# Virology

## A Laboratory Manual

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# PREFACE

This text was originally developed for a one-semester virology laboratory course. It is designed for the classroom with detailed experimental protocols and comprehensive lists of the materials required for each experiment. Since it is not possible to include all virological techniques in a single volume, the selected experiments are those that best illustrate key points of basic virology. The experiments are also designed to minimize the need for extensive preparation or sophisticated instrumentation. Our aim in preparing this text is not a complete treatise of virological methods, but the development of a practical and sequentially organized series of experiments.

More specifically, this laboratory text is designed to: (1) introduce the student to cell culture through a series of experiments that illustrate the basic principles involved in the routine passage and cryopreservation of cell lines; (2) demonstrate how to passage and preserve a virus stock in the laboratory; (3) assay and quantify the virus stocks produced in a number of different assay systems, in order to better appreciate what a given assay measures, its relative sensitivity, and its relationship to other virus assays; (4) introduce some of the simpler techniques in identifying a virus; (5) assay for viral antibody and perform immunofluorescent staining of virus-infected cells; (6) integrate many of the techniques previously mastered through the production and quantification of interferons; and (7) demonstrate some of the basic techniques of molecular biology that are now commonly used in the virology laboratory.

In this text, a given exercise not only demonstrates a principle of cell culture or virology, but also builds upon a previous experiment. This sequential organization, however, still allows for flexibility. Many more experiments are included than could be attempted in a normal semester, thus providing the instructor with choices within this sequential framework. The use, throughout this text, of only three cell lines and two viruses provides continuity and simplifies the preparation of the laboratory exercises.

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# 1

# INTRODUCTION TO THE VIROLOGY LABORATORY

## LABORATORY SAFETY

It is important to remember that whenever one works with an infectious agent there is the possibility of infection to oneself or to others by negligence. Furthermore, organisms that are referred to as "nonpathogenic" are still potential pathogens. For example, certain "non-human" viruses, such as Newcastle disease virus (NDV), have been known to cause conjunctivitis when inadvertently introduced into the human eye. Cell cultures can also be potentially hazardous since they can infect the laboratory worker with an endogenous or a latent virus. One should assume the same precautions when working with cell culture as when working with a virus. The following is a list of general laboratory safety procedures:

1. There must be NO EATING, DRINKING, SMOKING, OR APPLYING OF COSMETICS IN THE LABORATORY.
2. Unauthorized persons, particularly children and infants, should NOT be allowed in the laboratory.
3. NO MOUTH-PIPETING should be permitted due to the possibility of accidental ingestion of virus-contaminated materials, cell-contaminated products, or toxic chemicals. Automatic pipetors or pipeting bulbs must always be used.
4. Hands should be washed when coming in the laboratory, after handling cells or virus, and before leaving the laboratory.
5. When gloves have to be worn they should be used only for a specific task and taken off when it is done. One should NOT walk around touching doorknobs and surfaces with contaminated gloves. Contaminated gloves should be discarded in the decontamination pan.
6. Avoid touching the eyes, nose, mouth, or face while working in the laboratory.
7. Laboratory coats should be worn when doing cell culture or virus work and should be left in the laboratory. Laboratory coats should NOT be worn while eating or outside of the laboratory.

8. All materials should be clearly labeled with the user's name and the contents therein.
9. All contaminated materials (virus- or cell-contaminated) should be placed in decontamination pans and autoclaved prior to being washed or disposed. The addition of water to the decontamination pan prior to autoclaving prevents the formation of residues on glassware that needs to be recycled. Disposable materials, such as plasticware, can alternatively be placed in autoclavable biohazard disposable bags and autoclaved before being discarded.
10. Whenever working with syringes and needles, special containers (that are sealed and autoclavable) should be provided for their disposal. DO NOT replace the cap on the needle (this is where many accidental injections occur). Drop the uncapped syringe into the disposal container immediately after use. These containers are then autoclaved prior to disposal.
11. Viruses, cells, or their products, should NEVER be disposed of in the drainage system.
12. Stock solutions of suitable disinfectants (such as Osyl or Amphyl, from National Laboratories) should be available at each work station. Any spills should be promptly cleaned with the disinfectant and reported to the instructor.
13. Cages that contain infected animals should be disinfected or autoclaved, and the infected animal carcasses and tissues should be properly bagged and incinerated.
14. Prevention of aerosols so as to prevent inadvertent inhalation exposure is as follows:

    (a) When pipeting let the material slide down the side of the vessel, do not dispense in a dropwise fashion.

    (b) Do not expel the last amount of medium from a pipet.

    (c) Properly cap any material to be shaken or centrifuged.

    (d) Use a certified biohazard hood when performing procedures that create aerosols, such as grinding or homogenizing tissues.
15. Biological materials should NEVER be taken home from the laboratory.

## STERILIZATION

Cell culture medium provides a particularly rich environment for the growth of microorganisms. If contamination does occur, the doubling time of the contaminant is likely to be much faster then that of the

cultured cells. Thus, the sterility of all materials is an ongoing concern of the cell culture worker. Three common methods of sterilization are: *autoclaving, dry heat sterilization,* and *filtration.*

**Autoclaving** is used whenever possible, especially when dealing with liquid solutions and materials that should not dry out. The use of autoclave tape on all materials to be autoclaved permits the identification of those materials that have been autoclaved and prevents the accidental opening of contaminated materials. PLEASE NOTE that the color change on an autoclave tape is no guarantee of sterility but only that the given material was exposed to an autoclave cycle. Sterilization by an autoclave is primarily a function of temperature, pressure, and time. How the materials are packaged and loaded into the autoclave is also important since the steam must penetrate for sterilization to occur. The standard cycle is 121°C, 15 p.s.i., for 15 minutes. Large volumes of liquids (for example, 1-10 liter bottle as opposed to 10-1 liter bottles) may require longer sterilization times.

**Dry heat sterilization** is effective for glassware provided there are no rubber, plastic, or teflon-lined parts. The standard cycle is at 300°C for 2 hours. Proper loading of the oven is also very important so as to reduce insulating effects.

**Filtration** (using a 0.22 μm membrane filter) is used for aqueous solutions and medium components that are heat labile.

All the media and solutions used in cell culture must be tested for sterility prior to use. To test for bacterial contamination, tubes of liquid thioglycollate, trypticase soy broth, and blood agar plates are often used. If fungi are a problem, it may be necessary to plate some of the medium on Sabouraud's agar. Other tests and media can be used to determine contamination by mycoplasmas, and viruses. No one medium will detect all possible contaminants and it is important to realize the limitations of any given detection system.

## ASEPTIC TECHNIQUE

Even when sterile materials are available, proper aseptic technique must be rigorously enforced in order to avoid accidental contamination of cell cultures. Sterile transfer rooms and certified biohazard hoods are preferable for cell culture work, although individual plexiglass hoods equipped with a germicidal lamp and with accesses for gas, vacuum, and electrical outlets are adequate in many instances.

It is important to disinfect the working area with an appropriate disinfectant just prior to and after working. It is also important to be located away from high traffic areas. It is helpful to keep flasks and bottles open for as short a period as possible. Remember to flame the lips of the bottles, flasks, and tubes after opening, and to use only sterile pipets. If a sterile pipet accidentally touches a non-sterile

surface, it should be discarded and another one used in its place. All aseptic techniques should be strictly observed.

Separate laboratory coats should be used when working with animals and cell culture because of the possibility of contamination of the cell cultures with animal dander and droppings.

Any medium that appears cloudy should be brought to the instructor's attention since this solution may be contaminated. Contaminated materials should be autoclaved prior to disposal.

Sterility checks should be performed, when appropriate, on all materials used for a given experiment.

It is preferable to do routine cell passage work *before* virus work. If sterile rooms or hoods are available they should be equipped with germicidal U.V. lamps.

Remember that clean does not mean sterile, so that hands and clothing can be a source of contamination if they come into contact with sterile materials (ie: the lip of a sterile bottle or the tip of a sterile pipet).

## GLASSWARE

Whether glassware or plasticware is used in a given laboratory will depend to a great extent on the individual, the ease of recycling, and the comparative costs of the materials. Plastics presently manufactured are nontoxic to most cell lines and are of good optical quality. The plasticware should be described by the manufacturer as being "for tissue culture" and thus has been treated to promote cell adherence. Ordinary (bacterial) petri dishes are NOT suitable for tissue culture. Plastic products are disposable. Glassware on the other hand may be recycled thus minimizing costs.

Glassware made commercially is often toxic to a number of different types of cells. This toxicity problem can be solved in most cases by proper cleaning procedures. Since the cells grow by adherence to the glass surface this detoxifying step is very important.

A number of detergents may be satisfactory provided that the cleaning and rinsing procedures are adjusted accordingly. The following system is an example:

Wash the glassware in a Linbro "7x" cleaning solution, rinse 7 times with tap water, 7 times with deionized water, and 7 times with glass distilled water. The distilled glass water rinse is an important step of the cleaning cycle since piped water may contain pyrogens (by-products of bacterial growth) or dissolved metal ions that may interfere with cell growth. After being properly washed, the glassware should be dried and then autoclaved with the caps loosened. The sterile glassware is then dried in the oven to remove moisture. When the glassware is dry, the caps are tightened, and the glassware is shelved.

Some helpful notes regarding recyclable cell culture glassware are as follows:

1. Immerse all noncontaminated glassware in a Linbro "7x" cleaning solution immediately after use. Contaminated glassware should be placed in a decontamination pan and covered with water prior to autoclaving. Cell culture vessels should be brushed after autoclaving but before washing.
2. Glassware that has been in contact with toxic chemicals (such as formalin or picric acid) should be kept separate from the cell culture glassware or disposed of to prevent accidental use. As a general rule it is best to keep glassware used for cell culture separate from that used for other purposes.
3. Glass pipets should be acid dipped, thoroughly rinsed, cotton-plugged, placed in autoclavable containers, autoclaved, and oven dried.

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# 2

# INTRODUCTION TO CELL CULTURE

## CELL CULTURE

Viruses cannot replicate in synthetic media and require living cells as hosts. Until the 1950's, the only tools available to grow and study viruses were animals and embryonated eggs. Cell culture requires less space, is less expensive, and is more convenient than the use of animals or eggs, although eggs are still the method of choice in the large scale preparation of influenza virus vaccines. Developments in the cultivation of human and animal cells *in vitro* have been essential to advances in virology, genetics, and cell physiology. Cell culture allows the primary isolation of viruses, the performance of infectivity assays and biochemical studies, and the production of vaccines. The use of cell culture for virus replication is limited by the cell's sensitivity to the given virus, the method devised to detect virus replication, and the physical or biological conditions present that may interfere with virus replication.

Cultures of animal cells are usually divided into 3 classes: *primary cells*, *cell strains*, and *cell lines*. The ultimate source of cells for cell culture is the intact animal. The cells may be obtained from various organs and tissues of embryonic, infant, or adult origin. The cells thus obtained may be normal or cancerous. Regardless of their source, cultures obtained directly from the animal's organs and tissues, are referred to as **primary cell cultures**. To obtain a primary cell culture, tissues are cut up in small fragments and enzymatically digested, usually with trypsin, into constituent cells. The cell suspension and the appropriate growth medium are then added to a flat-bottomed vessel and placed in a $CO_2$ incubator. After a period of time the cells attach to the bottom of the flask and start dividing until a *monolayer* (a one-cell thick continuous layer) is formed. Cells that require a surface for attachment are referred to as *anchorage-dependent*. Certain cells (such as lymphoid cells) are not anchorage-dependent and are cultivated in suspension cultures.

Primary cell cultures retain some of the characteristics of the tissue from which they were derived and usually contain more than one cell type. With a few exceptions (such as nerve and muscle cells) cells in culture can be classified in two general morphological types. *Fibroblast-like cells* are thin and elongated, while *epithelial-like cells* are polygonal in shape and tend to form sheets. Most of the primary culture cells have a finite lifespan of 5-10 divisions *in vitro*. The

different cell types present in these types of culture make them sensitive to a wide range of viruses. Due to their limited lifespan, one cannot do long-term experiments with these cells.

Upon serial transfers of primary cells, a gradual selection may occur until a particular cell type becomes predominant. If these cells continue to grow at a constant rate over successive passages, these primary cells are referred to as a **cell strain** . These cells have a finite lifespan of less than 100 divisions *in vitro.* Diploid cell strains that retain their original diploid chromosome number and that are non-malignant (do not induce tumors in test animals) are useful in vaccine production.

If the cells in a cell strain undergo a transformation process (spontaneous or induced changes in karyotype, morphology or growth properties) that makes them "immortal" (able to divide indefinitely) they are called a **cell line.** It is not known how a diploid cell strain becomes a cell line, although this event may be mimicked by infection with oncogenic viruses or by exposure to chemical carcinogens. Cell lines often have abnormal chromosome numbers and may be tumorigenic when inoculated into susceptible animals. Cell lines that have been derived from tumors often do not exhibit *contact-inhibition* (inhibition of growth under crowded conditions), but rather continue to pile-up.

With some exceptions, the fully "normal" cell is considered to have its original number of chromosomes, have a finite lifespan, be anchorage-dependent, exhibit contact-inhibition, and to be non-malignant and non-transformed.

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# 3

# PREPARATION OF CELL CULTURE MEDIUM

## INTRODUCTION

Cells have complex nutritional requirements that must be met to permit their propagation *in vitro.* Different types of cells have different growth requirements and a number of chemically-defined formulations have been developed that support the growth of a variety of established cell lines. Table 3-1 describes some of the more commonly used cell culture media and some of their unique components. Although some serum-free media are available and some cell lines have been adapted to growing in such a medium, most cell lines require the addition of 5-10% serum as a supplement to promote cellular multiplication. Fetal Bovine Serum (FBS) is often the best to use although calf serum (CS) and horse serum are also used. The serum is heat-inactivated at 56°C for 30 minutes (to inactivate complement) prior to its addition to the cell culture medium. A pH indicator such as phenol red may be included in the original formulation to permit direct observation of the pH of the medium. The pH of the medium is adjusted by the dropwise addition of a sterile sodium bicarbonate solution that is used both as a nutrient and a buffering agent. Antibiotics such as penicillin and streptomycin are added to resist accidental bacterial contamination of the culture Once prepared, the cell culture medium has to be properly stored. For long-term storage, it should be frozen without $NaHCO_3$. On a short-term basis the medium should be kept at 4°C and warmed up to 37°C only for the time necessary to perform a given experiment. L-glutamine and antibiotics, in particular, have a short half-life at 37°C.

The medium that will be used throughout most of this text is Eagle's Minimum Essential Medium (E-MEM) with a phenol red indicator. E-MEM is a widely used cell culture medium. It was designed to support the monolayer growth of mouse L cells and HeLa cells with the addition of dialized serum. It is relatively simple and easy to prepare, and supports the growth of many different types of cells. E-MEM consists of 13 amino acids, 8 vitamins, glucose, and salts. E-MEM may be prepared from individual components provided that the chemicals are at least reagent grade and the water is glass-distilled. E-MEM is also commercially available in a powder form that is dissolved in glass-distilled water. The final solution has to be filter sterilized and dispensed into sterile containers. E-MEM may be obtained in a liquid form that does not require any filtration.

**Table 3.1** - "Characteristics of Commonly Used Cell Culture Media". Reprinted with the permission of Whittaker Bioproducts, Inc..

| Medium | Year Developed | Historical Development | Unique Components |
|---|---|---|---|
| **Medium 199** | 1950 | Formulated for nutritional studies on chick embryo muscle fibroblasts. | 1) Purines (adenine, guanine, thymine, uracil, xanthine, hypoxanthine)<br>2) Fat soluble compounds |
| **BME** | 1955 | Formulated for nutritional requirements of HeLa and mouse L cells. | |
| **EMEM** | 1959 | Formulated for cultivation of HeLa and L cells. Amino acid concentrations conform closely to the protein composition of human cells. Higher concentrations of nutrients permit longer periods between feedings. | 1) Vitamins 2-5X greater than BME<br>2) Amino acids greater than BME |
| **Alpha MEM** | 1971 | | 1) Vitamin $B_{12}$<br>2) Ascorbic acid<br>3) Non-essential amino acids<br>4) Sodium pyruvate<br>5) Lipoic Acid<br>6) D-Biotin |
| **DMEM** | 1969 | | 1) Types and quantities of amino acids greater than MEM<br>2) Vitamins 4X greater than MEM<br>3) Iron (Ferric nitrate) |
| **DMEM with high glucose** | Unknown | Cultivation of neural tissues | 1) Higher glucose concentration |
| **NCTC 109** | 1956 | Protein free medium for L-929 mouse cells | 1) Tween 80<br>2) Nucleic acid derivations<br>3) Additional vitamins<br>4) Coenzymes (DPN, Co A, TPN, FAD, UTP, TPP)<br>5) Reducing agents (Glutathione, ascorbic acid, L-cystine) |
| **NCTC 135** | 1964 | | 1) Same as NCTC 109 without L-cystine. |
| **McCoys 5A** | 1956 | Formulated for nutritional requirements of Walker 256 carcinoma. | 1) Asparagine<br>2) 3x normal glucose<br>3) Amino acids, all L-isomers<br>4) More folic acid |
| **RPMI** | 1964<br>1967<br>1968<br>1968 | #1629 (Modification of McCoy 5A) Cultivation of myeloblasts from human leukemia<br>#1640 Normal human leukocytes<br>#1634 Normal and neoplastic human hematopoietic cells<br>#1603 Multiple myeloma | |
| **Liebovitz L-15** | 1963 | Formulated for use without $CO_2$ atmosphere | 1) Free base forms of arginine, cysteine and histidine<br>2) High concentration of tyrosine<br>3) Galactose used instead of glucose<br>4) No $NaHCO_3$ |

The following protocol is for the preparation of a cell culture growth medium based on E-MEM. See the Appendix section for E-MEM preparation or ordering. Thioglycollate broth tubes are used as a routine indicator system for bacterial contamination of the medium.

## MATERIALS AND PREPARATION

- E-MEM 1 (95 ml) bottle/person with penicillin (100 units/ml) and streptomycin (100 µg/ml)
- Fetal Bovine Serum (FBS) heat-inactivated at 56°C for 30 minutes (5 ml)/person
- sterile 10% Sodium Bicarbonate ($NaHCO_3$) solution (10 ml)/person
- 1 liquid thioglycollate tube/person
- sterile 1 N HCl
- pipets
- decontamination pans

## EXPERIMENTAL PROTOCOL

1. Disinfect the working area with an appropriate disinfectant such as Osyl.
2. Using the bunsen burner and aseptic technique, transfer 5 ml of FBS to the 95 ml E-MEM bottle to obtain a solution of E-MEM with 5% FBS. The medium should be yellow or light orange.
3. Using a sterile 1.0 ml pipet add the sterile $NaHCO_3$ solution in a *dropwise* fashion until the medium is red, NOT PURPLE. After each addition, mix the contents before reading the color. If the color is purple, backtitrate with the dropwise addition of sterile 1 N HCl.
4. Sterility Test: transfer 0.5 ml of medium to a thioglycollate tube. Label the tube with your name, the date, and the medium being tested. Incubate at 37°C. Discard the negative tubes after 2 weeks. Positive tubes are indicative of a contaminated medium. The contaminated medium should be autoclaved and discarded, and new medium prepared.
5. Label the E-MEM medium with your name and the date and return to the refrigerator.
6. Label the sodium bicarbonate solution with your name.
7. Dispose of all pipets in a decontamination pan and disinfect your working surface.

NOTE: Whenever working with media and solutions remember to:

1. Examine the thioglycollate tube for possible contamination prior to using the medium in an experiment.
2. Label all materials.
3. Place on the appropriate shelf the solutions that can remain at room temperature.
4. Return the bottles of cell culture medium to the refrigerator when done with an experiment.
5. Warm all refrigerated media in a 37°C water bath prior to use with cell culture. The medium should be used as soon as possible as some of the medium components are labile even at room temperature.

## DISCUSSION QUESTIONS

1. Why should the cell culture medium be kept in the refrigerator?
2. Why should the cell culture medium be tempered to 37°C prior to addition to a cell monolayer?
3. Should you use your laboratory partner's cell culture medium that has been in the 37°C water bath since last night? Why?
4. Why do you need to worry about contamination even though penicillin and streptomycin are included in the culture medium?

## FURTHER READING

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# 4

# CELL GROWTH AND CELL PASSAGE

## INTRODUCTION

Different types of cells have different growth requirements, but in all cases the surrounding medium must supply all the essential nutrients. The temperature, pH, osmolality, and humidity must be kept within certain limits. Furthermore, toxic or inhibitory substances must not be present or allowed to accumulate. The addition of serum to basal medium as a source of macromolecular growth factors is essential to the growth of many cell types. **Anchorage-dependent cells** require a surface for attachment that is nontoxic and biologically inert.

Upon being seeded in a new culture vessel and attaching to the surface, the cells enter a *lag phase* during which there is no increase in cell number. In time, this is followed by a *log phase* of growth with an exponential increase in cell number and increased metabolic activity. The length of the log phase varies among cell types as different cell types divide at different rates (generation times). A *stationary phase* is reached when the nutrients are depleted and waste or toxic products accumulate. The cell number remains constant. This often happens when confluency is reached. The cell culture is termed **confluent** when the cells occupy all of the available growth area. Some cells are sensitive to contact inhibition (the inhibition of proliferation due to the cell-to-cell contact resulting from confluency) while other types, such as tumor cells, are not as sensitive. Cells become adversely affected and will eventually die if waste products are allowed to accumulate. Cells are *passaged* (subcultured) to maintain viability and optimum cell culture conditions. The **passage number** refers to the number of times the cells have been subcultured or transferred since the cell culture was obtained.

Different methods can be used to passage anchorage-dependent cells. The cells may be mechanically scraped from the cell culture surface with a rubber policeman (a wedge of soft rubber attached to the end of a glass rod). This removes the cells in clumps and can be damaging to some cells. The cell monolayer can also be treated with a calcium- and magnesium-free enzyme solution. The enzyme most often used is trypsin, although collagenase and pronase have also been used. The enzymatic treatment can be damaging to the cell surface and it is important to minimize the time of the cells' exposure to the enzyme. Ethylenediamine tetraacetic acid (EDTA), a chelating agent, is sometimes used alone or in combination with trypsin.

Cells that grow in suspension culture do not need to be trypsinized. The cell suspension is centrifuged, the spent medium is discarded, and the cells are resuspended in a small amount of fresh medium. A given quantity of these cells is then transferred into a new culture vessel with fresh medium.

When passaging cell cultures one can either make cell counts and transfer a given number of cells into a new vessel, or use a split ratio. The **split ratio** is not a dilution factor but rather refers to the fraction of cells transferred from the original vessel. A 1:5 split would result in the transfer of one fifth of the original cell population into each new culture flask, regardless of the final volume or size of the new flask. Although transferring a given cell number is a more accurate method, for routine cell passage, the use of split ratios works well and is less time consuming.

## *A. MECHANICAL PASSAGE OF CELLS*

### MATERIALS AND PREPARATION

- Flasks of various cell types to observe under the microscope
- 1-25 $cm^2$ confluent flask of $L_{929}$ cells/person
- E-MEM 5% FBS (as prepared in Chapter 3)
- sterile rubber policemen (cell scrapers) and pipets
- sterile 25 $cm^2$ flasks
- decontamination pans
- 37°C water bath (standard equipment for cell culture work)

### EXPERIMENTAL PROTOCOL

1. Observe the different cell types under the inverted microscope. Note the morphology, anchorage-dependence, health status, and percent confluency. Compare and contrast the various cell types.
2. Remove your E-MEM 5% FBS from the refrigerator and temper in a 37°C water bath.
3. Inspect the $L_{929}$ flask under the inverted microscope to make sure the cells are confluent . The cells should be elongated and firmly attached, and there should be no rounded or dead cells.
4. Aseptically decant the spent medium from the flask into the decontamination pan. The use of a paper towel at the bottom of the decontamination pan reduces splattering.

5. Aseptically add 3 ml of the warmed E-MEM 5% FBS to the cells.
6. Gently scrape the cell monolayer from the surface of the flask with a sterile rubber policeman.
7. Using a sterile pipet, pipet the cell suspension up and down 5 or 6 times to break up clumps of cells.
8. Aseptically add 5 ml of sterile MEM 5% FBS to the 25 $cm^2$ flasks that are to receive the cells.
9. Perform the required cell splits as determined by your instructor. Below are some examples of commonly used split ratios.

   For a 1:30 split (confluent within 6-7 days) transfer 0.10 ml of the original cell suspension to each new flask.

   For a 1:20 split (confluent within 5-6 days) transfer 0.15 ml of the original cell suspension to each new flask.

   For a 1:10 split (confluent within 4 days) transfer 0.30 ml of the original cell suspension to each new flask.

   For a 1:5 split (confluent within 2 days) transfer 0.60 ml of the original cell suspension to each new flask.
10. Cap the flasks tightly and tilt gently to evenly distribute the cells.
11. Use a permanent marker to label the new flasks with the cell type, passage number, split ratio, the date, and your name (fig. 4.1).
12. Place the flasks in the $CO_2$ incubator. Loosen the caps to allow $CO_2$ to enter.

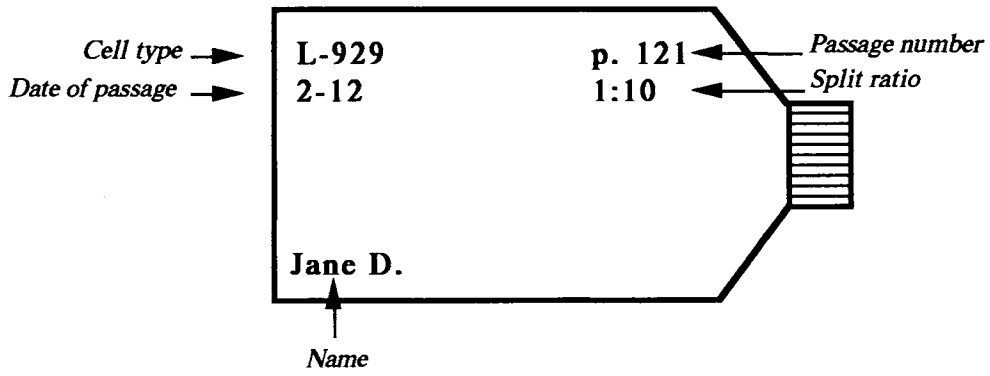


**Figure 4.1** Example of a labeled cell culture flask.

NOTE: At the end of each experiment it is important to replace all the media that require refrigeration in the refrigerator, to place all contaminated materials in the decontamination pans, and to disinfect the bench area.

## *B. ENZYMATIC TREATMENT OF CELLS: TRYPSINIZATION*

### MATERIALS AND PREPARATION

- Vero cells 1-75 $cm^2$/person or group
- Hanks' balanced salt solution (HBSS) without calcium & magnesium 20-25 ml/person
- 0.5% trypsin solution in HBSS without calcium & magnesium 5 ml/person or group
- sterile centrifuge tubes
- 1 each sterile 75 $cm^2$ flasks
- sterile pipets
- E-MEM 10% FBS
- decontamination pans
- 37°C water bath

NOTE: Trypsin solutions are susceptible to self-digestion. It is preferable to aliquot a freshly prepared trypsin solution into small quantities and to keep them frozen until needed.

### EXPERIMENTAL PROTOCOL

1. Warm the trypsin solution, HBSS, and E-MEM 10% FBS in a 37°C water bath.
2. Check the Vero cells under the inverted microscope.
3. Decant the spent medium and wash the Vero cells 3 times with 5 ml of HBSS without calcium or magnesium. All liquids should be decanted into the decontamination pan.
4. Add 5 ml of the 0.5% trypsin solution to the cell monolayer.
5. Place the flask in the $CO_2$ incubator for 5 minutes.
6. Gently shake the flask and check under the microscope for cell detachment.
7. Use a sterile pipet to transfer the contents of the flask to a sterile plastic centrifuge tube and centrifuge at 300 x g for 5 minutes.
8. Decant the supernatant into the decontamination pan and resuspend the pellet in 5 ml of E-MEM 10% FBS. Gently pipet up and down to break up cell clumps.
9. Add 10 ml of E-MEM 10% FBS to the 75$cm^2$ flask(s).

10. Do the appropriate cell split as determined by the instructor.

    Transfer 1 ml for a 1:5 (confluent within 2 days)

    Transfer 0.5 ml for a 1:10 (confluent within 3-4 days)

    Transfer 0.25 ml for a 1:20 (confluent within 6-7 days)

11. Cap the flasks and tilt gently to distribute the cells.
12. Label the flasks as indicated in Section A and place them in the $CO_2$ incubator. Loosen the caps to allow $CO_2$ to enter.

NOTE: The time of exposure to trypsin necessary for cell detachment will vary with different types of cells. This treatment is damaging to the cells and exposure to trypsin should be kept to the minimum amount of time required for the cells to detach. Frequent visual monitoring of the cells during trypsinization is required.

## DISCUSSION QUESTIONS

1. Cells are often routinely subcultered on a weekly basis. Why can a"healthier" cell culture sometimes be obtained by passaging the cells twice a week?
2. Why is a calcium- and magnesium-free medium necessary for trypsinization?
3. Why does EDTA alone work in releasing some cell types?
4. How does trypsin work to release cells?

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# 5

# COUNTING CELLS WITH A HEMACYTOMETER

## INTRODUCTION

Enumeration of cells is essential for cell culture work (ie. for the determination of multiplicity of infection and efficiency of plating, and when a specific number of cells is required to obtain a controlled cell density in a given time). Unless a large number of samples are routinely counted, a cell suspension is conveniently counted using a hemacytometer with a microscope. The hemacytometer (fig. 5.1) consists of two chambers that are covered by a cover slip. Each chamber is divided into a grid with nine large squares (fig. 5.2). Each large square is 1 mm x 1 mm (area of 1 $mm^2$) and the depth under the cover slip is 0.1 mm. The volume of each square in a given chamber is therefore 0.1 $mm^3$ or $10^{-4}$ $cm^3$. Since a $cm^3$ is equivalent to a ml, the number of cells per ml (cells/ml) of supension can be calculated by multiplying the average count per large square by $10^4$ and the reciprocal of the dilution factor. Red blood cell counts are done differently. For an explanation of a red blood cell count calculation, see fig 5.2 and its legend.

Errors that can lead to innacurate hemacytometer counts include: (1) inadequate dispersion of cells (presence of cell clumps in the sample), (2) inaccurate dilution of the cell suspension, (3) incomplete mixing of the cell suspension before filling the chambers, (4) improper filling of the chamber, and (5) improper cleaning of the chambers and cover slip (presence of air bubbles, grease and oils from fingers, or dirt particles).

The hemacytometer chamber and coverslip should be thoroughly cleaned with a mild detergent solution (such as 7x), rinsed with distilled water, rinsed with 95% ethanol, and wiped dry prior to addition of the cell suspension. The cell suspension should be pipetted up and down a few times to break up cell clumps and to provide a uniform suspension. Dilutions may be necessary to obtain the correct number of cells (each large square should have 20-60 cells). The hemacytometer chambers should be filled by capillary action. Overfilling or underfilling of the chambers will result in an inaccurate count. Once the chambers are properly loaded, the cell suspension should be allowed to settle before a count is taken. Since cell clumps will distribute as individual units, they are counted as one cell.

A total cell count does not distinguish between living and dead cells. The number of viable cells can be determined by staining the cell population with certain dyes such as trypan blue or erythrosine B. Living cells are able to exclude trypan blue and appear clear, whereas dead cells cannot and therefore appear blue. It is important to count the cells rapidly upon exposure to this dye since under certain conditions viable cells will begin to take up trypan blue over a period of time.

