cells. (a and f) mock; (b and g) 13-h postinfection; (c and h) 24-h postinfection; (d and i) 48-h postinfection; (e and j) 72-h postinfection. Panels a–e ×10 magnification. Panels f–j ×40 magnification. (From **ref. *2***, with permission.)

3. If bleeding occurs during extraction, the lesion might resolve as a consequence of the manipulation.
4. If a plug of cellular debris is used, aspiration through a needle is sufficient. However, if a lesion with surrounding tissue is used, the tissue in 1 mL 1 m*M* Tris must be dounce-homogenized.
5. Putting MCV particles in phosphate-containing buffers must be avoided if the particles are to be used in electron microscopy.
6. Whether or not to band-purify the virus after pelleting through a sucrose cushion through a gradient depends on how much virus is present. With some material from small lesions, there is only enough to put through a sucrose cushion. For epidemiological reasons, when possible, one tries not to mix preparations from different lesions because the virus from every individual lesion might be considered a separate strain. Although huge differences are not detected (i.e., large deletions) between viruses contained in lesions of one patient, we have found upon sequencing that there can be an extensive number of point mutations in certain genes. This may reflect the genetic drift as a result of several genome copies that are made in an individual lesion. Because complete genomes have not been sequenced from individual lesions, and the mutation rate varies with very high rates in mutational hotspots (seen in lesion-isolate sequencing of certain genes), it is not yet clear if there are sufficient differences to meet the definition of a new strain.
7. An alternative to sucrose gradient is to use an OptiPrep gradient (iodixanol in water). For example, a 60% (w/v) solution of iodixanol in water has a density of 1.32 g/mL.
8. MCV-infectious viral particles can be stored in 1 m*M* Tris buffer pH 8 at –70°C indefinitely.
9. Stocks of up to 10^9 viral particles/mL can be obtained from plugs of individual MCV lesions larger than 3-mm diameter.
10. As an alternative, albeit less accurate method than electron microscopy for particle quantitation, a sufficiently concentrated MCV suspension in 1 m*M* Tris might have a Tyndall effect measurable by spectrophotometry. As a rule of thumb, an OD of 1.0 at 260 nm corresponds to 10^{11} MCV viral particles.
11. Electron microscopy images of MCV viral particles isolated from lesional debris rich in MCV virions turn out especially well (**Fig. 4**). Additionally, reasonable quality immunoelectronmicroscopic images can be obtained if patient polyclonal sera and antihuman-secondary antibodies carrying gold particles (IgG conjugated to 6- or 10-nm gold particles) are used.
12. Standard negative-contrast stain of MCV on copper grids works best with ammonium molybdate as electron-dense contrasting agent.
13. Example calculation of viral particle concentration: Dilutions (10^5/5 μL; 10^6/5 μL; 10^7/5 μL) of PolybeadSelect® (2.1×10^{12} particles/mL) are made in 1 m*M* Tris. Vortex well. Add 5 μL of each particle solution to 5 μL viral particle suspension. Perform electron microscopy on each sample. *Raw estimate method*: Find Polybead-

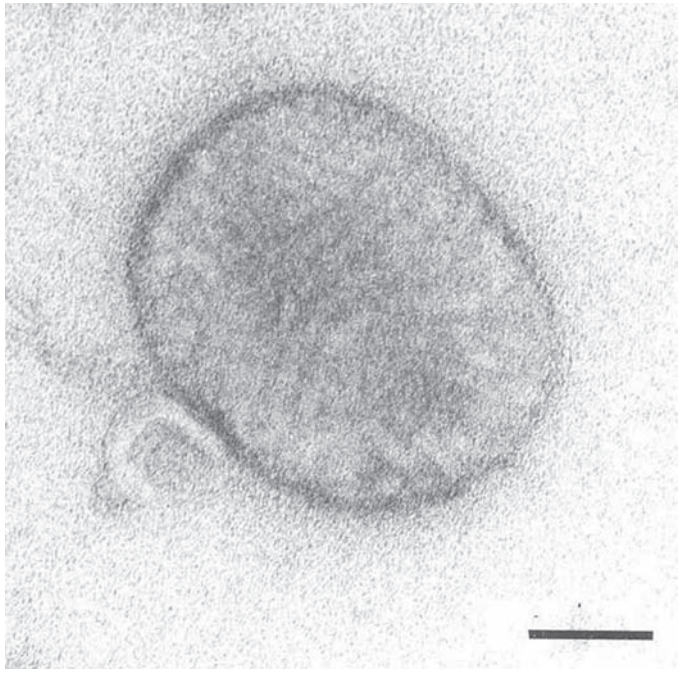

Fig. 4. Negative stain of MCV particle. Ammonium-molybdate technique. Semiattached piece of membrane to the left of the particle. Bar = 100 nm. (Taken from Melquiot, Krinsje-Locker, and Bugert, EMBL and University of Heidelberg, Department of Virology, 2000.)

Select® particle dilution closest to a 1:1 latex particle/MCV virus particle ratio. *Example 1:* the 10^7/5 µL PolybeadSelect® dilution shows about as many latex particles as MCV virions per selected grid area (e.g., what can be seen in any one viewing area or in one grid hexagon). There are 2×10^9 PolybeadSelect® particles in 1 mL of this particular dilution. Therefore, there are approx 2×10^9 MCV virion particle in 1 mL of this specific viral particle suspension. *Exact calculation method:* Count 10 individual grid-sections and determine mean relation between MCV virions and PolybeadSelect® particles. *Example 2:* There are a mean of 202 MCV particles in 10 individual selected grid areas (i.e., viewing areas or grid hexagons). There are a mean of 257 PolybeadSelect® particles of the 10^6/5 µL dilution in the same 10 areas. There are 2×10^8 PolybeadSelect® particles in 1 mL of this particular particle dilution. Thus, there are $(202/257) \times (2 \times 10^8) \approx 0.79 \times (2 \times 10^8) = 1.57 \times 10^8$ MCV particles in 1 mL of this specific MCV particle suspension.

