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Yoshihiro Kawaoka
Gabriele Neumann *Editors*

Influenza Virus

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Edited by

Yoshihiro Kawaoka

*Influenza Research Institute, Department of Pathobiological Sciences,
School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI, USA;
Department of Special Pathogens, International Research Center for Infectious Diseases,
Institute of Medical Science, University of Tokyo, Tokyo, Japan;
Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science,
University of Tokyo, Tokyo, Japan;
ERATO Infection-Induced Host Responses Project, Saitama, Japan*

Gabriele Neumann

*Influenza Research Institute, Department of Pathobiological Sciences,
School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI, USA*

Editors
Yoshihiro Kawaoka
Influenza Research Institute
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin–Madison
Madison, WI, USA

Department of Special Pathogens
International Research Center
for Infectious Diseases
Institute of Medical Science
University of Tokyo
Tokyo, Japan

Division of Virology
Department of Microbiology and Immunology
Institute of Medical Science
University of Tokyo
Tokyo, Japan

ERATO Infection-Induced Host
Responses Project
Saitama, Japan

Gabriele Neumann, Ph.D.
Influenza Research Institute
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin–Madison
Madison, WI, USA

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Preface

Reports of influenza-like illnesses date back to the Middle Ages, and outbreaks of influenza likely afflicted humans long before that. The first influenza virus was isolated in 1933 and influenza viruses have been among the most intensively studied viruses ever since. Influenza virus research led to the identification of the genetic material and viral proteins in the 1970s. The first influenza vaccine was approved in the USA in 1944. Live attenuated vaccine viruses were developed in the 1960s but did not become available for human use until 2003. Influenza virus research also led to the development of two classes of antivirals–ion channel and neuraminidase inhibitors, which were approved in the USA in 1966 and 1999, respectively. In 1999, a method of the artificial generation of an influenza virus was established. This system has been instrumental in the development of novel influenza vaccines, and in the understanding of viral pathogenicity and the functions of viral proteins. This book is intended to summarize the current techniques that have made this progress possible, ranging from protocols for virus isolation, growth, and subtyping to procedures for the efficient generation of any influenza virus. These techniques are used in numerous laboratories around the world and are, thus, the building blocks that underpin almost all influenza virus research.

Influenza viruses continue to threaten humans. Striking reminders include the influenza pandemic of 1918 that killed an estimated 40–50 million people worldwide and may even have affected the outcome of World War I, as well as the recent transmissions of highly pathogenic avian H5N1 influenza viruses to humans, resulting in a case fatality rate of approximately 60%. Are we prepared for future pandemics? The H1N1 pandemic in 2009 was unforeseen, and although vaccines to this novel strain were on the market within 6 months, an even shorter response time would be desirable. To cope with the emergence of novel strains and resistance to existing antivirals, researchers will more than ever have to rely on their ingenuity, expertise, and experience to better understand the mechanisms of influenza pathogenicity and to develop countermeasures. Solid knowledge of the standard techniques described in this book is a small, but important, step towards meeting these challenges.

Madison, WI, USA   *Yoshihiro Kawaoka*
                                                                                    Gabriele Neumann

Contents

Contributors

JUSTIN BAHL • *Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore, Singapore*

AMANDA BALISH • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

DORIS BUCHER • *New York Medical College, Associate Professor, Department of Microbiology and Immunology*

ZHONGYING CHEN • *MedImmune, Mountain View, CA, USA*

TONY COLGATE • *Novartis Vaccines and Diagnostics, Cambridge, MA, USA*

ALEXANDRA S. GAMBARYAN • *M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow, Russian Federation*

LARISA V. GUBAREVA • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA; WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Atlanta, GA, USA*

KATHY HANCOCK • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

AERON C. HURT • *WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory, Melbourne, VIC, Australia*

HONG JIN • *MedImmune, Mountain View, CA, USA*

JACQUELINE M. KATZ • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

YOSHIHIRO KAWAOKA • *Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI, USA*

GEORGE KEMBLE • *MedImmune, Mountain View, CA, USA*

ALEXANDER KLIMOV • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

SCOTT KRAUSS • *Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA*

EDWIN J.B. VELDHUIS KROEZE • *Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands*

THIJS KUIKEN • *Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands*

JIANHUA LE • *New York Medical College, Research Assistant Professor, Department of Microbiology and Immunology*

JONATHAN LIU • *MedImmune, Mountain View, CA, USA*

XIUHUA LU • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

RAMANUNNINAIR MANOJKUMAR • *New York Medical College, Research Assistant Professor, Department of Microbiology and Immunology*

MIKHAIL N. MATROSOVICH • *Institute of Virology, Philipps University, Marburg, Germany*

GABRIELE NEUMANN • *Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI, USA*

MARGARET OKOMO-ADHIAMBO • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA; WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Atlanta, GA, USA*

ALBERT D.M.E. OSTERHAUS • *Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands*

MAKOTO OZAWA • *Department of Pathobiological Sciences, University of Wisconsin–Madison, Madison, WI, USA*

BARBARA A. POKORNY • *New York Medical College, Supervisory Research Technician, Department of Microbiology and Immunology*

JARAD SCHIFFER • *Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

JEANMARIE SILVERMAN • *New York Medical College, Research Technician, Department of Microbiology and Immunology*

KIRSTEN ST. GEORGE • *Laboratory of Viral Diseases, Wadsworth Center, New York State Department of Health, Albany, NY, USA*

GAVIN J.D. SMITH • *Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore, Singapore*

KLAUS STÖHR • *Novartis Vaccines and Diagnostics, Cambridge, MA, USA*

HONG SUN • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

VIC VEGUILLA • *Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

DHANASEKARAN VIJAYKRISHNA • *Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore, Singapore*

DAVID WALKER • *Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA*

ROBERT G. WEBSTER • *Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA; Department of Pathology, University of Tennessee Health Science Center, Memphis, TN, USA*

DAVID E. WENTWORTH • *J. Craig Venter Institute, Rockville, MD, USA*

JOHN WOOD • *Novartis Vaccines and Diagnostics, Cambridge, MA, USA*

JOHN M WOOD • *National Institute for Biological Standards and Control, Potters Bar, UK (retired)*

BIN ZHOU • *J. Craig Venter Institute, Rockville, MD, USA*

Chapter 1

Influenza Viruses: An Introduction

Yoshihiro Kawaoka and Gabriele Neumann

Abstract

We provide a brief introduction into the genome organization, life cycle, pathogenicity, and host range of influenza A viruses. We also briefly summarize influenza pandemics and currently available measures to control influenza virus outbreaks, including vaccines and antiviral compounds to influenza viruses.

Key words: Influenza virus, Life cycle, Pathogenicity, Host range, Pandemic, Vaccine, Antiviral compound

1. Classification

Influenza viruses belong to the family *Orthomyxoviridae* that consists of five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, and *Isavirus* (reviewed in ref. (42)). Thogoto- and Isaviruses are not of medical relevance in humans and will therefore not be discussed in detail. Influenza C viruses infect humans but cause mild, typically symptomless, infections. Influenza A and B viruses cause annual epidemics, and influenza A viruses also cause pandemics at random intervals. Influenza A viruses are further classified into subtypes based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) proteins. Currently, 16 HA subtypes (H1–16) and nine NA subtypes (N1–9) are recognized. During the last century, influenza A viruses of the H1N1, H3N2, H2N2, and H1N2 subtypes have circulated in humans; currently, only viruses of the H1N1 and H3N2 subtypes are circulating in humans together with influenza B viruses. Viruses of other subtypes (H5N1, H7N7, H9N2) have sporadically infected humans, but have not caused widespread outbreaks due to their limited ability to spread among humans.

2. Genome Organization and the Viral Life Cycle

2.1. Genome Organization

Influenza A and B viruses contain eight segments of negative-sense (i.e., complementary in its orientation to mRNA), single-stranded RNA (reviewed in ref. (31)). The three largest segments and the fifth largest segments encode the components of the viral replication machinery, i.e., the polymerase proteins PB2, PB1, and PA, and the nucleoprotein protein NP. The PB1 segment of some influenza A viruses also encodes a second protein, PB1-F2, whose function is described in Subheading 3.4. The fourth and sixth largest segments encode the viral surface glycoproteins HA and NA. The NA segment of influenza B viruses encodes a second protein, NB, whose function is not fully understood. The seventh largest segment of influenza A and B viruses encodes the matrix protein M1 and the ion channel protein (termed M2 for influenza A viruses and BM2 for influenza B viruses); however, influenza A and B viruses differ in their coding strategies for M2 and BM2 by employing a splice mechanism (influenza A viruses) or an overlapping stop–start pentanucleotide (influenza B viruses). The smallest segment of influenza A and B viruses encodes the interferon antagonist NS1 from an unspliced mRNA and the nuclear export protein (NEP, formerly called NS1) from a spliced mRNA.

The viral RNAs contain conserved nucleotides at both ends that function as promoters for replication and transcription. The negative-sense viral RNAs (vRNAs) are transcribed into capped and polyadenylated mRNAs by the viral polymerase complex. During replication, the viral polymerase complex synthesizes a positive-sense copy of the viral RNA (termed cRNA), which serves as a template for the synthesis of vRNAs for virus propagation. In contrast to most other negative-sense RNA viruses, influenza virus replication and transcription take place in the nucleus of infected cells.

2.2. The Viral Life Cycle

The viral life cycle starts with the binding of virus particles to receptors on the cell surface. Binding is mediated by the HA protein that interacts with sialyloligosaccharides on proteins and lipids on the host cell surface. The virus is internalized by receptor-mediated endocytosis. The low pH in late endosomes triggers a conformation change in HA that leads to the fusion of the viral and endosomal membranes, and thus the release of viral ribonucleoprotein (vRNP) complexes (composed of vRNA and the polymerase and NP proteins) into the cytoplasm. After their import into the nucleus, replication and transcription (see previous section) lead to the amplification of vRNAs and the synthesis of mRNAs for viral protein synthesis. Late in the infection cycle, newly assembled vRNPs are exported to the cytoplasm with the help of the M1 and NEP proteins. These vRNPs are assembled with newly synthesized viral proteins at the plasma membrane, followed by the budding and release of influenza virions.

3. Functions of Viral Proteins in Pathogenicity and Host Range

3.1. HA

The HA protein is a major determinant of host range and pathogenicity (reviewed in refs. (13, 18)). The precursor protein (HA0) is posttranslationally cleaved into the HA1 and HA2 subunits. The HA proteins of highly pathogenic avian influenza viruses possess multiple basic amino acids at the HA cleavage site. The respective motifs are recognized by ubiquitous proteases, leading to systemic infection in poultry. By contrast, the HA proteins of avirulent influenza viruses contain a single basic amino acid at the cleavage site, which is cleaved by a limited number of proteases that are restricted to the respiratory and/or intestinal tract.

The HA protein affects host range through its receptor-binding specificity. It recognizes sialyloligosaccharides terminated by N-acetylsialic acid which is linked to galactose by an α2,6- or α2,3-linkage (NeuAcα2,6Gal or NeuAcα2,3Gal, respectively). Human influenza viruses have higher affinity for NeuAcα2,6Gal, while avian influenza viruses preferentially bind to NeuAcα2,3Gal. Correspondingly, epithelial cells in human trachea express primarily NeuAcα2,6Gal, while epithelial cells in duck intestine (the major replication site of avian influenza viruses) express predominantly NeuAcα2,3Gal. Interestingly, the epithelial cells in pig trachea express both types of sialyloligosaccharides, which likely explains why pigs can be infected by both human and avian influenza viruses.

3.2. PB2

The PB2 protein is a major determinant of pathogenicity in mammalian species. Lysine at position 627 (found in human influenza A viruses and in some highly pathogenic avian H5N1 influenza A viruses) confers efficient replication in the lower and upper respiratory tract of mammalian species (16, 17). Glutamic acid at this position (found in most avian influenza A viruses) allows efficient replication in the lower, but not upper respiratory tract of mammalian species. The ability to replicate efficiently in the upper respiratory tract of mammalian species may facilitate virus transmission.

3.3. NS1

The NS1 protein interferes with interferon-induced antiviral host cell responses (reviewed in refs. (9–11, 21, 24)). It prevents the efficient upregulation of transcription factors and interferon-β-stimulated genes. NS1 also interferes with the double-stranded RNA-dependent activation of 2′-5′ oligo(A) synthetase (28) and hence with the subsequent activation of RNase L, a critical factor in host cell innate immune responses. In addition, NS1 blocks the virus-induced activation of RIG-I (15, 20, 27, 30, 33), a major pathogen-recognition receptor. The NS1 proteins of different influenza A viruses differ in their ability to interfere with host immune responses.

3.4. PB1-F2

The PB1-F2 protein is encoded by a second reading frame in the PB1 segment of human and avian, but not swine, influenza A viruses. It induces apoptosis (3), most likely through the interaction with mitochondrial proteins. PB1-F2 also interacts with the PB1 protein to retain viral ribonucleoprotein complexes in the nucleus. A single amino acid change in PB1-F2 affected the virulence of a highly pathogenic avian H5N1 virus and of the pandemic "Spanish" influenza virus (see Subheading 4.1) (6), demonstrating the contribution of PB1-F2 to influenza viral pathogenicity.

4. Past and Present Influenza Pandemics

Pandemics are outbreaks of large, typically, global scale epidemics caused by viruses possessing HA proteins to which human populations have no preexisting immunity. The direct and indirect costs of influenza virus pandemics can be significant and typically put severe strains on health-care systems.

4.1. "Spanish Influenza" in 1918/1919

In 1918/1919, an estimated 20–50 million people worldwide died from infection of the "Spanish influenza" virus. Reverse genetics allowed the recreation of this virus from samples obtained from formalin-fixed, paraffin-embedded tissues of victims of the "Spanish influenza," or from lung tissue derived from a corpse of a "Spanish influenza" victim preserved in the permafrost of the Arctic (37). The "Spanish influenza" virus is an H1N1 virus of avian origin; it may have replicated in mammalian species for a limited period of time before causing the pandemic of 1918/1919. Studies with recreated "Spanish influenza" virus or variants thereof indicated that the HA (23, 32, 38, 39), the replication complex (32, 37, 40, 41), the NS1 protein (14), and the PB1-F2 protein (26) are critical for the virulence of the virus. However, the virus lacks signature amino acids typically associated with high pathogenicity (such as a multibasic cleavage site in HA or Lys at position 627 of the PB2 protein).

4.2. "Asian Influenza" in 1957

In 1957, reassortment of human H1N1 and avian H2N2 influenza A viruses resulted in a human influenza viruses possessing avian virus H2 HA, N2 NA, and PB1 segments (22, 35). This "novel" H2N2 virus caused the "Asian influenza" that killed an estimated 1 million people worldwide.

4.3. "Hong Kong Influenza" in 1968

In 1968, a pandemic H3N2 strain emerged which possessed avian virus H3 HA and PB1 genes in the background of a human influenza virus (22, 35). The death toll in the USA reached 33,800; the lower death toll as compared to previous pandemics likely resulted from preexisting antibodies to N2 NA in human populations.

4.4. "Swine-Origin H1N1" Pandemic in 2009

In February 2009, increased numbers of influenza-like illnesses were recorded in Mexico. Soon after, a novel influenza virus of the H1N1 subtype was identified as the causative agent of the outbreak. Genetic data indicate that this virus originated from pigs, although no influenza virus outbreaks in pigs were reported in the affected areas at the time of the outbreak. The novel virus possess six segments (PB2, PB1, PA, HA, NP, and NS) from originally avian/human/swine triple reassortant viruses that have now circulated in North American pig populations for more than a decade (12, 29). The NA and M segments are derived from a Eurasian avian-like swine virus. The outbreak spread rapidly and reached pandemic status in April of 2009. Most cases are mild and do not require hospitalization; however, certain risk factors including pregnancy and obesity seem to increase the risk of severe respiratory disease with potentially fatal outcome. Studies in mice, ferrets, pigs, and nonhuman primates suggest that the novel pandemic strain is more pathogenic in these animal models than contemporary human influenza viruses (19).

5. Interpandemic Periods

Antigenic drift, i.e., the accumulation of point mutations in the antigenic sites of HA and NA, creates variants that cause annual epidemics. Epidemics typically affect 10–20% of the population, but attack rates can be significantly higher in high-risk groups or specific age groups. Influenza virus epidemics are major causes of respiratory illness among schoolchildren and in nursing homes. Epidemics are also associated with access mortality, that is, an estimated 500,000 deaths worldwide (36,000 in the US) are attributed to influenza per year. The cumulative burden of epidemics during interpandemic years can reach that of pandemic outbreaks.

6. Interspecies Transmission of Influenza A Viruses

Host range restriction of influenza A viruses is primarily controlled by the HA and PB2 proteins (see Subheadings 3.1 and 3.2). Nonetheless, occasional interspecies transmission of influenza A viruses has been reported. Prime examples include the "Spanish influenza" virus (see Subheading 4.1) and the swine-origin H1N1 pandemic virus (see Subheading 4.4). Another example are highly pathogenic avian H5N1 viruses that emerged a decade ago (4, 5, 7, 36), are now endemic in poultry populations in Southeast Asia, and have caused local outbreaks in wild birds and poultry populations in three continents. These avian influenza viruses infect

humans and cause severe respiratory infections with a mortality rate of >60%. However, these viruses do not transmit among humans, in contrast to the swine-origin H1N1 pandemic viruses.

Other examples for the exchange of influenza A viruses between humans, pigs, and avian species exist; pigs may play a critical role in this process and influenza A virus reassortment since this species can be infected with both human and avian influenza viruses. In addition, occasional transmission of avian influenza viruses to horses and aquatic mammals has been reported. Overall, interspecies transmission appears to be rare and most cases are self-limiting.

7. Clinical Manifestations

Influenza A and B viruses cause respiratory infections that range from symptomless infections to life-threatening disease with potentially fatal outcome. Typical symptoms include cough, fever, sore throat, headache, malaise, chills, anorexia, coryza, and myalgia. The incubation period typically ranges from 1 to 4 days, followed by a symptomatic period of 3–4 days. The clinical outcome depends on the intrinsic properties of the virus, preexisting immunity, and the health status of the infected individual. Certain preexisting medical conditions including heart or lung disease, immunological disorders, renal failure, but also pregnancy and smoking, increase the risk of severe disease.

The severity of influenza virus infections is greatest with H3N2 viruses, followed by influenza B viruses, and H1N1 viruses; infections with influenza C viruses typically do not cause disease symptoms.

8. Influenza Virus Vaccines

In the USA, both inactivated and live attenuated vaccines for seasonal influenza are approved for use in humans. These vaccines include seasonal influenza A viruses of the H1N1 and H3N2 subtypes, and an influenza B virus. Due to the accumulation of amino acid mutations in HA and NA (antigen drift), the vaccine strains have to be replaced every 1–3 years with more recent strains.

Inactivated vaccine viruses possess at least the HA and NA genes of the circulating virus, and the remaining genes from A/Puerto Rico/8/34 (H1N1) virus which confer efficient growth in embryonated chicken eggs. After purification and concentration of the allantoic fluid, viruses are inactivated with formalin or β-propiolactone, and treated with detergents or ether for the production of split or subunit vaccines. These vaccines are administered intramuscularly or subcutaneously; they are ~60–80% efficacious in children and young adults but have lower efficacy in elderly populations.

Live attenuated vaccines elicit both humoral and cellular immune responses and may therefore be superior to inactivated vaccines. A live attenuated influenza vaccine is now licensed in the USA, but limited to people 2–49 years of age. This vaccine was generated by serial passage at low temperature of A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66, resulting in viruses that are attenuated, cold adapted, and temperature sensitive. Reassortment with circulating viruses leads to vaccine seed strains that possess the HA and NA genes of the circulating viruses in the genetic background of the attenuated viruses. Alternatively, reverse genetics methods are now approved for the generation of live attenuated viruses directly from cloned cDNAs.

9. Antiviral Compounds

At present, two classes of antiviral drugs—ion channel inhibitors and neuraminidase inhibitors—are licensed for use in humans.

Ion channel inhibitors (such as amantadine hydrochloride and rimantadine) block the ion channel formed by the influenza A virus M2 protein and thereby prevent the release of vRNP complexes into the cytoplasm of infected cells. Most circulating human H1N1 and H3N2 viruses (1, 2), the swine-origin H1N1 pandemic viruses (29), and some highly pathogenic avian H5N1 influenza viruses are now resistant to ion channel inhibitors. Thus, these compounds can no longer be used for the control of influenza A viruses.

Neuraminidase inhibitors including oseltamivir and zanamivir block the sialidase activity of the NA protein, which is critical for efficient release of viruses from infected cells. Human H1N1 viruses have recently developed resistance to oseltamivir (34), but they are still sensitive to zanamivir. In addition, low levels of oseltamivir resistance have been reported for highly pathogenic avian H5N1 viruses (8, 25) and pandemic swine-origin H1N1 influenza viruses.

10. Outlook

In this volume of "Methods in Molecular Biology," we present protocols that cover the basic techniques in influenza virology including diagnosis with subtyping, virus isolation, and titration; receptor-binding assays; the assessment of sensitivity to antiviral drugs; the use of animal models; the development and production of influenza virus vaccines; the artificial generation of influenza viruses from cloned cDNAs; and bioinformatics studies for phylogenetic and evolutionary analyses. These protocols will provide the audience with a comprehensive overview of the standard techniques in influenza virus research.

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Chapter 2

Influenza Virus Isolation

Scott Krauss, David Walker, and Robert G. Webster

Abstract

The isolation of influenza viruses is important for the diagnosis of respiratory diseases in lower animals and humans, for the detection of the infecting agent in surveillance programs, and is an essential element in the development and production of vaccine. Since influenza is caused by a zoonotic virus it is necessary to do surveillance in the reservoir species (aquatic waterfowls), intermediate hosts (quails, pigs), and in affected mammals including humans. Two of the hemagglutinin (HA) subtypes of influenza A viruses (H5 and H7) can evolve into highly pathogenic (HP) strains for gallinaceous poultry; some HP H5 and H7 strains cause lethal infection of humans. In waterfowls, low pathogenic avian influenza (LPAI) isolates are obtained primarily from the cloaca (or feces); in domestic poultry, the virus is more often recovered from the respiratory tract than from cloacal samples; in mammals, the virus is most often isolated from the respiratory tract, and in cases of high pathogenic avian influenza (HPAI) from the blood and internal organs of infected birds. Virus isolation procedures are performed by inoculation of clinical specimens into embryonated eggs (primarily chicken eggs) or onto a variety of primary or continuous tissue culture systems. Successful isolation of influenza virus depends on the quality of the sample and matching the appropriate culture method to the sample type.

Key words: Influenza virus, Virus isolation, Virus detection, Sample collection, Embryonated eggs, Tissue culture

1. Introduction

Influenza is caused by a zoonotic RNA virus that is perpetuated in the wild aquatic bird reservoir (1) and is occasionally transmitted via intermediate hosts (pigs, quails) to humans and other mammals; on rare occasions these viruses can evolve into highly pathogenic (HP) or pandemic strains (2, 3). To understand the evolution of influenza viruses it is necessary to do surveillance in the reservoir, intermediate, and mammalian hosts. The emergence of the pandemic H1N1 2009 influenza virus can be traced from humans to pigs and ultimately to wild aquatic birds (4). The emergence of the Asian

HP avian H5N1 influenza virus evolved along similar pathways but is different in acquiring a highly cleavable hemagglutinin (HA) molecule that is associated with high pathogenicity in gallinaceous poultry (5). The HP H5N1 influenza virus has to date not acquired the characteristics of consistent human-to-human transmission. The purpose of this chapter is to consider surveillance strategies for influenza virus from the perspective of the isolation of influenza viruses to elucidate the natural history, evolutionary strategies, pathogenesis, and the molecular basis of pathogenicity and transmissibility. The isolation of influenza viruses plays an important role in pandemic planning and vaccine preparation.

2. Materials

2.1. Collection of Specimens

Details of materials for collection of samples for influenza are given in the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf).

1. Cryovials, 2–3 mL.

2. Phosphate buffered saline (PBS), pH 7.2: For 10 L mix 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 or 21.7 g $Na_2HPO_4 \cdot 7H_2O$, and 2 g KH_2PO_4: if necessary adjust to pH 7.2 with 1 N HCl.

3. Transport media for egg inoculation: PBS, pH 7.2, autoclaved, 500 mL with 500 mL sterile glycerol with penicillin G (2×10^6 U/L) (Sigma, St. Louis, MO), streptomycin (200 mg/L) (Sigma), polymixin B (2×10^6 U/L) (X-Gen Pharmaceuticals, Inc., Long Island, NY), gentamicin (250 mg/L) (Hospira, Inc., Lake Forest, IL), nystatin (0.5×10^6 U/L) (Sigma), ofloxacin HCl (60 mg/L) (Sigma), sulfamethoxazole (200 mg/L) (Sigma) (see Note 1).

4. Transport media for tissue culture: Medium 199 with 0.5% BSA with penicillin G (2×10^6 U/L)) (Sigma), streptomycin (200 mg/L) (Sigma), polymixin B (2×10^6 U/L) (X-Gen Pharmaceuticals), gentamicin (250 mg/L) (Hospira, Inc.), nystatin (0.5×10^6 U/L) (Sigma), ofloxacin HCl (60 mg/L) (Sigma), sulfamethoxazole (200 mg/L) (Sigma) (see Note 1).

5. Swabs: cotton, polyester or Dacron tipped with plastic or wire shaft (see Notes 2 and 3).

6. Field Data Sheet.

7. Personal protective equipment.

8. Concentrated antibiotics: 1 mL contains 200,000 U penicillin G potassium salt (Sigma), 40 mg Streptomycin sulfate salt (Sigma), 20,000 U polymixin B for injection, USP (X-Gen

Pharmaceuticals), 4 mg gentamicin (Hospira, Inc.); store in 0.5 mL aliquots at –20°C.

9. 10% suspension of formaldehyde-fixed chicken red blood cells: one volume of fresh red blood cells is combined with nine volumes of sterile PBS and 37% formaldehyde solution is added dropwise with constant stirring to a formalin concentration of 1.5% and mixed at 4°C for 18–20 h with constant stirring. The mixture, which should be dark brown from reacting with the formaldehyde, is then washed five times with PBS to remove formalin. The fixed erythrocytes are diluted in PBS to a concentration of 10%.

2.2. Egg Inoculation

1. Egg sealant (wax, nail polish, household cement).

2. 1-cm³ syringe.

3. 27-gauge 1-1/2″ syringe needle.

4. 3-cm³ syringe with 21-gauge 1″ needle.

5. 10- to 11-day-old embryonated chicken eggs (7- to 8-day-old embryonated chicken eggs for influenza C).

6. 70% Ethanol.

7. PBS, pH 7.2: For 10 L mix 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 or 21.7 g $Na_2HPO_4 \cdot 7H_2O$, and 2 g KH_2PO_4: if necessary adjust to pH 7.2 with 1 N HCl.

8. Concentrated antibiotics: 1 mL contains 200,000 U penicillin G potassium salt (Sigma), 40 mg Streptomycin sulfate salt (Sigma), 20,000 U polymixin B for injection, USP (X-Gen Pharmaceuticals), 4 mg gentamicin (Hospira, Inc.); store in 0.5 mL aliquots at –20°C.

2.3. Tissue Culture Maintenance

1. Madin–Darby Canine Kidney Cells (MDCK), ATCC CCL-34.

2. Growth medium: Eagle's minimum essential medium (EMEM) (Invitrogen, Carlsbad, CA) with Earle's salts, L-glutamine and sodium bicarbonate augmented with fetal bovine serum (Thermo Scientific, Waltham, MA) added to a final concentration of 10% and Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 10 mL/L.

3. 0.5% Trypsin–EDTA (10×) (Invitrogen).

4. Trypsin Neutralizer (Invitrogen).

5. Sterile PBS.

2.4. Tissue Culture Infection

1. Madin–Darby Canine Kidney Cells (MDCK), ATCC CCL-34.

2. Infection medium: Eagle's minimum essential medium (EMEM) with Earle's salts, L-glutamine and sodium bicarbonate augmented with bovine serum albumin (Sigma) added to a final concentration of 4% and Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 10 mL/L.

3. Trypsin, TPCK-treated (Worthington Biochemical, Lakewood, NJ), diluted in ddH_2O to a concentration of 1 μg/mL, and sterile-filtered. Aliquot and store at –20°C.

4. PBS-ABC: 160 mL PBS-A (5×), 10 mL PBS-B (10×), 10 mL PBS-C (10×), 10 mL Antibiotic Antimycotic Solution (100×), Stabilized (Sigma), 1 mL gentamicin sulfate injection USP 80 mg/2 mL (Hospira, Inc.) in 810 mL sterile ddH_2O. (PBS-A (5×):100 g NaCl, 2.5 g KCl, 14.4 g Na_2HPO_4, 2.5 g KH_2PO_4, 0.2 g phenol red, bring to 2 L with ddH_2O and autoclave; PBS-B (10×): 13.2 g $CaCl_2·H_2O$, bring to 1 L with ddH_2O and autoclave; PBS-C (10×): 10 g $MgCl_2·6H_2O$, bring to 1 L with ddH_2O and autoclave).

3. Methods

The collection of samples for influenza virus surveillance depends on the species to be tested. Thus, influenza in the original avian reservoir species is primarily an intestinal tract infection ([6]); however, initial infection occurs in the respiratory tract making it necessary to collect both respiratory and fresh fecal samples. From domestic poultry the same samples can be collected as for wild aquatic birds. For influenza surveillance in mammals including humans, pigs, horses, cats, dogs, etc., respiratory tract samples are the primary source of virus but occasionally replication does occur systemically ([7]) and additional samples should be collected (see below).

Prospective surveillance for influenza is often done on apparently healthy birds and mammals ([8]). Protective equipment and clothing should be worn and biological safety and good hygiene practices followed. All samples to be collected must be identified with a tracking number, and information on species, date, sample type, field data, and health status recorded. Ideally, two samples of each specimen are recommended, but practical considerations frequently limit the sampling to one vial. The type of swab to be used depends on the animal and in principal should be large, but keeping in mind that the safety of the animal is paramount. The swab is fully saturated with the sample and immediately placed in plastic vials of transport media appropriate for the system used for isolation, i.e., for egg inoculation, glycerol saline transport medium is recommended and for tissue culture, bovine serum albumin (BSA) enriched tissue culture medium is recommended. The shaft of the swab is broken off and the vial closed and placed in ice (water based). The aim is to transport the sample to the laboratory on wet ice as rapidly as possible or in the field to move the sample vials to a liquid nitrogen dry shipper before the wet ice melts. If the samples cannot be processed immediately in the laboratory, they

are stored at –70°C until processed. On receipt in the laboratory, the samples are either stored at –70°C until processed or are thawed ready for inoculation. The samples must be thawed rapidly in a 37°C incubator (or water bath) until melted, and then held at 0°C on wet ice and injected into eggs or tissue culture.

One of the most frequently made mistakes in influenza surveillance is to microfilter (0.2 μm) the sample to remove bacteria—especially from fecal samples. It is important to rely on antibiotics to eliminate bacteria because filters frequently remove viruses.

Details of collection methods are given in the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf).

3.1. Samples to Collect for Virus Isolation

3.1.1. Avian Species (e.g., Chickens, Ducks)

1. Cloacal swab.
2. Tracheal swab.
3. Oropharyngeal swab—small birds where tracheal sampling is not feasible.
4. Fresh fecal sample (environmental).
5. Water trough sample—caged birds (e.g., song birds, live market poultry).
6. Tissue samples—from sacrificed or freshly dead birds (blood, brain, trachea, lungs, intestinal tract, pancreas, kidney, liver, spleen).
7. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same animal are necessary.

3.1.2. Mammalian Species (e.g., Pigs, Horses)

1. Nasal swabs.
2. Throat swab—oropharyngeal.
3. Tracheal swab (usually after slaughter).
4. Drinking water.
5. Rectal swab.
6. Fecal swab (environmental).
7. Tissue samples—from slaughtered or freshly dead animals (respiratory tract and lung samples, brain, intestinal tract, pancreas, kidney, liver).
8. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same animal are necessary.

3.1.3. Humans

1. Nasal swab.
2. Nasopharyngeal swab.
3. Throat swab.
4. Gargle (oropharyngeal wash).

5. Nasal wash.

6. Transtracheal aspirate.

7. Bronchioalveolar lavage.

8. Fecal sample.

9. Tracheal and lung tissue—postmortem.

10. Internal organs—postmortem.

11. Blood/serum sample—for serological diagnosis; two samples 21 days apart from the same person are necessary.

3.1.4. Isolation of Influenza from Large Volumes of Water

Low levels of influenza virus deposited by infected waterfowl can be concentrated from water (lake water, river water, etc.) using formalin treated erythrocytes (9).

1. To 1 L of the water sample add 5 mL of a 10% suspension of formaldehyde-fixed erythrocytes in PBS.

2. Set on ice and shake thoroughly at 10 min intervals for 1 h.

3. Aliquot into 250-mL centrifuge bottles.

4. Centrifuge in an RC-5B Plus Beckman centrifuge (or similar) for 5 min at $4054 \times g$ in a 4°C prechilled rotor chamber.

5. Discard all but 5 mL of the supernatant which is retained in the bottle.

6. Place bottle on ice and resuspend the pellet with the residual supernatant.

7. Transfer the resuspended pellet to a 50-mL plastic centrifuge tube and spin for 10 min at $4054 \times g$. Discard the supernatant.

8. Resuspend the pellet in a mixture of 0.3 mL antibacterial media and 0.3 mL PBS with antibiotics.

9. Transfer the mixture into a microcentrifuge tube and incubate for 1 h at 37°C. Mix by inverting the tube three or four times during the incubation period.

10. Inoculate three 10-day-old embryonated chicken eggs by the allantoic route (see Subheading 3.2.1) with equal volumes of the mixture.

11. Incubate eggs for 72 h at 35°C.

12. Chill eggs overnight at 4°C and harvest (see Subheading 3.2.2).

General Comments on Handling and Preparing Specimens for Culture

1. For samples where highly pathogenic (HP) avian influenza viruses of H5 or H7 subtype are endemic or suspected, the laboratory manipulation and virus isolation must be done in an approved biosafety level three enhanced (BL3+) laboratory wearing protective equipment (10). For other samples BL2 facilities are appropriate.

2. After the field samples are received in the laboratory they are handled in a class II biosafety cabinet.

3. Before the inoculation into fertile eggs and tissue culture, additional antimicrobial inhibitors are added. The sample is not centrifuged or filtered but injected directly into cultures.

4. Embryonated chicken eggs are tolerant of injection with fecal samples but they can be toxic to tissue cultures.

3.2. Virus Isolation in Egg Cultures

The embryonated egg most frequently used for influenza studies is from the chicken, but duck or other avian embryos also support the replication of influenza viruses (11). Fertile eggs are incubated at 38°C in a humidified atmosphere for 10 days. The eggs are candled with a bright candling lamp for fertility and are marked on the shell at the air sac-main vein connecting point. A major difference between avian and mammalian influenza viruses is that avian influenza viruses possess receptors on their HA molecule that are specific for α 2-3 sialic acid, while mammalian viruses possess predominantly α 2-6 sialic acid receptors (12). In the chicken embryo, the cells lining the allantoic cavity bear predominantly α 2-3 sialic acid while the amniotic cavity has predominantly α 2-6 bearing sialic acid (13, 14). Thus, avian influenza virus will replicate well in the cells lining the allantoic cavity, while mammalian influenza viruses will replicate preferentially in the amniotic cavity (Fig. 1), especially on initial isolation. However, since many mammalian influenza viruses will on initial isolation replicate in the allantoic cavity, both routes of inoculation are recommended for initial virus isolation. All influenza viruses that will replicate in the amniotic cavity of the chicken embryo can be adapted to replicate in the allantoic cavity— a necessary property for vaccine production in chicken embryos. It is noteworthy that this adaptation can result in the selection of antigenic variants that can have influence on the selection of vaccine strains (15). Since inoculation of the amniotic cavity is not as simple as inoculation of the allantoic cavity, it is recommended that those using this technique practice inoculation of the amniotic cavity using Coomassie brilliant blue stain (0.5%) followed by opening the egg to determine that the virus was injected into the amniotic cavity. The detailed methods and safety considerations are given on the WHO website in the document entitled "Laboratory-based surveillance of influenza virus infections—Part B Procedures" (http://whqlibdoc.who.int/publications/1982/a86910_partB.pdf).

The following points need to be considered during influenza virus isolation:

1. The kind of red blood cells (erythrocytes) to be used in the HA test needs to be considered. For initial detection of mammalian influenza viruses especially from humans, guinea pig or human erythrocytes should be used keeping in mind that these are nonnucleated cells and take longer to settle (13). Turkey or goose erythrocytes give broader detection of all influenza viruses than chicken erythrocytes (16), and like chicken erythrocytes
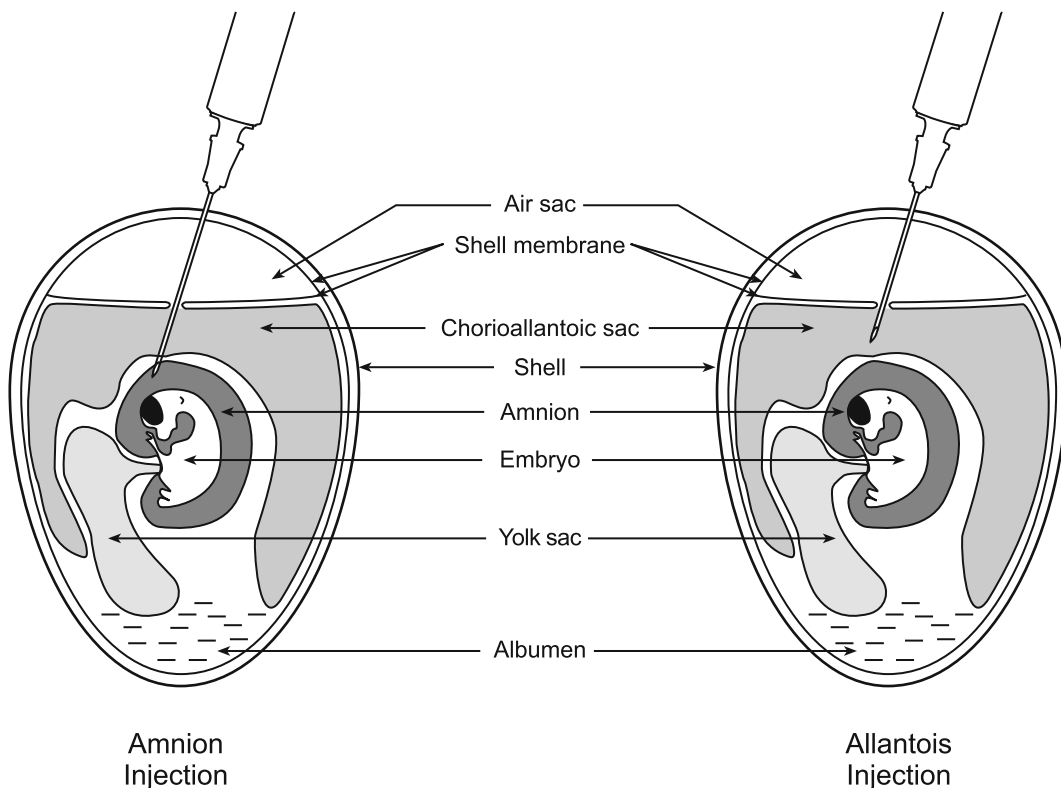
Fig. 1. Egg diagram illustrating the sites for injection of specimens into the amnion and allantois.

are nucleated and settle more rapidly than human or horse erythrocytes in hemagglutination assays. For some avian influenza viruses it is necessary to use horse erythrocytes, notably for HP H5N1 viruses from mammalian sources (17). Thus, for the initial detection of human and other mammalian influenza viruses, turkey, guinea pig, and horse erythrocytes can be used, and for avian influenza viruses, turkey and horse erythrocytes can be used.

2. Hemagglutination of erythrocytes can be caused by a number of agents including some parainfluenza viruses, bacteria, and by influenza viruses. It is important to note that some bacteria that do not kill chicken embryos can cause hemagglutination.

3. For rapid identification of the HA agent one of the rapid diagnostic kits (18) can be used. This will establish whether the HA agent is an influenza virus and will distinguish between influenza A and B. For identification of the subtype a hemagglutination inhibition (HI) test using specific immune sera is performed. If the available antisera do not identify the influenza subtype it is possible that the agent is a novel influenza virus, and sequencing of the HA and NA is recommended.

Additionally, the test results and the virus should be provided to the regional health or agricultural authority.

4. Two passages in embryonated eggs are recommended by WHO for mammalian influenza samples (particularly for humans). However, with the widespread use of molecular screening (real-time RT-PCR), experience has shown that a single passage in embryonated eggs detects almost all influenza viruses in field samples from apparently healthy avian species. If the RT-PCR assay is positive and the initial egg culture is negative for virus, then a second passage is recommended.

5. For short-term storage of virus cultures 4°C is satisfactory. Slow inactivation of infectivity occurs at 4°C permitting storage for several weeks (6). For long-term storage the virus cultures are stored at –70°C.

3.2.1. Egg Inoculation

1. All virus work should be done in a sterile biosafety cabinet that is cleaned and allowed to filter between samples to prevent cross-contamination. Add 0.5 mL of concentrated antibiotics to 50 mL sterile PBS.

2. Candle eggs to ensure viability of the embryo and to make a mark along the line of the air sac, preferably in an area free of blood vessels. Alternately, a small "X" may be used to indicate the site for injection.

3. Spray the eggs with a solution of 70% ethanol and pierce the shell in the area of the air sac just above the line (or at the "X").

4. For each egg to be injected, draw up 0.1 mL of PBS + antibiotics and 0.1 mL of sample into the syringe.

5. Hold the egg to a candling lamp to visualize the embryo (see Note 4) (Fig. 1). When inserting the needle into the amnion the embryo should move slightly. When this is observed inject 0.1 mL of inoculum, pause to allow the inoculum to finish flowing from the needle, and withdraw the syringe slightly (~1/4–1/2″) to inject 0.1 mL of inoculum into the allantois (see Note 5).

6. Seal the holes and incubate eggs at 35°C for approximately 3 days for influenza A and B, or 5 days for influenza C.

7. After incubation the eggs should be chilled at 4°C for 24 h (see Note 6).

3.2.2. Harvesting Virus from the Egg

1. Spray the eggs with 70% ethanol.

2. Use a forceps to crack the egg and remove the cap above the air sac. Sterility of the forceps can be maintained by placing them in a boiling pot between uses.

3. Use a sterile Scoopula™ to hold back the membrane of the egg and use a pipette to remove the allantoic fluid from the egg.

4. Locate the amnion by slowly inverting the egg over a beaker or another receptacle with a suitable disinfectant.

5. Use a 3-cm³ syringe with a 21 gauge 1″ needle to pierce the sac and draw as much amniotic fluid into the syringe as is possible. The fluid from each egg should be kept separate and tested individually for HA activity.

6. Any positive sample to be kept should be streaked on a blood agar plate (10% sheep blood) to check for sterility.

3.3. Isolation Procedures for Influenza Viruses in Tissue Cultures

Since many human influenza viruses cannot be isolated in embryonated chicken eggs it is necessary to utilize cell cultures for their isolation. A number of primary and continuous cell lines can be used for the isolation of influenza viruses. The most frequently used primary cultures are Cynomolgus or Rhesus monkey kidney cultures, while the most frequently used continuous cell lines are Madin–Darby Canine Kidney (MDCK) cells ([19]) or modified MDCK cells possessing an α 2-6 terminal sialic acid-Siat-1 gene ([20]). In addition to MDCK cells the continuous cell lines being used to produce influenza vaccines include Vero, PER.C6 ([21]), and EBx cells. Vero cells are a continuous cell line derived from African Green Monkey Kidney and have been used for over 30 years for the production of inactivated poliomyelitis vaccine, inactivated rabies vaccine, and oral poliomyelitis vaccine. PER.C6 cells were prepared from human fetal retinoblastoma and the cells were immortalized by transfection with an E1 minigenome of adenovirus ([21]). The EBx cell line developed from a duck embryonic stem cell line by Vivalis are licensed to GlaxoSmithKline.

A great advantage of using continuous cell lines is their flexibility. A qualified, fully validated cell line can be retrieved from the frozen state, expanded, and be available at any time. The difficulty of continuous tissue culture cell lines are that they are potentially tumorigenic or could potentially carry oncogenic agents that could theoretically transform host cells into cancer cells. Thus, for vaccine seed stocks for human vaccines, it has been necessary to obtain chicken egg isolated viruses. The WHO and the European Medical Evaluation Authority (EMEA) have set stringent requirements for the safety of continuous cell cultures for use in human vaccine preparation. Validated cell lines have now been approved in Europe for production of influenza vaccines.

Optimally, for influenza virus isolation, a universally available WHO validated cell line that replicates both α 2-3- and α 2-6-dependent influenza viruses (avian-like and human-like) to high titers without the selection of antigenic variants is what is needed. Investigations are ongoing both in WHO collaborating laboratories and in industry to achieve this goal.

Another issue with the use of cell cultures for isolation and production of influenza vaccines is the requirement for the addition

of trypsin in the medium for the cleavage activation of the HA molecule (22). The HA of most influenza viruses grown in tissue culture are not cleaved into HA1 and HA2 without the addition of exogenous trypsin and are therefore not infectious for subsequent cycles of replication. A limited number of influenza viruses including the highly pathogenic H5 and H7 strains do not require the addition of exogenous trypsin. For routine influenza virus isolation the source of the trypsin and animal sera used in influenza virus isolation is not an issue, but if the influenza viruses are to be used for human vaccine studies validated reagents must be used. Alternatively, synthetic tissue culture media can be used and crystalline trypsin produced by recombinant technology is an option.

Cell cultures inoculated with samples for influenza surveillance are usually incubated at 35°C in a humidified CO_2 incubator. While the body temperature of the avian host is ~42°C, of the pig ~39°C, and of the human ~37°C (23), a temperature of 35°C is permissive for influenza viruses from all hosts and is used for influenza isolation.

A brief outline of influenza virus isolation in cell culture is given below. Detailed information is available at the WHO website in the document entitled "Laboratory-based surveillance of influenza virus infections—Part B Procedures" (http://whqlibdoc.who.int/publications/1982/a86910_partB.pdf).

3.3.1. Tissue Culture Maintenance

Preparing Flasks from Frozen Cells

1. Transfer a vial of frozen cells to a 37°C water bath to thaw. Warm growth media at the same time.

2. Place thawed cells into a 15-mL conical centrifuge tube and bring up to volume with cold growth media, and centrifuge at 134–314×g for 8 min.

3. Aspirate media and replace with fresh cold growth media, pipetting up and down to break up clumps and dilute DMSO used for nitrogen storage.

4. Centrifuge again at 134–314×g for 8 min.

5. Resuspend pellet in 5–10 mL of warm growth media, pipetting up and down to break up clumps, and use a hemacytometer to count.

6. Seed a flask according to the number of cells stored.

Preparing Flasks from Confluent Cultures

1. MDCK cells are passaged when confluent with trypsin–EDTA to provide new maintenance cultures in 75 cm² flasks, usually twice per week. Generally a 1:16–1:20 split will yield a confluent 75 cm² flask in 3 days. A 24-well plate seeded with a 1:5 split should yield a confluent plate overnight.

2. Remove growth media and rinse cells with sterile PBS or HBSS.

3. Place 1× trypsin–EDTA onto the monolayer and place in 37°C incubator (see Note 7).

4. Check occasionally to see when cells have lifted from the floor of the flask.

5. Pipette cells into a conical tube and add sufficient Trypsin Neutralizer solution or growth media with fetal calf serum to neutralize the trypsin–EDTA.

6. Centrifuge for 5 min at 1,200 rpm.

7. Resuspend in 5 mL growth media, pipetting up and down to break up cells.

8. Subculture according to the dilutions given above for preparing flasks or plates (see Note 8).

Inoculation of Cell Culture

1. Cells should be approximately 80–90% confluent when infected with influenza virus.

2. Wash monolayer three times with PBS-ABC to ensure the removal of any serum and any nonspecific inhibitors it may contain. Leave the third wash on until just before infection.

3. Add TPCK-trypsin to the infection media at 1:1,000.

4. Dilute the virus in infection media or sterile PBS with antibiotics, usually at 1:100 or 1:1,000 depending on the virus.

5. Remove the third wash from the monolayer and add enough virus dilution to cover the cells and prevent drying (see Note 9).

6. After infection wash with PBS-ABC.

7. Add an appropriate amount of infection media based on the plasticware being used.

8. Incubate at 37°C with 5% CO_2 for influenza A (influenza B generally grows better at 35° or 33°C).

9. Check daily for CPE under low power magnification on the microscope (see Note 10). When CPE is detected (3–7 days), the medium is removed and tested for hemagglutinating agents with red blood cells (selection of erythrocytes follows the same "rules" as discussed above for egg isolation—Subheading 3.2). If using multiple wells it is advisable to check each well separately for HA.

4. Notes

1. Ofloxacin and sulfamethoxazole may be eliminated in areas of low antibiotic usage. Glycerol-based media ensures better long-term stability when cooling is not readily available but is not suitable for use with tissue culture.

2. Wooden shafts may contain toxins and formaldehydes.

3. Cotton or calcium alginate swab residues may inhibit PCR.

4. Using a candling lamp that allows the egg to be held upright may make this easier (e.g., Kuhl Corporation Jiffy Egg Candler FRZ-210Z).

5. Angling the egg slightly on the candling lamp may allow extra light to escape and aid in seeing the volume being injected.

6. In case of time constraint, the eggs may be placed in ice until thoroughly chilled.

7. Trypsin–EDTA (10×) may be aliquoted, stored at –20°C, and thawed and diluted to 1× with sterile PBS as needed.

8. Alternately, cells may be counted and dilutions made as follows: 2×10^6 cells/25 mL for 75 cm^2 flask, 1×10^6 cells/7 mL for 25 cm^2 flask, and 2×10^5 cells/mL (200 µL per well) for TC96- wellplates.

9. When using TC24-wellplates for infection the fluid may be drawn to the outside of the wells if the volume of inoculum is low ($\leq$100 µL), so care should be taken to use sufficient inoculum or rock the plates back and forth every 10 min to prevent drying.

10. If after 8 h the cell sheet is liberated from the plastic and the cells are rounded up, the usual cause is toxicity (e.g., fecal samples). The cells can be pelleted and immediately passed to new tissue culture.

Acknowledgments

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Chapter 3

Influenza Virus Titration, Antigenic Characterization, and Serological Methods for Antibody Detection

Alexander Klimov, Amanda Balish, Vic Veguilla, Hong Sun, Jarad Schiffer, Xiuhua Lu, Jacqueline M. Katz, and Kathy Hancock

Abstract

This chapter describes some commonly used methods of influenza virus titration, antigenic characterization, and serological methods by antibody detection. These methods are essential not only for virus characterization but also for identifying new antigenic variants, vaccine strain selection, and sero-epidemiologic studies of influenza virus transmission and prevalence. Virus titration methods such as the hemagglutination assay, 50% egg or tissue culture infectious dose, and plaque assay are employed to determine the amount of virus particles in a sample. The hemagglutination inhibition assay is a reliable, relatively simple and inexpensive technique to antigenically characterize isolates of influenza viruses. Serological methods such as virus neutralization and hemagglutination inhibition are the fundamental tools used in sero-epidemiologic studies of influenza virus transmission and prevalence and in the evaluation of vaccine immunogenicity. While serological methods rarely yield an early diagnosis of acute influenza virus infection, well-timed, paired acute, and convalescent serum samples may establish the diagnosis of a recent influenza infection even when attempts to detect the virus are negative.

Key words: Virus titration, Hemagglutination assay, Plaque assay, 50% Egg infectious dose, Hemagglutination inhibition assay, 50% Tissue culture infectious dose, Microneutralization, Virus neutralization, Neutralizing antibodies, Influenza

1. Introduction

There are a number of methods of influenza virus titration based on its biological characteristics, such as the virus' ability to agglutinate red blood cells (RBCs) of different species ([1]). The ability of infectious and noninfectious virus particles to agglutinate RBC is the basis of the hemagglutination assay (HA). The plaque assay, 50% tissue culture infectious dose ($TCID_{50}$), and 50% egg infectious dose (EID_{50}) are methods used to determine the amount of infectious

virus particles in a sample. Serological methods such as virus neutralization and hemagglutination inhibition (HAI) are the fundamental tools used in sero-epidemiologic studies of influenza virus transmission and prevalence and in the evaluation of vaccine immunogenicity. Both assays are based on the binding of antibodies to hemagglutinin (HA), the major surface glycoprotein of influenza. Neutralizing antibodies directed against HA are the major mediator of protective immunity against influenza.

Proper virus titration is essential for analysis of antigenic properties of major surface proteins of the virus, the hemagglutinin and the neuraminidase. Comparative antigenic analysis of different strains using standard antiserum, particularly from postinfection ferrets, plays an important role in monitoring antigenic evolution of influenza viruses of different types and subtypes. Antigenic characterization is an important tool in the selection of the most updated vaccine strains.

The standard method for influenza virus titration is based on the ability of the virus hemagglutinin (HA) to agglutinate RBCs of different species (see Note 1). Usually avian RBCs, such as from chickens or turkeys, are used in the hemagglutination assay (see Note 2). Avian RBC are small and nucleated, they settle fast and form a compact button on the bottom of the V-well microtiter plate in the absence of the virus, thus providing with clear endpoint dilution determination. Non-nucleated mammalian RBC (human, guinea pig, horse, etc.) appear as a "halo" or circle of settled cells on the bottom of the U-well microtiter plate control wells, making endpoint dilution reading difficult.

The hemagglutination (HA) assay is dependent on the amount of hemagglutinin on the surface of influenza viruses and not the ability of the virus to replicate (2). The HA assay is able to quantify viral particles regardless of infectivity. The HA endpoint is determined by the highest dilution of virus that causes complete hemagglutination. The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination. An HA unit (HAU) is defined as the amount of virus needed to agglutinate an equal volume of standardized RBCs (3). This "unit" of hemagglutination is an operational unit and not a measure of an absolute amount of virus. The influenza virus isolated in cell culture, such as the Madin–Darby Canine Kidney (MDCK) cell line or embryonated chicken eggs, is titrated by performing a twofold serial dilution of the isolated supernatant in a buffer using a 96-well microtiter plate. The first well contains only the isolate, the second and subsequent wells contain 50 μl of buffer and the diluted virus. The HAUs are used in the HAI assay to determine the standardized dilution of antigen needed to perform the assay.

The EID_{50} is a biological method to determine the amount of infectious virus in a sample by determining the highest dilution of the sample that can infect 50% of the embryonated chicken eggs.

This assay entails performing serial dilutions of the egg sample. To determine the dilution needed to produce a 50% positive result, the Reed–Muench method is used (4). The Reed–Muench method requires the use of three or more eggs per dilution to determine the 50% endpoint by performing a hemagglutination assay for each inoculated egg.

The plaque assay is based on the ability of influenza virus to form plaques in cell monolayer overlaid with agar or agarose (5, 6). The plaques are formed due to cytopathic effect (CPE) caused by the virus and death of the infected cells thus leading to the formation of circular zones of lysed cells on the monolayer. Only infectious virus particles should infect the host cell and be able to produce a plaque. Plaque forming units (PFU) is a quantitative measure of the amount of infectious virus in a sample by determining the number of plaques formed in a cell monolayer (usually MDCK cells are used). At a high dilution of virus stock, each plaque represents the zone of cells infected by a single virus particle. Therefore, the titer of a virus stock can be calculated in PFU per milliliter. The plaque assay is carried out by performing tenfold serial dilutions and then infecting the cell monolayer. An agarose overlay is placed in the wells of the infected cell monolayer. The agarose is removed; the virus inactivated with ethanol and crystal violet is added to the monolayer to visualize the plaques.

Specific antibody attachment to antigenic sites on the HA molecule interferes with the binding between the viral HA and receptors on RBCs. This effect inhibits hemagglutination and is the basis for the HAI assay. The HAI test, originally described by Hirst (7) and later modified by Salk (8), is currently performed in 96-well microtiter plates. Briefly, a standardized quantity of HA antigen is mixed with serial dilutions of antiserum, and RBCs are added to determine specific binding of antibody to the HA molecule. The presence of specific anti-HA antibodies will inhibit the agglutination, which would otherwise occur between the virus and the RBCs (7). This assay is reliable, relatively simple, and is an inexpensive technique. Limitations of the HAI test include the need to remove nonspecific agglutinins in some serum samples since they may cause false-negative results, the need to standardize the virus concentration each time a test is performed, and the need for specialized expertise in reading the results of the test.

The microneutralization test is a highly sensitive and specific assay for identifying influenza virus-specific, neutralizing antibodies in animal and human sera (9). The 2-day assay is performed in two stages. On day 1 of the assay (1) a virus-antibody reaction step, in which virus is mixed with dilutions of serum and time allowed for antibodies to react and (2) an inoculation step, in which the virus–serum mixture is inoculated into the appropriate host system, MDCK cells in this assay. An ELISA is performed on day 2 of the assay to detect virus-infected cells. The absence of infectivity

constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in the serum sample. The preferred serum samples in cases of influenza-like illness are paired acute and convalescent serum samples with the acute collected less than 7 days after symptom onset and the convalescent collected at least 14 days after the acute sample and ideally within 2–3 months of illness onset. A fourfold or greater rise in antibody titer demonstrates a seroconversion and is considered to be diagnostic. With single serum samples, care must be taken in interpreting low titers such as 20 and 40. Generally, knowledge of the antibody titers in an age-matched control population is needed to determine a minimum titer that is indicative of a specific antibody response to the virus utilized in the assay.