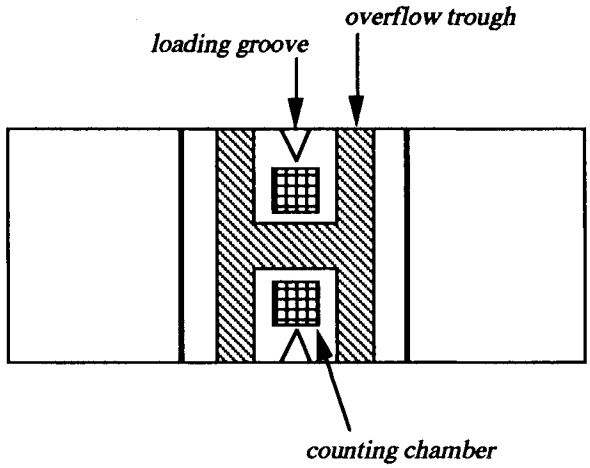


**Figure 5.1** The hemacytometer

## MATERIALS AND PREPARATION

- 1-25 $cm^2$ flask of $L_{929}$ cells/person
- HBSS
- Pipets
- Test tubes for dilutions
- Hemacytometers
- 95% ethanol
- Linbro "7x" solution and distilled water
- Lens paper
- Microscopes
- Hand counters
- Sterile Pasteur pipets and bulbs
- Trypan blue stain
- E-MEM 5% FBS

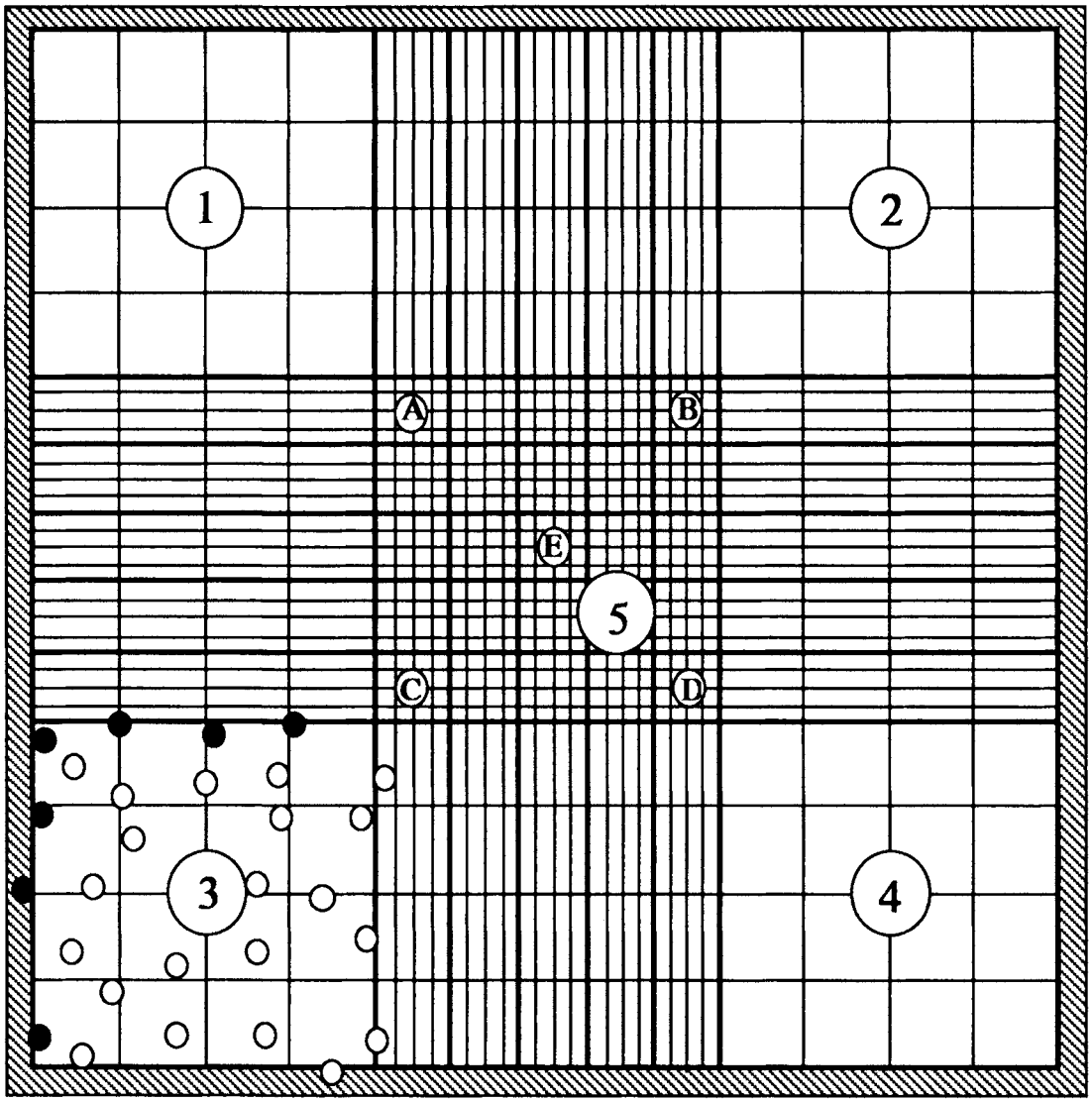


**Figure 5.2** Enlarged view of the hemacytometer counting chamber, as it would appear through the low power of the microscope. The cells in the five large squares (1-5) are counted and this number is divided by 5 to obtain the average number of cells per large square. This value is multiplied by $10^4$ and by the reciprocal of the dilution factor to obtain the number of cells/ml of the original cell suspension. Square #3 has a sample cell count. The open circles would be counted, whereas the dark circles indicate cells that would not be counted since they touch the top and left lines of that square.

Red blood cell counts are performed using the high dry objective of the microscope and counting the cells in 5 of the small squares (A,B,C,D, and E) of square #5. The cells/ml in the original suspension is calculated by multiplying the total number of cells in the 5 small squares by $5 \times 10^4$, and the reciprocal of the dilution factor.

## EXPERIMENTAL PROTOCOL

### *A. Cell Counts*

1. Thoroughly wash the hemacytometer and cover slip with the 7x solution and rinse with distilled water. Rinse with 95% alcohol and wipe dry with lens paper.
2. Decant the medium from the flask into a decontamination pan.
3. Wash the cells with 5 ml of HBSS. Decant into a decontamination pan.
4. Add 2 ml of E-MEM 5% FBS.
5. Gently scrape the cells. Pipet up and down to obtain a single cell supension.
6. Transfer 0.1 ml of the cell suspension to a dilution tube and add 0.4 ml trypan blue stain (1:5 dilution).
7. Gently swirl the cell suspension and remove a sample with a pasteur pipet.
8. Removing the bulb and using finger control, place the tip of the pasteur pipet in the V-shaped loading groove of the hemacytometer chamber. At about a 45° angle and by capillary action, let the cell suspension flow under the coverslip until the chamber is just filled but not overflowing into the overflow trough. Clean the hemacytometer and start over if the chamber is overfilled or air bubbles and dirt particles are present.
9. Load both chambers of the hemacytometer and allow the cells to settle for 1 minute before starting to count.
10. Use the low power objective on the microscope to count all the cells (stained and unstained) in each of the five large squares in one of the counting chambers (see fig. 5.2). Do the same for the second chamber. Take the average of these two values to obtain the *total cell count.*

    (a) Count the cells touching the right and bottom lines but do not count the cells touching the top and left lines. Remember that cell clumps are counted as one cell.

    (b) There should be 20-60 cells per large square (100-300 cells per 5 squares). If there are more, further dilute the cell suspension, and clean and reload the hemacytometer.

    (c) The cell counts of the two hemacytometer chambers should be within 10% of each other. If they are not, obtain further counts until the data cluster about a mean.

11. Following the same procedure as in step #10, count all the unstained (viable) cells in the 5 large squares of each chamber. Take the average of these two values to obtain the *viable cell count.*
12. The percent viable cells in the original suspension is determined as follows:

**% viable cells** = *100% x (viable cell count / total cell count )*

13. The number of cells per ml and the number of viable cells per ml, in the original cell suspension, can be calculated in the following manner:

**Total cells/ml** = *(total cell count / 5) x (1 / dilution) x* $10^4$

**Viable cells/ml** = *(viable cell count / 5) x (1 / dilution) x* $10^4$

NOTE: Trypan blue will bind to serum proteins and it is therefore important to dilute out the growth medium in HBSS prior to adding trypan blue. If there is excessive background staining the dye concentration may have to be increased or the cells centrifuged and resuspended prior to staining.

## *B. Preparation of Cell Suspensions*

Once a cell count is performed, the following formula is used to calculate the dilution necessary to obtain a desired volume of cells at a specific concentration (number of cells/ml).

$$V_1 \times C_1 = V_2 \times C_2$$

where:

$V_1$ is the volume in ml of diluted cell suspension desired.
$C_1$ is the concentration (number of cells/ml) desired.
$V_2$ is the volume in ml of the original cell suspension (to be calculated). This is the volume of the original cell suspension that will have to be diluted.
$C_2$ is the concentration (number of cells/ml) of the original cell supension.

So that, $V_2$ + **diluant** = $V_1$ at the desired concentration.

For example, if you need 15 ml of a 4 x $10^5$ cells/ml suspension for a given experiment, and if you have a cell suspension of 3 x $10^6$cells/ml, you can calculate the volume of the original cell suspension needed ($V_2$) in the following manner:

15 ml x (4 x $10^5$ cells/ml) = $V_2$ x (3 x $10^6$ cells/ml)
$V_2$ = 2 ml

Thus, 2 ml *($V_2$)* of the original cell suspension would be added to 13 ml of medium *(diluant)* to obtain 15 ml *($V_1$)* of 4 x $10^5$ cells/ml.

## DISCUSSION QUESTIONS

1. If the original cell suspension was diluted 1:10 prior to being loaded into the hemacytometer and the number of cells in the 5 large squares was 205, calculate the total number of cells/ml in the original cell suspension.
2. Assuming that of the 205 cells above, 10 were stained blue while 195 were unstained, calculate the percent viability.
3. Why might you expect a decrease in percent viability of cell suspensions that were stained with trypan blue and left for 30-45 minutes prior to being counted ? Does this reflect a true decrease in viability of the cell suspension ?
4. What is the reason for counting cells touching the right and lower boundaries of a square but not those touching the left and upper boundaries?
5. How would you prepare 10 ml of 6 x $10^5$ cells/ml from an original cell suspension containing 2 x $10^6$ cells/ml?

## FURTHER READING

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# 6

# PRIMARY CELL CULTURES

## INTRODUCTION

Primary cell cultures are cell cultures that are obtained directly from an animal's organs and tissues. They have a short "lifespan" in the laboratory since the culture dies off after a few passages. They usually contain a variety of cell types and support the growth of a variety of viruses. It should be noted that these primary cells may host some endogenous viruses and should be handled accordingly. The presence of an endogenous virus can also be an important variable when one is trying to isolate a virus from body fluids or tissue samples.

Primary cultures may be obtained from embryos or adult animals. Cells obtained from embryos are rapidly dividing cells that grow easily in vitro, are easily disaggregated and undergo mitosis soon after transplant. Chicken embryo cells (section A) are such an example. Cells obtained from an adult animal are more difficult to disaggregate and usually grow more slowly in culture. Sterility is often a problem with the adult animal since the normal microbial flora can contaminate the cell culture.

There are extreme differences in the tissue architecture of various organs and there is no "standard" procedure for preparing cell suspensions from tissues. A number of different techniques have been developed to obtain cell suspensions from various organs and these disaggregation methods can be broadly classified into 3 general categories: (1) *mechanical methods*, (2) *chemical methods*, and (3) *enzymatic digestion*. Most techniques for the preparation of primary cell cultures involve a combination of methods from two or even all three of these general categories.

**Mechanical methods** such as cutting, mincing, shearing and sieving of tissues can cause extensive damage to the cells and are normally used only in the initial stages of the disaggregation procedures to increase the surface area available to enzymatic digestion. **Chemical methods** usually involve the removal of divalent cations with or without the presence of a chelating agent such as ethylenediamine tetraacetic acid (EDTA). To maintain their integrity some tissues require the presence of divalent cations such as calcium and magnesium. If such divalent cations are omitted from the medium or a chelating agent is added, cellular dissociation is increased. **Enzymatic digestion** of tissues is performed with enzymes such as trypsin, collagenase, or pronase. The enzymatic treatment breaks down

the intercellular matrix and permits the release of individual cells. Under normal treatment some membrane damage also occurs but the cells are usually able to recover from this.

Since the disaggragation of tissues into single cell suspensions depends on the breakdown of the intercellular matrix, the method of choice will depend on the composition of this material as well as on the quantity and quality of cells desired. Larger numbers of cells are usually obtained by longer enzymatic digestions whereby healthier cells with a higher percentage of viability may be obtained by shorter exposure times and minimum mechanical trauma.

## *A. PRIMARY CHICKEN EMBRYO CELLS*

### MATERIALS AND PREPARATION

- embryonated eggs 10-12 days old
- candling lamp (a bright lamp which is shielded to make a beam opening of about 2 cm diameter)
- 70% ethanol
- sterile gauze
- sterile forceps
- 50 ml beaker with cotton soaked in ethanol (1/person)
- sterile petri dish
- sterile scissors (1/person)
- sterile 50 ml beaker (1/person)
- sterile HBSS with penicillin (100 units/ml) & streptomycin (100 ug/ml) (80-100 ml/person)
- sterile HBSS without calcium or magnesium
- sterile centrifuge tube (2/person)
- 0.5% trypsin in HBSS without calcium or magnesium (15 ml each)
- E-MEM 10% FBS
- sterile ehrlenmeyer flask (1/person)
- sterile 75 $cm^2$ flasks or 12-well dishes for plating the cells
- HBSS, trypan blue solution, and dilution tubes for cell counts
- 37°C shaker bath
- Egg holders
- sterile gauze filters (see fig. 6.2)

## EXPERIMENTAL PROTOCOL

1. Obtain an embryonated egg (10-12 days old)
2. Observe the embryo through a candling lamp. The vasculature should be evident. A lack of vasculature is indicative of a dead embryo and therefore of no use as a source of living cells. A dark or brown color may indicate that the egg is contaminated. Both the dead embryos and the contaminated eggs should be disposed of in a tied bag and sterilized prior to disposal.
3. Mark the location of the air sac with a pencil and then place the egg with the *pointed end down* in an egg holder.
4. Place warmed HBSS into a sterile petri dish to receive the embryo.
5. Sterilize the shell by washing with 70% ethanol and wipe with a sterile gauze.
6. Using sterile forceps, make a small hole at the top of the egg and gently peel off the shell through the air sac, to the shell membrane that covers the embryo. DO NOT YET REMOVE THE SHELL MEMBRANE THAT COVERS THE EMBRYO. See fig. 6.1.

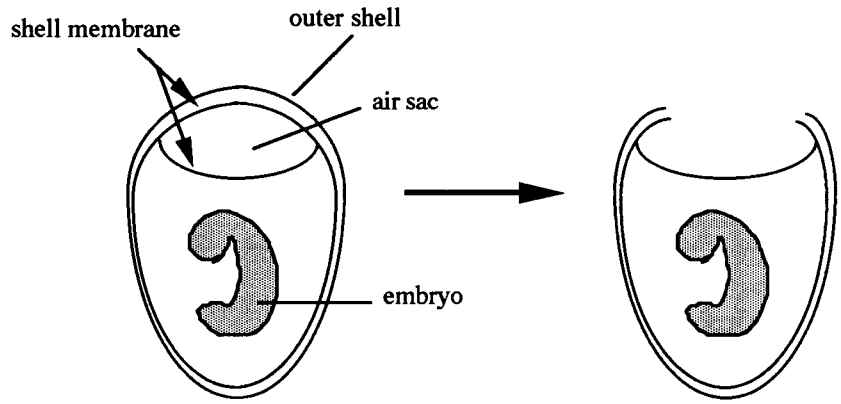


**Figure 6.1** Diagram of a chicken embryo with the top outer shell and shell membrane removed.

7. Sterilize the forceps by dipping them into a beaker containing cotton saturated with ethanol. Burn the excess ethanol from the forceps with a bunsen burner. Carefully remove the opaque shell membrane that covers the egg.
8. Ethanol-sterilize the forceps and then reach into the egg and remove the embryo (avoid taking out any yolk or membrane fragments). Place it in the petri dish with the HBSS.

9. Remove the head, wings, and legs with sterile scissors.
10. Place the torso in a small sterile beaker and mince the embryo for several minutes with the sterile scissors.
11. Mark the 10 ml volume mark on a sterile centrifuge tube.
12. Wash the cells in the beaker with 2-3 ml volumes of warmed HBSS and add to the centrifuge tube until the 10 ml mark.
13. Centrifuge at 300 x g for 10 minutes.
14. Discard the supernatant and wash the cells with 10 ml of HBSS two additional times.
15. After the third wash pour off the HBSS (at this point you should have about 2 ml of packed cells) and resuspend in 10 ml of HBSS without calcium or magnesium.
16. Transfer to a sterile ehrlenmeyer flask , add 10 ml of the 0.5% trypsin solution, and place in the shaker bath.

NOTE: Steps #15&16 should result in a 1:10 final volume of cells in a 50:50 solution of HBSS and 0.5% trypsin. If only 1 ml of cells is recovered, only add 5 ml of HBSS and 5 ml of the trypsin solution.

17. Incubate for 1 hour in the 37°C shaker bath.
18. Obtain a sterile centrifuge tube.
19. Filter the cells through a sterile gauze filter into the sterile centrifuge tube. Add more medium if necessary to wash the cells through the funnel. See figure 6.2.
20. Centrifuge the cells at 300 x g for 10 minutes.
21. Discard the supernatant and wash the cells 3 times with 10 ml of HBSS without calcium or magnesium for each wash.
22. After the final wash, decant the supernate and resuspend the pellet in 3-5 ml of E-MEM 10% FBS.
23. Perform a cell count (do not count red blood cells) and prepare a cell suspension of 4-5 x $10^6$ cells/ml in E-MEM 10% FBS.
24. Plate the cells in a sterile 75 $cm^2$ flask (10 ml/flask, confluent in 2-3 days) or in 12-well plates (1 ml/well), as indicated by your instructor.
25. Incubate the cells at 37°C and 5% $CO_2$.
26. Observe the cells daily and note growth rate and morphology. Check for contamination.

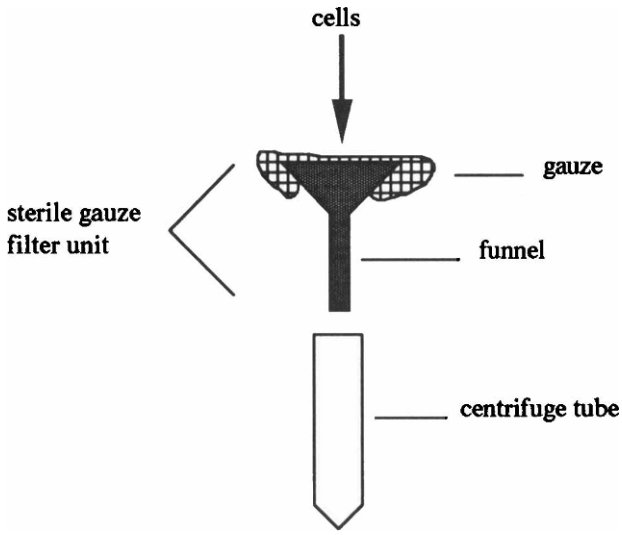


**Figure 6.2** Diagram of the sterile gauze filter unit used to filter the cell suspension into the centrifuge tube.

## *B. MOUSE SPLEEN CELL CULTURE*

### MATERIALS AND PREPARATION

- mice (1/person)
- HBSS with penicillin (100 units/ml) and streptomycin (100 ug/ml) (50ml/person)
- ice buckets with ice
- sterile forceps
- sterile 60 mm x 15 mm petri dishes (1/person)
- 70% ethanol
- RPMI 1640 10% FBS
- 16x125 mm sterile screw cap glass tubes (1/person)
- sterile 10 cc syringe with 26 gauge needle (1/person)
- sterile scissors (2/person)
- small beakers with 95% ethanol-soaked cotton (1/person)
- sterile 15 ml centrifuge tube (1/person)
- sterile rubber policemen (1/person)
- sterile towels (1/person)
- sterile pipets

- pasteur pipets and bulbs
- hemacytometers
- trypan blue solution
- $NH_4Cl$ lysing buffer, sterile
- 2-Mercaptoethanol solution (2-ME, 5 x $10^{-2}$, see Appendix), diluted 1:10 in RPMI 1640 10% FBS.
- Concanavalin A (Con A, purified; Sigma #C 5275). This is used as an interferon inducer (see chapters 32-35).

## EXPERIMENTAL PROTOCOL

1. Sacrifice the mouse by gasing with $CO_2$ or by anesthetic overdose.
2. Place the mouse with the abdomen facing up on a sterile towel and soak the abdomen with 70% ethanol.
3. Pick up the skin with the sterile forceps and with the sterile scissors perform a crosswise cut of the skin. Pin back the flaps to keep them out of the way from the exposed abdominal area. See fig. 6.3 below.

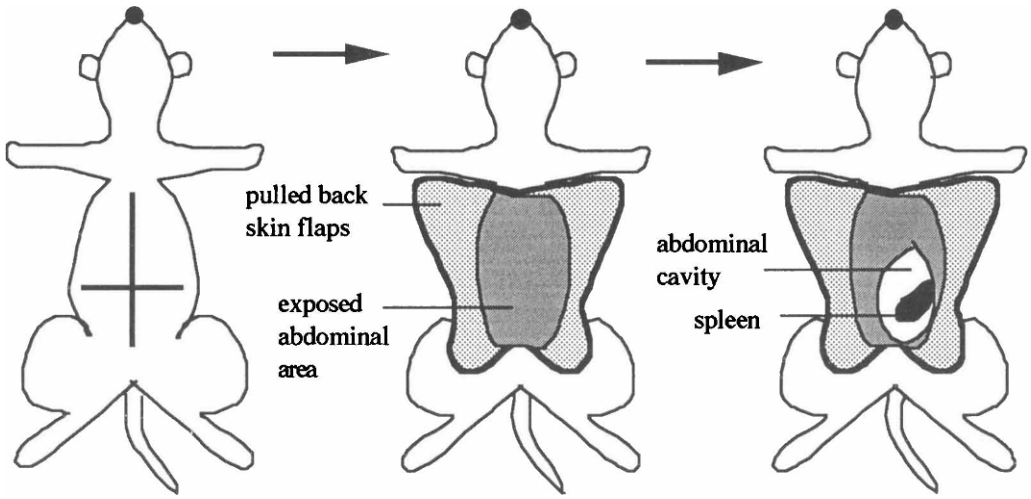


**Figure 6.3** Diagramatic representation of the incisions necessary to locate the spleen of a mouse.

4. Spray 70% ethanol on the exposed abdominal area.
5. Ethanol-sterilize the forceps and scissors by dipping them in the small beaker with ethanol-soaked cotton and igniting them with

the bunsen burner. Do not hold them in the flame for more then an instant since the extreme heat will ruin the temper of the metal. Simply ignite the ethanol and quickly remove. DO NOT TO REINSERT THE FLAMING SCISSORS INTO THE ETHANOL BEAKER. Wait for the flames to be extinguished before proceeding with the incision of the alcohol-soaked abdomen of the mouse.

6. Make an incision and cut the abdominal wall in the lower left quadrant of the abdominal area of the mouse, so as to expose the abdominal cavity and the spleen (see fig. 6.3).
7. Ethanol sterilize the forceps and scissors. Remove the spleen by lifting with the forceps and cutting away the connective tissue.
8. Place the spleen in a small sterile petri dish and add enough HBSS to keep the spleen wet. (If more than 1 spleen is needed to perform a given experiment, it is recommended to place the spleen-containing petri dishes on ice until all the spleens have been obtained).
9. With sterile scissors cut the spleen in two and gently loosen the cells with a sterile rubber policeman.
10. Aspirate the cells with a sterile 10 cc syringe fitted to a 26 gauge needle. Avoid picking up connective tissue.
11. Transfer the cell suspension into a sterile 15 ml centrifuge tube. Add HBSS to wash off the cells remaining in the petri dish and transfer to the centrifuge tube.
12. Centrifuge at 200 x g for 7 minutes.
13 Decant the supernatant and resuspend in 2 ml of $NH_4Cl$ lysing buffer.
14. Incubate at room temperature for 2 minutes.
15. Add 10 ml of HBSS and mix gently.
16. Centrifuge at 200 x g for 5 minutes. Decant the supernatant.
17. Wash the cells 2 times and resuspend the final pellet in 5 ml of RPMI 1640 with 10% FBS.
18. Perform a 1:5 (0.1 ml of cells + 0.4 ml of HBSS) dilution of the cells in a non-sterile test tube. Mix well and transfer 0.1 ml of this to a test tube containing 0.4 ml HBSS + 0.5 ml trypan blue solution. The final dilution is a 1:50. Determine the % viability and obtain a viable cell count.

19. Dispense the amount of the cell suspension required to yield 1 x $10^7$ cells/ml at the final volume required (10 ml) into a sterile centrifuge tube or flask.

    Add 0.1 ml of the 1:10 diluted 2 ME stock solution (= 5 x $10^{-3}$ M 2 ME).

    Add RPMI 1640-10% FBS to give the calculated final volume (10 ml) resulting in a final suspension containing 1 x $10^7$ cells/ml and 5 x $10^{-5}$ M 2-ME.

20. Aliquot 4 ml of the final suspension to each of 2 sterile round-bottomed 16 x 125 mm screw capped test tube.

21. *If performing this as an interferon induction experiment, to one tube add the required volume of Con A to have a final volume concentration of 1 μg/ml. See Chapter 32. This will be the experimental tube. The other tube will not receive any Con A and will be the control tube.*

22. Cap tighty, label, and place at 37°C on a roller apparatus.

23. *If performing the interferon induction experiment: Harvest the supernatant 48 hours later. Centrifuge the cell suspension at 600 x g for 10 minutes and pour off the supernatant in an appropriately labeled sterile tube. Freeze at -70°C until ready to assay.*

## DISCUSSION QUESTIONS

1. What are the advantages and disadvantages of using primary cell cultures?
2. What is the effect of the composition of the intercellular matrix on obtaining primary cell cultures?
3. Why is trypsin treatment necessary for preparation of chicken embryo cells but not for mouse spleen cells?
4. What are the possible consequences of the presence of an endogenous virus in a primary cell culture?

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# 7

# CRYOPRESERVATION OF CELLS

## INTRODUCTION

When cells are maintained at reduced temperatures, their metabolic rate is decreased and they require less frequent passaging. At extremely cold temperatures, destructive processes as well as normal metabolic processes are inhibited, and the cells are in a state of "suspended animation". Cells can be stored frozen at -70°C in the presence of glycerol but over a period of months to years there is a gradual loss of viability. Storage of mammalian cells in liquid nitrogen (-196°C) is presently the accepted procedure that results in indefinitely prolonged preservation of viability.

In the absence of a cryoprotective agent such as glycerol or dimethylsulfoxide (DMSO) the freezing process is lethal to most mammalian cells. The damage that occurs is caused by the mechanical injury due to ice crystal formation, altered concentration of electrolytes, changes in pH, dehydration, and protein denaturation. Procedures that have evolved to offset or minimize these damaging conditions are as follows:

1. Glycerol or DMSO is added to the cells to lower the freezing point and to protect the membrane from rupture.
2. A slowed cooling rate allows water to move out of the cells before freezing.
3. Storage of cells below -130°C retards ice crystal formation.
4. Cells are thawed rapidly when they are taken out of the frozen state in order to limit the cell damage that is thought to occur in the -50 to 0°C temperature range.

Different cell types may vary greatly in the response to the physical and chemical trauma of freezing and thawing. No single medium or procedure is optimum for all cell cultures. As a general rule most cell culture will respond well to the freezing procedure if (1) the medium contains 5-15% glycerol or DMSO, (2) freezing is performed under conditions that result in a 1-10° per minute cooling rate, (3) 5-25% FBS is added to the medium to further protect the cells during the freezing process, and (4) the cells are in a good state of nutrition and maximal viability at the time of freezing.

## *A. CRYOPRESERVATION*

### MATERIALS AND PREPARATION

- 1-25 $cm^2$ bottle of $L_{929}$ cells/person
- E-MEM-5% FBS with 10% glycerol (5 ml/person)
- HBSS for cell count (1 ml/person)
- trypan blue for viable cell count
- hemacytometer
- nonsterile tubes for cell counts
- sterile pipets
- 1 thioglycollate tube/person
- 2 sterile 5 ml ampules/person
- ampule sealer and forceps
- beaker with 95% ethanol
- sterile rubber policemen (cell scrapers)

### EXPERIMENTAL PROTOCOL

1. Observe the cells under the microscope. Cells should be healthy and not overconfluent. NOTE: when setting up a laboratory cell stock the culture medium is changed 24 hours prior to freezing to enhance the cells' metabolic activity.
2. Decant the medium and add 5 ml of E-MEM 5% FBS with 10% glycerol. (Alternatively, the cells could be trypsinized and then resuspended in freezing medium).
3. Scrape the cell monolayer with a sterile rubber policeman.
4. Pipet the cells up and down several times to break up the clumps and to get an even suspension.
5. Withdraw 0.6 ml of the cell suspension. Dispense 0.1 ml into a nonsterile test tube and the remainder into a thioglycollate tube as a sterility check.
6. Add 0.4 ml of trypan blue to the nonsterile tube with 0.1 ml of cells (1:5 dilution).
7. Load the hemacytometer and count the blue (non-viable) and the clear (viable) cells. Calculate the percent viability and determine the number of viable cells per ml.

8. Adjust the original cell suspension to obtain 4-5 ml of 2 x $10^6$ viable cells/ml in E-MEM with 5% FBS and10% glycerol.
9. Dispense 2 ml of the cell suspension into each of 2 sterile ampules.
10. Flame seal the ampules and properly label them.
11. Place the ampules in a beaker containing ethanol and transfer to a refrigerator for 1 hour. The ethanol is used as a bath to distribute the heat exchange from different parts of the ampule. It has a lower freezing point than water and will remain liquid at -70°C.
12. Transfer the beaker containg the ampules to a -20°C freezer for 1 hour.
13. Transfer the beaker containing the ampules from the - 20°C to the -70°C freezer for 1 hour. After the hour remove the beaker and place the ampules in an appropriately labeled freezer box at -70°C. The ampules should have a label with the following information: cell type, passage number, date, and initials of the person having performed this process.

NOTE: This procedure is compatible with maintenance of viability of $L_{929}$ cells and routine storage. Laboratory cell stocks should be frozen in liquid nitrogen for long term maintenance (see Further Reading references #1 & #2).

## *B. THAWING OF FROZEN CELLS*

### MATERIALS AND PREPARATION

- 1 ampule of frozen $L_{929}$ cells from section A
- E-MEM 5% FBS
- sterile centrifuge tubes (2/person)
- nonsterile tubes for cell counts
- HBSS & trypan blue for cell counts
- hemacytometers
- 2 sterile 25 $cm^2$ flasks/person
- sterile pipets

NOTE: Whenever a cell stock is frozen in the laboratory two ampules are thawed within 24 hours of the cryopreservation procedure to assess the viability of the cell stock thus preserved. In the following section only one of the ampules will be thawed

## EXPERIMENTAL PROTOCOL

1. Remove the ampule to be thawed from the -70°C freezer and immediately place in a 37°C water bath.
2. Open the ampule aseptically and transfer the cell suspension to a sterile centrifuge tube.
3. Centrifuge at 600 x g for 10 minutes. Discard the supernatant and resuspend in 10 ml of E-MEM 5% FBS.
4. Transfer 0.1 ml of the cell suspension to a small test tube and add 0.1 ml of trypan blue solution (1:2). Obtain a total and a viable cell count, and calculate the % viability. Compare that figure to the percent viability of the cell culture before freezing.
5. Dispense 5 ml of the cell suspension into each of 2 - 25 $cm^2$ flasks.
6. 24 hours later change the medium and observe the cells.

## DISCUSSION QUESTIONS

1. What is the purpose of the viable cell count performed following the thawing of the frozen cells? How might the percent viability vary over time in the freezer?
2. Why is it important that the laboratory cell stocks be backed up in liquid nitrogen?
3. Why change the medium after 24 hours?
4. In what ways would cryopreservation of cells be similar or different from cryopreservation of viruses?

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# 8

# INTRODUCTION TO VIRUS PASSAGE

## PROPAGATION OF VIRUS

There are various methods to propagate and harvest virus. The one common element to these procedures is the virus' requirement for living cells in order to replicate and produce progeny virus. Viruses are propagated in the laboratory in the following host systems: (1) *cell culture*, (2) *embryonated eggs*, and (3) *animals*.

**Cell culture** is the most convenient and widely used system for the isolation and propagation of many viruses. To passage virus, cell cultures (primary cells, cell strains, or established cell lines) are inoculated with virus. The cells are then monitored for the evidence of viral replication (ie. cytopathic effect, transformation, syncitia formation). The cells are freeze-thawed to release intracellular virus, and the cell culture medium is collected. Virus progeny are pooled and stored frozen at -70°C.

**Embryonated eggs** require less space than do animals and less preparation and upkeep than cell cultures. It is possible to work with eggs on a very large scale so that it is a popular method for the preparation of virus in bulk for vaccines. This method of virus propagation is still somewhat space and cost intensive because the infected eggs must be isolated. Many viruses do not grow in eggs but embryonated eggs are the method of choice for the propagation of paramyxoviruses and influenza viruses.

The **live animal**, especially the newborn mouse, is another commonly used host. Upon inoculation of the virus, the animals are monitored for signs of presence of the virus. The animals are then sacrificed and the necessary tissues are collected and frozen.

## PRESERVATION OF VIRUS

Once the virus has been harvested, it may be stored at 4°C for about 24 hours. Although viruses vary greatly in their sensitivity to heat (even room temperature), most viruses are heat labile and longer-term storage should be:

1. At temperatures of -196°C (liquid nitrogen) using tightly sealed containers and adding proteins (ie. bovine albumin, serum) to stabilize the virus during low temperature storage.

2. By lyophilization. The samples are rapidly frozen, lyophilized, and stored at 4°C or -20°C.
3. In 60% glycerol at -70°C (temperature of dry ice and low-temperature freezers).

Losses of viral infectivity can occur at any point during storage, freezing, thawing, or rehydration. In general, concentrated viral suspensions lose less infectivity, balanced salt solutions stabilize viruses during rehydration, and viruses are more stable when cell materials are not removed. Most viruses lose infectivity upon repeated freeze-thawing cycles. Unless a large amount of virus is needed, the virus suspension should be frozen in small aliquots and each time a vial is thawed a small mark or date should be recorded on the label. Depending on the virus and the suspending medium, a given aliquot should not be thawed more than 3-4 times. Depending on the virus, a 1/2 to 1 log reduction of infectivity can be expected with each freeze-thawing cycle.

## FURTHER READING

White, David O. and Frank J. Fenner. 1986. *Medical Virology.* Academic Press, Inc. Orlando, Fl., pp. 17-19 & 35-45.

# 9

# VIRUS REPLICATION IN CELL CULTURE

## INTRODUCTION

Cell culture is convenient for the small-scale propagation of viruses. One can normally obtain 10-100 times as many virions as the number of cells that were originally infected. Many details of the infection process are controllable to a high degree (including cell density, age, multiplicity of infection, medium composition, cell type, and length and temperature of adsorption). Many cell types from different species are available so that, with most viruses, it is not necessary to use live animals for virus propagation. For virus replication to occur, the cell type used must be permissive for the particular virus under study. Few viruses will grow in a wide variety of cells, and even within a given virus class, some viruses may grow in a particular cell type while others do not.

Virus growth in cell culture can be monitored through a variety of biochemical procedures that detect the increase in viral macromolecules and progeny virus. Most commonly, however, viral replication is followed through the appearance of cytopathic effect (CPE) in the infected cell culture. CPE (as rounded cells, granularity, vacuolization, syncytia formation, or focus formation) gradually becomes visible as the virus takes over the cell's machinery during the replication process.