14. Owing to the high GC content cycling conditions for PCR assays using MCV DNA as a template should denature at 96°C, and average annealing temperatures

should be approx 60°C. The standard ABI sequencing program (96°C for 2 min, 96°C for 30 s, 60°C for 4 min) × 25 cycles) works very well with most MCV templates.

15. Determination of the MCV type (by *Bam*HI), local insertion or deletion events (*Cla*I DNA fragment J), and for estimates of length differences in the MCV-inverted terminal repeats (*Bam*HI DNA fragments B and E, *Cla*I DNA fragment H, *Eco*RI DNA fragments G and H) ***(9)***.
16. Expect MCV DNA concentrations of 1 μg/100 μL to 1 μg/5 μL.
17. Efficiency of MCV mRNA synthesis is drastically reduced when DTT is used instead of 2-mercaptoethanol in the transcription buffer.
18. For synthesis of radioactively labeled MCV mRNA, the reaction is incubated for 180 min in a volume of 50 μL, and UTP is substituted by 0.2 m*M* [α-^{32}P]UTP (specific activity 1 to 3 × 10^3 Ci/mmol), then centrifuge at 2016*g* for 5 min to separate soluble and core-associated mRNA. Redissolve pellet in 50 μL Shand buffer. Add 1 mL 10% trichloroacetic acid to supernatant and/or pellet and filter onto nitrocellulose or glass fiber filter. Wash three times with 10% trichloroacetic acid. Measure incorporated radioactivity in a scintillation counter.
19. Infect cells at a multiplicity of infection (moi) of 1.
20. Typical CPE can be obtained after inoculation of MRC-5 cells after 13-h postinfection. CPE develops over the next 28 h and disappears after 72 h, leaving an intact monolayer of slightly square irregular-shaped instead of oblong fibroblasts (*see* **Fig. 3**).
21. An alternative to getting a specimen from a patient, one can use MCV-infected MRC-5 cells (*see* **Subheading 3.2.** and **Fig. 5**). Lysed MCV-infected MRC5 cells can be kept in guanidium isothiocyanate-containing buffers. As a negative control, MCV mRNA transcription can be inhibited by ultraviolet (UV) irradiation (total of 1200 W in a Stratagene UV crosslinker) and heating (56°C for 30 min in a water bath) of the virus inoculum before infection ***(2)***.
22. If RNA is extracted from cells in culture at different time-points using the Quiagen RNeasy procedure, MCV mRNA (e.g., copies of the MCV early gene mc148R) can be detected by mRNA-specific RT-PCR (e.g., using oligo dT primers) as early as 2 h postinfection in MCV-infected MRC-5 (*see* **Fig. 5**). Viral mRNA from human tissue specimen can be used for 5'-RACE (rapid analysis of cDNA ends: sensitive alternative to S1 mapping) and 3' analysis of MCV mRNAs in promoter location and termination signal studies ***(3)***. Using these (abortive) MCV cell culture systems, comparative analysis of the transcription kinetics of other MCV early genes and evaluation of the effect of transgenes and antibodies in various cell culture assays becomes possible. However, to date, only minimal amounts of intermediate- and late-MCV mRNA were detected in MCV-infected MRC-5 and HaCaT keratinocytes.

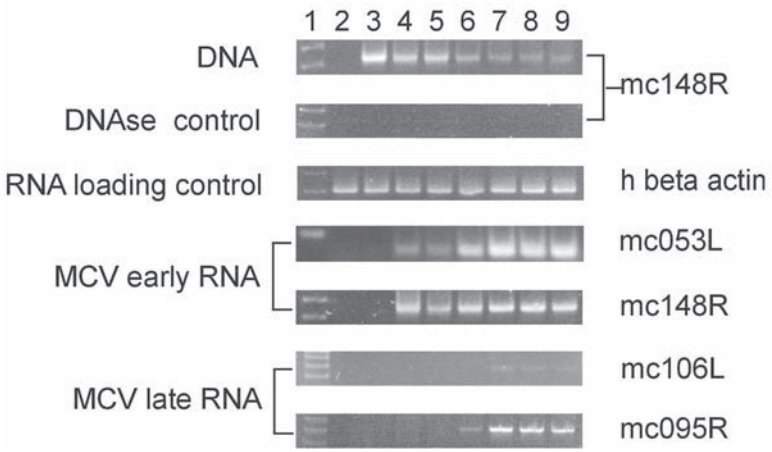

Fig. 5. Viral RNA analysis of MCV-infected MRC-5 cells. DNA and DNAse control of mc148R (early gene encoding MCV chemokine antagonist); RNA loading control (host cell β actin); MCV early RNA (mc148R and mc053L, early gene encoding MCV IL-18-binding protein); MCV late RNA (mc106L, late gene encoding a vaccinia virus A3L homolog), and mc095, late gene encoding a vaccinia virus D6R homolog. Lane 1: 100-bp ladder; lanes 2–6: 0, 2, 4, 72, and 120 h; lane 7: 7 d; lane 8: 9 d; lane 9: 14 d. (From **ref. *2***, with permission.)

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