Conventional neutralization tests for influenza viruses based on the inhibition of CPE formation and/or detection of hemagglutination activity in MDCK cell cultures are laborious and rather slow. Typically, CPE is read at days 3–7. The microneutralization assay described here uses an ELISA on day 2 of the assay to detect virus-infected cells. Studies have shown that neutralization assays using ELISA to detect virus-infected cells are less variable than neutralization evaluated using CPE and/or detection of hemagglutination activity, possibly because of the extended length of such assays, their use of an endpoint which is more difficult to measure than absorbance, and/or their use of preformed monolayers (10, 11). The steps involved in the influenza virus microneutralization assay are the following. Serially diluted sera are preincubated with a standardized amount of virus prior to the addition of MDCK cells. Serum neutralizing antibodies to influenza virus hemagglutinin inhibit the infection of MDCK cells with virus. After an overnight incubation, the cells are fixed and the presence of influenza A virus nucleoprotein (NP) in infected cells is detected by ELISA. The detection of NP indicates the absence of neutralizing antibodies at that serum dilution.

The microneutralization assay gives the most direct answer to the question of whether an individual has antibodies that can neutralize the infectivity of a given virus strain. The test has several additional advantages for detecting antibody to influenza virus. First, the assay primarily detects antibodies to the virus hemagglutinin and thus can identify functional, strain-specific antibodies in animal and human sera. Second, since infectious virus is used, the assay can be developed quickly upon recognition of a novel virus and is available before suitable purified viral proteins become available for use in other assays. However, the use of live virus in this assay also predicates the need for adherence to biosafety guidelines as outlined in the Biosafety in Microbiological and Biomedical Laboratories (www.cdc.gov/od/OHS/biosfty/bmbl5/bmbl5toc.htm).

2. Materials*

2.1. Hemagglutination Titration Assay

1. 96-Well microtiter plates (Nunc, Thermo Fisher Scientific): V-bottom for avian RBCs or U-bottom for mammalian RBCs.
2. 0.01 M Phosphate-buffered saline (PBS), pH 7.2–7.4 (Invitrogen, Corporation, Cat. # 4539).
3. Standardized RBCs (see Notes 1 and 2): 0.5% for avian and 0.75% for mammalian RBCs. Filter RBCs through sterile gauze, centrifuge at $200 \times g$ for 10 min at 4–8°C, aspirate off plasma, alsevers and buffy coat, add PBS, centrifuge and repeat twice. Resuspend RBCs in PBS and adjust to required concentration. Store at 4–8°C (see Note 3).

2.2. 50% Egg Infectious Dose

1. 9- to 11-day-old embryonated chicken eggs, candled to ensure that the embryos are viable (see Note 4).
2. Virus diluent: supplement 10 ml PBS with 1 ml penicillin–streptomycin solution (100 U/ml penicillin G and 100 μg/ml streptomycin) (Invitrogen, Cat. # 15140) and 0.2 ml 50 mg/ml gentamicin stock (10 μg/ml) (Invitrogen, Cat. # 15750-060). Filter with a 0.2-μm membrane filter and can be stored up to 2 months at 4–8°C.
3. Standardized RBCs (0.5% for avian and 0.75% for mammalian), filter through sterile gauze, centrifuge at $200 \times g$ for 10 min at 4–8°C, aspirate off plasma, alsevers and buffy coat, add PBS, centrifuge and repeat twice. Resuspend RBC in PBS to necessary concentration. Store at 4–8°C.
4. 96-Well microtiter plates (Nunc, Thermo Fisher Scientific): V-bottom for avian RBCs or U-bottom for mammalian RBCs.

2.3. Plaque Assay

1. 2× Plaque assay medium: Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with penicillin–streptomycin (100 U/ml penicillin G and 100 μg/ml streptomycin), L-glutamine 4 mM and HEPES buffer 50 mM. To sterilize, filter through a 0.2-μm membrane filter. Store for not more than 2 months at 4–8°C.
2. Lonza SeaKem Le Agarose (Thermo-Fisher Scientific, Cat. # 5004).
3. MDCK cells (ATCC# CCL-34) confluent in a 6-well tissue culture plates (Nunc, Thermo-Fisher Scientific, Cat. # 150239).
4. Plaque assay wash medium: supplement 490 ml of DMEM with 5 ml of penicillin–streptomycin (100 U/ml penicillin G and 100 μg/ml streptomycin). Filter through a 0.2-μm membrane filter. Can be stored up to 2 months at 4–8°C.
5. L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)–trypsin (2 μg/ml) working solution: supplement 10 ml of

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*Identification of particular products is provided as a guide to aid in the selection of equivalent, suitable products

DMEM with 20 mg TPCK-treated type XIII trypsin from bovine pancreas (Sigma-Aldrich, Cat. # T1426-100 mg). Sterilize through filtration. Store in small aliquots at –70 to –80°C until the earliest expiration date of the products.

6. 70% Ethanol.

7. Gram crystal violet primary stain (Becton, Dickinson, and Company, Cat. # 212525).

2.4. Hemagglutination Inhibition Assay

1. Receptor destroying enzyme (RDE) (II) "Seiken" (Denka Seiken Co, Ltd.), dissolved in physiological saline (0.85% NaCl).

2. 0.01 M PBS, pH 7.2–7.4.

3. 96-Well microtiter plates (Nunc, Thermo Fisher Scientific): V-bottom for avian RBCs or U-bottom for mammalian RBCs.

4. Standardized RBCs (0.5% for avian and 0.75% for mammalian): filter RBCs through sterile gauze, centrifuge at $200 \times g$ for 10 min at 4–8°C, aspirate off plasma, alsevers and buffy coat, add PBS, centrifuge and repeat twice. Resuspend RBCs in PBS to required concentration. Store at 4–8°C (see Note 3).

2.5. MDCK Cell Culture

1. MDCK medium prepared from DMEM (Invitrogen, Cat. # 11965-092), supplemented with 10% fetal bovine serum (FBS) (Hyclone Cat. # SH30070.03), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Cat. # 15140-122), and 2 mM l-glutamine (Invitrogen Cat. # 25030-081). Sterilize by filtration.

2. Solution of trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (Invitrogen, Cat. # 25300-054) (see Note 5).

2.6. TCID Assay to Determine Working Dilution of Virus for Microneutralization Assay

The materials needed for the TCID are the same as those listed for the microneutralization assay.

2.7. Microneutra lization Assay

1. Virus diluent prepared using DMEM, supplemented with 1% bovine serum albumin (BSA) (fraction V, protease free, Roche, Cat. # 03117332001, prepared as a 10% w/v solution in dH$_2$O, filter sterilized, and stored at 4–8°C) 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES (Invitrogen, Cat. # 15630-080). Prepare fresh for each assay. Sterilize by filtration.

2. PBS (0.01 M PBS, pH 7.2). Sterilize by autoclaving.

3. Fixative: Cold 80% acetone in PBS. Prepare and use 80% acetone in a BSC that is externally vented or in a chemical hood. Wear standard PPE which includes latex or nitrile gloves, lab coat, and safety glasses. Prepare the day before use and store

at $-20°C$. It is critical that the fixative is cold when used. After use, discard appropriately.

4. Wash Buffer: PBS + 0.3% Tween 20 (Sigma-Aldrich, Cat. P1379). Prepare fresh daily.

5. Antibody diluent: PBS + 0.3% Tween 20 + 5% w/v milk (nonfat dry milk). Prepare fresh daily. About 1 L is needed for 40 microtiter assay plates.

6. 1° antibody: Anti-influenza A NP mouse monoclonal antibody (Millipore, Cat. # MAB8257 and MAB8258, both purified Ig; mix equal quantities). Dilute in antibody diluent at optimum concentration as determined by titration (see Subheading 3.7.2, steps 5–11).

7. 2° Antibody: Goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (KPL, Cat. # 074-1802). Dilute in antibody diluent at optimum concentration as determined by titration (see Subheading 3.7.2, steps 1–4).

8. Substrate: σ-phenylenediamine dihydrochloride (OPD) in citrate buffer. Prepare citrate buffer by mixing one buffer capsule (Sigma-Aldrich Cat. # P4922) with 100 ml dH_2O. The contents of one capsule dissolved in 100 ml of dH_2O yields 0.05 M phosphate-citrate buffer, containing 0.03% sodium perborate as a substitute for H_2O_2, pH 5.0 at 25°C. Handle OPD tablets in a BSC that is externally vented or in a chemical hood. Wear standard PPE which includes latex or nitrile gloves, lab coat, and safety glasses. After the tablets are dissolved, the substrate may be used on the BSL-2 laboratory bench top. Prepare OPD immediately before use by adding OPD tablets (Sigma-Aldrich, Cat. # P8287) to citrate buffer, one tablet for each 20 ml of citrate buffer. After use, discard appropriately.

9. OPD stop solution (0.5 M sulfuric acid). Add 28 ml of concentrated sulfuric acid (18 M) to 972 ml dH_2O to obtain 0.5 M sulfuric acid. Prepare this solution in a BSC that is externally vented or in a chemical hood. After the addition of dH_2O, the stop solution may be used on the BSL-2 laboratory bench top. Wear standard PPE which includes latex or nitrile gloves, lab coat, and safety glasses. After preparation, store 0.5 M H_2SO_4 on bench top. After use, discard appropriately.

10. Influenza virus (see Note 6). The TCID (tissue culture infective dose) must be determined for each lot number of virus before beginning an MN assay. Virus propagated in MDCK cells or in the allantoic fluid of eggs must be stored at $-70°C$ or colder in single use aliquots. Thaw quickly shortly before use and put on ice. Once the virus dilution for the assay is made, any remaining virus in the thawed aliquot must be appropriately discarded. Virus that has been refrozen should not be used.

11. Serum samples for testing. All serum samples must be stored frozen at -20 to $-80°C$. Thaw serum samples rapidly in a 37°C

water bath. As soon as thawed, place on ice. Keep on ice or at 4–8°C until returned to freezer. If testing from the same aliquot the next day, it is acceptable to keep serum samples at 4–8°C overnight and return to freezer the next day. This method of rapid thawing and handling at 4–8°C minimizes antibody denaturation. Before use in the microneutralization assay, human serum samples must be heat-inactivated at 56°C for 30 min. Animal serum samples, for example positive and negative control sera, must be treated with RDE before use (see Subheading 3.4.1). RDE treatment includes a heat-inactivation step.

12. Purified mouse IgG (Thermo-Fisher Cat. # 31202) is needed for the determination of the working dilution of the 2° antibody (conjugate).

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3. Methods

3.1. Hemagglutination Assay

In the hemagglutination assay, serial virus dilutions are mixed with a constant amount of RBCs. The RBCs contain numerous receptors for virus hemagglutinin and agglutination occurs, if the virus concentration is high enough. If serum is added, the agglutination of RBCs by the influenza virus will be blocked; this is the bases of the HAI assay. Control antiserum and antigens should be included in every HAI assay to verify the specificity of the test.

1. An aliquot (100 μl) of each influenza virus isolated in tissue culture or embryonated chicken eggs (see Note 7) is placed in a well at the first row (row 1) of the 96-well microtiter plate (Fig. 1). Add 50 μl of PBS to rows 2 through 12. Perform a serial twofold dilution series down the 96-well plate transferring 50 μl from row 1 to row 2, etc., disposing of the final 50 μl from the last well. Add 50 μl of the standardized RBCs to all wells. Tap the plate gently to mix or use a mechanical plate shaker. Incubate the plates at room temperature (20–25°C) for the incubation time required for the RBCs used (Table 1).

2. After the incubation period, the HAU are observed for their endpoint of agglutination (Fig. 2). The RBCs will settle to the bottom of the V-well microtiter plate in negative samples, and in positive samples they will agglutinate. The wells in column 8 contain no virus. RBCs that agglutinate will evenly distribute within the well, whereas RBCs that do not agglutinate will form a button in the bottom of the V-well. The HA titer is the last dilution that shows complete hemagglutination activity (see Note 8).
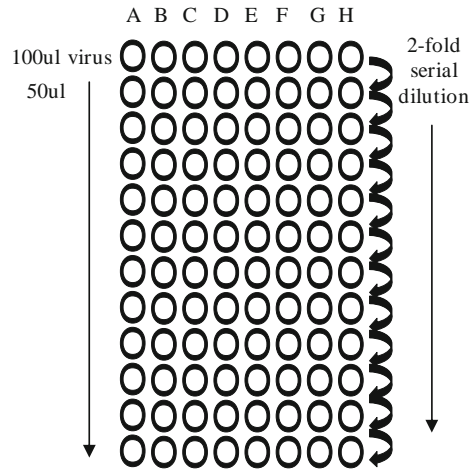
Fig. 1. The 96-well microtiter plate layout for the hemagglutination titration assay.

Table 1
Recommended time of incubation at 20–25°C for the hemagglutination assay with the use of different species of RBCs

	Avian		Mammalian	
	Chicken	Turkey	Guinea pig	Human type O
Concentration	0.5%	0.5%	0.75% v. 75%	0.75%
Microtiter plate	V	V	U	U
Incubation time	30 min	30 min	1 h	1 h
Appearance of control cells	Button[a]	Button[a]	Halo	Halo

[a]When tilted at a 45° angle the RBCs in the V-well plate will flow

3.2. Egg Infectious Dose Assay

1. Add 450 μl of the virus diluent to 10 vials labeled 1 through 10. Aliquot 50 μl of the influenza virus stock to be tested into the first vial, then perform a tenfold dilution by transferring 50 μl from vial 1 to subsequent vials, changing tips between each vial.

2. Inoculate three (or more) embryonated eggs with 100 μl of each virus dilution (see Note 9). Incubate eggs at 35–37°C for 48 h for influenza A and at 33–35°C for 72 h for influenza B. After incubation, chill eggs overnight to halt embryonic growth. Harvest eggs and perform a hemagglutination assay using 100 μl of allantoic fluid from each egg. Observe the endpoint of agglutination and record the results as positive or negative for agglutination.
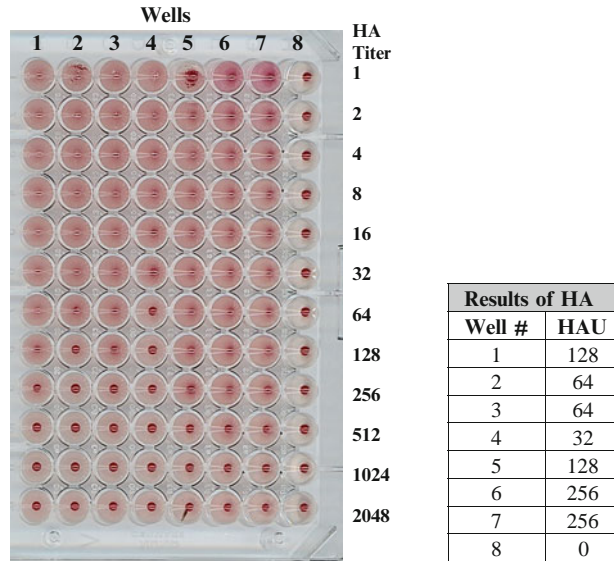
Fig. 2. Interpreting the hemagglutination titration assay.

Results of HA	
Well #	HAU
1	128
2	64
3	64
4	32
5	128
6	256
7	256
8	0

Table 2
Example of EID_{50} calculation using the Reed–Muench method

Log of virus dilution	Infected samples	Cumulative positive (A)	Cumulative negative (B)	Ratio of A/(A + B)	Percent infected (%)
–5	3/3	6	0	6/6	100
–6	2/3	3	1	3/4	75
–7	1/3	1	3	1/4	25
–8	0/3	0	6	0/6	0

Proportional distance formula:

$[(\%\text{positive value} > 50\% - 50\%)/(\%\text{positive value} > 50\%) - (\%\text{positive value} < 50\%)]$

$(75\%) - (50\%)/(75\%) - (25\%) = 0.5$

Log infectious dose50: $(-6) + (0.5 \times 1.0) = -6.5$

Infectious dose50 titer: $10^{6.5}$ ID50/0.1 ml or $10^{7.5}$ ID50/ml

3. Determine the EID_{50} titer per 100 μl by the Reed–Muench method (Table 2).

3.3. Plaque Assay

1. Pre-warm the 2× plaque assay medium in a 37°C water bath and prepare 1.6% (w/v) solution of agarose in a 56°C water bath.

2. Remove the growth medium from the 6-well MDCK tissue culture plates, wash cell monolayer with room temperature plaque assay wash medium (see Note 10). Do not pipette medium directly onto the monolayer as this may disrupt the cells.

3. Thaw virus in cool water. Perform tenfold dilution series starting at 10^{-1} and diluting virus samples down to 10^{-10} (or less) dilution in plaque assay wash medium. Changing tips between dilutions is required. Inoculate 6-well tissue culture wells in duplicate with 100 μl diluted virus samples and gently rock tissue culture plate to cover monolayer with inoculums. Incubate plates at 33–37°C for 30–60 min (see Note 11).

4. After incubation, wash wells twice with room temperature (20–25°C) plaque assay wash medium, taking care not to add medium directly onto the monolayer as this may disrupt the cells.

5. Add 1 μl of 2 mg/ml TPCK–trypsin working stock to 2× plaque assay medium. Mix (1:1) 2× plaque assay medium with Lonza SeaKem Le Agarose and immediately add 2 ml of this mixture to each inoculated well. Allow to solidify at room temperature (20–25°C). Incubate 6-well tissue culture plates at 33–37°C. Observe MDCK plates daily with an inverted microscope for plaque formation.

6. After 72 h, remove the agar plug from each well using sterile forceps. Pipette 2 ml of 70% ethanol into each well and incubate 20 min at room temperature (20–25°C) to fix the MDCK cell monolayer. Remove ethanol and add 1 ml crystal violet solution to each well. Incubate at room temperature (20–25°C) for 10 min to stain MDCK cell monolayer. Remove crystal violet solution and wash wells with water to rinse away excess stain solution. Allow plates to dry overnight at room temperature (20–25°C) prior to counting plaques.

7. Count plaques (Fig. 3) in each well and determine the PFU per milliliter using the following formula: PFU/ml = number of plaques × dilution factor × 10 (see Note 12). The PFU/ml calculation should be based on the dilution of virus sample that gives >10 plaques per well but is still countable. The number of plaques are counted, and then multiplied by the reciprocal of the amount

Fig. 3. Well of a 6-well plate indicating plaques visualized. The plaques will appear as clear circular areas on the background of the crystal violet-stained monolayer.

of samples added to the plate, then multiply by the reciprocal of the dilution factor. For example, if 50 plaques were counted in the 10^{-4} well that had 0.1 ml of sample added, 50 plaques $\times 10^{-1}$ ml sample $\times 10^{-4}$ dilution factor $= 5.0 \times 10^6$ PFU/ml.

3.4. Hemagglutination Inhibition Assay

3.4.1. Treatment of Sera

1. The serum samples should be treated with RDE (see Note 13) to remove nonspecific inhibitors of hemagglutination. Add 3 volumes of RDE to the tube with 1 volume of serum. Incubate in 37°C water bath for 18–20 h. Remove tubes from the 37°C bath and place them in a 56°C water bath for 30 min to inactivate the RDE (see Note 14).

2. Remove tubes from 56°C water bath and allow to equilibrate to room temperature (20–25°C). Add 6 volumes of physiological saline (0.85% NaCl).

3. To remove nonspecific agglutinins from the serum samples, add 1 volume of packed RBCs to 20 volumes of RDE-treated serum. Mix thoroughly and incubate at 4–8°C for 1 h shaking tubes periodically to resuspend cells. Centrifuge at $400 \times g$ for 10 min at 4–8°C.

3.4.2. Standardization of Antigens

1. Based on HAUs determined in the HA assay, standardize the antigens to 8 HAU/50 μl. For the HAI assay, the antigens will be 4 HAU/25 μl, since only 25 μl of standardized antigen is added to each well (1 ml of standardized antigen will test 5 sera). Homologous control antigens corresponding to the control antisera used in the assay should be included into the HAI assay. Calculate the antigen dilution dividing the HA titer by 8. Example: 64 HAU/8 = 8 HAU, thus add one part of antigen with titer 8 HAU to seven parts of PBS. To confirm that the antigen has 8 HAU/50 μl, make twofold dilutions of the standardized antigen with PBS and perform a back titration (see Note 15).

2. A back titration is performed similar to the hemagglutination assay. The standardized antigens should all equal to 8 HAU/50 μl. The titer is equal to 8 HAU when the first three dilutions of virus will show complete hemagglutination and the fourth and further dilutions will have partial or no hemagglutination. If the standardized antigens do not equal to 8 HAUs/50 μl, add antigen or PBS according to Table 3. After adjustment, perform another back titration to ensure all of the antigens have a titer of 8. Store the standardized antigens at 4–8°C.

3.4.3. The Hemagglutination Inhibition Assay Setup

1. Add 50 μl of the RDE treated serum to row 1 of the microtiter plate. Add 25 μl of PBS to rows 2 through 10 (see Note 16). Perform a serial twofold dilution mixing 3–5 times starting at row 1 and discarding the 25 μl of diluted serum into row 11.

Table 3
To adjust antigens based on the results of the back titration.
If the back-titration result is less that 8 HA units, add more
antigen and if greater than 8 HA units add buffer

Interpretation of HA units	ADD undiluted antigen	ADD volume of PBS
0	7 volumes	
1	5 volumes	
2	3 volumes	
2½	2 volumes	
4	Equal volume of antigen	
4½	½ volume	
8	No adjustment	
8½		½ volume
16		Equal volume
16½		2 volumes
32		3 volumes
32½		5 volumes

2. Add 25 μl of standardized antigen into all wells with diluted serum samples. Shake plate gently or use a mechanical plate shaker. Incubate at room temperature for 15–30 min.

3. Add 50 μl of standardized RBCs to all wells with serum–antigen mixtures. Shake plate gently or use a mechanical shaker. Incubate at room temperature for 30–60 min, depending on the RBCs used in the assay.

4. The interpretation: the HAI titer is the reciprocal of the last dilution of antiserum that completely inhibits hemagglutination (Fig. 4). The row 1 is read as 1:10, row 2 is read as 1:20, row 3 as 1:40, etc.

3.5. MDCK Cell Culture for TCID and Microneutralization Assays

MDCK cells were originally derived from a kidney of an apparently normal adult female cocker spaniel in September 1958 by S.H. Madin and N.B. Darby (12). The line of MDCK cells used here was obtained from Dr. John Wood at the National Institute for Biological Standards and Control (NIBSC) in the UK. This cell line originated from the Common Cold Laboratory in Salisbury UK at some point prior to 1985. This "London line" of MDCK cells exhibited greater sensitivity for isolation of virus from clinical
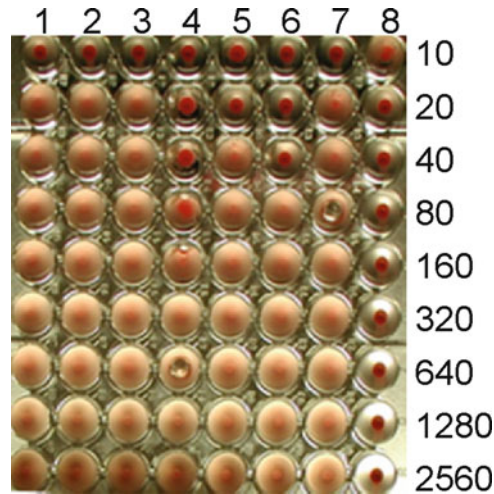
Fig. 4. Interpretation of an HAI assay. Columns 1, 2, 3, and 7 have HAI titers of 1:10. Column number 4 has an HAI titer of 80. Column 5 has an HAI titer of 20 and column 6 has an HAI titer of 40. Column 8 was the negative control.

specimens in studies during the mid-1980s than other MDCK cell lines. Other MDCK cell sublines may be used but should first be tested for their abilities to adhere to microtiter plates after being added as a suspension and to support infection of influenza viruses such that virus control wells achieve optimal ELISA O.D. values in the microneutralization assay.

Prior to the passage of MDCK cells, the cell monolayer should be 90–95% confluent. It is critical that the cells do not overgrow and enter stationary phase. Cells must be in log phase growth for maximum virus infectivity. Cells for use in an assay may be at 75–95% confluence. Split the confluent monolayer 1:10 2 days before use in an assay for optimum yield and growth. One T-162-cm² flask at 95% confluence should yield enough cells to seed about 4–5 microtiter plates. If the flasks are at 75% confluence, there will be enough cells to seed about four microtiter plates. A microneutralization assay testing 100 serum samples in duplicate against two viruses requires 40 microtiter assay plates and between 8 and 12 flasks of MDCK cells depending upon cell confluency.

1. To passage the MDCK cells, remove cell culture medium and gently rinse monolayer with 5 ml trypsin–EDTA. After removing, add another 5 ml trypsin–EDTA to cover the cell monolayer. Incubate at 37°C in 5% CO_2 until monolayer detaches, usually 3–10 min. As soon as most of the monolayer has been dislodged from the bottom of the flask, the remaining cells can be dislodged by gently tapping the side of the flask. Add 15 ml of MDCK medium to each flask containing trypsinized cells, bringing the total volume to 20 ml for each flask. Pipette the cells up and down about five times to separate from one another. Add cells to new 162-cm² tissue culture flasks, each

Table 4
Dilutions of cell suspensions for passage in T-162-cm² flasks

Dilution	Volume of cells to add to new flask (ml)	Number of cells to add to new flask	When confluent
1:5 (4 ml of the 20 ml)	4.0	$5–6 \times 10^6$	~24 h (1 day)
1:10 (2 ml of the 20 ml)	2.0	$1.75–2 \times 10^6$	~48 h (2 days)
1:20 (1 ml of the 20 ml)	1.0	$5–7 \times 10^5$	~72 h (3 days)

containing 30–50 ml MDCK medium, using the volumes of suspended cells described in Table 4. The dilutions shown refer to the proportion of suspended cells that are added to the new flask. These dilutions are based on optimal growth conditions and some variations may exist. Alternatively, the cells may be passed based on cells counts also shown in Table 4.

2. To use the MDCK cells in a TCID or MN assay (see Note 17), remove cell culture medium and gently rinse monolayer with 20 ml PBS. After removing, add 7 ml of trypsin–EDTA to cover the cell monolayer. Incubate at 37°C in 5% CO_2 until monolayer detaches. Add 7 ml of virus diluent to each flask. Pipette the cells up and down to separate the cells from one another. Remove cells and transfer to 50-ml conical tube. Combine cells from two or three flasks in one, 50-ml conical tube. Fill conical tube with virus diluent, centrifuge at $485 \times g$ for 5 min. This is the first wash.

3. Remove the supernatant and resuspend the cells in 10 ml of virus diluent. Pipette the cells up and down to separate the cells from one another. Combine all cells in one, 50-ml conical tube. Fill the conical tube with virus diluent and centrifuge again at $485 \times g$ for 5 min. This is the second wash.

4. Remove the supernatant and resuspend cells in 10 ml virus diluent. Pipette the cells up and down to separate the cells from one another. Determine the total volume of cells needed, 10 ml per microtiter plate. Divide the total volume needed by 3 to obtain the volume of virus diluent needed to resuspend the cells in. If the cells were less than 90% confluent, then decrease the amount of virus diluent added. Gently mix.

5. Count cells using a hemacytometer or a cell counter. Adjust the cell number to 1.5×10^5 cells/ml using virus diluent.

3.6. TCID Assay to Determine Working Dilution of Virus for Microneutralization Assay

The microneutralization assay requires that a standardized amount of virus is added to each serum-containing well of the microtiter plate to test for neutralizing antibodies. To quantify the amount of virus, a TCID for the microneutralization assay is determined. Generally, the virus working dilution is 200 or 400 times the $\log_{10}$

virus dilution at the cut-off point. We typically use 200 times the virus dilution at the cut-off point for avian influenza viruses such as H5N1 to obtain 100 TCID in 50 µl and 400 times the virus dilution for seasonal H3N2 and seasonal or pandemic H1N1 viruses to obtain 200 TCID in 50 µl (see Note 18). The minimum dilution of the virus stock for the microneutralization assay is 1:100. The virus titration is done on day 1 and the ELISA to detect viral-infected cells is done on day 2.

1. Rapidly thaw a vial of virus at 37°C and immediately place on ice. Virus should be thawed just before use for optimal infectivity. Test each virus at two or more different starting dilutions. Dilutions of 10^{-2}, 10^{-3}, and 10^{-4} in virus diluent are suggested depending upon how well the virus replicates in eggs or cells. Test each dilution on separate microtiter plates.

2. Add 100 µl of virus diluent to all wells, except column 1, of a 96-well microtiter plate. Add 146 µl of the virus starting dilution to all wells in column 1. Transfer 46 µl serially from column 1 through column 11. Change pipette tips between wells. After mixing, discard 46 µl from column 11. This dilution scheme results in $\frac{1}{2} \log_{10}$ dilutions. Dilutions will be 10^{-2}, $10^{-2.5}$, 10^{-3}, … 10^{-7} if the starting dilution was 10^{-2}. Column 12 contains virus diluent only (no virus) and is the cell control (CC).

3. Stack plates and cover with an empty plate. Place in 37°C, 5% CO_2 incubator for 1 h to mimic the virus + serum incubation step of the microneutralization assay.

4. Prepare MDCK cells for use in assay as described. Add 100 µl of diluted cells to each well of the microtiter plate. Each well will contain 1.5×10^4 cells. Incubate at 37°C in 5% CO_2 for 18–20 h.

5. The procedures for day 2 of the TCID determination, plate fixation, 1° antibody, 2° antibody, and substrate, are the same as these procedures for the microneutralization assay.

6. To analyze the data, calculate the median absorbance (O.D.) of the cell controls (column 12) and multiply by two to obtain the cut-off value. Prepare a table like Table 5. Once all test wells have been scored positive or negative for virus growth based on the cut-off value, the TCID of the virus suspension can be calculated by the method of Reed and Muench (4). Record the number of positive and negative values at each dilution. Select the region of the data where the transition from all wells positive at one dilution to all wells negative occurs. This region is shown in the box in Table 5. Calculate the cumulative numbers of positive wells at each dilution. The cumulative number positive is obtained by adding the positives at each dilution starting at the bottom. Calculate the cumulative numbers of negative wells at each dilution. The cumulative number negative is obtained by adding the

Table 5
Calculation of TCID by the Reed–Muench method

| Dilution | Observed value (O.D.) | | Cumulative value | | Ratio | % Positive |
	# Positive	# Negative	$\Sigma\uparrow$ Positive	$\Sigma\downarrow$ Negative		
10^{-2}	8	0				
$10^{-2.5}$	8	0				
10^{-3}	8	0				
$10^{-3.5}$	8	0				
10^{-4}	8	0	16	0	16/16	100
$10^{-4.5}$	7	1	8	1	8/9	89
10^{-5}	1	7	1	8	1/9	11
$10^{-5.5}$	0	8	0	16	0/16	0
10^{-6}	0	8				
$10^{-6.5}$	0	8				
10^{-7}	0	8				

negatives at each dilution starting at the top. Determine the ratio at each dilution as follows:

$$\text{Ratio} = \frac{\text{sum of cumulative positive}}{\text{sum of cumulative positive} + \text{sum of cumulative negative}}$$

Convert the ratio into percent positive. If the dilution with exactly 50% positive wells is identified, then the proportional distance does not need to be determined.

If not, then calculate the proportional distance between the dilution showing >50% positive and the dilution showing <50% positive as follows:

$$\text{Proportional distance} =$$

$$\frac{\%\,\text{positive value above } 50\% - 50}{\%\,\text{positive value above } 50\% - \%\,\text{positive value below } 50\%}$$

$$\times \text{ Correction factor for } \tfrac{1}{2}\log_{10} \text{ dilutions}$$

$$= \frac{89 - 50}{89 - 11} \times 0.5 = 0.5 \times 0.5 = 0.25$$

Calculate the MN TCID by adding the proportional distance to the dilution showing >50% positive. For the example in Table 2, add 0.25 to 4.5 to obtain $10^{-4.75}$. The virus working dilution that is 200 times the cut-off dilution is $10^{-4.75} \times 200 = 10^{-4.75} + 10^{2.30} = 10^{-2.45} = 1/10^{2.45} = 1{:}282$. This dilution will give 100 TCID per 50 µl. The virus dilution that is 400 times the cut-off is 1:141. This dilution will give 200 TCID per 50 µl.

3.7. Microneutralization Assay (see Note 19)

A virus titration, to determine the MN TCID and therefore the working dilution of the virus stock, must be done prior to running a microneutralization assay. The minimal acceptable working dilution of virus in the microneutralization assay is 1:100. For the microneutralization assay, the serum titration is combined with a standard amount of virus day 1 and the ELISA to detect viral-infected cells is done on day 2. The ELISA portion of the assay uses a mouse monoclonal antibody to influenza A virus nucleoprotein to detect virus replication in the MDCK cells. All serum samples are tested in duplicate against each virus. Each assay includes positive and negative control serum samples and a virus back titration. If animal serum samples are used as controls, then they must be RDE treated before use. Each microtiter plate includes four virus control (VC) wells and four cell control (CC) wells. See Fig. 5 for an overview of the microneutralization assay.
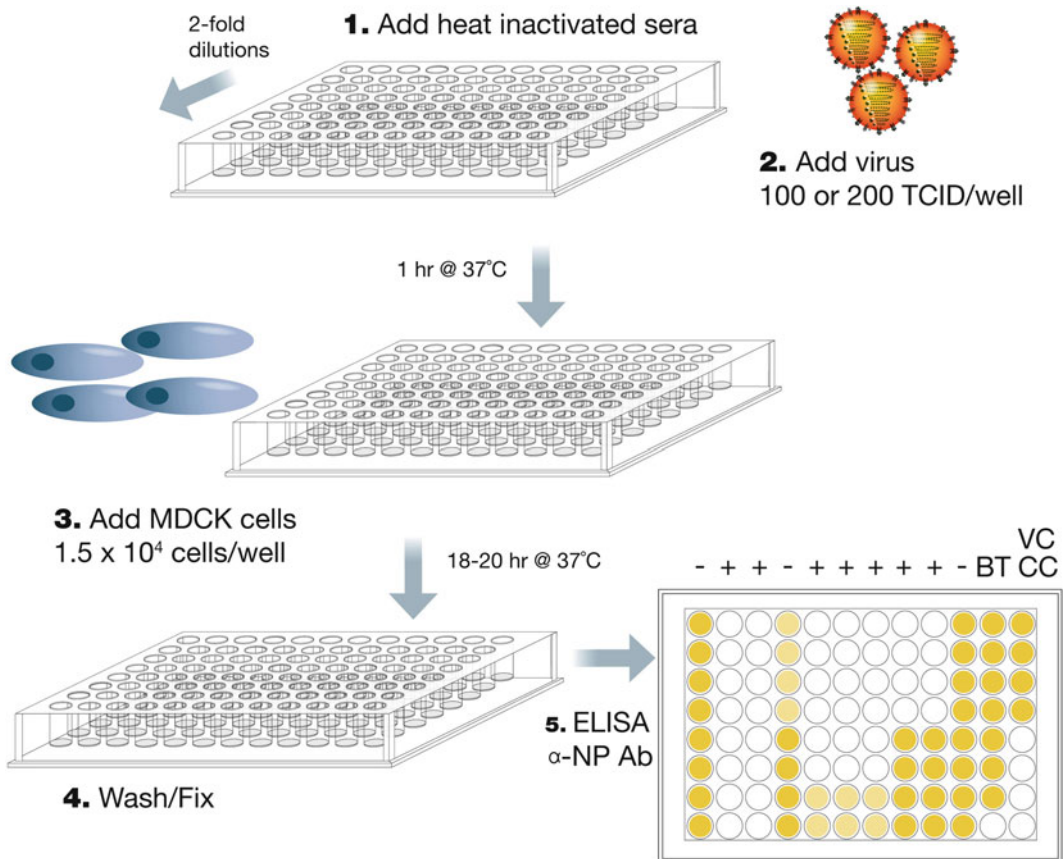
Fig. 5. Overview of microneutralization assay. The main steps of the microneutralization assay are shown. Steps 1 and 2, the neutralization portion of the assay and step *3*, the infection of MDCK cells with non-neutralized virus, are done on day 1. Steps *4* and *5*, the detection of virus infected cells using an ELISA, are done on day 2.

1. Quickly thaw frozen sera in 37°C water bath. Immediately after thawing, place sera in ice or proceed with heat inactivation for 30 min in a 56°C water bath. The heat-inactivated sera may be stored at 4°C overnight or at –20°C for a longer period of time.

2. Prepare initial serum dilutions of 1:5 using virus diluent. We recommend preparing the serial twofold dilutions of serum samples in Titertubes (Biorad, Cat. # 223-9390). To prepare serial dilutions for testing in duplicate against two viruses, add 50 μl of heat-inactivated sera to 200 μl of virus diluent. Add 125 μl of virus diluent to the other Titertubes and serially dilute transferring 125 μl. Discard 125 μl from the last Titertube after mixing. Add 125 μl of virus diluent to all Titertubes to make the serum dilutions 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. The total volume in each tube is 250 μl.

3. Transfer 50 μl of diluted sera from the Titertubes into four microtiter plates (two plates per virus) starting with row H and going to row A. Change the pipette tips after transferring the sera in row A to the microtiter plate. It is critical that the tips are changed before beginning to pipette the next set of serum samples. Cover and hold in 37°C, 5% CO_2 incubator while the diluted virus is being prepared. It is critical that the proper pH is maintained so that there will be no deleterious pH effects on the virus when it is added.

4. Dilute the virus in virus diluent to the correct working dilution as determined by the TCID assay. Five milliliters of diluted virus are needed per plate. Hold on ice. Add 50 μl of diluted virus to wells containing sera and the VC wells (A12, B12, C12, and D12). Do not add virus to the CC wells (E12, F12, G12, and H12) and do not add virus to the column of wells reserved for the virus back titration. Tap the plate gently to mix. Add 50 μl of virus diluent to the CC wells.

5. In each assay, include a back titration of the working dilution of virus. Add 50 μl of virus diluent to all wells in one column. Add 50 μl of the working dilution of virus to the first well and make serial twofold dilutions by transferring 50 μl from the first well to each successive well through well 12. Discard 50 μl from well 12. To avoid virus carryover, change pipette tips between wells. Add an additional 50 μl of virus diluent to all wells in the column to bring the total volume to 100 μl. Cover and incubate at 37°C, 5% CO_2 for 1 h. During this incubation, prepare the MDCK cells as described in Subheading 3.5.

6. Add 100 μl of diluted cells to each well of the microtiter plates. Each well contains 1.5×10^4 cells. Stack all plates in four plate stacks and cover each stack with an empty plate. Incubate at 37°C in 5% CO_2 for 18–20 h.

7. On the second day, after incubation, remove the medium from microtiter plates by carefully decanting into an autoclavable container. Add 200 μl PBS to each well to wash and remove PBS wash by carefully decanting. Tap inverted plate on laboratory paper towels.

8. Add 100 μl cold fixative to each well. Do not allow wells to dry out before the fixative is added. Stack and cover each stack with an empty plate. Incubate at room temperature (RT) for 10–12 min.

9. Remove the fixative by carefully decanting. Spray a paper towel with 70% EtOH and wipe exterior of plates. Let the plates air-dry inside the BSC for 10 min or until dry. After the microtiter plates have been wiped with EtOH and have air dried, they may be removed from the BSC.

10. Visually check the cell monolayer in each well and note any wells with reduced cell confluence on the process sheet. Compare the cell confluence to that observed in the CC. Cell toxicity due to the serum sample is most likely to be noticed in the wells with the lowest dilution of serum (see Note 20). Plates may be held at 4°C for up to 2 days before proceeding with the ELISA portion of the assay. Cover with an empty plate, place in plastic bags, and seal.

11. Dilute the 1° antibody (mouse anti-influenza A NP) in antibody diluent to a predetermined optimum working dilution. Mix well. Wash plates 3× with 300 μl wash buffer per well. Add 100 μl of diluted 1° antibody to each well. Stack plates and cover with an empty plate. Incubate for 1 h at RT.

12. Dilute 2° antibody (goat anti-mouse IgG HRP labeled) in antibody diluent to a predetermined optimum working dilution. Mix well. Wash plates 3× with 300 μl wash buffer per well. Add 100 μl diluted 2° antibody to each well. Stack plates and cover with an empty plate. Incubate for 1 h at RT.

13. Prepare citrate buffer. Wash plates 5× with 300 μl wash buffer per well. Prepare OPD substrate in citrate buffer immediately before use. Tap washed plates on a laboratory paper towel. Add 100 μl freshly prepared OPD substrate to each well. Incubate at RT until the color change in the VC wells (column 12, rows A–D) is intense and the corresponding color change in the CC wells is minimal. Add 100 μl stop solution to each well. The O.D. in the VC wells should be at least 0.8 and is typically in the range of 1.0–1.5 though higher is acceptable. The O.D. in the CC wells must be ≤0.2. The incubation time required to obtain the targeted O.D. may vary between viruses. Read the absorbance (O.D.) of wells at 490 nm using a microtiter plate spectrophotometer. See Fig. 6 for the image of a developed and stopped MN plate.
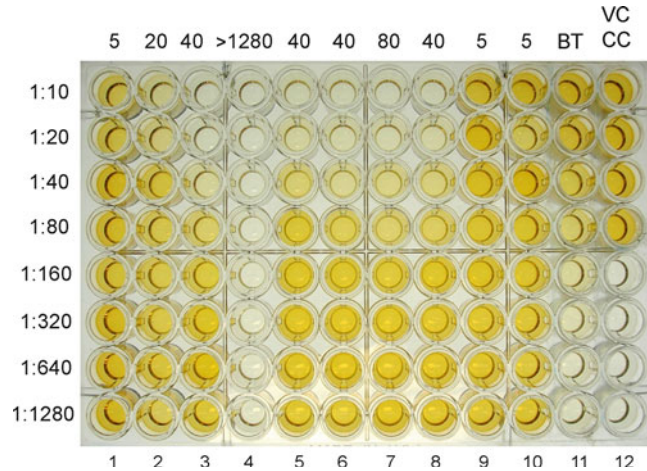
Fig. 6. Image of developed microneutralization assay plate. Ten serum samples were tested on this plate in columns 1 though 10 as noted at the bottom of the plate. The serum dilution is shown on the right-hand side of the microtiter plate. Column 11 shows the virus back titration (BT). The virus control (VC) is shown in the first four wells of column 12; the cell control (CC) in the second four wells of column 12. The titers obtained for these serum samples after data analysis are shown across the top of the plate.

14. The data are analyzed as follows: The VC and CC medians are determined for each plate. In order for the ODs on each plate to be considered valid, the CC must have a median OD of ≤0.2. The VC must have a mean OD of ≥0.8. The virus back titration is evaluated to determine if the working dilution of virus used in the assay contained the desired amount of virus. The cut-off value for the virus back titration is the mean of the VC median and the CC median. This is the same cut-off that is used to evaluate neutralizing antibody titers. The dilution of the first well below the cut-off value is the back titration titer. The dilutions in the back titration wells are beginning at well A: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. In general, back titration titers of 16, 32, and 64 are acceptable (see Note 21).

15. Neutralizing antibody titers are determined by calculating the cut-off value to determine a 50% neutralizing antibody titer for each plate based on the equation: (median O.D. of VC + median O.D. of CC)/2 = X, where X = the 50% cut-off value. All values below or equal to X are positive for neutralization. Read each column which contained diluted serum from the bottom, beginning at well H. Note the first well with an OD of less than the 50% cut-off. The reciprocal serum dilution corresponding to that well is the 50% neutralization antibody titer for that serum sample. Serum dilutions are well A 1:10, well B 1:20, well C 1:40, well D 1:80, well E 1:160, well F 1:320, well G 1:640, and well H 1:1,280. Serum samples with a titer of >1,280 may be repeated beginning at a 1:80 starting dilution to obtain an

endpoint titer. For an assay to be valid, the control serum samples must meet the following criteria: (1) the GMT of the duplicate titers of all negative control serum samples must be ≤10 and (2) the GMT of the duplicate titers of all positive serum samples must be ≥80 and must be within twofold of a previously determined GMT based on 10 or more assays done in duplicate. See Table 6 to troubleshoot potential problems associated with the interpretation of the microneutralization assay.

3.7.2. Determination of Working Dilution for Primary and Secondary Antibodies in the Microneutralization Assay

To detect all influenza nucleoprotein expressed in the MDCK cells, an excess of primary and secondary antibodies must be used. The working dilution, to ensure excess reagent, is determined as described below.

1. The working dilution of the secondary antibody, goat anti-mouse IgG HRP labeled, is determined first. Purified mouse IgG is used to coat microtiter plates at a concentration of 2 μg/ml in PBS, 100 μl/well. Incubate for 2 h or longer at room temperature with shaking using a mini-orbital shaker.

2. Wash plates 3× with 300 μl wash buffer per well. Prepare serial twofold dilutions of secondary antibody in antibody diluent buffer, typically beginning at 1:250 and continuing through 1:32,000. Add 100 μl of each dilution, in triplicate, to the coated wells. As a control, include a no conjugate control that contains antibody diluent only. Incubate for 30 min at room temperature with shaking.

3. Wash plates 3× with 300 μl wash buffer per well. Add 100 μl of OPD substrate in citrate buffer and develop until the color is intense in the wells with the highest concentrations of secondary antibody. Add 100 μl of stop solution to each well. Read the absorbance (OD) of wells at 490 nm using a microtiter plate spectrophotometer.

4. Analyze the data by graphing the mean OD of each dilution versus the secondary antibody dilution. It is expected that the curve will plateau at the lower dilutions. Choose the highest dilution in the plateau region. When a second lot of secondary antibody is evaluated, titrate both lots in the same assay to aid in the selection of a comparable working dilution.

5. The working dilution of the primary antibody, anti-influenza A NP mouse monoclonal antibody, is determined second. Test using two viruses, preferably of different subtypes. Add 50 μl of virus diluent to all wells in columns 1 through 8. Add 100 μl of virus diluent to all wells in columns 9 through 12.

6. Prepare the working dilution of each virus by diluting in virus diluent. Add 50 μl of the first diluted virus to all wells in columns 1 through 4. Add 50 μl of the second diluted virus to all wells in columns 5 through 8. Cover and hold in 37°C, 5% CO_2 incubator while the MDCK cells are being prepared.

Table 6
Potential problems associated with interpretation of the microneutralization assay

Problem	Possible cause(s)	Solution
Weak or no color in virus control (VC) wells	Problem with ELISA: using wrong antibodies or substrate Buffer solutions incorrect Too little virus used in assay or forgot to add virus to VC wells Virus inactivated during incubation step MDCK cells not optimal: too old (>30 passages), not in log phase growth, or contaminated	Check the antibodies and substrate Prepare fresh buffers Redetermine virus TCID or adjust the dilution of virus used or repeat and do not forget to add virus to VC wells Check incubator temperature and CO_2 level Thaw a new vial of cells, do not allow cells to enter stationary phase
Weak or no neutralization by positive control sera	Too much virus used in assay Serum deteriorated MDCK cells not optimal: too old (>30 passages), not in log phase growth, or contaminated	Redetermine virus TCID or adjust the dilution of virus used Obtain new antisera, check storage conditions Thaw a new vial of cells, do not allow cells to enter stationary phase
Neutralization by negative control sera	Nonspecific reaction or cross-reactivity Too little virus used in assay	Check the RDE treatment Check samples for cross-reactive antibodies Redetermine virus TCID or adjust the dilution of virus used
Nonspecific virus inactivation	Serum not heat inactivated or contains nonspecific viral inhibitors	Heat-inactivated serum (56°C, 30 min). Treat animal serum with RDE
MDCK cell monolayer toxicity	Serum not heat inactivated or is toxic to cells	Heat-inactivated serum (56°C, 30 min). Run serum toxicity check on normal MDCK cells and check microscopically for toxicity

7. Prepare the MDCK cells as described in Subheading 3.5. Add 100 μl of diluted cells to each well of the microtiter plates. Each well contains 1.5×10^4 cells. Stack all plates in four plate stacks and cover each stack with an empty plate. Incubate at 37°C in 5% CO_2 for 18–20 h.

8. Fix the plates as described in Subheading 3.7.1. As the plates are air drying, prepare serial twofold dilutions of anti-NP antibody in antibody diluent buffer, typically beginning at 1:250 and continuing through 1:32,000. Wash plates 3× with 300 μl wash buffer per well. Add 100 μl of 1:250 dilution to all wells in row A. Add the next dilution to row B and continue through row H. Following this design, each primary antibody dilution is tested against each virus in quadruplicate. There are cell controls, in quadruplicate, for each antibody dilution. Testing in triplicates is also acceptable. Incubate at room temperature for 1 h.

9. Dilute the 2° antibody (goat anti-mouse IgG HRP labeled) in antibody diluent to the optimum working dilution as determined above. Mix well. Wash plates 3× with 300 μl wash buffer per well. Add 100 μl diluted 2° antibody to each well. Stack plates and cover with an empty plate. Incubate for 1 h at RT.

10. Prepare citrate buffer. Wash plates 5× with 300 μl wash buffer per well. Prepare OPD substrate in citrate buffer immediately before use. Tap washed plates on a laboratory paper towel. Add 100 μl freshly prepared OPD substrate to each well. Incubate at RT until the color is intense in the wells with the highest concentrations of secondary antibody. Add 100 μl of stop solution to each well. Read the absorbance (O.D.) of wells at 490 nm using a microtiter plate spectrophotometer.

11. Analyze the data by graphing the mean O.D. of each dilution versus the primary antibody dilution for the wells with virus and the wells without (the cell controls). It is expected that the curve will plateau at the lower dilutions. Choose the highest dilution in the plateau region and also take into account background noise in the cell control. When a second lot of primary antibody is evaluated, titrate both lots in the same assay to aid in the selection of a comparable working dilution.

4. Notes

1. Blood from a farm should be prescreened, it is recommended to find a commercial source. The RBCs used for all testing should be as fresh as possible. The blood should be preserved in Alsever's or sodium citrate at a 1:1 dilution.

2. In the 1990s, human influenza A (H3N2) lost its ability to agglutinate chicken RBCs (13) RBCs such as turkey or guinea

pig RBCs are preferred when testing for currently circulating influenza types and subtypes.

3. Standardized RBCs may be used up to 1 week or until hemolyzed for the HA assay and the EID_{50}. RBCs used for the HAI assay should be made the day of the test for optimal sensitivity.

4. Use of dead, broken or nonembryonated eggs will result in little to no virus growth.

5. Aliquot trypsin–EDTA in 10 ml amounts and store at –20°C. Use half an aliquot, 5 ml, for each flask of cells to be passed or split.

6. One of the most critical components for a successful MN assay is the quality of the virus used. It is very important that care is taken to prepare a virus stock that is optimized for infectious virus and contains minimal noninfectious or defective interfering virus particles. Therefore, the virus stock should be generated with a high dilution of virus for the inoculum and the virus should be harvested when viral production is at its peak as judged by HA titer.

7. Keep virus stock on ice during the HA test to maintain virus infectivity.

8. The HAU will vary from RBC type to another.

9. All equipment and supplies coming into contact with the embryonated eggs must be sterile and proper sterile techniques should be used accordingly.

10. FBS inhibits viral entry and must be removed for efficient infection of cells.

11. Incubation for >1 h may cause the MDCK cell monolayer to dry out and result in reduced cell viability.

12. At low dilutions of virus, the monolayer may be completely destroyed, resulting in no or minimal purple staining in the wells.

13. The serum used in the test may contain sialic acid residues that may mimic the receptors of RBCs and may interfere with the RBC receptors for hemagglutinin. RDE, a culture of *Vibrio cholerae*, is an inhibitor inactivating reagent used to remove the nonspecific inhibitors. RDE is effective against alpha- and beta-inhibitors. Other serum treatments are heat and RDE, heat and periodate, trypsin, heat, and periodate, and absorption with Kaolin.

14. Treated serum should be stored at –20 to –40°C up to 1 year or to the date of expiration of the RDE.

15. The back titration should be performed the same day as the HAI test is performed. Back titration of hemagglutination antigen must be performed to confirm that the concentration of the antigen is 8, so an accurate hemagglutination-inhibition

titer of serum is obtained. Failure to standardize the control antigens and isolates to contain a titer of 8 can lead to false interpretations of the test.

16. The HAI test may be setup so the serum is added to column A and the PBS is added to columns B through G. Note that the HAI endpoint will be 1,280.

17. Use MDCK London line cells with a total passage number of no more than 25. It is recommended that the infectivity of the cells at higher passage numbers be monitored by examining virus infectivity. The acceptable number of passages may be adjusted based on these data.

18. Titration of virus yields a sigmoidal-shaped, 4-Parameter Logistic (4-PL) curve when O.D. versus virus $\log_{10}$ dilution is plotted. The current method of determining the virus working dilution uses a cut-off value for determining positivity that is twice the median O.D. of the cell control. This means that the data being evaluated are in the relatively flat region of the curve where small changes in the O.D. result in large changes in the virus dilution. We are currently evaluating the midpoint of the titration curve and determining how the midpoint virus dilution relates to the desired working dilution of virus. Preliminary data indicate that if the cut-off is selected by calculating the median O.D. in columns 1–11, selecting the one with the highest median absorbance as the maximum O.D. median, and the cut-off value is determined by the sum of the maximum O.D. median and the median O.D. of the cell controls divided by two, then the virus working dilution is 30 times the virus dilution at that midpoint. This yields a virus working dilution equivalent to 200 TCID. Other methods of accurately determining the correct amount of virus to use are also being evaluated.

19. The methods described here are essentially those used previously (Rowe et al.), but have been updated to improve the signal-to-noise ratio within the assay and to allow for improved methods of data analysis.

20. Serum samples that are hemolyzed, have a high lipid content, or have microbial contamination often are toxic to the MDCK cells, especially at the lower dilutions of serum. It is important to note instances of cell toxicity so that the lack of detection of NP is not interpreted as indicating the presence of neutralizing antibodies. Cell toxicity is detected by noting a marked decrease in the cell monolayer, especially at the 1:10 and 1:20 serum dilutions, following the overnight incubation of serum, virus, and cells and after acetone fixation.

21. While the back titration provides valuable information about the microneutralization assay, the more critical information is provided by the positive and negative control sera. It is very important to repeatedly test control antisera and establish a median value and upper and lower limits. RDE-treated ferret antisera from ferrets that have not been exposed to influenza should have a titer of <10. Positive control antibody titers that are higher than the upper limit may indicate that insufficient virus was used in the assay. Titers lower than the lower limit may indicate that too much virus was used in the assay.

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Diagnosis of Influenza Virus

Kirsten St. George

Abstract

The laboratory diagnosis of influenza uses a wide range of techniques including rapid immunoassays, immunofluorescence techniques, virus culture methods, and increasingly sophisticated molecular assays. The potential utility of each of these methods has changed over the years, most dramatically perhaps with the emergence of the pandemic H1N1 2009 influenza virus. While rapid immunoassays had previously been widely used in clinics and emergency departments, their poor detection sensitivity for the 2009 sub-type brought their application into question. Concerns were also raised about the detection sensitivities of antibody reagents used in immunofluorescence methods, and the safety of virus culture was initially questioned with regard to the newly emerged subtype. Early molecular detection techniques had been labor intensive, and required separate facilities in order to prevent contamination. Those techniques have largely been supplanted by more modern methods, most notably real-time reverse transcription PCR assays, which are currently the method of choice in many laboratories for the detection and subtyping of influenza viruses. Suspension and low-density array assays are also increasingly used, in an effort to detect larger numbers of viruses in a single assay, and microarrays have proven valuable for outbreak analysis and pathogen discovery. Each laboratory must assess the optimal methods for its situation and the best application of each technique, taking into account numerous factors including its budget, equipment, staff expertise, the patient population that it serves, the needs of its submitting clinicians, and its surveillance and public health responsibilities.

Key words: Nasopharyngeal, Oropharyngeal, Rapid Immunoassays, Chromatographic, Immuno-fluorescence, Monolayer, Real-time reverse transcription PCR

1. Introduction

The diagnosis of influenza in clinical and public health laboratories facilitates patient management decisions in clinical settings, and provides outbreak and surveillance data for public health policy and guidelines. Although type-specific testing was always considered important for surveillance data, its significance in clinical settings increased following the availability of anti-influenza drugs, specifically the adamantanes that are not effective against influenza B [1]. More recently, with the emergence of subtype-specific drug

resistance, concerns regarding novel subtypes of influenza, and the emergence of the pandemic (H1N1) 2009 virus, there has been a strong desire for the availability of methods capable of subtyping, in addition to methods for influenza detection and typing, in clinical as well as public health laboratories.

At present, the availability of rapid immunoassays or immunofluorescence testing materials that are subtype-specific is poor, but numerous commercial reagents and kits are in development and should be available in the near future. Most will utilize a methodology similar to that in the type-specific diagnostic devices currently in use. Culture methods have been streamlined considerably, although some laboratories choose to continue to perform conventional tube culture techniques. Some subtype-specific reagents for culture confirmation are already available and more should be obtainable soon. One ongoing challenge is the shifting growth characteristics of circulating influenza viruses, with variable cell line susceptibilities from strain to strain of influenza. Molecular methods for the diagnosis of influenza have undergone a number of developments. Early conventional reverse transcription PCR methods have largely been replaced with real-time techniques, except when sequence analysis is specifically needed. Instrumentation has become increasingly automated, multiplexing capacities have been expanded, and equipment is progressively more user friendly. The diagnostic methods for influenza described here, while not comprising an exhaustive list, are the most commonly used techniques currently in clinical and public health laboratories. Specific information and lists of commercial kits, reagents and equipment, are provided for information only, and are not intended as an endorsement of any of the products. The enumeration of detailed methodologies of the many diagnostic molecular techniques that can be used in laboratory-developed assays for the detection and subtyping of influenza is clearly beyond the scope of this chapter. Instead, the most commonly used extraction methods and equipment, commercially prepared master mix reagents, and real-time PCR instruments, are listed in tables, since automated extraction and real-time RT-PCR is currently the most widely used combination for influenza diagnosis. The individual laboratory should investigate which instrument(s) and kit(s) best suit its circumstances. Additionally, molecular assays are now available with all components included in the kit, and more are in development, including some designed specifically for use in low-complexity laboratories. Manufacturers have also developed suspension and low-density arrays with higher multiplexing capabilities than real-time instruments, enabling the detection and identification of many respiratory agents in a single test (2), and these are in use in some laboratories. Similarly, microarrays have been specifically developed for respiratory virus detection (3, 4) and can be used in investigations of outbreaks and emerging pathogens. However, such methods are not currently in routine

diagnostic use and detailed descriptions of techniques for the more complex assays are therefore beyond the scope of this chapter.