In the case of a non-cytopathogenic virus (one that does not cause CPE), the following methods may be useful in monitoring viral replication:

1. *Synthesis of viral nucleic acids and proteins* - indicating that the virus is replicating even though no apparent CPE can be observed.
2. *Hemadsorption* - the ability of cells infected with hemagglutinating viruses to adsorb erythrocytes.
3. *Immunofluorescence* - whereby a specific viral antibody that has been labeled with a fluorescent dye is used to stain the infected cell culture. If the virus is present, its specific antibody will bind, and fluorescence will be observed.
4. *Interference* - the process by which the multiplication of one virus in a cell inhibits the replication of a subsequently infecting virus.

In the following experiment, encephalomyocarditis virus (EMC) a small, non-enveloped, positive-strand RNA virus belonging to the family Picornaviridae will be used. The natural hosts of EMC virus are rodents. EMC virus hemagglutinates sheep and human red blood cells. EMC virus can be grown in eggs, cell culture, and animals. Experimental EMC virus inoculation of mice results in death with central nervous system involvement. Infection is usually inapparent in rodents infected in nature.

EMC virus will replicate in either L cells or BHK-21 cells. L cells (a mouse cell line) are used in most of the experiments because they are subcultured easily and grow well. The L cells are not used in the following experiment to passage the EMC virus because of the possibility of interferon production. The presence of this antiviral agent in the supernatant fluid along with the progeny virus could interfere with the results of some of the later experiments. The BHK-21 cells (a baby hamster kidney cell line) are used here to passage EMC. The virus replicates well in these cells with little interferon production since BHK-21 cells are poor interferon producers when exposed to EMC.

The BHK-21 cells are grown in MEM with 5% TPB and 5% FBS. The higher percentage of serum in this medium makes it a growth medium. For virus infection however, a maintenance medium (MEM 5% TPB and 2% FBS) is used. In this medium, the cells are not actively growing but are rather "maintained", preventing overgrowth of the cell monolayer while still allowing virus replication.

A high mutiplicity of infection (MOI) of the EMC is used for the infection process to obtain a single cycle of infection. Washing the cells after the time allowed for virus adsorption removes the original viral inoculum so that only the progeny virus will be harvested.

## MATERIALS AND PREPARATION

### Virus Inoculation

- BHK-21 cells in 25 $cm^2$ flasks (1/person)
- $10^{-3}$ dilution of EMC in GLB (0.7 ml/person)
- sterile PBS or HBSS (25 ml/person)
- sterile pipets
- MEM 5% TPB 2% FBS (5ml/person)

### Virus Harvest - 24 hours later

- sterile 15 ml conical centrifuge tube (1/person)
- sterile pipets
- 2 washed and sterile 1-dram vials/person

- Ethanol-dry ice bath: In an ice bucket with a lid, slowly add 1 volume of dry ice to 1-2 volumes of 95% ethanol. There will be a lot of foaming until the ethanol is cooled.

## EXPERIMENTAL PROTOCOL

### Virus Inoculation

1. Observe the BHK-21 cells under the microscope to make sure that the cells are healthy and not overconfluent.
2. Wash the BHK-21 cells two times with 5 ml of sterile PBS or HBSS to remove any dead cells.
3. Add 0.5 ml of a $10^{-3}$ dilution of EMC in GLB to the cell monolayer.
4. Incubate at 37°C for 30 minutes. Tilt the flasks gently every 10 minutes to allow virus adsorption, to promote even virus distribution, and to keep the cell monolayer moist.
5. Gently wash the cell monolayer 3 times with 5 ml of sterile PBS or HBSS to remove any unadsorbed virus. Carefully pour off the contents of each wash into a decontamination pan. A paper towel on the bottom of the pan will reduce splattering and virus aerosols.
6. Add 4 ml of prewarmed MEM 5% TPB 2% FBS to the cells. (This is a maintainance medium).
7. Incubate at 37°C in the $CO_2$ incubator with the caps loose.
8. Observe the cells at 24 hours post-infection. Compare the virus-infected cells to the mock-infected (no virus) control provided by the instructor. When complete CPE is observed tighten the cap and place the flask in the -70°C freezer. (If you are ready to proceed to virus harvest, freeze the flask in the ethanol-dry ice bath and go on to the following section)

### Virus Harvest

1. Thaw the virus-infected flask in cold tap water.
2. Freeze-thaw the cell monolayer 2 more times in an ethanol-dry ice bath to release the intracellular virus. Use cold tap water to thaw the cells and the dry ice/95% ethanol bath to freeze the cells.
3. Harvest the contents of the flask into a sterile 15 ml conical centrifuge tube.

4. Centrifuge at 600 x g for 15 minutes to pellet cellular debris.
5. Carefully pipet the supernatant fluid that contains the virus, and transfer 1-2 ml into each of 2 1-dram vials.
6. Label the vials as shown in fig. 9.1.
7. Place all the vials in a box and store at -70°C.

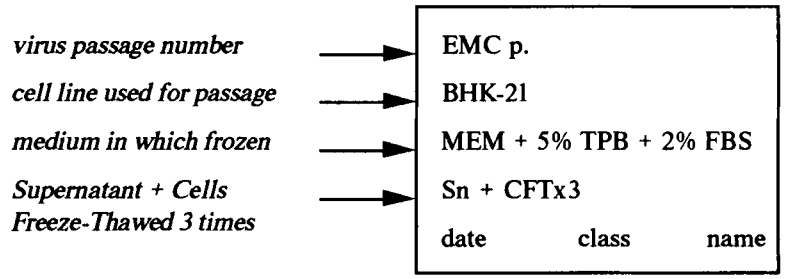


**Figure 9.1** Label of vial for freezing virus stock.

## DISCUSSION QUESTIONS

1. What is the purpose of washing the unadsorbed virus after virus infection?
2. What is the purpose of the freeze-thawing step during virus harvest?
3. What difference would it make if the cells in the bottle were sparse? overgrown?

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# 10

# VIRUS REPLICATION IN EMBRYONATED EGGS

## INTRODUCTION

Prior to the 1950's the standard host for the propagation of many viruses was the embryonated egg. A variety of embryonic cell types and routes of inoculation are available so that most viruses known at the time could be grown in the developing egg. Since then, cell culture has become widely adopted for the cultivation of viruses, but embryonated eggs are still used for the isolation of avian viruses, influenza viruses, and for vaccine production. The anatomy of the developing egg ( fig. 10.1) and the general function of its parts are as follows:

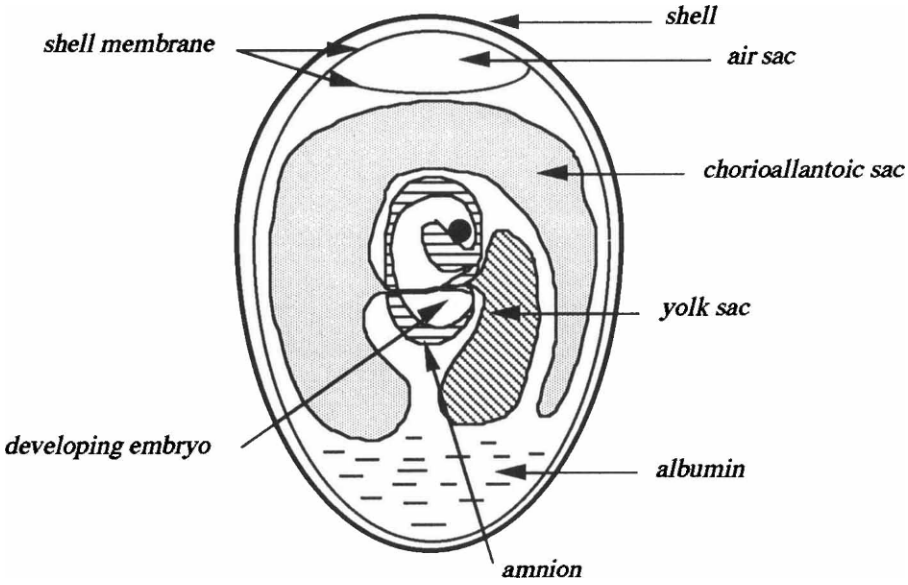


**Figure 10.1** The embryonated egg

1. The **air sac** is important to the developing embryo for respiration and for pressure adjustments.
2. The **shell** and **shell membrane** function both as a barrier and as an exchange system for gases and liquid molecules.

3. The **chorioallantoic sac** and its contents (allantoic fluid) remove waste products produced by the developing embryo. This membrane and its contents increases in size as the embryo grows.
4. The **yolk sac** is the source of nourishment for the developing embryo. As the embryo develops, the yolk sac decreases in size until it is completely absorbed into the digestive system of the mature embryo.
5. The **amnion** is a thin membrane that encloses the embryo and protects it from physical damage. It also serves as an exchange system and is best seen in the younger embryos.

Artificial incubation of the embryonated egg must be at 37°C with 62% humidity and forced air circulation. The humidity and air circulation are important to prevent dessication of the shell membrane that serves as an exchange system for the developing embryo. Once the hen lays the egg, and as long as the egg is not exposed to extremes of temperature, further development will be suspended until incubation occurs. The length of the period of incubation determines the age of the embryo.

The chosen route of inoculation and age of the embryo are determined by the given virus' selectivity for a certain membrane or developmental stage of the embryo. For example, Infectious bronchitis virus is propagated in the yolk sac of a 5-6 day old embryo whereas Rous sarcoma virus is inoculated on the chorioallantoic membrane of a 9-11 day old embryo and will produce pocks 5-10 days post-infection. Figure 10.2 briefly describes some of the commonly used routes of inoculation.

The following general procedure applies when a virus is grown in the embryonated egg:

1. The eggs are candled to determine the position of the embryo and its viability. Since viruses need living tissues to replicate, candling is an important first step. When candled, a healthy embryo has an orange color with evident vasculature. A dead embryo is clear yellow with a lack of vasculature. A black, brown or green cast is indicative of contamination and these eggs should be properly discarded.
2. The shell is disinfected and a hole is drilled or punched.
3. The virus is injected in the appropriate area of a 5-14 days old embryonated egg. The age chosen in this range depends on the route of inoculation desired since the various membranes and their contents vary in size as the embryo matures.
4. Embryos dying 16-24 hours post-injection are discarded since death is usually then due to injection trauma or contamination.
5. 2-5 days post-injection, viral growth can be recognized by such criteria as death of the embryo, pocks, or hemagglutination.

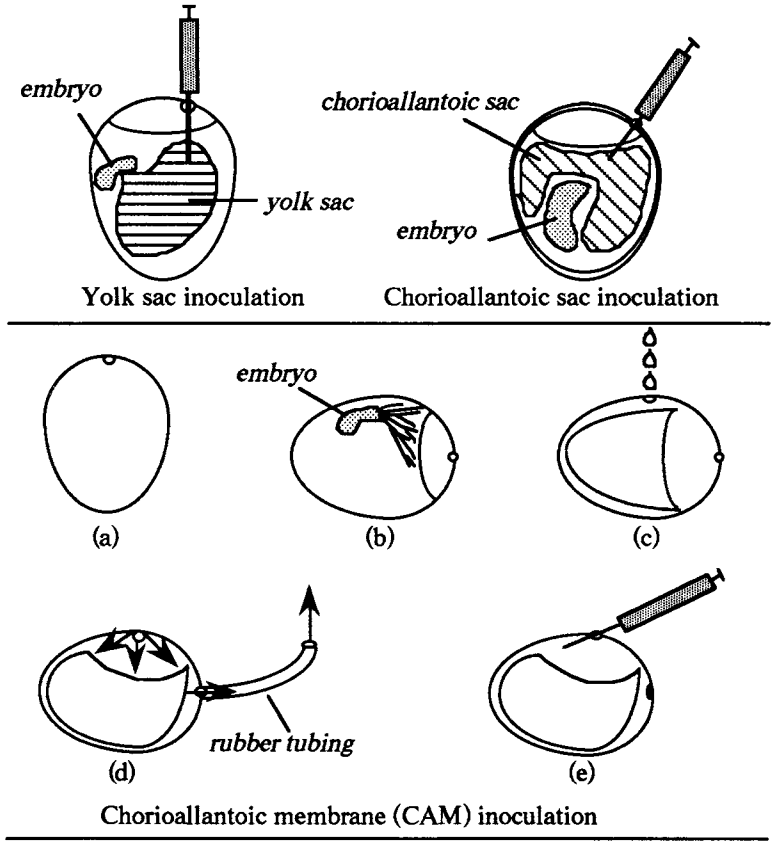


**Figure 10.2** Common routes of inoculation of embryonated hen eggs.

*Yolk sac inoculation* - A 5-6 day old embryonated egg is candled to determine the position of the embryo and the air sac. A hole is punched half way up the air sac and away from the embryo. The inoculum is delivered using a 22 gauge, 1 and1/2 inch needle inserted straight down into the yolk sac. The hole is sealed and the egg is incubated blunt end up.

*Chorioallantoic sac (CAS) inoculation*- A 9-11 day old embryonated egg is candled to determine the injection site that is 5-6 mm above the bottom margin of the air sac and one-third turn the distance away from the embryo. A hole is punched and the inoculum is delivered using a tuberculin syringe. The hole is sealed and the egg is incubated blunt end up.

*Chorioallantoic membrane (CAM) inoculation*- A 10-12 day old embryonated egg is candled to determine the position of the embryo and air sac. (a) A hole is punched on the top of the blunt end and the shell membrane is pierced with a sterile needle. (b) The egg is laid on its side and a second hole is punched where the embryo was located. (c) Drops of sterile saline are allowed to seep into the CAM to loosen it. (d) Gentle suction is applied while a piece of rubber tubing is held firmly against the air sac hole. This moves the egg contents into the air sac and creates an artificial air space under the hole in the side of the egg. (e) The blunt end hole is sealed and a tuberculin syringe is used to slowly deliver the inoculum in the cavity just created. The hole is sealed and the egg is incubated in a horizontal position with the side hole facing up.

In this experiment Newcastle disease virus (NDV) will be passaged in embryonated chicken eggs. NDV is a paramyxovirus that causes a highly contagious and sometimes fatal infection in chickens and other birds. Human infection may occur and results in conjunctivitis and laryngitis. NDV is relatively heat stable in that it may remain infectious for up to a month at room temperature. NDV is an enveloped virus and has hemagglutination, neuraminidase, hemolytic and cell fusion abilities. Early infection can be detected by hemadsorption. Although this virus may be grown in a variety of avian and mammalian cells, it is readily propagated in embryonated eggs.

NDV will be grown in the chorioallantoic sac of a 9-11 days old embryonated hen egg. An older embryonated egg is chosen in this case since the chorioallantoic sac gets larger as the embryo grows. In this time span the chorioallantoic sac is large enough to be readily located but the embryo is not so large as to make candling difficult.

## MATERIALS AND PREPARATION

### Virus Inoculation

- 1 egg/person
- egg holders
- candling lamp (a bright lamp which is shielded to make a beam opening of about 2 cm in diameter)
- hole puncher
- syringes- 1 tuberculin syringe preloaded with 0.1 ml of $10^{-3}$ dilution of NDV in GLB (1/person)
- melted paraffin wax or collodion (Mallinckrodt)
- 70% ethanol
- gauze to wipe off the eggs
- pencils or markers to mark the injection site
- gloves (1 pair/person)

### Virus Harvest

- virus-infected eggs
- egg holders
- 2 sterile forceps/person
- 1 sterile pasteur pipet with bulb/person
- 2 sterile screw capped vials/person
- 70% ethanol and gauze
- gloves (1 pair/person)

## EXPERIMENTAL PROTOCOL

### Chorioallantoic Sac (CAS) Inoculation of Embryonated Eggs with NDV

1. Candle the egg to determine viability and the point of inoculation. This is best done in a darkened room by setting the embryonated egg in front of the candling lamp whose diaphragm is set so that the embryo can be seen clearly. An orange-colored egg with evident vasculature is indicative of a healthy embryo.
2. While candling, mark with a pencil the point for the chorioallantoic sac inoculation. This point is determined in the following manner. With the pointed end down, rotate the egg until the shadowed embryo and its blood vessels are facing you. Rotate the egg one-third turn away from you. Mark the injection site with a pencil so that it is facing you (1/3 away from the embryo) and 5 mm above the margin of the air sac. See figure 10.3.

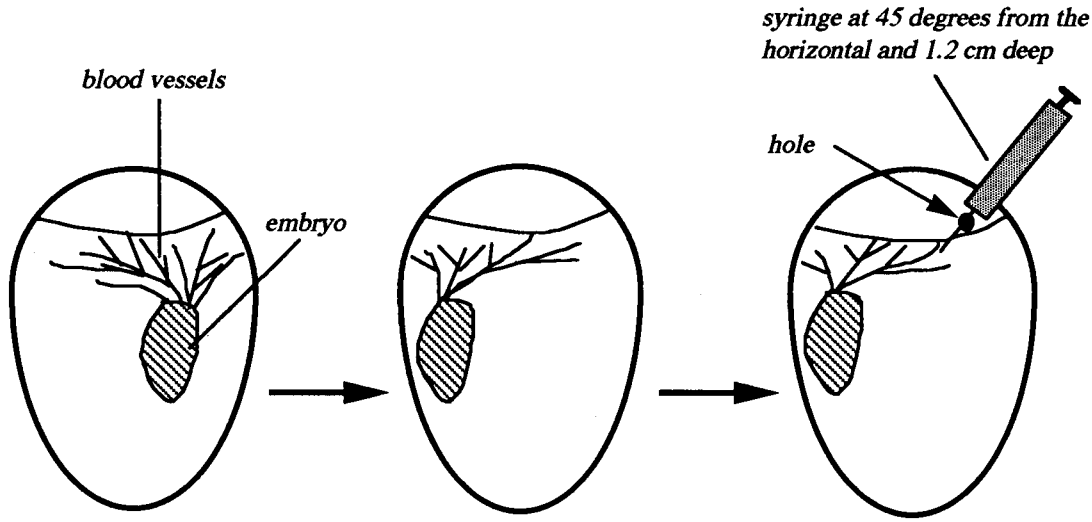


**Figure 10.3** Determination of the site for chorioallantoic sac inoculation of NDV.

3. Sterilize the egg by wiping the surface with a 70% ethanol soaked gauze or cotton.
4. Punch a hole in the shell at the point previously marked.

5. Wearing gloves, inject 0.1 ml of the virus dilution. This injection should be performed at a 45°angle from the horizontal and a total depth of 1.2 cm. DO NOT recap the needle, and immediately dispose of the syringe and cap by dropping them in the appropriate container.
6. Thoroughly seal the hole with melted paraffin wax (or collodion), by putting a drop or two on the hole and letting it harden for 1 minute. Label the egg with your name, date, and virus inoculated.
7. Place the eggs with pointed end down in the egg holders, dispose of your gloves in the decontamination pan, and return the eggs to the egg incubator.
8. Candle the eggs 16-18 hours post-inoculation to observe for dead embryos. This is evidenced by degradation of vasculature and total lack of movement from the embryo during candling. These deaths can be attributed to inoculation trauma or to contamination, and these eggs should be sterilized prior to disposal
9. 48 hours later, promptly remove the egg from the incubator and place it at 4°C (for 4- 24 hours), until the virus can be harvested.

## Virus Harvest

1. Wearing gloves, place the egg with the pointed end down in the egg holder, and sterilize the blunt end with 70% ethanol.
2. Using sterile forceps poke a small hole in the blunt end of the egg into the air sac, and gently remove enough of the shell to be able to insert a pair of forceps and a pasteur pipet. DO NOT PIERCE THE SHELL MEMBRANE COVERING THE EMBRYO. See figure 10.4 part A.
3. Using another pair of forceps (or by ethanol-sterilizing the pair used to open the shell) remove the shell membrane that is covering the embryo.
4. Move the forceps down the side of the egg about 1.5 cm between the chorioallantoic membrane and the shell. Poke a hole in the chorioallantoic membrane at that point and push back on the membrane to create a cavity where the allantoic fluid can accumulate. See figure 10.4 part B. Do not puncture the yolk sac. Do not collect albumin (thick, sticky, transluscent fluid).
5. While still holding the membrane back, use a sterile pasteur pipet to remove the allantoic fluid. The fluid should be clear. If it is cloudy, it is contaminated and should not be harvested. (If any blood is present in the sample, centrifuge the allantoic fluid at 500 x g for 10 minutes.)

6. Aseptically transfer the fluid into sterile and properly labeled vials. The label should include the following information: your name, class, date, the virus harvested, virus passage number, and method of passage (ie: CAS inoculation). Dispose of the gloves in the decontamination pan.
7. Place the vials in a proper container in the -70°C freezer.

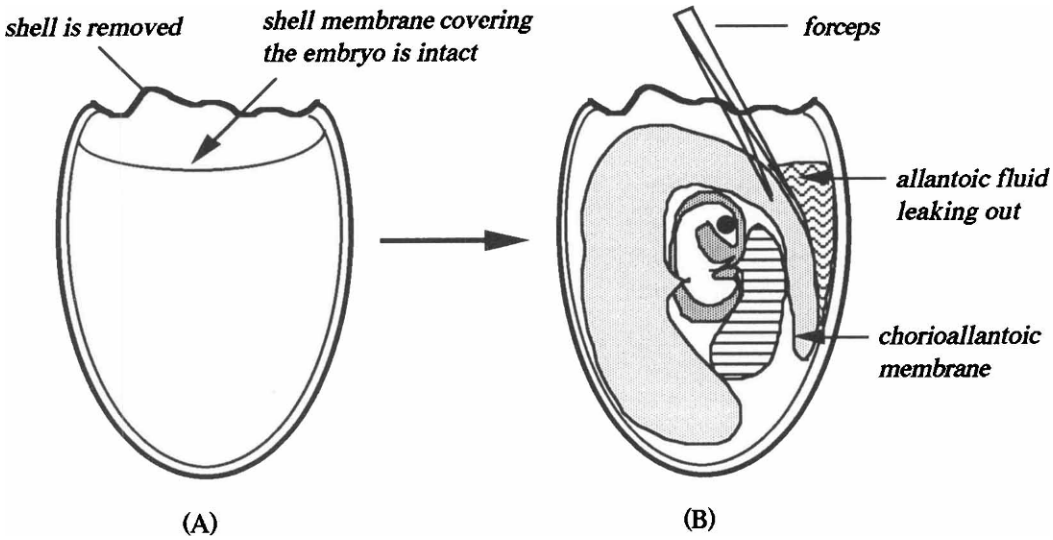


**Figure 10.4** Harvest of NDV from the chorioallantoic sac. (A) The shell is removed but the shell membrane covering the embryo is left intact. (B) The shell membrane has been aseptically removed and the sterile forceps have made a hole in the chorioallantoic membrane. By holding the membrane back with the forceps, a cavity is created where the allantoic fluid is allowed to accumulate.

## DISCUSSION QUESTIONS

1. Why is age of the embryo an important factor in virus inoculation?
2. What are some important parameters in passaging viruses in embryonated eggs?
3. What is the purpose of candling?
4. Would you expect a higher yield of virus from dying or from dead embryos? Why?
5. Would you expect a difference in the yield of virus as a result of the length of the 4°C incubation (4 hours versus 24 hours)? Why?

## FURTHER READING

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# 11

# INTRODUCTION TO QUANTAL VIRUS ASSAYS

## QUANTAL ASSAYS

The most important property of a virus is its infectivity - or ability to invade a cell and parasitize that cell to replicate itself. To measure infectivity, one could look at any virus-cell interaction indicative of virus replication, and develop an assay to obtain a titer for a given virus stock- where a **titer** is defined as a given number of infectious virus units per unit volume, and an **infectious unit** is the smallest amount of virus that produces some recognizable effect in the host system employed. In the following chapters (12-18) a number of virus-cell interactions will be utilized to obtain a titer for the virus samples produced in the previous exercises (chapters 9-10).

There are 2 basic types of infectivity assays : (1) *Quantal Assays*, and (2) *Quantitative Assays.* Quantal assays do not count the number of infectious virus particles present in the inoculum but instead obtain a value for the virus titer where the measurement is based on an all-or-nothing principle; is there CPE or not? Is the animal dead or alive ? In quantitative assays (described in chapters 15-16) the number of infectious virus particles present in the original suspension can be quantified.

To perform quantal assays, serial dilutions of virus are inoculated in (1) cell culture, (2) eggs, or (3) animals. Adequate time is allowed for viral infection and replication to occur in the inoculated hosts, and observations are made.The "endpoint" is an arbitrary selection of a given effect of the virus on the indicator system (ie: death of the animal in an $LD_{50}$). At each dilution each host or test unit is scored as being infected or uninfected. The proportion of infected to uninfected host, as determined by death, CPE, or other criterion, is recorded. At the higher virus dilutions the hosts are unaffected, and at the lower virus dilutions the hosts are all infected. The viral titer is calculated using the intermediate dilutions where only some of the hosts are scored as being infected, and the *titer,* is defined as *the reciprocal of the dilution of virus that infects 50% of the inoculated hosts.* The infectivity titer is thus expressed as the 50% infectious dose ($ID_{50}$). The virus dilution where 50% of the inoculated hosts are infected can be interpolated using the Reed and Muench method. At this virus dilution each infected host would contain 1 $ID_{50}$. When cell lines are the indicator system, the titer is expressed as the 50% tissue culture infective dose

or $TCID_{50}$. When experimental animals are used, the lethal dose that kills 50% of the inoculated animals is referred to as the $LD_{50}$. If eggs are the indicator system, the 50% egg infective dose is termed the $EID_{50}$.

This method of virus titration is useful only when measuring large differences in viral titer. For example, given 6 replicates and serial 10-fold dilutions, the range of uncertainty could be at least 36-fold between the minimum and maximum values for the titer obtained (see reference #1). The accuracy of this type of assay is affected by the number of replicates and the dilution interval. The greater the number of replicates per virus dilution, and the smaller the dilution interval, the greater the precision.

Factors that affect the quantification of viruses include:

1. The host species or cell line in which the virus is being assayed. Human influenza viruses do not readily infect chickens.
2. Intraspecies differences in sensitivity to a given virus. C57Bl/6 mice are resistant to intravenous challenge with (herpes simplex virus-1) HSV-1 while AKR and A/J mice are susceptible.
3. Cell line history. Semliki forest virus (SFV) produces good plaques in $L_p$ strain of $L_{929}$ cells, but no plaques in $L_{929}$ cells.
4. Route of inoculation. Intracranial inoculation is the route of choice for replication of arboviruses.
5. Virus passage history. EMC virus passaged in $L_{929}$ cells induces interferon, and therefore there would be both viral particles and interferon present in the viral stock suspension.

## REED AND MUENCH CALCULATION OF THE 50% ENDPOINT:

The method of Reed and Muench is widely used to calculate the 50% endpoint. By accumulating the infected and non-infected test units over the whole dilution range, the effective test population is enlarged beyond the actual number of test units on either side of the 50% endpoint (see table 11.1). This method is simple and easy to use.

In table 11.1 the dilution that would correspond to the 50% endpoint lies somewhere between the $10^{-6}$ (66.7% infected) and $10^{-7}$ (14.3% infected) dilutions. The **proportionate distance** between these two dilutions is calculated in the following manner:

$$\frac{\textit{(\% positive above 50\%)} - \textit{50\%}}{\textit{(\% pos. above 50\%) - (\% pos. below 50\%)}} = \textit{proportionate distance}$$

$$\frac{66.7\% - 50.0\%}{66.7\% - 14.3\%} = 0.3 = \text{proportionate distance}$$

Given that the log of the dilution above 50% is -6, the proportionate distance, as calculated previously, is 0.3, and the log of the dilution factor is -1 (serial 10-fold dilutions were used), the 50% endpoint is now calculated in the following way:

*(log dilution above 50%) + (proportionate distance x log dilution factor) = log $ID_{50}$*

$$(-6) + (0.3 \times -1.0) = -6.3$$

$$ID_{50} = 10^{-6.3}$$

This is the end-point dilution, namely the dilution that will infect 50% of the test units inoculated. The reciprocal of this number yields the titer in terms of infectious dose per unit volume. If the inoculum added to an individual test unit was 0.1 ml, the titer of the virus suspension would therefore be:

$$10^{6.3}\ TCID_{50}/0.1\ ml = 10 \times 10^{6.3}\ TCID_{50}/ml = 10^{7.3}\ TCID_{50}/ml$$

**Table 11.1**
Sample data used to determine the 50% endpoint using the Reed and Muench method.

| Log of virus dilution | Infected test units | Cumulative infected (A) | Cumulative non-infected (B) | Ratio of A/(A +B) | Percent infected |
|---|---|---|---|---|---|
| - 5 | 5/5 | 9 | 0 | 9/9 | 100 |
| - 6 | 3/5 | 4 | 2 | 4/6 | 66.7 |
| - 7 | 1/5 | 1 | 6 | 1/7 | 14.3 |
| - 8 | 0/5 | 0 | 11 | 0/11 | 0 |

Infected test units would be wells exhibiting obvious CPE in a $TCID_{50}$, dead animals in an $LD_{50}$, or infected eggs in the $EID_{50}$. Five test units were inoculated at each dilution. The cumulative infected column is calculated based on the assumption that the 1 test unit that was infected at the $10^{-7}$ dilution of virus would also have been infected at a $10^{-6}$ dilution. Therefore, at $10^{-6}$ there would be 4 (1 at $10^{-7}$+ 3 at $10^{-6}$) cumulative infected units. Similarly, at $10^{-5}$ the cumulative infected units would be 1 (at $10^{-7}$) + 3 (at $10^{-6}$) + 5 (at $10^{-5}$) = 9. A similar argument is made to calculate the cumulative non-infected column. Here the assumption is that test units which were not infected by a given dilution of virus would also be uninfected by a higher dilution of the virus.

## VIRUS DILUTIONS

Whenever one is performing an assay to determine the titer of a given virus suspension, serial dilutions of the virus stock must be prepared. These serial dilutions are commonly done using factors of 2, 5, or 10.

The smaller the factor, the more precise the titer, but when a broad range of dilutions are needed, 10-fold dilutions are most commonly used.

The first thing that needs to be determined is how much volume of each virus dilution will be needed to perform the assay. For each dilution one will need the *exact amount necessary for infection* in addition to *the amount to be transferred* to perform the next dilution, *plus a small additional volume*. This additional volume accounts for the volume that is undeliverable by some pipets. For example: if 0.1 ml of a given dilution is to be added to each of 5 wells and 0.1 ml is needed for the next dilution, the exact amount needed would be 0.6 ml, and the needed additional volume would be 0.1-0.2 ml. Thus for each dilution one would need a minimum of 0.7-0.8 ml of suspension.

Using the previous example of 0.1 ml of virus per well, 5 wells per dilution, and if the dilution range to be tested was $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$, the following dilution sequence could be used:

tube#1 - 0.1 ml of virus + 0.9 ml diluent - ($10^{-1}$)
tube#2 - 0.1 ml of tube#1 + 0.9 ml diluent - ($10^{-2}$)
tube#3 - 0.1 ml of tube#2 + 0.9 ml diluent - ($10^{-3}$)
tube#4 - 0.1 ml of tube#3 + 0.9 ml diluent - ($10^{-4}$)

Once the dilution scheme has been determined the actual dilutions are performed in the following manner:

1. Label the tubes and dispense 0.9 ml of diluent with a 1.0 ml pipet in tubes #1-4.
2. Using a *new* 1.0 ml pipet, add 0.1 ml of the virus stock suspension to the first tube. Touch the tip of the pipet to the side of the tube and the desired amount (0.1 ml in this case) should be allowed to run down the side. This pipet should not otherwise touch any other side of the tube nor the liquid therein, and should be properly discarded after delivering the required volume.
3. Use a *new* pipet to mix the contents of this first tube by drawing up and releasing the virus suspension 6-7 times.
4. Once mixed, 0.1 ml is withdrawn and transferred to tube #2, again being careful to only touch one side of the tube and to discard the pipet in a decontamination pan.
5. Use a *new* pipet to mix the contents of tube #2 and to transfer 0.1 ml of the mixed suspension to tube #3.

This same procedure would be followed until all the dilutions are completed. Figure 11.1 is a diagram of the dilution scheme used in our example.

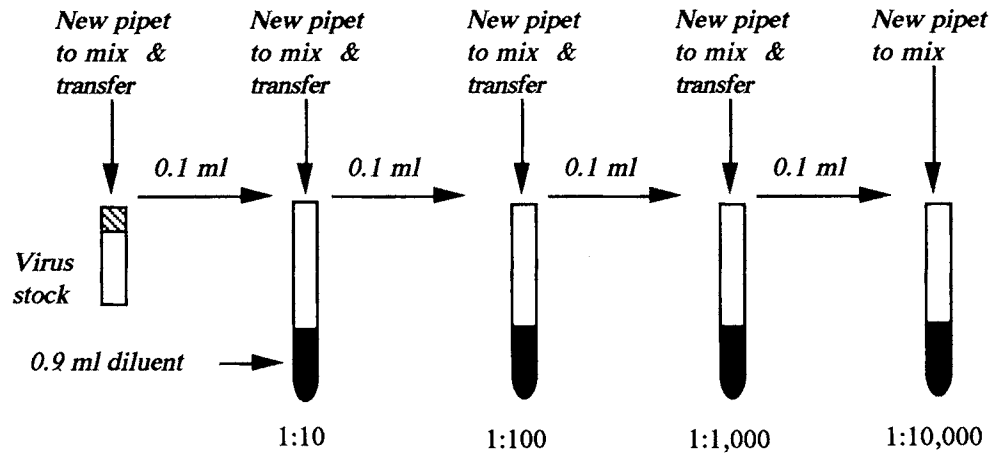


**Figure 11.1** - Diagram of a sample dilution.

It is very important to use a new pipet for each transfer and to thoroughly mix each virus dilution. A used pipet will have a large number of virus particles on its outer surface. If the pipet is not discarded after each transfer, millions of virus particles will be carried along resulting in a large dilution error. Transfer from an improperly mixed suspension will also lead to dilution errors. Errors made in virus dilutions result in an erroneous titer and a loss of time and supplies.

## FURTHER READING

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# 12

# $TCID_{50}$

## INTRODUCTION

A variety of interactions are possible between a virus and a cell. The outcome in any particular case is largely determined by the genetic makeup of the virus and that cell. The result of the interaction of a virus with a susceptible cell system can be any of the following:

1. A *cytocidal infection* whereby the infecting virus causes death of the infected cell. In this case lethal cellular damage is caused by the effects of the products of the viral genome on the cell or its regulation.
2. A *steady-state infection* in which a noncytocidal virus (one that does not cause cell death) is continuously produced by the cells in culture. In such cells large amounts of infectious virus may be constantly produced while the cells' multiplication and metabolism are not altered.
3. *Transformation* whereby the infection of a cell culture by an oncogenic (cancer-causing) virus results in morphological, biochemical, and biological changes in the infected cell. These changes may then lead to formation of a tumor.

The $TCID_{50}$ assay measures infectious cytocidal virions. The host cells are grown in confluent monolayers to which various dilutions of the virus are added. The virus replicates and the progeny virus that is released into the supernatant fluid is free to infect any other cell. The cytopathological damage is allowed to develop usually over a period of days (depending on the given virus and the cells). At that time the cells are stained or observed microscopically and the results - presence or absence of CPE - are recorded. *The $TCID_{50}$ is defined as that dilution of virus required to infect 50% of the cell cultures inoculated.* It is a quantal assay of viral infectivity in that each infected well yields only one piece of information- namely, is there CPE or not? The $TCID_{50}$ titer does not tell how many infectious units may be present in a given sample, but only that this dose or dilution will produce CPE in 50% of the cell cultures inoculated.

The rate of cellular changes and the patterns of CPE induced by different viruses vary greatly and depend upon (a) the type of cell culture system used, (b) the properties of a given virus strain, and (c) the concentration of virus in the specimen.

In the following experiment BHK-21 cells are infected with EMC virus. The normal BHK-21 monolayer consists of adherent elongated cells positioned close together. The EMC virus-induced CPE is characterized by loss of adherence of many of the cells, with the rounded-up cells floating singly or in sheets in the supernatant. The cells that remain attached are abnormal, rounded and granular.

Once CPE is obtained and the results are recorded, the virus infectivity titers are calculated using the Reed and Muench method for the determination of the 50% endpoint (see chapter 11 or the Appendix section).

## MATERIALS AND PREPARATION

- 1 96-well plate/person (sterile, flat-bottom)
- BHK-21 cells (1 confluent 75 $cm^2$ flask/person)
- rubber policeman
- E-MEM 5% TPB 2% FBS (20 ml/person)
- hemacytometer
- nonsterile tubes for cell counts
- pasteur pipets & HBSS for cell counts
- small sterile flask or tube to prepare cell suspension (1/person)
- 9 sterile dilution tubes/person
- EMC virus stock
- sterile pipets
- decontamination pans
- gloves

## EXPERIMENTAL PROTOCOL

1. Observe the BHK-21 cells under the microscope. They should be confluent and healthy.
2. Pour off the medium and add 2 ml of E-MEM 5% TPB 2% FBS to the flask.
3. Scrape the cells with a rubber policeman and pipet up and down to break up the cell clumps.
4. Withdraw 0.1 ml of the cell suspension and dilute with 0.9 ml of PBS.
5. Perform a cell count.

6. Prepare 4.5 - 5.0 mls of 1.0 x $10^5$ cells/ml.
7. Thaw the EMC virus stock in a 37°C water bath.
8. Wearing gloves, perform serial 10-fold dilutions of the virus in E-MEM 5% TPB 2% FBS (0.1 ml virus + 0.9 ml media) through $10^{-9}$.
9. Add 0.1 ml of medium to each well H 1-5 (the cell control wells). See figure 12.1.
10. Add the 0.1 ml/well of the virus dilutions in replicates of 5 as shown in figure 12.1. By starting with the $10^{-9}$ (most dilute) dilution and working your way up to the $10^{-3}$ dilution a single pipet may be used throughout.
11. Mix the cell suspension. Add 0.1 ml of the 1.0 x $10^5$ cells/ml suspension to each wells, starting with the cell controls and again working up from the most dilute virus dilution wells.

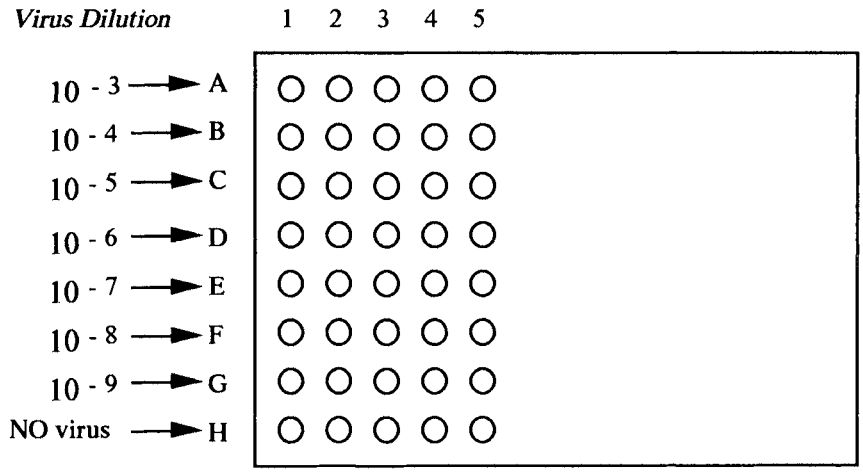


**Figure 12.1** Diagram of the experimental protocol for the $TCID_{50}$

12. Dispose of all contaminated materials and gloves in the decontamination pan.
13. Incubate the plates at 37°C in the $CO_2$ incubator and monitor by microcopic observation the wells daily for 4 days. Record the gradual development of CPE in the wells in the following manner:
    0 - *No CPE*
    1 - *Partial CPE ( less than 50% of the cells exhibit CPE)*
    2 - *About 50% of the cells exhibit CPE*
    3 - *About 75% of the cells exhibit CPE*
    4 - *The monolayer is totally destroyed*

14. Calculate the $TCID_{50}$ counting all the wells with 1-4 CPE as positive.
15. Dispose of all infected plates in the decontamination pan.

## DISCUSSION QUESTIONS

1. Why were the 5 cell control wells (no virus wells) included in this experiment?
2. Describe the gradual development of CPE in the virus infected wells.
3. Why should a 1+ CPE be recorded as positive well for the $TCID_{50}$ in a cell culture?
4. Does one $TCID_{50}$ unit correspond to one infectious virus particle?
5. Envision the same experiment (step #8 of the Experimental Protocol) using dilution factors of 2 and 5. How would you do these dilutions (what volumes would you use) ?
6. Take the raw data in Table 11.1 and calculate the $TCID_{50}$ using 2 and 5 as dilution factors.

## FURTHER READING

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# 13

# $LD_{50}$

## INTRODUCTION

The $LD_{50}$ is an infectious quantal assay that is similar in nature to the $TCID_{50}$, except that a different indicator system is utilized. The endpoint in the $TCID_{50}$ is CPE in cultured cells, whereas in the $LD_{50}$ the endpoint is the virus-induced death of the animal. *The $LD_{50}$ titer is defined as the reciprocal of that dilution per unit volume of virus stock that will kill 50% of the inoculated animals.* Although only infectious virions will infect and kill, the $LD_{50}$ titer gives no measure of the number of infectious virus particles present in a given dilution.

Factors that influence the $LD_{50}$ include:

1. *The age of the animal.* Typically, very young and very old animals are more susceptible to infectious disease.
2. *The health status of the animal.* The animals should be healthy and kept in facilities that would keep their exposure to infectious agents to a minimum.
3. *The strain of the animal.* Different strains of an animal species may have genetically-based differences in susceptibility or resistance to a given virus. For example, C57/Bl mice are more susceptible to EMC virus peritoneal inoculation than are CD-1 mice. Using the same EMC virus stock, the $LD_{50}$ titer obtained using these two strains of mice can differ by as much as 100-fold.
4. *The route of inoculation.* The route of inoculation chosen may affect the outcome of the viral infection. For example, the C3H/Ten and ICR mice are more resistant to Semliki Forest virus injected intraperitoneally than when the virus is injected intracranially.
5. *The strain and passage history of the infecting virus.* Different virus strains may differ in their virulence.
6. *The size of the inoculum.* The size of the inoculum has to be adjusted so that it allows the animal to live long enough so that the introduced virus may replicate and produce progeny virus. Too large an inoculum may overwhelm the animal so that it dies before progeny virus has been produced.

7. *The statistical accuracy of a given assay.* For example, the smaller the dilution interval and the larger the number of animals per experimental group, the greater the accuracy of the results obtained.

In this particular exercise, CD-1 mice are inoculated intraperitoneally with varying dilutions of EMC virus. Within 2-3 days, the infected mice exhibit symptoms of the disease that include ruffled fur, paralysis, closed eyes and a general loss of alertness. In the animals injected with lower virus dilutions these symptoms then progress to death, while in the higher dilutions some of the mice may recover. The control animals do not receive any virus but are injected intraperitoneally with the diluent. This group of mice is used to control (1) for possible deaths due to a toxic compound or pathogen, other than the infecting virus, which might be present in the suspension, and (2) for deaths due to injection trauma.

## VIRUS INOCULATIONS IN ANIMALS

All materials and equipment to be used for inoculation must be sterile and the procedures should be done with care to protect the person doing the inoculations as well as to minimize discomfort to the animal.

The needle selected should have as fine a gauge (diameter of opening) as possible (the higher the gauge number the smaller the diameter) while still permitting efficient delivery of the inoculum, and the syringe should accomodate enough inoculum for a number of animals. A commonly used combination of needle and syringe is the "tuberculin syringe" - a 1ml syringe attached to a 26 gauge 5/8 inch needle.

### Loading the syringe:

The suspension to be inoculated should be as homogeneous as possible since the presence of clumps or solid particles can clog the needle. Wearing gloves, remove the needle cap and draw the suspension into the syringe. Once filled, the syringe is placed with needle up and an alcohol soaked piece of cotton or gauze (held with forceps) is placed over the needle. The barrel is gently tapped to release trapped air bubbles, and the plunger is then depressed until all air is removed from the syringe and needle (see fig. 13.1). The contaminated gauze should be discarded in a decontamination pan and the needle cap *very carefully* replaced to avoid an accidental prick. This can be done easily by placing the cap on the benchtop and gently sliding the needle into it. Once the cap covers the needle, hold the syringe upright and tighten the cap.

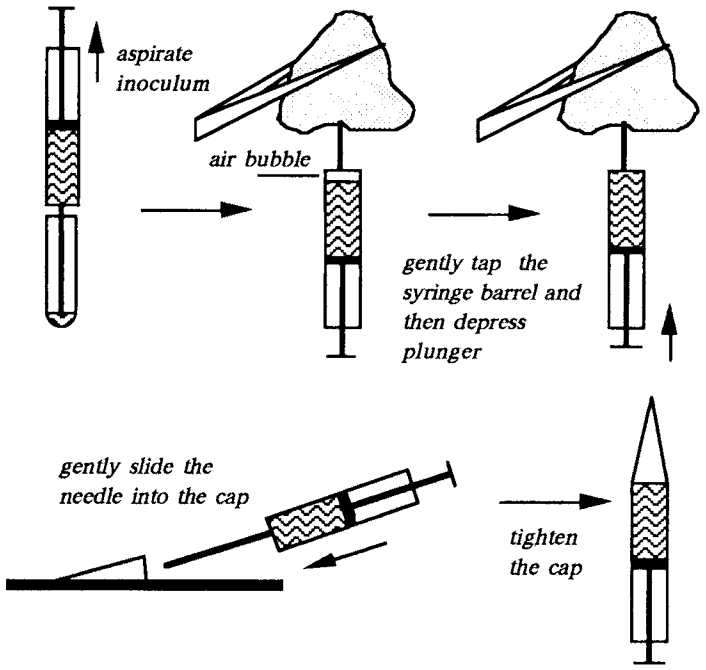


**Figure 13.1** Loading of a syringe

## Routes of inoculation:

A variety of routes of inoculations are used depending on the virus and the animal to be infected. Some of the more common routes are as follows:

1. *Intraperitoneal (ip).* The material is injected into the peritoneal cavity of the animal. If possible the animal is slanted upside down during the injection so that the internal organs will slide toward the head and away from the injection site.
2. *Intravenous (iv).* The suspension is injected directly into the blood stream of the animal. The marginal ear vein is usually chosen for rabbits while mice and rats are inoculated in the tail vein. This injection should be done in the direction of the blood flow. To determine this, the vein is firmly pressed to constrict blood flow and the side that blanches is the side toward which blood is flowing. If veins are not clearly visible they can be enlarged by gentle warming, rubbing, or the application of xylene. The xylene should be washed off thoroughly after inoculation. The needle is inserted at a low angle through the skin. Once in the vein there should be very little resistance and the plunger is easily depressed. If properly performed, as the material

is injected, a blanching of the vein is observed and there is no accumulation of liquid in the surrounding tissues.

3. *Subcutaneous (sc).* The skin is lifted between the thumb and forefinger and the material is injected right under the skin with the needle almost horizontal to the skin at a depth of 1/8 -1/4 inch. When done properly a raised area or "bleb" is formed at the site of injection.
4. *Intramuscular (im).* This injection is usually done in one of the large body muscles such as the thigh. The area to be injected is held firmly and the needle is inserted in a quick jab almost perpendicular to the skin. The material is slowly expelled and the needle is withdrawn.
5. *Intracranial (ic).* The needle is inserted vertically through the cranial wall at a depth of about 2 mm.
6. *Footpad.* The material is injected directly into a footpad of the animal.

**Injection of the animal:**

Excess fur is shaved off the animal, the injection site is thoroughly disinfected with 70% alcohol or tincture of iodine, and the injection is performed (see the previous section on routes of inoculation). Inoculated animals should be marked with picric acid solution or other such colored dye and placed in a properly labeled cage. The label should contain all pertinent information such as the number of animals, their age and sex, the date of inoculation, the material inoculated, the route of inoculation, and the dilution and volume injected. All animals inoculated with a given dilution should be placed in the same cage and if an infectious agent was used these cages must be kept isolated from the rest of the animal colony. Any dead animal should be recorded, promptly removed and cervically dislocated prior to disposal in a proper bag for incineration. If they are not incinerated within 1 day they should be kept at 4°C until incineration is possible.

After using the syringe dispose of it in an autoclavable disposable container specifically labeled for that purpose. Autoclave and dispose of it. Do NOT recap the needle and do NOT just drop the syringes in a decontamination pan.

## MATERIALS AND PREPARATION

- $10^{-5}$-$10^{-10}$ dilutions of EMC virus stock in GLB (1.5 ml of each dilution)
- sterile GLB to inoculate control group (1.5 ml)
- gloves (1 pair/person)

- 1 ml tuberculin syringe (7)
- 35 8-12 week old CD-1 mice (5 mice/dilution)
- 7 cages with food and water
- plastic isolator or hood for infected mice
- picric acid solution or other dye to mark inoculated animals
- swabs to apply picric acid
- sterile cotton or gauze, and forceps
- 70% ethanol
- wire racks (1/group)
- disposal containers for used syringes
- decontamination pans

## EXPERIMENTAL PROTOCOL

1. Label the cages correctly with the following information: the date, name, virus inoculated, route of inoculation, and virus dilution.
2. Wearing gloves properly load the syringe as explained in the introduction section of this chapter, and demonstrated by the instructor. Each syringe should be filled with 1 ml of a given virus dilution (1 syringe will contain 1 ml sterile GLB). Each individual or group (as determined by instructor) will be responsible for injection of a given dilution into 5 animals.
3. Intraperitoneal (ip) injection of mouse: Following the demonstration by the instructor, pick up a mouse by the tail and place it on a wire rack while still holding the tail with the right hand. The mouse will grasp the wire on the rack and pull forward. As it does so, quickly grasp the skin of its neck between the thumb and first two fingers of the left hand. Tuck the tail under the little finger and turn the mouse with the abdomen facing up and the head slanted down. The mouse should be held snugly (without hurting it) and have only limited movement. *The mouse will bite if not held securely.* Thoroughly disinfect the abdominal area with a sterile gauze saturated with 70% ethanol. With the bevel up, insert the needle into the lower right half of the abdominal area. Do not plunge too deeply to avoid damaging the abdominal organs. Inject 0.2 ml of the suspension and remove the syringe. Disinfect the site of injection with 70% ethanol, mark the head or back of the mouse with picric acid and place the animal in the previously labeled cage. Treat similarly the other 4 mice of this dilution group. (5 mice will be injected with 0.2 ml of sterile GLB without any virus. These are the control group.)

4. Place all the infected mice is a plastic isolator or a hood and observe 2 times daily for 12 days. Record symptoms of the disease and the animals in the group as being well, sick, or dead. *Wearing gloves*, remove dead animals from the cage, cervically dislocate with a pair of large forceps, and place them in a bag to be incinerated.
5. Dispose of virus dilutions, gloves, and all other contaminated materials into autoclavable biohazard bags. Seal them and place in the decontamination pan. Thoroughly wipe down the bench area with disinfectant.
6. Using the Reed and Muench (see chapter 11 or the Appendix), calculate the $LD_{50}$ titer.

## DISCUSSION QUESTIONS

1. What is the purpose of the 5 mice in the control group?
2. Why should the infected mice be kept isolated?
3. What is the relationship between the $TCID_{50}$ titer and the $LD_{50}$ titer of the stock EMC virus? Can 1 $TCID_{50}$ kill an animal? Is it possible for an animal to survive a dose of 2 $LD_{50}$?
4. Given an identical stock of virus, The $LD_{50}$ obtained in two different laboratories varied by 100-fold. How could you explain such results?

## FURTHER READING

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# 14

# $EID_{50}$

## INTRODUCTION

The $EID_{50}$, like the $TCID_{50}$ and $LD_{50}$, is an infectious quantal assay where each infected host (the embryonated egg) only yields one piece of information - is the indicator system infected or not infected? The endpoint chosen to determine infection may be death of the embryo or another recognizable endpoint. The $EID_{50}$ assay may also be performed by quantifying virus in each embryonated egg. For example, the $EID_{50}$ for influenza virus is often determined by measuring the presence or absence of hemagglutination in the allantoic fluid of each embryonated egg.

To perform an $EID_{50,}$ groups of embryonated eggs are inoculated with serial dilutions of the virus. The infected eggs are incubated to permit viral replication and to allow for the development of the appropriate response. The *$EID_{50}$ titer* can then be calculated using the Reed and Muench method (see chapter 11 or Appendix), and *is defined as the reciprocal of that dilution of virus per unit volume that results in death (or other observable endpoint) in 50% of the inoculated eggs.*

A variety of tissues are available for virus replication within the embryonated egg. The chosen route of inoculation will depend on the individual virus and its preferential site of replication.

In this experiment serial dilutions of NDV will be used to inoculate the chorioallantoic sac of 10-12 day old embryonated eggs. The observable endpoint will be the presence or absence of hemagglutination in the allantoic fluid of the infected eggs, and the $EID_{50}$ will be calculated using the Reed and Muench method.

## MATERIALS AND PREPARATION

### Injection of eggs with virus

- 36 viable embryonated eggs (4 eggs/virus dilution and 4 controls) per person or per group, as determined by instructor.
- egg holders
- candling lamp
- 9 sterile syringes (1 ml tuberculin syringe with 25 gauge needle) preloaded with 0.5 ml of a given NDV dilution ($10^{-3}$-$10^{-10}$ NDV in

GLB with penicillin 100 units/ml and streptomycin 100 µg/ml). One syringe will contain 0.5 ml sterile GLB with penicillin and streptomycin for the controls.

- hole puncher
- melted parafin wax or collodion, and applicator sticks
- 70% ethanol and sterile gauze
- pencils to mark injection site
- gloves (1 pair/person)
- container for syringe disposal
- decontamination pans
- egg incubator

### Determination of hemagglutination activity

- virus-infected eggs and egg-holders
- sterile forceps
- sterile pasteur pipets and bulb
- sterile vials or test tubes
- 70% ethanol and gauze
- gloves
- non-sterile, round-bottom 96-well plates
- 1% sheep red blood cells suspension in PBS (see Appendix)
- PBS
- virus samples (allantoic fluid from infected eggs)
- pipets
- automatic pipetor and tips
- decontamination pans with and without biohazard bags

## EXPERIMENTAL PROTOCOL

### Injection of eggs with virus

1. Candle the egg to determine viability and the point of inoculation. This is best done in a darkened room by setting the embryonated egg in front of the candling lamp whose diaphragm is set so that the embryo can be seen clearly. An orange-colored egg with evident vasculature is indicative of a healthy embryo. Do not use cracked or infertile eggs.
2. While candling, mark the point for the chorioallantoic sac inoculation with a pencil. This point is determined in the

following manner. With the pointed end down, rotate the egg until the shadowed embryo and its blood vessels are facing you. Rotate the egg one-third turn away from you. Mark the injection site with a pencil 1/3 away from the embryo and 1/4 inch above the margin of the air sac. See fig.14.1

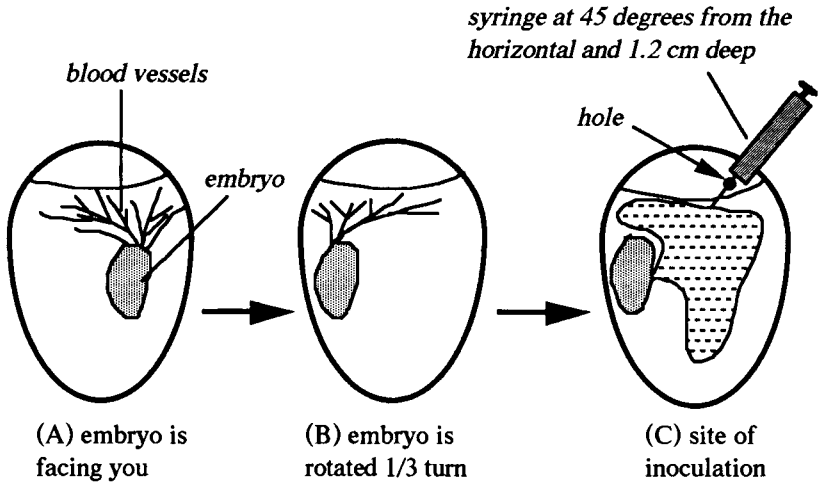


**Figure 14.1** Determination of the site for chorioallantoic sac injection of NDV.

3. Sterilize the eggs by wiping the surface with a 70% ethanol-soaked gauze or cotton.
4. Punch a hole in the shell of the eggs at the point previously marked. It is not necessary to completely penetrate the shell. All that is needed is a chip in the outer shell that will permit an easy inoculation with the syringe.
5. Wearing gloves, inject 0.1 ml of the virus dilution into each of 4 eggs. This injection should be performed at a 45°angle from the horizontal and a total depth of 0.5 inch. DO NOT recap the needle, and dispose of the syringe and cap in the appropriate container. 4 eggs will be inoculated each with 0.1 ml sterile GLB as controls.
6. Wipe the injection site with a 70% ethanol-soaked gauze.
7. Thoroughly seal the hole with melted paraffin wax or collodion, and label the egg with your name, date, and virus dilution .
8. Place the eggs in the egg holders and dispose of the gloves and other contaminated materials in the decontamination pan. Return the eggs to the egg incubator.

9. Candle the eggs 16-24 hours post-inoculation to observe for dead embryos. These deaths can be attributed to inoculation trauma or to contamination, and these eggs should be discarded in a sealed biohazard bag placed in a decontamination pan and sterilized. The remaining eggs should be returned to the egg incubator for an additional 24 hours.

### Determination of hemagglutination activity

1. 48 hours post-injection, remove the eggs from the incubator and refrigerate at 4°C for 4-24 hours.
2. Label the vials or test tubes and collect the allantoic fluid from the infected eggs as described in chapter 10 (pp.50-51). Use only one vial or test tube per egg and discard the egg and its remaining allantoic fluid in a biohazard bag in the decontamination pan.
3. Label the 96-well plate and transfer 50 µl of each sample per well.
4. Add 50 µl of the 1% sheep red blood cell suspension.
5. Leave the plates undisturbed at 4°C for 90 minutes.
6. Record the wells as being "+" or "-" for hemagglutination (see chapter 18) and dispose of the plates in a decontamination pan.
7. Calculate the $EID_{50}$ using the Reed and Muench method (see chapter 11 or Appendix).

## DISCUSSION QUESTIONS

1. Why should the eggs be incubated with the pointed end down after a chorioallantoic sac inoculation? Under which circumstances would you want to incubate the egg lying on its side?
2. Can a virus infect an embryo without killing it?
3. Under what circumstances would an $EID_{50}$ assay be preferable over other assays such as the $TCID_{50}$, for titrating virus?

## FURTHER READING

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# 15

# INTRODUCTION TO QUANTITATIVE ASSAYS

## QUANTITATIVE ASSAYS

The quantitative assay quantifies the number of virus particles in an inoculum. A bacterial colony count, in bacteriology, is an example of a quantitative assay. Each viable bacterial cell originally plated gives rise to a single colony, thus the colony count is a direct estimate of the number of viable bacterial cells originally seeded on the agar plate. In virology, the same principle applies when one counts the number of plaques on a cell monolayer (plaque assay). Other quantitative viral assays include : pock assays, infectious center assays, transformation assays, and direct particle counts with an electron microscope. Virus particles measured in quantitative assays may be either *infectious* (as in the plaque assay where only infectious virus particles give rise to plaques), or *"non-infectious"* (as in an electron microscope count where all particles are counted, whether infectious or not).

The **plaque assay** (chapter 16) was introduced to animal virology in the 1950's, as a modification of the bacteriophage plaque assay. In this assay, virus is added to a cell monolayer and allowed to adsorb. A solid gel is then added that restricts the spread of viral progeny to neighboring cells, resulting in the formation of a *plaque* - localized area of virus-induced cell pathology.

The **pock assay** is a variation of the plaque assay involving the titration of viruses (such as poxviruses) on the chorioallantoic membrane of chicken embryos rather than in cell culture. Serial dilutions of the virus are inoculated on the chorioallantoic membrane of eggs. Within 3 to 7 days, progeny virus spreads to adjacent cells and gives rise to localized lesions or *pocks*. The membranes are harvested and the pocks counted. Knowing the virus dilutions used, a titer can be obtained since each pock originated from one infectious virus particle.

The **infectious center assay** is a modification of the plaque assay. This assay measures the number of infected *cells* present- it does not measure the number of infectious virus particles. In an infectious center assay, a population of freshly infected cells is harvested and a single cell suspension is obtained. These cells are then serially diluted and plated on a fresh cell monolayer. An overlay is added and the monolayer is incubated. Over a period of time, the progeny virus spreads from the originally infected cell to the fresh neighboring cells, and forms a plaque. The plaques are counted, and knowing the dilution plated, a titer *(of infected cells)* is obtained.

**Transformation assays** allow the quantification of infectious, noncytocidal (viruses that do not cause CPE) transforming viruses. Certain oncogenic viruses, such as Rous sarcoma virus (RSV), do not cause cell death, but rather transform the cells they infect. Such transformed cells possess many properties similar to malignant tumor cells. They do not exhibit contact inhibition, they grow in an haphazard and unrestrained fashion, they multiply while suspended in soft agar, and when $10^6$ cells are introduced in an animal, a tumor develops. Thus, transformation assays look for (1) the unrestricted growth of cells in cell culture, (2) the ability of cells to grow in soft agar, or (3) the development of tumors *in vivo* by injection of $10^6$ cells.

Methods, other than transformation assays, used to assay for *noncytocidal* viruses are : **hemadsorption** (chapter 19) and **interference**, the process by which the replication of a virus in a cell inhibits the replication of a second virus subsequently infecting the cell. Rubella virus was discovered by viral interference studies.

The **direct particle count** utilizes the electron microscope. In this case both infectious and non-infectious virus particles are counted since defective virus particles can not always be distinguished by morphology alone. In any given preparation, a certain proportion of virions are non-infectious due to a lethal genetic alteration or to inactivation during preparation or storage.

As in the previous section on quantal assays (chapters 11-14) the virus stocks produced in chapters 9 & 10 will be titered using the plaque assay. One should keep in mind that the various assays have different sensitivities and measure different viral properties. Thus, one should not directly compare the numerical titer obtained by hemagglutination to a plaque assay titer. These two assays measure different viral properties and have nothing in common except that they each provide a way of titrating a given virus. Therefore, it is not surprising that for a given virus, different titers may be obtained for each assay system used. It should be noted that changing conditions (such as using a different cell line) within a single assay method can also alter the results obtained. Comparisons under similar conditions within a given assay system, however, are useful and valid.

## FURTHER READING

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# 16

# PLAQUE ASSAYS

## INTRODUCTION

The plaque assay is an *infectious quantitative assay* that quantifies the number of infectious virus particles present in a virus suspension.

In the plaque assay, dilutions of a virus sample are added to confluent cell monolayers and allowed to adsorb. The unadsorbed virus is washed off and an overlay medium is added. The overlay medium insures that the spread of progeny virus is restricted to the surrounding cells so that a **plaque** - *localized area of virus-induced cytopathology* - can develop. After a period of incubation that allows for viral replication and infection of adjacent cells, a stain overlay is added. This stain overlay contains a vital dye (stains only living cells) such as neutral red or tetrazolium. The viable cells are stained with the dye and the plaques are clear. When using a methylcellulose overlay, crystal violet is used to stain the monolayer. This is not a vital dye but since there are no intact cells in the plaque area, plaques show up as clear areas with the cell monolayer stained purple. For statistical reasons 20-100 plaques per well is an ideal number to count, although the actual number is limited by the size of the well in which the plaque assay is performed and by the plaque size (which is a function of the virus-cell interaction and can be different in variants of the same virus type).

The plaque assay is a very sensitive assay in that, theoretically one plaque is initiated by infection from a single infectious virus particle, and the number of plaques (and thus the number of virions) can be quantified. The infectivity titer is expressed as the number of plaque forming units per ml (pfu/ml), and is obtained in the following manner:

*plaque number x reciprocal of dilution x reciprocal of volume in ml*

For example, if there is a mean number of 35 plaques from monolayers infected with 0.1 ml of a $10^{-6}$ virus dilution , there are :

$$35 \times 10^{6} \times 10 = 3.5 \times 10^{8} \text{ pfu/ml}$$

However, since virus samples are inevitably heterogeneous populations, infectivity is not an all-or-nothing phenomenon and is dependent on the assay conditions. In segmented viruses like influenza, two or more *non-infectious* virus particles adsorbing to the

same cell can complement each other to produce infectious virus progeny (and plaques) if their defects are in different genes. This can even happen at high dilutions, as sometimes virus particles may clump. Thus, in practice, the plaque titer obtained may not always accurately represent the number of infectious particles present.

Factors that may affect the results obtained in a given plaque assay are as follows:

1. The sensitivity of the cells to infection by that virus, as well as the health status of the cells. A virus sample can be infectious in different cell types with different levels of efficiency. For example, vaccinia virus can produce 5-fold more plaques on CV1 cells than on primary chicken embryo fibroblast cells.
2. The required time of adsorbtion of virus may vary for different virus-cell systems, since the adsorbtion time is a function of both the virus and the cell.
3. The type of agar used in the overlay. Autoclaved Difco agar contains sulfated polysaccharides that can be inhibitory to the replication of certain viruses, including EMC virus. Thus, the choice of overlay composition may affect the time of incubation necessary for plaque formation (ie: 48 versus 24 hours being necessary for the development of plaques). The use of Noble agar, methylcellulose, agarose, or the addition of DEAE-dextran to Difco agar would overcome this inhibition.
4. The time of incubation necessary for the development of plaques may vary in different virus-cell systems. There must be enough time to allow for the formation of discrete visible plaques, but not enough time as to allow the plaques to fuse together.
5. The staining procedure is affected by the concentration of neutral red in the overlay. At high stain concentrations crystals interfere with the viewing of the plaques, and at low stain concentrations the plaques are difficult to visualize.
6. Plaques are not always visible within 4 hours of addition of the stain overlay and a longer staining time may be necessary.
7. Only cytopathogenic viruses (viruses that result in CPE) will form plaques, and the cells must form a confluent monolayer for the discrete plaques to develop.
8. Requirement for proteolytic cleavage. In some cell types, a virus may cause plaques in the presence, but not in the absence, of an exogenous protease (ie: trypsin).

Because of all the above-mentioned, it is essential that one be familiar with both the virus and the host cell under investigation.

An important application of the plaque assay is the *plaque reduction bioassay* (chapter 33). In this case, dilutions of the interferon preparation to be tested are added to confluent cell monolayers that are later infected with a standard dose of challenge virus (adjusted to yield a countable number of plaques.) The remainder of the steps are the same as in the plaque assay. The titer in this case is expressed as the plaque depressing dose 50 ($PDD_{50}$), and is the reciprocal of that dilution of sample that will cause a 50% reduction in plaque number as compared to virus controls. The plaque reduction bioassay can be useful in titering neutralizing antibodies and antiviral drugs, as well as interferon preparations.

## *A. PLAQUE ASSAY OF EMC VIRUS USING AN AGAR OVERLAY*

In this section, a plaque assay titer will be determined for EMC virus. This will be done using $L_{929}$ cells and an agar overlay medium.