In general, diagnostic laboratory work on clinical samples from patients with influenza, suspected influenza, or influenza-like illness should be conducted in a BSL2 laboratory, with all sample manipulations performed inside a biosafety cabinet (5). For molecular testing, a sample aliquot that has been transferred into lysis buffer such that any virus present in the sample is inactivated can then be further handled outside a cabinet. Additional higher-level containment should be used for samples from suspected novel influenza cases, such as highly pathogenic avian influenza, and virus culture should not be attempted on such samples unless the laboratory is appropriately licensed.

2. Materials

2.1. Specimen Collection and Transport

1. Polyester or Dacron tip swab with aluminum or plastic shaft. Flocked swabs are also acceptable (Copan Diagnostics, Murrieta, CA) (see Note 1).

2. Vial of viral transport medium or universal transport medium such as M4, M4-RT, or VTM. (Multiple suppliers, e.g., Remel, Lenexa, KS; Copan Diagnostics).

3. Alternatively, specimen collection device specifically supplied by immunoassay kit for use with that test.

4. Cold packs or dry ice, if specimen is to be shipped to another location for testing.

2.2. Rapid Immunoassays for Antigen Detection

Rapid immunoassays for detecting and distinguishing influenza A and B are invariably performed with commercially supplied kits (see Notes 2 and 3): some of those most commonly used are listed in Table 1. Test kits contain all of the required materials and reagents including, typically:

1. Capture antibodies immobilized on chromatographic paper, housed inside a plastic test device.

2. Detection (anti-influenza) antibody conjugates with particles for visualization.

3. Wash buffers and diluents.

4. Positive and negative controls.

5. Specimen collection devices, either included with the kit or sold by the company as a separate item specifically for use with the test kit.

Table 1
Commonly used rapid immunoassay kits for influenza A and B identification[a]

Test kit	Manufacturer
Binax NOW® Influenza A & B	Binax (Scarborough, MN)
Directigen™ EZ Flu A+B	Becton Dickenson (Sparks, MD)
X/pect™ Flu A & B	Remel (Lenexa, KS)
3-M™ Rapid Detection Flu A+B Test[b]	3M (St. Paul, MN)
TRU FLU®	Meridian Bioscience (Cincinnati, OH)
Quick Vue™ Influenza A+B Test	Quidel (San Diego, CA)

[a] All kits detect and distinguish influenza A and B
[b] Uses an automated reader

2.3. Antigen Detection by Direct Immunostaining

1. Vortex.
2. Sterile capped polypropylene tubes.
3. Sterile PBS.
4. Teflon-coated microscope slides (or similar) with wells.
5. Acetone (see Note 4).
6. Commercial immunofluorescence antibody kit for influenza antigen detection (see Table 2) or individual anti-influenza antibodies and conjugate, which must be cross-titrated for determination of the optimal working concentrations (also see Note 5).
7. Humidified chamber and 35–37°C incubator (alternatively, humidified 35–37°C incubator).
8. Mounting fluid.
9. Coverslips.
10. Epifluorescence microscope.

2.4. Rapid Culture Methods

1. Rapid culture for influenza viruses can be performed in various cell types, including primary Rhesus monkey kidney (pRhMK) cells, Madin–Darby canine kidney (MDCK) cells, mink lung (ML) epithelial cells, or mixed cell lines specifically designed for respiratory virus culture (6).
2. Suitable culture vessels include glass shell vials and tissue culture plates with various well sizes. In diagnostic laboratories, rapid culture is commonly performed in commercially supplied cell monolayers grown on round coverslips which are placed in the bottom of glass shell vials or 24-well tissue culture plates.

Table 2
Commonly used commercial immunofluorescence kits for influenza antigen detection

Test kit	Manufacturer	Purpose	IF method	Detection specificity
Bartels Viral Respiratory Screening and Identification Kit	Bartels/Trinity Biotech (Carlsbad, CA)	Direct specimen detection and culture confirmation	Indirect	Detects and distinguishes seven respiratory viruses, including influenza A and B
D³ *Ultra*™ 2009 H1N1 Influenza A Virus ID Kit	Diagnostic Hybrids (Athens, OH)	Direct specimen detection and culture confirmation	Indirect	Detects 2009 H1N1 influenza A virus
D³ *Ultra*™ DFA Respiratory Virus Screening and ID Kit	Diagnostic Hybrids	Direct specimen detection and culture confirmation	Indirect	Detects and distinguishes seven respiratory viruses, including influenza A and B
D³ *Ultra*™ 8 DFA Respiratory Virus Screening and ID Kit	Diagnostic Hybrids	Direct specimen detection and culture confirmation	Indirect	Detects and distinguishes eight respiratory viruses, including influenza A and B
D³ Duet™ DFA Influenza A/Respiratory Virus Screening Kit	Diagnostic Hybrids	Culture confirmation	Direct	Detects seven respiratory viruses with dual fluorophores
D³ FastPoint™ L-DFA™ Influenza A/Influenza B Virus Identification Kit	Diagnostic Hybrids	Direct specimen detection	Direct	Detects and distinguishes influenza A and B

(continued)

Table 2
(continued)

Test kit	Manufacturer	Purpose	IF method	Detection specificity
Light Diagnostics™ Influenza A & B DFA Kit	Light Diagnostics/Millipore (Billerica, MA)	Culture confirmation	Direct	Detects and distinguishes influenza A and B
Light Diagnostics™ Respiratory Viral Screen DFA Kit	Light Diagnostics/Millipore	Direct specimen detection and culture confirmation for influenza viruses	Direct	Detects and distinguishes seven respiratory viruses, including influenza A and B
Light Diagnostics™ Simulfluor® Respiratory Screen Reagent	Light Diagnostics/Millipore	Direct specimen detection and culture confirmation	Direct	Detects seven respiratory viruses
Light Diagnostics™ Simulfluor® Flu A/Flu B	Light Diagnostics/Millipore	Direct specimen detection and culture confirmation	Direct	Detects and distinguishes influenza A and B with dual fluorophores
PathoDx® Respiratory Virus Panel Kit	Remel (Lenexa KS)	Culture confirmation	Direct	Detects and distinguishes seven respiratory viruses, including influenza A and B

3. Temperature-controlled centrifuge with biohazard-sealed rotor or buckets that will hold the culture vessels.

4. Penicillin (100 U/ml) and streptomycin (100 µg/ml).

5. Maintenance medium for the cultures. The type will vary depending on the cultures, generally, Eagles MEM with 15% tryptose phosphate broth.

6. Fresh acetone.

7. Sterile PBS.

8. Humidified chamber.

9. Fine tip forceps.

10. Commercial immunofluorescence antibody kit for influenza antigen detection (see Table 2), or individual anti-influenza antibodies and conjugate (which must be cross-titrated for determination of the optimal working concentrations).

11. 35–37°C incubator.

12. Microscope slides.

13. Mounting fluid.

14. Epifluorescence microscope.

2.5. Conventional Virus Culture

1. Conventional culture for influenza diagnosis, although less common than rapid culture, is usually performed with commercially supplied pRhMK or MDCK cells in tubes, and requires the addition of trypsin in serum-free medium when non-primary cell lines are used. As for rapid culture methods, cells must be obtained fresh each week; 85–90% confluence of monolayers is optimal for virus isolation.

2. Penicillin (100 U/ml) and streptomycin (100 µg/ml).

3. Maintenance medium for the cultures, commonly Eagle's MEM with 15% tryptose phosphate broth and 2 µg/ml TPCK trypsin, if non-primary cell lines are used.

4. Sterile PBS.

5. 33°C incubator.

6. Microscope and tube support for viewing.

2.6. Molecular Diagnosis

At present, the most commonly used molecular technique for the diagnosis of influenza is real-time RT-PCR, preceded by automated extraction of nucleic acid from the specimen.

1. The most widely used extraction methods are listed in Table 3.

2. Master mix preparations from commercial suppliers are used in diagnostic laboratories, and those most frequently used for influenza real-time assays are listed in Table 4.

3. The real-time instruments generally used for influenza diagnostic testing are shown in Table 5.

Table 3
Extraction methods and instruments commonly used for molecular influenza testing

Manufacturer	Instrument	Chemistry/Kit	Sample capacity
Qiagen	BioRobot MDx	QIAamp Virus BioRobot MDx Kit	96
	QIAxtractor	Reagent Pack VX	96
	QIAsymphony	Virus/Bacteria Kit	96
	QIAcube	QIAamp Viral RNA	12
	QIAcube	RNeasy	12
	EZ1 XL	Virus Kit	14
	EZ1	Virus Kit	6
	Manual[a]	QIAamp Viral RNA	
	Manual[a]	RNeasy	
Roche	MagNA Pure	DNA and Viral Nucleic Acid	96
	MagNA Pure LC	Nucleic Acid Isolation Kit I	32
	MagNA Pure Compact	RNA Isolation Kit	8
bioMerieux	easyMAG	NucliSENS	24
	miniMAG	NucliSENS	12
	Manual[a]	NucliSENS	

[a] Not automated

Table 4
Commercial real-time RT-PCR master mix preparations commonly used for influenza detection

Manufacturer	Reagent name	Available kit size
Applied Biosystems	AgPath-ID™ One-Step RT-PCR Kit	100×50 µl reactions 500×50 µl reactions $1,000 \times 50$ µl reactions
	TaqMan® One-Step RT-PCR Master Mix Reagents Kit	200×50 µl reactions $1,000 \times 50$ µl reactions
Bio-Rad	iScript One-Step RT-PCR Kit for Probes	50×50 µl reactions 200×50 µl reactions
Invitrogen	Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (without ROX)	100×50 µl reactions 500×50 µl reactions
	Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (with ROX)	100×50 µl reactions 500×50 µl reactions
Qiagen	QuantiTect Probe RT-PCR Kit	200×50 µl reactions $1,000 \times 50$ µl reactions
Quanta BioSciences	qScript™ One-Step qRT-PCR Kit	50×50 µl reactions 200×50 µl reactions
	qScript™ One-Step qRT-PCR Kit, ROX™	50×50 µl reactions 200×50 µl reactions

Table 5
Real-time PCR instruments commonly used for influenza diagnosis

Manufacturer	Instrument	Multiplexing capability	Format	Light source	Comments
Applied Biosystems	StepOne Plus	4	96 wells	Blue LED	
	StepOne	3	48 wells	Tungsten Halogen	
	7500	5	96 wells	Tungsten Halogen	
	7500 Fast	5	96 wells	Tungsten Halogen	
	7500 Fast DX	5	96 wells	Tungsten Halogen	FDA approved[a]
	7900HT	4	96, 384 wells and micro fluidic cards	Argon laser	
	ViiA7	6	96, 384 wells and micro fluidic cards	Tungsten Halogen	
	7000	4	96 wells	Tungsten Halogen	No longer manufactured
	7300	3	96 wells	Tungsten Halogen	
	7700	4	96 wells	Argon laser	No longer manufactured
Bio-Rad	CFX96	5	96 wells	LED	
	CFX384	5	384 wells	LED	
	MiniOpticon	2	48 wells	Blue-Green LED	
	MyiQ2	2	96 wells	Halogen	
	iQ5	5	96 wells	Halogen	No longer manufactured
	iQ4	4	96 wells	Halogen	No longer manufactured
Roche	LightCycler 1.5	3	32 capillary tubes	LED	CE approved
	LightCycler 2.0	6	32 capillary tubes	LED	CE approved
	LightCycler 480	5	96 and 384 well	Xenon Lamp	

(continued)

Table 5
(continiued)

Manufacturer	Instrument	Multiplexing capability	Format	Light source	Comments
Agilent technologies	Mx3000P	4	96 wells	Tungsten Halogen	
	Mx3005P	5	96 wells	Tungsten Halogen	
Cepheid	SmartCycler	4	16 wells	LED	
Qiagen	Rotor-Gene Q	6	96 wells	LED	
Eppendorf	Mastercycler	2	96 wells	LED	
3M	Integrated cycler	4	96 wells	Laser	FDA approved[b]

LED Light emitting diode

[a]FDA approval is coupled to the CDC nolecular influenza assays

[b]FDA approval is coupled to the Quest Simplexa Influenza A H1N1 (2009) assay

4. Cold racks for tubes.

5. Adjustable micropipettes and aerosol barrier tips.

6. Appropriate disposable plastics for extraction and PCR instruments.

7. Nuclease-free microcentrifuge tubes.

8. Microcentrifuge.

3. Methods

3.1. Specimen Collection and Transport

1. Specimens should be collected as soon as possible after the onset of the patient's symptoms, preferably within 1–3 days.

2. Optimal specimens for the detection of influenza virus are nasopharyngeal aspirates, swabs, and washes; oropharyngeal swabs; and nasal swabs, washes, and aspirates. Deeper respiratory system specimens, such as tracheal swabs and aspirates, bronchial swabs and aspirates, and bronchoalveolar lavage, can be collected in the event of suspected influenza pneumonia. Additionally, tissue specimens can be collected for analysis at autopsy.

3. To prevent drying and to help preserve any virus present, specimens should be placed immediately in 1–3 ml of sterile virus transport medium, and immediately placed on cold packs for transport to the laboratory, or refrigerated.

4. If delays in transport of more than a few days are anticipated, specimens should be frozen at –70°C, and subsequently shipped on dry ice.

3.2. Rapid Immunoassays for Antigen Detection

1. The recommended specimen types for each commercial kit are listed by the manufacturer in the package insert.

2. Specimens are mixed with diluent (provided in the kit) to disrupt any virus present in the specimen and release viral antigens.

3. Antibody conjugate, which binds to virus antigen, is added.

4. When the combined specimen/conjugate is applied to the chromatographic paper housed inside the test device, the sample migrates along the test paper, to the reaction area, where the detection antibody is bound.

5. The antigen–antibody conjugate complex binds to the capture antibody on the paper.

6. Wash buffer can be applied to remove residual specimen matrix.

7. The conjugated particle forms a visible band or spot at the position of the capture antibody, so the reaction can be read by eye.

3.3. Antigen Detection by Direct Immunostaining

A number of commercial kits are available for the direct detection of influenza virus antigens in the cells present in respiratory secretions (see Table 2 and Note 6). Alternatively, the laboratory can purchase anti-influenza antibodies and conjugates, and develop its own test in-house. In either case, the method can use a direct immunofluorescence procedure, with an anti-influenza antibody conjugate, or an indirect procedure utilizing an unlabeled anti-influenza antibody followed by a labeled conjugate. For the staining of cells from respiratory samples, conjugates are most commonly FITC labeled, and results are read with an epifluorescence microscope. When a commercial kit is used, the method is detailed in the manufacturer's instructions. Procedures are much the same for laboratory-developed assays, except that the use of the latter requires optimization of the two reagents by cross-titration of the antibody solutions on positive material for determination of their ideal working concentrations. These titrations must be repeated for each new batch of antibody received. A common procedure for direct fluorescent antigen detection is given below.

1. Vortex the specimen with swab left inside the vial of VTM.
2. Remove 1 ml of specimen from vial, transfer to another sterile polypropylene tube, and cap the tube.
3. Centrifuge at $700 \times g$ for 5 min.
4. Aspirate supernatant and resuspend pellet in 1 ml of PBS.
5. Centrifuge at $700 \times g$ for 5 min.
6. Aspirate all but 200 µl of supernatant and then resuspend cell pellet.
7. Place one drop of cell suspension into each well of slide and allow to air dry.
8. Fix cells in fresh acetone for 5 min.
9. Remove slide from acetone, allow to air dry completely.
10. Add first antibody to cell spot, sufficient to cover it completely (extend the liquid beyond the edge of the well) and prevent its drying out during incubation.
11. Place slide in humidified chamber, seal, and incubate for 30 min at 35–37°C.
12. Wash with PBS.
13. If indirect immunofluorescence is being performed, add second antibody (conjugate), incubate for 30 min, and then wash with PBS.
14. Add a drop of mounting fluid, coverslip, and examine under epifluorescence optics at 200–400× magnification.
15. Infected cells will contain apple-green speckled fluorescence (see Notes 7–9). Noninfected cells will contain no fluorescence, but will stain red with the Evans Blue counterstain that is included in all commercial conjugate reagents.

3.4. Rapid Culture Methods for Influenza Virus

Rapid culture methods for influenza viruses are based on centrifugation-enhanced culture, coupled with the detection of viral antigens by immunofluorescence, usually prior to the development of cytopathic effect (CPE). The basic method below can be applied to all appropriate cell types, culture vessels, and stains for the detection of influenza viruses. Cells can be either purchased commercially or prepared in-house; for optimal infectivity, they should be 85–90% confluent at the time of inoculation. Cultures can be held at room temperature to prevent growth beyond confluency prior to use.

1. On the day of inoculation, if cultures are not already at 35–37°C, transfer them to incubator for 2 h.
2. Vortex specimens with swab in for 30–60 s.
3. Remove swab with forceps, squeezing fluid against the side of the tube.
4. Add antibiotics to specimen (0.1 ml per 1 ml of specimen).
5. Remove the shipping medium from cell culture vials, without touching the monolayer.
6. Add 0.3 ml of specimen to the monolayer.
7. Centrifuge the culture vessel at $700 \times g$ for 60 min, with temperature set to hold at 33°C.
8. Add 1 ml of maintenance medium (type of medium varies, depending on the cell type being used).
9. Incubate the culture at 33°C for 16–24 h.
10. Observe for visible CPE, toxicity, and contamination.
11. Aspirate the maintenance medium from the vials without disturbing the monolayer.
12. Gently add 1 ml of PBS per vial, and then aspirate carefully without disturbing the monolayer.
13. Add 1 ml of cold, fresh acetone, cover the vial, and fix for 10 min.
14. Decant acetone, gently rinse with PBS, and decant PBS.
15. Stain with fluorescently labeled antibody for influenza (as per manufacturer's protocol, or see Subheading 3.3 above).
16. Remove the coverslip with fine-tipped forceps and place on a microscope slide with mounting fluid (invert the coverslip).
17. Read the slide with fluorescence microscopy at 200–400× magnification.
18. Positive results should appear as apple-green speckled fluorescence in the cytoplasm and/or nucleus of the infected cells.
19. Negative (noninfected) cells should stain red with the Evans Blue counterstain present in all commercial conjugates.

3.5. Conventional Virus Culture

1. Vortex specimen with swab in for 30–60 s.

2. Remove swab with forceps, squeezing fluid against the side of the tube.

3. Add antibiotics to specimen (0.1 ml per 1 ml of specimen).

4. Decant medium from cell culture tubes (at least two tubes per specimen).

5. Inoculate 0.2 ml of prepared specimen per tube.

6. Incubate for 1 h at 37°C.

7. Check tubes for toxic reaction to inoculum. If present, draw off inoculum.

8. Add 1 ml medium per tube and incubate at 33°C.

9. Check under microscope on day 1 and change medium if no cytopathic effect (CPE) is observed.

10. Check under microscope thereafter three times a week for CPE effect, and change medium every 7 days if no CPE is present.

11. If CPE is observed, confirm with either rapid immunoassay, immunofluorescence (see Note 10), or molecular assay for influenza.

12. If no CPE is observed, check monolayer with hemadsorption (HA) assay on day 7 and again before discarding on day 14 (see Chapter 3 in this volume for HA method).

13. If HA assay is positive, check for the presence of influenza with either rapid immunoassay, immunofluorescence, or molecular assay for influenza (see Note 11).

3.6. Molecular Diagnosis (see also Notes 12–14)

1. Extract nucleic acid (see Table 3), transfer eluates to nuclease-free tubes, and store refrigerated or freeze at –70°C if you are not performing the test within 4 h.

2. Thaw all PCR reagents and hold in cold blocks or on ice.

3. Prepare assay mixtures according to manufacturer's instructions with master mix (see Table 4), primers and probes, and dispense into the PCR reaction tube.

4. Add extracted patient samples, along with positive and negative controls, to wells or tubes of PCR reaction vessel that have been already loaded with assay mixture.

5. Seal reactions and transfer to PCR instrument (see Table 5).

6. Typical cycling conditions for one-step influenza real-time RT-PCR assays in a 96-well instrument are reverse transcription at 50°C for 30 min, inactivation at 95°C for 2 min, then 45 cycles of 95°C for 15 s and 55°C for 30 s. However, cycling temperatures and times must always be optimized for the individual primer and probe combination.

4. Notes

1. Do not use cotton or calcium alginate swabs, or swabs with wooden shafts, for the collection of specimens for influenza diagnostic testing, because they may contain substances that inactivate the virus, decrease the immunoassay reaction, and inhibit PCR.

2. For all commercial kits, components should be stored, and procedures should be performed, according to the manufacturer's instructions. In general, reagents are supplied at working strength and dilution results in decreased detection sensitivity.

3. Rapid immunoassays are convenient and their use requires minimal technical skill, but they are considerably less sensitive than immunofluorescence, culture, or molecular assays, for the detection of influenza, especially pandemic (H1N1) 2009. They have also been found to have occasional issues with false-positive reactions, which may be lot specific.

4. Acetone is hygroscopic and should be kept in tightly sealed containers. Moisture in the acetone fixative used in fluorescence microscopy can produce a hazy appearance.

5. Most commercial antibody reagents contain sodium azide, which is poisonous and can also cause the production of explosive metal azides. If discarded into a drain, these reagents must be flushed with large volumes of water.

6. The laboratory can also test tissue sections for the presence of influenza antigens, although such methods are usually performed in specialized pathology laboratories rather than virology laboratories. These methods, which commonly employ immunoperoxidase or immunoalkaline phosphatase rather than immunofluorescence techniques, require expertise in tissue sectioning, mounting, and dewaxing of sections, and following the usual antibody staining procedure, the application of a color-producing substrate. Additional labels such as streptavidin and biotin can be used to amplify the reaction chemically further. An advantage is that stained sections can be stored long term without deterioration of the resulting colored product. These methods have been extensively described elsewhere (7).

7. Numerous artifacts may be observed when immunofluorescence assays are read, either for direct detection or for culture confirmation. Drying of the conjugate at the edge of the well will cause a strong fluorescence where the reagent has dried onto the slide. Cell debris and other small particles can nonspecifically adsorb the fluorescent conjugate. While such particulate matter can fluoresce brightly, it is usually readily identifiable on the basis of morphology. A low-level milky fluorescent haze across the cell spot can indicate insufficient

washing of the slide, or residual mucus in the specimen when the cells were spotted. Yellow or yellow-green fluorescence is nonspecific.

8. Fluorescence will fade relatively quickly, especially with exposure to light. Results should be read within a few hours of staining, and slides should be protected from light immediately after staining. Microscopic examination for an extended period of time will cause fading of fluorescence.

9. For the detection of antigens directly in cells from respiratory specimens, cell spots should contain at least 20 columnar epithelial cells, to be considered an adequate sample for analysis. If fewer are present and no positive cells observed, the test should be considered invalid and repeated on a larger volume of sample (more than 1 ml from the VTM tube), or a new specimen should be collected from the patient.

10. For culture confirmation testing, the cell spot should contain at least 200 cells.

11. Other viruses, including parainfluenza viruses, may produce positive hemadsorption (HA) reactivity. If a HA test is positive but influenza confirmation tests are negative, confirmatory tests for these other viruses can be performed.

12. The diagnosis of influenza with real-time molecular assays requires training in the operation of the specialized equipment used for these assays and should not be performed by inexperienced personnel.

13. The specificity of a molecular assay, for type or subtype of influenza, is determined by the genomic target of the primers and probes. Most influenza A detection assays use primer/probe combinations that target the matrix gene, while subtyping assays commonly target the hemagglutinin genes.

14. Numerous primer and probe sequences for influenza assays have been published in the peer-reviewed literature and are publicly available. Primer and probe concentrations should be optimized, and each lot of reagents should be checked for reactivity by comparison with the previous lot, prior to use. Different master mix reagents should be tested with individual primer/probe sets and PCR instruments for optimization of assays.

Acknowledgments

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Chapter 5

Solid-Phase Assays of Receptor-Binding Specificity

Mikhail N. Matrosovich and Alexandra S. Gambaryan

Abstract

Influenza virus attachment to sialic acid-containing molecules on the cell surface initiates the infection. The spectrum of functional receptors on target cells and decoy receptors on cells and epithelial mucus varies substantially between animal species leading to variations in the receptor-binding specificity of viruses circulating in these species. Analysis of the receptor specificity of different animal and human influenza viruses can give insight into factors and mechanisms that determine viral host range, tissue and cell tropism, replication efficiency, and pathogenesis. Knowledge of viral receptor specificity may also be useful for the development of more efficient influenza vaccines and anti-influenza drugs.

A majority of known receptor specificity assays measure influenza virus binding to sialic acid-containing natural and synthetic compounds (receptor analogues). Here, we describe protocols of two solid-phase enzyme-linked receptor-binding assays which are technically similar to standard ELISA. Each assay determines binding of the virus immobilized in the wells of 96-well plate to receptor analogues in solution. In the direct binding assay, the virus binds to either synthetic biotinylated sialylglycopolymers or to peroxidase-labeled sialylglycoprotein fetuin (Fet-HRP); the apparent association constants of the virus–receptor complexes are calculated from the Scatchard plots of the binding data. In the fetuin-binding inhibition assay, the virus is incubated with a mixture of unlabeled receptor analogue and standard preparation of Fet-HRP; the association constant for analogue is calculated based on the level of its competition with Fet-HRP.

Key words: Receptor specificity, Influenza virus, Solid-phase receptor-binding assay, Competitive inhibition, Sialic acid, Peroxidase-labeled fetuin, Sialylglycopolymer

1. Introduction

Influenza viruses attach to target cells via multivalent interactions of the viral envelope protein hemagglutinin (HA) with sialyloligosaccharide moieties of cellular glycoconjugates traditionally called viral receptors. The interactions of the virus with cellular receptors and extracellular inhibitors in the target tissues determine viral host range, tissue tropism, and pathogenicity (for reviews, see refs. 1–5).

First assays of influenza virus receptor-binding specificity were based on either virus-mediated agglutination of erythrocytes from different species or hemagglutination inhibition by natural and chemically modified glycoprotein inhibitors (for review, see ref. 6). A major advance was associated with utilization of specific sialyltransferases by Paulson and colleagues to generate resialylated erythrocytes with defined structure of terminal sialic acid motifs (7). Studies using this assay for the first time demonstrated that receptor specificity of influenza virus depends on the viral host species as avian and equine viruses preferentially bound to Neu5Acα2-3Gal-terminated receptors (avian-type receptors), whereas swine and human viruses preferentially bound to the alternative (human-type) terminal receptor moiety Neu5Acα2-6Gal (reviewed in ref. 8). Another useful assay type took advantage of the fact that glycolipids, unlike sialylglycoproteins, contain only one oligosaccharide chain per molecule and can be purified to homogeneity. Furthermore, the hydrophobic ceramide component easily anchors glycolipids in various assay media for binding studies. Several different assay formats have been developed to characterize influenza virus binding to gangliosides (sialic acid-containing glycolipids) immobilized on solid phase (for reviews, see refs. 2, 3). The most recent new assay platform, glycan microarrays, comprises a library of structurally defined sugars printed on glass or cellulose slides (9, 10). The capacity of the arrays to provide highly detailed profiles of influenza virus binding to sialyloligosaccharides has been demonstrated (11–13). This powerful assay can greatly facilitate studies on the fine receptor specificity of avian and mammalian influenza viruses. However, the assay is relatively expensive and technically demanding, making its routine use in general virological laboratories impossible.

The solid-phase enzyme-linked assays analogous to sandwich ELISA were developed in the early 1990s (14, 15). These assays were easy to perform, sensitive, quantitative, and allowed comparison of large panels of viruses for their binding to sialylglycoproteins and to low molecular mass sialic acid compounds, such as free Neu5Ac, 3′-sialyllactose and 6′-sialyl-*N*-acetyllactosamine (16–22). Binding experiments using structurally defined monovalent sialosides provided valuable information about HA–receptor interactions, but could not account for the effects of presentation of the sialyloligosaccharide moiety in the context of the receptor macromolecule as a whole and for polyvalency of virus–receptor interactions. In addition, these experiments require large amounts of expensive sialosides because of the low binding affinity of the virus for monovalent receptors. These pitfalls were overcome with the development by Nicolai Bovin and colleagues of synthetic sialylglycopolymers (SGPs), monospecific macromolecular probes which comprised multiple copies of sialyloligosaccharide moieties attached to soluble hydrophilic polymeric carrier (23, 24).

Analyses of the virus binding to monovalent sialosides and to corresponding SGPs have been proven to be useful for characterizing the receptor-binding properties of influenza viruses from different hosts (25–27). In particular, by comparing the viral binding to a panel of SGPs that harbored the same Neu5Acα2-3Gal motif in a context of different oligosaccharide core sequences, Gambaryan, Bovin, and colleagues were able to specify the recognition of the inner parts of sialyloligosaccharide sequences by viruses from different avian species (28–31). Unexpectedly, these studies revealed significant distinctions between duck, gull, and chicken viruses raising an intriguing question about the role of these distinctions in the interspecies transmission of avian viruses.

The protocols described in this chapter are based on our long-term working experience with the solid-phase enzyme-linked receptor-binding assays. Two assay types are described, a direct binding assay and a competitive fetuin-binding inhibition (FBI) assay. At the first stage of each assay, the virus is adsorbed in the wells of 96-well ELISA plates coated with sialylglycoprotein fetuin. In the direct binding assay (Subheading 3.2), the immobilized virus is allowed to interact with dilutions of labeled macromolecular receptor analogues. Two types of such analogues are described: monospecific synthetic biotinylated SGPs 3′SL-PAA-biot and 6′SLN-PAA-biot (Subheading 3.2.1) and monospecific peroxidise-labeled preparations of fetuin, 3-Fet-HRP, and 6-Fet-HRP (Subheading 3.2.2). After non-bound material is removed by washing, the binding of labeled receptor analogues is evaluated using standard colorimetric assay of peroxidise activity. The binding data are converted into Scatchard plots, and association constants of virus–analogue complexes are determined from these plots (Subheading 3.2.1.1). In the FBI assay (Subheading 3.3), the virus is incubated with a mixture of unlabeled receptor analogue and standard preparation of Fet-HRP; the association constant of the analogue is calculated based on the level of its competition with Fet-HRP (Subheading 3.3.2.1).

2. Materials

2.1. Preparation and Optimization of Reagents

2.1.1. Viruses

Suspensions of influenza A and B viruses propagated in either embryonated hen's eggs or cell culture (see Chapter 2 in this book) with hemagglutination titer higher than 32 are required (see Notes 1 and 2). Viruses in allantoic fluid (AF) or culture fluid (CF) can be stored at 4°C for up to 4 weeks. It is recommended to add protease inhibitors (e.g., Complete Mini, Roche) and 0.02% sodium azide. For longer storage, either keep in aliquots at –20°C or prepare partially purified virus concentrates and store in 50% glycerol at low temperature as described in Subheading 3.1.1.

2.1.2. Fetuin-Coated *96-Well Plates*	1. Flat-bottom 96-well ELISA plates. 2. 5% solution of fetuin (Sigma, Cat No F3004) in water. Store in aliquots at −20°C. 3. Phosphate-buffered saline (PBS), 0.02 M, pH 7.2–7.4. 4. 8–12-channel pipette or 8-channel dispenser (0.2 ml).
2.1.3. Desialylated BSA *(BSA-NA) (see Note 3)*	1. BSA powder or solution, for example, Sigma, Cat. No. A3059 or A0336, respectively. 2. PBS. 3. Neuraminidase from *Vibrio cholerae* (Sigma, Cat. No. N7885). 4. PEN-STREP, 100× (Sigma).
2.1.4. Standard Fet-HRP *and High-Avidity Fet-HRP*	1. Fetuin from fetal calf serum (Sigma, Cat. No. F3004). 2. 1 and 0.1 M sodium carbonate buffers, pH 9.3. 3. Horseradish peroxidase, lyophilized powder, absorbency ratio (RZ) not lower than 3. 4. Bidistilled water (see Note 4). 5. Sodium periodate ($NaIO_4$), reagent grade. 6. Sephadex G-25 column, 5 ml, equilibrated with water. 7. Sodium borohydride ($NaBH_4$), reagent grade. 8. 1 M Tris–HCL buffer, pH 6.0. 9. Glycerol, reagent grade. 10. 0.1 M Tris–HCL buffer, pH 7.2. 11. Sephacryl S-200 column, 60 ml, equilibrated with 0.1 M Tris–HCL buffer, pH 7.2.
2.1.5. Monospecific *3-Fet-HRP and 6-Fet-HRP*	1. Items 3–10 described in Subheading 2.1.4. 2. 0.1 M sodium carbonate buffer, pH 9.3. 3. Asialofetuin from fetal calf serum (Sigma, Cat. No. A1908). 4. Slide-A-Lyzer Dialysis Cassettes, 10,000 MWCO (Pierce) or dialysis tubing (10–30 kDa). 5. PBS. 6. 1 M solution of $MgCl_2$ in water. 7. CMP-sialic acid (Calbiochem, Cat. No. 233264). Prepare 4 mM solution in water, store in aliquots at −20°C. 8. Alfa-2,6-(*N*)-sialyltransferase from rat liver (WAKO Chemicals). 9. Rat recombinant alfa-2,3-(*N*)-sialyltransferase (Merck).
2.1.6. Determination of *Working Dilutions of* *Fet-HRP Preparations*	1. Stock solutions of Fet-HRP, high-avidity Fet-HRP (ha-Fet-HRP), 3-Fet-HRP, or 6-Fet-HRP (see Subheadings 3.1.4 and 3.1.5). 2. Human influenza virus with good binding to fetuin, for example, A/X31 (H3N2), A/Chile/1/83 (H1N1), or A/Taiwan/1/86 (H1N1).

3. Avian influenza virus, for example, A/duck/Ukraine/1/63 (H3N8) or A/duck/Czechoslovakia/56 (H4N6).

4. Fetuin-coated plates.

5. Assay solutions TBS, BS,WS, RS, SS, and stop solution (see Subheading 2.1.8)

6. 8–12-channel pipette or 8-channel dispenser (0.2 ml).

7. 8-Position multiwell plate washer/dispenser manifold (Sigma, Cat. No. M2656).

8. Microplate reader (450 nm).

2.1.7. Determination of Working Dilutions of Viruses for Adsorption

1. Fet-HRP or ha-Fet-HRP (see Subheading 3.1.4).

2. Non-purified virus suspensions or partially purified viruses (see Subheadings 2.1.1 and 3.1.1).

3. Fetuin-coated plates.

4. Assay solutions TBS, BS, WS, RS, SS, and stop solution (see Subheading 2.1.8).

5. 8–12-channel pipette or 8-channel dispenser (0.2 ml).

6. 8-Position multiwell plate washer/dispenser manifold (Sigma, Cat No M2656).

7. Microplate reader (450 nm).

2.1.8. Solutions Used in Receptor-Binding Assays

1. Tris-buffered saline (TBS): 0.02 M, pH 7.2–7.4 (diluent for virus suspensions).

2. 10% (w/v) stock solution of tween-80 in PBS.

3. Stock solution of neuraminidase inhibitor, either oseltamivir carboxylate (Roche) or zanamivir (GlaxoSmithKline) (see Note 5). Prepare 1 mM stock solution in water, store in aliquots at –20°C.

4. 1% (w/v) stock solution of 3,3′,5,5′-tetramethylbenzidine (TMB). Dissolve TMB in dimethyl sulfoxide (reagent grade), store in aliquots at –20°C.

5. Washing solution (WS): 0.01% tween-80 in PBS. Cool to 4°C before use.

6. Blocking solution (BS): 0.1% solution of BSA-NA in PBS. Prepare from 5% stock solution (see Subheading 3.1.3).

7. Reaction solution (RS): PBS containing 0.02% tween-80, 0.1% BSA-NA (see Subheading 3.1.3), and 1 μM neuraminidase inhibitor. Store at 4°C for up to 5 days.

8. Substrate solution (SS). Add 0.1 ml of 1% stock solution of TMB to 10 ml of 0.05 M sodium acetate buffer pH 5.5. Add 10 μl of 30% hydrogen peroxide (Sigma, Cat. No. H0904) and mix.

Prepare SS immediately before use, do not store. Any commercial peroxidase substrate can be used as an alternative to SS described here.

9. Stop solution: 3% (v/v) H$_2$SO$_4$ in water.

2.2. Direct Binding Assays

1. Items 1–7 described in Subheading 2.1.7 are common for both assay variants.

2. Materials specific for each of the two assay variants are described below (Subheadings 2.2.1 and 2.2.2).

2.2.1. Binding to Biotinylated Sialylglycopolymers

1. Biotinylated SGPs 3′SL-PAA-biot and 6′SLN-PAA-biot (Lectinity Holding, Inc., Moscow, Russia) (24). These SGPs contain 20 mol% of 3′-sialyllactose (Neu5Acα2-3Galβ1-4Glc) and 6′-sialyl-N-acetyllactosamine (Neu5Acα2-6Galβ1-4GlcNAc), respectively, and 5 mol% of biotin attached to poly[N-(2-hydroxyethyl)acrylamide] backbone. SGPs are available with average molecular mass of either 30 or 1,500 kDa. The 30 kDa SGPs are suitable for a majority of applications; however, they bind weakly if at all to many non-egg-adapted H3N2 human viruses isolated after 1992 (32); for these viruses as well as for other low-avidity viruses, high-molecular mass SGPs (1,500 kDa) should be used.

   Prepare 0.1 mM stock solutions (with respect to sialic acid) by dissolving SGPs in bidistilled water; store in aliquots at –20°C.

2. Peroxidase-labeled streptavidin, for example, Pierce, Cat. No. 21126.

2.2.2. Binding to 3-Fet-HRP and 6-Fet-HRP

1. 3-Fet-HRP and 6-Fet-HRP (see Subheading 3.1.5)

2.3. Fetuin-Binding Inhibition Assay

1. Items 1–7 listed in Subheading 2.1.7.

2. *Receptor analogues*. A variety of natural and synthetic sialic acid-containing compounds can be used depending on availability and goals of the study. Below, we list some of the commercially available compounds of defined chemical structure with references to reports describing their use for characterization of viral receptor-binding specificity in FBI assay.

   (a) *Monovalent compounds* (contain one sialic acid residue per molecule) (14, 15, 18–22, 25, 26).

   - 3′ sialyllactose, sodium salt (3′SL, Neu5Acα2-3Galβ1-4Glc) (Sigma, Cat. No. A8681).

   - 6′-sialyl-N-acetyllactosamine, sodium salt (6′SLN, Neu5Acα2-3Galβ1-4GlcNAc) (Sigma Cat. No. 37966).

     Prepare 40 mM stock solutions of 3′SL and 6′SLN in water, store in aliquots at –20°C.

- N-acetylneuraminic acid (α,βNeu5Ac) (Sigma, Cat. No. A0812). Prepare 800 mM stock solution, which will contain 40 mM of binding-competent α-anomeric form (αNeu5Ac) [19]. Dissolve 250 mg of α,βNeu5Ac in 0.75 ml of 1 M solution of NaOH, adjust to neutral pH with 10 M NaOH, adjust volume to 1 ml with water. Store in aliquots at –20°C.

(b) *SGPs* [20, 25, 27–31, 33, 34]

Non-labeled poly[*N*-(2-hydroxyethyl)acrylamide]-based sialylglycopolymers are produced by Lectinity Holding, Inc., Moscow, Russia [23, 24]. Standard SGPs contain 20 mol% of a sialyloligosaccharide moiety (~98% purity) linked to the 30 kDa polymeric carrier. Several sialyloligosaccharides that were particularly useful in the studies on species-specific distinctions in receptor specificity of avian influenza viruses [14, 29–31, 34] are listed below.

Neu5Acα2-3Galβ1-4GlcNAcβ	3′SLN
Neu5Acα2-3Galβ1-4(6-HSO$_3$)GlcNAcβ	Su-3′SLN
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ	SLe^x
Neu5Acα2-3Galβ1-4(Fucα1-3)-(6-HSO$_3$)GlcNAcβ	Su-SLe^x
Neu5Acα2-3Galβ1-3GlcNAcβ	SLe^c
Neu5Acα2-3Galβ1-3GalNAcα	STF
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ	SLe^a
Neu5Acα2-6Galβ1-4GlcNAcβ	6′SLN

Prepare 1 mM stock solutions with respect to sialic acid by dissolving SGPs in bidistilled water; store in aliquots at –20°C.

3. Methods

3.1. Preparation and Optimization of Reagents

3.1.1. Viruses

Avian viruses and most egg-adapted mammalian viruses can be used without purification.

Many non-egg-adapted human and swine viruses and some laboratory-derived receptor-binding mutants do not bind efficiently to fetuin-coated plates from allantoic or cultural fluid. To solve this problem, such viruses must be partially purified from egg- or cell-derived protein and concentrated as described below.

1. Clarify allantoic or culture fluid by low-speed centrifugation ($5,000 \times g$, 20 min).
2. Pellet the virus by high-speed centrifugation ($120,000 \times g$, 1 h).

3. Resuspend the virus pellet thoroughly in 50% glycerol-0.1 M Tris–HCL (pH 7.2) at 1% of the original volume. Remove remaining cellular debris by centrifugation for 5 min at $1,000 \times g$.

4. Prepare aliquots. Store one aliquot for routine work at –20°C and store the rest at –80°C.

3.1.2. Fetuin-Coated 96-Well Plates

1. To coat 25 plates, prepare 510 ml of a 10 μg/ml working solution of fetuin in PBS.

2. Add 0.2 ml of fetuin solution to each well, cover plates, and incubate at 4°C overnight.

3. Flick plate content into a waste container. Rinse and flick the plates three times with 0.35–0.4 ml of tap water and once with distilled or deionized water. Strike the plates sharply onto paper towel to remove water droplets.

4. Let plates air-dry for at least 3 h at room temperature in a clean bench area.

5. Store in a moisture-proof bag at room temperature away from light and heat for up to several months.

3.1.3. Desialylated BSA (BSA-NA) (see Note 3)

1. Prepare 50 ml of 5% solution of BSA in PBS, add 1 ml PEN-STREP and adjust pH to 7.3–7.5.

2. Add 1 unit of *Vibrio cholerae* neuraminidase and incubate for 24 h at 37°C.

3. Incubate for at least 24 h at 60°C to inactivate the neuraminidase.

4. Aliquot and store at –20°C.

3.1.4. Synthesis of Peroxidase-Labeled Fetuin (Fet-HRP)

Synthesis of the four following preparations is described.

1. Standard Fet-HRP is routinely used for the FBI assay (Subheading 3.3) with the majority of avian and mammalian influenza viruses.

2. High-avidity Fet-HRP which is prepared from heat-aggregated fetuin is routinely used in all assay variants for determination of working dilutions of viruses for adsorption (see Subheading 3.1.7). High-avidity Fet-HRP can also be used for direct binding assay and FBI with viruses that show insufficient avidity for the standard Fet-HRP.

3-4. Monospecific Neu5Acα2-3Gal- and Neu5Acα2-6Gal-containing peroxidase-labeled fetuin preparations (3-Fet-HRP and 6-Fet-HRP) are primarily used for differentiation between virus preference for the type of Neu5Ac-Gal linkage in the direct binding assay (Subheading 3.2.2).

3.1.4.1. Standard Fet-HRP

1. Dissolve 2 mg of fetuin in 0.6 ml of 0.1 M sodium carbonate buffer, pH 9.3.

2. Dissolve 4 mg of HRP in 0.9 ml of bidistilled water.

3. Add 0.1 ml of freshly prepared 0.2 M $NaIO_4$ solution in water to the solution of HRP. Close the tube, mix, and incubate for 20 min in the dark at room temperature.

4. Desalt reaction mixture by filtration through 5-ml Sephadex G-25 column, elute with water. Collect major colored fraction (1.2–1.4 ml) containing oxidized HRP.

5. Add collected solution of oxidized HRP to the solution of fetuin. Mix, incubate for 4 h in the dark at room temperature.

6. Transfer reaction mixture to a 10-ml tube with cap to avoid potential losses due to formation of foam at the next steps. Add 0.1 ml of freshly prepared 5 mg/ml solution of $NaBH_4$ in water, mix, and incubate for 30 min on ice. Add 0.2 ml of another freshly prepared solution of $NaBH_4$, incubate for 30 min on ice.

7. Slowly add 1/5 volume of 1 M Tris–HCL pH 6 on ice. Caution: this will destroy residual $NaBH_4$ and may result in foaming. Leave overnight at 4°C to ensure complete hydrolysis of $NaBH_4$ before the next step.

8. Separate Fet-HRP from nonconjugated peroxidase by chromatography on Sephacryl S-200 column in 0.1 M Tris buffer pH 7.0. Collect and combine major HRP-containing fractions eluted in the void volume of the column. Add equal volume of glycerol, mix, and aliquot. Store one aliquot for routine work at –20°C and store the rest at –80°C.

3.1.4.2. High-Avidity Fet-HRP

1. Dissolve 15 mg of fetuin in 0.3 ml water (5% solution) in a 0.4-ml capped tube. Incubate for 4 h at 90°C, vortex tube each 30 min (see Note 6). Heating leads to aggregation of fetuin and increases avidity of its binding to influenza viruses.

2. Dissolve 15 mg HRP in 1 ml water in a 2-ml tube.

3. Add 0.1 ml of freshly prepared 0.4 M $NaIO_4$ solution in water to the solution of HRP. Mix and incubate for 20 min in the dark at room temperature.

4. Desalt reaction mixture by filtration through 5-ml Sephadex G-25 column, elute with water. Collect major colored fraction (1.2–1.5 ml) containing oxidized HRP.

5. Combine solution of oxidized HRP with solution of heat-aggregated fetuin, add 0.1 ml of 1 M carbonate buffer, pH 9.3. Mix, incubate for 4 h at room temperature in the dark.

6. Follow the instructions for the steps 6–8 in Subheading 3.1.4.1.

3.1.5. Monospecific 3-Fet-HRP and 6-Fet-HRP

1. Monospecific Neu5Acα2-3Gal- and Neu5Acα2-6Gal-containing peroxidase-labeled fetuin preparations 3-Fet-HRP and 6-Fet-HRP are primarily used for differentiation between virus preference for the type of Neu5Ac-Gal linkage in the direct binding assay (Subheading 3.2.2). Dissolve 15 mg of asialofetuin in 0.3 ml of 0.1 M sodium carbonate buffer, pH 9.3 in a 2-ml tube.

2. Dissolve 15 mg HRP in 1 ml of water.

3. Add 0.1 ml of freshly prepared 0.4 M NaIO$_4$ solution in water to the solution of HRP. Mix and incubate for 20 min in the dark at room temperature.

4. Desalt reaction mixture by filtration through a 5-ml Sephadex G-25 column, elute with water. Collect major colored fraction (1.2–1.4 ml) containing oxidized HRP.

5. Add solution of oxidized HRP to the solution of asialofetuin. Mix, incubate for 4 h at room temperature in the dark.

6. Follow the instructions for the steps 6–7 in Subheading 3.1.4.1.

7. Dialyze HRP-labeled asialofetuin against PBS, pH 7.0 at 4°C.

8. Collect dialyzed product. Add MgCl$_2$ to 2 mM and CMP-Neu5Ac to 1.5 mM.

9. Divide the mixture into two aliquots (about 1.6 ml each) for the separate preparation of 3-Fet-HRP and 6-Fet-HRP. Add 2,3-(*N*)-sialyltransferase to one aliquot to a final concentration 20 mU/ml. Add 2,6-sialyltransferase to another aliquot to 80 mU/ml. Incubate for 16 h at 37°C (see Note 7).

10. Dialyze 3-Fet-HRP and 6-Fet-HRP against 0.1 M Tris–HCL buffer, pH 7.2 at 4°C.

11. Collect dialyzed products. Add equal volume of glycerol, mix, and aliquot. Store one aliquot of each preparation for routine work at –20°C and store the rest at –80°C.

3.1.6. Determination of Working Dilutions of Fet-HRP Preparations

1. Dilute virus suspensions in TBS to hemagglutination titer 128.

2. Dispense 0.05 ml of diluted human and avian viruses into wells of a fetuin-coated plate. Use two replicate 12-well columns for each virus and preparation of Fet-HRP. Use two replicate 12-well columns for the negative control (0.05 ml of TBS).

3. Incubate at 4°C overnight (see Note 8).

4. Empty wells by vacuum suction using 8-positon manifold. Wash the plate three times with 0.2 ml of PBS.

5. Add 0.2 ml of the blocking solution (BS). Incubate for 1 h at room temperature or leave under BS at 4°C overnight.

6. Prepare 12 serial twofold dilutions of Fet-HRP stock in RS starting from 1/100.

7. Remove blocking solution; wash plate twice with 0.2 ml of ice-cold WS. Add 0.05 ml of each Fet-HRP dilution to duplicate virus-coated and non-coated wells (see Note 9).

8. Incubate at 4°C for 1 h.

9. Wash five times with 0.2 ml of ice-cold WS (see Note 10).

10. Add 0.1 ml of freshly prepared substrate solution; incubate for 30 min at room temperature away from direct light.

11. Stop the reaction by adding 0.05 ml of 3% H_2SO_4.

12. Read plate on a microplate reader at 450 nm and transfer the absorbency data from the reader into Microsoft Excel or another spreadsheet program.

13. Prepare plots of absorbency (A_{450}) versus Fet-HRP dilution in semilogarithmic scale. As illustrated in Fig. 1, avian viruses typically show saturated binding curves with the plateau at low Fet-HRP dilutions. Binding plots of human influenza viruses often lack a clear-cut plateau because these viruses have low avidity for fetuin. The optimal working dilutions of the Fet-HRP depend on specific application and on viruses included in the study as outlined below.

    – For virus detection (Subheading 3.1.7) use the highest dilution of Fet-HRP that still shows high values of A_{450} with both avian and human virus.

    – For the direct binding assay with all preparations of Fet-HRP and for titration of Fet-HRP and ha-Fet-HRP in the binding inhibition assay (Subheading 3.3.1) use dilutions that cover the range of absorbencies from 0.1 to the beginning of the plateau.
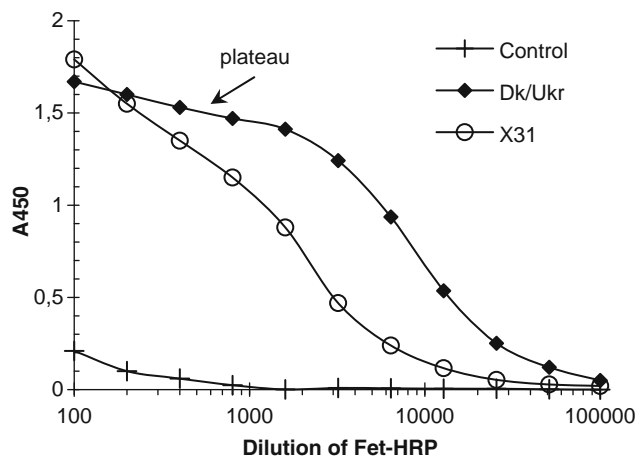
Fig. 1. Determination of working dilutions of Fet-HRP preparations (Subheading 3.1.6). Serial twofold dilutions of Fet-HRP were incubated in the wells of 96-well plate coated with viruses A/X31 (H3N2) (*open circles*) and A/Duck/Ukraine/1/63 (H3N8) (*filled diamonds*). Wells without virus were used for a control of nonspecific binding of Fet-HRP to the plate (*plus symbols*).

- In any assay, avoid using dilutions of Fet-HRP that produce significant nonspecific binding ($A_{450} > 0.2$) in the control virus-non-coated wells.

3.1.7. Determination of Working Dilutions of Viruses for Adsorption

1. Prepare eight serial twofold dilutions of viruses in TBS (0.05 ml each) in eight consecutive wells of fetuin-coated plate (two replicate rows for each virus). For non-concentrated allantoic or culture fluid, start from undiluted material; for concentrated viruses, start from 1/50 dilution. Use four to eight wells of a plate for a negative control (0.05 ml TBS).

2. Incubate overnight at 4°C (see Note 11).

3. Empty wells by vacuum suction using 8 position manifold. Wash the plate three times with 0.2 ml of PBS.

4. Add 0.2 ml of blocking solution. Incubate for 1 h at room temperature or leave under BS overnight at 4°C.

5. Remove blocking solution; wash twice with 0.2 ml of WS.

6. Add 0.05 ml of working dilution of ha-Fet-HRP in RS as determined in Subheading 3.1.6.

7. Follow the instructions for the steps 8–12 in Subheading 3.1.6.

8. Prepare plots of absorbency (A_{450}) versus virus dilution (Fig. 2). For routine work, choose maximal dilution of the virus that still saturates the plate (end of absorbency plateau, see Fig. 2). If plateau is not reached, choose the dilution corresponding to A_{450} in the range from 0.4 to 2.5 (optimally 1.5–2).

3.2. Direct Binding Assays

3.2.1. Binding to Biotinylated Sialylglycopolymers

This assay was initially introduced to study phenotypic differences between mammalian H1, H2, and H3 viruses and their avian precursors (26). Afterwards, the assay was used for a number of different applications, among them, comparison of the receptor-binding phenotypes of human and swine influenza viruses isolated in cell culture and eggs (27, 32), analysis of effects of antibody-escape mutations on receptor specificity of H9N2 virus (35), detection of receptor specificity changes during adaptive evolution of the influenza virus in the mouse lung (36). The assay is particularly useful for characterization of non-egg-adapted human and swine influenza viruses, many of which do not bind to fetuin.

1. Determine optimal dilutions of viruses to be compared as described in Subheading 3.1.7.

2. Dispense 0.05 ml of chosen virus dilutions in TBS into the wells of two replicate fetuin-coated plates to be used with either 3′SL-PAA-biot or 6′SLN-PAA-biot. Use at least two replicate rows per virus and two rows for the negative control, 0.05 ml of TBS (Fig.3, left panel) (see Note 12).

3. Incubate, wash, and block virus-coated plates (see steps 2–4 in Subheading 3.1.7).
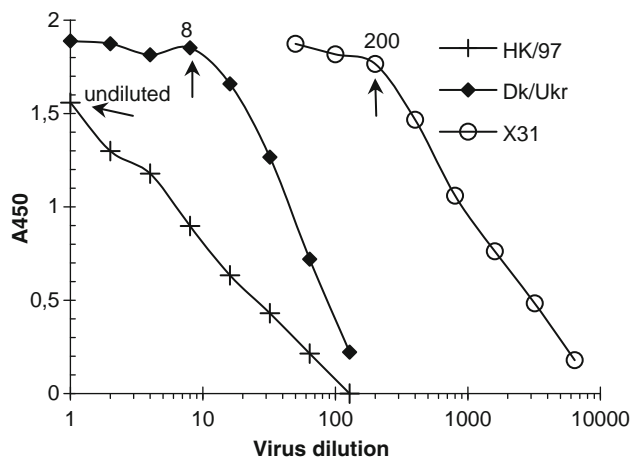
Fig. 2. Determination of working dilutions of viruses for adsorption (Subheading 3.1.7). Serial twofold dilutions of viruses were adsorbed in the wells of fetuin-coated plate and incubated with Fet-HRP followed by washing and detection of peroxidise activity in the wells. Viruses A/Hong Kong/156/97 (H5N1) (HK/97, *plus symbols*) and A/Duck/Ukraine/1/63 (H3N8) (*filled diamonds*) were adsorbed from allantoic fluid; A/X31 (H3N2) (*open circles*) was partially purified before adsorption (see Subheading 3.1.1). *Arrows* depict dilutions of the virus stocks that were chosen for the assay.

4. Prepare eight serial twofold dilutions of biotinylated SGPs (see Note 13). To study avian viruses, start titration from 0.1 µM of 3′SLN-PAA-biot and 1 µM of 6′SLN-PAA-biot. For human viruses, start from 1 µM of 3′SLN-PAA-biot and 0.1 µM 6′SLN-PAA-biot, respectively. If both human and avian viruses are tested on the same plate, start from 1 µM of each SGP. At least 0.65 ml of each dilution per 96-well plate is required.

5. Remove blocking solution; wash twice with 0.2 ml of ice-cold washing buffer.

6. Add 0.05 ml dilutions of 3′SLN-PAA-biot (Plate 1) and 6′SLN-PAA-biot (Plate 2) (see Fig. 3, right panel).

7. Incubate for 1 h at 4°C.

8. Wash five times with 0.2 ml of ice-cold WS.

9. Fill each well with 0.05 ml of peroxidase-labeled streptavidin diluted 1/1,000 in RS (see Note 14).

10. Incubate, wash, and determine peroxidase activity in the wells; save the absorbency data (see steps 8–12 in Subheading 3.1.6).

3.2.1.1. Analysis of Results

1. Using either Microsoft Excel or another spreadsheet software, calculate mean values of A_{450} for each dilution of SGP in the negative control without the virus. Subtract these values from the values of A_{450} in virus-coated wells that were incubated with corresponding dilutions of SGP. Use background-corrected values of A_{450} in all subsequent calculations.
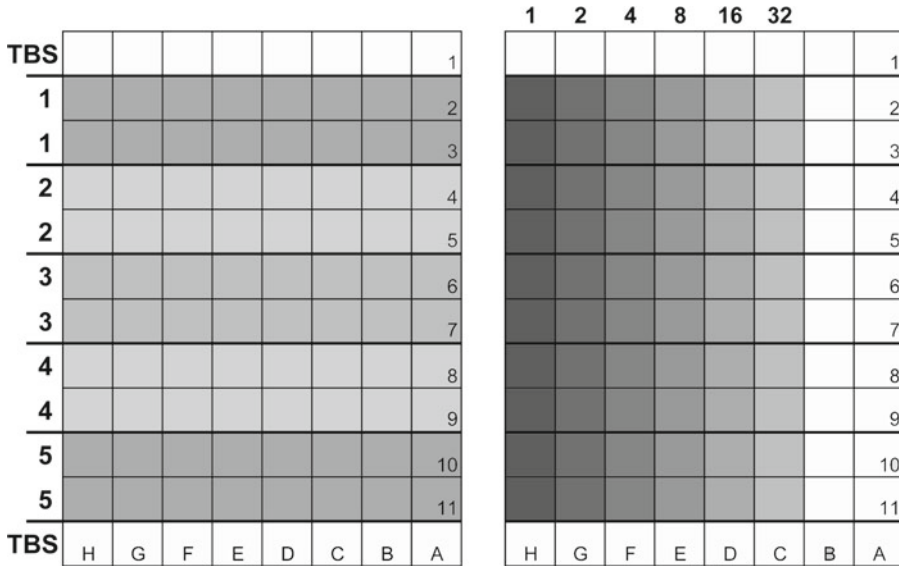
Fig. 3. Layout of the direct binding assay (Subheading 3.2.1). *Left panel*: Virus adsorption in the fetuin-coated plate with five virus specimens numbered 1–5 (two replicates of each) and two replicate rows of control wells incubated with TBS instead of the virus. Prepare two replicate plates to be used with two SGPs (either 3′SL-PAA-biot or 6′SLN-PAA-biot). *Right panel*: Titration of SGP in the virus-coated plate. Numbers on top depict serial twofold dilutions; the concentration of the first dilution is chosen as described in the text.