## MATERIALS AND PREPARATION

### Setting up the Cells

- $L_{929}$ cells
- 1 12-well plate/person
- E-MEM with 5% FBS
- sterile rubber policemen
- sterile pipets to set up cells
- hemacytometers, nonsterile tubes, and Pasteur pipets
- PBS
- decontamination pans

### Infection of $L_{929}$ cells with EMC

- 1 12-well plate/person with confluent monolayers (from day 1)
- EMC virus stocks
- ice buckets with ice
- 6 sterile dilution tubes/person (for the virus dilutions)
- sterile PBS and sterile towels
- sterile GLB
- 48°C water bath
- microwave or boiling water to melt agar
- sterile pipets
- decontamination pans

- 2 sterile 1 oz. bottles to combine Unit I and II/person
- 17 ml Difco agar overlay/person (see experimental protocol for individual components)
- gloves

### Staining and counting of plaques

- 12 well plates overlaid with agar
- 17 ml stain overlay/person (see experimental protocol for components)
- 48°C water bath
- microwave or boiling water to melt agar
- sterile pipets
- sterile 1 oz. bottle/person
- 5% neutral red stock solution (Appendix) wrapped in foil
- table lamp to count plaques
- decontamination pans
- gloves

## EXPERIMENTAL PROTOCOL

### Setting up the cells

1. Decant the spent growth medium from the $L_{929}$ cells.
2. Add fresh medium (E-MEM with 5% FBS) to the $L_{929}$ cells
3. Scrape the cells with a rubber policeman and pipet up and down to break up clumps
4. Do a viable cell count and prepare 14 ml of 1.25 x $10^5$ cells/ml cell suspension
5. Dispense 1 ml of this 1.25 x $10^5$ cells/ml suspension into each well of a sterile plastic 12-well plate.
6. Place the cells at 37°C in the $CO_2$ incubator for 48 hours.

### 48 hours later : Infection of cells with EMC

1. Observe the cells under the microscope. The cell monolayers must be healthy and confluent.
2. Prepare the agar overlay (You will need 17 ml of Difco agar overlay). Keep everything properly labeled.

### Agar Overlay

| | | |
|---|---|---|
| E-MEM | 8.3 ml | |
| FBS | 1.7 ml | UNIT I |
| TPB (tryptose phosphate broth) | 1.7 ml | |
| AGAR (3% in 1x HBSS and autoclaved) | 5.0 ml | UNIT II |

* Sterile $NaHCO_3$ solution (Appendix) to adjust the pH

a. Melt the agar and maintain in the 48°C water bath
b. Prepare Unit I and equilibrate at 48°C in the water bath
c. Combine Unit I & II in a sterile 1 oz. bottle and adjust the pH
d. Keep in the water bath until step #7
e. If there are clumps in the overlay, *prepare another overlay.*

3. Using gloves, prepare $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions of EMC in sterile GLB Prepare 1 ml of each dilution. Keep the dilutions on ice until ready to use
4. Discard the old medium in the 12-well plates and wash the cells 2 times with sterile PBS (1 ml/wash).
5. Label the plates carefully with your name, the date, the virus, and the dilutions used. Add 0.1 ml of each virus dilution to triplicate wells of each plate. Remember to add 0.1 ml *sterile GLB* to the cell control wells (do not add virus to these wells).

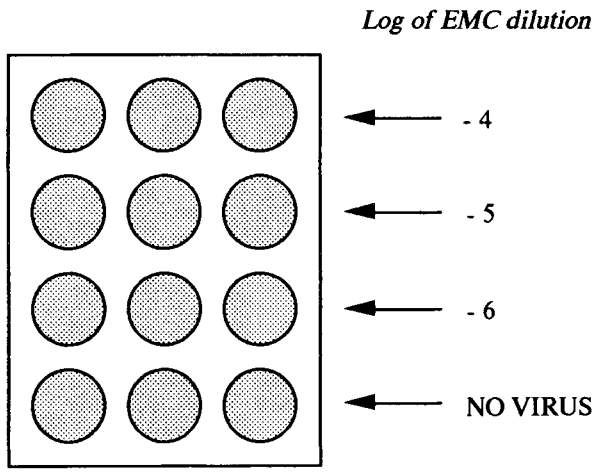


**Figure 16.1** Diagram of experimental set-up of the plaque assay

6. Allow the virus to adsorb for 30 minutes in the incubator. Tilt the plates every 10 minutes for even virus distribution and to prevent monolayer dessication.
7. Wash the cell monolayers two times with sterile PBS to remove unadsorbed virus. Blot the edges of the plates on a sterile towel.
8. During the last 5 minutes of virus adsorbtion, remove the agar overlays from the water bath. This permits the overlays to cool slightly and minimizes cell death. Do not let it cool too long or the agar will start to harden. The agar should be warm but not uncomfortable to the touch.
9. Add 1 ml of the appropriate overlay/well. Allow the agar to solidify. Incubate the plates *inverted* at 37°C for 48 hours.

**48 hours later - Staining and counting of plaques**

1. Prepare 17 ml stain agar overlay/person

**Stain Agar Overlay**

| | | |
|---|---|---|
| E-MEM | 8.3 ml | |
| FBS | 1.7 ml | UNIT I |
| TPB | 1.7 ml | |
| AGAR (3% in 1x HBSS and autoclaved) -- 5% neutral red (sterile) | 5.0 ml | UNIT II |

   a. Melt agar and maintain at 48°C in the water bath
   b. Prepare Unit I and equilibrate at 48°C
   c. Combine Units I & II in a sterile 1 oz. bottle
   d. Add 0.2 ml of the 5% neutral red solution

2. Using gloves add 1 ml of stain overlay/well Let it cool slightly before adding the stain overlay to the cell wells.
3. Keep the 12-well plate covered until the overlay hardens (use a paper towel or some foil to prevent photosensitization of the cells due to the presence of Neutral Red in the overlay).
4. Incubate the plates *inverted* at 37°C in the $CO_2$ incubator.
5. After 4-5 hours of incubation count.the plaques in a darkened room with a table lamp. Use a marker to dot the plastic underside of the plate as you count plaques. If the plaques are not large enough, continue incubating until they become visible.
6. Record your results and discard the plates into decontamination pans.

### Calculation of titer:

Select the dilution with a good number of discrete countable plaques (about 20-80 would be a good number for a well in a 12-well plate). Take the mean number of plaques for the three monolayers at that dilution, and multiply by the reciprocal of the dilution and the reciprocal of the volume added (see the Introduction section for a sample calculation). The value thus obtained will be the number of pfu (plaque forming units)/ml. There should be 10-fold differences in plaque counts at successive 10-fold dilutions.

## *B. PLAQUE ASSAY USING A METHYL CELLULOSE OVERLAY*

The following experiment is a variation of the standard plaque assay procedure from the previous section. The basic principle of the plaque assay however remains the same. Namely, upon infection of a confluent cell monolayer with the virus, an overlay is added that restricts the progeny virus to infection of adjacent cells until a plaque is formed. In this experiment, the virus-cell system is different as is the composition of the overlay medium, and the addition of a stain overlay is omitted. Upon the development of plaques due to infection by *NDV* or *EMC virus*, the *Vero cell* monolayer is fixed with formalin, the methyl cellulose overlay is removed, and the monolayer is stained with a crystal violet solution. The cell monolayer will stain purple and the plaques will be clear.

## MATERIALS AND PREPARATION

### Setting up the cells

- Vero cells
- E-MEM 10% FBS (30 ml/person)
- 0.5% trypsin in HBSS without calcium or magnesium (5 ml/person)
- HBSS without calcium or magnesium (15 ml/person)
- sterile centrifuge tubes (1/person)
- 12-well plates (2/person)
- pipets and decontamination pans

### Infection with virus and addition of overlay

- NDV and EMC virus
- GLB for virus dilutions (16 ml/person)
- 12 well-plates with confluent Vero cells from day 1

- HBSS (25-30 ml/person)
- sterile dilution tubes
- methyl cellulose overlay (50 ml/person, see Appendix)
- 44°C water bath
- pipets and decontamination pans
- gloves

### 3-4 days post infection: Staining of plaques

- 12-well plates of Vero cells infected with NDV or EMC virus
- 10% formalin in PBS (25 ml/person)
- 1% crystal violet in 70% methanol (25 ml/person)
- decontamination pans and pipets
- hypodermic needles or spatulas to lift overlay
- container for disposal of hypodermic needles
- gloves

## EXPERIMENTAL PROTOCOL

### Setting up the cells

1. Wash the Vero cells 3x with 5 ml of HBSS without calcium or magnesium.
2. Add 5 ml of 0.5% trypsin to the cell monolayer and incubate at 37°C in the $CO_2$ incubator for 5 minutes. Periodically shake the flask and check for cell detachment.
3. Add 5 ml of E-MEM 10% FBS and pipet up and down to break up cell clumps.
4. Transfer these contents to a sterile centrifuge tube and centrifuge at 300 x g for 5 minutes.
5. Decant the supernatant and resupend the pellet in 5 ml E-MEM 10% FBS.
6. Do a viable cell count and prepare 25-27 ml of 5 x $10^5$ cells/ml.
7. Dispense 1 ml of the cell suspension into each well of the 12 well-plates.
8. Incubate the 12-well plates at 37°C and 5% $CO_2$ for 48 hours.

### 48 hours later : Infection with virus and addition of methyl cellulose overlay

1. Make dilutions of NDV and EMC virus in GLB. A minimum of 0.5 ml each of the $10^{-5}$, $10^{-6}$, and $10^{-7}$ dilutions will be needed for each virus.
2. Wash the Vero cell monolayers with 1 ml HBSS.
3. Label the plate with the dilutions used in the various wells and add 0.1 ml of each dilution in triplicate to the cell monolayers. (See figure 16.2). Add 0.1 ml GLB to the virus control wells.

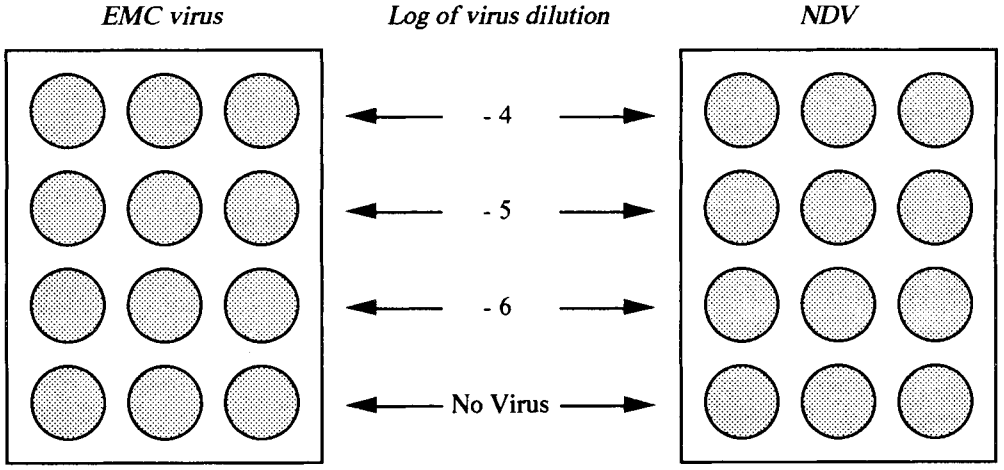


**Figure 16.2** Experimental set-up for the plaque assay of NDV and EMC virus on Vero cells using a methyl cellulose overlay.

4. Let the virus adsorb for 1 hour in the $CO_2$ incubator at 37°C.
5. Add 2 ml of methyl cellulose overlay/well.
6. Incubate at 37°C in the $CO_2$ incubator for 3-4 days.

### 3-4 days later : Staining of plaques

1. The plaques should be visible macroscopically by holding up the plate up to a light. If the plaques are not visible, incubate an additional 24 hours.
2. Add 1 ml/well of 10% formalin (to fix the monolayer) for 30 minutes.
3. With the tip of a hypodermic needle or using a spatula, gently lift and remove the overlay into the decontamination pan.

4. Carefully wash the cell monolayer with tap water. Discard the washes into the decontamination pan.
5. Add 1 ml of 1% crystal violet per well and let it stain for 30 minutes.
6. Wash the cell monolayers with tap water until the excess crystal violet has been removed.
7. Invert the plates over absorbent paper and allow to dry.
8. Count the plaques and determine the titer expressed as pfu/ml

**Calculation of titer:**

Select the dilution with a good number of discrete countable plaques (about 20-80 would be a good number for a well in a 12-well plate). Take the mean number of plaques for the three monolayers at that dilution, and multiply by the reciprocal of the dilution and the reciprocal of the volume added (see the Introduction section for a sample calculation). The value thus obtained will be the number of pfu (plaque forming units)/ml. There should be 10-fold differences in plaque counts at successive 10-fold dilutions.

## DISCUSSION QUESTIONS

1. Why is the plaque assay an *infectious quantitative* assay?
2. Compare the results you obtained for the $TCID_{50}$, and plaque assays. How does the plaque assay differ from the $TCID_{50}$ assay? Which assay is more sensitive? Which assay would you prefer to use to screen a large number of samples?
3. If CPE is normally observed in a cell line upon infection by a virus but no plaques were obtained in your plaque assay, does this mean that the titer should be recorded as 0 pfu/ml? Justify your answer.
4. If EMC had been titered using both the neutral red and methyl cellulose plaque assays, would you expect to obtain exactly the same pfu/ml titer for both sections of this chapter? Justify your answer.
5. How would you modify the experimental protocol to make this an infectious center assay?
6. Since crystal violet (unlike neutral red) stains both living and dead cells indiscriminately, why do plaques appear as clear zones by this method?

## FURTHER READING

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# 17

# ASSAYS OF OTHER VIRUS PROPERTIES

## INTRODUCTION

The hemagglutination assay (chapter 18) and the hemadsorption test (chapter 19) are similar in nature since they are both based on the ability of a virus-coded hemagglutinin to hemagglutinate red blood cells. These assays are different however, in that for hemagglutination to occur there has to be a direct interaction between the virus particle that contains the hemagglutinin on its surface and the red blood cell, whereas for hemadsorption the interaction is between the red blood cell and a virus-coded hemagglutinin that is present on the cell membrane of the virus-infected cell.

In the hemagglutination assay, serial dilutions of the virus suspension are prepared and red blood cells are added. Upon incubation the wells are observed for the presence or absence of hemagglutination at each virus dilution. The hemagglutination assay depends on the ability of the hemagglutinating virus particles in a sample to interact with the red blood cells. No virus replication is required, the hemagglutinating virus particles may or may not be infectious, and therefore *the hemagglutination assay does not measure virus infectivity.* The hemagglutinating titer would be the same whether a virus sample contained infectious virions or inactivated virus particles (provided that the hemagglutinin was not affected).

In the hemadsorption test, a red blood cell suspension is added to wells containing virus-infected cells. If the virus is replicating in these cells and the virus-coded hemagglutinin is being incorporated in the host cell membrane, the red blood cells will adsorb to the surface of the virus-infected cell - **hemadsorption**. *The hemadsorption test does measure virus infectivity* because virus replication and incorporation of the virus-coded hemagglutinin in the host cell membrane are required for hemadsorption to occur. The number of virus particles present in a virus sample is not quantified since one is only looking for the presence or absence of hemadsorption in the wells containing virus-infected cells. The hemadsorption test is very useful as it permits the early recognition of viral infection in cell culture and the recognition of replication of non-cytopathogenic viruses (viruses that do not cause CPE).

# 18

# HEMAGGLUTINATION ASSAY

## INTRODUCTION

Hemagglutination is the aggregation of red blood cells (RBCs) in the presence of hemagglutinating virus particles. This phenomenon is due to the presence of outer surface proteins on the hemagglutinating virus that recognize and attach to cellular surface receptors expressed by RBCs.

The hemagglutination assay (HA) assay is one of the most common indirect methods for quantifying virus particles in suspension. In contrast to the $ID_{50}$ assays, it does not give any measure of viral infectivity since viral replication is not required in this assay. The HA assay is not a very sensitive assay since a large minimum number of virus particles are necessary to obtain macroscopic agglutination, but it is convenient because of its rapidity and ease of titration of a large number of samples. Although not all virus types are hemagglutinators, many viruses have been shown to agglutinate red blood cells (RBCs). The conditions under which agglutination occurs, however, vary from virus to virus. For instance, some viruses will only agglutinate RBCs of certain animal species, while other viruses are not so particular. Some viruses (togaviruses) agglutinate RBCs only under carefully defined pH and ionic strength, while other viruses only require a saline solution. The incubation temperature may also be an important parameter. Influenza viruses possess both hemagglutinin and neuraminidase surface glycoproteins that protrude through the virus envelope. The hemagglutinin surface glycoproteins are responsible for the attachment of the virus to the N-acetylneuraminic acid residues present on the surface of the RBC membrane. The neuraminidase surface glycoproteins, however, possess enzymatic activity that results in the cleavage of the N-acetylneuraminic acid residues on the RBC and the release of the virus particle. Because the enzymatic activity of the neuraminidase is extremely low at 4°C, at low temperatures hemagglutination is favored over receptor destruction.

For the phenomenon of HA to occur, a virion must attach simultaneously to two RBCs, creating a cross-bridge. At low virus concentrations, the amount of RBC cross-bridging is minimal (see Figure 18.1) and the RBC's fall to the bottom of the well, slide toward the center, and form a small, well-defined pellet. At sufficiently high viral concentrations, large aggregates of cross-bridged RBCs are formed that cannot slide to the center, but instead form a thin lattice on

the bottom of the well. In cases of very large virus excess hemagglutination may not be observed because the cellular receptors are saturated and there is no opportunity for cross-bridges to form (not shown in Figure 18.1).

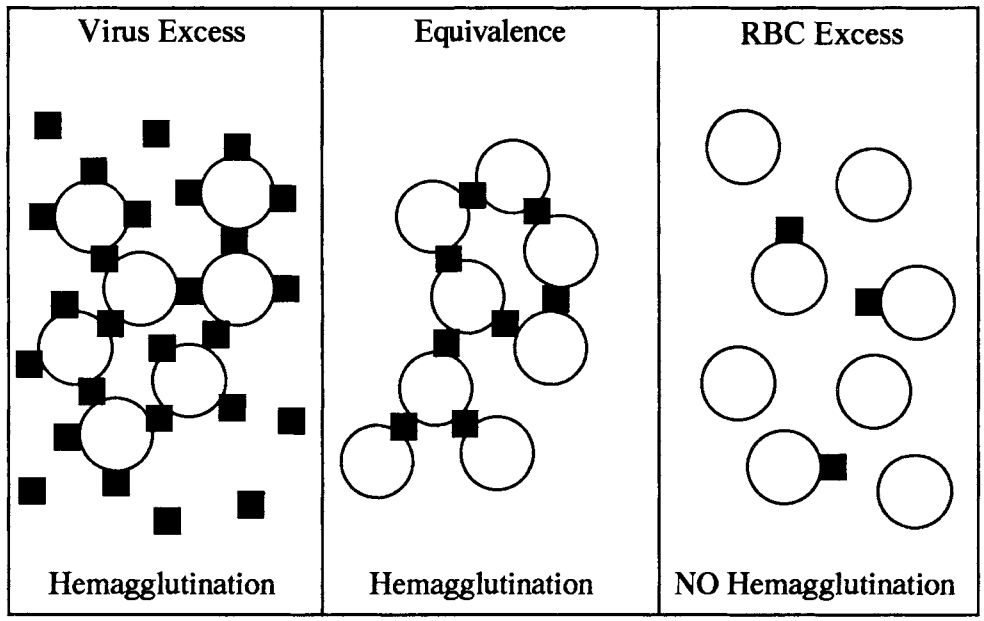


**Figure 18.1** Graphic representation of the hemagglutination phenomenon. The left panel shows the effect of high virus concentration and the right panel the effect of low virus concentration. The middle panel shows an area of equivalence where the number of virus particles equals the number of RBCs in the suspension.

The hemagglutination assay is usually done by endpoint titration. Serial two-fold dilutions of the virus suspension are mixed with a RBC suspension containing a known RBC concentration. The endpoint of the titration is *the last dilution of virus showing complete agglutination* and *is said to contain 1 HA unit.* The **HA titer** is defined as *the reciprocal of the highest dilution of virus that causes complete agglutination*, and is expressed as the number of hemagglutinating units (HA units) per unit volume. For a practical illustration of HA titer determination, see Figure 18.2. Notice that the endpoint of this assay is the last well with *complete* hemagglutination.

Theoretically, viruses are multivalent (have more than 1 binding site) with respect to hemagglutination. In practice, however, only two RBCs can be cross-bridged by a single virus because RBCs are so much larger than viruses. Therefore, within the virus dilution sequence a zone of equivalence should exist wherein the number of hemagglutinating particles just equals the number of RBCs (Figure 18.1, center panel). This is the minimum amount of virus needed for complete hemagglutination, and the HA titer is the reciprocal of this

dilution. At the endpoint of the virus titration, the number of hemagglutinating particles nearly equals the number of RBCs in the well. There is an error margin inherent in this assay since a 2-fold difference in titer (1 well) is not considered significant. This error margin should be kept in mind in making calculations and comparing titers.

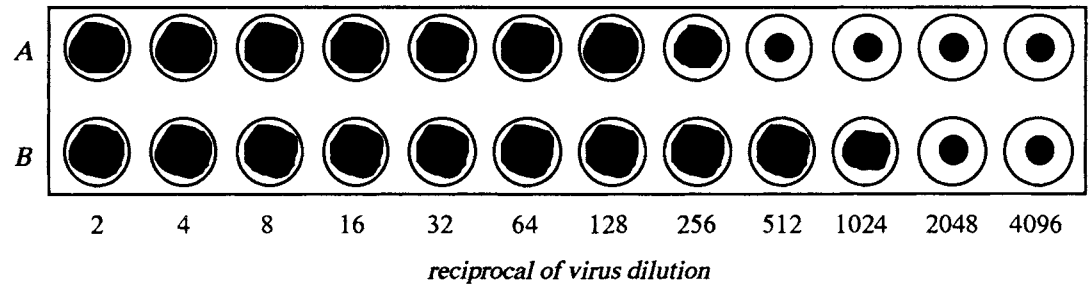


**Figure 18.2** Diagram of a sample EMC hemagglutination assay. Serial doubling dilutions of two EMC virus stocks, A and B, were done through 12 wells. Then an equal volume of the RBC suspension was added. Sample A caused *complete* hemagglutination until dilution 1:128 , and sample B until 1:512. The 1:256 (Sample A) and 1:1024 (Sample B) dilutions show wells with only *partial* agglutination. The RBSs in the remaining wells form well-defined buttons indicative of *no* hemagglutination. If 0.1 ml of a given virus dilution was added per well, the HA titer for Sample A would be 128 HA units/0.1 ml (reciprocal of the last dilution showing *complete* hemagglutination per unit volume), or 1280 HA units/ml. Similarly, the titer for Sample B would be 512 HA units/0.1 ml or 5120 HA units/ml.

Given the HA titer (where the number of RBCs approximates the number of HA particles), and the number of RBCs in the well, an estimate of the number of hemagglutinating virus particles can be obtained. Given that a 0.3% RBC suspension resulted in an HA titer of 512 HA units per 0.1 ml, we can calculate the approximate number of hemagglutinating virus particles in the original suspension. A 10% RBC solution is defined as $8 \times 10^8$ RBCs/ml. Therefore, a 0.3% solution contains $2.4 \times 10^7$ RBCs/ml. We added 0.1 ml of the RBC suspension to each well so there were $2.4 \times 10^6$ RBCs in the well. Thus, at a 1:512 dilution there were approximately $2.4 \times 10^6$ hemagglutinating virus particles in the well. We used 0.1 ml of virus, which means the virus concentration in the well was $2.4 \times 10^7$ virus particles per milliliter. When multiplied by the dilution used (512), this gives $1.2 \times 10^{10}$ hemagglutinating virus particles per milliliter of the original stock suspension. There is an error margin inherent in this assay since a 2-fold difference in titer is not considered significant. This error margin should be kept in mind in making these calculations.

In this manner, the number of hemagglutinating particles in a suspension can be approximated. This does not imply that there are $1.2 \times 10^{10}$ *infectious* virus particles in the stock solution. *The HA assay does not measure infectivity.* The number of hemagglutinating virus

particles calculated in this manner will always exceed the number of infectious units in a virus suspension. Many animal viruses have particle to infectious unit ratios that exceed 1.0, due to the presence in any given virus sample of non-infectious virus particles. Therefore, the particle to infectious unit ratio can generally be regarded as a measure of the probability with which a virus particle can establish a productive infection, or the degree of defectiveness of the virus population.

In this experiment, the HA assay will be used to obtain a titer for the EMC virus prepared in chapter 9. As a positive control, an EMC control virus stock will be provided by the instructor. Four different RBC concentrations will be used for each virus.

## MATERIALS AND PREPARATION

- Washed sheep red blood cells
- EMC-HA buffer (50 ml/person)
- EMC virus stock from chapter 9 (Experimental Sample)
- EMC virus stock provided by the instructor (Control Sample)
- 1 nonsterile 96-well round bottom microtiter plate/person
- 1 ml pipets, disposable plastic gloves, decontamination pans
- Ice buckets
- Mechanical 100 µl pipetor (optional)

## EXPERIMENTAL PROTOCOL

**NOTE :** Although blood products are screened for a number of disease agents, they should be handled as if they contained pathogenic agents. When performing this assay be sure to wear plastic gloves, place all used materials in the decontamination pans, and promptly clean your hemacytometer and any spills with disinfectant.

### A. Preparing a 10% RBC stock solution

1. Make a 1:4000 dilution of the washed RBC stock solution (Appendix) in EMC-HA buffer as follows:
   a. *0.1 ml of stock RBCs + 0.3 ml EMC-HA buffer (1:4)*
   b. *0.1 ml of (a) + 0.9 ml EMC-HA buffer (1:40)*
   c. *0.1 ml of (b) + 0.9 ml EMC-HA buffer (1:400)*
   d. *0.1 ml of (c) + 0.9 ml EMC-HA buffer (1:4000)*

2. Using a hemacytometer and the high power objective, determine the number of cells/ml in the 1:4000 tube (see the legend of figure 5.2 - red blood cell count). Calculate the RBC concentration in the original suspension.
3. Prepare 2 ml of a 10% RBC solution in EMC-HA buffer. A 10% RBC solution = 8 x $10^8$ cells/ml.
4. From your 10% stock solution, prepare 4 ml of each of the following RBC solutions : 0.1% RBCs., 0.3% RBCs, 0.5% RBCs, and 0.7% RBCs in EMC-HA buffer. Keep all the RBC suspensions on ice.

**B. EMC-HA assay**

1. Thaw the virus stocks in a 37°C water bath and keep the stocks on ice.
2. Add 0.1 ml of EMC-HA buffer to each well of the 96-well plate.
3. Add 0.1 ml of your EMC virus stock to wells B1, D1, F1, and H1. Return the virus stock to the ice bucket. See fig.18.3.
4. Using another tip for the mechanical pipetor, do serial two-fold dilutions (0.1 ml + 0.1 ml) through 11 wells of column B. Discard the last 0.1 ml from row 11 into the decontamination pan. *Do not add any virus to row 12.*
5. Change pipet tip and repeat the dilution sequence for columns D, F, and H. *As above, do not add any virus to row 12.*
6. Add 0.1 ml of the *control* EMC virus stock to wells A1, C1, E1, and G1. Return the virus stock to the ice bucket.
7. Repeat the dilution sequence as in steps 4 and 5 above, remembering not to add any virus to row 12.
8. Using a new pipet or pipet tip, add 0.1 ml of the 0.1% RBC solution to all the wells in columns G and H; 0.3% RBC solution to columns E and F; 0.5% RBC solution to columns C and D ; and 0.7% RBC solution to columns A and B.
9. Allow the plate to stand undisturbed for 90 minutes.
10. Return the virus stocks to the (- 70°C) freezer for storage.
11. Record your results (you may wish to draw them on Figure 18.3) and place the plates in the decontamination pan.

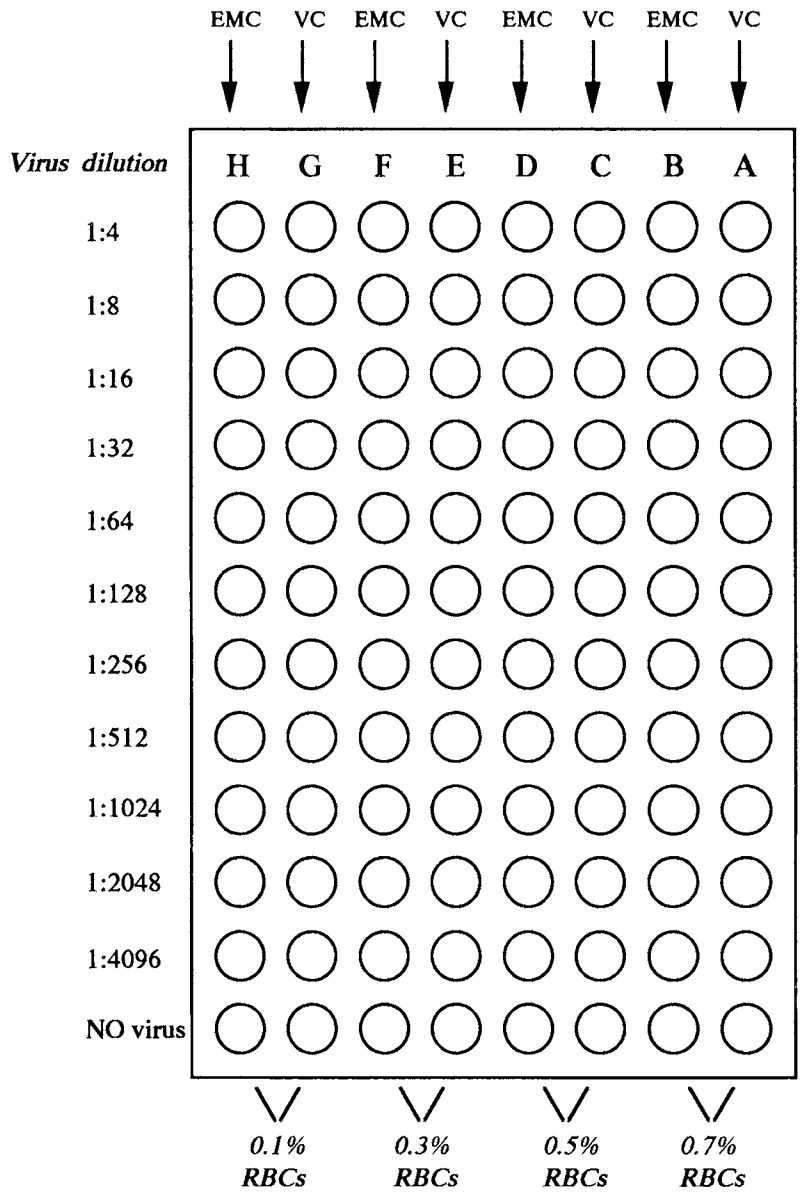


**Figure 18.3** Representation of the experimental set up of the hemagglutination assay. EMC stands for the EMC virus prepared in chapter 9. VC stands for Virus Control ,the EMC virus provided by the instructor as a positive control.

## DISCUSSION QUESTIONS

1. Which RBC concentration gave the highest HA titer? Why do different RBC concentrations result in different HA titers?
2. Using the results of each of your virus titrations, calculate the number of hemagglutinating virus particles in the original virus stock. Do you get different results when various RBC concentrations are used? (Remember that the variability of this assay is a 2-fold dilution).
3. How many virus particles constitute 1 HA unit? Would you expect this to vary among different viruses?
4. Calculate the virus particle to infectious unit ratio for your EMC virus stock using the data you have collected to date. Does this ratio vary with different assays? Explain your results.

## FURTHER READING

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# 19

# HEMADSORPTION

## INTRODUCTION

Cultured cells infected with viruses that bud from the cytoplasmic membrane (such as the orthomyxoviruses, paramyxoviruses, and togaviruses) have the ability to adsorb red blood cells. This phenomenon of the red blood cells adsorbing to the surface of the infected cell is referred to as *hemadsorption.* Hemadsorption results from the incorporation into the cell's plasma membrane of a virus-coded hemagglutinin.

An advantage of this technique is that it enables one to recognize infection by noncytopathogenic viruses (viruses that do not cause CPE), or to recognize an early stage of infection by cytopathogenic viruses (viruses that cause CPE). If sequential time points are taken post-infection, the actual time of incorporation of the virus-coded hemagglutinin into the cell membrane can be approximated. Thus, the hemadsorption assay can give useful information about the replication cycle of a given virus.

A modification of this assay is the *hemadsorption-inhibition assay.* In this case, serial dilutions of a sample containing an antiviral agent or an antiviral antibody are mixed with a constant amount of virus. The cells are then infected, and later observed for hemadsorption at the various antibody dilutions. By recording the presence or absence of hemadsorption at a given sample dilution, it is possible to determine a hemadsorption inhibition titer for the antibody or antiviral agent being tested. This technique is very similar in principle to the hemagglutination yield-reduction assay (chapter 35), and to the hemagglutination-inhibition antibody assay (chapter 28).

Hemadsorption is dependent upon (1) the sensitivity of the cells to the given virus , (2) temperature (if a neuraminidase is present), (3) pH, (4) RBC type, species, and concentration, and (5) the presence of nonspecific inhibitors of hemagglutination in certain serum samples. With respect to the red blood cells, not only will RBCs from certain species hemadsorb only to cells infected with a specific virus type, but old RBCs can cause non-specific hemadsorption and yield false results.

In this experiment Vero cells will be infected with NDV and EMC virus. At various times post infection, sheep red blood cells will be added and the cells will be observed for evidence of hemadsorption, as compared to the controls (uninfected cells). NDV buds from the

cytoplasmic membrane of the infected cell and causes hemadsorption while EMC virus does not.

This hemadsorption experiment does not yield any viral titer. To obtain an infectivity titer, the addition of sequential virus dilutions and the determination of an endpoint would be required. This experiment was placed in this section because of its relationship to the HA assay although it could have easily been included in the virus characterization section (chapters 20-25).

## MATERIALS AND PREPARATION

### Setting up the cells

- Vero cells
- HBSS (Hanks' Balanced Salt Solution) without calcium or magnesium (20-25 ml/person)
- 0.5% trypsin in HBSS without calcium or magnesium, 5 ml/person (see Appendix)
- sterile centrifuge tubes
- 9 - 35 mm sterile tissue culture dishes/person
- E-MEM with 10% FBS ( about 25 ml/person)
- hemacytometers, nonsterile tubes, PBS, trypan blue, and pasteur pipets for cell counting
- sterile pipets
- decontamination pans.

### Virus infection: 24-48 hours later

- GLB for virus dilutions (about 4 ml/person)
- viruses (NDV and EMC)
- plastic gloves
- sterile pipets and decontamination pan
- sterile PBS (1-2 ml/person) for the washing step
- EMC-HA buffer (see Appendix)
- E-MEM with 5% FBS (15 ml/person)
- 0.5% sheep RBCs in EMC-HA buffer (1 ml/person)
- 0.5% sheep RBCs in PBS (2 ml/person)
- **** : The RBC suspensions should be refrigerated until ready to use. (See chapter 18 for preparation of the RBC suspension)

## EXPERIMENTAL PROTOCOL

### Setting up the cells

1. Set up 9 dishes with $1x10^5$ Vero cells/ml, 1.5 ml per well . (Use the individual 35 mm sterile petri dishes and E-MEM 10% FBS). Vero cells have to be trypsinized. Refer to chapter 5 - Section B.
2. Incubate 24 hours. (If a 48 hours incubation is more convenient, use $5x10^4$ cells/ml, 1.5 ml per dish).

### Virus infection: 24-48 hours later

1. Observe the Vero cells under the microscope. They should be healthy and 50-80% confluent.
2. Prepare 1 ml of a $10^{-3}$ dilution of each virus (EMC and NDV) in GLB.
3. Infect 3 wells with NDV ( 0.2 ml of $10^{-3}$ NDV per well )
   Infect 3 wells with EMC ( 0.2 ml of $10^{-3}$ EMC per well )
   Leave 3 wells *uninfected* ( 0.2 ml of GLB - *no virus* )
   See figure 19.1 below.

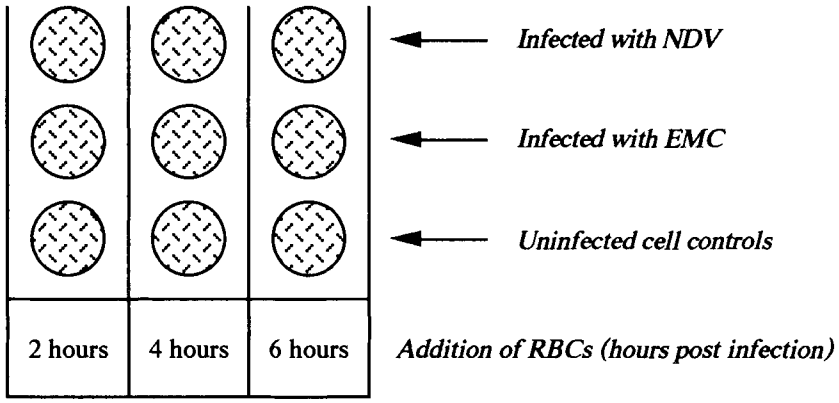


**Figure 19.1** Diagram of hemadsorption protocol for infection of Vero cells with EMC and NDV. Note the 2,4, and 6 hours post infection addition of RBCs. If BHK-21 cells are used instead of Vero cells,these time points for the addition of the RBCs change to 16, 24, and 36 hours post infection.

4. Adsorb virus for 1 hour in the incubator, swirling every 10-15 minutes (to maximize virus adsorbtion and to prevent monolayer dessication).
5. Decant the unadsorbed virus into the decontamination pan .
6. Wash all the dishes 1x with 1 ml of sterile PBS. (After the washing step, decant the PBS into the decontamination pan).

7. Add 1.5 ml of E-MEM 5% FBS to each well of washed cells.
8. Incubate for 2, 4, and 6 hours .

## Addition of red blood cells

**NOTE** : Although blood products have been screened for a number of disease agents, they should be handled as if they contained pathogenic agents. When performing this part of the assay be sure to wear plastic gloves, place all used materials in the decontamination pans, and promptly clean your hemacytometer and any spills with disinfectant.

1. At the appropriate times, select 3 of the dishes (1 NDV-infected, 1 EMC virus-infected, and 1 control).
2. Empty the medium into the decontamination pan and add 0.2 ml of 0.5% RBC in PBS, for the NDV and control dishes . Use the 0.5% RBC in EMC-HA buffer for the EMC-infected well . (Remember to swirl the RBC suspension before adding it to the wells).
3. Incubate at 37°C for 10 minutes.
4. Remove supernatant, add fresh PBS or EMC-HA buffer and examine under the microscope .

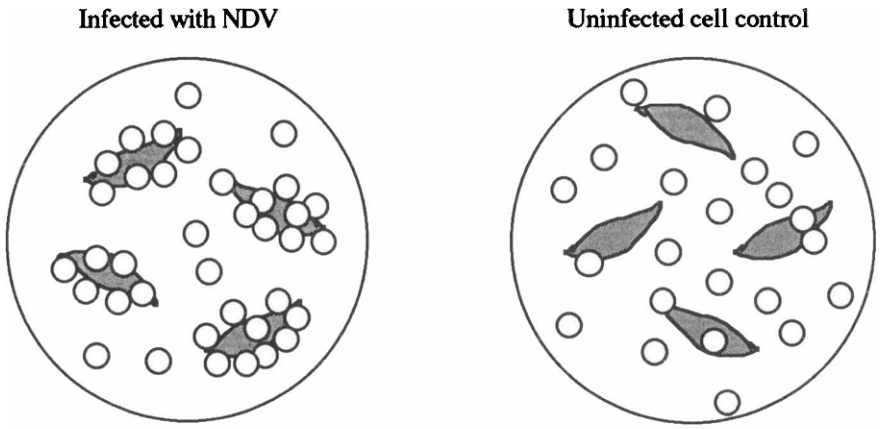


**Figure 19.2** Representation of a hemadsorption-positive and hemadsorption-negative well as observed under the microscope.

5. Compare the infected dishes to the control dishes . Record a given time point as being "+" or "-" hemadsorption for a given virus. (*Always compare to cell control*). See figure 19.2.
6. Dispose of all materials into the decontamination pans.

## DISCUSSION QUESTIONS

1. Correlate your microscopic observations with what is known to be happening at the cellular level during viral infection.
2. Compare and contrast HA (previous chapter) and hemadsorption in terms of what you observe and what you are measuring.
3. Why is is there no calcium or magnesium in the buffers?
4. Establish an experimental protocol to obtain a hemadsorption titer for NDV. What would be your endpoint and how would you determine the titer?

## FURTHER READING

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# 20

# INTRODUCTION TO VIRUS CHARACTERIZATION

## VIRUS CHARACTERIZATION

A number of methods for virus isolation and characterization are available. Once obtained, the specimen is usually inoculated in various cell cultures, embryonated eggs, or newborn mice.

When taken in conjunction with clinical symptoms, the patterns of virus infection in cell culture are often very helpful to obtain a presumptive identification of a virus isolate. The type of cytopathic effect (CPE) produced by a virus and its rate of spread in a particular cell culture is particularly important. Some viruses, such as the enteroviruses, cause an initial rounding up of the cells into highly refractile elliptical bodies that stand out against an otherwise normal monolayer (early stages of infection). Other viruses cause the appearance of grape-like clusters of rounded cells, of inclusion bodies, or of giant multinucleated cells. However, it should be kept in mind that there are many more viruses than recognizable types of CPE, and more than one virus will produce a given pattern of CPE.

The presence of viruses that cause little or no CPE may be identified by their ability to cause adsorption of red blood cells to the surface of the infected cell (hemadsorption - chapter 19). The ability of a given virus to hemagglutinate red blood cells (hemagglutination - chapter 18) can also be useful in the identification of certain viruses.

When some viruses (such as poxviruses) are grown on the chorioallantoic membrane of embryonated eggs, the morphological appearance of the pocks produced can be used to differentiate between these viruses.

Inoculation of animals is most helpful in identification of coxsackie viruses in that the type of paralysis induced by the virus in the suckling mouse can give a clue to the coxsackie virus group involved.

When the presence of the virus has been detected, and a presumptive identification has been made, serologic tests are performed to obtain definitive identification.

If a new agent is suspected the virus isolate can be classified by determining:

1. *The nucleic acid type.* Whether the nucleic acid is DNA or RNA can be determined to by using structural analogs of naturally occuring pyrimidines that interfere with DNA synthesis. Viruses

susceptible to the action of these structural analogs are considered to be DNA viruses. Other techniques used to determine nucleic acid type include acridine orange staining and the Feulgen reaction. Under the fluorescent microscope, a yellowish green inclusion in the nucleus (with acridine orange staining), or a bright red inclusion in the cell (with the Feulgen reaction) is often indicative of a DNA virus.

2. *The size and shape of the virus.* Virus size and shape can be determined directly by electron microscopy. When this is not available the size can be indirectly estimated by using filters of varying pore sizes.
3. *The sensitivity of the virus to lipid solvents.* The sensitivity to lipid solvents indicates the presence of a viral lipid envelope.
4. *The stability of the virus at pH 3.* Most viruses do not tolerate extremes in pH and this test is most useful in identifying viruses, such as the enteroviruses, that can withstand the conditions present in the gastrointestinal tract.
5. *The sensitivity of the virus to heat.* This test helps to further differentiate between viruses at a temperature (56°C) where protein denaturation is rapid and many viruses quickly lose infectivity.

In the following chapters (21-25) The NDV and EMC stocks produced in chapters 9 & 10 are characterized. The one-step growth curve (chapter 21) provides important information about the replication cycle of these viruses. Their size range is approximated in chapter 22. Their thermostability is determined in chapter 23, their sensitivity to lipid solvent in chapter 24, and their sensitivity to low pH in chapter 25.

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# 21

# VIRAL REPLICATION: ONE-STEP GROWTH CURVE

## INTRODUCTION

The replication cycle of a virus consists of a number of sequential steps involving the adsorption, penetration, replication of the parent virus, and release of progeny virus. The one-step growth curve provides valuable information regarding the replication cycle of a given virus in a given cell system and is a common experimental procedure for studying viral replication. The one-step growth curve describes the production of progeny virus over a period of time following infection under one-step conditions. One-step conditions exist when all the cells are infected simultaneously so as to prevent secondary cycles of infection. This is usually achieved by using a high MOI which facilitates a practically synchronous initiation of infection of all available cells. The unadsorbed virus is then washed off or neutralized with antibody.

At various times after infection samples of supernatant fluid and of disrupted cells are assayed for infectious virus. The yield of *extracellular virus* is determined by assaying the supernatant medium. The *intracellular (cell-associated) virus* titer is determined in disrupted cells from which the supernatant medium was previously removed. The *total virus* at any given time point is the sum of the extracellular and the intracellular virus. When these titers are plotted as a function of time a growth curve is obtained in which the following stages can be recognized (fig. 21.1):

1. *Adsorption and penetration.* Upon addition of the virus to the cell culture, the processes of virus adsorption and penetration occur. This is the time period during which the parent virus attaches to the cell surface and the viral nucleic acid enters the cell.
2. *Eclipse period.* After adsorption of the virus, there is a time period during which there are no demonstrable virus particles present even in disrupted cells. During this stage the main processes of viral replication are initiated, including the production of viral nucleic acid and proteins.
3. *Maturation.* The period during which infectious virus may be recovered intracellularly but not yet in the extracellular fluid. During this time the nucleic acid genome and capsid components

are assembled into progeny virus. The time period from the beginning of the eclipse period to the end of the maturation period is referred to as the *latent period* of virus replication.

4 *Release* or rise period during which there is a rapid rise in the extracellular virus titer until a constant titer is reached at the end of the replication cycle. If the number of cells originally infected is known (ie: from an infectious center assay or by determining the number of cells in the monolayer), the *virus yield* can be calculated by determining the average number of infectious units produced per infected cell. The release period is the final step of the replication cycle. With non-enveloped viruses, the infected cell usually disintegrates and the progeny virus that has been accumulating inside the cell is released. In this case, the amount of cell-associated virus exceeds the amount of extracellular virus until the very end of the replication cycle (fig. 21.1).

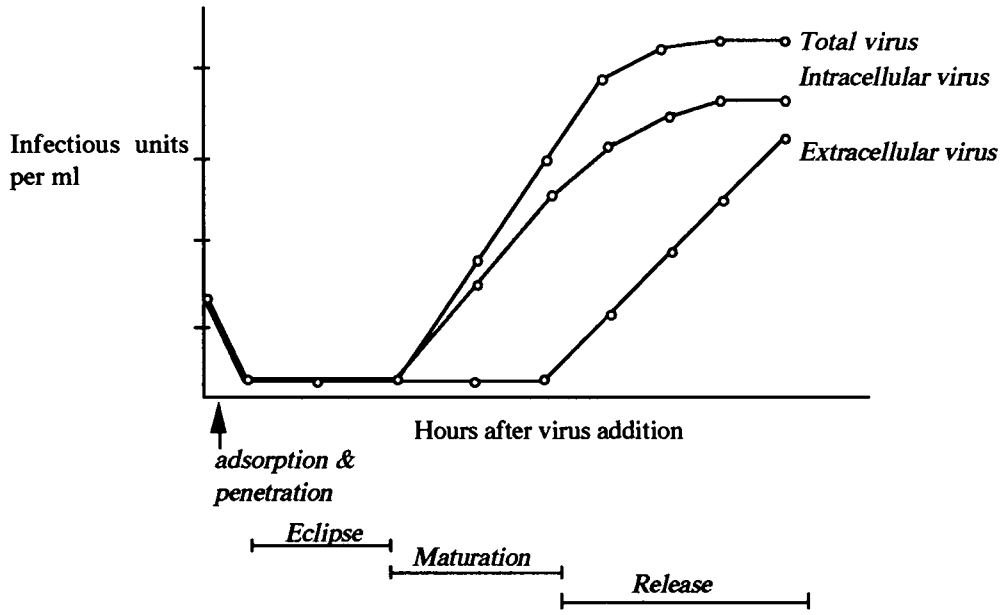


**Figure 21.1** A one-step growth curve for a non-enveloped virus.

Enveloped viruses acquire their envelopes by budding through cellular membranes (ie: cell membrane, nucleus, or endoplasmic reticulum). Usually, these viruses are not fully mature and infectious within the cell until they are enveloped. In such a growth curve the amount of extracellular virus may exceed the amount of cell-associated virus during the replication cycle (fig. 21.2).

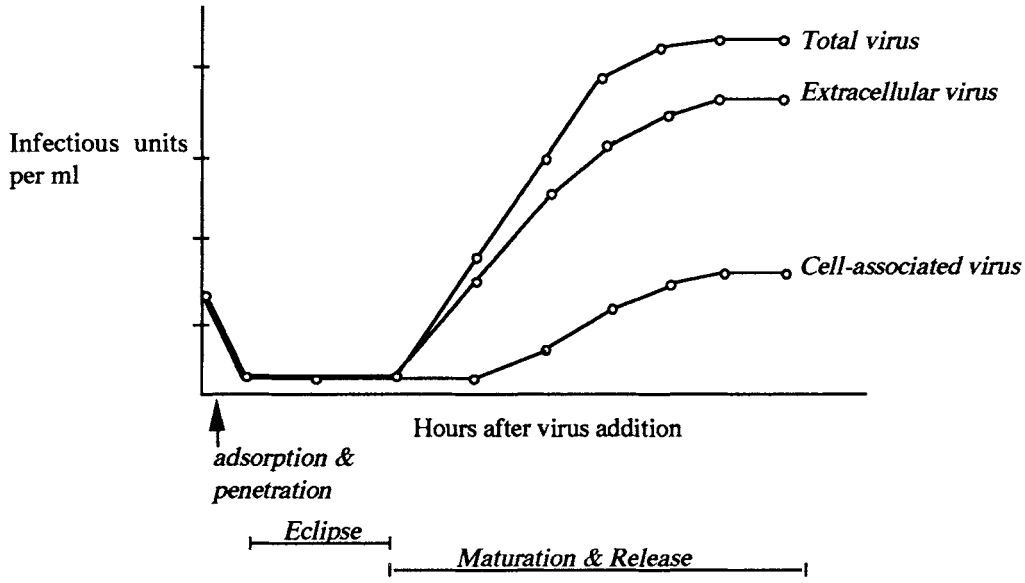


**Figure 21.2** A one-step growth curve for an enveloped virus budding from the cytoplasmic membrane. Following adsorption and penetration, there is an eclipse period but no independent maturation period since maturation occurs concurrently with release of the progeny virus.

With many virus types, the final stage of virus maturation does not occur until after the virus particle is released from the host cell. Such an example would be the human immunodeficiency virus (HIV), a retrovirus, where cleavage of the viral glycoprotein is done by a virus-coded protease after virus release. An interesting feature of the later stages of the replication cycle of the paramyxoviruses (such as NDV) is that they require proteolytic cleavage of their surface glycoprotein spikes to become infectious. This cleavage is performed by a host protease and frequently occurs upon virus release. Cells with the protease release infectious virus whereas cells without the protease release non-infectious virus. The non-infectious virus particles still have the ability to become infectious however, if an exogenous protease (such as trypsin) is added.

The length of the various stages of virus replication outlined above vary depending on both the virus and the host cell.

In the following experiment a one-step growth curve is obtained for EMC and NDV using Vero cells. At various times after adsorption the supernatant samples are collected and cell samples are disrupted by 3 cycles of freeze-thawing. As they are collected, the samples are frozen until they can be assayed for presence of infectious virus. The growth curve for each virus may then be obtained by plotting the titer in terms of infectious units per ml as a function of time.

## MATERIALS AND PREPARATION

### One-step growth curve

- 20 (35x10 mm) individual dishes with confluent Vero cells/group
- E-MEM 2% FBS (50 ml/group)
- sterile PBS (130 ml/group)
- sterile centrifuge tubes (40/group)
- sterile tubes to collect samples (40/group)
- GLB to prepare virus dilutions
- cotton-plugged sterile pasteur pipets with bulbs to collect samples
- NDV & EMC stock viruses
- ethanol-dry ice bath for freeze-thaw cycles
- gloves and decontamination pans

### $TCID_{50}$ assay of samples

- 4 96-well plates/pair (flat-bottom and sterile)
- BHK-21 cells (2-3 confluent 75 $cm^2$ bottle/group)
- rubber policemen
- E-MEM 5% TPB 2% FBS
- hemacytometer
- nonsterile tubes for cell counts
- Pasteur pipets & PBS for cell counts
- 1 small sterile flask to prepare cell suspension (1/group)
- sterile dilution tubes and sterile pipets
- samples generated in one-step growth curve
- gloves and decontamination pans

## EXPERIMENTAL PROTOCOL

1. Determine the number of cells in a sample well
2. Perform the appropriate dilutions of NDV and EMC in GLB, so as to obtain an MOI of 5-10 (per 0.1 ml of virus).
3. Decant the spent medium from the individual dishes and add 0.1 ml of NDV to each of 10 wells, and 0.1 ml of EMC to the remaining 10 wells.
4. Let the virus adsorb for 1 hour in the $CO_2$ incubator, gently tilting the wells every 10-15 minutes.

5. Wash the cell monolayers 3 times with sterile PBS to remove unadsorbed virus, add 1 ml of E-MEM 2% FBS to each well, and place the wells in the $CO_2$ incubator.
6. At 2, 3, 4, 5, 6, 18, 24, 28, 42, and 48 hours post-infection (or as determined by the instructor), perform steps# 7-11 for each time point (see figure 21.3).

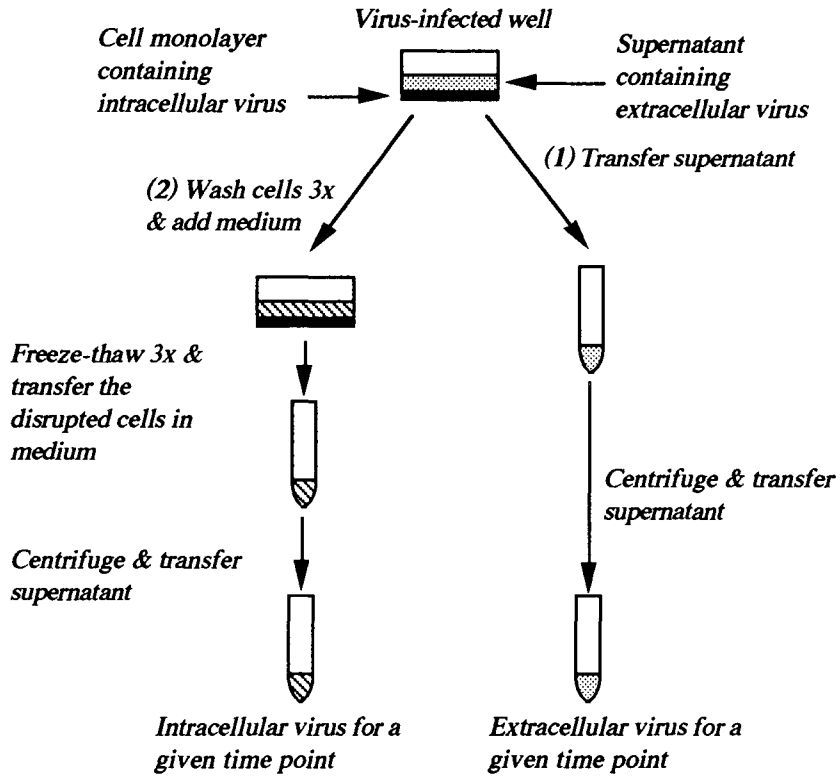


**Figure 21.3** Experimental protocol for the recovery of the intracellular and extracellular virus samples at a given time point.

7. At a given time point, remove 1 NDV-infected dish and 1 and EMC virus-infected dish from the incubator. Using a sterile Pasteur pipet aseptically aspirate the supernatant fluid and transfer it to a properly labeled sterile centrifuge tube. These samples are the extracellular virus for that time point.
8. Wash the cell monolayer 3 times with sterile PBS and add 1 ml of E-MEM 2% FBS to each dish
9. Freeze-thaw the cell monolayers 3 times using the ethanol-dry ice bath to freeze, and the 37°C water bath to thaw.

10. Transfer the contents of the freeze-thawed dishes to sterile centrifuge tubes. These are the intracellular virus samples for that time point.
11. Centrifuge all the tubes (intracellular and extracellular samples) to remove cellular debris, transfer to properly labeled sterile tubes, and place at -70°C until ready to assay.
12. Titrate all the samples using a plaque assay or $TCID_{50}$ assay. If using the $TCID_{50}$ assay, refer to figure 21.4 for an illustration of the experimental set up.
13. Create a one-step growth curve for each virus by plotting the infectious units/ml as a function of time. Label the eclipse, maturation, and release stages of viral replication.
14. Dispose of all the gloves and other contaminated materials in the decontamination pans.

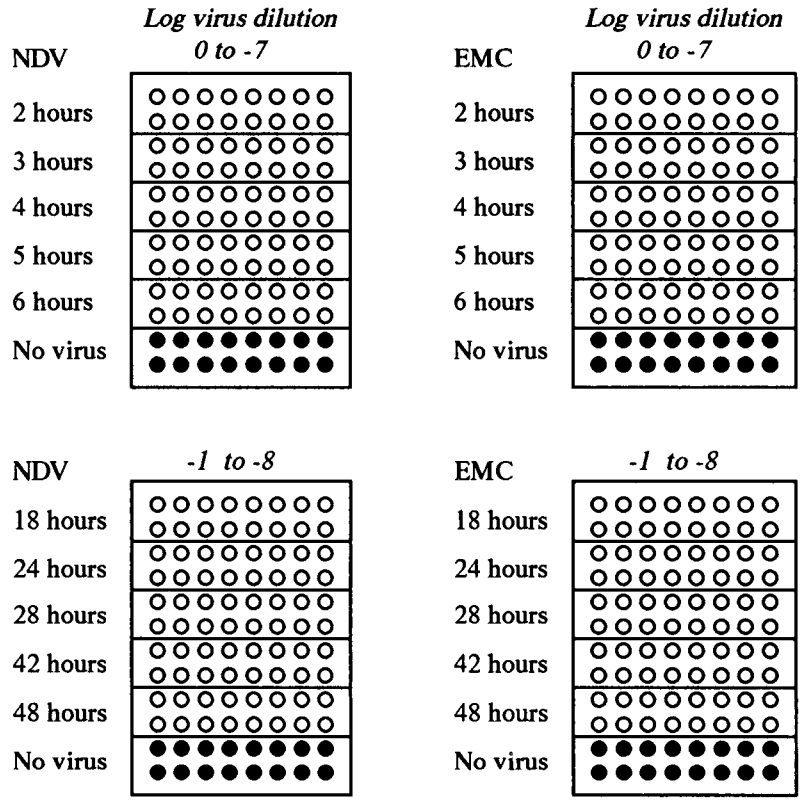


**Figure 21.4** Experimental set up for a $TCID_{50}$ assay to titrate virus samples obtained from the one-step growth curve. 4-96 well plates of BHK-21 cells are used and each dilution for a given time point is done in duplicate. The early time point samples are diluted $10^{0}$-$10^{-7}$, while the later time points are diluted $10^{-1}$-$10^{-8}$. The dark wells indicate virus control wells where no virus is added.

## DISCUSSION QUESTIONS

1. Knowing that NDV is an enveloped virus that buds through the cell plasma membrane and that EMC is a non-enveloped virus, what growth curves would you expect? Does your data agree with this?
2. If another cell line had been used to replicate NDV and no infectious virus could be recovered when the samples were titrated, does it necessarily follow that NDV does not replicate in that cell line? Justify your answer.
3. What information can be gathered about the mode of replication of a given virus when performing a one-step growth curve?
4. What fraction of cells would you expect to be infected with a MOI of 1? What are the disadvantqages of using an MOI that is too low? too high?

## FURTHER READING

Fenner, Frank, Peter A. Bachmann, E. Paul Gibbs, Frederick A. Murphy, Michael J. Studdert, David O. White. 1987. *Veterinary Virology* . Academic Press Inc. Orlando, Fl., pp. 55-88.

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# 22

# DETERMINATION OF VIRUS SIZE

## INTRODUCTION

Size is a useful parameter in the classification of viruses. Size can be determined directly by electron microscopy, or indirectly by using membranes with varying pore sizes. When using membranes, the virus suspension is centrifuged to remove cell debris and other smaller particulate matter is removed by filtering the virus suspension through a 300 µm filter. The resulting virus suspension is then passed through a 100 µm and a 50 µm filter. The filtrates are all titered to determine the activity remaining upon each filtration step. A virus is classified as large if it does not go through the 100 µm filter, as being of medium size if it goes through the 100 µm but not through the 50 µm filter, and as small if it goes through the 50 µm filter.

Determination of size by filtration assumes that a virus particle is spherical. When it is not, some virus particles may go through but the pores become occluded by virus particles lying sideways and there is a large partial drop in activity. Thus, when determining the size of a virus by filtration, one must use care until the exact configuration of the virus under study is known. Furthermore, some viruses may adsorb to untreated membranes and this would also lead to an erroneous size determination. Adsorption applies to all membranes regardless of the pore size. Adsorption can be prevented by pretreating the filter membranes with protein prior to filtering the virus.

In the following experiment the size ranges of NDV and EMC are determined.

## MATERIALS AND PREPARATION

- EMC & NDV stocks (4 ml/group of 3)
- 300 µm sterile Millipore filter (2/group of 3)
- 100 µm sterile Millipore filter (2/group of 3)
- 50 µm sterile Millipore filter (2/group of 3)
- sterile plastic syringes & 18 gauge needles (6/group of 3)
- 5% sterile calf serum (CS) in sterile PBS (20 ml/group of 3)
- sterile tubes to collect filtrates (7/group of 3)
- decontamination pans and gloves

### Titration of Samples

- 2 96-well plates/group of 3 (sterile, flat-bottom)
- BHK-21 cells (1-2 confluent 75 $cm^2$ flask/group of 3)
- rubber policeman
- E-MEM 5% TPB 2% FBS
- hemacytometer
- nonsterile tubes for cell counts
- Pasteur pipets & HBSS for cell counts
- small sterile flask or tube to prepare cell suspension (1/group of 3)
- sterile dilution tubes
- virus samples
- sterile pipets
- decontamination pans
- gloves

## EXPERIMENTAL PROTOCOL

1. Filter 3 ml of 5% CS in PBS through each Millipore filter to pretreat the membranes to prevent virus adsorbtion. To do this, attach the 18 gauge needle to the syringe and aspirate the 5% CS. Carefully recap and remove the needle, and attach the Millipore filter.
2. Aspirate 3 ml of the centrifuged NDV virus suspension through the 18 gauge needle. Carefully recap and remove the needle, and attach the 300 µm filter. Collect the filtrate in a sterile tube. This is the 300 µm filtrate. (The 1 ml remaining in the original tube is the unfiltered virus sample). See figure 22.1.
3. Remove 2 ml of the 300 µm filtrate (as in step #2) and filter through the 100 µm filter. Collect the filtrate in a sterile tube. This is the 100 µm filtrate.
4. Remove 1 ml of the 100 µm filtrate (as in step #2) and filter it through the 50 µm filter. Collect the filtrate in a sterile tube. This is the 50 µm filtrate.
5. Repeat steps 2-4 for EMC virus.
6. Titrate the samples generated above by $TCID_{50}$ assay as illustrated in figure 22.2.

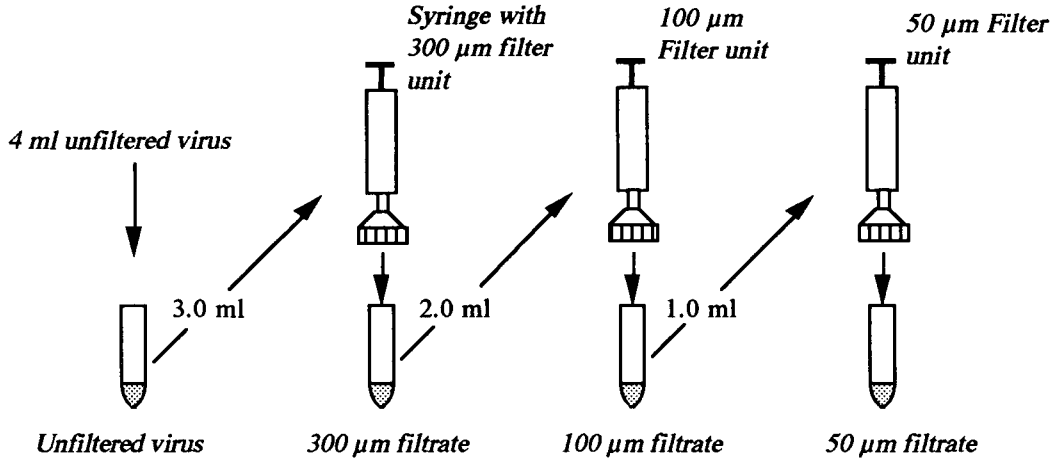


**Figure 22.1** Schematic representation of the filtration scheme for size determination.

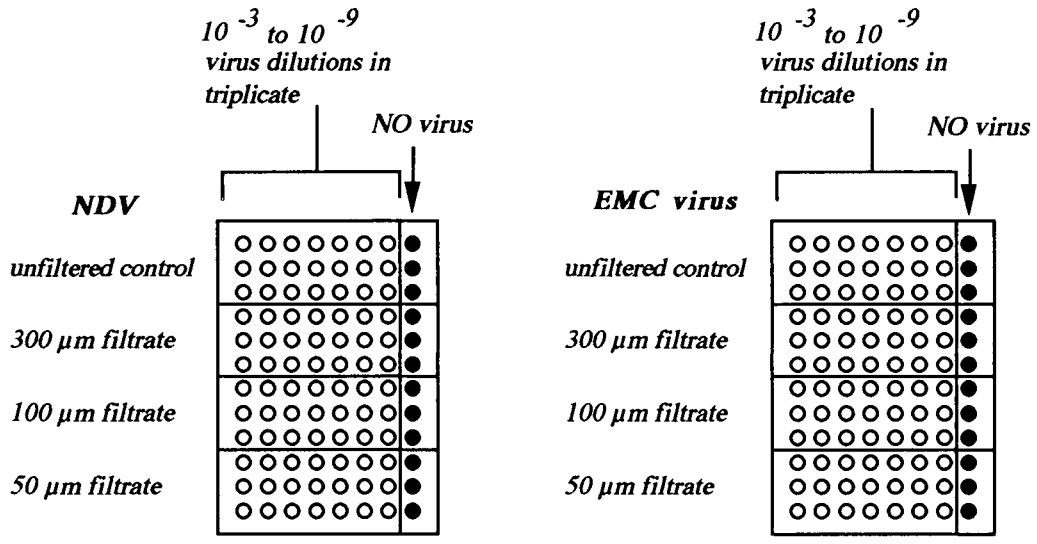


**Figure 22.2** Diagram of the $TCID_{50}$ titration of the samples.

7. Dispose of all contaminated materials in the decontamination pans.
8. Compare the infectivity titers of the unfiltered virus and of each of the filtrates to determine the size range of EMC and NDV.

## DISCUSSION QUESTIONS

1. Given the following about an unknown virus: there is no loss of activity upon passage through the 300 μm filter, there is a 1 log decrease in activity upon passage through the 100 μm filter, and there is a total loss of the remaining activity upon passage through the 50 μm filter. What would be your conclusions?
2. In the above example, should you rule out adsorption? Why?

## FURTHER READING

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Revozzo, G. C. and C. N. Burke. 1973. *A Manual of Basic Virological Techniques.* Prentice-Hall, Inc. Englewood Cliffs, N.J., pp. 141-144.

# 23

# ASSESSING THERMOSTABILITY

## INTRODUCTION

Viruses vary widely in their response to heat. Some viruses are quickly inactivated at higher temperatures, whereas others are much more heat stable. Knowledge of the thermostability of a given virus can predict if a given procedure will result in destruction or preservation of viral activity.