2. Calculate concentration of SGP (C) in μM sialic acid for each dilution.

3. Calculate values of A_{450}/C for each virus-coated well of the plate.

4. Prepare Scatchard plots (A_{450}/C vs. A_{450}) for each virus and each replicate titration of SGP (Fig. 4). Draw trendlines (if required, ignore experimental points with A_{450} less than 0.1).

5. Determine intercept of the trendlines with the Y and X axes (Y_o and A_{max}, respectively). Because 3′SL-PAA-biot and 6′SLN-PAA-biot have identical molecular masses and molar contents of sialic acid (20%) and biotin (2.5%), we assume that Scatchard plots for these SGPs should have identical values of A_{max}. Therefore, the trendline for a weakly binding polymer is drawn to have the same A_{max} as the trendlines of the high-avidity counterpart (see Fig. 4).

6. Calculate apparent association constants, $K_{ass} = Y_o/A_{max}$. Average values of K_{ass} from the replicate performed on the same day. Higher values of K_{ass} reflect a stronger binding (see Notes 15 and 16).

3.2.2. Binding to
3-Fet-HRP and 6-Fet-HRP

Two monospecific preparations of fetuin are made by resialylation of the same original HRP-labeled asialofetuin (see Subheading 3.1.5). As a result, 3-Fet-HRP and 6-Fet-HRP differ only by the type of
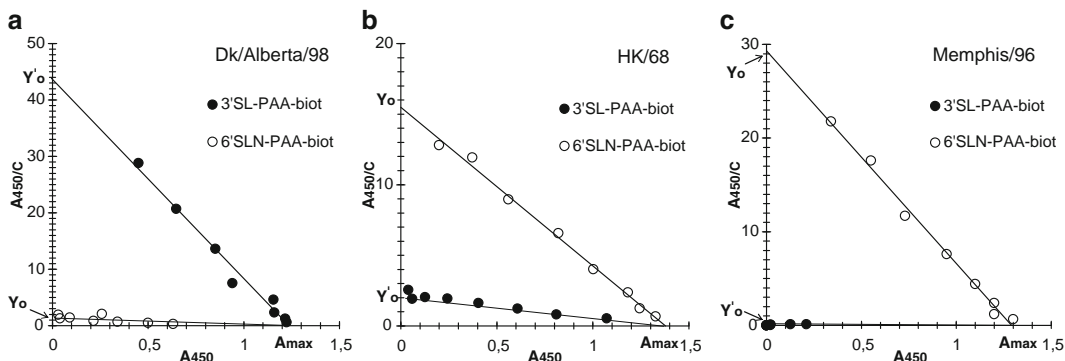
Fig. 4. Scatchard plots for the direct binding of biotinylated SGPs 3′SL-PAA-biot (*filled circles*) and 6′SLN-PAA-biot (*open circles*) to influenza viruses A/Duck/Alberta/119/98 (H1N1) (**a**), A/Hong Kong/1/68 (H3N2) (**b**), and A/Memphis/14/96-M (H1N1) (**c**). A_{max}, Y_o, and Y'_o depict intercepts of the trendlines with the axes. Note that the trendlines for the weak binding of 6′SLN-PAA-biot to Dk/Alberta/98 and of 3′L-PAA-biot to Memphis/96 are drawn to have the same A_{max} with the high-avidity SGP counterpart. Apparent association constants (in μM^{-1}) can be determined from the plots as the ratio $Y_o / A_{max.}$

the glycosidic linkage between the terminal Neu5Ac residue and the penultimate Gal residue ($\alpha 2$-3 or $\alpha 2$-6). Direct binding assay with this pair of receptor analogues was previously employed to characterize changes in the receptor specificity during egg adaptation of seasonal human H1N1 influenza virus (37).

1. Prepare two replicate plates coated with the viruses (see steps 1–3 in Subheading 3.2.1).

2. Prepare eight serial twofold dilutions of 3-Fet-HRP and 6-Fet-HRP in RS that cover the optimal range of concentrations determined in Subheading 3.1.6. At least 0.65 ml of each dilution per 96-well plate is required.

3. Remove blocking solution; wash twice with 0.2 ml of ice-cold washing buffer.

4. Add 0.05 ml of 3-Fet-HRP dilutions (see Note 9).

5. Repeat steps 3 and 4 to fill the replicate plate with 6-Fet-HRP.

6. Incubate, wash, and determine peroxidase activity in the wells; save the absorbency data (see steps 8–12 in Subheading 3.1.6).

7. Analyze the data and calculate association constants as described for SGPs (see Subheading 3.2.1.1) taking concentration of 3-Fet-HRP and 6-Fet-HRP in stock solutions for 1,000 arbitrary units (U). Higher values of K_{ass} reflect a stronger binding.

3.3. Fetuin-Binding Inhibition Assay

This assay is based on the competition between non-labeled receptor analogue and standard Fet-HRP preparation for the binding site on a solid-phase immobilized virus (14, 15). The assay is performed in two steps. First, Fet-HRP is titrated in a direct binding assay to determine the optimal working concentration. Then, the binding inhibition test is performed using chosen dilution of Fet-HRP.

3.3.1. Determination of Working Concentration of Fet-HRP

1. Prepare 96-well plates coated with the viruses (see Fig. 5 and steps 1–3 in Subheading 3.2.1). Make one replicate plate for titration of Fet-HRP and one replicate plate per each receptor analogue to be tested in FBI.

2. Prepare eight serial twofold dilutions of Fet-HRP in RS that cover the optimal range of concentrations determined in Subheading 3.1.6. At least 0.65 ml of each dilution per 96-well plate is required.

3. Remove blocking solution; wash twice with 0.2 ml of ice-cold washing buffer.

4. Add 0.05 ml of Fet-HRP dilutions (see Note 9).

5. Incubate, wash, and determine peroxidase activity in the wells; save the absorbency data (see steps 8–12 in Subheading 3.1.6).

6. Prepare Scatchard plots and determine A_{max} as described in Subheading 3.2.1.1.

7. For each virus and each Fet-HRP dilution, calculate parameter $\alpha = (A_{max} - A_{450})/A_{max}$, where α is the portion of remaining free binding sites on the virus and, A_{450} is the absorbency at a given concentration of Fet-HRP and A_{max} is the absorbency at infinite concentration of Fet-HRP (see Fig. 6).

Fig. 5. Layout of the binding inhibition assay (Subheading 3.3.2). *Left panel*: Virus adsorption in the fetuin-coated plate with five virus specimens numbered 1–5 (two replicates of each) and two replicate rows of control wells incubated with TBS. Prepare separate replicate plate for titration of Fet-HRP and for each receptor analogue to be tested in FBI. *Right panel*: Incubation of dilutions of non-labeled receptor analogue in working solution of Fet-HRP in the wells of virus-coated plate. Numbers on top depict serial twofold dilutions of the analogue in rows 2–11; the concentration of the first dilution is chosen as described in the text. Rows 1, 12 and columns A, B are incubated with working solution of Fet-HRP (controls of nonspecific binding and specific binding without inhibitor, respectively).
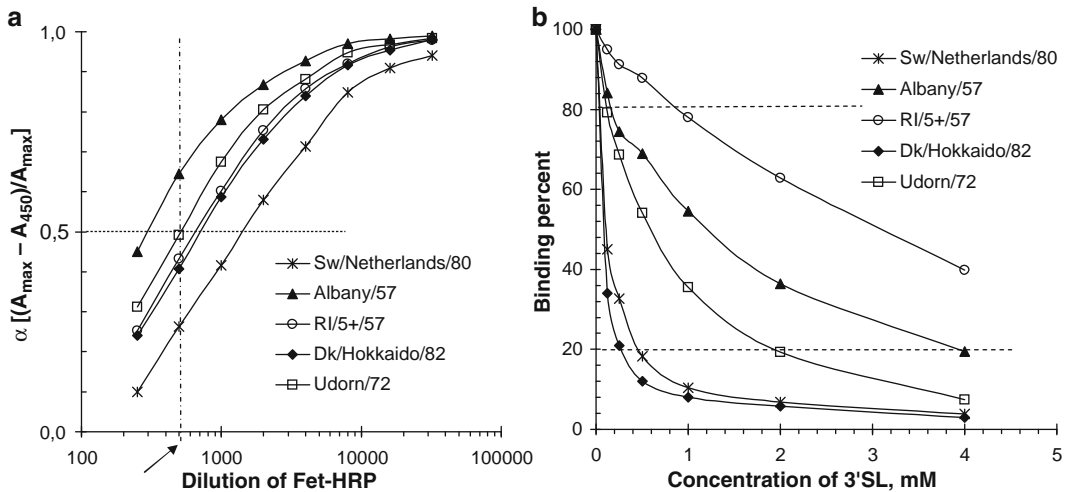
Fig. 6. Example of the binding inhibition assay: determination of association constants (K_{ass}) with 3′-sialyllactose for viruses A/Swine/Netherlands/3/80 (H1N1), A/Albany/7/57 (H2N2), A/RI/5+/57 (H2N2), A/Duck/Hokkaido/7/82 (H3N?), and A/Udorn/307/72 (H3N2) (data from ref. 26). The *charts* show data for one of two replicate rows (see Fig. 5). (**a**) Determination of working dilution of Fet-HRP; *arrow* depicts chosen working dilution (1/500). (**b**) Inhibition of Fet-HRP binding by 3′-sialyllactose. Experimental points with binding between 20 and 80% (*dotted lines*) were used for calculation of apparent association constants as described in Subheading 3.2.2.1.

8. Find dilution of Fet-HRP that corresponds to α around 0.5 for a majority of viruses to be tested simultaneously (see Fig. 6a). Record actual value of α for each virus at the chosen dilution; these values will be used for calculation of apparent association constants in the binding inhibition assay.

3.3.2. Binding Inhibition Assay: Determination of Association Constants

1. Prepare working solution of Fet-HRP in RS (6–7 ml per 96-well plate) using dilution determined in Subheading 3.3.1.

2. Prepare six serial twofold dilutions of non-labeled sialic acid-containing compounds in the working solution of Fet-HRP. For the monovalent analogues, start from 4 mM concentration, for sialylglycopolymers, start from 0.02 mM (see Note 17). At least 0.55 ml of each dilution per 96-well plate is required. Cool on ice.

3. Use virus-coated plates prepared at the step 1 of Subheading 3.3.1. Remove blocking solution; wash twice with 0.2 ml of ice-cold washing buffer.

4. Fill the plate with 0.05 ml per well of either working solution of Fet-HRP or its mixtures with non-labeled receptor analogue (see Fig. 5, right panel).

5. Incubate for 1 h at 4°C, wash, and determine peroxidase activity in the wells; save the absorbency data (see steps 8–12 in Subheading 3.1.6).

3.3.2.1. Analysis of Results

1. Using Microsoft Excel or another spreadsheet program, calculate mean value of A_{450} in the negative control (no virus). Subtract this value from the absorbency values in virus-coated wells. Use background-corrected values of A_{450} in all subsequent calculations.

2. Calculate mean value of absorbency in virus-coated wells without inhibitor (A_0). Taking A_0 for 100%, calculate percentages of Fet-HRP binding (B) in the presence of the inhibitor: $B_i = 100 \times A_i / A_0$ (see Fig. 6b).

3. For each experimental point with B_i in the range from 20 to 80%, calculate value of association constant for the virus–inhibitor complex:

$$Kass = (100\text{-}B_i)/\alpha^* B_i^* C_i,$$

where B_i is the percent of Fet-HRP binding at inhibitor concentration C_i, α is a portion of free binding sites determined for this virus and working dilution of Fet-HRP (see Subheading 3.3.1).

4. Calculate mean value of K_{ass} and standard deviation using values of K_{ass} determined at different concentrations of the inhibitor. Higher values of K_{ass} reflect a stronger binding.

3.3.3. Binding Inhibition Assay: Determination of Binding Patterns

This is a variant of FBI, which was previously used to compare virus binding to a panel of 5–10 SGPs containing the same terminal Neu5Acα2-3Gal moiety but differing by the structure of penultimate sugar residue(s). The patterns of binding to such receptor analogues were found to differ between duck, gull, and poultry viruses indicating that these viruses have different receptor specificity (29–31, 34) (for an example, see Fig. 7).

The only difference from the assay variant described in Subheading 3.3.2 is that each virus is tested for binding inhibition
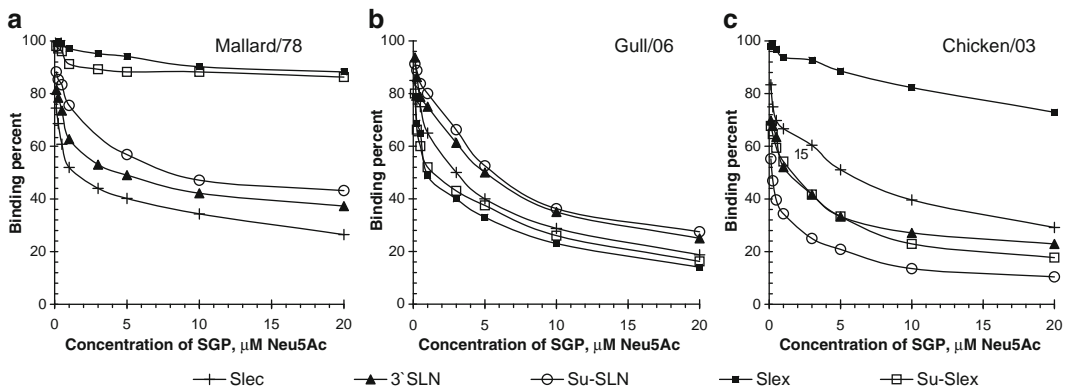
Fig. 7. Example of the binding inhibition assay: determination of binding patterns for viruses A/Mallard New York/670/78 (H4N6) (**a**), A/Gull/Moscow/3100/06 (H6N2) (**b**), and A/Chicken/Vietnam/NCVD-11/03 (H5N1) (**c**) (data from ref. 31). SGPs: SLe^c-PAA (*plus symbols*), 3′SLN-PAA (*filled triangles*), Su-3′SLN-PAA (*open circles*), SLe^x-PAA (*filled squares*), and Su-SLe^x-PAA (*open squares*).

by all SGPs on the same 96-well plate. This assay format serves the goal of focusing on the distinctions in binding of different analogues to a single virus, rather than on binding of the same analogue to different viruses (Subheading 3.3.2).

1. Determine working concentration of Fet-HRP exactly as described in Subheading 3.3.1.

2. Prepare virus-coated plates for FBI. For each virus, fill rows 2–11 of the fetuin-coated plate with 0.05 ml of chosen virus dilutions in TBS. Fill row 1 with 0.05 ml TBS (control of non-specific binding). One plate is sufficient to test two replicate titrations of five different SGPs. Fill additional plates for more replicates or more SGPs. Incubate, wash, and block virus-coated plates (see steps 2–4 in Subheading 3.1.7).

3. Prepare working solution of Fet-HRP in RS (6–7 ml per 96-well plate).

4. Prepare eight serial twofold dilutions of each SGP in the working solution of Fet-HRP starting from 0.02 mM (see Note 17). At least 0.15 ml of each dilution is required for two replicate titrations of one SGP with each virus. Cool on ice.

5. Use virus-coated plates prepared at the step 2. Remove blocking solution; wash twice with 0.2 ml of ice-cold washing buffer.

6. Fill rows 1 and 12 of the plate with 0.05 ml per well working solution of Fet-HRP without SGPs (controls of nonspecific binding and 100% binding (A_0), respectively). Fill rows 2–11 with dilutions of SGPs in working solution of Fet-HRP.

7. Incubate for 1 h at 4°C, wash, and determine peroxidase activity in the wells; save the absorbency data (see steps 8–12 in Subheading 3.1.6). Analyze the data and determine K_{ass} as described in Subheading 3.3.2.1 (see Note 18).

Figure 7 shows example of FBI with three different avian viruses and five SGPs. The assay reveals the following species-specific features of duck, gull, and highly pathogenic chicken viruses (31). The duck virus displays the highest binding to SLe^c and does not bind to fucosylated sialyloligosaccharides SLe^x and Su-SLe^x. The gull virus efficiently binds to fucosylated receptors SLe^x and Su-SLe^x. The H5N1 chicken virus binds strongly to sulfated sialyloligosaccharides Su-3′SLN and Su-SLe^x.

4. Notes

1. Egg-grown human and swine influenza viruses acquire adaptive mutations in the HA that alter their receptor-binding properties (25, 32, 37). To avoid egg-adaptation artifacts, these viruses must be isolated and propagated solely in cell culture (MDCK, CACO-2, VERO).

2. Non-inactivated viruses are preferable for the binding studies. If required, the viruses can be inactivated by beta-propiolactone (BPL), formaldehyde, or UV-treatment. For example, buffer the virus-containing suspension with 20 mM sodium bicarbonate to prevent change of the pH during hydrolysis of BPL, add one volume of BPL per 2,000 volumes of virus suspension and incubate for 3 days at 4°C. Limited studies suggest that inactivation does not affect receptor-binding characteristics of the viruses (12, 21); however, this conclusion may not be applicable to all viral strains.

3. Some preparations of commercial BSA may contain sialylglycoproteins that can bind to influenza viruses and interfere with the binding assay. To avoid potential interference, we routinely desialylate BSA using bacterial neuraminidase. Alternatively, BSA can be tested for the presence of inhibitors in the FBI assay; preparations of BSA lacking the inhibitors can be used without desialylation.

4. During the synthesis of HRP-labeled fetuin, use water double distilled in glass for all solutions. Water distilled in metal tanks and deionized water may inhibit the enzyme and the reaction intermediates (38).

5. Addition of NA inhibitors to the binding buffer prevents desialylation of non-labeled receptor analogues and Fet-HRP by the viral neuraminidase. The assays can be performed without adding NA inhibitors, but the sensitivity will be reduced.

6. Some preparations of fetuin may form gel during heating. It is recommended to test a new batch of fetuin by heating 0.05 ml aliquot of 5% fetuin solution in tightly closed 0.2-ml tube. If gel is formed, check the pH of the solution using pH indicator paper and raise pH by one to two units by adding 0.01 M NaOH.

7. Binding avidity of the preparations of resialylated 3/6-Fet-HRP may vary depending on aggregation state, efficiency of HRP labeling, and level of sialic acid incorporation. It is recommended to perform a preliminary analytical resialylation experiment using a range of concentrations of 2,3- and 2,6-sialyltransferases (from 10 to 100 mU/ml) and to choose the concentration of the enzymes that result in Fet-HRP preparations with high binding avidity with respect to avian (3-Fet-HRP) and human (6-Fet-HRP) viruses.

8. Evaporation of liquid from the wells during incubation can result in a high background and erroneous results. This effect is particularly critical for incubations with solutions containing HRP or biotinylated SGPs. To avoid errors, perform all incubations in a humid chamber, for example, in a closed plastic box lined with wet paper towel.

9. To prevent drying of the wells between each step, do not leave empty wells at room temperature and humidity for more than 10–15 s. If risk of drying exists during addition of the next reagent, perform this operation while keeping the plate on ice.

10. Good washing is critical at most assay steps, especially, after incubation with Fet-HRP or streptavidin-HRP. Inefficient washing will result in falsely elevated and variable absorbency readings.

11. Instead of overnight incubation at 4°C, 1–2 h incubation at room temperature can be used for virus adsorption. This may result in a slightly lower optimal dilution of the virus.

12. For adequate comparison of viral binding to two or more receptor analogues (3′SL-PAA-biot and 6′SLN-PAA-biot, 3-Fet-HRP and 6-Fet-HRP in direct binding assay; non-labeled analogues in FBI), it is essential that replicate plates are treated identically at all assay steps including washings and incubation with HRP substrate. However, the range of concentrations of receptor analogues used may differ.

13. Other dilution steps can also be used. For example, 1.5-fold dilutions can be useful to work within the optimal range of absorbency (from 0.2 to 2.5) and thus to increase the accuracy of the assay. Three- or fourfold dilutions can be used to cover a broader range of concentrations in preliminary experiments with new virus strains.

14. Indicated working dilution of S-HRP is approximate. Optimal dilution of S-HRP stocks must be determined experimentally.

15. Association constants determined in the direct binding assay are apparent constants and depend on the assay conditions. Some of these conditions, such as washing efficiency at different stages of the assay, are not easily reproduced. Therefore, it is not advisable to average absolute values of K_{ass} from the replicate experiments performed on different days.

16. Values of K_{ass} determined in the same experiment may vary depending on the amount of virus adsorbed in the plate wells. Therefore, absolute values of K_{ass} for different viruses can only be compared when corresponding Scatchard plots have similar values of A_{max} (example of an adequate comparison of three viruses is shown in Fig. 4). If values of A_{max} differ more than 1.5-fold, repeat the assay after adjusting virus dilutions during the adsorption step. To improve accuracy of the assay, one can determine values of K_{ass} at two to three different virus dilutions and average the data.

17. Indicated starting concentrations of receptor analogues are approximate and may vary depending on the strength of virus–inhibitor complex. Optimally, the starting concentration of the

inhibitor should be 4–6 times higher than the concentration required for 50% inhibition of binding.

18. Instead of calculating K_{ass}, one can determine concentration of 50% inhibition of Fet-HRP binding (IC_{50}) for each receptor analogue. This will not affect viral binding pattern (relative binding avidity of the same virus for different receptor analogues determined in the same experiment).

Acknowledgments

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The Chemiluminescent Neuraminidase Inhibition Assay: A Functional Method for Detection of Influenza Virus Resistance to the Neuraminidase Inhibitors

Margaret Okomo-Adhiambo, Aeron C. Hurt, and Larisa V. Gubareva

Abstract

Neuraminidase inhibitors (NAIs) represent a newer class of anti-influenza drugs. Widespread natural or acquired resistance to NAIs is a major public health concern as it limits pharmaceutical options available for managing seasonal and pandemic influenza virus infections. Molecular-based methods, such as pyrosequencing, sequencing, and PCR are rapid techniques for detecting known genetic markers of resistance, but they are unable to identify novel mutations that may confer resistance, or subtle differences in the susceptibility of viruses to the NAIs. This chapter describes the chemiluminescent neuraminidase (NA) inhibition (NI) assay, a functional method used for assessing influenza virus susceptibility to NAIs. The assay generates IC_{50} values (drug concentration needed to reduce the NA enzymatic activity by 50%) which are determined by curve-fitting analysis. Test viruses showing elevated IC_{50} values relative to those of NAI-sensitive reference viruses of the same antigenic type and subtype are further analyzed by pyrosequencing or conventional sequencing to identify known markers of NAI resistance or new changes in the NA. The criteria for NAI resistance are currently not well defined and tend to vary by laboratory and NI assay, therefore harmonization of NI assay conditions and interpretation of results across surveillance laboratories is necessary to improve the NAI susceptibility testing and analysis.

Key words: Neuraminidase inhibitor, Oseltamivir, Zanamivir, Chemiluminescence, Inhibition, Sialidase

1. Introduction

Antiviral drugs play an important role in the control of influenza virus infections. Prior to 1999, only adamantane derivatives (amantadine and rimantadine) were available for the prophylaxis and treatment of influenza infections. Adamantanes target the proton channel formed by the M2 protein of influenza A viruses and are not effective against influenza B viruses (1). The recent emergence and spread of adamantane resistant viruses has greatly diminished the usefulness of this class of drugs (2, 3). The neuraminidase

inhibitors (NAIs) are a newer class of drugs active against both influenza A and B viruses. Currently, inhaled zanamivir and oral oseltamivir are the only FDA-approved NAIs for use against type A and B influenza infections (4, 5). Peramivir, developed as an intravenous (IV) formulation, was prescribed in the USA under an emergency use authorization (EUA) during the 2009 H1N1 pandemic (6) and is now licensed in Japan and South Korea (7). In addition, R-125489 (8) developed as an inhaled prodrug laninamivir (CS-8958) is licensed in Japan.

NAIs mimic the natural substrate of NA, sialic acid (*N*-acetylneuraminic acid), and competitively bind to the conserved NA active site. They target the viral enzyme's key function of destroying the neuraminic acid-containing receptors. When influenza viruses are propagated in the presence of NAIs, progeny virions remain attached to the cell membrane and to each other, thus limiting the spread of infection to neighboring cells (4, 9). Prior to 2007, only low levels of resistance to NAIs were detected (10–12), although detailed studies of virus variants collected following oseltamivir treatment of young children revealed the presence of markers of resistance in 18% of the tested samples (13). Moreover, the recent analysis of the NA crystal structure revealed the differences in the structure of the N1 enzyme (14). Noteworthy, amino acid changes that prevent induced fit of the NAI in the N1 enzyme active site have a potential to cause resistance to oseltamivir and/or zanamivir. It is essential, therefore, to determine the NAI susceptibility of each new virus strain, especially those carrying the N1 subtype enzyme in the phenotypic (functional) assay, even if the amino acid sequence of the NA active site is unchanged compared to the NAI-susceptible viruses (15), because amino acid changes that lie outside the active site can affect the induced fit required for tight binding of NAI.

Since 2008, global surveillance of seasonal influenza isolates showed a marked increase and rapid spread of oseltamivir resistant seasonal influenza A(H1N1) viruses throughout both the Northern and Southern Hemispheres (16–21). The oseltamivir-resistant seasonal H1N1 viruses disappeared from circulation in 2010; however, there is a growing evidence of emerging oseltamivir resistance in the 2009 pandemic H1N1 viruses, which are already resistant to the adamantane class of drugs. It is, therefore, critical to conduct surveillance on NAI susceptibility of influenza viruses circulating in different parts of the world, especially those carrying the N1 enzyme (2009 pandemic H1N1, avian H5N1, and others).

Cell culture-based assays would be desirable for initial screening in antiviral susceptibility studies due to their ability to detect a broad range of resistant phenotypes (22). However, the interpretation of NAI susceptibility in cell culture-based assays is unreliable and complicated by several factors unique to NAIs (23). Alternatively, functional assays such as the NA inhibition (NI) assay

are appropriate for NAI susceptibility testing since they allow detection of drug resistant viruses with established (e.g., H275Y in N1 subtypes) or novel changes in the targeted enzyme. The most commonly used functional assays for assessing influenza virus susceptibility to NAIs are the chemiluminescent (17, 24–26) and fluorescent (27, 28) NI assays.

Both the chemiluminescent and fluorescent NI assays utilize small synthetic substrates (29–31) and are based on the ability of the NA to cleave off neuraminic acid moieties from natural or synthetic conjugates. The chemiluminescent NI assay utilizes a 1,2-dioxetane derivative of neuraminic acid as substrate (29), while the fluorescent assay uses methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA) (31). Both assays require the use of grown viruses; however, even in the absence of drug pressure, commonly used cell cultures such as the Madin–Darby canine kidney (MDCK) cells have been shown in some instances to provide a growth advantage to particular virus variants, including those with mutations in the NA and thus may alter virus susceptibility after propagation (32, 33). Initial titration of propagated virus samples is typically done to obtain a working virus dilution in the linear portion of the NA enzyme activity curve. The two assays utilize similar principles where test viruses are preincubated with the NAI followed by the addition of a substrate, then accelerator (chemiluminescent assay) or stop solution (fluorescent assay) followed by the reading of a signal. Analysis of the emitted luminescence or fluorescence signals generates IC_{50} values for test and reference viruses.

The chemiluminescent NI assay provides an advantage over the fluorescent assay when assessing drug susceptibility in samples with low virus load or limited volume (29) and has been shown to provide greater linearity of its signal and higher sensitivity in measuring NA activity compared to other substrates (24–27). Based on these factors as well as its reproducibility, ease of automation, and shorter assay time, the chemiluminescent NI assay was selected by Neuraminidase Inhibitor Susceptibility Network (NISN) for evaluation of NAI susceptibility of influenza virus isolates circulating globally (25), although the fluorescent assay was also utilized. However, when viral sample permits, the fluorescent NI assay is preferable for detecting resistance as it typically offers better discrimination between NAI-susceptible and -resistant viruses when compared with the chemiluminescent NI assay (28). The fluorescent assay is also less expensive than the chemiluminescent NI assay. Nevertheless, NAI-resistant mutants can accurately be detected by either NI assay; hence the decision on which method to use as a primary assay depends on the particular goals and needs of the surveillance laboratories, local conditions, and other factors. At times, an array of assays needs to be employed to characterize resistance caused by a novel mutation(s).

The reagents used in the chemiluminescent NI assay described in this chapter are available as a manufactured kit, the NA-Star® Influenza Neuraminidase Inhibitor Reagent Kit which provides a rapid and sensitive quantitation of influenza NA activity in a 96-well microplate format. The kit provides all necessary assay reagents (with exception of the NAIs and the reference virus strains) and plates, enabling improved assay standardization and more accurate comparison of results from one laboratory to another. The NAIs are not commercially available and need to be requested from the respective manufacturers, while the reference control virus strains can be obtained from influenza virus surveillance laboratories or ATCC. The kit also includes a protocol, provided by the manufacturer; however, there are minor differences between the manufacturer's protocol and that described in this chapter.

The IC_{50} values generated in the chemiluminescent NI assay provide valuable information for the detection of NAI resistant viruses, but IC_{50} values should not be used to draw a direct correlation with the drug concentrations needed to inhibit virus replication in the infected human host; as clinical data to support such inferences are limited. Assessment of NAI susceptibility of virus in the chemiluminescent NI assay, reinforced by NA sequencing in the virus isolate and its matching clinical specimen provides a reliable and reasonably comprehensive approach to the identification of NAI-resistant isolates for surveillance purposes.

2. Materials

2.1. Critical Equipment

1. 96-Well plate reader for luminescence with injectors [such as the Victor™ (PerkinElmer, Waltham, MA)].
2. Class II biological safety cabinet.
3. Freezer (−80°C) for long-term storage of virus isolates.
4. Refrigerator (4°C) for short-term storage of thawed virus isolates.
5. 37°C Incubator (cabinet) with thermometer.
6. Microcentrifuge (with rotor for 1.5–2 ml centrifuge tubes).
7. Vortex mixer.

2.2. Software

Software such as Robosage (in-house, GlaxoSmithKline) (25), JASPR (in-house, CDC) (34), or others such as Graphpad Prism and Sigmaplot (35) for curve fitting and calculation of the IC_{50} (drug concentration required to inhibit NA enzyme activity by 50%).

2.3. Viruses

1. Test virus isolates, propagated in cell culture (e.g., MDCK cells, ATCC, Manassas, VA) or in embryonated chicken eggs. Viruses are stored at −80°C.

2. Reference (control) virus strains, propagated in MDCK cells (ATCC, Manassas, VA) and stored at –80°C. Preferably, a pair of references including NAI-sensitive and NAI-resistant viruses.

Influenza viruses pose a biosafety hazard, therefore protocols described in this chapter must be performed in a containment laboratory according to institutional requirements.

2.4. Reagents

1. NAIs:
   - Zanamivir (GlaxoSmithKline, Uxbridge, UK).
   - Oseltamivir carboxylate, active metabolite of the prodrug oseltamivir (Hoffman-La Roche, Basel, Switzerland).

   NAIs are acquired from their respective manufacturers via Material Transfer Agreements.

2. NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, Foster City, CA) which includes:
   - NA-Star® Buffer (Part No. 4374345). Stored at 4°C.
   - NA-Star® Substrate (Part No. 4374347). Stored at 4°C.
   - NA-Star® Accelerator (Part No. 4374346). Stored at 4°C.
   - 96-Well NA-Star® Detection Microplates (Part No. 4374349).

2.5. Other Materials

1. 1.5-ml Eppendorf tubes (Fisher Scientific, Pittsburgh, PA).
2. Assorted pipettes and pipette tips (Rainin, Oakland, CA).
3. Reagent reservoirs (ISC BioExpress, Raleigh, NC).
4. 15- and 50-ml Falcon conical tubes (Fisher Scientific, Pittsburgh, PA).

3. Methods

3.1. Reagent Preparation

This protocol describes the preparation of working dilutions of NA-Star® Substrate and drugs (NAIs) for use in consecutive protocols. *Note*: the NA-Star® Buffer and NA-Star® Accelerator do not require further preparation.

1. Prepare a 1:1,000 working dilution of NA-Star® Substrate as follows:
   - Briefly spin the tube containing undiluted NA-Star® Substrate in a microcentrifuge to settle the contents.
   - To make 20 ml of diluted substrate, for example, add 20 μl NA-Star® Substrate to 19.98 ml NA-Star® Buffer.

Dilute appropriate amounts of substrate depending on the number of viruses to be tested (see Note 1).

- Mix by vortexing.

2. Prepare 10 ml of 20 mM stock solutions of zanamivir and oseltamivir as follows:

- Weigh 66.5 mg of zanamivir (MW: 332.31) or 70.66 mg GR121167X (MW: 350.331); 56.9 mg of oseltamivir carboxylate (MW: 284.35) or 77.3 mg of D-tartrate salt of oseltamivir carboxylate (MW: 386.44) (see Note 2).

- Transfer weighed NAI powder into a 15-ml conical tube, labeled appropriately.

- Add 10 ml of molecular grade distilled water to each tube containing respective NAIs.

- Agitate each tube until the NAIs are dissolved. This will be the initial stock solution of 20 mM for each NAI.

- Filter solution through 0.22-μm filter.

- Aliquot 20 mM NAI solution in 1 ml volumes and store at –20°C.

3. Prepare a 50 μM working solution of each NAI by diluting the 20 mM stock solution of NAI as follows.

- Add 25 μl of the 20 mM NAI stock solution into 9.975 ml of molecular grade distilled water.

- Mix by vortexing.

- Aliquot the 50 μM stock solution in 1 ml volumes.

- Store at –20°C.

4. Preparation of 2× half-log$_{10}$ dilutions of the NAIs as follows:

- Serially dilute the 50 μM working concentrations of zanamivir and oseltamivir carboxylate in NA-Star® Buffer as shown in Table 1. If higher (or lower) volumes of the inhibitors are prepared, the respective volumes of buffer and NAI shown in Table 1 can be scaled up (or down) by a common factor.

- Transfer the NAI dilutions to columns 1–10 of a 12-column reagent reservoir. Add NA-Star® Buffer to columns 11 and 12. Seal the reservoir with an adhesive plate seal.

- The diluted NAIs can be stored at 4°C for up to 1 month.

3.2. Determining Background Activity for the Plate Reader

The background activity of reagents used in a particular luminometer (plate reader) and 96-well plate should be determined prior to testing viruses in the chemiluminescent NI assay. Background activity needs to be determined only once for each plate reader/96-well plate combination and the value determined can be used for all consecutive runs using the respective plate reader/96-well

Table 1
Preparation of 2× half-log$_{10}$ dilutions of NAI

Dilution no.	Buffer volume (ml)	NAI volume	Resulting NAI concentration (nM)	Final NAI concentration (nM)[a]
1	21.6	900 μl of 50 μM NAI	2,000	1,000
2	16.2	7.5 ml from dilution 1	633	316
3	16.2	7.5 ml from dilution 2	200	100
4	16.2	7.5 ml from dilution 3	63.4	31.7
5	16.2	7.5 ml from dilution 4	20	10.0
6	16.2	7.5 ml from dilution 5	6.3	3.18
7	16.2	7.5 ml from dilution 6	2	1.01
8	16.2	7.5 ml from dilution 7	0.64	0.32
9	16.2	7.5 ml from dilution 8	0.20	0.10
10	16.2	7.5 ml from dilution 9	0.06	0.03

[a]The final concentration of NAI in the assay reaction volume, which accounts for the twofold dilution (25 μl of the virus dilution combined with 25 μl of NAI). The final concentration *does not* account for 10 μl of NA-Star substrate and 60 μl of NA-Star accelerator. However, some laboratories do account for the 10 μl of NA-Star substrate when calculating the final concentration of NAI in the reaction mix

plate combination. This will allow calculation of the approximate NA activity required to be within the linear range for the chemiluminescent NI assay.

1. Prepare to determine the background activity for a particular plate reader and 96-well plate as follows:
    - Remove NA-Star® Buffer and NA-Star® Accelerator from 4°C storage and allow reagents to reach room temperature.
    - Prepare 1:1,000 dilution of NA-Star® Substrate (see Subheading 3.1, step 1).

2. Add 50 μl NA-Star® Buffer to all wells of the 96-well plate.

3. Using a 200 μl 8-channel pipette or electronic repeater pipette, add 10 μl of the 1:1,000 dilution of NA-Star® Substrate to each of the 12 columns.
    - Since all wells are identical and only contain NA-Star® Buffer, the same tips can be used to dispense the substrate. Ensure that the pipette tips are in the bottom of each well and monitor the volume in the pipette tip to guarantee correct dispensation of the substrate. After the addition of the substrate, tap the plate gently on each side to mix the buffer and substrate.

4. Incubate the 96-well plate containing NA-Star® Buffer and NA-Star® Substrate for 30 min at room temperature.

5. Set up the plate reader in accordance with the manufacturer's instructions.

6. Place the 96-well plate onto the plate reader and measure luminescence.

7. Average the luminescence values determined for each of the 96 wells of the plate. This is the background activity of NA-Star® Buffer and NA-Star® Substrate in that type of 96-well plate. Multiply this value by 60. This is the target value for NA activity. Selecting a virus dilution in the NA activity portion of the assay which corresponds to this target value will provide a signal-to-background ratio of approximately 30:1 (NA activity: background). The linear range of NA activity for the chemiluminescent NI assay is between 10:1 and 40:1.

3.3. Neuraminidase Activity Assay for Determining Virus Dilution

Measuring the neuraminidase (NA) activity of each virus prior to performing the inhibition portion of the assay (see Subheading 3.5 and Note 3) improves the reproducibility of IC_{50} data and allows the user to select a working virus dilution that provides NA activity within the linear range for the NI assay. Too low or too high level of NA activity may skew IC_{50} values.

1. Prepare to perform NA activity for a total of eight viruses on a single 96-well plate as follows:
   - Remove test and reference viruses from –80°C storage and allow to thaw at room temperature in a class II biological safety cabinet.
   - Remove NA-Star® Buffer and NA-Star® Accelerator from 4°C storage and place at room temperature.
   - Prepare 1:1,000 dilution of NA-Star® Substrate in NA-Star® Buffer for the appropriate number of plates to be tested for NA activity and NI assays (see Subheading 3.1, step 1).
   - Set up the plate reader in accordance with the manufacturer's instructions.

2. Perform a series of twofold dilutions of test and reference viruses in NA-Star® Buffer (see Fig. 1), within a class II biological safety cabinet, as follows:
   - In a 96-well white plate, add 80 μl NA-Star® Buffer to all wells in column 1.
   - Add 50 μl NA-Star® Buffer to the remaining wells of the plate (columns 2–12). Column 12 will be a blank control.
   - Prepare an initial 1:5 dilution of the batch of eight viruses by adding 20 μl of the virus to 80 μl NA-Star® Buffer
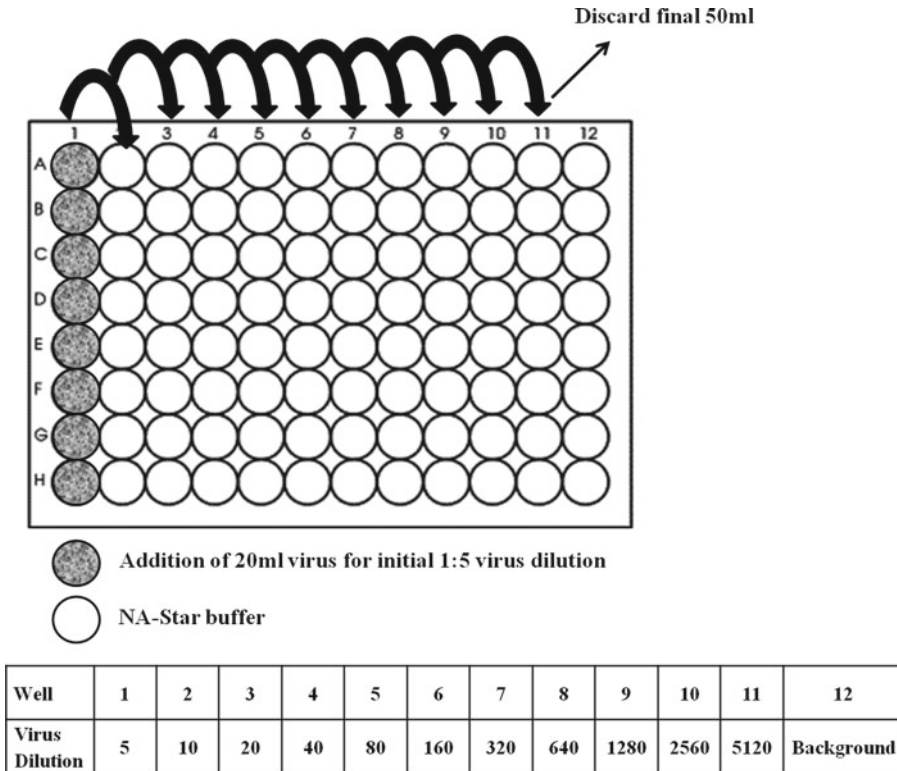
Fig. 1. Twofold serial dilution of test and reference viruses (8 viruses per plate).

in the well in column 1, rows A to H, using one row per virus.

- Using an 8-channel 200-μl multichannel pipette set at 50 μl volume, perform serial twofold dilutions from columns 1 to 11. Pipette the virus suspension in column 1 up and down (three times) and transfer 50 μl from the wells in column 1 to the wells in column 2. Repeat this for columns 2–11 and discard the last 50 μl from column 11. Column 12 should only contain 50 μl of NA-Star® Buffer as a blank control (background).

3. Using an 8-channel 200-μl pipette, add 10 μl of NA-Star® Substrate (diluted 1:1,000) to each well, starting from column 12 toward column 1 (see Note 4).

- If using an 8-channel 200-μl electronic repeater pipette, aspirate 120 μl of the NA-Star® Substrate into each pipette tip (enough to dispense 12 times). Visually inspect the pipette tips to ensure that they hold equal the volume of the substrate. Dispense 10 μl of NA-Star® Substrate to each column, starting from column 12 toward column 1.

> Ensure that the pipette tips touch the bottom of each well to guarantee correct dispensation of the substrate.
>
> - After the addition of the NA-Star® Substrate, tap the plate gently on each side to mix the virus and substrate.

4. Incubate the plates containing virus and substrate at room temperature (~20–22°C) for 30 min.

5. Set up the plate reader in accordance with the manufacturer's instructions.

6. Following 30 min incubation, place the 96-well plate on the plate reader and measure NA activity (see Note 5).

7. Determine the well with the NA activity (light signal) which corresponds to the target level of NA activity (as determined in Subheading 3.2) for the plate reader/96-well plate combination being used. The virus dilution which is equal to the target signal should be selected for the NI assay.

   - For example: If the background activity is 250 relative luminescence units (RLU), 60 times this background is 15,000 RLU, therefore the virus dilution with the NA activity closest to 15,000 RLU should be selected for the NI assay.

3.4. Diluting Viruses

Viruses to be tested in the chemiluminescent NI assay are appropriately diluted prior to the assay, to allow NA activity to be measured within the linear range for the assay.

Perform virus dilution in a class II biological safety cabinet as follows:

1. Using the dilution factor determined in the NA activity assay, dilute each virus to be tested in NA-Star® Buffer, in 1.5 ml centrifuge tubes as shown in Table 2. Prepare a volume of 400 µl of each diluted virus for each NAI to be tested.

2. Vortex centrifuge tubes containing diluted virus suspension for about 5 s and briefly spin down in microcentrifuge.

3. Transfer each diluted virus from centrifuge tubes to corresponding section of a 12-channel V-bottom dilution reservoir.

4. Only add eight viruses to each of the 12-channel reservoirs as only eight viruses are assayed per 96-well plate. *Note*: If testing is done in duplicate, only four viruses could be tested per plate.

5. Table 2 provides a summary of volumes of virus and buffer needed to prepare dilutions similar to those made for the NA activity assay (see Notes 6–8).

3.5. Chemiluminescent Neuraminidase Inhibition Assay

A total of eight viruses may be assayed on a single 96-well white plate, one virus per row. Inclusion of NAI-sensitive and NAI-resistant reference viruses is recommended (see Subheading 2.3, step 2).

Table 2
Virus dilutions for the NI assay

Virus dilution	One NAI		Two NAIs		Three NAIs	
	Virus (μl)	Buffer (μl)	Virus (μl)	Buffer (μl)	Virus (μl)	Buffer (μl)
1:5	80	320	160	640	240	960
1:10	40	360	80	720	120	1,080
1:20	20	380	40	760	60	1,140
1:40	10	390	20	780	30	1,170
1:80	5	395	10	790	15	1,185
1:160	3	397	5	795	8	1,192
1:320	1	399	3	797	3	1,197
1:640	[a]	[a]	1	799	2	1,198

[a] When making virus dilutions, it is not advisable to make dilutions greater than 1:600 or pipette volumes less than 2 μl into 1 ml (or greater volume) due to possible pipetting error. If a dilution greater than 1:600 is needed, then a two-step dilution should be performed. For example, if a 1:1,000 dilution is required, first make a 1:100 dilution followed by a 1:10 dilution of the initial 1:100 dilution

It is good laboratory practice to run the NI assay in duplicate, to reduce testing errors. The settings of curve-fitting software such as JASPR require that NI assay testing of different NAIs be conducted on separate 96-well plates.

1. Aliquot the respective diluted NAIs (see Subheading 3.1, steps 2–4) and NA-Star® Buffer into 96-well plates as follows:

   • To the wells in column 12, add 50 μl NA-Star® Buffer as a blank (background) control.

   • To the wells in column 11, add 25 μl of NA-Star® Buffer. This is a control for uninhibited (100%) NA activity. The activity of this well also will be used to determine the signal to background (S/B) for each virus tested in the NI assay. This helps ensure that the appropriate amount of virus was used and that the NA activity is within linear range.

   • To the wells of columns 1–10, add 25 μl of the corresponding drug dilution. Add the drug starting with the lowest concentration in column 10 through to the highest concentration in column 1. The same tips can be used if adding from lowest to highest drug concentrations.

2. Add diluted viruses to the plates containing NAI as follows:

   • Using an 8-channel pipette, add 25 μl of the diluted viruses to appropriate rows of the 96-well plates containing NAIs,

starting at column 11 toward column 1 (from lowest to highest NAI dilution). Change tips between each addition of virus dilution. Following addition of the virus dilutions, tap the plates gently on each side to mix virus and NAIs.

- If using an 8-channel 200 µl electronic repeater pipette, set it to dispense 25 µl 6 times. Aspirate the diluted virus and visually inspect the volume in the pipette tips. Dispense 25 µl of the diluted viruses to *columns 11 to column 6*, visually inspect tips to ensure that remaining volume is equal for each tip. Change tips, reset the pipetter and aspirate another 150 µl (6×25 µl) of virus diluted and visually inspect the volume in the pipette tips. Dispense 25 µl of the diluted viruses from *columns 5 to column 1* as described above, then discard tips, including the excess volume of virus dilution. It is not critical that pipette tips touch drug solution in the wells while using a repeater pipette when adding diluted virus starting from low-drug concentration. It is better to use technique with tips touching to the side, bottom, or corner of the well while dispensing diluted virus rather than dispensing into the air.

- Incubate the plates at 37°C for 30 min.

3. Add substrate to the wells containing virus and NAI as follows:

- Using an 8-channel pipette add 10 µl of NA-Star® Substrate (diluted 1:1,000) to the bottom of each well, starting at column 12 toward column 1 (from lowest to highest NAI dilution). Change tips between each addition of substrate.

- If using an 8-channel 200-µl electronic repeater pipette, set it to dispense 10 µl 12 times, then aspirate 120 µl of the substrate. Visually inspect the volume in the pipette tips. Dispense 10 µl of NA-Star substrate to each column, starting from column 12 toward column 1. Ensure that the pipette tips are in the bottom of each well to guarantee correct dispensation of the substrate.

- After the addition of the NA-Star® Substrate, tap the plate gently on each side to mix the virus and NA-Star® Substrate.

4. Incubate the plates at room temperature for 30 min.

5. Set up the plate reader in accordance with the manufacturer's instructions.

6. Following the 30 min incubation, place the 96-well plate containing substrate, virus, and NAI on the plate reader and measure NA activity.

3.6. Data Analysis   Susceptibility of the tested virus isolates to NAIs is assessed based on the IC_{50} value, the drug concentration required to inhibit NA

enzyme activity by 50% which is determined through curve fitting by plotting raw data (RLUs) against drug concentration (nM) (see Note 9).

1. The curve-fitting software, Robosage (in-house, GlaxoSmithKline) generates IC_{50} values using the equation: $y = V_{max}(1 - (x/(K+x)))$, where V_{max} is the maximum rate of metabolism, x is the inhibitor concentration, y is the response being inhibited, and K is the IC_{50} for the inhibition curve (i.e., $y = 50\%$ V_{max} when $x = K$).

2. The JASPR software (in-house, CDC), can also be used for curve fitting and IC_{50} calculation purposes. Curve fitting in JASPR is done using the equation: $V = V_{max}(1 - ([I]/(K_i + [I])))$, where V_{max} is the maximum rate of metabolism, $[I]$ is the inhibitor concentration, V is the response being inhibited, and K_i is the IC_{50} for the inhibition curve.

3. The JASPR and Robosage software compute comparative IC_{50} values (see Fig. 2), with JASPR providing a faster and higher throughput method of IC_{50} determination and a more user-friendly format. Attention must be paid to IC_{50} values and curve shapes generated by both these software; the inhibition curves should always be visually inspected to ensure that all data points are on or close to the curve.

4. Notes

1. When diluting the NA-Star® Substrate (see Subheading 3.1, step 1), take note that each 96-well plate used in the NA activity assay (see Subheading 3.3) and chemiluminescent NI assay (see Subheading 3.5) requires approximately 1.2 ml of NA-Star® Substrate; therefore, appropriate amounts of substrate should be diluted, depending on the number of viruses to be tested. The final concentration of NA-Star® Substrate used in the assay is 1.67 μM. A fresh working dilution of substrate should be prepared daily.

2. When reconstituting NAIs (see Subheading 3.1, step 2), always check the molecular weight (MW) of NAI compounds to ensure that appropriate drug weights and water volumes are used in reconstitution. A reliable molarity calculator should be used to determine appropriate volumes, such as the Tocris Molarity Calculator (36). Additionally, NAIs are also available as prodrug form. Oseltamivir carboxylate is the active compound of the ethyl ester prodrug oseltamivir phosphate (Tamiflu, MW 410.4), therefore, oseltamivir carboxylate, and not oseltamivir phosphate, should be used in the NI assay.
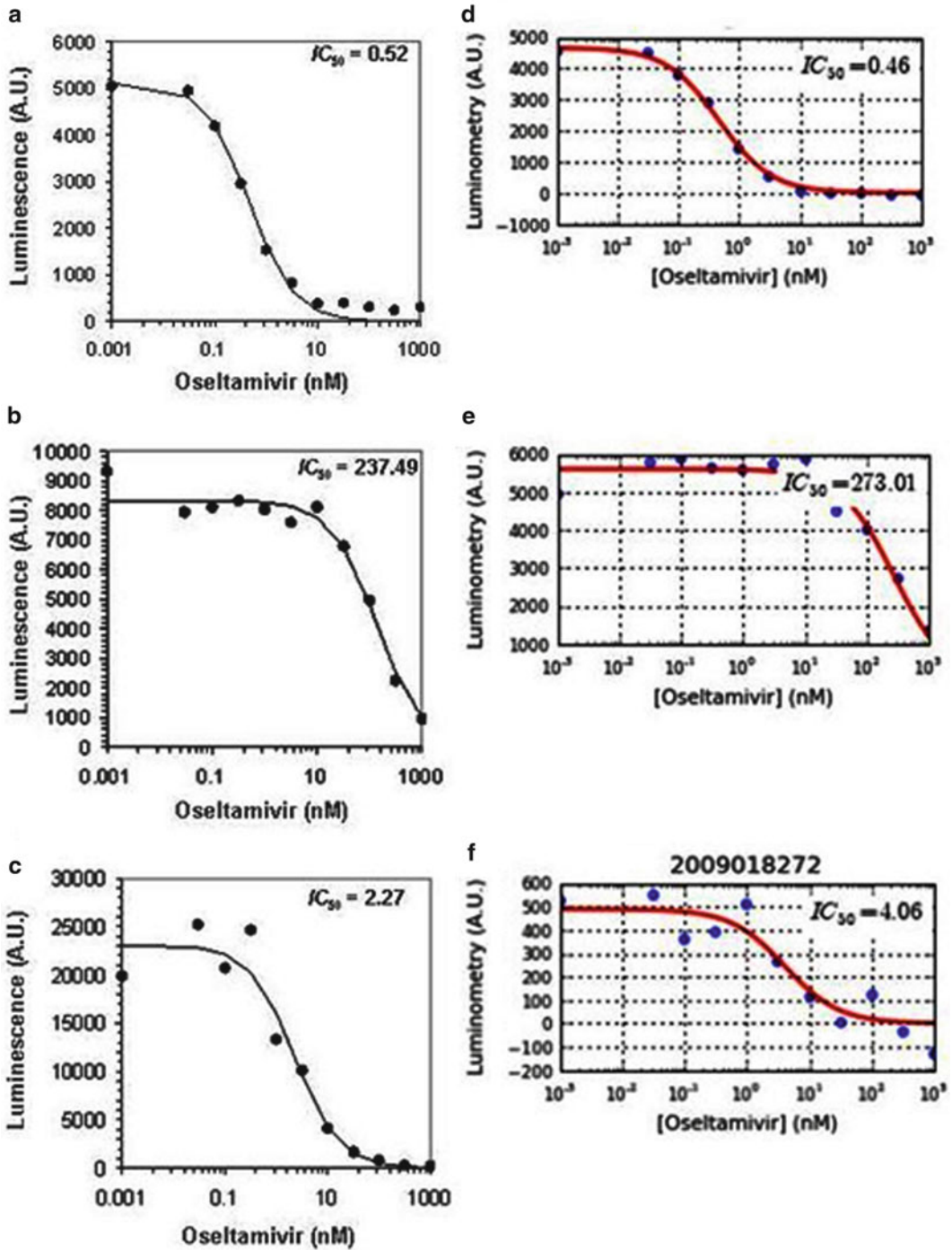
Fig. 2. Chemiluminescent NA inhibition assay: NA activity inhibition curves and IC$_{50}$ values for oseltamivir generated with the use of either Robosage (**a**–**c**) or JASPR (**d**–**f**) softwares: an oseltamivir sensitive A/H1N1 virus (**a** and **d**), an oseltamivir-resistant virus with the H275Y mutation in the NA (**b** and **e**) and examples of curves with scattered points may lead to inaccurate IC$_{50}$ values (**c** and **f**).

3. Repeated freeze/thaw of test and reference viruses or extended time at 4°C may alter NA activity for particular viruses, thus the NA activity (see Subheading 3.3) and chemiluminescent NI assays (see Subheading 3.5) should be conducted on the same day.

4. If testing multiple plates (each with eight viruses) in the NA activity (see Subheading 3.3) and chemiluminescent NI assays (see Subheading 3.5), the addition of NA-Star® Substrate needs to be staggered to account for the time needed to dispense NA-Star® Accelerator and read each plate. For example, with the Victor ³V plate reader, it takes approximately 7 min to dispense the accelerator and to read each plate; therefore, the addition of NA-Star® Substrate to each plate should be staggered by approximately 7 min.

5. Chemiluminescence is the *emission* of *light* as a result of a chemical reaction that does not involve a change in temperature. NA activity is measured as light emission, which is triggered by the addition of 60 μl of NA-Star® Accelerator into each reaction well. The light emission from each well is recorded 0.1 s after the addition of the NA-Star® Accelerator.

6. When diluting viruses (see Subheading 3.4), if phenol red is present in virus growth media, the lowest dilution of virus that should be used is 1:5 so that the quenching effect caused by the presence of phenol red is minimized.

7. Some laboratories use a dilution calculator spreadsheet for accurately determining virus dilutions (see Subheading 3.4) following NA activity assessment.

8. When diluting viruses (see Subheading 3.4), if viruses are to be tested with more than one NAI, multiply 400 μl (volume required for 1 NAI) by the number of NAIs to be tested. For example, if testing two NAIs (zanamivir and oseltamivir) carboxylate, prepare 800 μl of the working dilution for each virus to be tested. If testing three NAIs (zanamivir, oseltamivir carboxylate, and peramivir), prepare 1,200 μl of the working dilution for each virus to be tested.

9. The critical parameters measured in the chemiluminescent NI assay (see Subheading 3.6) for each test virus include an IC_{50} value (drug concentration that reduces NA enzymatic activity by 50%), a fold difference in IC_{50} (IC_{50} test virus/IC_{50} sensitive reference virus), and a signal-to-background (S/B) ratio. A significantly elevated IC_{50} value in the test virus compared to the reference virus is an important indicator of potential NAI resistance.

   The IC_{50} values provide valuable information for detecting resistant viruses as well as for comparing potencies of different NAIs. However, elevated IC_{50} values alone are not sufficient criteria for defining NAI resistance and should be combined with the detection of known (e.g., H275Y) or novel molecular

markers of resistance in the NA genes by conventional sequencing (17), pyrosequencing (37, 38), or other genotypic assays.

IC_{50} values may be affected by assay conditions (substrate concentration, buffer, etc.) and they typically vary by influenza types/subtypes for the different NAIs. Therefore, it is essential to establish baseline IC_{50} values for a particular NAI, NI assay, as well as NA type and subtype. This allows detection of viruses with slightly reduced susceptibility to NAIs (mild outliers). Statistical analysis is typically used to identify such mild outliers, whereas extreme outliers can be easily detected due to a great increase in IC_{50} values compared with an NAI-sensitive control (17, 25, 34). It is essential to confirm, when possible, that the NA change(s) responsible for the elevated IC_{50} values of the virus isolate is also present in the matching clinical specimen.

10. Certain problems may arise when performing the chemiluminescent NI inhibition assay, such as a lack of or weak NA activity (signal), which may be caused by a variety of factors including (1) using the wrong virus dilution and thereby adding too little virus to the assay, (2) adding too little NA-Star® Substrate to the assay, (3) using reagents that are too cold for the chemistry to be accurately executed, (4) using insufficient incubation times, or (5) not adding NA-Star® Accelerator to the assay. These problems are mainly technical and can be solved by identifying and correcting the error that the operator may have made while performing the assay. This would include rechecking NA activity data and recalculating virus dilutions, repeating NA activity determination if necessary, repeating the NI assay with correct addition of substrate to the bottom of each well, ensuring that all reagents used are at room temperature, correct incubation times are adhered to and enough volume of accelerator is present in the plate reader. A weak signal may also be the result of too low NA content in the virus preparation used in the NI assay, which can be corrected by regrowing the virus isolate and retesting it.

Conversely, an abnormally high signal may also be encountered as a result of using the wrong virus dilution or adding too much NA-Star® Substrate to the assay. This can be remedied by rechecking NA activity data and recalculating virus dilutions, repeating NA activity if necessary or repeating the NI assay with the addition of correct volume of substrate to the bottom of each well.

An abnormally high IC_{50} value generated in the chemiluminescent NI assay may result from factors such as (1) adding too little virus to the assay, (2) incorrect concentrations of NAI within the drug dilutions, (3) testing virus samples that contain a mixed infection with another pathogen that has NA activity (e.g., parainfluenza viruses). An abnormally high IC_{50}

values for influenza A viruses could also be encountered when samples contain a mix of influenza A and influenza B viruses. These problems may be solved by repeating the NI assay using sufficient amounts of virus, checking the possible mix of influenza A and B in virus samples by real-time PCR analysis, repeating the assay using correctly prepared drug dilutions and performing real-time PCR analysis with primers specific for the detection of parainfluenza viruses, respectively.

Acknowledgments

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The Fluorescence Neuraminidase Inhibition Assay: A Functional Method for Detection of Influenza Virus Resistance to the Neuraminidase Inhibitors

Aeron C. Hurt, Margaret Okomo-Adhiambo, and Larisa V. Gubareva

Abstract

Neuraminidase inhibitors (NAIs) are presently the only effective antiviral drugs for treatment and chemoprophylaxis of influenza A and B infections, due to the high prevalence of resistance to the adamantane class of drugs among influenza A(H3N2) and A(H1N1) viruses, including the 2009 pandemic H1N1 strain. The limited pharmaceutical options currently available for control of influenza infections underscore the critical need for surveillance on NAI susceptibility of influenza viruses circulating globally. This chapter describes the fluorescent neuraminidase (NA) inhibition (NI) assay, a functional method used for assessing influenza virus susceptibility to NAIs. The IC_{50} (drug concentration needed to reduce the NA enzymatic activity by 50%) values generated in this assay are used to evaluate the NAI-susceptibility of test viruses relative to those of sensitive reference viruses of the same antigenic type and subtype. Test viruses with significantly elevated IC_{50}s are further analyzed by pyrosequencing or conventional sequencing to identify known markers of NAI resistance or novel changes in the NA. The harmonization of NI assay conditions and interpretation of results across surveillance laboratories is necessary to improve NAI susceptibility testing and analysis.

Key words: Neuraminidase inhibitor, Oseltamivir, Zanamivir, Fluorescence, Inhibition

1. Introduction

Antiviral drugs are essential in the management of infections caused by seasonal and pandemic influenza viruses. Adamantanes (M2 ion channel blockers) and neuraminidase inhibitors (NAIs) are classes of drugs licensed for prevention or treatment of influenza virus infections (1). However, adamantanes are not effective against influenza B infections (2), and widespread resistance to this class of drugs among influenza A(H3N2) and A(H1N1) viruses has compromised their effectiveness (3, 4).

Two NAIs, oseltamivir and zanamivir, licensed in many countries worldwide, are antiviral drugs effective against influenza A and B infections, including the recently emerged 2009 pandemic H1N1 (5). An investigational NAI, peramivir, in intravenous (IV) formulation was prescribed in the USA under an emergency use authorization (EUA) during the 2009 H1N1 pandemic (6), and is now licensed in Japan and S. Korea (7). Another neuraminidase inhibitor, R-125489 (8), developed by Daiichi Sankyo and Biota as an inhaled long-acting prodrug, laninamivir (CS-8958) was approved in Japan.

NAIs competitively bind to the highly conserved NA active site which comprises of catalytic and framework residues (9). Mutations at this site arise from single amino acid changes (10) which confer resistance to NAIs in a drug- and virus type/NA subtype-specific manner. Resistance to NAIs among circulating influenza viruses was low (<1% worldwide) (11–13), but the 2007–2008 and 2008–2009 influenza seasons were marked by an emergence of oseltamivir-resistant seasonal influenza A(H1N1) viruses with the H275Y mutation in the NA (13–18), emphasizing the critical need for sustained NAI susceptibility surveillance of influenza viruses circulating globally.

Assessment of NAI susceptibility is primarily performed in functional neuraminidase inhibition (NI) assays that allow detection of drug-resistant viruses with established mutations (e.g., H275Y in N1 subtypes) and/or novel mutations. The most commonly used NI assays, the chemiluminescent (11, 13, 19, 20) and fluorescent (21, 22), both utilize small synthetic substrates, namely, a 1,2-dioxetane derivative of neuraminic acid (23) and methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA) (24), respectively. Both assays also require the use of grown viruses; however, commonly used cell cultures such as the Madin–Darby canine kidney (MDCK) cells have been shown, in some instances, to provide a growth advantage to particular virus variants, including those with mutations in the NA and thus may alter virus susceptibility after propagation (25, 26). When compared to the chemiluminescent NI assay, the fluorescent NI assay requires samples with high virus load or volume, but is less costly and typically offers better discrimination between NAI-susceptible and -resistant viruses (22, 23). The introduction presented in Chapter 6 provides additional background information on these two most commonly utilized neuraminidase inhibition assays.

The fluorescent NI assay discussed in this chapter describes procedures used by the WHO Collaborating Centre for Reference and Research on Influenza in Melbourne, Australia, and requires laboratory preparation of buffers and other necessary assay reagents. The fluorescent NI assay was recently developed as a commercial kit, the NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems, Foster City, CA) that provides ready for use assay reagents, enabling improved assay standardization and comparison

of results from one laboratory to another. The WHO Center for Surveillance, Epidemiology, and Control of Influenza in Atlanta, Georgia, USA, primarily utilizes this commercial kit, with a few modifications, for assessing influenza virus antiviral susceptibility to NAIs.

2. Materials

2.1. Critical Equipment

1. 96-well plate reader for fluorescence with an excitation wavelength of 360 nm and slit width of 2.5 nm and an emission wavelength of 448 nm and slit width of 20 nm (e.g., Thermo Ascent FL fluorometer).
2. Class II biological safety cabinet.
3. Freezer (–80°C) for long-term storage of virus isolates.
4. Refrigerator (4°C) for short-term storage of thawed virus isolates.
5. 37°C incubator (cabinet) with thermometer.
6. Microcentrifuge (with rotor for 1.5–2-mL centrifuge tubes).
7. Vortex mixer.

2.2. Software

Software such as Robosage (in-house, GlaxoSmithKline) (20), JASPR (in-house, CDC) (5) or others such as Graphpad Prism and Sigmaplot (27) for curve fitting and calculation of the IC_{50} (drug concentration required to inhibit NA enzyme activity by 50%).

2.3. Viruses

1. Test virus isolates, propagated in cell culture (e.g., MDCK cells, ATCC, Manassas, VA) or in embryonated chicken eggs. Viruses are stored at –80°C.
2. Reference (control) virus strains, propagated in MDCK cells (ATCC, Manassas, VA) and stored at –80°C. Preferably, a pair of references including NAI-sensitive and NAI-resistant viruses.

Influenza viruses pose a biosafety hazard, therefore protocols described in this chapter must be performed in a containment laboratory according to institutional requirements.

2.4. Reagents

1. Neuraminidase inhibitors (NAIs):
   (a) Zanamivir (GlaxoSmithKline, Uxbridge, UK).
   (b) Oseltamivir carboxylate, active metabolite of the prodrug oseltamivir (Hoffman-La Roche, Basel, Switzerland)
      NAIs are acquired from their respective manufacturers via Material Transfer Agreements.
2. 2-(4-Methylumbelliferyl)-a-D-*N*-acetylneuraminic   acid (MUNANA) (Sigma Cat. No. M8639).

3. 2-[*N*-morpholino]ethanesulphonic acid (MES) (free acid) (Sigma Cat. No. M8250).

4. 1 M Calcium Chloride (CaCl$_2$).

5. Absolute Ethanol (APS AJAX Fineham Cat. No. 214-2.5L GL).

6. Surfactant-Amps-NP-40 (10% solution) (Pierce Cat. No. 28324).

7. 0.824 M Sodium Hydroxide (NaOH(aq)).

2.5. Other Materials

- Nunc 96-well Clear MaxiSorp plates, polystyrene, untreated (Cat. No. 456529).

- Sarstedt 96-well "U"-bottom microtest plates, polystyrene (Cat. No. 82 9923 154).

- Corning Tube filter, pore size 0.22 μm, cellulose acetate (Cat. No. 430320).

- Beckman Coulter 96-well reservoir (Cat. No. 609681).

- Assorted pipettes and pipette tips (Rainin, Oakland, CA).

- Reagent reservoirs (ISC BioExpress, Raleigh, NC).

- 15- and 50-ml Falcon conical tubes (Fisher Scientific, Pittsburgh, PA).

3. Methods

3.1. Reagent Preparation

This protocol describes the preparation of the assay buffer, substrate, and drugs (NAIs) for use in consecutive protocols.

1. Preparation of 1× and 2× strength Assay Buffer:

  (a) To prepare 2× Assay Buffer (2× AB):

   - Measure out the following reagents:
     – 13 g MES.
     – 8 mL 1 M CaCl$_2$.
     – 992 mL Distilled Water.
   - Mix components to homogeneity and then add 10 M sodium hydroxide to adjust to a pH of 6.5.
   - Then filter the buffer using a sterile cellulose acetate filter of pore size 0.2 μm.

  (b) To prepare 1× Assay Buffer (1× AB), make a 1:1 v/v dilution of 2× AB in distilled water.

  (c) 1× and 2× AB can be stored at room temperature for a period of 2 years.

2. Preparation of NA inhibitors:

Prepare NI drugs at concentrations of 0.03, 0.3, 3, 30, 300, 3,000, and 30,000 nM in 2× AB. These concentrations correspond to 0.01, 0.1, 1, 10, 100, 1,000, and 10,000 nM in the final assay volume (see Notes 1 and 2).

(a) Zanamivir:

Make 300 μM solution of compound zanamivir (MW = 332.32) by adding 5 mg to 50.13 ml of 2× AB. From this stock solution make serial 1:10 dilutions to achieve the concentrations stated above.

(b) Oseltamivir carboxylate (D-tartrate salt):

Make 300 μM solution of compound oseltamivir phosphate (MW = 386.44) by adding 5.81 mg to 50 ml of 2× AB. From this stock solution make serial 1:10 dilutions to achieve the concentrations stated above.

(c) Store all inhibitor dilutions at 2–8°C.

(d) Stock solutions must be replaced after 12 months and dilutions prepared from the stock after 6 months.

3. Preparation of MUNANA substrate stock and working solution.

(a) Prepare 2.5 mM MUNANA stock by addition of 20 mL distilled water to 25 mg MUNANA and store in 800 μl aliquots at –20°C.

(b) Working strength substrate (0.3 mM) is prepared by addition of 720 μl 2.5 mM MUNANA to 5.28 mL 1× AB which is sufficient volume for one plate.

(c) Keep working strength MUNANA on ice unless used immediately, any leftover material should be discarded.

4. Preparation of Stop Solution.

(a) Prepare by adding 2.225 ml 0.824 M NaOH to 11.0 ml absolute ethanol (1 plate equivalent).

(b) Stop solution must be prepared fresh for each experiment due to precipitation on storage.

3.2. Neuraminidase Activity Assay for Determining Virus Dilution

This procedure is used to determine the appropriate virus dilution for use in the NA inhibition assay (see Note 3). Figure 1 illustrates the plate set up for this procedure.

1. Add 120 μl of neat virus to rows A–H in column 1 of a U-bottom 96-well plate.

2. Add 60 μl of virus diluent (1× AB with 0.1% v/v NP-40) to rows A–H columns 2–12.

3. Using a multichannel pipette transfer 60 μl of virus to column 2, mix and transfer 60 μl to column 3.

Mix and transfer 60µl of virus and 1XAB   1XAB
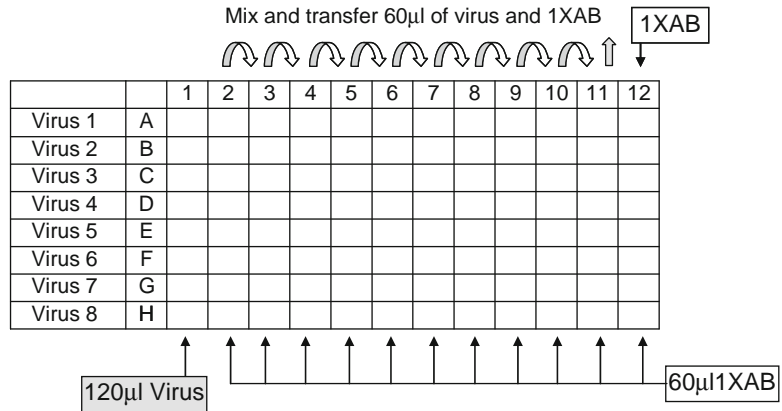
Fig. 1. Twofold serial dilution of viruses (8 viruses per plate).

4. Continue making 1 in 2 dilutions of each virus until column 11 (ensuring 60 µl is discarded from this column), and then leave column 12 containing virus diluent only.

5. After preparing the virus dilutions in a U-bottom plate, transfer 50 µl to the corresponding wells of a 96-well clear MaxiSorp plate and then add 50 µl of working strength MUNANA substrate to each well using a multichannel pipette. Tap the plate by hand to mix and then incubate for 1 h at 37°C. Cover the plate with a lid to prevent evaporation.

6. Following the incubation period, the reaction is stopped by the addition of 100 µl per well of stop solution.

7. The plate can then be read on the fluorometer.

8. Microsoft Excel should be used for the processing of results.

9. Adjust the fluorescence readings of the plate by averaging the 1× AB values and subtracting from each virus fluorescence value. Then draw a graph plotting virus dilution against fluorescence units. From the sigmoidal curve produced for each virus it is possible to select the dilution that corresponds to the midpoint of the linear section of the curve. At this virus dilution a linear relationship between NA activity and MUNANA substrate exists, and this is the appropriate dilution for use in the subsequent NA inhibition assay.

3.3. Diluting Viruses

1. Virus dilutions are most conveniently made in Beckman Coulter 96-well reservoir, which allows the viruses to be arranged in a 96-well plate format and therefore accessible by a 12-tip multichannel pipette.

2. Viruses should be diluted in 1× AB before adding NP-40 at 0.1% v/v.

Table 1
Virus dilutions for the NI assay

Virus dilution required	Virus volume (μl)	1× AB volume (μl)	Surfactant-amps-NP-40 (10%) (μl)
1:10	100	890	10
1:20	50	940	10
1:30	33	957	10

3. To test a virus against two drugs (e.g., oseltamivir and zanamivir) requires 1,000 μl of volume. Therefore for a 1/10 dilution of virus, 100 μl of virus is added to 890 μl of 1× AB and then 10 μl of 10% NP-40 is added.

4. Because a range of virus dilutions are typically used in normal assay (e.g., 1:10, 1:20, 1:30), it is easier to make the dilutions (see Table 1) and then add 10 μl of 10% NP-40 to all samples with a multichannel pipette. Column 12 of each row should contain only 1× AB (with NP-40).

3.4. Fluorescence Neuraminidase Inhibition Assay

1. Add 50 μl of NI drug at dilutions ranging from 0 (2× AB only) to 30,000 nM to rows A to H, respectively, in a 96-well clear MaxiSorp plate. Figure 2 illustrates the plate set up for this procedure.

2. Following the addition of the inhibitor dilutions, add 50 μl per well of control and test viruses from the 96-well deep well block prepared in Subheading 3.3. The deep well block should also contain 1× AB in column 12 for blank readings in the absence of virus. See Fig. 2 below for plate plan. It is recommended that control viruses be run on two plates for each assay (e.g., on the first and the last plate) (see Note 4).

3. Tap the plate by hand to mix. Cover the plate and incubate at room temperature for 45 min.

4. Following incubation add 50 μl of working strength MUNANA substrate to each well using a multichannel pipette. Tap the plate by hand to mix and then incubate for 1 h at 37°C. Cover the plate with a lid to prevent evaporation.

5. Following this incubation period, the reaction is stopped by the addition of 100 μl per well of stop solution.

6. The plate should then be read on the fluorometer.

		1	2	3	4	5	6	7	8	9	10	11	12
0 (2XAB)	A												
0.03 nM	B												
0.3 nM	C												
3 nM	D												
30 nM	E												
300 nM	F												
3,000 nM	G												
30,000nM	H												
		Control A	Control B	Control C	Test Virus 1	Test Virus 2	Test Virus 3	Test Virus 4	Test Virus 5	Test Virus 6	Test Virus 7	Test Virus 8	No Virus 1XAB

Fig. 2. Plate set up for fluorescence NI assay.

3.5. Data Analysis

Susceptibility of the tested virus isolates to NAIs is assessed based on the IC_{50} value, the drug concentration required to inhibit NA enzyme activity by 50%, which is determined through curve fitting by plotting raw data (relative fluorescent units; RFUs) against drug concentration (nM) (see Note 5).

1. The curve fitting software, Robosage (in-house, GlaxoSmithKline) generates IC_{50} values using the equation: $y = V_{max} \times (1 - (x/(K+x)))$, where V_{max} is the maximum rate of metabolism, x is the inhibitor concentration, y is the response being inhibited, and K is the IC_{50} for the inhibition curve (i.e., $y = 50\%$ V_{max} when $x = K$).

2. The JASPR software (in-house, CDC), can also be used for curve fitting and IC_{50} calculation purposes. Curve fitting in JASPR is done using the equation: $V = V_{max} \times (1 - ([I]/(K_i + [I])))$, where V_{max} is the maximum rate of metabolism, $[I]$ is the inhibitor concentration, V is the response being inhibited, and K_i is the IC_{50} for the inhibition curve.

3. The JASPR and Robosage software compute comparative IC_{50} values (Fig. 2), with JASPR providing a faster and higher throughput method of IC_{50} determination, and a more user friendly format. Attention must be paid to IC_{50} values and curve shapes generated by both these software; the inhibition curves should always be visually inspected to ensure that all data points are on or close to the curve.

4. Notes

1. When reconstituting NAIs (see Subheading 3.1, step 2), always check the molecular weight (MW) of NAI compounds to ensure that appropriate drug weights and water volumes are used in reconstitution. A reliable molarity calculator should be used to determine appropriate volumes, such as the Tocris

Molarity Calculator (28). Additionally, NAIs are also available as prodrug form. Oseltamivir carboxylate is the active compound of the ethyl ester prodrug oseltamivir phosphate (Tamiflu, MW 410.4); therefore, oseltamivir carboxylate, and not oseltamivir phosphate, should be used in the NI assay.

2. For quality control purposes, newly prepared inhibitors should first be tested prior to their use in regular assays to confirm correct preparation. To do this run control viruses in triplicate on a single plate for each set of drug dilutions. Mean IC_{50} values of the controls need to be within the acceptable range for each virus.

3. Repeated freeze/thaw of test and reference viruses or extended time at 4°C may alter NA activity for particular viruses, thus the NA activity (see Subheading 3.2) and fluorescent NI assays (see Subheading 3.4) should be conducted on the same day.

4. If testing a large number of viruses (see Subheading 3.4), it is *not* necessary to include control viruses on *every plate;* however, it is recommended that they be included on at least TWO plates, and a "no virus" (1× AB) negative control must be included on each plate.

5. The IC_{50} values (see Subheading 3.5) provide valuable information for detecting resistant viruses as well as for comparing potencies of different NAIs. However, elevated IC_{50} values alone are not sufficient criteria for defining NAI resistance and should be combined with detection of known (e.g., H275Y) or novel molecular markers of resistance in the NA genes by conventional sequencing (13), pyrosequencing (29, 30), or other genotypic assays.
IC_{50} values may be affected by assay conditions (substrate concentration, buffer, etc.) and they typically vary by influenza types/subtypes for the different NAIs. Therefore, it is essential to establish baseline IC_{50} values for a particular NAI, NI assay, as well as NA type and subtype. This allows detection of viruses with slightly reduced susceptibility to NAIs (mild outliers). Statistical analysis is typically used to identify such mild outliers, whereas extreme outliers can be easily detected due to a great increase in IC_{50} values compared to a NAI-sensitive control (5, 13, 20). It is essential to confirm, when possible, that the NA change(s) responsible for the elevated IC_{50} values of the virus isolate is also present in the matching clinical specimen.

Disclaimer

Acknowledgments

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Chapter 8

Animal Models

Edwin J.B. Veldhuis Kroeze, Thijs Kuiken, and Albert D.M.E. Osterhaus

Abstract

Five well-established animal models in influenza research are discussed in a schematic fashion. Although there are clear parallels between these models, like viruses used, housing and handling conditions under biosafety conditions, routes of virus inoculation, sampling strategies, and necropsy techniques (mostly elaborated on in Subheading 4), each of these models involves specific differences in their practical applicability that need thorough assessment depending on the scientific question raised. In other words, there is no universal animal model for influenza and depending on the actual question to be answered the model and the experimental conditions should be carefully selected.

Key words: Influenza, Animal model, Mouse, Ferret, Guinea pig, Macaque, Chicken, Duck, Methods

1. Introduction

Several animal models for human and animal influenza have been established to answer questions related to the pathogenesis of influenza and to the development and testing of intervention strategies like the use of vaccines and antiviral drugs. The most commonly used animal species are the laboratory mouse (*Mus musculus*; predominantly BALB/c-strain (1–3) and C57BL/6J-strain (4–8)), the ferret (*Mustela putorius furo*) (9–14), and the cynomolgus macaque (*Macaca fascicularis*) (15–20), which all exhibit respiratory tract lesions to a lesser or greater extent comparable to those commonly observed in humans and animals with influenza. Several of these models can also be used to study questions related to transmission of influenza viruses. Especially ferrets and, more recently, also guinea pigs (*Cavia Porcellus*) (21–28) are used in influenza transmission studies. Other mammalian species often used in influenza models include the cotton rat (*Sigmodon hispidus*) (29), the domestic pig (*Sus scrofa domestica*) (30–33), the golden

hamster (*Mesocricetus auratus*) (34), and the domestic cat (*Felis catus*) (35, 36). Frequently used avian animal models are the White Leghorn chicken (*Gallus gallus domesticus*) for pathogenicity studies (35, 37) and vaccine studies (38), and various species of ducks (Anseriformes) for studies on the geographical spread of influenza (39, 40). In addition, several of these animal models are used to answer basic scientific and practical questions. Each animal model has its advantages and disadvantages and limitations that must be considered in relation to the research question concerned (see Table 1).

Table 1
Advantages and disadvantages of several animal species as a model for human influenza (modified from refs. 50, 51)

	Pro	Con
Laboratory mouse	– Pathology of 1918 H1N1 and HPAI H5N1 influenza viral pneumonia comparable to humans – Low costs (purchase, maintenance, and reproduction) – Well-characterized genetics; microarray and knockouts readily available – Minimal host variability and background pathology of inbred SPF strains – Many available molecular biological reagents	– Anatomy and histology of respiratory tract and pattern of influenza virus attachment dissimilar to humans – Seasonal human influenza viruses need adaptation to the mouse to replicate sufficiently to cause respiratory lesions – Unsuitable for live-attenuated vaccines – Unsuitable for transmission experiments – Reservations about extrapolative value of data – Need of animal handling experience
Ferret	– Pathology of influenza viral pneumonia comparable to humans – Anatomy and histology of respiratory tract moderately similar to humans and similar pattern of influenza virus attachment – Susceptible for human and avian influenza viruses – Suitable for transmission experiments – Apt animal size for blood and tissue sampling	– Genetically outbred, resulting in host response variability to viral challenge – No SPF animals, so need to confirm Aleutian disease and initial influenza seronegative status – Appear more susceptible to developing influenza pneumonia than humans – Systemic disease in avian influenza different from humans – Ferret microarrays unavailable, although canine chips prove practical cross-hybridization – Few molecular biological reagents available – Need of animal handling experience

(continued)

Table 1
(continued)

	Pro	Con
Cynomolgus macaque	– Pathology of influenza viral pneumonia comparable to humans – Anatomy and histology of respiratory tract and immune response similar to humans – Absence of systemic disease in avian influenza similar to humans – Microarray readily available – Many available molecular biological reagents and cross-reaction with human reagents – Good extrapolative value of data	– Genetically outbred, resulting in host response variability to viral challenge – No SPF animals, thus need to confirm initial influenza seronegative status – High costs (purchase and maintenance) – Ethical concerns – Need of animal handling experience
Guinea pig	– Suitable for transmission experiments	– Pathology of influenza viral pneumonia dissimilar to humans – Usually no clinical symptoms after virus challenge – Need to confirm initial influenza seronegative status – Reservations about extrapolative value of data – Need of animal handling experience
Chicken/duck	– Monitoring vectors of avian influenza (ducks) – Confirming pathogenicity of avian influenza viruses (chickens)	– Pathology of influenza viral pneumonia dissimilar to humans – Need to confirm initial influenza seronegative status in pathogenicity studies

2. Materials

2.1. Mice

1. *Tissue Homogenate Medium* (storage condition: +4°C, for maximum of 2 weeks):
   Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml nystatin.

2. *MDCK Infection Medium* (storage condition: +4°C, for maximum of 2 weeks): 500 ml Eagles minimal essential medium, 10 ml Hepes buffer (1 M), 5.7 ml Sodium bicarbonate solution (7.5%), 5.0 ml l-Glutamine (200 mM), 5.0 ml Penicillin (10,000 IU/ml) and Streptomycin (10,000 µg/ml) solution,

350 μl Trypsin 2.5% (10×), 4.3 ml BSA fraction V (35%), 5.0 ml Amphotericin B (0.25 μg/ml).

3. *RNAlater*, storage conditions: stabile at +4°C for 4 weeks, or long term at –20 to –80°C.

2.2. Ferrets

1. *Transport Medium for virology samples* (storage conditions: +4°C for 2 weeks, and –20°C for 52 weeks):
   430 ml General Virus Transport Medium [500 ml Eagles minimal essential medium, 10 ml Hepes buffer (1 M), 5.7 ml Sodium bicarbonate solution (7.5%), 5.0 ml L-Glutamine (200 mM), 5.0 ml Penicillin (10,000 IU/ml) and Streptomycin (10,000 μg/ml) solution, 4.3 ml BSA fraction V (35%), 5.0 ml Amphotericin B (0.25 μg/ml)], 50 ml Penicillin (10,000 IU/ml) and Streptomycin (10,000 μg/ml) solution, 8.6 ml BSA fraction V (35%), 20 ml Amphotericin B (0.25 μg/ml).

2. *MDCK Infection Medium* (see Subheading 2.1.2).

3. *RNAlater*, storage conditions: stabile at +4°C for 4 weeks, or long term at –20 to –80°C.

2.3. Non-human Primates

1. *Transport Medium for virology samples* (see Subheading 2.2.1).

2. *MDCK Infection Medium* (see Subheading 2.1.2).

3. *RNAlater*, storage conditions: stabile at +4°C for 4 weeks, or long term at –20 to –80°C.

2.4. Guinea Pigs

1. *PBS-BA-PS*: Viral stock diluted in phosphate-buffered saline (PBS) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.3% bovine serum albumin (BSA).

2.5. Chickens/Ducks

1. *Transport medium for virology samples*: Hank's balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 μg/ml gentamicin.

3. Methods

3.1. Mice (Mus musculus)

1. Laboratory mouse strains commonly used are C57BL/6J (H-2b) and BALB/c with a specified pathogens-free (SPF) status, mostly females 6–8 weeks of age.

2. Animals are group housed in age-matched study groups in standard filter-top cages or in isolator units (see Notes 1–3).

3. General anesthesia is induced by means of inhalation of 3% isoflurane in O_2 (see Note 4), and it is also induced when mice are immunized intramuscularly.

4. Blood sampling is performed from the tail vein. Serum samples are collected at predefined time points and tested (after treatment with cholera filtrate and heat inactivation at 56°C) for the presence of anti-HA antibodies via the hemagglutination inhibition (HI) assay and for virus-neutralizing antibodies using the (micro-) virus neutralization (VN) assay.

5. Monitoring for clinical signs and body weight loss (see Note 5) is performed at predefined regular intervals as indicators of disease.

6. Vaccines or other appropriate treatments are administered intramuscularly in the hind legs (maximum injection volume used is 100 μl i.m. equally divided over both hind legs).

7. Virus challenge is performed by intranasally instilling a volume of 50 μl phosphate-buffered saline (PBS) that contains 10^2–10^4 median tissue culture infective dose ($TCID_{50}$) of mouse-adapted H1N1 strains (e.g., influenza virus A/PR/8/34). Non-mouse-adapted (highly pathogenic avian influenza virus) HPAIV H5N1 (e.g., A/Vietnam/1194/04) are intranasally inoculated at a dose of 10^3 $TCID_{50}$ in 50 μl PBS (see Note 6). Control animals are sham challenged in the same way with the matching volume of PBS.

8. Virus shedding is monitored postinfection in daily serial nose washings and at necropsy. At necropsy the nasal passages are washed and nasal turbinates are sampled for histopathology, and virus titers are determined by standard procedures in MDCK cells (see Note 6).

9. Euthanasia of laboratory mice is performed under general anesthesia by means of exsanguination from orbital puncture or by cervical dissociation at predefined time points, or according to predefined clinical criteria.

10. During necropsy lungs are examined for macroscopic changes and snap frozen (see Note 7). They may alternatively be intratracheally inflated with and immersed in 10% neutral-buffered formalin for fixation and disinfection, and subsequently routinely processed (see Note 8) for histopathological examination by light microscopy. Additionally, immunohistochemistry (IHC) may be carried out to visualize influenza A virus' nucleoprotein (NP) (see Note 9). Spleens can be harvested for the detection of virus-specific CD8+ cytotoxic T lymphocytes (CTL) by tetramer-staining to assess vaccine-induced T-cell responses (see Note 10).

11. Lung virus titers ($TCID_{50}$) are determined in snap-frozen lungs collected at necropsy (see Note 11); the titers of clarified supernatants of homogenized lungs in tissue homogenate medium or MDCK infection medium are determined by standard procedures in MDCK cells (see Note 6).

12. A microarray assay to assess differences in mRNA expression profiles can be performed using tissue (lung) samples collected at necropsy and immediately stored in RNAlater (see Note 12).

3.2. Ferrets (Mustela putorius furo)

1. Studies are carried out with male and/or female outbred ferrets, approximately 8 months of age, with body weights ranging from 0.8 to 1.5 kg.

2. Ferrets are housed in study groups of 6–10 animals. For transmission experiments, naïve ferrets are placed in a transmission enclosure adjacent to an inoculated ferret at 1 day postinfection. The animals in these cages are separated by two relatively closely spaced stainless steel grids to allow airflow exchange but to prevent direct contact and fomite transmission (see Notes 1–3). Nose and throat swabs are taken from the inoculated and exposed ferrets at predefined daily time points to assess potential horizontal virus transmission (see Subheading 3.2, step 8).

3. General anesthesia is induced by intramuscular injection in the hind legs with a mixture of ketamin 12.5 mg/kg body weight and medetomidine–HCl 7.5 μg/kg body weight (which can be antagonized by atipamezole–HCl 0.5 mg/kg body weight), i.m. injection of ketamin 25 mg/kg body weight is used for blood sampling, euthanasia, and swabbing throat, nose, and rectum (see Note 4).

    Anesthesia of ferrets can alternatively be induced by means of inhalation of 5% isoflurane in O_2 (41).

4. Blood sampling and i.v. administration of drugs are performed from the jugular vein. Ferrets are screened before use for the presence of serum antibodies against Aleutian disease, and against circulating seasonal and other relevant influenza virus strains. Only seronegative animals are used (see Note 13). Peripheral blood mononuclear cells (PBMC) can be collected and isolated from blood samples, and a T-cell proliferation assay can be performed to assess vaccine-induced T-cell responses (see Note 14).

5. Monitoring for clinical signs, like labored breathing, reduced activity, and body weight loss is performed at predefined regular intervals as indicators of disease. The body temperature is recorded at regular intervals, ranging from every 5–15 min, using a temperature-logging device implanted in the peritoneal cavity 14 days prior to the start of the experiment.

6. Vaccines or other appropriate treatments are administered intramuscularly in the hind legs (maximum injection volume used is 1 ml i.m. equally divided over both hind legs). Certain vaccines (see Note 15) are instilled intranasally (0.5 ml equally divided over both nostrils).

7. Virus challenge is performed by intratracheal instillation of 10^5–10^7 $TCID_{50}$ influenza virus (dosage depending on the virus used) suspended in 3 ml phosphate-buffered saline (PBS). The challenge virus can also be administered by intranasal instillation of 10^5–10^7 $TCID_{50}$ influenza virus (dosage depending on the virus used) suspended in 0.5 ml PBS (equally divided over both nostrils) (see Note 6). Control animals are sham challenged in the same way with the matching volume of PBS.

8. Virus shedding is monitored by collection of pharyngeal, nasal, and occasionally rectal swabs in transport medium for virology samples, collected every day or at other predefined time points post challenge until euthanasia or death. Viral shedding from the upper respiratory tract is analyzed by measuring the infectivity titers ($TCID_{50}$) in nasal/throat swabs taken from the ferrets (see Note 16).

9. Euthanasia of ferrets is performed under general anesthesia by means of exsanguination from cardiac puncture.

10. During necropsy a thorough macroscopic examination is performed, this includes weighing of the animal and selected organs and assessment of gross pulmonary lesions. The lungs are weighed, and the left lung is collected during necropsy, inflated with and immersed in 10% neutral-buffered formalin for adequate fixation and disinfection. Lung sectioning for histopathology is done in a standardized way not guided by the presence of gross lesions. These sections are routinely processed for examination by light microscopy (see Note 8). Further immunohistochemistry (IHC) can be applied to visualize influenza A virus' nucleoprotein (NP) (see Note 9).

11. Lung virus titers ($TCID_{50}$) are determined, in pooled homogenized (in MDCK infection medium) small lung sections collected and weighed at necropsy (see Note 11), by standard procedures in MDCK cells (see Note 6). Lung samples from each animal are collected at the cranioventral, craniodorsal, caudoventral, and caudodorsal segments of the right lung (in a standardized way, not guided by the presence of gross lesions), and weighed (each sample ≈ 0.1 g, so total averaged weight of sampled lung tissue ≈ 0.4–0.5 g per animal). Virus titers of additional tissues like nasal mucosa, tonsils, tracheobronchial lymph nodes, trachea, bronchi, brain, spleen, liver, kidney, pancreas, intestines, and plasma can be determined in a similar way.

12. A microarray assay to assess differences in mRNA expression profiles can be performed using tissue (lung) samples collected at necropsy and immediately stored in RNAlater (see Note 12).

3.3. Non-human Primates

1. Cynomolgus macaques (*Macaca fascicularis*), male and/or female, adolescent to young adults (approximately 3 years), colony bred in captivity, body weight ranging from 3.5 to 4.5 kg are most frequently used in influenza infection experiments.

2. The macaques are group housed in study cohorts, either in harem groups (1 dominant male with several females) and/or in male groups (peer group of young adults). Commercial macaque food is supplemented with fresh fruits (see Notes 1–3).

3. General anesthesia is induced by intramuscular injection in one of the legs of a mixture of ketamin 1.0 mg/kg body weight and medetomidine–HCl 0.1 mg/kg body weight (which can be antagonized by atipamezole–HCl 0.25 mg/kg; i.m.) (see Note 4).

4. Blood sampling and intravenous drug administration is usually performed from the inguinal vein. Before start of the experiments animals are screened before use for the presence of serum antibodies against circulating seasonal and other relevant influenza virus strains by HAI assays. Only seronegative animals are used (see Note 17).

5. Monitoring for clinical signs, like labored breathing, reduced activity, and body weight loss is performed at predefined regular intervals as indicators of disease. The body temperature is recorded at predefined regular intervals (see Subheading 3.2, step 5 and Note 18).

6. Vaccines or other appropriate treatments are administered intramuscularly in the legs (maximum injection volume used is 1.0 ml equally divided over both legs). Certain vaccines (see Note 15) are instilled intranasally (volume usually used is 1.0 ml equally divided over both nostrils).

7. Virus challenge is performed by intratracheal instillation of 10^4–10^7 $TCID_{50}$ influenza virus suspended in 3–4 ml PBS (dosage depends on the virus applied) propagated in monkey kidney (MK) cells or MDCK cells. Virus challenge can also be performed by intranasal instillation of 10^4–10^7 $TCID_{50}$ influenza virus (dosage depending on the virus applied) suspended in 1.0 ml PBS (equally divided over both nostrils) (see Note 6). Intranasal or intratracheal inoculation can be combined with application of the virus suspension on the tonsils and conjunctivae (17). Control animals are sham challenged in the same way with the matching volume of PBS.

8. Virus shedding is monitored by collecting serial swabs (see Subheading 3.2, step 8 and Note 16), and/or by collecting lung lavage fluids (LLF) every day or every other day post challenge until euthanasia or death. The samples are analyzed for

the presence of cell-free virus by inoculating MDCK cells with serial tenfold dilutions of the swab fluids and/or LLF obtained after removing cells by low-speed centrifugation, and virus titers ($TCID_{50}$) are determined according to standard procedures (see Note 6).

9. Euthanasia of macaques is performed under general anaesthesia by means of exsanguination from cardiac puncture.

10. During necropsy a thorough macroscopic examination is performed and lung tissues are sampled (see Subheading 3.2, step 10).

11. Lung virus titers ($TCID_{50}$) are determined as described (see Notes 6, 11 and Subheading 3.2, step 11). Virus titers of additional tissues and plasma can be determined in a similar way.

12. A microarray assay to assess differences in mRNA expression profiles can be performed using tissue (lung) samples collected at necropsy and immediately stored in RNAlater (see Note 12).

3.4. Guinea Pigs (Cavia porcellus)

1. Female guinea pigs of the outbred Hartley strain, approximately 8 weeks of age with body weights of 300–350 g, are used predominantly (21–28) in influenza virus transmission models. Primary human influenza A virus isolates replicate in both upper and lower respiratory tract of guinea pigs without further adaptation. The animals shed high levels of virus in nasal secretions that can be transmitted via direct contact and/or droplets and aerosols, to nearby sentinel guinea pigs (21). Unadapted A/Panama/2007/99 (H3N2) inoculated Hartley guinea pigs do not show clinical signs. However, this is dependent on the guinea pig strain (21). Other viruses such as a human isolate of highly pathogenic avian influenza H5N1 did cause slight temporary lethargy in Hartley strain guinea pigs (22). Also, the dose of virus used proved important as intranasal challenge with high dose of influenza A virus H3N2 in juvenile Hartley guinea pigs resulted in ruffled fur and reduced activity (23). Since infected guinea pigs do not produce burst expulsions like cough or sneeze, the extrapolation of guinea pig transmission data to humans seems to be complicated (24).

2. Guinea pigs in transmission settings are housed each in a standard rat cage (Ancare R20 series) with an open wire top and (modified) open wire side, and are usually kept on a 12 h light/dark cycle. Two cages, one containing the infected animal and the other containing the exposed animal, are placed with the wire grids facing each other at variable distances without possible physical contact in an environmental chamber (Caron model 6030) with supporting unidirectional airflow. During transmission experiments, strict measures are to be implemented to prevent aberrant cross contamination between cages. This

includes handling of sentinel animals before inoculated animals, changing gloves and sanitizing work surfaces between animal handlings (21, 24, 28) (see Notes 1–3).

3. General anesthesia is induced by a mixture of ketamin (30 mg/kg body weight) and xylazine (2 mg/kg body weight) administered intramuscularly in the gluteal muscles (21) (see Note 4).

4. Blood sampling is performed from the metatarsal vein. The animals are screened before use for the presence of serum antibodies against circulating seasonal and other relevant influenza virus strains by HAI assays. Only animals seronegative for influenza A virus antibodies are used (21).

5. Monitoring of body weight loss and body temperature is performed at predefined regular intervals. The body temperature is recorded with subcutaneously implanted telemetric transponders (21) (see Note 19).

6. Vaccines are administered intramuscularly in the gluteal muscles (maximum injection volume used is 0.5 ml i.m. equally divided over both sites), or instilled attenuated virus vaccines of 10^6 PFU in 300 μl intranasally (equally divided over both nostrils) (25) (see Note 20).

7. Virus challenge is performed by intranasal instillation of a virus stock diluted in a volume of 300 μl PBS-BA-PS (equally divided over both nostrils) (21). Inoculation doses reported are 10^6 EID_{50} for IAV H1N1 and HPAI H5N1 (26) and 1×10^3 p.f.u. for IAV H3N2 and H1N1 (24). Intragastric inoculation of HPAI H5N1 has been performed by means of gavage catheter, 10^6 EID_{50} in 2 ml PBS (22) (see Note 21). Control animals are sham challenged in the same way with the matching volume of PBS(-BA-PS).

8. Virus shedding and transmission are monitored every day or every other day postinoculation. Both challenged and naïve animals undergo nasal washing by instilling 1 ml PBS-BA-PS into the nostrils and collection of liquid runoff draining it on a sterile petri dish, collected in tubes and centrifuged for 5 min at $2,000 \times g$ and 4°C, supernatants can be stored at –80°C before analysis. Virus titers ($TCID_{50}$) are determined according to standard procedures in MDCK cells (21).

9. Euthanasia of guinea pigs is performed by means of intraperitoneal injection of sodium pentobarbital (21), or by intracardiac injection with Beuthanasia-D solution 1 ml/kg (26), or by exposure to CO_2 gas (27).

10. During necropsy lungs are collected and weighed (21).

11. Lung virus titers ($TCID_{50}$) are determined in supernatants of tissue homogenates tested by standard procedures in MDCK cells or in embryonated chicken eggs (21, 22) (see Note 22).

3.5. Chickens/Ducks

1. In most cases, specified pathogens-free (SPF) White leghorn chickens (*Gallus gallus domesticus*) are used; aged 4–10 weeks in pathogenicity studies (see Note 23), or 1-day-old chicks as influenza virus infected prey animals in infection experiments in carnivores (e.g., cats).

   Several duck species, including the mallard (*Anas platyrhynchos*) ([40](#)), males and females, usually between 8 and 11 months of age, are used in infection and transmission experiments. The ducks are captive bred and housed indoors since hatching to avoid the risk for inadvertent avian influenza virus infection.

2. Chickens and ducks are usually group housed in study groups of 4–8 animals (see Notes 1–3).

3. General anesthesia is induced by means of inhalation of 5% isoflurane in O_2 (see Notes 4).

4. Blood sampling is performed from the jugular or ulnar veins. Serum samples (and cloacal and oropharyngeal swabs) are collected 1 week before inoculation. Serum is analyzed by means of a commercial influenza A virus antibody ELISA kit for detection of antibodies against NP. The swabs are tested by RT-PCR. Only seronegative and RT-PCR negative animals are used.

5. Monitoring for clinical signs like labored breathing, diarrhea, and body weight loss, as indicators of disease is performed at predefined regular intervals.

6. Vaccines or other appropriate treatments are administered subcutaneously or intramuscularly (pectoral muscle), with volumes adapted to body weight (see Note 24).

7. Virus challenge is performed by intratracheal instillation of 2.5×10^4 $TCID_{50}$ of, e.g., HPAIV H5N1 virus in 1-day-old chicks in transmission experiments to carnivores. To this end the chicks are euthanized at 1 day postinoculation. In pathogenicity studies 8 weeks old chickens are inoculated intranasally with, e.g., 10^4 EID_{50} HPAIV H5N1 in 0.3 ml PBS ([42](#)). Ducks in pathogenicity and excretion studies are inoculated with, e.g., 10^4 $TCID_{50}$ HPAIV H5N1 in PBS, 1.5 ml intratracheally and 1.5 ml intraoesophaeally (see Note 6). Control animals are sham challenged in the same way with the matching volume of PBS-diluted sterile allantoic fluid.

8. Viral shedding is monitored by daily collection of cloacal and oropharyngeal swabs stored in 1 ml transport medium. Viral RNA is isolated from 200 µl supernatant using the MagNA Pure LC system and influenza A virus is detected by a TaqMan assay. Infectious virus titers ($TCID_{50}$) can be determined by standard procedures using MDCK cell cultures (see Note 6).

9. Euthanasia of these avian species is performed under general anesthesia by means of exsanguination from cardiac puncture.

10. During necropsy organs are evaluated and sampled. Tissue sections are routinely processed for examination by light microscopy (see Note 8). Additional immunohistochemistry can be applied to visualize the presence of NP (see Note 9). Avian-specific tissues collected at necropsy include the caudothoracic or abdominal airsac, proventriculus, and bursa of Fabricius.

11. Lung virus titers ($TCID_{50}$) are determined in weighed and homogenized (in 3 ml of transport medium) samples (see Note 11) by standard procedures in MDCK cells (see Note 6). Virus titers of additional tissues and plasma can be determined in a similar way. RNA isolation from homogenized tissues suspensions and swabs is performed by using a MagNa Pure system. Real-time RT-PCR assays are performed using TaqMan EZ RT-PCR Core Reagents Kit to detect the hemagglutinin gene of HPAIV (H5N1).

4. Notes

1. All animal experiments are approved by an independent expert governmental and/or institutional animal ethics and welfare committee.

2. Laboratory animals have access to fresh species-specific commercial food pellets, with or without supplements, which meet their individual nutritional requirements and fresh water ad libitum.

3. Animal experiments (including housing, sample handling, and laboratory work) dealing with Biosafety level 3 (BSL-3) classified pathogens are performed under Biosafety level 3 (+) (BSL-3 (+)) conditions. Animals are housed in negatively pressurized and high efficiency particulate air (HEPA)-filtered biocontainment isolation units (usually glove boxes). Guinea pigs also can be housed in high efficiency particulate air-filtered isolation units under BSL-3 (+) conditions when infected with, e.g., HPAIV H5N1 (22).

4. General anesthesia is applied for performing virus challenges (intranasal, intratracheal, intraesophageal, intragastric), taking nose/throat/rectal swabs, blood samplings, nasal washes, subcutaneous or intraperitoneal implantation of telemetric transponders, and euthanasia (21).

5. According to protocol laboratory mice are euthanized if they show a postinfection weight loss exceeding 20% (8).

6. Challenge viruses can be either propagated in the allantoic cavity of 10- or 11-day-old embryonating chicken eggs (e.g., mouse-adapted A/PR/8/34) or propagated in MDCK or MK cell cultures (e.g., influenza A viruses to challenge ferrets). Allantoic fluid is harvested 3 days after inoculation and infectious virus titers ($TCID_{50}$) are determined in MDCK cells. After completion of cytopathic changes cell culture supernatants are collected and cleared by low-speed centrifugation, and infectious titers are similarly determined in MDCK cells. The virus-containing fluids are diluted with phosphate-buffered saline (PBS) to obtain final titers needed for inoculation.

   Infectious virus titers ($TCID_{50}$) can be assessed using MDCK cell cultures. 100 μl of tenfold serially diluted culture supernatants are inoculated in quadruplicate or quintuplicate on MDCK cells grown confluent in 96-well microtiter plates (10^5 cells per well) in culture medium [minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin]. Before infection the cells are washed with PBS twice. After 1 h at 37°C, cells are washed twice with infection medium [MEM supplemented with 4% bovine serum albumin (BSA) fraction V, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4 μg/ml trypsin] in a humid 5% CO_2 atmosphere at 37°C for 6–7 days. 50 μl volumes of these culture supernatants are serially diluted twofold in PBS in round bottom plates and added 25 μl PBS and 25 μl of a 1% suspension of turkey erythrocytes. After 1 h incubation at 4°C the haemagglutination patterns are read. HA activity is used as an indicator for infection of the cells in individual wells (43). Infectious virus titers are calculated according to the method of Spearman–Karber (44) or Reed–Muench (45) and expressed as log $TCID_{50}$ per ml. Infectious virus titers can alternatively be assessed using 10-day-old embryonating chicken eggs reported as log median egg infectious dose (EID_{50}) per ml.

   Usually a large animal experiment is preceded by a dose-finding study that includes fewer animals than the definitive experiment to determine the optimal challenge dose of a particular virus, e.g., four different challenge groups of six mice each (46).

7. Collected at necropsy, entire lungs of mice or lung (or other organs) portions of larger animals are snap frozen (on dry ice with ethanol) and stored at –70°C/–80°C pending further processing.

8. During necropsy the lungs are inflated with and submersed in 10% neutral-buffered formalin for a minimum of 2 days to allow for adequate tissue fixation and virus inactivation. Lungs and other organs collected at necropsy are placed in 10%

neutral-buffered formalin with an approximate ratio of 1 parts tissue to 9 parts formalin. Then tissue sections are routinely processed and paraffin embedded and sectioned at 3–4 μm, deparaffinized with xylene and rehydrated with alcohol and stained with haematoxylin and eosin (HE).

9. Sequential slides are cut at 3–4 μm and stained using an immunoperoxidase method with a monoclonal antibody directed against the NP of influenza A virus. A horseradish peroxidase-labeled goat anti-mouse (e.g., anti-IgG2a) is used as secondary antibody. Endogenous cellular peroxidase is blocked with 3% hydrogen peroxide. The peroxidase is revealed using 2,3-diaminobenzidine as a substrate, resulting in a deep dark brown granular staining in the nuclei of influenza virus infected and replicating cells, followed by counterstaining with haematoxylin. The peroxidase activity alternatively can be revealed by incubating slides with 3-amino-9-ethylcarbazole (AEC) for 10 min resulting in deep red nuclear precipitates in infected cells. Lung sections from domestic cats with experimental HPAI H5N1 virus are simultaneously stained and thus used as positive controls. Negative controls are performed in absence of the primary antibody, substitution of primary antibody by an irrelevant monoclonal antibody of the same isotype, and testing of sham-inoculated animals (18).

10. Erythrocytes are removed from single-cell splenocyte suspensions (obtained by using 100 μm cell strainers) with ery-lysis buffer. The cells are washed with 0.5% bovine serum albumin (BSA) or 2% fetal calf serum (FCS) in PBS and stained for flow cytometry with antibodies: CD3e-PerCP, CD8^b.2-FITC, ToPro 3-APC, and APC or PE-labeled H-2Db-PE tetramer with the NP$_{366-374}$ epitope ASNENMETH. Cells are analyzed on FACSCalibur with a high throughput sampler in combination with PlateManager and CellQuest Pro software (8).

11. Tissues are weighed and subsequently homogenized in 1 ml of medium by means of the FastPrep system with two ¼ in. (≈6.4 mm) diameter ceramic balls or four 3.0 mm diameter steel balls and then resuspended by adding an extra 2 ml medium (and can be subsequently frozen at –80°C until further analysis). The suspension is then clarified by centrifugation (10 min at $500 \times g$). Virus titers in the supernatant are determined (see Note 6) and data are expressed as log TCID$_{50}$ per gram of tissue. Alternatively, a Polytron PT2100 can be used to homogenize tissues in 3 ml of medium.

12. Several small fresh tissue samples (≈0.1 g) are collected at necropsy; non-lesional versus lesional, and challenged versus placebo-treated animals, and directly stored in RNAlater for subsequent total RNA isolation, purification, labeling, and hybridization on GeneChip Arrays. GeneChip Arrays are readily

available for laboratory mice but not for ferrets yet, however, amplified RNA can be hybridized to Affymetrix GeneChip Canine Genome 2.0 Array (41). Arrays are also readily available for the Rhesus macaque (*Macaca mulatta*) that bears a close genetic resemblance to the Cynomolgus macaque and can be used successfully (47). Among others, differences in gene expression levels of inflammatory mediators like interferons (IFN) and other cytokines and chemokines can be assessed.

13. Since ferrets with a specified pathogens-free (SPF) status are not readily available, previous field infections with circulating influenza viruses may have occurred which may interfere with vaccine efficacy and virus challenge studies because of possibly induced cross-immunity against the vaccine or challenge virus. Similarly, infection with Aleutian disease virus (ADV; a parvovirus) can cause interference by means of an altered immune competent status (48).

14. PBMCs are isolated from blood samples, collected 4 weeks after the first and second vaccination in EDTA tubes by density gradient centrifugation using lymphoprep (can be cryopreserved at –135°C), labeled with 0.3 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS for 5′ at 37°C, washed twice and resuspended in RPMI 1640. 10^5 cells per well are seeded into a 96-well plate with or without the immunogenic compound (200 ng HA content) or phytohaemagglutinin (PHA) (1 μg/ml) and incubated at 37°C/5% CO_2 for 6 days. After 2 days, 100 μl supernatant of Concanavalin A-stimulated ferret lymph node cells is added. After 4 days cells are washed and stained with a monoclonal antibody against human CD8 (OKT-8)-Pacific Blue. Cells are stained with LIVE/DEAD Aqua Fixable Dead Cell Stain to exclude dead cells. Next cells are fixed and permeabilized with Cytofix and Cytoperm, and stained with an Alexa Fluor 647-labeled mAb specific for human CD3 (PC3/188A). These CD3- and CD8-specific mAb cross-react with ferret CD3 and CD8. Data are acquired using a FACSCanto-II and analyzed with FACS Diva software. The proliferation of CD3 + CD8– (and CD3 + CD8+) cells is calculated by subtracting the control CD3 + CD8-(+) CFSE^{low} cells from the HA-stimulated CD3 + CD8-(+)CFSE^{low} cells (9).

15. When a vaccine based on a "live" attenuated virus is used that needs limited replication within the nasal epithelium to induce an adequate protective immunity, it is usually applied intranasally.

16. Swabs collected for virus shedding can be stored at –80°C until further analysis. Individual swabs are homogenized and resuspended briefly on vortex in 3 ml transport medium for virus

isolation. Viral titers ($TCID_{50}$) are determined (see Note 6) and data are expressed as log $TCID_{50}$ per ml of swab medium.

17. Additional confirmation of the absence of virus-specific anti-bodies can be performed by immunofluorescence assay using MDCK cells infected with influenza A or B viruses (43).

18. Changes in body temperature can individually be calculated by subtracting the mean day (4-h) and night (4-h) temperatures measured on 4 successive days during the period before the challenge from the mean day (4-h) and night (4-h) temperatures measured after infection (15).

19. Since guinea pigs generally do not exhibit clinical signs when challenged with influenza A viruses (21), clinical observations are not always performed routinely.

20. Both intramuscular and intranasal vaccinations are to be administered without bovine serum albumin (BSA) in the vaccine preparation to avoid hypersensitivity during virus challenge, since this may occur after repeated exposure to this antigen (25).

21. Virus stocks are propagated in the allantoic cavity of 10-day-old embryonating chicken eggs, and the infected allantoic fluid was titered and reported as 50% egg-infective dose (EID_{50}), calculated according to the method of Reed–Muench (22, 45).

22. The lung viral load is determined by plaque assay of tenfold serial dilutions on MDCK cells and expressed as $\log_{10} TCID_{50}$ or by limiting dilution in embryonating chicken eggs and expressed as $\log_{10} EID_{50}$ (21, 22).

23. The intravenous pathogenicity index (IVPI) of influenza viruses is determined using 6-week-old specified pathogen-free (SPF) White Leghorn chickens by intravenous injection in the ulnar vein of 0.1 ml of 10^6 $TCID_{50}$ virus. Clinical signs are monitored for 10 consecutive days, and the animals are considered diseased when displaying a single symptom, such as depression, cyanosis of the comb or wattles, respiratory involvement, diarrhoea, oedema of the face/head, and nervous signs, and considered severely sick if they displayed two or more clinical signs. The index is calculated as the mean score per bird per observation (29).

24. Injected volumes of vaccines are correlated to the body weight of birds; <1.4 kg: 0.25 ml, 1.4–7 kg: 0.5 ml, >7–12 kg: 0.75 ml, >12–44 kg: 1.25 ml, >44 kg: 2.5 ml (49).

Supplementary Note. Supplier(s) of, standard laboratory material and pharmaceuticals are Lonza The Netherlands, Sigma-Aldrich The Netherlands, ICN The Netherlands; RNAlater: Ambion USA; veterinary anesthetics: Eurovet The Netherlands, Orion Pharma Finland, Pfizer The Netherlands; temperature loggers are Starr-Oddi Iceland; the MagNA Pure LC system: Roche diagnostics The

Netherlands; influenza A antibody ELISA kit: EVL The Netherlands; GeneChips: Affymetrix USA; RPMI: Cambrex USA; Lymphoprep: Axis-Shield Norway; Cytofix, cytoperm, cell strainers and Facs software: BD pharmingen The Netherlands; human monoclonal CD8 antibody: eBiosciences USA; labeled human monoclonal CD3 antibody: Santa Cruz Biotechnology USA; Fastprep system: MP biomedicals Europe; Polytron PT2100: Kinematica AG Switzerland.