Most viruses are stable at room temperature, at least for short periods of time, when they are suspended in complex media. Differences occur in the 50-60°C range where there is rapid protein denaturation. The standard test uses constant exposure in a 56°C water bath. Samples are removed at given time points and all the samples thus obtained are titrated. When this data is plotted as a function of time a heat-inactivation curve is obtained for this virus. A given virus is considered heat-labile if there is a 1 log (or greater) decrease in infectivity following exposure at 56°C for 1 hour.

When present in high concentrations monovalent (ie: sodium) and divalent (ie: magnesium and calcium) cations stabilize certain viruses (ie: enteroviruses) to heat-inactivation. Other viruses are not stabilized and thus quickly inactivated (ie: arboviruses).

In the following experiment EMC virus and NDV will be heated to 56°C to determine their thermostability and to obtain a thermal inactivation curve for each virus.

## MATERIALS AND PREPARATION

### Thermostability

- NDV & EMC stocks (1.4 ml of each virus/person)
- small sterile test tubes (12/person)
- sterile pipets
- test tube racks
- 56°C water bath
- ice buckets with ice
- decontamination pans
- gloves

### $TCID_{50}$ titration of samples

- 2 96-well plates/person (sterile, flat-bottom)
- BHK-21 cells (1-2 confluent 75 $cm^2$ flask/person)
- rubber policeman
- E-MEM 5% TPB 2% FBS
- hemacytometer
- nonsterile tubes for cell counts
- Pasteur pipets & HBSS for cell counts
- small sterile flask or tube to prepare cell suspension (1/person)
- sterile dilution tubes
- virus samples
- sterile pipets
- decontamination pans
- gloves

## EXPERIMENTAL PROTOCOL

1. Dispense 0.2 ml of each virus into 6 small sterile test tubes.
2. Place 5 tubes of each virus in the 56°C water bath, making sure that the virus suspension in the tube is submerged. The remaining tube is not exposed to 56°C (it is the time 0 point) and is kept on ice.
3. At intervals of 15 minutes, 30 minutes, 45 minutes, 1 hour, and 2 hours, remove one tube of each virus from the water bath and place it on ice.
4. Titrate the samples thus obtained using a $TCID_{50}$ or plaque assay. See figure 23.1 for an illustration of the experimental set-up of the $TCID_{50}$ assay.
5. Dispose of all contaminated materials in the decontamination pans.
6. Compare the 1 hour and 0 time points. A 1 log decrease in activity is indicative of heat lability. Plot the titers of the various time points as a function of time to obtain a thermal inactivation curve for each virus.

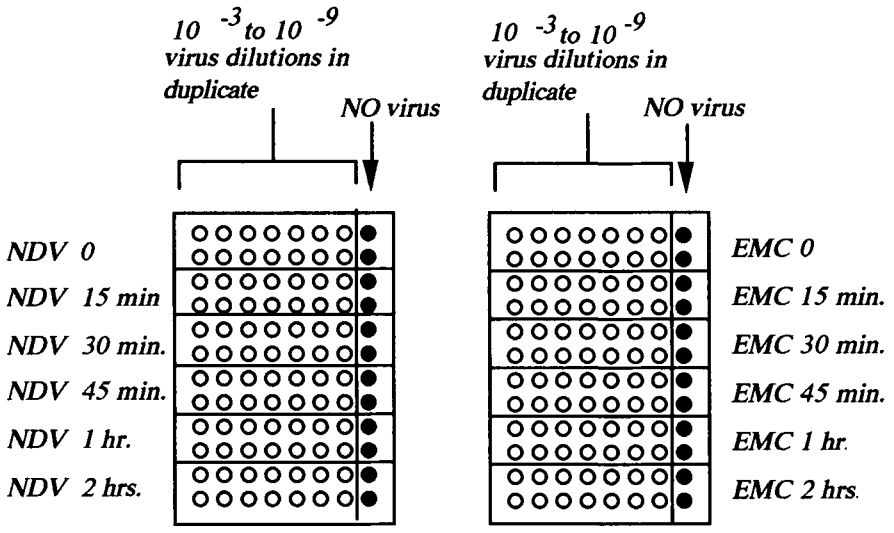


**Figure 23.1** Diagram of the $TCID_{50}$ titration of the samples.

## DISCUSSION QUESTIONS

1. Under what circumstances would one be interested in obtaining a thermal inactivation curve at lower temperatures (ie: 25-27°C or 4°C)?
2. Under what circumstances would one be interested in obtaining such a curve at temperatures higher than 56°C?
3. What is the difference between a "temperature-sensitive" and a "thermolabile" virus? Can a virus be called thermolabile but not temperature-sensitive, or vice-versa?
4. What is the effect of increasing serum content on the heat-inactivation of a virus sample?

## FURTHER READING

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Wallis, C. and J. Melnick. 1961. Stabilization of poliovirus by cations. *Texas Reports on Biology and Medicine*, 19:683-700.

Wallis, C., C. Yang, and J. L. Melnick. 1962. Effects of cations on thermal inactivation of Vaccinia, Herpes simplex, and Adenoviruses. *J. Immunol.*, 89:41-46.

# 24

# SENSITIVITY TO LIPID SOLVENTS

## INTRODUCTION

Enveloped viruses are usually susceptible to the action of lipid solvents such as ether or chloroform. Exposure to these solvents results in a marked decrease in virus infectivity (except for the poxviruses that respond variably to ether). Non-enveloped viruses do not exhibit such a decrease in infectivity when treated similarly.

Ether and chloroform fragment the cell membrane that envelops the virus and expose the nucleic acid and protein core. A given virus is considered sensitive if exposure to the lipid solvent results in a 1 log decrease in infectivity as compared to unexposed controls.

In the following experiment EMC virus and NDV are exposed to chloroform for 10 minutes at room temperature and titrated. Any infectious assay (ie: plaque or $TCID_{50}$) may be used to determine the titer of these samples following their exposure to the lipid solvent.

## MATERIALS AND PREPARATION

### Exposure to lipid solvent

- 2 ml each of EMC virus and NDV/person
- chloroform ($CHCl_3$), reagent grade (1.5 ml/person)
- HBSS (1.5 ml/person)
- sterile tightly capped glass tubes for samples (8/person)
- test tube racks
- sterile pasteur pipets and bulbs
- sterile pipets and decontamination pans
- gloves
- glass jar for disposal of chloroform

**NOTE**: *Chloroform is flammable, volatile, a skin irritant, and a suspected carcinogen.* Use glass pipets, wear gloves, and avoid breathing the fumes. Dispose of the chloroform in the designated labeled glass jar. Chloroform must NOT go into the decontamination pan.

### $TCID_{50}$ titration of samples

- 1 96-well plate/person (sterile, flat-bottom)
- BHK-21 cells (1 confluent 75 $cm^2$ flask/person)
- rubber policeman
- E-MEM 5% TPB 2% FBS
- hemacytometer
- nonsterile tubes for cell counts
- Pasteur pipets & HBSS for cell counts
- small sterile flask or tube to prepare cell suspension (1/person)
- sterile dilution tubes
- virus samples
- sterile pipets
- decontamination pans
- gloves

## EXPERIMENTAL PROTOCOL

1. Add 0.5 ml of chloroform to 1.0 ml of virus. Do this for EMC and NDV. These are the treated tubes.
2. Add 0.5 ml of chloroform to a tube containing 1 ml HBSS. This will be the choroform control.
3. Add 0.5 ml of HBSS to the other 1 ml of EMC and NDV. These are the control tubes. See Figure 24.1.

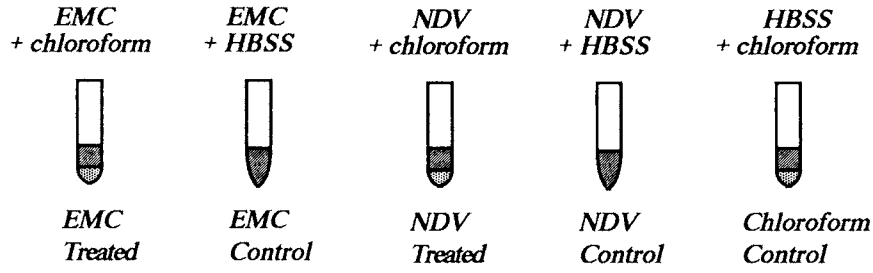


**Figure 24.1** Schematic representation of steps 1, 2 ,& 3 of Experimental Protocol.

4. Cap the tubes and shake manually for 10 minutes.
5. Centrifuge at 1000 x g for 5 minutes.

6. Remove the uppermost clear phase with a sterile pasteur pipet and transfer it to another sterile tube. (The bottom phase contains the chloroform and the middle layer is interphase). There are no such phases in the control tubes with HBSS. The entire contents of the control tubes should be transferred to the sterile tubes after centrifugation. (Remember that there is a 1:3 dilution of the virus in the control tubes when these samples are titrated.)
7. Titrate the samples using a plaque or $TCID_{50}$ assay (fig. 24.2) Determine the sensitivity of the viruses to chloroform.The titer has to drop at least 1 log, as compared to the untreated control, for a given virus to be considered susceptible.

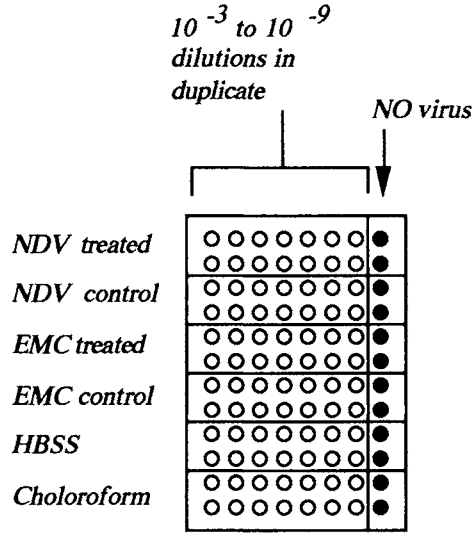


**Figure 24.2** $TCID_{50}$ titration.

8. Dispose of the chloroform in the designated glass jar labeled "waste chloroform". The instructor will dispose of the collective waste chloroform according to the institutional guidelines for the disposal of organic wastes. Place all other contaminated materials in the decontamination pans.

## DISCUSSION QUESTIONS

1. Why does removal of the host-derived envelope destroy viral infectivity?
2. Why is it necessary to separate the aqueous phase from the chloroform phase in steps #4 and 5?

3. Knowing that EMC is non-enveloped while NDV is enveloped, what results would you expect? Does your data agree with your prediction?
4. After centrifugation in which phase are the "de-enveloped" virus particles? Why?

## FURTHER READING

Andrewes, C. and D. Horstmann.1949. The susceptibility of viruses to ethyl ether. *J. Gen. Microbiol.*, 3:290-297.

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# 25

# SENSITIVITY TO LOW pH

## INTRODUCTION

Viruses vary in their stability to low pH. As a general rule, enteric viruses are stable to low pH since they are able to withstand the acidic conditions in the gastrointestinal tract.

Stability to low pH will be determined by exposing the virus samples to pH 3.0 for 60 minutes. A decrease of 1 log or more in infectivity will be considered indicative of sensitivity to low pH. A retention of activity or of any decrease less than 1 log will be indicative of stability.

In the following experiment, EMC virus and NDV are exposed to pH 3.0 conditions for 60 minutes to determine their sensitivity to low pH. Upon exposure the samples are titrated in a suitable indicator system to determine sensitivity to low pH

## MATERIALS AND PREPARATION

### Exposure to low pH

- NDV & EMC (0.3 ml of each virus/person)
- sterile Glycine-HCl buffer, pH 3.0 (3 ml/person)
- sterile HBSS adjusted to pH 7.2 with $NaHCO_3$ (3 ml/person)
- sterile test tubes (8/person)
- test tube racks
- sterile pipets
- decontamination pans

### $TCID_{50}$ titration of samples

- 1 96-well plate/person (sterile, flat-bottom)
- BHK-21 cells (1 confluent 75 $cm^2$ flask/person)
- rubber policeman
- E-MEM 5% TPB 2% FBS
- hemacytometer
- nonsterile tubes for cell counts
- pasteur pipets & HBSS for cell counts

- small sterile flask or tube to prepare cell suspension (1/person)
- sterile dilution tubes and sterile pipets
- virus samples
- decontamination pans and disposable gloves

## EXPERIMENTAL PROTOCOL

1. Add 0.9 ml of pH 3.0 buffer to each of 2 sterile tubes, and 0.9 ml of the pH 7.2 buffer to each of the other 2 sterile tubes.
2. Add 0.1 ml of EMC virus to one tube with pH 3.0 buffer and to one tube with pH 7.2 buffer. Do the same for NDV. See figure 25.1. This step results in a 1:10 dilution of the virus sample.

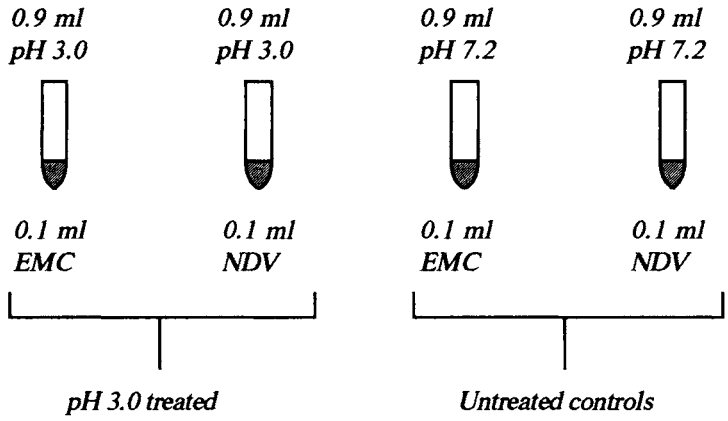


**Figure 25.1** Diagram of the virus treatment protocol.

3. Gently mix the contents of the tubes and incubate the virus suspensions at room temperature for 60 minutes.
4. Titrate the samples using a $TCID_{50}$ assay (see figure 25.2). Refer to chapter 12 for experimental protocol. Remember that the virus samples were already diluted 1:10 in step #2 above. Dilute the buffer controls in medium as you would a virus sample. These controls are very important since they can indicate a possible toxic effect of the buffer on the cells. A drop of 1 log of activity in the treated virus sample, as compared to the untreated control, is indicative of low pH sensitivity.
5. Dispose of all contaminated materials in the decontamination pans.

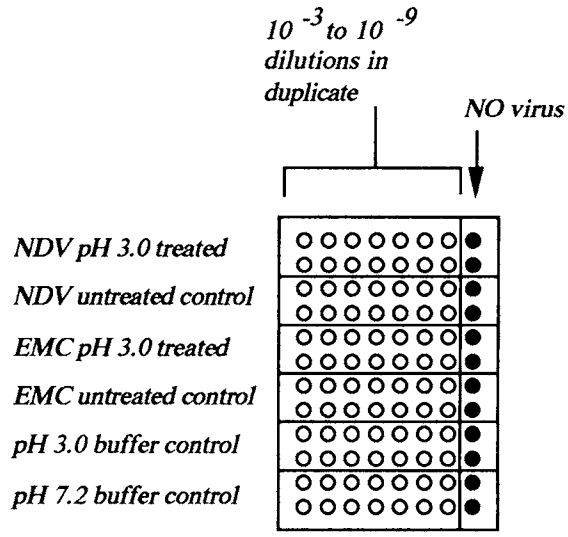


**Figure 25.2** Diagram of the $TCID_{50}$ titration.

## DISCUSSION QUESTIONS

1. Why would it be important for enteric viruses to be stable to low pH ?
2. What is the purpose of the dilution performed after the 60 minute exposure to the low pH ?
3. Would you expect EMC or NDV to be pH sensitive? Justify your answer.
4. What is a possible mechanism of pH-mediated inactivation?

## FURTHER READING

Ketler, A., V. Hamparian, and M. Hilleman.1962. Characterization and classification of ECHO 28-rhinovirus-coryzavirus agents. *Proc. Soc. Exptl. Biol. Med.*, 110:821-831.

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# 26

# INTRODUCTION TO TECHNIQUES FOR DETECTION OF VIRAL ANTIBODY

## INTRODUCTION

There are numerous serological techniques used to diagnose viral infections. These techniques may be used to accurately identify an unknown virus isolate utilizing a known reference antibody, or may utilize a panel of known viral antigens in order to test for viral antibody in a patient suspected of having a disease of viral etiology. Testing for antibody in a patient's serum requires testing of both acute and convalescent serum samples. An acute serum sample is obtained at the onset of disease and convalescent serum samples are obtained 2 to 3 weeks later. A fourfold or greater increase in antibody titer is required for diagnosis of infection. These assays can be used to determine the effectiveness of viral vaccines by quantifying the specific antibody response following administration of the vaccine and the booster vaccination. The World Health Organization uses these techniques to monitor the incidence of influenza virus throughout the world. The hemagglutination-inhibition antibody assay is useful to study the antigenic variation occurring constantly with the influenza viruses.

Diagnostic virology utilizes the following techniques, either singly or in combination, in order to accurately identify a viral isolate, determine the antigenic relationship of different viral isolates, and to identify and quantify viral antibody: *virus neutralization* (Ch. 27), *hemagglutination-inhibition* (Ch. 28), *immunofluorescence* (Ch. 29), *enzyme-linked immunosorbent assay* (ELISA) (Ch. 30), *complement fixation, immunoelectron microscopy, immunodiffusion, counter-immuno-electrophoresis, radioimmunoassay* (RIA), *immuno-peroxidase staining, single radial immunodiffusion, hemolysis in gel* and *immune adherence hemagglutination.*. Each of the above assays has advantages and disadvantages. Properties to be considered before selecting one or a combination of assays include: sensitivity, specificity, simplicity, expense and time required.

The following chapters (27-30) will utilize four different methodologies to demonstrate antibody to EMC virus. These protocols will all utilize rabbit antibody to EMC virus in order that titers and results using the different methods may be compared. However, these methods may also be used to demonstrate the presence of antibody in the serum of surviving mice from the EMC $LD_{50}$ experiment in Chapter 13. However, due to the variable titers obtained in mice from

this experiment, it is recommended that antibody obtained by immunization of rabbits be compared in this Section.

## FURTHER READING

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# 27

# ENCEPHALOMYOCARDITIS VIRUS NEUTRALIZATION

## INTRODUCTION

Viral neutralization may be performed for virus identification, to determine the antigenic relationship of different viral isolates, and the identification and quantification of viral antibody to virus. Known reference antiserum is necessary for virus identification and known infectious virus is necessary for identification and quantification of viral antibody. Neutralization assays are based on the principle that viral antibody will bind specifically to the virus with a resultant neutralization of viral infectivity. Virus neutralization tests may utilize any assay that measures viral infectivity. These *in vitro* assays may include inhibition of plaque formation, cytopathic effect (CPE), or hemadsorption, while *in vivo* assays may utilize incubation of specific antiserum with infectious virus prior to challenge of test animals (or by administration of antiserum following virus infection). Requirements for viral neutralization vary with different viruses. These requirements include a suitable *in vitro* assay system consisting of susceptible indicator cells for plaque formation, CPE, or hemadsorption (for those viruses capable of interacting with red blood cells). The viral neutralization assay is sensitive and specific, but also more complex, time consuming and expensive than many other assays. Neutralization of viral infectivity correlates with protection from disease and for many applications, an assay of neutralization of infectivity is appropriate.

## MATERIALS AND PREPARATION

- E-MEM 10% FBS
- E-MEM 2% FBS
- GLB: Gelatin (5 g/L) and lactalbumin hydrolysate (2.5 g/L) in HBSS with penicillin (100 units/ml), streptomycin (100 µg/ml) and sodium bicarbonate
- Trypsin-EDTA (1X): Gibco #610-5300, 0.05% trypsin and 0.02% EDTA
- trypan blue stain (0.4%): Gibco # 630-5250
- HBSS with calcium and magnesium

- HBSS without calcium and magnesium
- $L_{929}$ cells
- Tissue culture cluster 12 (Costar): 12-well plates (6 per person).
- Tissue culture cluster 24 (Costar): 24-well plate (1 per person)
- EMC virus
- antibody to EMC virus (see Appendix)
- normal serum control
- methyl cellulose overlay medium (290-300 ml per person)
- formalin: 10% in phosphate buffered saline (290-300 ml per person)
- crystal violet: 1% in 70% methanol (145-150 ml per person)
- Biohazard Hood: virus assay conducted under biohazard hood

## EXPERIMENTAL PROTOCOL

### Day1

1. Wash $L_{929}$ cell monolayers using HBSS without calcium and magnesium; add trypsin-EDTA (1X) to stock cell monolayer; remove after 30 seconds and add HBSS with calcium and magnesium.
2. Prepare a single cell suspension of $L_{929}$ cells in E-MEM 10% FBS and determine the viable cell count using trypan blue.
3. Prepare 38 ml of a 5.0 x $10^5$ cells/ml suspension in E-MEM 10% FBS. Dispense 0.5 ml of the prepared cell suspension into each well of the (6) 12-well plates to be used for the plaque assay. Add 0.5 ml E-MEM 10% FBS to all wells for a final volume of 1 ml. Sequentially label both the top and bottom of all plates in case lids get separated from the wells during the staining procedure.
4. Incubate cells at 37°C in 5% $CO_2$ until a confluent monolayer is obtained (24 hours).

### Day 2

5. Prepare a dilution of EMC virus in E-MEM 2% FBS that will yield plaque counts easily quantified (20 per well of a 12-well plate, 20 pfu/100 µl).
6. Heat serum sample containing antibody to EMC virus, and the normal serum control at 56°C for 30 minutes.

7. Prepare 1 ml of a 1:50 dilution of the antibody-containing serum in HBSS, and 1 ml of a 1:50 dilution of the normal serum in HBSS.
8. Add 500 µl of HBSS to each well of a 24-well plate. Add 500 µl of the 1:50 dilution of the antibody-containing serum to the first well (this makes it a 1:100 dilution). Using a 500 µl pipetor, perform serial 2-fold dilutions through a 1:102400 dilution. Discard the last 500 µl into the decontamination pan. Repeat this procedure for the normal serum sample. The virus control (VC) wells receive 500 µl of HBSS (NO serum). See figure 27.1.

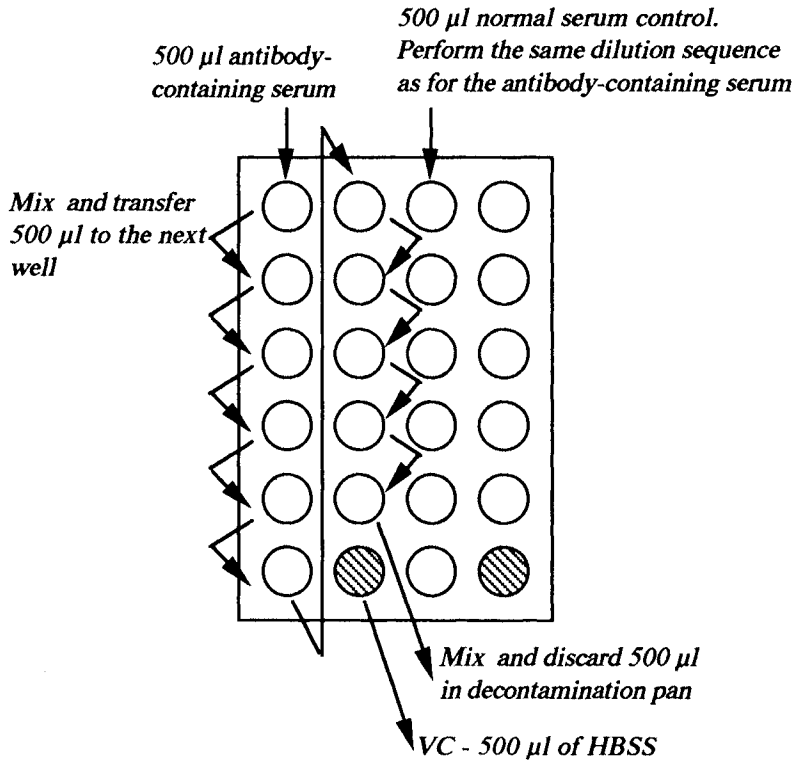


**Figure 27.1** Illustration of step #8 of the experimental protocol.

9. Add 500 µl of the prepared EMC virus suspension to each well of the 24-well plate.
10. Gently mix and incubate all samples for 1 hr at 25°C.
11. Decant the medium from the cell monolayers.
12. Transfer 200 µl antibody/virus (serum/virus) of each dilution, in triplicate, to the cell monolayers. See figure 27.2.
13. Add 200 µl virus control to each of the virus control wells.

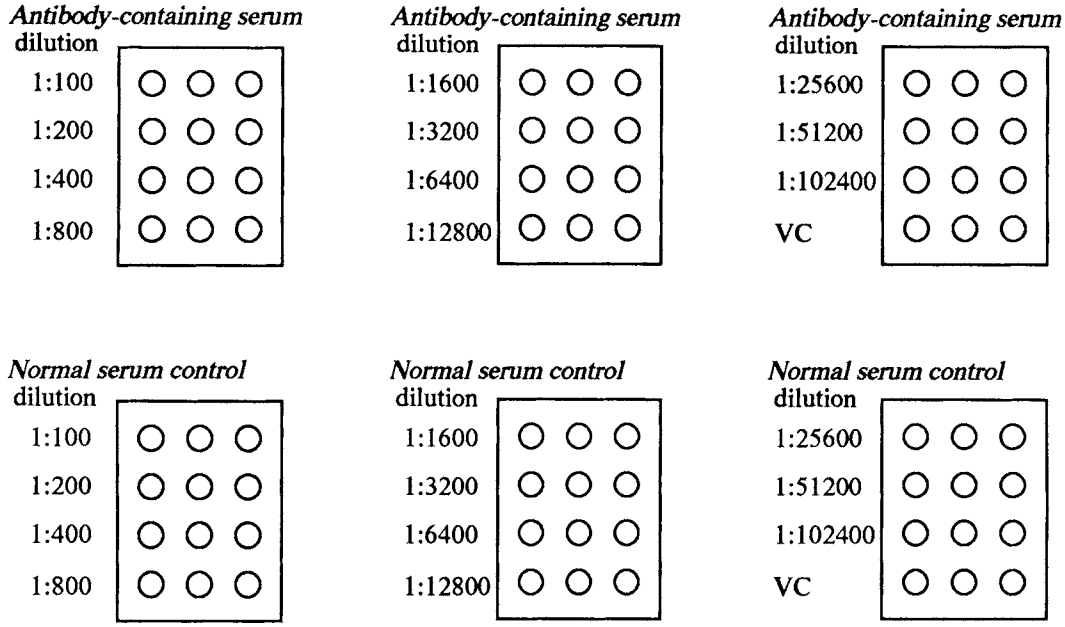


**Figure 27.2** Diagram of the experimental set-up of the plaque assay.

14. Adsorb the virus for 1 hr at 37°C. Tilt the plates every 10 minutes.
15. Wash cells in 1 ml HBSS (with $Ca^{++}$ and $Mg^{++}$).
16. Pour off HBSS, add 2.0 ml methyl cellulose overlay medium per well, and incubate at 37°C in 5% $CO_2$ for 20-22 hours.

### Day 3

17. Remove the overlay medium and discard into a decontamination pan. Add 2.0 ml formalin per well for 30 minutes to fix cells.
18. Remove formalin and add 1.0 ml crystal violet (1% in 70% methanol) for 30 minutes to stain cell monolayer.
19. Wash cell monolayer under tap water until all excess stain is removed and invert on absorbent towels to air dry.
20. Count and record plaque numbers.

## Interpretation of data

Positive neutralization is determined as the highest antibody dilution which demonstrates > 80% inhibition of plaque formation[1].

## DISCUSSION QUESTIONS

1. Discuss mechanisms by which antibody neutralizes virus infectivity.
2. Discuss cases where viral antibody is positive in one assay but negative for neutralization of viral infectivity.
3. Discuss how antibody directed against the hemagglutinin and the neuraminidase of influenza viruses may provide clues to the functions of these glycoproteins.

## FURTHER READING

[1]Hsiung, G.D. 1973. *Diagnostic Virology - An Illustrated Handbook.* Yale University Press, New Haven and London. page 22.

# 28

# HEMAGGLUTINATION-INHIBITION ASSAY

## INTRODUCTION

The hemagglutination-inhibition (HI) test may be used for virus identification, to determine the antigenic relationship of different virus isolates, and for the identification and quantification of viral antibody to virus. This assay is used to monitor the antigenic variation of influenza A viruses. One requirement is that the viral antigen must be able to agglutinate erythrocytes or red blood cells in a process known as *hemagglutination.* Known reference antiserum is necessary for virus identification and known viral antigen is necessary for identification and quantification of viral antibody to virus. Genera or species within the following families of viruses are capable of hemagglutination: adenoviridae, bunyaviridae, coronaviridae, flaviviridae, orthomyxoviridae, paramyxoviridae, parvoviridae, picornaviridae, poxviridae, reoviridae, rhabdoviridae, and togaviridae. Requirements for hemagglutination vary with different viruses. These requirements include species of erythrocyte, pH of diluent, and the temperature of incubation. Hemagglutination is caused by virus-coded protein peplomers located on the outer surface of the virus. Hirst in 1941 observed the agglutination of red blood cells from a torn blood vessel of a chicken embryo while passaging influenza virus in the allantoic fluid. Hirst recognized the importance of this serendipitous discovery and described hemagglutination as a method of virus assay. The HI assay is based on the binding of antibody to the viral hemagglutinin with a resultant inhibition of viral hemagglutination. The HI assay is sensitive, specific, simple, inexpensive, rapid and therefore widely used for virus identification as well as for identification and quantification of viral antibody to virus.

## MATERIALS AND PREPARATION

- Sheep red blood cells (SRBC)
- viral antigen: EMC virus
- antibody against EMC virus
- Alsever solution: Gibco #670-5190
- 96-well microtiter plates (U bottom): Falcon #3911
- saline solution 0.85%: Fisher #SS442-10

- Titertek pipetor: Flow Laboratories
- Potassium periodate 0.01 M (255 mg per 100 ml saline): Sigma #P-0517
- 0.6% glycerol in saline: Sigma #G-9012
- EMC-HA buffer (see Appendix)

## EXPERIMENTAL PROTOCOL

### Sheep Red Blood Cells

1. Wash blood three times in Alsever solution by centrifugation at 300 x g for 10 minutes.
2. Prepare 0.5% SRBC v/v in EMC-HA buffer by making a 1:100 dilution (1.0 ml packed RBC plus 99.0 ml saline) followed by a 1:2 dilution of this suspension. The final volume of 0.5% SRBC suspension to be made depends upon the number of assays to be performed.

**NOTE:** Although blood products are screened for a number of disease agents, they should be handled as if they contained pathogenic agents. When performing this assay, be sure to wear plastic gloves, place all used materials in the decontamination pan, and promptly clean any spills with disinfectant.

### Titration of Hemagglutinin Antigen (HA)

1. Mark 2 vertical rows of the round-bottom microtiter plate.
2. Add 50 µl EMC-HA buffer to wells 2 through 12 in vertical row A. Do not put any EMC-HA buffer in well A-1. Add 50 µl EMC-HA buffer to *all* the RBC control wells (vertical row B).
3. Perform a 1:10 dilution of the viral antigen in EMC-HA buffer. Add 100 µl of the diluted viral antigen to well A-1.
4. Prepare serial, twofold dilutions of viral antigen by adding 50 µl antigen from well 1 to well 2, mixing with Titertek pipetor, and repeating this process through well 12. Discard the last 50 µl in the decontamination pan.
5. Add 50 µl washed, well-suspended, 0.5% SRBC to each well of the dilution series and to the RBC control wells.
6. Mix by manually.

8. The last well with complete hemagglutination is the end point of the titration. This dilution contains one hemagglutinin antigen (HA) unit/50µl.
9. Determine the dilution of antigen which will contain 4 HA units/25 µl by dividing the end-point dilution by 8. This dilution will be used in the hemagglutination-inhibition (HI) test.

   For example:
   End point dilution of antigen titration = 2560
   Dividing by 8: 2560/8 = 320.

   Therefore, a 1:320 dilution of the original antigen will contain 8 HA units per 50 µl or 4 HA units per 25 µl.
10. Store the diluted test antigen at 4°C.

## Back Titration of EMC Hemagglutinin Antigen (HA) for the Hemagglutination-Inhibition (HI) Test (Antigen Control)

1. Add 50 µl EMC-HA buffer to wells 1 through 5 (vertical) and in SRBC control wells.
2. Add 50 µl of diluted test antigen (containing 4 HA units/25 ul) to well 1.
3. Prepare a twofold dilution series in vertical row. Start by transferring 50 µl solution from well 1 to 2 and continue through row 5. Discard the last 50 µl in the decontamination pan.
4. Add 50 µl 0.5% SRBC to each well in the dilution series and to the SRBC control wells.
5. Mix by manual agitation.
6. Cover and incubate at room temperature for 1 hour.
7. If the proper dilution of "test antigen" has been made, wells 1 through 3 will show complete hemagglutination and wells 4 and 5 will have partial or no hemagglutination.

### Adjustments

If complete hemagglutination is found in well 2 but not in well 3, two-fold more antigen is required. Perform a 1:160 dilution instead of a 1:320 dilution (see step #9).

If complete hemagglutination is present in well 4 but not well 5, two-fold less antigen is required. Perform a 1:640 dilution instead of a 1:320 dilution (see step # 9).

## Treatment of Serum for Destruction of Nonspecific Inhibitors

Sera from many animal species contain nonspecific inhibitors and/or nonspecific agglutinins. These inhibitors can lead to erroneous HI results and must be destroyed prior to performing the HI test.

1. Samples should first be heat inactivated at 56°C for 30 minutes.
2. Additional inactivation is accomplished as follows:

   a. Add 100 µl sample and 300 µl potassium periodate
   b. Vortex gently
   c. Incubate at room temperature for 15 minutes
   d. Add 600 µl 0.6% glycerol
   e. Mix gently

   The resultant solution is a 1:10 dilution of the original sample.