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Chapter 9

Influenza Virus Surveillance, Vaccine Strain Selection, and Manufacture

Klaus Stöhr, Doris Bucher, Tony Colgate, and John Wood

Abstract

As outlined in other chapters, the influenza virus, existing laboratory diagnostic abilities, and disease epidemiology have several peculiarities that impact on the timing and processes for the annual production of influenza vaccines. The chapter provides an overview on the key biological and other factors that influence vaccine production. They are the reason for an "annual circle race" beginning with global influenza surveillance during the influenza season in a given year to the eventual supply of vaccines 12 months later in time before the next seasonal outbreak and so on. As influenza vaccines are needed for the Northern and Southern Hemisphere outbreaks in fall and spring, respectively, global surveillance and vaccine production has become a year round business. Its highlights are the WHO recommendations on vaccine strains in February and September and the eventual delivery of vaccine doses in time before the coming influenza season. In between continues vaccine strain and epidemiological surveillance, preparation of new high growth reassortments, vaccine seed strain preparation and development of standardizing reagents, vaccine bulk production, fill–finishing and vaccine release, and in some regions, clinical trials for regulatory approval.

Key words: Influenza, Vaccine, Manufacturing, Standardization, Surveillance, Influenza vaccine strains, High growth reassortant, Single radial immunodiffusion, Potency assay

1. Before Vaccine Production: Global Influenza Strain Surveillance, Variant Selection, and Strain Decision Making Klaus Stöhr

1.1. Why Is Global Influenza Surveillance Needed?

Introduction of influenza immunization after world war II was a success; but only for 2 years until a drifted influenza H1N1 variant virus emerged in 1947 and the vaccine effectiveness for preventing disease in healthy adults plunged from around 80% to below 35–50% in the UK and 0% in the US army. This drop was caused by the emergence of a new, dominating influenza H1N1 variant virus against which the then vaccine virus protected only partially. Consequently, the new virus variant was used for subsequent vaccine production (1–3). In order to ensure that the vaccine always

contains the prevalent influenza variant viruses, a global institutional network was created in the late 1940s. It had to take into account the biological and other realities of the influenza virus and the disease it causes (Table 1). The network has evolved into a global institution composed of WHO Global and National Influenza laboratories and several specialized national centers that have working relationships with several vaccine manufacturers and national and regional licensing agencies. The key deliverables of these institutions are recommendations on which variant viruses should be used for vaccine production, preparation of vaccine candidate virus and vaccine calibrating reagents, and vaccine registration and licensing (5). Although each of these organizations follow their rules and standards, there is no formal treaty or contract that governs the seamless cooperation of these institutions; but the function of each of them in a concerted fashion is conditional for the timely delivery of effective influenza vaccines every year.

1.2. Sampling, Detection of Variant Viruses, and Their Genetic and Antigenic Analyses

The backbone for global virus sampling is the approximately 135 National Influenza Centers that operate in more than 77 countries (http://www.who.int/influenza/gisrs_laboratory/national_influenza_centres/en/; accessed 10 Jan 2012). They receive samples from patients with respiratory tract infections taken in affiliated local hospitals or by local physicians. In addition, some National Influenza Centers coordinate national networks of virological laboratories from which they receive already pre-tested virus samples. These laboratories use a set of reagents (http://www.who.int/influenza/resources/documents/manual_diagnosis_surveillance_influenza/en/index.html; accessed 10 Jan 2012) and laboratory techniques (see also other chapters on influenza virus isolation, characterization) to verify

(a) Whether an influenza virus is present in the clinical sample
(b) If it is antigenically significantly different from the variant virus contained in the influenza vaccine

If the latter is the case, a so-called low reactor variant virus is detected and virus supernatant from egg or cell-culture isolation or preferably the original cotton swabs are sent to one of the 5 WHO Collaborating Centers for Human Influenza Surveillance (http://www.who.int/influenza/surveillance_monitoring/updates/en/; accessed 10 Jan 2012). Depending upon the annual outbreak intensity and other factors, more than 200,000 specimens are estimated to be investigated globally by the National Influenza Centers and more than 15,000 virus samples are sent to the WHO CCs for further investigation every year. In addition, the WHO Global Influenza Program located in Geneva, collects routinely epidemiological information from national disease surveillance (http://gamapserver.who.int/GlobalAtlas/home.asp; accessed 14 July 2010).

Table 1
Biological and other realities of influenza and impact on public health and vaccine production

Biological and other realities	Public health and other consequences	Impact on vaccine production
• Best protection derives from virus neutralizing antibodies but they wane quickly (within months) below the level considered protective	• Annual revaccination needed shortly before influenza season. Vaccine required in NH before Nov/December and in SH before April/May	• Vaccine production must start in Jan and Sep to ensure supply ahead of the NH and SH outbreaks, respectively
• Recurrent, annual seasonal (winter) outbreaks in Northern and Southern Hemisphere; influenza viruses circulate at low levels year around in Equatorial region	• WHO global influenza virus surveillance needed to detect newly emerging dominating variant viruses	• High-growth reassortants derived from the dominating wild viruses are to be available 1 month before production begins (to allow for seed virus preparation)
• Frequent, often annual, emergence of dominant drift variant influenza viruses in humans	• Vaccine must be produced with currently circulating or emerging dominant virus to maintain high vaccine efficacy	• Standardizing reagents (serum) must always be updated when strains change. Reagents are needed early after beginning of production to measure strain yield and to calibrate antigen before bulk blending and vaccine formulation
• Three influenza viruses are concurrently circulating in humans: H1; H3; and B viruses	• Wild viruses must be adapted in laboratory to become high-growth reassortants (different technologies available)	• Formulated and filled vaccine doses are needed already in June for clinical trials
• Influenza viruses grow in eggs but require adaptation for high yield which is condition for efficient production	• Clinical trials are to be executed during the narrow window between availability of first vaccine doses and annual immunization campaigns	• Companies begin production at one's own risk before WHO recommendations are available. They start production with the strain, that is, the least likely to be updated by WHO
• Some regulatory agencies require small clinical trials before approval to market after a strain has changed in the influenza vaccine		• WHO makes recommendation on vaccine candidate strains only in Feb and Sep to allow time for robust virus surveillance in countries
		• High-growth reassortants are often prepared from various "candidate" variant viruses before a dominant virus is identified to save time. Nevertheless, HGR are sometimes not immediately available after strain recommendations are made
		• Companies will switch production to next vaccine strain without knowing how much of the first one has already been produced
		• Standardizing reagents are prepared in sheep which takes 1–2 months and requires antigen from virus production for initiation. Therefore, it is often only available late in the production process and becomes rate limiting; particularly when some doses are needed very early because of requirements to conduct clinical trials before licensing

Table 2
Criteria to be fulfilled by influenza vaccine candidate viruses

Antigenically different viruses (supported by other findings)
Differ from the vaccine virus strains as characterized by the HI test supported by clinical and epidemiological information and (a) genetic characterization and (b) serological tests with sera from subjects immunized with current seasonal influenza vaccine

Dominant influenza viruses
Although many different variant viruses of, e.g., H1 and H3 subtypes and B virus lineages emerge and can be isolated throughout the year, most virus variants presence is short lived and geographically limited. Only very few gain global significance and will dominate the next influenza seasonal outbreaks. To detect those, samples from different continents are required. As surveillance is ongoing simultaneously in several WHO centers with different geographical reach, exchange of information and candidate viruses and their antisera raised in ferrets is vital. Phylogenic trees generated from sequencing of parts of the HA and NA surface protein help detect clustering of antigenically related variant viruses (e.g., Fig. S1-3 in Ref. (4)). In addition, WHO Headquarters assesses the epidemiological and clinical relevance of these new variant viruses routinely through data obtained from national authorities (http://www.who.int/influenza/surveillance_monitoring/updates/en/; accessed 10 Jan 2012)

Suitable for vaccine preparation
To comply with regulatory requirements, vaccine companies require virus isolates that (a) are antigenically and genetically similar to those identified by WHO as becoming dominant; (b) have a known passage history isolated on approved egg substrates (With more influenza vaccine producers switching over to cell-culture production, standards are also developed by regulatory agencies for viruses isolated in cell culture.); (c) have acceptable growth characteristics (grow well in hens eggs)

The overarching task of the WHO Collaborating Centers is to detect influenza viruses that fulfill all of the below criteria (see Table 2):

1. Antigenically *significantly* different from the current vaccine virus strains
2. Are or likely to become the *dominant* influenza viruses during the next influenza season
3. *Suitable* for vaccine strain preparation

To this end, the WHO CCs and affiliated laboratories carry out a range of activities and perform various laboratory tests with viruses from national influenza centers. They include several of the below efforts but are not necessarily limited to:

(a) Preparation of egg isolates including initial assessment of their growth characteristics that could subsequently be used to prepare high growth reassortants (see section 2 of this chapter).

(b) Hemagglutination Inhibition Tests of isolates for antigenic analyses.

To this end, the viruses from the National Influenza Centers are tested for how well their hemagglutinating ability is inhibited by a panel of ferret sera raised against the vaccine virus and

other past and currently circulating variants. Results are compiled in so-called HI-tables and more recently in antigenic maps (6). In addition, human sera are tested from subjects of different age (healthy adults; elderly) and locations (often USA, Europe, and Japan) who were immunized with the current influenza vaccine.

(c) Preparation of ferret sera from low-reacting variant viruses (see previous bullet) and distribution between WHO Collaborating Centers for HI-testing to low-reacting variant viruses.

(d) Genetic analyses of portions of the hemagglutinin glucoprotein. Results are captured in phylogenetic trees compiled based on the number of amino acid differences discovered in the HA of the viruses. Clustering of influenza viruses with reduced HI-titers is valuable supporting evidence on the emergence of a new dominant variant virus.

(e) Preparation of reagents test kits for National Influenza Centers and affiliated laboratories for initial virus analyses and detection of variant viruses (low reactors) and potentially new subtypes. The test kits contain diagnostic reagents including polyclonal sera, monoclonal antibodies, and viral antigens for relevant influenza strains. These kits are updated and distributed annually to ensure standardized analysis of current strains and submission of antigenic variants to WHO Collaborating Centers for detailed analysis.

(f) Assessing neuraminidase activity to detect antiviral resistance.

1.3. WHO Recommendations on Influenza Viruses for Vaccine Production

The WHO recommendation's meetings (Mid Feb; Mid Sep) are timed to both allow for sufficient virological and disease surveillance AND for influenza vaccine production, delivery, and immunization ahead of the next influenza season in the Northern and Southern Hemisphere. This tight time schedule requires compromises; e.g., vaccine manufacturers are forced to begin production at risk often in January usually with the strain they believe is the least likely one updated by WHO in February. That has resulted several times in the waste of 6–8 weeks of vaccine production. Likelihood of these losses can be reduced by close interactions between WHO Collaborating Centers and vaccine manufacturers during the weeks leading to the WHO strain selection meetings. On the other hand, duration of productive influenza surveillance may also be short with the influenza season often only beginning around end December or July, respectively, leaving little time for international exchange of strains, preparation of ferret sera, or development of high growth reassortants. Because of these constraints, there have been instances in the past when no suitable vaccine virus was available and production had to commence with the available seed virus of the preceding year (e.g., 2003).

2. Preparation of High Growth Reassortants by "Classical" Reassortment Method

Jianhua Le,
Ramanunninair
Manojkumar,
Barbara A. Pokorny,
Jeanmarie Silverman,
and Doris Bucher

2.1. Introduction

Influenza vaccines have been prepared with influenza virus grown in embryonated eggs since the 1940s ([7, 8]). In many cases, the human isolate of influenza virus grew poorly in eggs in spite of multiple passages, jeopardizing production of sufficient doses of vaccine to meet demand. In 1969, Edwin D. Kilbourne proposed that high-yielding (hy) or high-growth reassortant (hgr) viruses could be developed by coinfection of A/PR/8/34 (PR8), along with the current wild-type (wt) "target" virus with resultant "recombinants," later found to be reassortants ([9]). In this process selection against the surface antigens of PR8 is made with antisera to PR8. The antisera neutralizes any virus which has the surface antigens of PR8, resulting in virus reassortants which have the correct surface antigens. At the same time, selection for growth occurs with reassortant viruses with the property of high growth in eggs out-competing slower growing viruses. Since 1971, the type A influenza component for the majority of influenza vaccines has been produced using hy reassortants ([10, 11]).

Generation of hy reassortant vaccine seed viruses for influenza A viruses requires incorporation of the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), from wt "target" virus with one to six of the remaining genes from the hy donor virus. H3N2 hy reassortants are generated using PR8 (H1N1) as the hy donor virus to allow a clear antigenic distinction between H3N2 and H1N1 viruses, permitting ready selection with H1N1 antisera. Influenza A virus H1N1 hy reassortants are generated using an H3N2 hy reassortant donor virus which is 6:2, that is, the H3N2 donor has six "internal" genes or those genes which encode proteins other than the surface antigens from PR8 along with the two genes for H3 hemagglutinin and N2 neuraminidase. We currently use NYMC X-157 [a 6:2 hy reassortant containing the six internal genes from PR8 and the HA and NA genes from the wt virus, A/New York/55/2004]. X-157 was developed in our laboratory and used as the H3N2 component of the 2005–2006 seasonal influenza vaccine for the northern hemisphere.

2.2. Procedure

The development of hy reassortants proceeds as follows:

Step 1: Amplification of wt virus target

An egg isolate of wt "target" virus is provided to us by the CDC. This initial wt virus is amplified in 10–12-day-old embryonated SPF Premium eggs (Specific Pathogen-Free, Charles River, CT) with inoculation at a dilution of 10^{-1}–10^{-3} depending on the initial virus titer [all dilutions are made with sterile phosphate-buffered saline (PBS) with added 0.025% gentamicin (Sigma-Aldrich, St. Louis, MO)]. Incubation proceeds for 42–46 h at

35°C. After chilling at 4°C for several hours or overnight, the allantoic fluids are harvested and the hemagglutination (HA) titer determined with the use of chicken erythrocytes (0.5% in PBS).

Step 2: Coinfection of wt and hy donor viruses

Allantoic fluids for the wt and hy donor viruses are co-inoculated at equal volumes (50 μl each; total volume 100 μl) into 10–12-day-old SPF eggs at 10^{-1} and 10^{-4} dilutions, respectively (PBS with 0.025% gentamicin). The mixture is allowed to stand at room temperature for 30 min before injecting into the eggs. Generally reassortant viruses are developed independently in parallel in eight different egg lineages, with sequential egg-to-egg passage through each of the steps. In addition, control viruses, including wt and hy donor, are each independently passaged in four egg lineages.

Steps 3–5: Antibody selection

After 42–48 h of incubation at 35°C followed by refrigeration, the allantoic fluids are harvested from the embryonated eggs. Allantoic fluids containing progeny virus (100 μl) are incubated with anti-PR8 or anti-X-157 antisera or antibody (100 μl) at dilutions from 1:10 to 1:40 in order to eliminate virus containing the HA and NA from the donor virus. The mixture is incubated at room temperature for 15 min before injecting into the eggs. This antibody selection step is repeated twice. Growth of the resultant viruses is monitored by HA titer at each step. Following step 5, the antigenic identity of the hemagglutinin and neuraminidase is assessed by hemagglutination inhibition (HI) activity and neuraminidase inhibition (NI) activity. The parental source of the genes is determined by RT-PCR/restriction fragment length polymorphism (RFLP) analyses.

Step 6: Amplification

An additional passage in eggs is performed at 10^{-3}–10^{-4} dilution without antisera or antibody to amplify the virus. The harvested virus following this step is evaluated by both serological tests (HI and NI) and RT-PCR/RFLP analyses.

Steps 7 and 8: Cloning by limiting dilution

The reassortants with the highest HA titer (following step 6) and a gene constellation closest to 6:2 (6 genes from PR8 and the two surface antigens, HA and NA, from wt virus) are subjected to two sequential steps of cloning by limited dilution with cloning at dilutions ranging from 10^{-5} to 10^{-9}. Two to three eggs are used for each dilution level.

Step 9: Final amplification

The final hy reassortants are amplified @10^{-5} dilution in a minimum of 20 eggs per reassortant. Based on HA titers the harvested

allantoic fluids are pooled and the final serology and gene analyses are performed. Sterility testing is performed by streaking the sample on blood agar plates and incubating for 48 h at 35°C.

2.3. Notes

(a) The PR8 and X-157 antisera is developed against sucrose gradient-purified virus in rabbits (Pocono Rabbit Farm & Laboratory, Canadensis, PA). Antisera against PR8 and X-157 surface glycoproteins, is prepared by immunization of rabbits with HA and NA solubilized from sucrose gradient-purified virus with 7.5% N-octylglucopyranoside, centrifuged to remove viral cores followed by dialysis of the supernatants (12). Antibodies are purified on Protein G columns (Thermo Fisher Scientific, Rockford, IL). All antisera are pretreated with 100 Units/mL of receptor-destroying enzyme (RDE; Lonza, Walkersville, MD) to remove nonspecific inhibitors of hemagglutination (13).

(b) Hemagglutination (HA) Assay for viral titer determination is carried out in "V"-bottom 96-well microtiter plates using chicken erythrocytes (cRBC) standardized to 0.5% in PBS (pH 7.2).

(c) Hemagglutination inhibition (HI) Assay is performed to insure that the HA antigen was obtained from the wt strain. The assay is performed in "U"-bottom microtiter plates as per standard protocol (14).

(d) Neuraminidase inhibition (NI) Assay is performed to insure that the NA is obtained from the wt strain. The assay is performed according to the procedure described in the WHO Manual (15) with modifications (16).

(e) RT-PCR is performed using the Takara One Step RNA PCR Kit (Takara Bio Inc., Otsu, Shiga, Japan) as per manufacturer's recommendations. Briefly 2 μg of vRNA is added to the following mixture containing 10× One Step RNA PCR Buffer, 5 mM $MgCl_2$, 1 mM dNTP, 0.8 U RNase Inhibitor, 0.1 U AMV RTase XL, 0.1 U AMV-Optimized *Taq*, 0.4 μM each of forward and reverse primers (Integrated DNA Technologies Inc., Coralville, IA); the primer sequences are available on request (manuscript in preparation), and RNase-free H_2O up to a total volume of 50 μl. RT-PCR parameters used are as follows: 55°C for 30 min, 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 1 min (HA, NP, NA, M, and NS gene segments) or 60°C for 1 min (PB2, PB1, and PA gene segments), 68°C for 2 min and a final extension at 72°C for 10 min. The reactions are performed on an Eppendorf Mastercycler®. The amplified RT-PCR products are visualized on a 2% agarose-TAE/EtBr gel.

(f) PCR products are gel purified in a 2% low-melt agarose gel using QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's recommendations.

(g) RFLP is carried out in a 10 µl volume reaction mixture which contains purified DNA and restriction enzymes with their respective buffer. The reactions are carried out as per the manufacturer's recommendations. Both the hy donor and the wt viruses are digested along side with the reassortants as positive controls (17).

3. The Manufacture of Inactivated Influenza Vaccine

3.1. Introduction

Egg-based influenza vaccine has been produced for about 60 years. Initial vaccines were relatively crude and reactogenic due to the high content of egg protein remaining in the finished vaccine. The basic method of virus propagation in eggs has changed little over the 60 years apart from the use of mechanized egg-handling systems as the number of eggs processed has increased. Quality control of the eggs, egg supply, and virus seed preparation has also significantly improved over the years. High-speed ultracentrifugation, sucrose density gradient virus purification methods developed and introduced in the late 1960s and early 1970s removed egg protein from the product and significantly improved the vaccine. Subsequent downstream processing steps to produce split and surface antigen (subunit) vaccines introduced in the 1970s and early 1980s further improved vaccine purity and again significantly reduced reactogenicity. These process improvements and the introduction over the years of good manufacturing practice (GMP), Quality systems, and regulatory control have resulted in the high-quality inactivated influenza vaccines currently available. In recent years mammalian cell-based inactivated vaccines have started to be licensed for manufacture and use in Europe.

3.2. Vaccine Production Process

The processing steps are similar in most inactivated influenza vaccines currently on in production,

- Vaccine seed preparation and virus propagation,
- Allantoic fluid harvesting, clarification and virus inactivation,
- Sucrose density gradient virus purification,
- A virus-splitting process,
- Dia-filtration and sterilizing filtration to concentrated monovalent pool stage,
- Formulation of monovalent pools into trivalent vaccine,
- Fill–finish activities.

Manufacturers may perform these processing steps in a differ-ent sequence in the downstream purification and inactivation stages, to follow the specific evolution of different vaccine brands. Vaccines are however standardized by the content of the influenza surface protein hemagglutinin (HA) for each vaccine strain. The other surface protein, neuraminidase (NA) must be present in the vaccine but is not standardized.

(a) Vaccine seed preparation and virus propagation
Master and working seed is produced, from WHO recom-mended reassortant or "wild-type" viruses by serial passage at high dilution in specific pathogen-free (SPF) eggs. Following quality assurance (QA) release, suitably diluted working seed is inoculated into the allantoic cavity of com-mercial pre-incubated 11-day-old embryonated eggs which are then incubated for a further 72°h, normally at 35°C for Influenza A strains and 33°C for Influenza B strains to enable the virus to replicate.

(b) Allantoic fluid harvesting, clarification, and virus inactivation
The eggs are chilled to +4°C which kills the embryo and sets the major blood vessels to aid the harvest process as influenza virus will haemadsorb to red blood cells. The allantoic fluid is collected in a harvesting vessel, usually by a machine that removes the top of the egg and aspirates the fluid via a harvest-ing probe that enters the egg. Once harvested the fluid passes through a clarification centrifuge and is sometimes concen-trated by dia-filtration. The virus is inactivated by the addition of Formaldehyde or beta-Propiolactone for a time and tem-perature validated to kill the influenza virus. Validation studies are also required to evaluate the inactivation process on avian leucosis virus and a range of Mycoplasma bacteria.

(c) Sucrose density gradient virus purification
The virus is removed from the allantoic fluid by continuous flow into the rotor of an operational ultracentrifuge containing a sucrose density gradient. Figure 1 shows the isopycnic influ-enza virus band with polyacrylamide gel electrophoresis (PAGE) clearly demonstrating the separation of the egg pro-teins from the influenza virus proteins. The virus protein frac-tion is collected from the stationary centrifuge rotor, once all of the allantoic fluid has passed through the ultracentrifuge.

(d) Virus-splitting process
Webster and Laver (18) demonstrated that pyrogenicity was reduced by disruption of the influenza particle by sodium deoxycholate. Similar results were obtained by disruption with diethyl ether (19) and Tween–ether mixture (20). This tech-nology has been the basis for current split vaccines.

Brady and Furminger (21) demonstrated that Triton N101, a nonionic surfactant, could be used to strip the HA
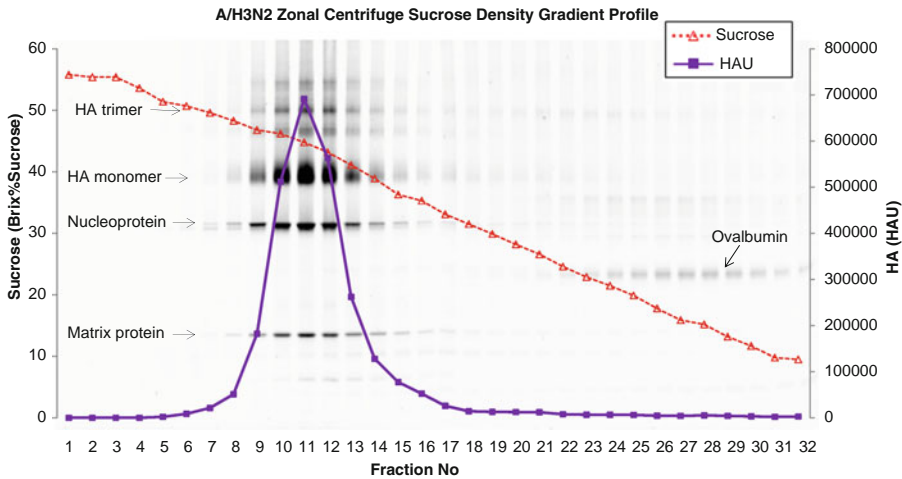
Fig. 1. A/H3N2 Zonal centrifuge sucrose density gradient profile.

and NA from the virus particle and these virus surface proteins could be separately purified by an additional ultracentrifugation step. Subsequently the ionic surfactant, cetyltrimethylammonium bromide (CTAB) was used to solubilize the influenza virus lipid layer and release the surface proteins. Sandoz patent (*Ref.: US Patent 4,064,232 Dec. 20 1977*). This technology is used to produce current <u>surface</u> antigen, but often called <u>subunit</u> vaccines.

(e) Dia-filtration and sterilizing filtration to concentrated monovalent pool stage

Once the virus has been split, residual splitting agent has to be removed by additional "downstream processing." There is usually also a dia-filtration stage to remove sucrose and a change to the final buffer either before or after the splitting stage. The sterilizing filtration stage is completed as close as technically possible to the final monovalent pool stage for sterility assurance, as these concentrates have to be held until potency values are known, which is dependent on single radial immunodiffusion (SRD) potency reagents (see Chapter 9.4).

(f) Formulation of monovalent pools into trivalent vaccine

Once SRD reagents are available and the potency of individual monovalent pools has been determined, it is possible to formulate the bulk trivalent vaccine. Currently, each dose of vaccine has to contain a minimum of 15 µg of HA for each strain and it must be stable for 1 year.

(g) Fill–finish activities

Product can either be filled in multi-dose vials or prefilled syringes depending on market requirements. Prefilled single use syringes are preservative free. Multi-dose vials however must contain a preservative which is normally thiomersal.

(h) Mammalian cell-based inactivated vaccines

New influenza cell-culture vaccines currently on the market or in late stage development replace embryonated eggs for virus propagation with a "qualified" mammalian cell line. Both Vero (monkey kidney cells) and canine kidney cells (Madin–Darby canine kidney) are in use. The downstream processing methods may differ slightly but in principle the production stages are unaltered. The biggest hurdle initially has been to convince regulators that the cell system is as safe as eggs and companies have had to make significant investments to "qualify" their specific cell lines.

(i) Pandemic vaccines

The majority of pandemic vaccines are produced in the same facilities used for seasonal vaccine. Pandemic vaccines require different antigen dosage and immunization schedule. Different adjuvants of varying safety profiles are used in some vaccines to help reduce antigen per dose, increase cross-reactivity, breath of immune response, boostability and antibody persistence (22).

4. Reagents Preparation for Potency Testing of Influenza Vaccines

John M. Wood and Una Dunleavy

4.1. Introduction

Current inactivated influenza vaccines are approved with a minimum content of 15 μg hemagglutinin (HA) per dose/strain. As antigen content in the final monobulk material may vary greatly depending upon individual strain growth characteristics and several factors particularly in the downstream process, a reproducible test for vaccine potency standardization of the HA content in the vaccine is needed.

The single radial immunodiffusion (SRD) assay was developed for influenza vaccine potency assessment in the late 1970s (23) and became established in 1979 after vaccine clinical trials in the USA and the UK first demonstrated that HA antigen content of inactivated influenza vaccines as measured by SRD correlates well with vaccine immunogenicity (24). The assay depends on the availability of an antiserum reagent (usually sheep) and a calibrated antigen standard for each vaccine component, and these reagents are supplied worldwide by four essential regulatory laboratories (ERLs) (http://www.who.int/influenza/gisrs_laboratory/collaborating_centres/en/; accessed 28 Jan 2012).

The influenza HA reacts with antibody to HA in an agarose gel to produce a precipitin ring and the size of the ring depends on the amount of HA. The HA content of an influenza vaccine is calculated by comparison of the precipitin ring formed by the vaccine with that formed by the antigen standard.

4.2. Materials

(a) SRD antigen and antiserum reagents are supplied by ERLs. They should be used according to the supplied instructions for use.

(b) Low melting temperature Seakem ME agarose (Lonza Biologicals, Slough, UK) is dissolved in PBS containing 10% (w/v) sodium azide at a concentration of 0.1% (w/v). The agarose is dissolved by boiling for approximately 20 min and stored at room temperature in single use aliquots (see Subheading 4.3.b). A microwave can also be used to dissolve the agarose.

(c) Glass plates 120×120 mm.

(d) Perspex mold to cover glass plates with either circular (90 mm diameter) or square (103 mm diameter) internal dimensions.

(e) Stainless steel punch with internal diameter of 4 mm.

(f) Template for punching holes in agarose gel, either 4×4 or 6×6 design (see 4.4.a).

(g) Whatman 1 filter paper approximately 15 cm diameter.

(h) Absorbent paper towels.

(i) 10% (w/v) Zwittergent 3–14 (Calbiochem, La Jolla, USA).

(j) Coomassie brilliant blue (BDH, Poole, UK) dissolved in destain at 0.3% (v/v).

(k) Destain solution: methanol, distilled water, and acetic acid (ratio 5:5:1).

(l) Racks to hold glass plates in stain and destain.

4.3. Methods

(a) Duplicate SRD gels will be needed for each vaccine strain being tested.

(b) Melt two aliquots of agarose for one duplicate test using a boiling water bath or a microwave. These instructions assume that a circular gel is prepared with a capacity of 16 holes (also termed wells), i.e., four antigens tested at four dilutions (see 4.4.b).

(c) When molten, pipette 13 ml aliquots of agarose into each of the two bottles pre-warmed to 56°C and allow agarose to adjust to 56°C (approximately 15 min) (see 4.4.c).

(d) Prepare two glass plates by wiping with molten agarose and allowing plates to dry. Place a mold on each plate and seal the inside edges of molds with molten agarose.

(e) Place the glass plates on a level table, add the required volume of antiserum to the 13 ml agarose, mix gently, and add agarose/antiserum mixture to each mold avoiding bubbles. Allow to set for about 30 min and then remove the mold and cover with a petri dish lid. The gels can be used immediately or stored at 2–8°C for up to 1 week before use.

Table 3
Dilution schedule for SRD assay

	Volume added at each dilution (µl)			
	1:1	3:4	1:2	1:4
Antigen	500	150	100	50
PBS	0	50	100	150

(f) Using the 4×4 template, punch wells in the gels using the stainless steel punch connected to a suction device. Allow wells to dry for about 15 min and gels are ready to use.

(g) Prepare an antigen standard reagent for each strain being tested according to supplied instructions (see 4.4.d).

(h) In tubes, add 450 µl of either antigen standard or vaccine followed by 50 µl of a 10% solution (w/v) of Zwittergent detergent. Carefully mix by vortexing and leave for 30 min at room temperature. Repeat for duplicates.

(i) Prepare a dilution series for each duplicate detergent-treated antigen using PBS according to the schedule in Table 3.

(j) Add 20 µl of each antigen dilution into the allocated well in an SRD gel according to a randomization scheme. One of the duplicate dilution series will be added to one of the duplicate gels and the second set of duplicate dilutions will go into the second set of gels. Where a vaccine is being tested, antigen dilutions will be added to SRD gels containing antiserum to each of the vaccine strains.

(k) Allow wells to empty, cover gels with a petri dish lid, and incubate for 18 h in a humid environment at 20–25°C (see 4.4.e).

(l) Remove petri dish lid, wet gel with water, and cover with a filter paper. Cover gel and filter paper with absorbent paper towels and a 600 g weight for about 30 min. Dry the gels in warm moving air until the filter paper can be easily removed.

(m) Stain and destain the gels until SRD rings can be easily distinguished from the background. Dry the stained gel.

(n) Measure the diameter (d) of each SRD ring (see 4.4.f).

(o) By converting each d into d^2 and comparing d^2 and antigen dilutions for the antigen standard and each vaccine, construct dose–response slopes using a suitable statistical program so that slopes of antigen standards can be compared with those of vaccines. The ratio of the slopes is used to calculate the vaccine HA concentration (see 4.4.g).

4.4. Notes

(a) Holes in gel should be spaced at approximately 8 mm from neighboring holes so that SRD rings do not overlap. It is important to avoid using the edge of a gel due to variability in gel thickness in this region.

(b) If other gel sizes and well formats are used, it is important to adjust agarose volume so that wells will hold 20 μl of antigen.

(c) If molten agarose is not allowed to adjust to 56°C, there may be denaturation of sheep antibodies when this is added.

(d) Antigen standards are usually freeze-dried and will need reconstitution in distilled water. The SRD assay conditions are usually designed to test vaccines at HA concentrations of 30 μg HA/ml so the antigen standard should be diluted to this concentration.

(e) The 18 h incubation time is sufficient for SRD rings of diameter 7.5–8.0 mm to be formed by vaccines containing 30 μg HA/ml provided antiserum instructions are followed. For assays of antigens with higher antigen concentrations, either a longer incubation is needed or more antiserum should be incorporated into the agarose.

(f) The measuring devices can range from a simple calibrated micrometer scale to a semi-automated image analyser.

(g) Slope ratio or parallel line analysis can be used for statistical analysis to evaluate assay validity and to calculate vaccine potency. Suitable "Combistats" programs can be obtained from EDQM in the EU (http://combistats.edqm.eu/). In the slope ratio analysis, vaccine potency = slope of vaccine ÷ slope of antigen standard.

(h) The most common problems occurring in SRD assays are

- SRD rings not reaching theoretical size at equilibrium. This results in the SRD assay underestimating vaccine potency. SRD rings can either be made smaller by increasing antigen dilution, increasing antiserum concentration or allowing longer than 18 h incubation.

- Chemicals added to a vaccine (e.g., formaldehyde, some adjuvants) can interfere with antigen diffusion. It may be necessary to assay before the chemical is added.

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Chapter 10

Genetic Engineering of Live Attenuated Influenza Viruses

Hong Jin, Zhongying Chen, Jonathan Liu, and George Kemble

Abstract

The first live attenuated influenza vaccine (LAIV) was licensed in the USA in 2003; it is a trivalent vaccine composed of two type A (H1N1 and H3N2) and one type B influenza virus each at 10^7 fluorescent focus units (FFU). Each influenza vaccine strain is a reassortant virus that contains the hemagglutinin (HA) and neuraminidase (NA) gene segments from a wild-type influenza virus and the six internal protein gene segments from a master donor virus (MDV) of either cold-adapted A/Ann Arbor/6/60 or B/Ann Arbor/1/66. MDV confers the cold-adapted, temperature-sensitive, and attenuation phenotypes to the vaccine strains. The reassortant vaccine seeds are currently produced by reverse genetics and amplified in specific pathogen-free (SPF) 9–11 days old embryonated chicken eggs for manufacture. In addition, MDCK cell culture manufacture processes have been developed to produce LAIV for research use and with modifications for clinical and/or commercial grade material production.

Key words: Live attenuated influenza vaccine, Plasmid rescue, Reverse genetics, Embryonated chicken eggs, Serum-free medium, MDCK cell culture

1. Introduction

Individual LAIV strains in the seasonal vaccine are 6:2 reassortant viruses that contain the HA and NA gene segments from circulating influenza viruses to induce protective immune responses, and the six internal protein gene segments (PB1, PB2, PA, NP, M, and NS) from cold-adapted (*ca*) A/Ann Arbor/6/60 or *ca* B/Ann Arbor/1/66 (the master donor virus for LAIV (MDV)). In addition to conferring the temperature-sensitive (*ts*), cold-adapted (*ca*), and attenuation (*att*) phenotypes [1, 2], MDV also allows the reassortant viruses to replicate efficiently in embryonated chicken eggs. Due to constant antigenic drift of the circulating influenza viruses, the individual strains of the vaccine are updated on an

annual basis such that the vaccine antigen matches the antigen of the predominant circulating strains. The classical reassortment method was used in the past to select reassortant vaccine viruses by coinfection of a wild-type virus and MDV followed by selection of the 6:2 reassortants by genotyping a number of virus progeny. Plasmid-based reverse genetics of influenza virus was available in 1998 to allow influenza viruses produced from transfected 12 or 8 plasmid cDNAs (3–5). The reverse genetics system allows rapid generation of the reassortant vaccine viruses, evaluation of amino acid sequence on vaccine virus antigenicity, immunogenicity, and protective efficacy in animal models. In addition, the system allows introduction of specific changes or manipulation of viral genomes to study viral protein function and virus–host interactions. Since the eight-plasmid rescue system requires minimal numbers of plasmids and is efficient for making reassortant vaccine strains, this system is being used for vaccine seed production.

Eight influenza virus gene segments were individually cloned into the pol I–pol II expression plasmid pAD3000 vector for virus rescue in human and simian cell lines. This plasmid is a derivative of pHW2000 containing two opposingly oriented transcription units: human polymerase I promoter and a murine pol I transcription terminator for the expression of a viral RNA, a human cytomegalovirus major immediate-early RNA polymerase II promoter, and a SV40 polyadenylation signal for the expression of a viral mRNA. cDNAs are cloned into the *Bsm*BI restriction enzyme cloning sites between the two transcription units (5). The plasmid pAD4000 vector is used for cloning of all eight influenza gene segments for virus rescue in Madin–Darby canine kidney (MDCK) cells where the human polymerase I promoter is replaced by a canine polymerase I promoter (6).

We have developed methods for creating recombinant viruses in different cell substrates, such as 293T, Vero, and MDCK cells; each with its own features and utility for specific applications. 293T cells cocultured with MDCK cells are highly efficient for rapidly generating a large number of recombinants, but they were less desirable for vaccine manufacture due to the regulatory challenges associated with these cells. Vero cells are appropriate for manufacturing vaccines, yet the virus rescue process using these cells is less efficient than 293T cells. The Vero rescue method is mainly used for preparing recombinant vaccine seeds for vaccine production in specific pathogen-free (SPF) eggs.

Human influenza viruses normally bind to cell surface receptors containing glycans with terminal sialic acids in α2–6 linkage (human-like receptor). Cultivation of influenza viruses in embryonated chicken eggs frequently results in the change at or near the receptor-binding regions of the HA protein to preferentially bind

to glycans with terminal sialic acids in α2–3 linkage (avian-like receptor), which allows the virus to replicate more efficiently in eggs. However, the changes in the HA protein may result in alteration of vaccine antigenicity (7) and could also affect vaccine immunogenicity and efficacy (8, 9). Coupled with the newly developed cell culture-based manufacture technology (6, 10, 11), MDCK cells can be used as a single-cell line to rescue recombinant vaccine strains, produce bulk vaccine, and avoid the use of eggs and other cells throughout the entire manufacturing process. Through a combination of different rescue techniques and production of virus stocks in eggs or cells, a number of questions can be addressed to understand the impact of changes at the cell- or egg-adaptation sites, the intrinsic properties of the viral replication machinery, and enables production of recombinants for further development toward clinical use.

2. Materials

2.1. cDNA Cloning of Eight Influenza Segments

1. Virus RNA extraction kit: QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA).
2. RT-PCR kit: SuperScript ™III one-step RT-PCR system with Platinum® Taq High Fidelity (Invitrogen, Carlsbad, CA).
3. PCR product purification: QIAquick PCR Purification Kit (Qiagen).
4. Restriction Enzyme: *Bsm*BI (New England Biolabs, Ipswich, MA).
5. Gel extraction: QIAEX® II Gel Extraction System (Qiagen).
6. DNA ligation: Rapid DNA ligation kit (Roche Applied Science, Indianapolis, IN).
7. Animal source-free competent *E. coli* cells: Veggie NovaBlue Singles Competent Cells (Novagen, Madison, WI).
8. Plasmid preparation: QIAprep Spin Miniprep Kit and QIAGEN plasmid Maxi kits (Qiagen).
9. Primers for influenza type A HA and NA cloning:
    (a) Bsm-A-HA-F (for influenza A HA): tattcgtctcaggg AGC AAA AGC AGG GG.
    (b) Bsm-A-HA-R (for influenza A HA): atatcgtctcgtatt AGT AGA AAC AAG GGT GTT TTT.
    (c) Bsm-A-NA-F (for influenza A NA): tattcgtctcaggg AGC AAA AGC AGG AGT.
    (d) Bsm-A-NA-R (for influenza A NA): atatcgtctcgtatt AGT AGA AAC AAG GAG TTT TT.

2.2. Plasmid Rescue of Influenza Virus by Lipid Transfection	1. 293T cells (ATCC, Manassas, VA).
	2. MDCK cells (ATCC).
	3. MEM growth medium: Minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS).
	4. Opti-MEM I-AB: Opti-MEM I (Invitrogen Cat. No. 31985) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin.
	5. TransIT-LT1 (Mirus Cat. No. MIR 2305, Madison, WI), stored at 4°C.
	6. TPCK-trypsin (Sigma Cat. No. T1426, St. Louis, MO): 1 mg/ml in 0.001 N HCl, filtered with 0.20 µM filter, aliquoted into 1 ml/vial, and stored at –20°C.
	7. Cell incubators: $37 \pm 1°C$ or $33 \pm 1°C$, $5 \pm 1\%$ CO_2, humidified.

2.3. Plasmid Rescue of Influenza Virus in Vero Cells by Electroporation

1. Vero cells: Serum-free Vero cells are cultured in OptiPro SFM (Invitrogen, Cat. No. 12309) supplemented with 4 mM L-glutamine (Invitrogen, Cat. No. 25030-081) (see Note 1). Vero cells are normally used within ten passages from the thawing of the frozen cells.

2. BioRad Gene Pulser Xcell electroporator or equivalent (BioRad, Hercules, CA).

3. Other reagents and supplies:

   (a) Opti-MEM I (Invitrogen, Cat. No. 31985).

   (b) Lima bean trypsin inhibitor (Worthington Biochemical, Cat. No. 2830, Lakewood, NJ).

   (c) TrypLE™ Select (Invitrogen Cat. No. 12563) (see Note 2).

   (d) DPBS without Mg^{2+}/Ca^{2+} (DPBS⁻, Invitrogen Cat. No. 14190).

   (e) DPBS with Mg^{2+}/Ca^{2+} (DPBS⁺, Invitrogen Cat. No. 14040).

   (f) 0.8 mm Minisart Syringe filter; Membrane material: cellulose acetate (Sartorius Cat. No. 16592K).

   (g) Gene Pulser Cuvette, 0.4 cm (BioRad Cat. No. 1652088).

   (h) Sucrose–phosphate–glutamate (SPG) 10×: 2.18 M sucrose–110 mM potassium phosphate buffer–50 mM monosodium glutamate, pH 7.0.

2.4. Plasmid Rescue of Influenza Virus in MDCK Cells by Promofectin

1. Opti-MEM I (Invitrogen, Cat. No. 31985).

2. Lima bean trypsin inhibitor (Worthington Biochemical, Cat. No. 2830, Lakewood, NJ).

3. TrypLE™ Select (Invitrogen Cat. No. 12563).

4. DPBS⁺ and DPBS⁻ (Invitrogen).

5. PromoFectin Transfection Reagent (PromoKine Cat. No. PK-CT-2000-100, Heidelberg, Germany).

Table 1
Serum-containing cell growth medium components

Component	Volume required to prepare 2,000 ml	Vendor
Minimal essential medium	1,760 ml	Hyclone, Logan, UT
Fetal bovine serum	200 ml	JRH, Lenexa, KS
200 mM L-glutamine	20 ml	JRH, Lenexa, KS
100× nonessential amino acid	20 ml	JRH, Lenexa, KS

2.5. Producing Stocks of Recombinant Influenza Virus in Embryonated Chicken Eggs

1. Embryonated chicken eggs from Charles River SPAFAS (Franklin, CT).
2. Humidified egg incubator, at 37 ± 1°C for primary egg incubation, 33 or 31 ± 1°C for secondary incubation.

2.6. Producing Stocks of Recombinant Virus in MDCK Cells

1. MDCK cells, e.g., ATCC (CCL-34) or an MDCK cell clone isolated from ATCC's MDCK cells through limiting dilution (10).
2. Viruses: 6:2 reassortant viruses produced by plasmid rescue or wild-type influenza viruses.
3. 1× TrypLE™ Select and 10× TrypLE™ Select (Invitrogen) stored at 4°C until use.
4. Lima bean trypsin inhibitor (Worthington, Lakewood, NJ) stored at 4°C until use.
5. SPG 10×: 2.18 M sucrose–110 mM potassium phosphate buffer–50 mM monosodium glutamate, pH 7.0.
6. Serum-containing cell culture medium and supplementary components (all except FBS are stored at 4°C until use) (see Note 3) (Table 1).
7. Virus infection medium.
   (a) Add 40 ml of 200 mM L-glutamine to 1,930 ml of DMEM.
   (b) Add 20 ml of 45% D-glucose and 6.1 ml of 10× TrypLE™ Select to above mixture.
   (c) Mix and stored at 4°C until use (up to 2 months).

3. Methods

3.1. cDNA Cloning of Eight Influenza Segments

Viral RNAs are extracted from influenza viruses using QIAamp Viral RNA Extraction Kit. The corresponding cDNA fragments are amplified by RT-PCR using the primers that contained the

exact termini of the vRNAs and an additional restriction enzyme cleavage site for cloning purposes. The cDNAs are digested with the restriction enzymes, gel purified, and ligated into the *Bsm*BI-digested pAD3000, transformed into the competent *E. coli* strain. Individual colonies are amplified and the plasmid DNAs are prepared by QIAprep Spin Miniprep Kit and examined by restriction enzyme digestion analysis and sequencing analysis. The HA and NA cDNA plasmids and the six internal protein gene segments of MDV that were identified to contain the desired sequences are then processed for plasmid purification by Qiagen Maxiprep Kit and used for plasmid rescue as described below (2, 5).

3.2. Plasmid Rescue in 293T Cells by Lipid Transfection

1. Mix 5×10^5 cells/ml of 293T cells and 2×10^5 cells/ml of MDCK cells in MEM medium supplemented with 10% FBS. Seed 2.5 ml of cell suspension per well of the TC-6 plate and incubate the plates in a 37°C cell incubator overnight. The cell density should be approximately 80% confluency at the time of transfection.

2. Wash cell monolayers with pre-warmed Opti-MEM I-AB twice. Add 3 ml of Opti-MEM I-AB per well 60 min prior to transfection.

3. Prepare DNA and Lipid mixture. Mix 1.0 μg of each plasmid DNA (PB2, PB1, PA, NP, M, NS, HA, and NA) in a 1.5-ml Eppendorf tube and add Opti-MEM I-AB to the plasmids to the volume of 100 μl. In a separate tube, add 16 μl of TransIT-LT1 (2 μl/μg of plasmid DNA) into 84 μl of Opti-MEM I-AB to a final volume of 100 μl and mix completely by gentle pipetting three times. After 5–10 min incubation at room temperature, add 100 μl of TransIT-LT1 and Opti-MEM I-AB mixture to 100 μl of plasmid and Opti-MEM I-AB mixture for a combined volume of 200 μl. Mix completely by gently pipetting five times, and after 30–45 min, add 800 μl of Opti-MEM I-AB into the vial.

4. Aspirate medium supernatant from the cells, add 1 ml transfection mixture to one well of cells and incubate the plates at 33°C for 6–18 h.

5. Aspirate the transfection mixture from the cells, replace with 2 ml of pre-warmed Opti-MEM I-AB containing 1.0 μg/ml TPCK-trypsin to each well, and incubate at 33°C for 3–7 days.

6. Virus from the transfected cell culture supernatant (P0) can be amplified in MDCK cells or embryonated chicken eggs as described in Subheadings 3.5 and 3.6.

3.3. Plasmid Rescue in Vero Cells by Electroporation

1. Prepare plasmid DNA: Aliquot 3.0 μg of each plasmid (PB1, PB2, PA, NP, M, NS, HA, and NA) into a sterile 1.5-ml Eppendorf tube for each electroporation reaction. Add sterile

water to a total volume of 250 µl. Add 27.5 µl of 3 M sodium acetate (pH 5.2) and 700 µl of cold (–20°C) ethanol. Cap and mix by inverting the tube for at least three times and place the tube at –70°C for 1–2 h or on dry ice for 15–30 min. Centrifuge the Eppendorf tube for 15 min at $18,000 \times g$ at room temperature. Discard the supernatant into a waste container. Add 0.5 ml 70% (v/v) ice-cold ethanol and invert. Centrifuge again for 10 min at $18,000 \times g$ and carefully remove the supernatant with a micropipette tip. Repeat centrifugation for 1 min at $18,000 \times g$. Remove residual supernatant with a micropipette tip. Be careful not to discard the DNA pellet which may or may not be visible at the bottom of the microcentrifuge tube. Air dry the pellet briefly in the BSC or on the bench until all of the residual ethanol has evaporated. Resuspend the dried DNA pellet in 20 µl sterile water for each electroporation.

2. Prepare Vero cells: The day prior to transfection, split Vero cells and seed 10^7 cells/T-225 flask in 100 ml of OptiPro SEM supplemented with 4 mM L-glutamine and incubate in the 37°C cell incubator overnight.

3. DNA transfection by electroporation: Remove Vero cell flasks from the incubator. Trypsinize Vero cells with 5-ml/T-225 flask of TrypLE™ Select and transfer the cells to a 50-ml centrifuge tube using a 10-ml pipette. Wash the T-225 flask with approximately 10 ml DPBS⁻ and transfer to the 50-ml centrifuge tube containing the Vero cell suspension. Pellet the cells by centrifugation at $400 \times g$ for 5 min and discard the supernatant. Resuspend the cell pellet in 150 µl of Opti-MEM I per T-225 flask. Remove 10 µl cell suspension and dilute 1:40 to determine cell density and viability. Add approximately 5.0×10^6 cells to each 0.4-cm sterile BioRad aluminum electrode cuvette and add Opti-MEM I to the cuvette to bring the total volume to 300 µl. Tap the cuvette gently to mix well. Add DNA mix (20 µl) and electroporate the cells at a setting of 220 V, 950 µF (the time constant should be 34–45 ms) using a BioRad Gene Pulser Xcell electroporator. Immediately after electroporation, add 700 µl of Opti-MEM I to the Vero cell suspension in the cuvette using a 1-ml pipette, and mix gently by pipetting. Transfer the electroporated Vero cells from each cuvette to one well of six-well plate (TC-6). Wash the cuvette with 1 ml of Opti-MEM I and transfer to the same well. Incubate the transfected Vero TC-6 plate in the 33°C cell incubator. Change medium after overnight incubation by aspirating the supernatant and replace with 2 ml of Opti-MEM I containing 1:33 TrypLE™ Select and continue incubation 33°C cell incubator for 2–6 days.

4. Three days after electroporation, collect 1 ml of the supernatant, add 1 ml fresh Opti-MEM I containing 1:33 TrypLE™ Select

to the cells, and continue incubation. Process the supernatant by adding 0.11 ml 10× SPG and passing through a 0.8-μm syringe filter pre-wetted with Opti-MEM I with 1× SPG. Aliquot 0.5 ml per tube, label, and store at –60°C or below. Five days after electroporation, harvest all of the supernatant and process the supernatant as described above. Virus from the transfected cell culture supernatant (P0) can be amplified in MDCK cells or embryonated chicken eggs as described in Subheadings 3.5 and 3.6.

3.4. Plasmid Rescue in MDCK Cells by Promofectin

1. Remove the MDCK culture flasks from the 37°C incubator and discard medium. Wash cell monolayer once with 15 ml DPBS⁻. Add 5-ml/T-225 flask of TrypLE™ Select and incubate in the 37°C incubator for approximately 15–20 min to completely detach the cells. Inhibit trypsin activity by adding 5-ml/T-225 flask of Lima bean trypsin inhibitor in DPBS⁺. Resuspend the cell suspension and transfer the cell suspension to a 50-ml tube using a 10-ml pipette. Wash the flask with 10 ml DPBS⁻ and transfer to the 50-ml tube with the MDCK cell suspension. Pellet the cells by centrifugation at $400 \times g$ for 5 min and discard the supernatant.

2. Resuspend the cell pellet in 20 ml of pre-warmed MEM supplemented with 10% FBS. Remove 2×0.1 ml samples of cell suspension and count the cells. Dilute cells to 1.5×10^5 viable cells/ml with pre-warmed MEM supplemented with 10% FBS and seed TC-6 plates with 2 ml of the diluted cells per well. Incubate the TC-6 plates in the 37°C incubator for approximately 24 h. The optimal cell density should be 50–60% after overnight incubation.

3. Aliquot 2.5 μg of each of the eight plasmids (PB1, PB2, PA, NP, M, NS, HA, and NA) into a sterile Eppendorf tube for each transfection. Dilute the DNA mix into 600 μl of Opti-MEM I. Vortex gently and spin down briefly.

4. Dilute 48 μl of PromoFectin solution into 600 μl of Opti-MEM I in a sterile Eppendorf tube. Vortex gently and spin down briefly. Add the diluted PromoFectin solution to the diluted DNA solution made in step 3 and immediately vortex. Spin down briefly. Incubate the PromoFectin/DNA mix at room temperature for 25–30 min.

5. Drop wise add 200 μl/well (total six wells) of the PromoFectin/DNA mixture to the MDCK TC-6 plates while gently swirling the plate. Centrifuge the TC-6-well plate at $280 \times g$ for 5 min at room temperature. Minimize mixing, gently transfer the plate to the 33°C cell incubator, and incubate for 4–5 h.

6. Gently remove the supernatant from each well of the TC-6-well plates. Add 1.5 ml of Opti-MEM I containing 1:33 TrypLE™ Select to each well and incubate in the 33°C incubator.

7. One day after transfection, examine the transfected cells microscopically and record the cell density. Add 1.0 ml of Opti-MEM I containing 1:33 TrypLE™ Select to each well and incubate in the 33°C incubator.

8. Three days after transfection, examine the transfected cells microscopically for the appearance of CPE and record. Add 1.0 ml of Opti-MEM I containing 1:33 TrypLE™ Select to each well and incubate in the 33°C incubator.

9. Remove 50 µl of supernatant from the well(s) which exhibit >50% CPE or where >50% of the cells have detached for the HA assay. Harvest all the supernatants from the wells with the highest HA titers. Centrifuge the supernatant at $500 \times g$ for 5 min. Collect the supernatant and add 11% volume of 10× SPG (final 1× SPG). Aliquot in 2-ml cryovials and store at –60°C or below.

10. Examine the transfected plates by microscope daily for the appearance of CPE until 7 days after transfection. If the CPE is not obvious on day 7, harvest 1 ml of the supernatant and infect MDCK cells in TC-6-well plate. If no virus detected after 7 days of incubation, discard the plate.

3.5. Production of Recombinant Virus Stock in Eggs

1. Incubate SPAFAS eggs at 37°C for 10–11 days in the primary egg incubator.

2. Candle eggs under lamp and mark the inoculation site at the position of allantoic cavity, spray egg surface with 70% alcohol, inoculate each egg with 0.1 ml of the filtered transfected cell supernatant using a 1-ml sterile syringe, seal the inoculation site with hot wax, and incubate at 33°C for 2–3 days.

3. Chill the eggs at 4°C for 12–24 h. Harvest the allantoic fluid from each egg with a sterile pipette into a 15-ml tube. Aliquot 0.5 ml for hemagglutination (HA) testing. Select HA positive fluids, add 0.11 volume of 10× SPG (final 1× SPG), aliquot viruses into sterile 2-ml screw-cap tubes, label, and store at –60°C or below.

4. To amplify large amount of viruses in eggs, inoculate each egg with 10^3–10^4 PFU/egg of virus, incubate at 33°C for 2–3 days and harvest allantoic fluid as above.

5. Titrate virus by plaque assay (PFU), 50% tissue culture infectious dose (TCID50) or fluorescence focus assay (FFA) in MDCK cells.

3.6. Production of Influenza Virus Stock in MDCK Cells

3.6.1. Prepare MDCK Cells for Virus Infection

1. Add 35 ml of pre-warmed (37°C) cell growth medium to a T-75 tissue culture flask.

2. Thaw quickly one vial of frozen MDCK cells (1×10^7 cells/vial) in a 37°C water bath and transfer the content of the vial to the cell growth medium in the T-75 tissue culture flask.

3. Place the T-75 tissue culture flask in a humidified incubator at 37°C and 5% CO_2, and incubate for 2–3 h.

4. Remove the T-75 tissue culture flask from the incubator and examine for cell attachment under a light microscope. Make sure at least 50% of the cells are attached to the bottom of the T-75 tissue culture flask. If the cells are not attached, repeat steps 1–4 with a new vial of cells.

5. Return the tissue culture flask to the incubator and continue incubation for 3–4 days to allow the cells reach over 80% confluence before virus infection.

3.6.2. Subculture of MDCK cells

1. Remove a tissue culture flask of growing cells from the incubator and examine cell morphology and confluent level. The cells should be firmly attached to the bottom of the tissue culture flask with uniform epithelial cell appearance and ideally reach more than 80% confluence in the first 3–4 days postseeding.

2. Remove the cell culture supernatant by aspiration with a pipette.

3. Add 5 ml/T-75 tissue culture flask or 10 ml/T-225 tissue culture flask of 1× TrypLE™ Select to the flask and incubate at 37°C for approximately 15 min.

4. Tap the flask against the palm of a hand and look at the bottom of the flask under a light source. When the bottom surface is covered with moving cell clumps (this can be verified by examining the flasks under a light microscope), mix the TrypLE™ Select and cell mixture with equal volume (5 ml/T-75 tissue culture flask or 10 ml/T-225 tissue culture flask) of lima bean trypsin inhibitor. Pipette up and down the cell suspension with a 5-ml pipette at least ten times to disburse the large cell clumps.

5. Count the cells using a hemocytometer and calculate the cell concentration in the cell suspension.

6. Add cell growth medium to new tissue culture flask(s) at the following volume: 35 ml/T-75 tissue culture flask or 100 ml/T-225 tissue culture flask.

7. Add appropriate volume of the cell suspension in step 4 based on the cell count calculated in steps 5 to make the final cell concentration at 5×10^4 cells/ml (1.75×10^6/cell T-75 or 5×10^6/cell T-225 flask) in the new tissue culture flask(s).

8. Place the new tissue culture flasks in an incubator and incubate the cells at 37°C and 5% CO_2 for 3–4 days to allow the cells reach at least 80% confluence before virus infection.

3.6.3. Virus Infection

1. On the day of infection, calculate the amount of virus stock required for infection using the following equation:

Volume (in μL) of virus stock = (total cell number x MOI */ titer of virus stock) x 1,000

where MOI is the multiplicity of infection. It is normally expressed as virus infectious unit per cell and may range from 10^{-6} to 1 fluorescent focus unit/cell.

2. Mix the virus stock with virus infection medium to prepare the virus inoculum.

3. Place the virus inoculum on ice until use.

4. Examine the tissue culture flasks to be infected under a light microscope. Note the cell morphology and confluence level.

5. Take one flask, trypsinize and count the cells as described in steps 2–5 in Subheading 3.6.2.

6. Aspirate the cell culture media out of the flask with a pipette.

7. Wash the cell monolayer by adding 5 ml/T-75 tissue culture flask or 15 ml/T-225 tissue culture flask of DPBS⁻, rinse the cells and then aspire the buffer out of the flask.

8. Repeat the above cell-washing procedure one more time.

9. Add 35 ml/T-75 tissue culture flask or 100 ml/T-225 tissue culture flask of virus inoculum to the cells.

10. Place the infected flasks in an incubator and incubate at 33°C and 5% CO_2 for 3–4 days.

11. Centrifuge the tissue culture fluid collected from the infected flasks at 13,000 rpm for 2 min and collect the supernatant.

12. Mix 1 part of 10× SPG buffer with nine parts of the harvested supernatant.

13. Aliquots the above mixture in a volume of 0.5–10 ml in size in cryovials and store at –60°C freezer until use.

14. Take one aliquot and determine the virus titer by an infectivity assay such as FFA (12) or TCID50 assay (13).

4. Notes

1. Serum-free Vero cells can be replaced by regular Vero cells that are cultured in serum-containing medium.

2. TrypLE™ Select can be replaced with regular trypsin for cell splitting and by TPCK-trypsin for virus infection.

3. Both serum-containing and serum-free cell culture media can be used to culture the MDCK cells and grow influenza viruses.

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Chapter 11

Influenza A Virus Molecular Virology Techniques

Bin Zhou and David E. Wentworth

Abstract

Molecular biological techniques for genomic analysis and for creation of recombinant viruses are critical tools in our efforts to understand and combat influenza A viruses. These molecular virology approaches are used in diagnostics, basic research, molecular epidemiology, bioinformatics, and vaccine development. The majority of the techniques used to study this segmented negative-sense RNA virus begin by purifying RNA from the virus, or infected cells, and converting it to cDNA, then to dsDNA, and amplifying that dsDNA using reverse transcription in combination with the polymerase chain reaction (RTPCR). The RTPCR amplicons can be probed, sequenced, or cloned into a variety of vectors for further analysis and to create recombinant influenza A viruses by plasmid-based reverse genetics. To accelerate the amplification and cloning process, we developed multi-segment-RTPCR (M-RTPCR) techniques that efficiently amplify the eight genomic viral RNA segments (vRNAs) of influenza A virus in a single reaction, irrespective of the virus strain. The M-RTPCR amplicons are ideal for nucleotide sequence analysis and cloning full-length vRNAs into plasmids or other vectors designed for protein expression or reverse genetics. Therefore, we also developed modified reverse-genetics plasmids that are designed to rapidly clone M-RTPCR products, or other full-length vRNA amplicons, using recombination-based techniques. The combination of M-RTPCR and recombination-based cloning confers sensitivity, speed, fidelity, and flexibility to the analysis and rescue of any strain/subtype of influenza A virus, without the need for in vitro propagation. The specific topics described in this chapter include purification of high-quality viral RNA, genomic amplification using two different M-RTPCR schemes, sequencing vRNA amplicons, and cloning vRNA amplicons into our modified reverse-genetics plasmids, or commercially available plasmids.

Key words: Influenza A virus, RTPCR amplification, M-RTPCR, Cloning, Recombination, Ligase independent, Reverse genetics, Sequencing

1. Introduction

The influenza A virus genome (~13.5 kb) is composed of eight negative-sense vRNAs that range in size from 0.89 to 2.3 kb and encode up to 11 proteins (1). Influenza A virus diagnostics, bioinformatics/genomic analysis, basic research, and vaccine creation by reverse genetics are all facilitated by a variety of molecular virology techniques. These techniques include the purification of high-quality

vRNA, RTPCR procedures that convert the vRNAs to dsDNAs and amplify the dsDNA, cloning RTPCR amplicons for a variety of downstream applications (e.g., sequencing, protein expression, or reverse genetics), and methods that enable rapid nucleotide sequence analysis. Although a plethora of approaches for RTPCR amplification and/or detection of a specific vRNA, or small regions within a vRNA, have been developed, the extensive sequence diversity found among influenza A viruses hindered the development of a single method for the full-length amplification of the eight vRNAs that compose the viral genome.

A few approaches have been developed for single-reaction genomic amplification (2–4) or for rapid cloning into reverse-genetics plasmids (5, 6). These powerful techniques (2–6) represent significant advances that have improved influenza A virus vRNA amplification, cloning, and reverse genetics; however, each has limitations such as the need to multiplex many different oligonucleotide primers or require at least eight separate RTPCR reaction vessels. Although we strongly encourage people to read these papers, the primary topics covered in this chapter are recently developed techniques that enable the amplification of the entire genome of any influenza A virus in a single RTPCR reaction, and the subsequent use of these amplicons either for nucleotide sequencing or for cloning into commercially available plasmids, or modified reverse-genetics plasmids to rapidly create recombinant influenza viruses (7). M-RTPCR is a very robust method that can be used for historic, contemporary, or novel emerging strains of influenza A virus, regardless of their diverse genotypes. Some of the advantages of M-RTPCR include (1) prior sequence information is not required; (2) 1 primer set amplifies the entire segmented genome in a single reaction; (3) genomic amplification directly from swab specimens, which provides more accurate sequence information and clones that are free from artifacts selected by virus propagation in the laboratory; (4) its ideally suited for high-throughput DNA sequencing and array platforms; (5) could be a valuable method in the field, because different subtypes/strains/reassortants can be identified easily by differences in the mobility of amplicons in standard agarose gel electrophoresis; (6) provides a universal platform to speed cloning into reverse-genetics plasmids or other vectors (e.g., protein expression). We describe two different primer sets that are optimized for efficient genomic amplification by M-RTPCR. One primer set was designed for sequencing or cloning of the amplicons into commercial plasmids and the other set was primarily designed for cloning into modified reverse-genetics plasmids. We modified a 12-plasmid-based reverse-genetics plasmid (pHH21 (8)) and a bidirectional 8-plasmid-based reverse-genetics plasmid (pDZ (9)) to facilitate cloning of full-length vRNA amplicons produced by M-RTPCR (or other approaches) via

ligase-independent in vitro recombination (we refer to Chapter 12 for a detailed discussion of reverse-genetics approaches). Similar modifications could be made to any reverse-genetics plasmid or other vectors to accelerate cloning of influenza A virus vRNAs.

2. Materials

2.1. Purification of Viral RNA

1. RNeasy Mini Kit, catalog # 74104 (QIAGEN, Valencia, CA); if using the kit for the first time add ethanol to buffer RPE as described by the manufacturer.
2. Additional RNase-free 1.5-ml snap-cap centrifuge tubes.
3. Molecular biology grade ethanol (100%).
4. Biosafety cabinet for working with infectious materials (e.g., virus cultures).
5. Refrigerated microcentrifuge at 4°C is recommended.

2.2. Influenza A Virus Genomic Amplification by Multi-segment RTPCR

1. Superscript III HF RT-PCR kit catalog # 12574-035 (Invitrogen, Carlsbad, CA).
2. Make 10 μM stocks of the oligonucleotide primers for your use only (see Note 1).
   MBTuni primer set (see Note 2):

(a) MBTuni-12	5′-ACGCGTGATC<u>AGC*A*AAAGCAGG</u>-3′
(b) MBTuni-12.4	5′-ACGCGTGATC<u>AGC*G*AAAGCAGG</u>-3′
(c) MBTuni-13	5′-ACGCGTGATC<u>AGTAGAAACAAGG</u>-3′

Uni/Inf primer set (see Note 3):

(d) Uni12/Inf-1	5′-GGGGGG<u>AGC*A*AAAGCAGG</u> 3′
(e) Uni12/Inf-3	5′-GGGGGG<u>AGC*G*AAAGCAGG</u>-3′
(f) Uni13/Inf-1	5′-CGGGTTATT<u>AGTAGAAACAAGG</u>-3′

The region of primers that is complimentary to influenza A virus vRNA or full-length positive sense RNAs is underlined.

3. Aliquot the 2× RT-PCR buffer, primers, and enzyme mix into RNase-free tubes which will be for individual use only.
4. Temperature cycler.
5. RNase-free, DEPC-treated water.
6. Molecular biology grade ethanol (100%).
7. RNase-free 0.2-ml PCR tubes or plates.

2.3. Purification of Amplicons for Nucleotide Sequencing or Cloning	1. QIAquick PCR purification kit catalog # 28104 (QIAGEN, Valencia, CA). 2. Sodium acetate, 3 M, pH 5.0, filtered through a 0.22 μm membrane. 3. Microcentrifuge.
2.4. Sanger Sequencing of M-RTPCR Amplicons	1. Virus strain-specific primers. 2. Access to institutional DNA sequencing core or commercial provider. 3. DNase-free 1.5-ml snap-cap tubes.
2.5. Cloning of M-RTPCR Amplicons into Reverse-Genetics Plasmids by Recombination-Based Cloning Techniques	1. QIAprep Spin Miniprep Kit catalog # 27106 (QIAGEN, Valencia, CA). 2. *Pst*I-HF restriction endonuclease catalog # R3140S (New England Biolabs, Ipswich, MA). 3. In-Fusion Advantage PCR Cloning Kit catalog # 639620 (Clontech, Mountain View, CA). 4. Buffer TE (pH 8.0). 5. One Shot TOP10 chemically competent *Escherichia coli* catalog # C4040-03 (Invitrogen, Carlsbad, CA). 6. 37°C Heat block/incubator (shaking preferred). 7. 42°C Water bath/heat block (water bath preferred). 8. 10-cm Diameter LB agar plates containing 100 μg/ml of ampicillin. 9. S.O.C. broth. 10. Ice bucket with wet ice.
2.6. TOPO TA Cloning Influenza Virus Amplicons	1. TOPO TA cloning kit (Invitrogen, e.g., pcDNA™3.3-TOPO® TA Cloning Kit catalog # K8300-01). Includes chemically competent *E. coli*. 2. Items 5–10 from Subheading 2.5. 3. DNA Clean and Concentrator™-5, catalog # D4013 (Zymo Research Corporation, Orange, CA) [optional].

3. Methods

3.1. Purification of Viral RNA	Isolation of RNA free of contaminants and RNases may be the most critical step for the amplification of dsDNA copies of full-length RNAs representing each of the eight vRNAs that compose the genome of influenza A virus. There are many RNA isolation kits and "home-brews" available to choose from, and most of these can be successfully employed for the isolation of high-quality RNA.

In fact, it is the laboratory researchers' attention to detail and maintenance of an RNase-free environment that is critical for RTPCR of genomic-length fragments. That being said, we find that the use of QIAGEN products for low- to high-throughput RNA extraction work well, and we recommend using Qiamp vRNA extraction kit or RNeasy RNA extraction kit because they offer the most reproducible results for multiple researchers that have different sources of virus-containing starting material. This section focuses on the RNeasy extraction procedure as it is very simple and works well for the isolation of viral RNA from tissue culture supernatants, allantoic fluid, or primary swab specimens, and it can be used for low-, medium-, and high-throughput procedures (see Note 4).

3.1.1. Important Factors for RNA Purification

Avoid Contamination

- Most downstream techniques employ very sensitive PCR procedures so extreme care should be used to avoid DNA contamination of the tubes, solutions, etc.

- If possible work in a dedicated "clean-BSC" within a clean room in which nucleic acid isolation is conducted, but the use of PCR amplification products, plasmids, and/or virus amplification is avoided.

- Clean the bench area and replace any bench liners in preparation for noninfectious work. (Recommend 1–10% bleach, followed by 70% ETOH).

- RNases are stabile, ubiquitous, and on our skin so use gloves (recommend nitrile).

- Use RNase/DNase-free tubes and handle them as little as possible.

- Label all RNA isolation and plasticware containers so all members of lab are aware to keep these items free of contaminating RNases or nucleic acids (e.g., RNA only, recommend storing tubes and other plasticware in the original containers that each user individually controls).

- Pour tubes from bag or beaker onto saran wrap, pickup, close lid, label, and rack.

- Use filter tips for pipetting.

- Use dedicated tube openers to open microcentrifuge tubes, rather than hands.

Other Points for Consideration

- *Biosafety*. Influenza viruses are human and animal pathogens that are transmitted via aerosols and direct contact, so always work with infectious virus in a certified biosafety cabinet using appropriate personal protective equipment and precautions.

- Check off protocol steps when they are completed.

- Always setup an extraction negative control(s) to identify contamination.

Read through RNA cleanup protocol, pg 56 RNeasy mini Handbook (version 04/2006). Follow safety guidelines for reagents and solutions (Buffer RLT contains high concentration of guanidine salt).

3.1.2. RNA Purification Procedure

1. Aliquot enough RLT for the total number of RNA purifications being done into a 50-ml conical tube (e.g., 3.6 ml for 9 samples and 1 negative control).

2. Add 350 μl of RLT to each labeled individual snap-cap tube.

3. Add 100 μl of virus-containing allantoic fluid, culture supernatant, or swab specimen and pipette five times to mix (*If you have less than 100 μl, adjust the volume up to a final of 100 μl using RNase-free ddH2O*).

4. Cap and mix well and incubate 5–10 min at room temperature.

5. Setup RNeasy Mini spin columns in 2-ml collection tubes (supplied) in a stable rack (make sure to label the column appropriately).

6. Add 250 μl molecular biology grade ethanol (100%) to the diluted RNA, and mix well by pipetting eight times, and transfer the sample (~700 μl) immediately to the spin columns placed in a 2-ml collection tube (setup in step 5).

7. Close the lid gently and centrifuge for 15 s at $8,000 \times g$. Discard the flow-through and return spin column to the same collection tube. (*Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Initial centrifugation should be done at (20°C) and elution should be done at 4°C*).

8. Add 500 μl Buffer RPE to the spin column. Close the lid and centrifuge for 15 s at $8,000 \times g$. Discard the flow-through.

9. Add 500 μl Buffer RPE to the spin column. Close the lid and centrifuge for 2 min at $8,000 \times g$.

10. Place the RNeasy spin column into a new 2-ml collection tube (supplied in kit), and discard the old collection tube with the flow-through.

11. Close the lid and centrifuge at maximum speed (12,000–$16,000 \times g$) for 1 min.

12. Carefully remove the column after centrifugation and place the RNeasy spin column into a new 1.5-ml collection tube (supplied in kit).

13. Add 30 μl RNase-free water to the center of the spin column membrane. Close the lid gently and centrifuge for 1 min at 12,000–$16,000 \times g$ at 20–25°C to elute the vRNA. (The caps of the tubes are likely to break off during this step, so be prepared to transfer the eluted RNA to a new well-labeled tube).

14. Keep these tubes at 4°C or on ice while working in the lab and store the RNA at –80°C for future use. (The RNA concentration is usually so low that the spectrophotometer is not useful for determining the concentration).

3.2. Influenza A Virus Genomic Amplification by Multi-segment RT-PCR

This section describes the use of M-RTPCR to simultaneously amplify the complete genome using 1 set of three oligonucleotide primers (7). There are two sets of primers (3 primers/set) that have been optimized for efficient M-RTPCR. These 5′-tailed primers are complimentary to the conserved 5′- and 3′ termini of each vRNA segment, which base pair to form the promoters for influenza virus RNA transcription and replication.

3.2.1. Important Factors

Avoiding contamination

- As discussed for RNA purification, PCR procedures are very sensitive and once a lab begins to use M-RTPCR, amplicons and clones of all influenza A virus vRNAs are being processed throughout the workspace. Therefore, unidirectional workflow should be strongly considered. Keep reaction setup areas of the laboratory separate from analysis and cloning areas.

- Virtually all other considerations listed for RNA purification are also applicable for RTPCR.

- Keep primer stocks and reagents well maintained (proper storage etc.) and free from contamination.

3.2.2. M-RTPCR Procedure

1. Isolate the influenza RNA from 100 µl of allantoic fluid, tissue culture supernatant, or swab material as described in Subheading 3.1, or thaw on ice if stored at –80 (see Note 5).

2. Thaw either the MBTuni or the Uni/Inf primer set (described in Subheadings 2 and 4).

3. Thaw Superscript III HF RT-PCR reagents and mix by vortexing (except for the enzyme), then place on ice.

4. Determine the volume of RNA (usually use 2.5 or 5 µl) to be used per reaction, the volume of DEPC-treated water to be added to the master mix, and the volume of master mix to be added to each tube from the total reaction volume (typically a 25 µl reaction is sufficient for cloning and one should double the volumes below for 50 µl reactions for subsequent sequencing).

Typical reaction planning example for a total reaction volume of 25.0 µl:

If the volume of RNA per M-RTPCR reaction tube is 2.5 µl
Then volume of DEPC-treated ddH$_2$O added to Master Mix should be 8.5 µl
And the volume of Master Mix (per tube) should be 22.5 µl

5. Make up the Master Mix on ice. Add H_2O first and the enzyme last.

Typical master mix example using Uni/Inf primer set (see Note 6):	
Per reaction	Total volume for 25 reactions
8.5 μl DEPC-treated ddH$_2$O	212.5 μl DEPC ddH$_2$O
12.5 μl 2× RT-PCR buffer	312.5 μl 2× RT-PCR buffer
0.20 μl, 10 μM Uni-12/Inf1	5.0 μl, 10 μM
0.30 μl, 10 μM Uni-12/Inf3	7.5 μl, 10 μM
0.5 μl, 10 μM Uni-13/Inf1	12.5 μl, 10 μM
0.5 μl RT/HiFi enzyme mix	12.5 μl RT/HiFi enzyme mix

6. Add 22.5 μl of Master Mix to 24 PCR tubes (0.2 ml) at 4°C (see Note 7).

7. Add 2.5 μl of RNA template (including negative RNA purification control) or ddH$_2$O (PCR negative control) to tubes (see Note 8).

   Make sure to record which tube # corresponds to each sample in your notebook.

8. Place reaction tubes into a temperature cycler that is paused at 42°C (warm start).

9. Select the 3 stage-cycling parameters:
   42°C/60 min; 94°C/2 min, then 5 cycles of (94°C/30 s; 44°C/30 s; 68°C/3 min), followed by 23–31 cycles of (94°C/30 s; 57°C/30 s; 68°C/3 min) and then hold at 4°C (see Note 9).

10. Analyze 5 μl of the M-RTPCR reactions by 0.8% agarose gel electrophoresis. An example of the results obtained from optimal (A) and typical (B) M-RTPCR amplifications are shown in Fig. 1. Differences in migration of the HA and NA segments are sometimes easily detected prior to sequencing [compare lanes 1–4 (H1N1) with lanes 5–10 (H7N2)] (Fig. 1b). The polymerase vRNAs are very similar in size (PB2 and PB1 are 2.3 kb and PA is 2.2 kb) so they migrate together.

11. Store amplicons at –20°C.
    This procedure typically yields 50–80 ng/μl of DNA and this is related to the amount and quality of the template RNA.

3.3. Purification of Amplicons for Nucleotide Sequencing or Cloning

The M-RTPCR (and standard RTPCR) amplicons can be used directly in various applications such as enzymatic digestion, subtyping with specific primers, and can be directly cloned (particularly if you are using TOPO TA described below). However, to obtain optimal sequencing quality and cloning efficiency, the amplicons should be purified using commercially available PCR cleanup columns such as the QIAGEN QIAquick PCR purification kit used in this protocol
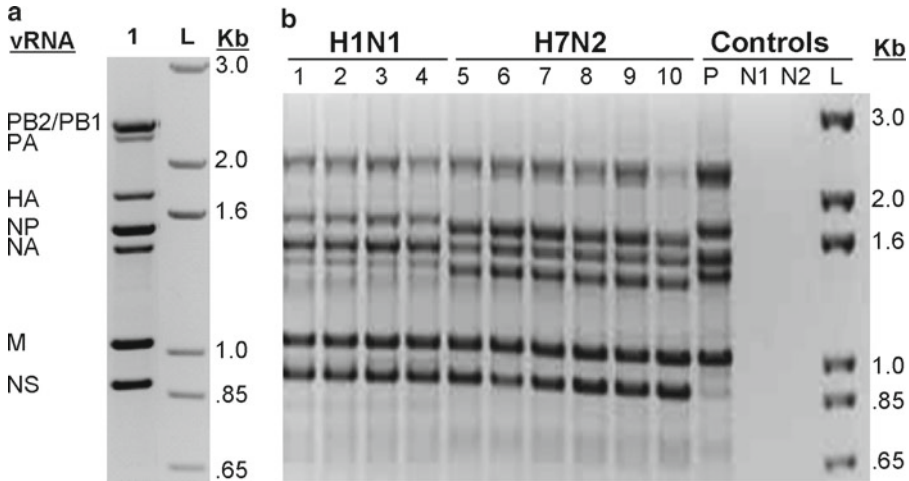
Fig. 1. M-RTPCR amplification of human and avian influenza A viruses. The viral genomes of a 2009 pandemic H1N1 virus (**a**) and a series of hemagglutination-positive specimens isolated swabs collected from live bird markets in New York (**b**) were amplified by M-RTPCR with the Uni/Inf primer set, and 5 μl from each of the reactions was analyzed by agarose gel electrophoresis (negative image of the ethidium bromide-stained gels are shown). The vRNA segment corresponding to the amplicon is listed to the left of *panel A*. The amplicons in *panel B* lanes 1–10 were subsequently sequenced and the subtype of viruses corresponding to lanes 1–4 was determined to be H1N1 and lanes 5–10 were determined to be H7N2 (note the slight shift in the migration pattern of the HA and NA vRNA amplicons). L, 1 kb + DNA ladder (Invitrogen); P, positive control for M-RTPCR (A/USSR/77, H1N1); N1, RNA purification negative control; and N2, M-RTPCR ddH$_2$O template control.

(see Note 10). Due to the high specificity of the M-RTPCR amplification, purification from the agarose gel is generally not necessary and will result in significant loss of the amplicons.

3.3.1. DNA Amplicon Purification Procedure

Before starting, read through the QIAGEN MinElute Handbook (03/2008) and be sure that all buffers are at the appropriate working concentrations.

1. Add 24 ml of 100% molecular biology grade ethanol into the buffer PE (concentrate) bottle to obtain 30 ml working solution of buffer PE.

2. Add 5 volumes (100 μl) of buffer PB to 1 volume (20 μl) of the M-RTPCR products and mix. Add 1 μl of 3 M sodium acetate (pH 5) and mix.

3. Place a QIAquick spin column in a 2-ml collection tube (provided).

4. Apply the sample to the column and centrifuge for 1 min at 16,000×*g*.

5. Discard flow-through and place the column back into the same 2-ml tube.

6. Add 0.75 ml buffer PE into the column, let it stand for 2 min, and centrifuge for 1 min at 16,000×*g*.

7. Discard flow-through and place the column back in the same tube. Centrifuge the column for an additional 1 min at $16,000 \times g$.

8. Make sure that no liquid is on the side or bottom of the column and place it in a clean 1.5-ml microcentrifuge tube.

9. Add 30 µl buffer EB to the center of the column and ensure that it completely covers the membrane, let the column stand for 1 min and centrifuge for 1 min at $16,000 \times g$ (see Note 11).

10. Measure the concentration of the purified DNA with spectrophotometer and/or run 2 µl on an agarose gel with known standards to estimate/confirm the DNA concentration.

11. Use for subsequent procedures or store at –20°C.

3.4. Sanger Sequencing of M-RTPCR Amplicons

Sanger-based nucleotide sequencing reactions are routinely carried out by institutional cores or commercial services. Therefore, this section focuses only on the factors important for initial reaction setup and the sequencing reaction method is not discussed in detail. For our Sanger approach, outlined below, the DNA is sequenced on an Applied Biosystems (ABI, by Life technologies, Carlsbad, California) 3730xl or ABI 3130xl sequencer using BigDye v3.1 Terminator Kits, and the results are analyzed with ABI Sequence Analysis 5.2 using the KB basecaller (see Note 12). For the typical automated DNA sequencing approaches used to be successful, two factors are crucial (1) the template should be of high purity and (2) the template must be accurately quantified. Failure to meet either of these criteria may result in poor or no useful sequence data.

The M-RTPCR amplicons can be sequenced directly after purification in Subheading 3.4, using virus-specific primers. Ideally, a combination of spectrophotometry and agarose gel electrophoresis of the purified amplicons will give the best estimation of the amount of the DNA to use for each sequencing reaction. However, we found that the following conditions work reasonably well, given the primers match the template.

3.4.1. Sanger-Sequencing Procedure (See Note 13)

1. Amplify the genome using M-RTPCR (recommend 50 µl reactions) or any region of the genome using strain-specific primers and purify them as described above (Subheading 3.4).

2. To sequence the PB2, PB1, and PA segments, mix 5 µl (~200 ng) of purified M-RTPCR amplicons with 3.3 pmol of virus sequence-specific primer for one sequencing reaction.

3. To sequence the HA, NP, NA, M, and NS, mix 2 µl (~80–100 ng) of purified M-RTPCR amplicons with 3.3 pmol of sequence-specific primers for one sequencing reaction.

4. If you are sequencing small regions of amplified DNA, a good rule of thumb is to multiply the length of the product by 0.1 to determine the number of nanogram to mix with 3.3 pmol of primer.

M-RTPCR will amplify any influenza A virus and it can be used on specimens for which sequence information is not available. Two approaches can be easily used to initially determine the probable virus lineage and subtype so that strain-specific primers can be identified for subsequent sequencing.

1. Sequence the M-RTPCR products using a conserved M-segment primer (M-217, 5′-TCACGCTCACCGTGCCC AG-3′) and analyze the results using Blast (10).

2. Gel purify each segment using commercially available kits and use one of the M-RTPCR primers (e.g., MBTuni-12) per sequencing reaction to partially sequence each gene (for small gene segments such as NS and M, the use of both amplification primers in two separate reactions will provide complete coverage of the gene).

3.5. Recombination-Based Cloning of M-RTPCR Amplicons into Reverse-Genetics Plasmids

The strict requirement for precise initiation and termination of influenza vRNA-like transcripts that is required for reverse genetics dramatically limits the restriction endonucleases available for cloning DNA copies of influenza vRNAs into reverse-genetics plasmids. Thus, cloning techniques independent of restriction sequence and ligation significantly simplify and accelerate this process. Therefore, we developed modified reverse-genetics plasmids that can be used to directly incorporate M-RTPCR amplicons (or more traditional vRNA amplicons) based on recombination between identical sequences in the termini of the M-RTPCR amplicons and the linearized reverse-genetics plasmids (7).

3.5.1. Rational and Design of Recombination-Based Bidirectional Reverse-Genetics Plasmids pBZA15 and pBZ61A18 for Use in Eight-Plasmid-Based Rescue of Influenza A Viruses (See Note 14)

We modified a bidirectional reverse-genetics plasmid pDZ (9) by inserting a linker of 22 bp, to introduce an In-Fusion™ cloning site between the existing RNA polymerase I promoter and terminator, creating pBZ61A15 (Fig. 2). Some influenza A virus vRNAs have a cytosine substitution at position 4 of the 3′ terminus; therefore, pBZ61A18, which has a guanosine substituted for the adenosine in the fourth position of the complement of the 3′-terminal promoter element of influenza A virus was also created. These reverse-genetics plasmids were designed for cloning dsDNA copies of any influenza A virus vRNA segment using recombination between short regions (15–19 nucleotides) of identity between the plasmid and influenza A virus amplicons using commercially available enzymes. Ligase-independent recombination-based cloning can be accomplished using "home-brew" approaches (11); however, the In-Fusion kit (Clontech) is recommended because of its efficiency and reproducible results. Amplicons of each of the eight vRNA segments that are present after M-RTPCR can be cloned simultaneously into the *Pst*I-linearized pBZ61A15 or pBZ61A18 plasmids in a single reaction by using In-Fusion cloning (see Note 15). Alternatively, any amplicon of an influenza A vRNA segment, which is appropriately

Recombination-based Reverse-genetics Plasmid   **M-RTPCR Amplicons**
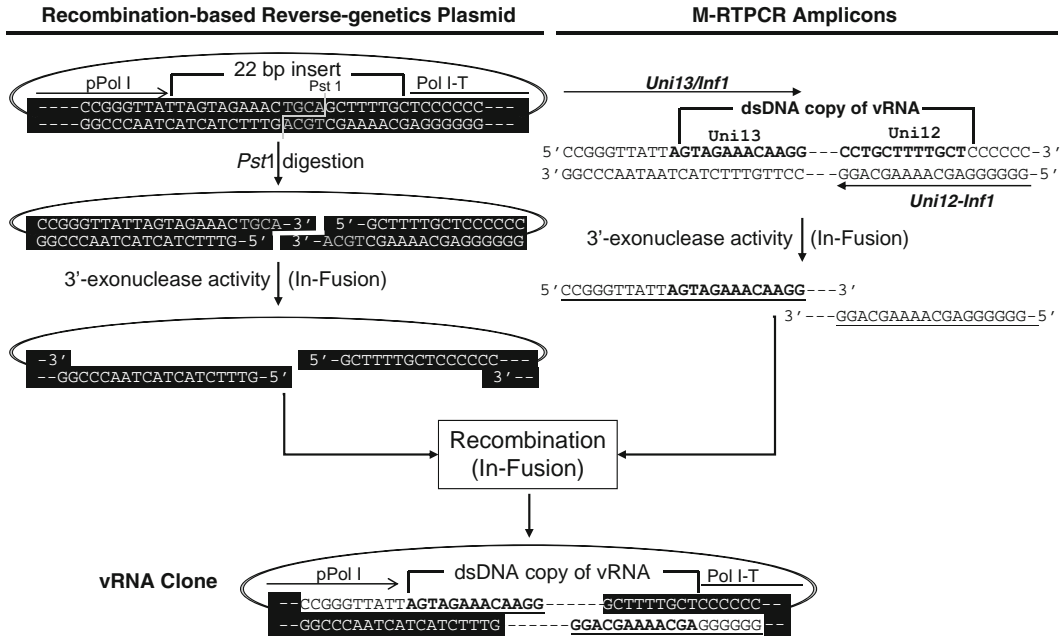
Fig. 2. Recombination-based cloning of M-RTPCR amplicons into reverse-genetics plasmids. Schematic diagram of the procedure used to clone M-RTPCR amplicons into our recombination-based reverse-genetics plasmid pBZ61A15. The M-RTPCR amplicons (1 segment shown) contain a dsDNA copy of a vRNA segment flanked by ten (5′) or six (3′) bp that are derived from the 5′-tails incorporated into the M-RTPCR primers (Uni13/Inf1 and Uni12/Inf1). The 13-conserved nucleotides at the 5′ termini of all influenza vRNAs (Uni13) and the 12-conserved nucleotides found at the 3′ termini (Uni12) are shown in *bold type*. To generate the reverse-genetics plasmid, a 22 bp fragment containing 9 bp that correspond to the 5′ termini of all vRNAs, followed by 4 bp (shown in gray) to create the *Pst*1 site, followed by 9 bp that correspond to the 3′ termini of all vRNAs, was inserted between an RNA polymerase I promoter (pPol I) and terminator (Pol I-T). The RNA polymerase II promoter and poly A signal, which flank the Pol I promoter and terminator in an opposing orientation, in this plasmid are not shown. The plasmid is digested with *Pst*1 and purified. The M-RTPCR amplicons and *Pst*1 linearized plasmid are mixed and treated with In-Fusion enzyme, and exonuclease activity exposes the complementary nucleotides at the termini leading to annealing and recombination (*underlined* nucleotides are the primer sequences used in M-RTPCR). The clones generated produce mRNAs from the RNA polymerase II promoter and authentic vRNAs are transcribed by RNA polymerase I upon transfection into appropriate host cells.

tailed at the 5′ terminus (6–9 nt), can be cloned into these pBZ plasmids (or any properly designed plasmid) using the procedures outlined below.

3.5.2. Preparation of Linearized Reverse-Genetics Plasmids

1. Prepare pBZ61A15 and pBZ61A18 plasmids with any commercial kits (e.g., QIAprep Spin Miniprep Kit) or standard alkaline lysis methods.

2. Digest 5 μg of each plasmid with 200 U (10 μl) of restriction endonuclease *Pst*I, in a 300 μl reaction volume, at 37°C for 6 h.

3. Analyze 10 μl of the digestion by agarose gel electrophoresis to ensure complete digestion.

4. Purify the digested products using the QIAquick PCR purification kit following the procedure described in Subheading 3.3, but elute with 50 μl buffer EB instead of 30 μl.

5. Measure DNA concentration using spectrophotometer and dilute DNA to 50 ng/μl, aliquot multiple vials each containing 20 μl diluted DNA.

6. Store vials at –20°C.

Typically ~4 μg of linearized plasmid DNA will be recovered from 5 μg of starting material.

3.5.3. Cloning of Amplicons into pBZ61A15 and pBZ61A18 or Other Similarly Modified Reverse-Genetics Plasmids (See Note 16)

1. Amplify the influenza A virus genome by M-RTPCR using the Uni/Inf primer set, as described in Subheading 3.2, using 28–30 temperature cycles total (see Note 17) and purify as described in Subheading 3.3.

2. Mix 200 ng of purified M-RTPCR amplicons with 100 ng of linearized pBZ61A15 and pBZ61A18 in two separate 200 μl PCR reaction tubes. Bring the volume to 7 μl each with ddH$_2$O (see Note 18).

3. Add 2 μl of 5× In-Fusion reaction buffer and 1 μl of In-Fusion enzyme into each tube and pipette to mix completely but gently.

4. Use a temperature cycler to incubate the mixture at 37°C for 15 min and 50°C for 15 min, followed by 4°C hold.

5. Dilute the mixture with 40 μl TE buffer (pH 8), pipette to mix, and leave on ice.

6. Thaw competent cells on wet ice, add 3 μl of each diluted mixture into a corresponding vial of cells. Tap the vials gently to mix. Keep the vials on wet ice.

7. Incubate on ice for 30 min. Tap the vials every 10 min.

8. Incubate vials at 42°C (water bath preferred) for exactly 30 s, followed by immediate incubation on wet ice for 2 min.

9. Add 450 μl of room temperature S.O.C. into each vial.

10. Incubate the transformation mixture for 1 h at 37°C (shaking at 250 rpm is desirable).

11. Spread 20 and 200 μl of each transformation onto two labeled LB Agar plates containing 100 μg/ml of ampicillin (or appropriate antibiotic).

12. Incubate the plates inverted at 37°C for 16–20 h. Typically there are thousands of colonies on the 200 μl plate and hundreds of colonies on the 20 μl plate.

13. Screen for clones using standard PCR amplification across the insert region, or enzymatic digestion to identify the colonies containing the correct clones. If the nucleotide sequence is not determined for the 4th position of the 3′ terminus of vRNA, then PB2, PB1, and PA clones should be selected from the pBZ61A18 vector, whereas HA, NP, NA, M, and NS should be selected from the pBZ61A15 vector.

3.6. TOPO TA Cloning Influenza Virus Amplicons

Although the In-Fusion and other ligase-independent cloning systems are amenable to any plasmid vector (including other reverse-genetics plasmids), there are a number of situations in which it is desirable to clone fragments into commercially available plasmids developed for specific functions (e.g., sequencing, RNA transcription, or protein expression). The TOPO TA Cloning Systems available (Invitrogen) are fast, efficient, and reliable for cloning any RT-PCR products, including M-RTPCR amplicons, into a wide range of plasmids (see Note 19). This system takes advantage of the fact that *Taq* DNA polymerases (including the high-fidelity version we recommend for M-RTPCR) add single non-templated nucleotides to the 3′ termini of amplicons [often these are 3′ adenosine (A) overhangs]. The vectors are provided as linearized plasmids with 3′ deoxythymidine (T) overhangs that is bound to topoisomerase I, which efficiently ligates the inserts and plasmid. The 3′ A overhangs of the PCR product complement the 3′ T overhangs of the vector and allow for fast ligation (5 min).

3.6.1. TOPO TA Cloning Procedure

1. Amplify the influenza A virus genome by M-RTPCR using either primer set as described in Subheading 3.2, using 28–30 temperature cycles total. Alternatively, amplify a specific segment using virus-specific terminal primers or segment-specific universal primers described by Hoffmann et al. (5) (see Note 20).

2. Concentration and purification of amplicons (optional, see Note 21).

   (a) Prepare buffers for Zymo Research, Inc. DNA Clean and Concentrator™-5 as directed by manufacturer.

   (b) Add 40–50 μl of M-RTPCR, or other RTPCR reaction, to 1.5-ml tube.

   (c) Add 250 μl of the DNA Binding Buffer and mix briefly by vortexing.

   (d) Transfer mixture to the Zymo-Spin™ Column in a collection tube.

   (e) Centrifuge at 12,000–16,000×g for 30 s. Discard the flow-through.

   (f) Add 200 μl Wash Buffer to the column, and centrifuge at 12,000–16,000×g for 30 s.

   (g) Repeat wash step.

   (h) Add 10 μl of ddH$_2$O directly to the column matrix.

   (i) Transfer the column to a 1.5-ml microcentrifuge tube and centrifuge at 12,000–16,000×g for 30 s to elute the DNA.

3. Add 4 μl of amplicons from step 1, or purified amplicons from step 2, to a 0.2-ml PCR tube or a 1.5-ml tube.

4. Add 1 μl of salt solution (provided in kit).

5. Add 1 μl of the TOPO plasmid, mix by pipetting, and incubate 5 min at 22–25°C, then place on ice or store at –20°C.

6. Transform chemically competent Top10 *E. coli* (supplied) with 2–4 μl of the TOPO reaction as described in Subheading 3.5.3, steps 6–13.

4. Notes

1. Two (MBTuni-12 and MBTuni-12.4, or Uni12/Inf-1 and Uni12/Inf-3) of the three primers used in each set are nearly identical except for a single nucleotide (italicized). The *A* or *G* is used because the 3′ termini of the vRNA segments vary at the 4th position (U or C), and this is dependent on the segment and the virus lineage. Often the polymerase segments have a C at this position and most other segments have a U, however this has not been extensively studied. It is recommended that the two primers be synthesized independently rather than synthesizing a primer with an R at this position (e.g., 5′-ACGCGTGATCAGC*R*AAAGCAGG-3′), so that the ratio of the two primers can be controlled.

2. Universal influenza A virus primers with 5′-tails that contain *Mlu*1 and *Bcl*1 restriction enzyme sites (*Mlu*1 is very rare in influenza A virus). The MBTuni primer set generally yields more robust amplification of all segments, but the ratio of small segment to large segments is greater than the Uni/Inf set. This primer pair is recommended for sequencing of the amplicons and can also be used for cloning of full-length segments into standard plasmids via ligation, TOPO TA, or ligase-independent cloning approaches.

3. The Uni/Inf primer set was designed primarily for cloning influenza A virus gene segments into reverse-genetics plasmids using ligase-independent cloning procedures, but can also be used for other downstream cloning and sequencing approaches. This primer set typically yields more uniform amplification of each gene segment.

4. If starting with cloacal swabs, we recommend isolation of virus by inoculation of eggs or TRIzol (Invitrogen) extraction with 10 μg of carrier tRNA.

5. Keep RNA template on ice (4°C) at all times.

6. Unequal concentrations of the Uni12/Inf1 and Uni12/Inf3 are used to improve amplification of the larger vRNAs (PB1, PB2, and PA). If using the MBTuni primer set, the same volumes should be used for MBTuni-12 and MBTuni-12.4.

7. The master mix was calculated for one additional reaction to account for small losses during pipetting.

8. The use of negative controls for both the RNA isolation process and the M-RTPCR reaction itself can help to identify the source of contamination if it occurs.

9. There are a number of variables that need to be considered and incorporated into the cycling parameters. The warm start reduces nonspecific amplification products. The regions of the oligos that hybridize with the influenza vRNAs are very short and A:T rich; therefore, 3–8 cycles with a lower annealing temperature (43–45°C) are required to incorporate the tailed primers into cDNA and for initiation of second strand synthesis. Finally the total number of cycles used varies depending upon the ultimate use of the amplicons. If the amplicons will be used for sequencing, 36 cycles (5 with an annealing temperature at 44°C and 31 with an anneal temperature of 57°C) are typically used. If the amplicons will be used for cloning purposes, 28 cycles (5 with an annealing temp at 44°C and 23 with an anneal temperature of 57°C) are typically used. Finally, the type of cloning procedure to be used should also be considered, if the products will be incorporated into TA-based cloning plasmids it is advisable to add a 10 min hold at 68°C between the 3rd stage and the hold at 4°C, because this will increase the percentage of the amplicons that have incorporated non-templated 3′ adenosines.

10. We also find that the columns from Zymo Research, Inc. described later (Subheading 3.6.1) are very good for purification, particularly if you need to concentrate the amplicons.

11. The elution is very sensitive to pH. If using water to elute, make sure that the pH > 7. DEPC-treated water sometimes can have a quite acidic pH. Therefore, we recommend following QIAGEN's guidelines and use Tris–HCl pH 8.0 for elution of the DNA. We avoid TE because the EDTA can have inhibitory effects in some enzymatic reactions.

12. Some minor adjustments based upon the equipment and reagents used may be required, so you should discuss the sequencing of PCR products with your sequencing service provider.

13. Although M-RTPCR amplicons are well-suited for a high-throughput genomic sequencing using Sanger, or massively parallel technologies such as 454 sequencing, this section describes approaches for use by typical academic-type laboratories. Also, to sequence a complete genome one would need to setup ~10 M-RTPCR reactions. Another approach for complete genome sequencing is to use M-RTPCR for a first-round amplification and then use the amplicons as templates for overlapping PCR reactions with multiple primer sets. Primers for

this type of approach were developed as part of the NIAID influenza genome sequencing project and can be found on the J. Craig Venter Institute website (http://gsc.jcvi.org/projects/ msc/influenza/infl_a_virus/primers.shtml).

14. A modified 12-plasmid reverse-genetics plasmid (pG26A12) derived from pHH21 was also produced and is described by Zhou et al. (7). We also refer to Chapter 12 for more details about the various reverse-genetics approaches.

15. The In-fusion enzyme has 3′-exo nuclease activity, so the 3′-overhang left from *Pst*I digestion will be removed in the reaction and will not interfere with the recombination of the identical sequences between the amplicons and plasmids (Fig. 2). In a previous version, we used *Stu*I (7) as the linearization enzyme; however, *Pst*I is a more efficient enzyme than *Stu*I.

16. Amplicons from segment-specific RTPCR reactions that have the same 5′-tails as the Uni/Inf primers can also be cloned into these pBZ plasmids. Additionally, virtually any plasmid (including most reverse-genetics plasmids) can be modified by the insertion of the In-Fusion cloning sites similar to those we have designed.

17. To reduce/limit mutations created during PCR, we use fewer temperature cycles for cloning procedures. Three–five cycles with an annealing temperature of 44°C are required to tail the DNA copies but the second stage can be reduced and this will reduce total yield but improve fidelity.

18. It is advisable to include the following controls in the In-Fusion reaction and transformation: (1) linearized vector only for In-Fusion reaction followed by transformation, (2) amplicon only for In-Fusion reaction followed by transformation, (3) pUC19 (included in the competent cell kit) control for transformation efficiency, and (4) mock-transformed bacteria as a control.

19. We recommend these systems primarily because they are straightforward and provide reproducible results.

20. The MBTuni primer set is recommended for this type of cloning because these primers contain restriction enzyme recognition sequences that are convenient for colony screening and subcloning into other plasmids.

21. Although concentration and purification of amplicons is not required for cloning RTPCR amplicons into TOPO TA plasmids, we find that this can increase the number of positive colonies and thereby reduce screening efforts.

Acknowledgments

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Chapter 12

Reverse Genetics of Influenza Viruses

Gabriele Neumann, Makoto Ozawa, and Yoshihiro Kawaoka

Abstract

The ability to modify influenza viruses at will has revolutionized influenza research. Reverse genetics has been used to generate mutant or reassortant influenza viruses to assess their replication, virulence, pathogenicity, host range, and transmissibility. Moreover, this technology is now being used to generate approved influenza virus vaccines and develop novel vaccines to combat seasonal and (future) pandemic influenza viruses. Several variations of the original system have been established, all of which are considerably robust and efficient.

1. Introduction

Scientists faced several challenges in generating influenza viruses from plasmids. The genome of influenza viruses comprises 8 (influenza A and B viruses) or 7 (influenza C viruses) negative-strand viral RNAs (vRNAs), all of which are required to generate functional viruses. The negative-strand vRNA is not a template for translation and is therefore noninfectious; hence, the components of the viral replication machinery (i.e., the three polymerase subunits PB2, PB1, and PA, and the nucleoprotein NP) are required for viral replication and transcription. Thus, to artificially generate influenza A viruses, 12 components are needed—eight to synthesize the eight vRNAs, and four to synthesize the polymerase and NP proteins. These components must be delivered into the nucleus, the site of influenza virus replication and transcription. In addition, the artificially generated vRNAs need to mimic authentic influenza vRNAs, which do not possess 5′-cap and 3′-polyA structures. This is achieved by inclusion of the RNA polymerase I
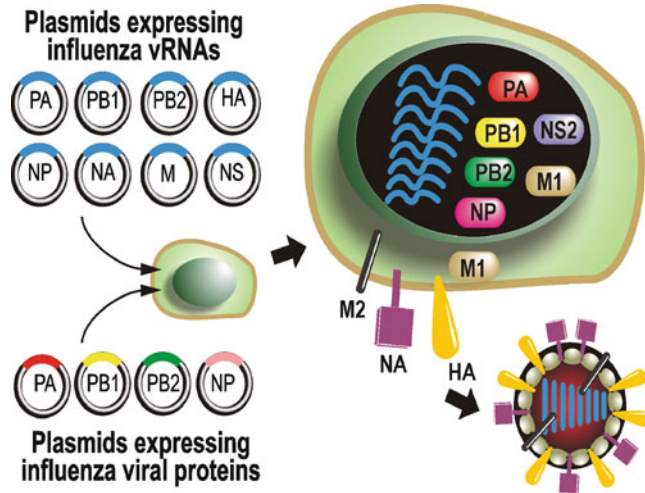
Fig. 1. Artificial generation of influenza viruses (reverse genetics). Cells are transfected with eight plasmids for the transcription of the eight viral RNAs (vRNAs), and with four plasmids for the expression of the polymerase and NP proteins. The polymerase and NP proteins amplify the transcribed vRNAs, resulting in the expression of all of the viral proteins and the amplification of the vRNAs, leading to influenza viruses. Figure modified after Neumann and Kawaoka, 2001, Virology 287:243–250.

transcription system, in which influenza viral cDNAs are inserted between the RNA polymerase I promoter and terminator sequences (1). RNA polymerase I is an abundant nuclear enzyme that transcribes non-capped and non-polyadenylated ribosomal RNAs. This transcription system thus allows the artificial generation of authentic influenza vRNAs in the nucleus of plasmid-transfected cells. The PB2, PB1, PA, and NP proteins are provided by standard RNA polymerase II-driven protein expression plasmids (1) (Fig. 1).

This basic approach is described in detail in this chapter. Variations of this theme include (1) vectors that produce both the vRNA and mRNA from one template (2); (2) vectors that combine RNA polymerase I transcription units (3); (3) an adenovirus system for cell transduction, rather than plasmid transfection (4); and (4) a T7 RNA polymerase transcription system that uses this enzyme and a ribozyme sequence for vRNA synthesis (5). These variations are briefly described in Subheading 4.

2. Materials

2.1. Influenza Viral RNA Extraction

1. "RNase Away" (Molecular Bio-Products, Inc., San Diego, CA, or Sigma-Aldrich, St. Louis, MO).
2. Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA).

3. Add β-Mercaptoethanol to buffer RLT before use: 10 μl β-Mercaptoethanol per 1 ml buffer RLT; buffer RLT with β-Mercaptoethanol is stable for 1 month.

4. QIAshredder (Qiagen, Valencia, CA).

2.2. Viral cRNA Synthesis	1. PCR Nucleotide Mix (dNTP; Roche, Basel, Switzerland).
	2. SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).
	3. RNasin Ribonuclease Inhibitor (Promega, Madison, WI).
2.3. PCR Amplification of Viral cDNAs	1. Phusion DNA polymerase (Finnzymes, Espoo, Finland).
2.4. Cloning of Viral cDNAs into RNA Polymerase I Transcription Vector	1. *Bsm*BI and *Bsa*I (NEW ENGLAND BioLabs, Ipswich, MA).
	2. DNA Ligation Kit, Version 2.1 (Takara, Otsu, Japan).
2.5. Generation of Protein Expression Plasmids	1. Protein expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) or pCAGGS-MCS (Subheading 3.5, step 4)
2.6. Transfection of Eukaryotic Cells with Plasmids for vRNA and Protein Synthesis	1. DMEM (Lonza, Basel, Switzerland).
	2. Fetal bovine serum (Invitrogen, Carlsbad, CA).
	3. Pen–Strep (Invitrogen, Carlsbad, CA).
	4. L-Glutamine 200 mM (Invitrogen, Carlsbad, CA).
	5. OPTI-MEM (Invitrogen, Carlsbad, CA).
	6. TransIT-LT1 Transfection Reagent (Mirus, Madison, WI).
2.7. Amplification of Influenza Viruses	1. Newborn calf serum (Sigma, St. Louis, MO).
	2. MEM (Invitrogen, Carlsbad, CA).
	3. Bovine serum albumin (Invitrogen, Carlsbad, CA).
	4. Pen–Strep (see above).
	5. L-Glutamine 200 mM (see above).
	6. MEM Vitamin Solution (Invitrogen, Carlsbad, CA).
	7. MEM Amino Acids (Invitrogen, Carlsbad, CA).
	8. Trypsin, TPCK-Treated (Worthington, Lakewood, NJ).
2.8. Amplification of Influenza Viruses That Require Exogenous Proteases for HA Cleavage	1. Trypsin, TPCK-Treated (see above).

3. Methods

The generation of influenza viruses can be divided into the following steps: (1) The extraction and amplification of the viral RNAs. Here, efficient RNA extraction and RT-PCR protocols are of critical importance. (2) Sequence analysis of the viral RNAs. (3) Cloning of the viral cDNAs into the appropriate plasmid vectors for vRNA or protein synthesis. This step requires the selection of vectors and restriction enzymes, the establishment of efficient ligation and transformation protocols, and the selection of *E. coli* clones possessing plasmids with viral cDNA inserts. (4) Transfection of cells with several plasmids. For highly efficient influenza virus generation, it is essential to use cell lines that can be transfected efficiently, and to optimize the parameters of transfection. (5) Amplification of generated viruses, where virus yield is dependent on the cell line used and its trypsin requirements.

3.1. Influenza Viral RNA Extraction

1. RNA-destroying enzymes (RNases) are commonly found on work surfaces such as benches, racks, pipettes, etc., and on human skin. Sterile gloves are, therefore, recommended for the RNA extraction process. In addition, work surfaces may be cleaned with "RNase Away" or similar reagents.

2. It is also recommended to keep the sample chilled throughout the RNA extraction procedure.

3. Mix the predetermined volume of virus stock (see Note 1) with 350 μl of buffer RLT (Qiagen RNeasy Mini Kit). The protocol outlined here is based on the Qiagen RNeasy Mini Kit (Qiagen); however, other RNA extraction kits or procedures may be used.

4. Transfer lysed virus solution onto a QIAshredder spin column placed in a 2-ml collection tube.

5. Centrifuge for 2 min at highest speed, preferably at 4°C.

6. Add 350 μl of 70% ethanol to lysate in collection tube and mix by pipetting.

7. Place one RNeasy mini column in a 2-ml collection tube and transfer lysate.

8. Centrifuge for 15 s at >8,000 × g and discard flow-through.

9. Add 700 μl buffer RW1 to RNeasy column. Centrifuge for 15 s at >8,000 × g as described above.

10. Transfer RNeasy column to new 2-ml collection tube.

11. Add 500 μl buffer RPE to RNeasy column. Centrifuge (see step 8) and discard flow-through.

12. Add 500 µl buffer RPE to RNeasy column. Centrifuge for 2 min at >8,000×g to dry RNeasy column membrane.

13. Transfer RNeasy column to 1.5-ml microtube (not supplied with extraction kit).

14. Pipet 30–50 µl of RNase-free H$_2$O (supplied with the Qiagen RNeasy Mini Kit) onto membrane.

15. Centrifuge for 1 min at >8,000×g to elute RNA.

16. Store RNA at –80°C until further use.

17. We typically do not determine the RNA concentration or 260/280 nm ratio at this point.

3.2. Viral cDNA Synthesis

1. The following protocol is based on SuperScript III Reverse Transcriptase (Invitrogen). Other commercially available enzymes or kits for reverse transcription may be used.

2. Mix the following components on ice:

Extracted RNA (see Subheading 3.1)	10 µl
dNTP mix (Roche, 10 mM each)	2 µl
"Universal" primer (10 µM, see Note 2)	1 µl
RNase-free H$_2$O (supplied with Qiagen RNeasy Mini Kit)	13 µl

3. Incubate at 65°C for 5 min.

4. Immediately place tube on ice.

5. Add the following components:

5× buffer (provided with enzyme)	8 µl
DTT (provided with enzyme)	2 µl
RNase inhibitor (Promega)	2 µl
SuperScript III (Invitrogen)	2 µl
(Total volume: 40 µl)	

6. Incubate at 50°C for 60 min, followed by 15 min at 70°C.

7. Place tube on ice.

8. The resulting cDNA can now be used for PCR amplification or stored at –20°C.

3.3. PCR Amplification of Viral cDNAs

1. The twelve 3′-terminal nucleotides and the thirteen 5′-terminal nucleotides of influenza viral RNAs are highly conserved among the eight segments and among influenza A viruses. The highly conserved sequences are followed by segment-specific

nucleotides that are also highly conserved among influenza A viruses. Based on this information, Hoffmann et al. (6) described segment-specific oligonucleotides that can be used for most influenza A viruses:

```
Segm.           Forward Primer                              Reverse Primer

 PB2    TATTGGTCTCAGGGAGCGAAAGCAGGTC        ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT

 PB1    TATTCGTCTCAGGGAGCGAAAGCAGGCA        ATATCGTCTCGTATTAGTAGAAACAAGGCATTT

  PA    TATTCGTCTCAGGGAGCGAAAGCAGGTAC       ATATCGTCTCGTATTAGTAGAAACAAGGTACTT

  HA    TATTCGTCTCAGGGAGCAAAAGCAGGGG        ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT

  NP    TATTCGTCTCAGGGAGCAAAAGCAGGGTA       ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT

  NA    TATTGGTCTCAGGGAGCAAAAGCAGGAGT       ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT

   M    TATTCGTCTCAGGGAGCAAAAGCAGGTAG       ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT

  NS    TATTCGTCTCAGGGAGCAAAAGCAGGGTG       ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT
```

The sequences complementary to the influenza viral sequences are in boldface. Segment-specific sequences are underlined. The recognition sites for the restriction endonucleases *Bsm*BI (CGTCTCN$_{1/5}$) and *Bsa*I (GGTCTCN$_{1/5}$) are italicized. For more details on the cloning strategy, refer to Fig. 2 and see Notes 3 and 4.

2. To the synthesized cDNA (Subheading 3.2, step 7), add the following components:

H$_2$O	32.0 μl
5× buffer (provided with the enzyme)	10.0 μl
dNTPs (provided with the enzyme)	1.0 μl
Forward primer (10 μM)	2.5 μl
Reverse primer (10 μM)	2.5 μl
cDNA (from Subheading 3.2)	1.0 μl
Phusion DNA polymerase (Finnzymes)	1.0 μl

3. PCR amplification:

One cycle:	30 s at 98°C
35 cycles:	10 s at 98°C 30 s at 55°C 1.5 min at 72°C
One cycle:	5 min at 72°C
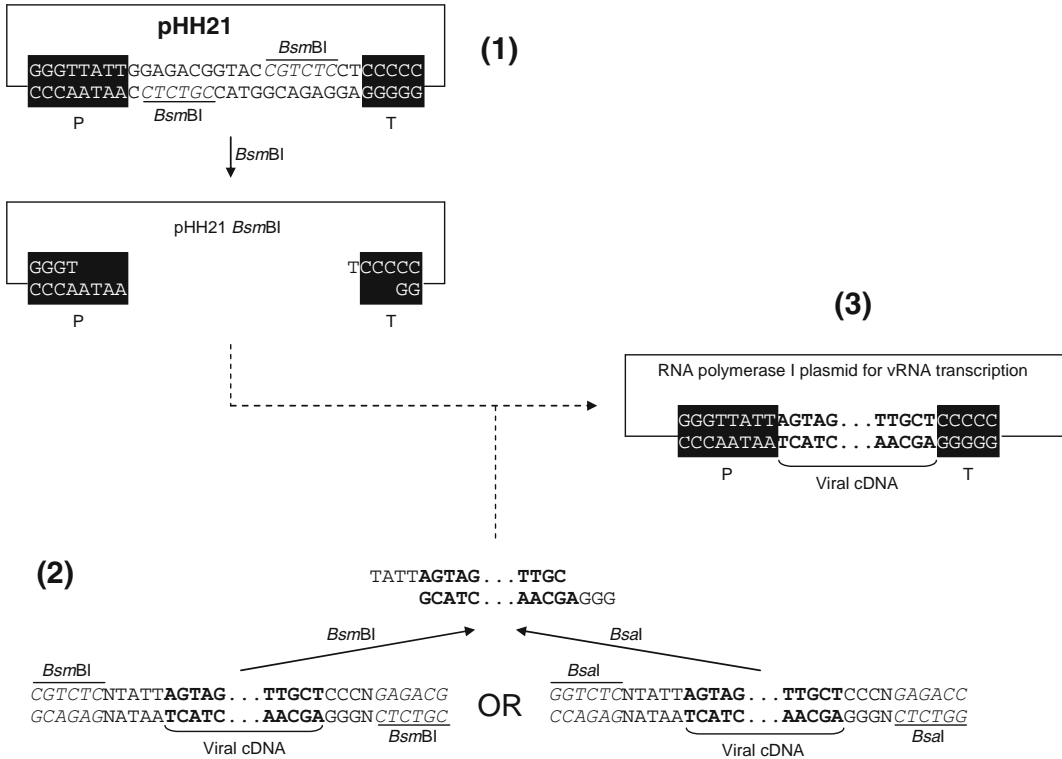
Fig. 2. Cloning strategy for the generation of RNA polymerase I plasmids for vRNA transcription. (1) Vector preparation. Vector pHH21, which contains polymerase I promoter (P) and terminator (T) sequences, is incubated with *Bsm*BI, yielding the vector fragment. (2) Insert preparation. Influenza viral RNAs are RT-PCR amplified with oligonucleotides possessing *Bsm*BI (*left panel*) or *Bsa*I (*right panel*) restriction sites. Incubation with *Bsm*BI or *Bsa*I results in inserts with the same 5′- and 3′-terminal nucleotide overhangs, which can be ligated with the vector fragment. (3) Cloning product. In the resulting construct, the influenza viral cDNAs are nucleotide-specific fused between the promoter and terminator sequences, yielding transcripts that are identical to influenza viral RNAs. The schematic diagram illustrates that (1) the insert can be inserted in only one orientation, (2) several restriction endonucleases, including *Bsm*BI, *Bsa*I, or *Bbs*I (not shown here) can be used to amplify viral RNAs, and (3) the *Bsm*BI sites in the vector are eliminated in the cloning process. *Black boxes*: RNA polymerase I promoter (P) or terminator (T) sequences. Influenza viral sequences are in *boldface*. Recognition sites of restriction endonucleases are *italicized* and *underlined*.

4. Purify the PCR product on agarose gel: Elute purified PCR product in 20 μl H$_2$O.

3.4. Cloning of Viral cDNAs into an RNA Polymerase I Transcription Vector

1. Incubate PCR products with *Bsa*I at 50°C for at least 4 h or with *Bsm*BI at 55°C overnight.

   (a) For *Bsm*BI, incubation overnight is recommended because of its low activity.

   (b) Increased efficiency can also be achieved by incubating with *Bsm*BI for several hours, adding more enzyme, and then continuing the incubation at 55°C.

2. Incubate the RNA polymerase I transcription vector (i.e., pHH21) with *Bsm*BI as described above (see Notes 5 and 6).

3. Gel-purify vector and insert.

4. Ligation:

pHH21 treated with *Bsm*BI (100 ng/μl)	1 μl
Influenza viral cDNAs treated with *Bsm*BI or *Bsa*I (100 ng/μl)	4 μl
DNA Ligase (Takara)	5 μl
Incubate for 30 min at 16°C	

5. Transform ligation mixture into competent *E. coli* cells (JM109 or DH5α *E. coli* strains are best, but other strains may be used).

6. Inoculate transformed *E. coli* cells on agar plates containing 100 μg/ml ampicillin (note that pHH21 encodes the ampicillin-resistance gene).

7. Incubate plates overnight at 37°C.

8. Pick individual *E. coli* colonies and characterize by restriction analysis and/or PCR analysis.

   (a) Note that the cloning strategy results in the elimination of the *Bsm*BI recognition sequences in the vector; hence, clones cannot be characterized by using *Bsm*BI (see Fig. 2 and Note 3).

   (b) Note that the cloning strategy results in the predetermined orientation of the insert; hence, the orientation of the inserts does not need to be assessed (see Fig. 2 and Note 3).

9. For clones that possess an influenza viral cDNA insert, carry out sequence analysis of the insert:

   (a) If the sequence of the influenza viral segment is already known, sequence analysis will confirm the absence of unwanted mutations introduced by RT-PCR.

   (b) Otherwise, at least three individual clones should be sequenced to establish a consensus sequence.

10. For a clone that matches the consensus sequence and does not possess unwanted mutations, prepare plasmid DNA (>1 μg) for DNA transfection.

   (a) The quality of the DNA affects the transfection efficiency and the use of commercially available kits (such as the Qiagen Plasmid Midi Kit or Qiagen Plasmid Maxi Kit, or other comparable kits) is recommended.

   (b) To avoid cross-contamination of plasmid preparations, plasmid DNAs should be prepared individually.

3.5. Generation of Protein Expression Plasmids

1. In addition to the eight RNA polymerase I plasmids for the transcription of the eight influenza viral RNA segments, plasmids for the expression of the influenza viral PB2, PB1, PA, and NP proteins must be prepared (see Note 7).

2. Design oligonucleotides that amplify the PB2, PB1, PA, and NP open-reading frames.

3. As a template for PCR amplification, use the respective

   (a) viral cDNAs (Subheading 3.2, step 7) or

   (b) RNA polymerase I plasmids (Subheading 3.4, step 10).

4. Clone PCR products into a protein expression vector, such as pCAGGS-MCS (7) or pcDNA3.1(+) (Invitrogen).

5. Sequence the resulting constructs to confirm the absence of unwanted mutations introduced by PCR.

3.6. Transfection of Eukaryotic Cells with Plasmids for vRNA and Protein Synthesis

1. Seed ~5×10^5 to 1×10^6 293T (human embryonic kidney) cells/well of a six-well plate (see Note 8).

2. Incubate cells for 24 h in regular growth medium (DMEM, 10% fetal bovine serum, 1× penicillin/streptomycin, 2 mM L-glutamine). Cells should be 40–60% confluent at the time of transfection.

3. Premix DNAs for transfection:

   (a) Use 1 μg each of the PB2, PB1, PA, and NP protein expression plasmids (see Note 9).

   (b) Use 0.1 μg each of the eight RNA polymerase I plasmids for the transcription of influenza viral RNA (see Note 9).

   (c) This gives a total of 4.8 μg of DNA to be transfected.

4. Transfection:

   (a) Add 200 μl OPTI-MEM (Invitrogen) to an eppendorf tube.

   (b) Add 2 μl transfection reagent (TransIT-LT1, Mirus) per μg of DNA (see Note 9).

   (c) Incubate for ~5 min at room temperature.

   (d) Add premixed DNAs.

   (e) Incubate transfection mixture for 15–30 min at room temperature.

5. Wash cells twice with OPTI-MEM; add 2 ml of OPTI-MEM; then, add transfection mixture dropwise to cells:

   (a) Note that 293T cells detach easily; therefore, wash cells carefully and add transfection mix gently.

   (b) Although optional, growth medium does not need to be aspirated and the cells do not need to be washed.

6. Controls should include the following:

   (a) Untreated 293T cells.

   (b) 293T cells treated with transfection reagent to monitor cytotoxic effects of the transfection reagent.

(c) 293T cells transfected with plasmids for vRNA synthesis but not plasmids for protein synthesis; no virus should be recovered from this control.

(d) 293T cells transfected with plasmids for protein synthesis but not plasmids for vRNA synthesis; no virus should be recovered from this control.

7. Incubate cells for 48 h at 37°C and 5% CO_2; typically, no cytopathic effect (CPE) is observed (see Note 9).

8. Collect virus-containing supernatant from transfected cells.

9. Optional:

(a) Spin down supernatant for 5 min at room temperature to pellet 293T cells.

(b) Transfer supernatant to fresh tube.

10. Store virus-containing supernatant at –80°C until further use (ideally store sample in aliquots).

3.7. Amplification of Influenza Viruses

1. Seed 10^6 MDCK (Madin–Darby canine kidney) cells/well of a six-well plate in growth medium (MEM containing 5% newborn calf serum, 1× penicillin/streptomycin, 4 mM L-glutamine, 1× MEM vitamin, and 1× MEM amino acids).

2. Incubate cells for 24 h at 37°C and 5% CO_2; cells should be confluent at the time of infection.

3. Prepare tenfold dilutions of virus-containing supernatant collected from transfected 293T cells:

(a) Dilute supernatant in MEM/BSA (MEM containing 0.25% bovine serum albumin, 1× penicillin/streptomycin, 4 mM L-glutamine, 1× MEM vitamin, and 1× MEM amino acids).

(b) The following dilutions should be tested: undiluted—10^{-6}.

4. Wash MDCK cells twice with PBS.

5. Add 300 μl of (un)diluted virus-containing supernatant to MDCK cells.

6. Infect MDCK cells for 1 h at 37°C and 5% CO_2.

7. Wash MDCK cells three times with PBS to remove virus inoculum.

8. Incubate cells with MEM/BSA.

(a) Although avian and some human influenza viruses do not require exogenous proteases such as trypsin for HA cleavage, addition of trypsin (0.5–1 μg/ml TPCK trypsin; Worthington) may facilitate virus replication (for further information on trypsin requirements and

amplification of viruses that require exogenous pro-
teases, see Subheading 3.8).

9. Observe cells daily for CPE:

   (a) CPE is indicative of virus replication.

   (b) For undiluted virus-containing supernatant, CPE typically
        appears within 48 h of infection.

   (c) For higher dilutions, CPE typically appears within 72 h of
        infection.

10. When ~80% of MDCK cells are lysed, harvest virus-containing
    supernatant.

11. Spin down supernatant for 5 min at room temperature to
    pellet floating cells.

12. Transfer virus-containing supernatant to fresh tube.

13. Store virus-containing supernatant at –80°C until further use
    (ideally store samples in aliquots).

14. Before the virus is used for further studies, its sequence should
    be confirmed.

15. Before the virus is used for further studies, virus stocks should
    be grown and their titers be determined (these techniques are
    described in Chap. 3). Also, an aliquot of the virus stock should
    be resequenced to confirm the authenticity of the virus and the
    presence of the mutation(s) introduced.

*3.8. Amplification
of Influenza Viruses
That Require
Exogenous Proteases
for HA Cleavage*

1. The proteolytic cleavage of HA into its HA1 and HA2 sub-
   units is critical for influenza virulence. HA cleavability is mainly
   determined by the amino acid sequence at the HA cleavage
   site: multiple basic amino acids are recognized by ubiquitous
   proteases, while a single basic amino acid is recognized by a
   limited number of proteases.

2. Most cell culture systems require the addition of trypsin to
   cleave the HA proteins of human influenza viruses.

3. Trypsin [0.5–1 μg/ml TPCK trypsin (Worthington)] may be
   added to

   (a) Transfected 293T cells for 1 h before the supernatant is
        collected (Subheading 3.6, step 7).

   (b) The collected supernatant (Subheading 3.6, step 8), followed
        by incubation for 1 h at 37°C.

4. MDCK cells may be incubated in the presence of trypsin
   (Subheading 3.7, step 8).

5. All other steps of virus generation and amplification are carried
   out as described in Subheadings 3.6 and 3.7.

4. Notes

1. The titers of most virus stocks range from 10^6 to 10^8 plaque-forming units/ml. Typically, 100–500 μl of such a virus stock is used for RNA extraction.

2. The twelve 3′-terminal nucleotides of influenza viral RNAs are conserved and a "universal" primer can therefore be used to amplify all eight viral RNAs.

3. The use of so-called type IIs restriction endonucleases (such as *Bsm*BI and *Bsa*I) adds significantly to the flexibility of the cloning system (see Fig. 2);

   (a) Type IIs restriction endonucleases allow nucleotide-specific fusion of any two DNA segments without the introduction of mutations or unwanted nucleotides.

   (b) For type IIs restriction endonucleases, the cleavage site is downstream of the recognition site. As a result, the four-nucleotide overhangs at the 5′ and 3′ ends differ from each other and result in directed cloning of the insert (i.e., the insert is inserted in the desired orientation).

   (c) *Bsm*BI or *Bsa*I can be used even if the influenza viral segment to be amplified possesses internal recognition sites for these enzymes. Since the four-nucleotide overhangs will differ from each other, efficient three-segment ligation is possible.

   (d) Alternatively, other type IIs restriction endonucleases that create four-nucleotide overhangs (such as *Bbs*I) can be used for the amplification and cloning of influenza viral cDNAs.

   (e) In the presented cloning strategy, the *Bsm*BI or *Bsa*I sites will be deleted upon ligation of the vector and insert. These restriction endonucleases cannot, therefore, be used to characterize the resulting constructs.

4. For the NA segment, the segment-specific nucleotides vary among the neuraminidase subtypes (N1–N9). For subtype-specific oligonucleotides, refer to Hoffman et al. (6).

5. The procedure described in Subheading 3 outlines the original RNA polymerase I system with eight RNA polymerase I plasmids for vRNA synthesis, and four protein expression plasmids for the synthesis of the viral PB2, PB1, PA, and NP proteins (1). The following modifications have since been established.

   (a) RNA polymerase I/II system (2): In this system, vRNAs and mRNAs are derived from the same template, eliminating the

need for separate protein expression plasmids and reducing the number of plasmids required from 12 to 8; hence, Subheading 3.5 can be skipped.

(b) "Tandem" RNA polymerase I system (3): In this system, RNA polymerase I transcription units are cloned in tandem, so that all eight vRNAs can be derived from one plasmid. This reduced number of plasmids required for virus generation provides an advantage for cell lines that cannot be transfected efficiently, such as African green monkey kidney (Vero) cells. However, the large size of the plasmid (~22.5 kb) makes cloning and handling cumbersome.

(c) T7 RNA polymerase system (5): In this system, influenza viral cDNAs are flanked by the T7 RNA polymerase promoter and ribozyme sequences.

(d) Adenovirus system (4): In this system, RNA polymerase I transcription units are encoded by replication-incompetent adenoviruses, which allows highly efficient gene transfer into cell lines that are not readily transfected, such as Vero cells (Fig. 2).

6. pHH21 contains the *human* RNA polymerase I promoter sequence. Due to the species-specificity of the RNA polymerase I system, the human promoter may not be efficient in non-human cells. However, *avian* (8) and *canine* (9, 10) RNA polymerase I transcription systems have also been established.

7. The protein expression plasmids need not match the virus to be rescued. For example, plasmids expressing the PB2, PB1, PA, and NP proteins of A/WSN/33 (H1N1) virus can be used to generate, for example, H5N1 or H3N2 viruses. Since the mRNAs transcribed from the protein expression plasmids will not be incorporated into viruses, no reassortants are generated. However, rescue efficiencies may be higher if viral RNAs are transcribed and replicated by proteins derived from the same virus.

8. 293T cells can be transfected efficiently and are therefore used for plasmid transfection. However, 293T cells do not optimally support influenza virus replication. Nonetheless, we have had great success with this approach and typically transfect 293T cells, followed by passage of the supernatant in MDCK cells. In alternative protocols, 293T and MDCK cells are co-seeded (2).

9. If virus cannot be rescued, or the rescue efficiency is low, the following modifications can be tried:

(a) Test different amounts and/or different ratios of protein expression plasmids (11).

(b) Test different amounts of RNA polymerase I plasmids for vRNA synthesis.

     (c) Generate and use not only plasmids expressing the PB2, PB1, PA, and NP proteins, but also plasmids expressing the HA, NA, M1, M2, and NS2 proteins (1).

     (d) Test different amounts and/or ratios of different transfection reagents.

     (e) Incubate transfected cells for up to 96 h.

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Chapter 13

Genetic Analysis

Gavin J.D. Smith, Justin Bahl, and Dhanasekaran Vijaykrishna

Abstract

Genetic analysis of sequence data is central to determining the evolutionary history and molecular epidemiology of viruses, particularly those such as influenza A virus that have complex ecosystems involving multiple hosts. Here we provide an outline of routine phylogenetic analyses of influenza A viruses including multiple sequence alignment, selecting the best-fit evolutionary model and phylogenetic tree reconstruction using Neighbor joining, Maximum likelihood, and Bayesian inference.

Key words: Sequence alignment, Phylogeny, Evolution, Natural selection, Neighbor joining, Maximum likelihood, Bayesian inference

1. Introduction

In recent years, much research has focused on the evolutionary history and molecular epidemiology of influenza A viruses (1). Current efforts in influenza surveillance and sequencing have resulted in phenomenal amounts of data being made publicly available. The recent emergence of the H1N1/2009 pandemic virus provided a timely example of the importance and power of phylogenetic analysis to inform both the planning and response to newly emerged viruses (2). This chapter focuses on the phylogenetic analyses of influenza A virus genomic sequence data to infer their evolutionary history. Basic strategies used to characterize these will be described: from dataset design to alignment and tree building.

The relationships of rapidly evolving RNA viruses like influenza A are best represented by phylogenetic trees (3). Phylogenetic analysis establishes the evolutionary history and relationship between genes by inferring the common history of the genes. To achieve this, homologous regions (e.g., the coding region of the

hemagglutinin (HA) gene), are compared in a multiple sequence alignment. Influenza A viruses have a small segmented genome most of which is protein coding (4). This makes multiple sequence alignment easy when compared to other more diverse and sparsely sampled viruses such as coronaviruses. However, influenza A viruses undergo frequent genetic mixing, termed reassortment, and caution must be used during analyses and interpretation of the whole genomes. Inappropriate analysis and/or dataset design can lead to inaccurate results and erroneous interpretations of the molecular epidemiology of influenza A viruses.

Three tree building methods; Neighbor joining (NJ), Maximum likelihood (ML), and Bayesian inference (BI) are described here. These methods are all statistically consistent; however, they are presented in order of increasing statistical robustness and computational cost. These methods require an explicit evolutionary model as accurate and realistic substitution models allow for greater robustness. A model testing and selection tool is also described. While the methods and examples provided here are specific for influenza A viruses, the platforms, programs, and methods are easily portable to other virus families.

2. Materials

These instructions were written with Mac OS X in mind, but should also be applicable to other operating systems. A list of databases and programs and from where they can be obtained is provided below—where possible we have used freely available programs.

2.1. Dataset Design

1. NCBI GenBank Influenza Virus Resource at NCBI—http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html (5) (see Note 1).

2.2. Multiple Sequence Alignment

1. Multiple sequence alignment based on fast Fourier transform (MAFFT). Available as a standalone program (Linux/UNIX, Mac OS X, Windows) or online—http://align.bmr.kyushu-u.ac.jp/mafft/software/ (6, 7).
2. Se-Al (Mac OS X)—http://tree.bio.ed.ac.uk/software/seal/ (see Note 2).

2.3. Selecting the Best-Fit Evolutionary Model

1. jModelTest: phylogenetic model averaging (JAVA program that will run on Linux, Mac OS X, and Windows)—http://darwin.uvigo.es/software/jmodeltest.html (8).

2.4. Phylogenetic Reconstruction

1. Neighbor joining (NJ). PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods) (Mac OS X, UNIX, Windows, or DOS-based formats)—http://paup.csit.fsu.edu/ (9).

2. Maximum likelihood (ML). GARLI: Genetic Algorithm for Rapid Likelihood Inference (Linux/UNIX, Mac OS X, and Windows) http://garli.nescent.org (10).

3. Bayesian inference (BI). MrBayes: Bayesian Inference of Phylogeny (Linux/UNIX, Mac OS X, and Windows)—http://mrbayes.csit.fsu.edu/ (11).

4. Viewing and manipulating trees. Figtree (Linux/UNIX, Mac OS X, and Windows)—http://tree.bio.ed.ac.uk/software/figtree/.

3. Methods

3.1. Data Formats and Dataset Design

3.1.1. FASTA and NEXUS Formats

Numerous data formats are used in phylogenetic analysis, but the most common are FASTA (or Pearson) and NEXUS. FASTA is a text-based format for representing nucleotide or amino acid sequences that is easy to manipulate and process using simple text programs. A sequence in FASTA format consists of a single-line description that starts with a ">" (greater-than) followed by lines of sequence data. The description line most commonly contains the virus name. When a sequence is downloaded from public sequence databases as FASTA format, the description line most often includes additional information associated with the sequence (i.e., accession number, gene information, or collection information). The sequence data that follows the description line may be of multiple lines, for example:

```
>A/mallard/Alberta/300/1977(H4N3)

AGCAAAAGCAGGTGCGAGATGAATCCAAATCAGAAGATAATAACAATCGGTGTAGTGA

ATACTACTCTATCA

>A/pintail duck/New York/155/1982(H4N3)

AGCAAAAGCAGGTGCGAGATGAATCCAAATCAGAAGATAATAACAATCGGGGTAGTGA

ATACCACTCTGTCA
```

NEXUS format has been extensively used in sequence analysis and is accepted by many different software packages (12). NEXUS format is composed of a number of separate blocks and standardized commands. These blocks can either be public or private blocks. Public blocks are utilized by multiple programs and house information about taxa (which in this case are viruses), morphological and molecular characters, genetic distances, genetic codes, assumptions, datasets, or phylogenetic trees. In contrast, private blocks contain information relevant to single programs such as PAUP*, GARLI, or MrBayes. The format is naturally extensible and flexible, and can be created from scratch using a text editor or exported from other software that creates NEXUS formatted files (e.g., Se-Al: File>Export>File Format).

The first line in a NEXUS format file is "#NEXUS". This tells the software program that the file is in NEXUS format and will contain information in blocks that start with "begin" and end with "end"—with each remark separated by a semi-colon (;). Small variations exist in how different computer programs read the NEXUS format and these are most often associated with the treatment of special characters in taxa names. For example, "/" is unrecognized by PAUP* unless defined in the DATA block. In order to resolve these commonly encountered issues taxa names are often read enclosed in single quotation marks (i.e., 'A/mallard/ Alberta/300/1977(H4N3)'). However, single quotes are not recognized by MrBayes. Comments can be included in the NEXUS file but must be enclosed within square brackets "[ ]", which informs the program to ignore the enclosed text. An example of NEXUS format is as follows:

```
#NEXUS
```

[This comment, in square brackets, will not be read by the program]

```
Begin DATA;
  Dimensions ntax=2 nchar=72;
  Format datatype=NUCLEOTIDE gap=-;
  Matrix
'A/mallard/Alberta/300/1977(H4N3)'
AGCAAAAGCAGGTGCGAGATGAATCCAAATCAGAAGATAATAACAATCGGTGTAGTG
AATACTACTCTATCA
'A/pintail duck/New York/155/1982(H4N3)'
AGCAAAAGCAGGTGCGAGATGAATCCAAATCAGAAGATAATAACAATCGGGGTAGTG
AATACCACTCTGTCA
;
End;
```

A great advantage of a NEXUS file is that a block of instructions can be placed at the end of the file that can instruct programs, such as PAUP*, when conducting analyses. This will be addressed in Subheading 3.4.

3.1.2. Influenza Specific Dataset Preparation Criteria

1. It is critical that reference sequences, obtained from public databases, are included in an analysis of virus sequences generated through sampling from surveillance studies or an outbreak. This provides a framework to understand the diversity of any newly generated sequence data in relation to previous work.

2. While it is always good to start from the largest possible dataset (i.e., all available sequences in public databases), this is usually not feasible due to computational limitations, or in some cases

it may not be necessary. In general the criteria below can be used during taxon sampling.

3. *BLAST search results:* Initial dataset size can be reduced using tools such as a BLAST search. BLAST identifies sequences from a database (usually GenBank) that have the highest percentage similarity to the query sequence. It is important to note that the results of a BLAST search may not represent the closest related sequences in terms of phylogenetic relationships. Only a phylogenetic analysis will provide information on the relatedness of gene sequences. Furthermore, the results of a BLAST search are listed by highest similarity and earliest released sequence—if there are 10 BLAST hits with identical percentage similarity, these results will be listed from oldest to newest. Merely choosing the top BLAST hits is therefore not a suitable method for designing a dataset.

4. *Gene segment:* When analyzing surface protein genes (HA or neuraminidase, NA) it is essential that you include only the subtype that is under study, or generate individual datasets for each subtype under study. However, in the case of internal gene segments (PB2, PB1, PA, NP, M, and NS), all subtypes of influenza A virus sequences can be aligned together. This is particularly important if one wishes to detect reassortment of gene segments between viruses of different subtypes.

3.2. Multiple Sequence Alignment

Multiple sequence alignments form the basis for all subsequent analyses, such as evolutionary model selection and tree building. Influenza A viruses have a small genome consisting of primarily protein-coding regions, therefore alignment is fairly simple. But incorrect alignments (especially for datasets with greater genetic diversity) may lead to dramatically misleading results; therefore, extreme caution should be taken to maximize the correctness of the alignment (see Note 3).

3.2.1. Multiple Sequence Alignment Using MAFFT

1. For downloading, installation, and basic usage instructions please see the MAFFT Web site (see Note 4).

2. MAFFT contains many modes of alignments where speed and accuracy are inversely proportional—from fast and inaccurate to slow and accurate.

3. MAFFT provides command aliases for all of these fast and slow methods.

4. Run MAFFT through the Terminal window (Finder > Applications > Utilities > Terminal).

5. cd to your working folder and type "mafft" to start the program (see Fig. 1). You will be first asked to specify the input file (i.e., H5_align.fas) and then the output file (e.g., H5_align_out.fas). You will then be asked to specify a number of alignment criteria.

Fig. 1. The MAFFT terminal window.

For influenza genes we can use the defaults, which specifies the FFT-NS-2 (Fast but rough) strategy (see Note 3).

6. Upon completion, the aligned sequence is stored within the output file. Manual inspection and optimization is necessary for most alignments as no alignment program is perfect.

3.2.2. Optimization of Multiple Sequence Alignment Using Se-Al

1. Double-click the Se-Al icon (Finder > Applications > Utilities > Terminal) to open the program. From the Se-Al menu select "File > Open" and select the MAFFT output file (i.e., H5_align_out.fas).

2. The main areas of the alignment that are likely to need manual optimization are the start and end of the alignment where not all sequences in public databases are incomplete at the 5′ and 3′ terminals. Other areas that may be misaligned include the connecting peptide of the HA, and the NA stalk that may have a deletion. However, it is important that the entire alignment be visually inspected and any misalignments corrected.

3. For nucleotide sequences of protein-coding genes there are three visualization modes: nucleotides: edit an alignment of single bases; codons: edit an alignment of codons in any reading frame; and translation: edit an alignment of inferred amino acids translated using various genetic codes (influenza A virus genes utilize the universal genetic code) (see Note 5).

4. Alignments can be edited by selecting a block of sequences and sliding the block relative to the other sequences wherein gaps will open up behind the block. In addition, sequences and their labels can be edited in a separate window by double-clicking on the virus name.

5. In order to ease visualization of the aligned file use block colors (select "Alignment > Use Block colors"). This is especially useful when optimizing codon alignments, as amino acids are colored based on size and polarity.

6. Shifting reading frames, reversing, and complementation can be done independently to any sequence or to the whole alignment and can be reversed.

7. Alignments from Se-Al can be exported in numerous formats, including FASTA and NEXUS. From the menu select "File > Export" and a new dialogue window will open where the export format can be selected (see Note 6).

3.3. Selecting an Evolutionary Model

jModelTest uses hierarchical likelihood ratio tests (hLRTs) and the Akaike Information Criterion (AIC) to find the evolutionary model that best fits the particular sequence alignment that you are analyzing (8) (see Note 7). If you modify your dataset you should recalculate the evolutionary model.

1. Double-click the jModelTest.jar file to open the program console as shown in Fig. 2.
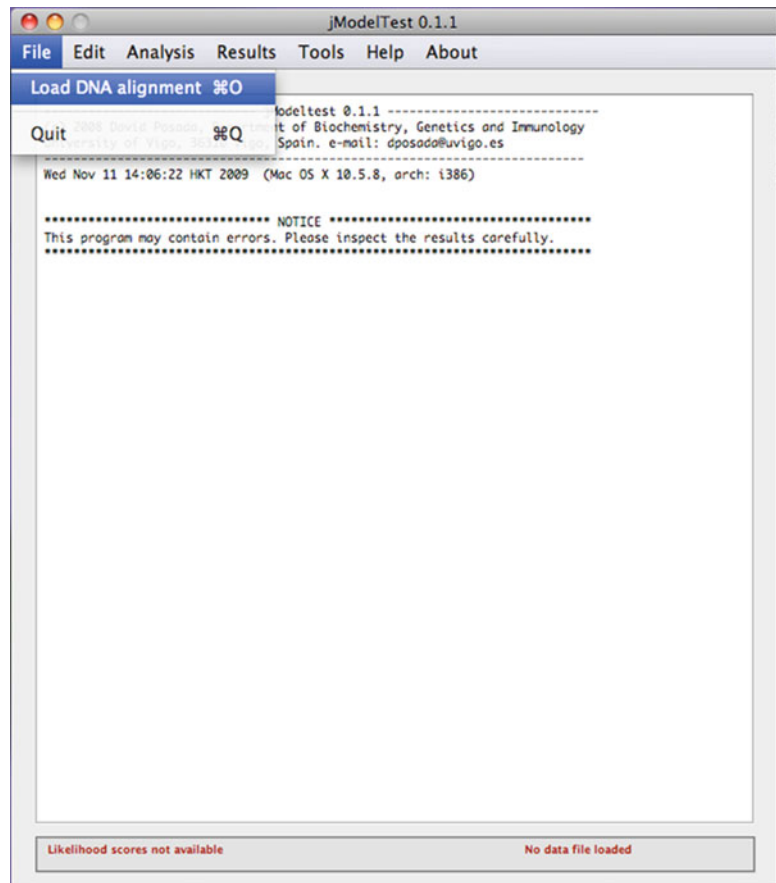
Fig. 2. The jModelTest console.
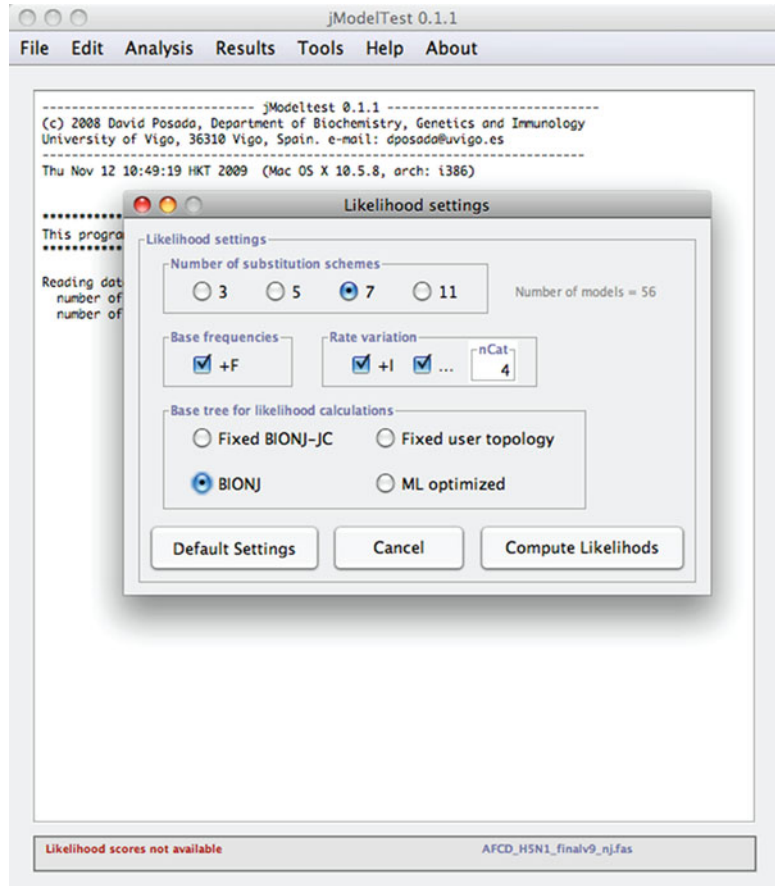
Fig. 3. Settings for the likelihood calculations.

2. From the menu select "File > Load DNA alignment" and choose the FASTA format alignment that was exported from Se-Al (i.e., "H5_align_out.fas") (see Note 8).

3. Then select "Analysis > Compute likelihood scores" and a window will open with options for calculating likelihood scores.

4. Change "Number of substitution schemes" to 7 and "Base tree for likelihood calculations" to BIONJ (see Fig. 3), and then click "Compute likelihoods" and a progress bar will open.

5. The program will test 56 models in a hierarchical manner and will take approximately 20–30 min on a modern computer for an alignment of 100 full-length HA sequences. Details of the tests will also appear in the main console window (see Note 9).

6. Now select "Analysis > Do AIC calculations …," and a window will open with options for the AIC settings. Check "Write PAUP* block" and then click "Do AIC calculations" (see Fig. 4).
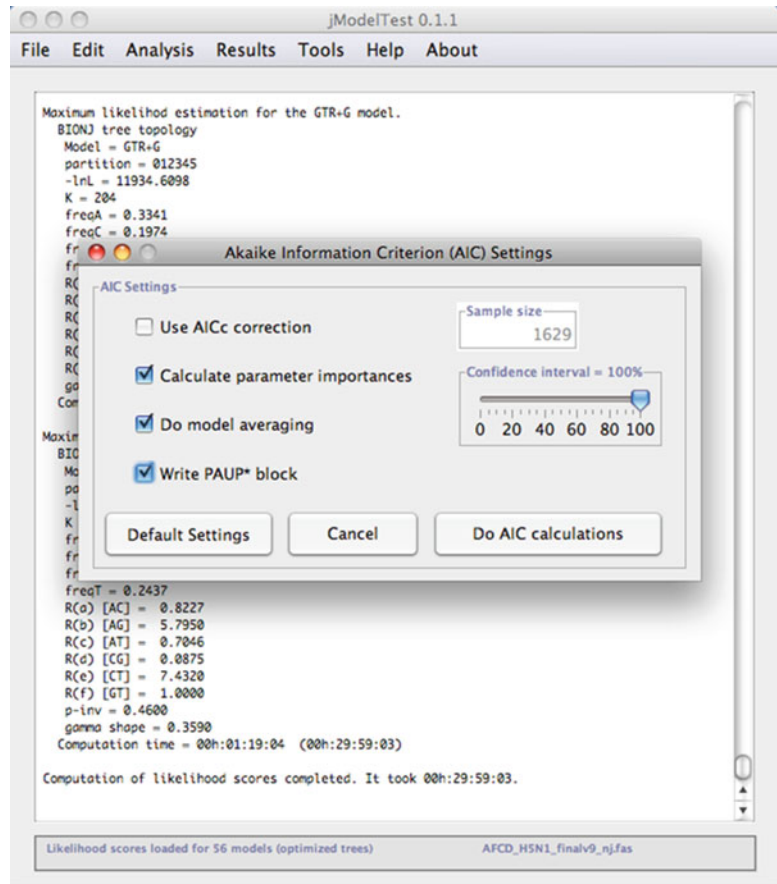
Fig. 4. Settings for AIC model selection.

7. Results of the AIC calculations will be summarized in the jModelTest console, but you will need to scroll up a bit (see Fig. 5). For the example used here, the model selected is the GTR+I+G with the precise variables defined. These variables will be used in subsequent analyses.

8. Directly below the description of the selected model a "PAUP* Commands Block" is provided (see Fig. 5). This block will be used in Subheading 3.4.1 "Neighbor joining in PAUP*."

9. The default settings for GARLI and MrBayes use the GTR+I+G model. The execution of these programs will be described using this model. For details on using alternative models refer to the program manuals.
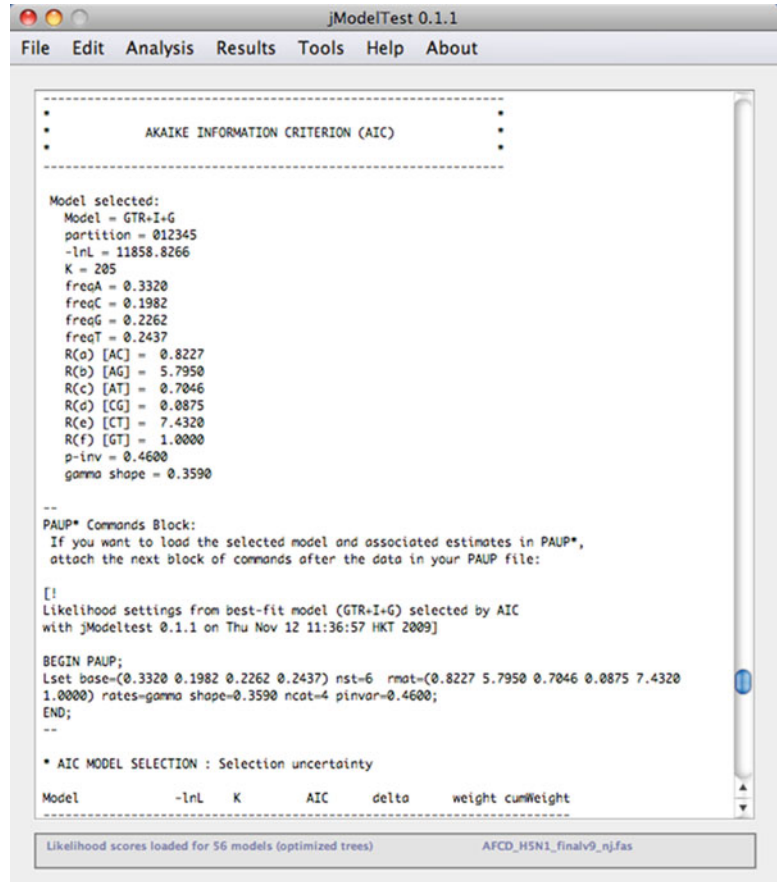
Fig. 5. Results of the AIC model selection.

3.4. Phylogenetic Reconstruction

3.4.1. Neighbor Joining in PAUP*

PAUP* is one of the most widely used phylogenetic software packages. The software is not free but is powerful, flexible, and reliable. The manual included with this program contains an extensive command reference that is very useful.

1. First, convert the FASTA file "H5_align_out.fas" to Nexus format using the program Se-Al, saving the file as "H5_align_out.nex" (see Subheading 3.2.2).

2. Open the file "H5_align_out.nex" in a text editor, scroll to the end, and paste the "PAUP* Commands Block" from Subheading 3.3. The PAUP* block should read (see Note 10) as follows:

```
BEGIN PAUP;

Lset base=(0.3320 0.1982 0.2262) nst=6 rmat=(0.8227 5.7950

0.7046 0.0875 7.4320) rates=gamma shape=0.3590 ncat=4

pinvar=0.4600;

END;
```
(see Note 10).
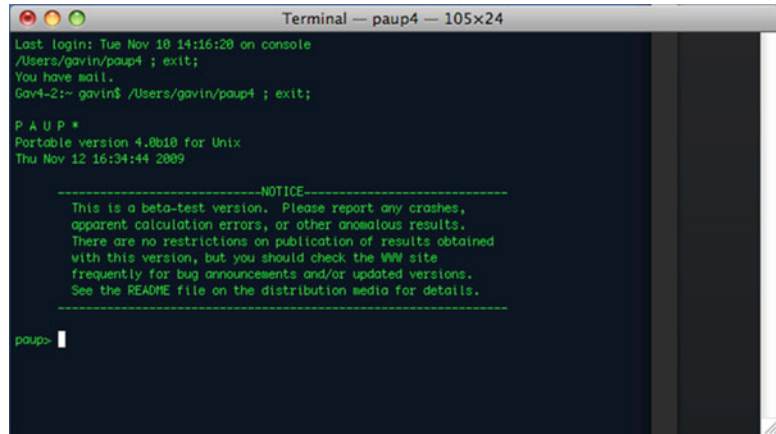
3. This only describes the evolutionary model, so we need to include some further commands for calculating and saving the NJ tree. The PAUP* block should now read as follows:

```
BEGIN PAUP;

Lset base=(0.3320 0.1982 0.2262) nst=6 rmat=(0.8227

5.7950 0.7046 0.0875 7.4320) rates=gamma shape=0.3590

ncat=4 pinvar=0.4600;

set crit=dist; dset dist=ml;
```
[sets the search criterion to Distance method and specifies the use of the model]
```
nj breakties=random;
```
[starts the NJ analysis]
```
savetrees file=H5_align_out.tre brlens=yes;
```
[saves the NJ tree with branch lengths]
```
boot search=nj nreps=1000;
```
[runs a bootstrap of the dataset with 1,000 replicates]
```
savetree file=H5_align_out_BS.tre savebootp=both
from=1 to=1;
```
[saves the bootstrap consensus tree]
```
END;
```

4. Make sure that the PAUP* executable and the Nexus file are contained in the home directory, although the path can be included in the execute command used in PAUP*. Please note that there is no graphical interface for the program in Mac OS X and that the UNIX version needs to be used.

5. Double-click the PAUP* executable to open the program in the Terminal (see Fig. 6). At the command prompt type "execute filename.nex." In the example used here the file name is "H5_align_out.nex."

6. As all the necessary commands were placed within the NEXUS file the analyses should run without any further input. Once the calculations are completed, the NJ and bootstrap trees will be saved in the same directory that contains the NEXUS file.

3.4.2. Maximum Likelihood in GARLI

Genetic algorithm for rapid likelihood inference (GARLI) performs phylogenetic searches on aligned sequence datasets using the maximum-likelihood criterion. Common substitution models are implemented in GARLI to calculate the likelihood scores. The model parameters can be fixed or estimated and used to optimize branch lengths, tree topology, and maximize the likelihood. The genetic algorithm used by GARLI to perform maximum likelihood

Fig. 6. The PAUP* terminal window.

searches is generally faster than PAUP* and, when searches have been run for sufficient length, the likelihood scores should be comparable. GARLI maximum likelihood tree search optimization is faster than PAUP* maximum likelihood analysis but slower than the neighbor joining method described above.

1. For downloading, installation, and basic usage instructions please see the GARLI Web site (see Note 11).

2. The best-fit evolutionary model can also be specified as a "GARLI block" in the NEXUS file; however, the format is different for the PAUP* block described above. For the model from Subheading 3.3 the GARLI block will read (see Note 12) as follows:

```
BEGIN GARLI;

e 0.3320 0.1982 0.2262 0.2436 r 0.8227 5.7950 0.7046 0.0875

7.4320 a 0.3590 p 0.4600;

END;
```

3. So replace the PAUP* block in the NEXUS file and rename the file to "H5_align_garli.nex."

4. It is then necessary to modify the GARLI configuration file (garli.conf) to specify the analysis file. The configuration file is in the folder "example" that is included with the program download. This file can be opened in any text editor.

5. On lines 2 and 6 of "garli.conf" change the field "datafname = rana.nex" to "datafname = H5_align_garli.nex" and "ofprefix = rana.nuc.GTRIG" to "ofprefix = H5.nuc.GTRIG." This specifies the correct analysis file.

6. It is also necessary to modify Lines 25–31 of the configuration file, specify that the parameters of the evolutionary model have been fixed using the GARLI block:

```
datatype = nucleotide
ratematrix = fixed
statefrequencies = fixed
ratehetmodel = gammafixed
shape = fixed
numratecats = 4
invariantsites = fixed
```

7. It is advisable to conduct multiple runs of GARLI to ensure that an optimal result is obtained. The default is 2 replicates but this can be increased by modifying "searchreps = 2" in the configuration file.

8. Ensure that the "garli.conf" file is in the same folder as the GARLI executable.

9. Open the Terminal window and cd to the folder containing the executable file "Garli0.96b8."

10. Type "./Garli0.96b8" and the program will begin to run.

11. Once completed, the example dataset took about 15 min, the best tree will be saved in the file "H5.nuc.GTRIG.best.tre" in the same folder. In the terminal window, the likelihood of both runs is provided, which should be similar values.

12. To conduct an ML bootstrap then simply modify the third last line of the "garli.conf" file to read "bootstrapreps = 0" to "bootstrapreps = 500" or "bootstrapreps = 1,000" (see Note 13).

3.4.3. Bayesian Inference in MrBayes

MrBayes is a program for the Bayesian estimation of phylogeny. Bayesian phylogenetics has been gaining prominence and acceptance in evolutionary science, especially with dramatically improved computational power. Importantly, Bayesian phylogenetics sample many equivalent trees in the tree space in contrast to neighbor joining and maximum likelihood methods, which will produce only a single tree. The phylogenetic tree produced is based on all of these trees, which is known as the posterior probability distribution of trees. This computation is analytically difficult and for large datasets impossible. MrBayes uses a simulation method known as Metropolis-coupled Markov Chain Monte Carlo (or MCMC) to overcome this difficulty.

1. For downloading, installation, and basic usage instructions please see the MrBayes Web site (see Note 14).

2. The best-fit evolutionary model is specified with a "MrBayes block" in the NEXUS file; however, the format is different for the PAUP* and GARLI blocks described above. For the model

from Subheading 3.3 the MrBayes block will read (see Note
15) as follows:

```
BEGIN MRBAYES;

Lset nst=6  rates=invgamma;
Prset statefreqpr=dirichlet(1,1,1,1);

mcmcp ngen=1000000 samplefreq=100 printfreq=100 nchains=6
savebrlens=yes;
mcmc;

END;
```

3. Replace the PAUP* block in the NEXUS file and rename the
   file to "H5_align_bayes.nex."

4. Double-click the MrBayes executable file to open the program.
   At the prompt type "exec H5_align_bayes.nex" to execute the
   file (see Fig. 7).

5. The MrBayes block specified one million generations, with
   trees sampled every 100 generations. Also, the default setting
   for MrBayes is to conduct two independent runs. This will
   result in 10,000 trees per run, or a total of 20,000 trees.

6. MrBayes will save two types of files for each independent run,
   the tree files and the run statistics, that have the extensions
   "run1.t and run2.t" and "run1.p and run2.p," respectively.
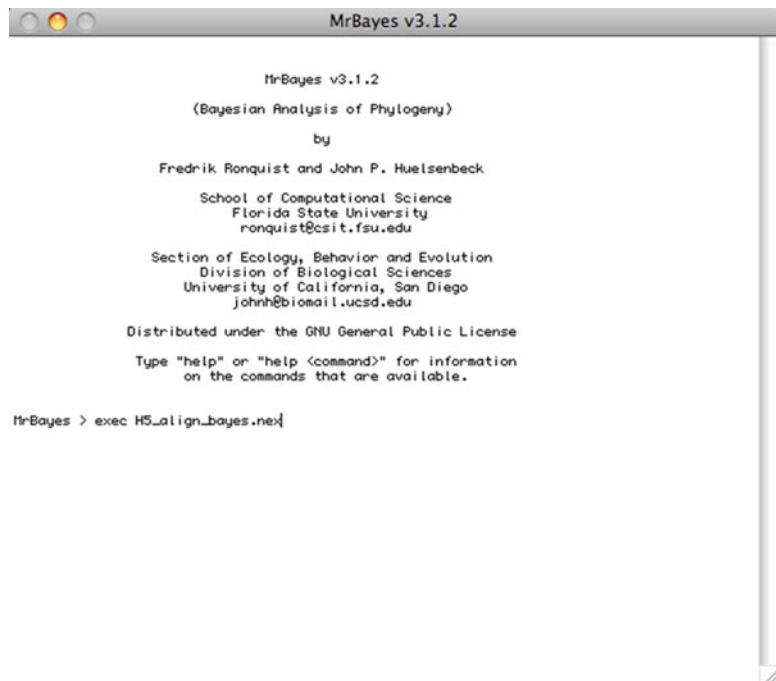
Fig. 7. The MrBayes program window.

7. The Bayesian posterior probability is assessed from these trees *after* suboptimal trees calculated at the start of the run are discarded. The portion of the trees that are discarded are referred to as the "*burnin*."

8. Once the analysis has finished running, you will be prompted on whether to continue the analysis or not. First, it is necessary to check whether the two independent runs have converged by looking at the "average standard deviation of split frequencies" that is provided in the MrBayes window. As the two runs converge onto the stationary distribution, the average standard deviation of split frequencies is expected to approach zero, reflecting the fact that the two tree samples become increasingly similar. A value <0.01 is considered adequate indication that the two runs have converged.

9. Alternatively, the program Tracer can be used. Double-click the Tracer icon to open the program. This has the advantage that the burnin can also be determined though visual inspection.

10. Click on the "+" sign near the top left of Tracer, this will open another window to select the necessary files. Navigate to the MyBayes folder and select the run statistic files "H5_align_bayes.nex.run1.p" and "H5_align_bayes.nex.run2.p" (see Fig. 8).

11. Tracer will automatically assign a burnin of 10%. Double-click the burnin value in the "Traces files" box and change this to zero for both files. Select both files in the "Traces files" box and then click on the LnL statistic in the "Traces" box—a graph of both tree likelihoods will appear to the right (see Fig. 8).
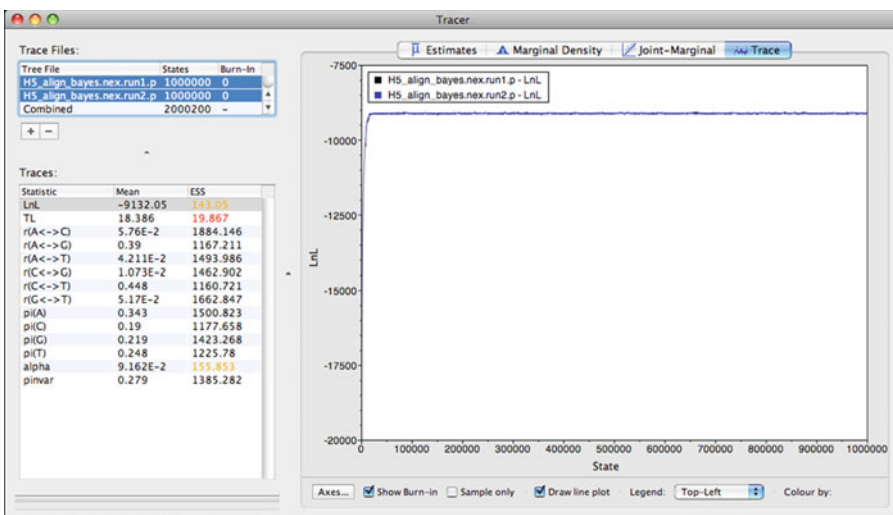
Fig. 8. The Tracer program window.

Fig. 9. State frequencies and summarizing trees in MrBayes.

12. For the example dataset used here, an appropriate burnin is 1,000 (10%) of sampled trees.

13. If the two runs have not converged, you can continue the analysis by typing "Yes" and then entering an additional 1 million generations.

14. If the two runs have converged, then type "No" and then type "sumt burnin=1,000"—or whatever burnin you have determined from visual inspection in Tracer. This will take some time and once completed, a consensus tree with Bayesian posterior probabilities will be saved as "H5_align_bayes.nex.con" (see Note 16) (Fig. 9).

3.5. Tree Visualization with FigTree

Figtree (http://tree.bio.ed.ac.uk/software/figtree/) is a graphical viewer with various tree-editing capabilities that produces publication-quality figures in multiple graphic file formats.
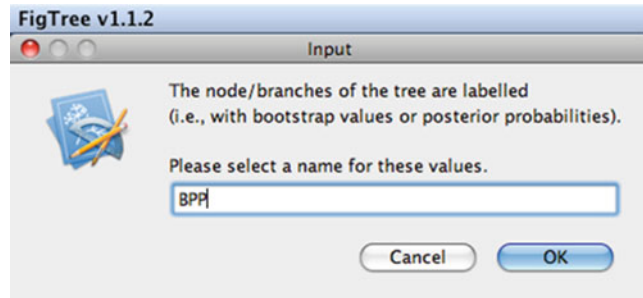
Fig. 10. Figtree: tree input.

1. For downloading, installation, and basic usage instructions please see the Figtree Web site.

2. As an example, we will use the "H5_align_bayes.nex.con" file that was generated using the sumt command in MrBayes (see Subheading 3.4.3).

3. Double-click Figtree executable to start the application. To open a tree file use File > Open and then select the tree file.

4. Upon opening tree files, the users are prompted to enter names for undefined labels that exist in the tree file. In this case, >95% Bayesian posterior properties are stored in the tree file (Fig. 10).

5. The Figtree window (Fig. 11) is divided into the top menu panel, a left control panel, and the center tree panel. The top menu and the left panels can be used to edit the tree, color branches or leaves, and most importantly root the tree using an appropriate outgroup or using midpoint (see Note 17).

6. Using the File > Export Graphic (or) File > Print > PDF > Save as PDF menu options the tree graphic can be exported in various graphic file formats.

4. Notes

1. Other resources are available including the Influenza Research Database (http://www.fludb.org) (13) and the Influenza Primer Design Resource (http://www.ipdr.mcw.edu) (14).

2. Other programs for multiple sequence alignment and optimization include ClustalW/X (15) and BioEdit (16). There are also many online alignment tools, including those provided at http://align.genome.jp/.

3. Many multiple alignment programs also produce a guide tree that is used to conduct the multiple sequence alignment.
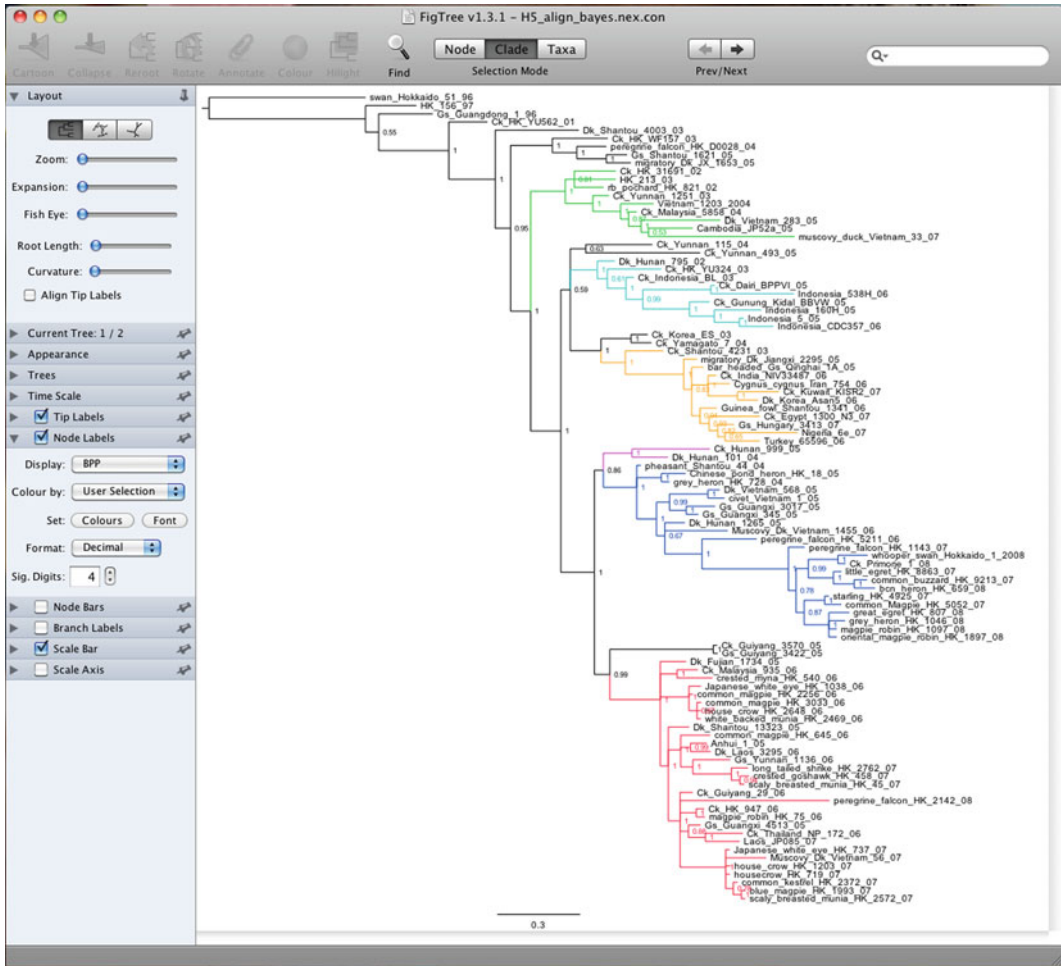
Fig. 11. Figtree.

This tree is unlikely to be accurate and must not be used to infer phylogenetic relationships or reproduced in publication.

4. The instructions we give here are for the locally installed program; however, the program can also be run from the Web page.

5. To ease the optimization of the multiple sequence alignment, shift all the sequences to the correct reading frame and translate the sequences to amino acids (select "Alignment > Alignment type > Amino Acids").

6. If you have loaded a DNA sequence alignment but wish to export the amino acid sequences, these must be converted to amino acid in the sequence inspector window first. Then, in the export file dialogue box, click the "Export alignment as viewed" tab.

7. jModelTest is a Java version of the earlier ModelTest ([17]). There is also a MrModeltest ([18]) that was specifically designed for only testing those models that could be implemented in the program MrBayes. Both these programs are faster than jModeltest, but both run from the terminal window and are more difficult to use.

8. jModelTest appears to have a problem loading NEXUS files. If you get an error with a particular file format, then just try a number of different file formats—as is usually the case, FASTA works fine.

9. In comparison, testing 56 models using ML trees took 1 h 20 min in jModelTest. If you reduce the number of substitution schemes to 3 (i.e., 24 models) and use BIONJ trees, the analysis will run in 10–15 min.

10. There is an error in the "Lset" command output from jModelTest that will cause an error when executing the file in PAUP* and other programs. Rather than having four numbers for "base = (n1 n2 n3 n4)" this should be "base = (n1 n2 n3). To fix this simply delete the last number. A similar error is made with "rmat = (n1 n2 n3 n4 n5 n6). There should only be five numbers in the "rmat" and the last number should be deleted.

11. The instructions given here are for the Mac OS X Intel multithreaded v0.96, which does not have a graphical user interface, but this version is capable of distributing the analysis over multiple processors.

12. In the GARLI block "e" is equivalent to "base" in the PAUP* block, "r" to "rmat," "a" to "shape," and "p" to "pinvar." Details of GARLI configuration settings are available at the following site: https://www.nescent.org/wg_garli/GARLI_Configuration_Settings.

13. Maximum likelihood bootstraps are very computationally intensive and even using GARLI will take days to complete 1,000 bootstraps for a dataset of 100 full-length HA genes.

14. The instructions given here are for the Mac OS X executable. However, this version runs on a single processor and is quite slow. It is therefore preferable to compile the program source code on a UNIX system, as this version is capable of distributing the analysis over multiple processors.

15. MrModeltest writes a MrBayes block, but jModeltest does not unfortunately. Commands for a MrBayes block are explained in detail in the MrBayes manual.

16. If virus names in the NEXUS file are overly long (e.g., ≥20 characters), this may cause the sumt command to hang indefinitely.

17. Extensive scientific literature illustrates the importance of using an appropriate outgroup taxon. As a rule of thumb, the best outgroup is a taxon that falls within the sister group of the ingroup under study. In the above example, for the Asian highly pathogenic avian influenza H5N1 virus ingroup, a low pathogenic H5 virus "Swan/Hokkaido/51/96" is used as an outgroup.

Acknowledgments

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