## Hemagglutination Inhibition (HI) Test

1. Perform HA and Back-Titrations of all viruses to be tested.
2. Add 25 µl EMC-HA buffer to wells 2 through 12.
3 Add 50 µl antiserum (or sample) to well 1.
4. Prepare serial twofold dilutions (using 25 µl antisera) through well 12.
5. Add 25 µl antigen (4 HA units/25 ul) to wells 1 through 12.
6. Mix plate by manually.
8. Cover and incubate for 1 hour at 25°C.
9. Add 50 µl washed 0.5% SRBC to each well.
10. Prepare Controls:
    a. *Serum control*: to test for agglutinins: 25 µl of the lowest dilution of serum + 25 µl EMC-HA buffer + 50 µl 0.5% SRBC
    b. *SRBC control*: 50 µl EMC-HA buffer + 50 µl 0.5% SRBC
    c. *Antigen control*: 50 µl antigen + 50 µl 0.5% SRBC
11. Incubate for 1 hour at 25° C.

## Interpretation

1. The RBC and serum controls should show a negative pattern.
2. The antigen control should be positive for hemagglutination.

3. The Sample antibody titers are determined as the inverse of the dilution at which no hemagglutination is detected, that is, where hemagglutination inhibition has occurred.

## DISCUSSION QUESTIONS

1. Why is the HI assay important in monitoring epidemic strains of influenza virus ?
2. Discuss the limitations of the HI assay in monitoring epidemic strains of influenza virus.

## FURTHER READING

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# 29

# IMMUNOFLUORESCENT STAINING OF EMC VIRUS-INFECTED CELLS

## INTRODUCTION

Immunofluorescent staining is one of the simplest, highly specific and most rapid means of identifying an unknown virus isolate. Albert H. Coons and colleagues established the principle of fluorescent antibody techniques and the application of these techniques that are now so widely used in numerous fields of medical research. The studies by Coons et al. utilized the specific reactivity of labeled antibody to locate and identify antigen *in situ.* The technique of immunofluorescence is the simplest means of identifying unknown virus isolates. Virus isolates are used to infect a cell monolayer. The infected cell monolayer is then stained by either the direct or the indirect immunofluorescent method.

Direct immunofluorescent staining is most often used for demonstrating virus in clinical materials. This technique utilizes an unlabeled antigen (such as a clinical specimen) stained with a fluorescent-labeled (such a fluorescein isothiocyanate - FITC) viral antibody to yield a fluorescent-labeled product. Direct immunofluorescent staining requires a labeled specific antibody for each virus to be identified. This method is more specific and has less background staining than the indirect method.

Indirect immunofluorescent staining requires an additional step. Unlabeled antigen is incubated with an unlabeled antibody (primary antibody) to yield an unlabeled product that is then incubated with a labeled anti-globulin (secondary antibody) to yield a labeled product. The indirect method is more sensitive, requires labeled antibody only against certain species (the species of the primary antibody), but less specific than direct immunofluorescent staining.

## MATERIALS AND PREPARATION

- E-MEM 10% FBS
- E-MEM 2% FBS
- GLB:Gelatin (5 g/L) and lactalbumin hydrolysate (2.5 g/L) in Hanks' balanced salt solution supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and sodium bicarbonate

- Trypsin-EDTA (1X): Gibco #610-5300, 0.05% trypsin and 0.02% EDTA
- trypan blue stain (0.4%): Gibco # 630-5250
- HBSS
- HBSS without calcium and magnesium
- 8-Well Chamber Slides (Labtek, Cat # 4808)
- PBS-NRS (Phosphate buffered saline, 2% Normal Rabbit serum, 0.01% $NaN_3$)
- rabbit antibody to EMC virus
- $L_{929}$ cells
- Fluorescein-labeled goat anti-rabbit antibody (Sigma # F 9887))
- Mounting solution: 9:1 saline:glycerol
- Pasteur pipets

## EXPERIMENTAL PROTOCOL

1. Transfer 5 x $10^4$ $L_{929}$ cells in 0.1 ml E-MEM 10%FBS into each well of an 8-well chamber slide and incubate for 24 hr at 37°C in 5% $CO_2$ .
2. Decant the medium. Add 0.1 ml EMC virus diluted 1:1000 in GLB and incubate for 1 hr at 37°C in 5% $CO_2$. Tilt the slides every 10 min assuring distribution of the virus and fluid over the cells. Prepare mock-infected controls by adding GLB to cell controls.

   Note: Optimal MOI and therefore dilution of virus must be determined for each cell line used.
3. Remove non-adsorbed virus by gently applying HBSS using a Pasteur pipet. It is advisable to add the HBSS down the sides of the plastic wells rather than directly to the cells.
4. Incubate at 37°C in 5% $CO_2$ for 8, 12, 16 and 20 hours.
5. Gently remove the medium from the wells and wash cells 3 times with HBSS using a Pasteur pipet.
6. Preadsorb the cells with PBS-NRS for 30 minutes at 4°C.
7. Gently remove the medium from the wells using a Pasteur pipet.
8. Add 0.1 ml of EMC virus antibody and incubate for 45 minutes at 4°C. Tilt the slides every 10 minutes for adequate interaction of antibody with the virus-infected cells.
9. Wash the cells 3 times with cold PBS-NRS.

10. Add 0.1 ml fluorescein-labeled antibody and incubate for 45 minutes at 4°C.
11. Wash the cells 5x with cold PBS-NRS.
12. Allow the slides to air dry under a stream of warm air.
13. Add mounting solution, cover slip, and examine microscopically.

## DISCUSSION QUESTIONS

1. Discuss the advantages and disadvantages of immunofluorescence as a diagnostic tool in the clinical laboratory.
2. Was the protocol in this chapter direct or indirect immunofluorescent staining? How could you change that ?

## FURTHER READING

Coons, A.H., H.J. Creech and R.N. Jones. 1941. Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* 47:200-202.

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# 30

# ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

## INTRODUCTION

Antibody specific for a specific antigen (antigen-antibody interactions) may be detected by many diverse types of immunological assays. These assays include modifications to either identify an unknown antibody with a known antigen or an unknown antigen with a known antibody. Among these assays are included assays that utilize an antibody conjugated to a fluorochrome, an isotope, or an enzyme. Attachment of a fluorochrome (such as fluorescein isothiocyanate) to a primary antibody may be used in a direct method to identify an antigen of interest. An indirect method may be used by incubating the primary antibody with the antigen in question followed by identification with a fluoresceinated anti-immunoglobulin (directed against the species of the primary antibody - i.e., anti-mouse immuno-globulin, see Ch. 29). Fluoresceinated antibody may also be used in conjunction with a flow cytometer to accurately and rapidly identify cells bearing antigens recognized by the labeled antibody. This technique may be extended to separate desired populations of cells bearing these antigens. Live stained cells may be passed through a fluorescence activated cell sorter (FACS) that quantifies the fluorescence intensity of each cell and sorts the cells according to the differential fluorescence.

Antibody may be conjugated to an isotope in similar fashion as the conjugation to fluorochromes. The Nobel prize was awarded to Rosalyn Yalow in 1977 for her pioneering work with Solomon Berson in discovering the basis of the radioimmunoassay (RIA). This research detected extremely small amounts of hormone. The RIA has been used to detect minute quantities of many different biological substances (antigens) and less often used to detect antibodies. This technique has been extended to include the radio-allergosorbent test (RAST) to detect antigen-specific IgE in a RIA when the ligand is a labelled anti-IgE antibody and the radio-immunosorbent test (RIST) which is a competetion RIA for total serum IgE.

Antibody may also be conjugated to an enzyme and utilized to detect either antigen or antibody in an enzyme-linked immunosorbent assay (ELISA). The ELISA assay was developed independently in 1971 by Engvall and Perlmann and by van Weeman and Schuurs. The ELISA assay is highly sensitive and accurate. This assay most often uses alkaline phosphatase, horseradish peroxidase or ß-galactosidase

conjugated to an antibody (or an antigen) that when supplied with the proper colorless chromogenic substrate results in a strongly colored compound after degradation and thus a very sensitive detection system. Commonly used substrates include p-nitrophenyl phosphate for alkaline phosphatase and o-phenylenediamine for peroxidase conjugates.

Numerous variations of ELISA have been used to successfully detect antigen or antibody. The sensitivity of immuno-assays has been enhanced by utilizing the proteins avidin and biotin. Avidin has a molecular weight of 68,000 and is a tetraner consisting of four identical subunits. Avidin has a high affinity to the vitamin biotin. Biotin reacts with avidin to form a bond that is approximately a million times stronger than most antigen-antibody complexes. Thus, an amplified more sensitive immunoassay results from the utilization of avidin (or streptavidin) and biotin.

The ELISA immunoassay then consists of antigen attached to a solid support such as a 96-well plate. Nonspecific binding is prevented by addition of a blocking buffer followed by the addition of primary antibody. A linking "bridge" antibody that is highly biotinylated and directed against the primary antibody is then added and functions as the secondary antibody. A labeling enzyme such as horseradish peroxidase conjugated directly to avidin or streptavidin is added and binds to the biotin of the secondary antibody. The colored product then occurs after the addition of substrate.

ELISA immunoassays are therefore performed in various configurations to detect either antigens or antibodies and may be configured to suit the needs and sensitivity of a particular situation.

## MATERIALS AND PREPARATION

- STE buffer
- Sucrose (25% in STE buffer)
- Coating carbonate-bicarbonate buffer
- PBS-Tween 20 (0.05%), pH 7.4
- 1% BSA
- 2.5 M $H_2SO_4$
- Substrate (ortho-phenylenediamine)
- Reaction stopping solution (see Appendix)
- Positive Reference Serum (rabbit antibody to EMC, see Appendix)
- Negative Reference Serum (normal rabbit serum)
- Horseradish peroxidase (HRP) - conjugated goat anti-rabbit IgG (Zymed #62-6122; Sigma 9169)

- Immulon II plate, Dynatech Laboratories, Inc., also distributed by Fisher Scientific.
- ELISA spectrophotometer
- Biohazard Hood

## EXPERIMENTAL PROTOCOL

The following protocol will allow ELISA assay for rabbit antibody to EMC virus. The protocol may be altered to detect mouse antibody or human antibody to EMC virus by substituting HRP-goat anti-mouse IgG or HRP-goat anti-human IgG in the following method.

### Preparation of EMC Virus Antigen

1. Grow BHK-21 cells to confluency in 150 $cm^2$ cell culture flasks. Dilute EMC virus in GLB and add 1.0 ml of a $10^{-3}$ dilution to half of the flasks. Add 1.0 ml of GLB to control flasks to prepare control antigen. Adsorb the virus for 1 hour at 37°C. Remove virus and add 15 ml E-MEM-5% TPB 2% FBS per flask and incubate at 37°C for 24 hours in 5% $CO_2$. See Chapter 9.
2. Pool the supernatant fluids from EMC virus infected and mock infected cell cultures and centrifuge at 600 x g for 15 minutes to remove cell debris. Pooled supernatant fluids constitute viral antigen and control antigen.
3. Concentrate virus by sedimentation onto a sucrose cushion: Add 5 ml 25% sucrose per tube and then fill the tube with pooled supernatant fluid. Centrifuge at 100,000 x g at 4°C for 120 minutes.
4. Suspend the concentrated viral antigen and the mock-infected control antigen in 0.5 ml carbonate-bicarbonate buffer and pool corresponding samples.
5. Determine hemagglutination titer and plaque titer as described in Chapters 16 and 18.
6. Aliquot in 0. 5 ml amounts and store at - 70°C.

### Titration of Viral Antigen

Each new lot of viral antigen must be titrated to determine the optimum concentration of antigen to be used in ELISA assays. This is determined by checkerboard titration of dilutions of the viral antigen run against positive and negative laboratory reference sera. Dilutions of viral antigen and control antigen from 1:50 through 1:1000 are

measured vertically against positive and negative reference sera. The highest dilution of antigen that gives a reading greater than 1.0 with the positive reference serum and a reading of less than 0.2 with the negative reference serum is chosen for use in subsequent ELISA assays.

1. Viral antigen is coated onto 96-well plates by passive adsorption Coat wells of a 96-well Immulon II plate by adding 100 µl EMC antigen diluted in coating buffer per well. Coat overnight at 4°C or 2 hrs at 20°C.
2. Remove excess viral antigen into decontamination pans and wash 3 times with PBS-Tween 20 (0.05%).
3. Block with 100 µl 1% BSA per well; incubate for 2 hr at room temperature.
4. Wash 3 times with PBS-Tween 20 (0.05%).
5. Add 100 µl of an optimum dilution of positive and negative reference sera and incubate 1 hr at room temperature.
6. Wash 3 times with PBS-Tween 20 (0.05%). Use 5 min intervals between washes.
7. Make dilutions (1:1000 through 1:128,000) of secondary antibody HRP conjugate in 1% PBS. Add 100 µl of each dilution to the appropriate wells.
8. Incubate secondary antibody HRP conjugate dilutions for 1 hr at room temperature.
9. Wash with PBS-Tween 20 (0.05%) three times. Use 5 minutes intervals between washes.
10. Incubate with 100 µl substrate solution for 30 minutes IN THE DARK at room temperature. Stop incubation with 50 µl 2.5 M $H_2SO_4$.
11. Read results at 492 nm on an ELISA spectrophotometer.
12. Plot OD vs. amount of viral antigen. Choose an amount of viral antigen in the plateau region of the curve.

## Titration of HRP-Conjugated Goat Anti-Rabbit IgG

Each new lot number of HRP-conjugated goat anti-rabbit IgG must be titrated before use in ELISA assays. This is determined by titration of the HRP-conjugated secondary antibody against optimum antigen (as determined in step #12 above) and the reference positive serum.

1. Coat each well of a 96-well Immulon II plate with 100 µl optimum viral antigen diluted in coating buffer as determined above. Incubate overnight at 4°C or 2 hrs at 20°C.
2. Remove excess viral antigen into decontamination pans and wash 3 times with PBS-Tween 20 (0.05%).
3. Block with 100 µl 1% BSA per well; incubate for 2 hours at room temperature.
4. Wash 3 times with PBS-Tween 20 (0.05%).
5. Add 100 µl of an optimum dilution of positive reference sera and incubate 1 hr at room temperature.
6. Wash 3 times with PBS-Tween 20 (0.05%). Use 5 minutes intervals between washes.
7. Make dilutions (Choose dilution range from results obtained in the "titration of viral antigen" section) of HRP-conjugated secondary antibody in 1% PBS. Add 100 µl of each dilution to the appropriate wells.
8. Incubate HRP-conjugated dilutions secondary antibody for 1 hr at room temperature.
9. Wash with PBS-Tween 20 (0.05%) three times. Use 5 minutes intervals between washes.
10. Incubate with 100 µl substrate solution for 30 minutes IN THE DARK at room temperature. Stop incubation with 50 µl 2.5 M $H_2SO_4$.
11. Read results at 492 nm on an ELISA spectrophotometer.
12. Plot OD vs.amount of HRP-conjugated secondary antibody and choose a dilution of the secondary antibody that gives a response in the plateau region of the curve.

## ELISA Determination of Antibody

1. Viral antigen is coated onto 96-well plates by passive adsorption. Coat wells of a 96-well Immulon II plate by adding 100 µl EMC antigen diluted in coating buffer per well. Coat overnight at 4°C or 2 hrs at 20°C.
2. Remove excess viral antigen into decontamination pans and wash 3 times with PBS-Tween 20 (0.05%).
3. Block with 100 µl 1% BSA per well; incubate for 2 hr at room temperature.
4. Wash 3 times with PBS-Tween 20 (0.05%).

5. Add 100 µl of each dilution of sample serum diluted in 1% BSA and incubate 1 hr at room temperature.
6. Wash 3 times with PBS-Tween 20 (0.05%). Use 5 minutes intervals between washes.
7. Add 100 µl of the optimum dilution of HRP-conjugated secondary antibody (as determined in step #12 of the previous section) to all wells.
8. Incubate secondary antibody HRP conjugate dilutions for 1 hour at room temperature.
9. Wash with PBS-Tween 20 (0.05%) three times. Use 5 minutes intervals between washes.
10. Incubate with 100 µl substrate solution for 30 minutes IN THE DARK at room temperature. Stop incubation with 50 µl 2.5 M $H_2SO_4$.
11. Read results at 492 nm on an ELISA spectrophotometer.

NOTE: Include both positive and negative reference control sera. Include reagent control that includes everything except sample.

## DISCUSSION QUESTIONS

1. Discuss reasons to choose a concentration of reactant that falls on the plateau region of data resulting from checkerboard titrations.
2. Discuss ways to increase O.D.readings of ELISA while using more dilute concentrations of reactants.
3. What is the purpose of the Tween 20 in the ELISA?
4. Discuss the controls required in the ELISA and the purpose of each.

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# 31

# ASSAYS OF ANTIVIRAL AGENTS AND INTERFERONS

## INTRODUCTION

Animal viruses utilize the metabolic processes of the host cell for replication and therefore many compounds effective in the inhibition of viral replication are also cytotoxic. Paul Ehrlich first stated the aim of chemotherapeutic compounds to exert antimicrobial activity without toxicity to the host. The *chemotherapeutic index* of a compound is the ratio of the minimal toxic dose to the minimal therapeutic dose. This index is high for approved antibacterials and is in general low for antiviral agents.

There are numerous potential specific sites of action for antiviral agents. Broad sites of action include the major steps in the viral replication cycle and include: attachment, penetration, uncoating, transcription, translation, replication of viral nucleic acid, maturation and release. The ideal site of antiviral action is a biochemical process unique to the replication of the virus. Viral diseases result in tremendous levels of morbidity and mortality each year. There is tremendous research efforts directed at the discovery and evaluation of antiviral agents. Efforts to discover effective antiviral agents have increased due to the epidemic of acquired immunodeficiency syndrome associated with the human immunodeficiency virus (HIV).

There are numerous *in vitro* assays to assess the effectiveness of a potential antiviral agent. These assays may quantify a reduction in the replication of a challenge virus added to an *in vitro* cell culture system. Replication of the challenge virus may be quantified by (a) measuring infectious virus produced, (b) measuring a challenge virus product such as hemagglutinin or an enzyme associated with the virus such as neuraminidase or RNA-directed DNA polymerase, or (c) measuring the resultant consequences of a virus-cell interaction. The type of virus-cell interaction measured depends both on properties of the virus and the cell culture assay system utilized. The antiviral effectiveness of a potential chemotherapeutic agent may be evaluated by looking for a decrease in any quantifiable virus-cell interaction. Examples of virus-cell interactions that may be utilized for assay of antiviral agents include:

**Cytopathic effect (CPE):** CPE is produced by lytic infection of cells by the replication of cytopathogenic viruses in which the cells are

killed. CPE may be observed microscopically in stained or unstained cell preparations.

**Inclusion bodies**: Inclusion bodies are formed in cells after infection by certain, but not all, viruses. Inclusion bodies may consist of aggregates of virions or cellular components altered as a result of the viral infection. Inclusion bodies are observed after staining and microscopic observation. Inclusion bodies are characteristic and diagnostic of certain viruses. The inclusion bodies may be intracytoplasmic or intranuclear, acidophilic or basophilic, single or multiple, or may be characterized by shape.

**Syncytia**: Certain viruses interact with cells by causing cell fusion resulting in the formation of syncytia. Syncytia are large cells containing many nuclei and are also called polykaryocytes or giant cells. Paramyxoviruses and herpesviruses interact with cells and form syncytia.

**Interference**: Interference refers to the phenomenon whereby infection of cells with one virus will be resistant to infection with the same or an unrelated virus. Viral interference was used to demonstrate the original isolation of rubella virus.

**Hemadsorption**: This virus-cell interaction is specific to a number of viruses that mature by budding from cytoplasmic membranes and that contain glycoprotein peplomers named hemagglutinin. The hemagglutinin incorporated in the cytoplasmic membrane adsorbs erythrocytes added to the cell monolayer and thus serves as an early indicator of viral infection.

**Transformation**: Certain virus families interact with cells to effect cellular transformation. Transformed cells produce tumors when injected into nude mice or into the same strain of animal from which the transformed cells were derived. Transformed cells may demonstrate new cell surface antigens, demonstrate altered growth properties, exert changes in membrane fluidity, lose contact inhibition, have the ability to grow in soft agar, and attain the ability to be passaged *in vitro* for extended periods. Transformation of cells with DNA viruses is a non-lytic or non-productive infection. Transformation of cells by RNA viruses is however usually a productive infection with the release of infectious progeny virus.

**Plaque formation**: The plaque assay was developed by Dulbecco in 1952. The plaque, representing a focal area of viral cytopathic effect, may be used to quantify the resultant effect of the interaction of one virus with one cell.

**Pock formation**: Pock formation may be used to quantify the virus-cell interaction of a poxvirus with the chorioallantoic membrane of the chicken embryo. Progeny virus spread to adjacent cells resulting in a localized lesion called a *pock*.

**Focus formation**: Localized areas of transformed cell growth may be used to measure the infectivity of certain oncogenic viruses, such as Rous sarcoma virus.

**Interferon production**: Interferon was discovered by Isaacs and Lindenmann in 1957. Interferons are proteins produced by all nucleated cells as a result of interaction with a virus or interferon inducers. These cellular proteins have different degrees of species specificity and fall into three antigenic and biological types - alpha, beta and gamma. Interferons have pleiotropic effects on cells. The three major biological roles of interferons include their antiviral, antitumor and immunomodulatory roles. Interferons are not directly antiviral, i.e., direct interaction of interferon with a virus does not inhibit viral replication. Interferons are produced by cells and then induce an antiviral effect in other cells.

Assays of antiviral agents must be designed to detect and quantify both direct-acting antivirals and those agents such as interferon that exert their antiviral activity by inducing an antiviral state in other cells. A direct-acting antiviral compound is added to the *in vitro* assay system at the same time the challenge virus is added. Antiviral activity is then measured by a decrease in viral replication or by a decrease in any virus-cell interaction chosen for assay. If a given chemical antiviral agent inhibits a certain specific event in viral replication, it may work only on viruses utilizing this specific event in their replication scheme. In this case, many viruses must be screened in order to determine the full spectrum of antiviral activity. Testing an indirect-acting antiviral agent (such as interferon) that induces an antiviral state requires a different assay system. Interferon must be added to cells in culture for a time, usually 24 hours, prior to addition of the challenge virus.

The selection of indicator cells is an important consideration in these assays. Interferon is relatively species specific and therefore human interferon must be assayed with human cells and mouse interferon assayed using mouse cells. Thus, cells of the same species as the interferon to be assayed should be used in order to maximize assay sensitivity. Cells chosen should also demonstrate a high sensitivity to interferon and also be susceptible to the challenge virus.

The selection of challenge virus is another important consideration. Interferon is not *virus* - specific and therefore a single challenge virus is sufficient for assay of biological activity. The ideal challenge virus should be sensitive to interferon while not inducing large amounts of interferon in the chosen assay system. Passage of the challenge virus should be performed in a species other than that of the interferon species to be assayed. This prevents carry-over of bioactive interferon in the challenge virus fluid. For example, measuring human interferon with a challenge virus grown in human cells would result in the addition of both virus and human interferon to the assay system at a stage where only virus is required. Addition of challenge virus passaged in hamster cells and containing hamster interferon would not affect an assay for human interferon activity tested with human cells.

Thus, many assay systems are available to quantify the effectiveness of antiviral agents. The assay of choice will require a thorough

understanding of the virus chosen for evaluation, as well as the assay objective. Assays may be chosen to screen antiviral compounds (ie: CPE-reduction bioassay) or chosen to yield precise mechanistic information (ie: inhibition of thymidine kinase or neuraminidase activity).

## FURTHER READING

Dulbecco, R. 1952. *Proc. Nat. Acad. Sci. U.S.A.* 38:747.

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Isaacs,A. and J. Lindenman. 1957. Virus interference. I. The interferon. *Proc. R. Soc. Lond.* (Biol) 147:258-267.

Isaacs,A., J. Lindenman and R.C. Valentine. 1957. Virus interference. II. Some properties of interferons. *Proc. R. Soc. Lond.* (Biol) 147:268-273.

White, D. O. and F. J. Fenner. 1986. *Medical Virology.* Academic Press, Inc., Orlando, pp.303-324.

# 32

# PRODUCTION OF INTERFERON

## INTRODUCTION

Interferon may be produced in the laboratory by a number of different procedures using different cells and different interferon inducers. Fibroblast cells infected with virus produce interferon that is classified as primarily beta with a small amount of alpha type. Peripheral blood lymphocytes infected with virus produce predominantly alpha interferon. Peripheral blood lymphocytes or spleen cells induced with mitogens such as concanavalin A, phytohemagglutinin (PHA), or staphylococcus enterotoxin A results in the production of gamma interferon (Johnson et al. , 1981). Gamma interferon was produced in Chapter 6 by the addition of concanavalin A (Con A) 1.0 µg per ml to mouse spleen cells and harvesting the supernatant fluid 48 hours later. The exercise in this section details how to produce and partially purify virus-induced fibroblast interferon.

## MATERIALS AND PREPARATION

### Preparation of crude interferon

- E-MEM 2% FBS (10 ml/person)
- GLB
- HBSS
- NDV
- $L_{929}$ cells in 75 $cm^2$ flasks (1/person)
- 15 ml sterile plastic centrifuge tubes (2/person)
- dilution tubes
- pipets
- decontamination pans

### Perchloric acid treatment of crude interferon

- 2 N perchloric acid
- 5N KOH. For small volumes (3-20 ml) of crude interferon, dilute 1:2 in distilled water to obtain 2.5N KOH.

- 0.1 N HCl
- 15 ml sterile plastic centrifuge tubes (2/person)

NOTE: reagents must be stored and used in a chemical safety hood.

## EXPERIMENTAL PROTOCOL

### Preparation of crude interferon

1. Prepare a $10^{-2}$ dilution of NDV in GLB.
2. Remove media from $L_{929}$ cells and wash cells two times with HBSS.
3. Add 1.0 ml of a $10^{-2}$ dilution of NDV to a 75 $cm^2$ flask of confluent $L_{929}$ cells and incubate at 37°C for 1 hour (gently rotate flask every 15 minutes in order to distribute virus and to prevent dessication of the cell monolayer).
4. Carefully remove virus and wash cell monolayer three times with HBSS.
5. Add 10 ml E-MEM 2% FBS.
6. Incubate cells at 37°C for 24 hours.
7. Collect the supernatant fluid in a plastic centrifuge tube and centrifuge at 5000 x g for 15 minutes to remove cells.
8. Collect the supernatant fluid (crude interferon) in another plastic centrifuge tube. Discard the centrifuge tube with the cell pellet in the decontamination pan.
9. Treat with perchloric acid (see below). Alternatively, the crude interferon tube can be labeled and stored at -20°C until a later time.

### Perchloric acid treatment of crude interferon

Crude interferon is treated with perchloric acid in order to (a) partially purify the interferon preparation and (b) to inactivate the virus used to induce the interferon. Viral interferon inducers must be inactivated and removed from the interferon preparation. Virus not inactivated and removed from an interferon preparation may interfere with the subsequent assay of interferon activity. Virus in an interferon preparation may destroy the cells used in the assay of interferon and thus alter the titer of this preparation. The contaminating virus may also infect indicator cells and interfere with the subsequent infection by

challenge virus. This result would indicate protection of indicator cells by interferon when the cells were protected as a consequence of interference, rather than interferon. It is also important to remove the contaminating viral inducer if the interferon is to be administered *in vivo* for tests to evaluate the therapeutic effectiveness of the preparation in animals.

1. Add 7. 5 ml of 2 N perchloric acid per 100 ml of interferon preparation (See table 32.1 below).

**Table 32.1**
Reagent volumes for the perchloric acid treatment of crude interferon.

| crude interferon (ml) | 2N $HClO_4$ (ml) | 2.5N KOH (ml) | 5.0N KOH (ml) |
|---|---|---|---|
| 3 | 0.25 | 0.18 | - |
| 5 | 0.38 | 0.30 | - |
| 6 | 0.50 | 0.36 | - |
| 8 | 0.625 | 0.48 | - |
| 10 | 0.75 | 0.60 | - |
| 15 | 1.13 | 0.90 | - |
| 20 | 1.50 | 1.20 | - |
| 40 | 3.0 | - | 1.2 |
| 80 | 6.0 | - | 2.4 |
| 120 | 9.0 | - | 3.6 |
| 160 | 12.0 | - | 4.8 |
| 200 | 15.0 | - | 6.0 |
| 240 | 18.0 | - | 7.2 |
| 280 | 21.0 | - | 8.4 |

2. For small volumes (3-20 ml), place the tubes at 4°C for 30 minutes with gentle mixing every 5-10 minutes. Place larger samples on a magnetic stirrer in the cold room for 30 minutes (critical).
3. Centrifuge for 15 minutes at 1000 x g.
4. Transfer the supernatant fluid to a plastic centrifuge tube and neutralize with 2.5N KOH if the volume of crude interferon is small (3-20 ml) or with 5N KOH for larger volumes. Add dropwise (approximately 3 ml per 100 ml) until the color changes to orange-pink (see Table 32.1 above). If necessary - back titrate with 0.1 N HCl
5. Place in freezer (- 20°C) for at least 30 minutes.
6. Centrifuge for 15 minutes at 1000 x g.
7. Place supernatant fluid in sterile plastic tube. Cap tightly.
8. Label properly with interferon type, cell type, interferon inducer, volume and date.
9. Store at - 20°C.

## DISCUSSION QUESTIONS

1. Discuss the differences in properties between alpha, beta and gamma interferons. Discuss the types of interferon made in this exercise.
2. Discuss the properties of an ideal viral interferon inducer.
3. Discuss the potential of endogenous interferon as a prophylactic and therapeutic approach to combat viral and neoplastic disease.
4. Compare and contrast interferons to hormones.
5. Discuss the major biological functions of interferons.

## FURTHER READING

Johnson, H.M., F. Dianzani, and J.A. Georgiades. 1981. Large-scale induction and production of human and mouse immune interferons. In: *Methods in Enzymology*, Volume 78 Part A,"Interferons", S. Pestka (ed.). Academic Press, New York, NY, pp. 158-162.

Kawade, Y. and Y. Yamamoto. 1981. Induction and production of L cell interferon. In: *Methods in Enzymology*, Volume 78, Part A, "Interferons", S. Pestka (ed.). Academic Press, New York, NY, pp. 139-143.

# 33

# PLAQUE-REDUCTION BIOASSAY

## INTRODUCTION

The biological activity of interferon may be quantified by any assay measuring the result of a virus-cell interaction. The plaque assay was developed by Dulbecco in 1952. The plaque, representing a focal area of viral cytopathic effect, may be used to quantify the resultant effect of the interaction of one virus with one cell.

The **plaque-reduction bioassay** measures the effect of interferon on multicycle virus replication that is responsible for the formation of a plaque. The reduction in numbers of plaques is proportional to the amount of interferon incubated with the indicator cells prior to addition of challenge virus. In this assay, dilutions of interferon are added to confluent monolayers of indicator cells and incubated for 20-24 hours to allow for the development of an antiviral state in these cells. Interferon itself has no direct antiviral activity. A dilution of virus that results in 30-50 plaques is then added to cell monolayers. Cells not protected by interferon will demonstrate plaque formation while those cells treated with interferon will have a reduced number of plaques, proportional to the amount of interferon used in the overnight incubation. This assay depends on the quantitative nature of the plaque assay and is sensitive, precise and reproducible.

## MATERIALS AND PREPARATION

- $L_{929}$ cells
- EMC virus passaged in BHK-21 cells
- Laboratory standard interferon preparation with known interferon titer
- E-MEM 10% FBS
- E-MEM 2% FBS
- GLB
- Trypsin-EDTA (1X): Gibco #610-5300, 0.05% trypsin and 0.02% EDTA
- Trypan blue stain (0.4%): Gibco # 630-5250
- HBSS
- HBSS without calcium and magnesium

- Formalin (10% in phosphate buffered saline)
- Crystal violet: 1% in 70% methanol.
- Biohazard Hood: virus assay conducted under biohazard hood
- Tissue culture cluster 12 (Costar): 12-well plates
- Methyl cellulose overlay medium (See Appendix)
- Interferon samples from chapters 6 & 32

## EXPERIMENTAL PROTOCOL

### Day 1

1. Wash $L_{929}$ cell monolayers using HBSS without calcium and magnesium; add trypsin-EDTA (1X) to stock cell monolayer; remove after 30 seconds and add HBSS *with* calcium and magnesium.
2. Prepare a single cell suspension of $L_{929}$ cells in E-MEM 10% FBS and determine the viable cell count using trypan blue.
3. Plate 5.0 x $10^5$ $L_{929}$ cells/ml; 0.5 ml per well in 12-well plates. 56 ml of cell suspension (for 9 plates) will be needed to assay the interferon preparations from chapters 6 & 32. Only 36 ml of cell suspension (for 6 plates) will be needed if only one interferon preparation is assayed. Sequentially label both the top and bottom of all plates in case lids get separated from the wells during the staining procedure.
4. Add 0.5 ml of E-MEM 10% FBS to all the wells. Incubate cells at 37°C in 5% $CO_2$ until a confluent monolayer is obtained (24 hours).

### Day 2

5. Prepare twofold dilutions of each interferon sample using E-MEM 2% FBS. 4 ml of each dilution of 1:4 through 1:4096 will be needed for each interferon sample including the laboratory interferon standard.
   This can be done in the following manner:

| | | | |
|---|---|---|---|
| 2 ml interferon sample | + 6 ml | E-MEM 2% FBS | (1:4) |
| 4 ml of 1:4 dilution | + 4 ml | " | (1:8) |
| 4 ml of 1:8 dilution | + 4 ml | " | (1:16) |
| 4 ml of 1:16 dilution | + 4 ml | " | (1:32) |

   continued through a 1:4096 dilution

6 Add dilutions of interferon to triplicate wells of cells (1 ml/well). Virus controls receive 1 ml E-MEM 2% FBS per well and no interferon. See figure 33.1.

7. Incubate cells at 37°C in 5% $CO_2$ for 24 hours.

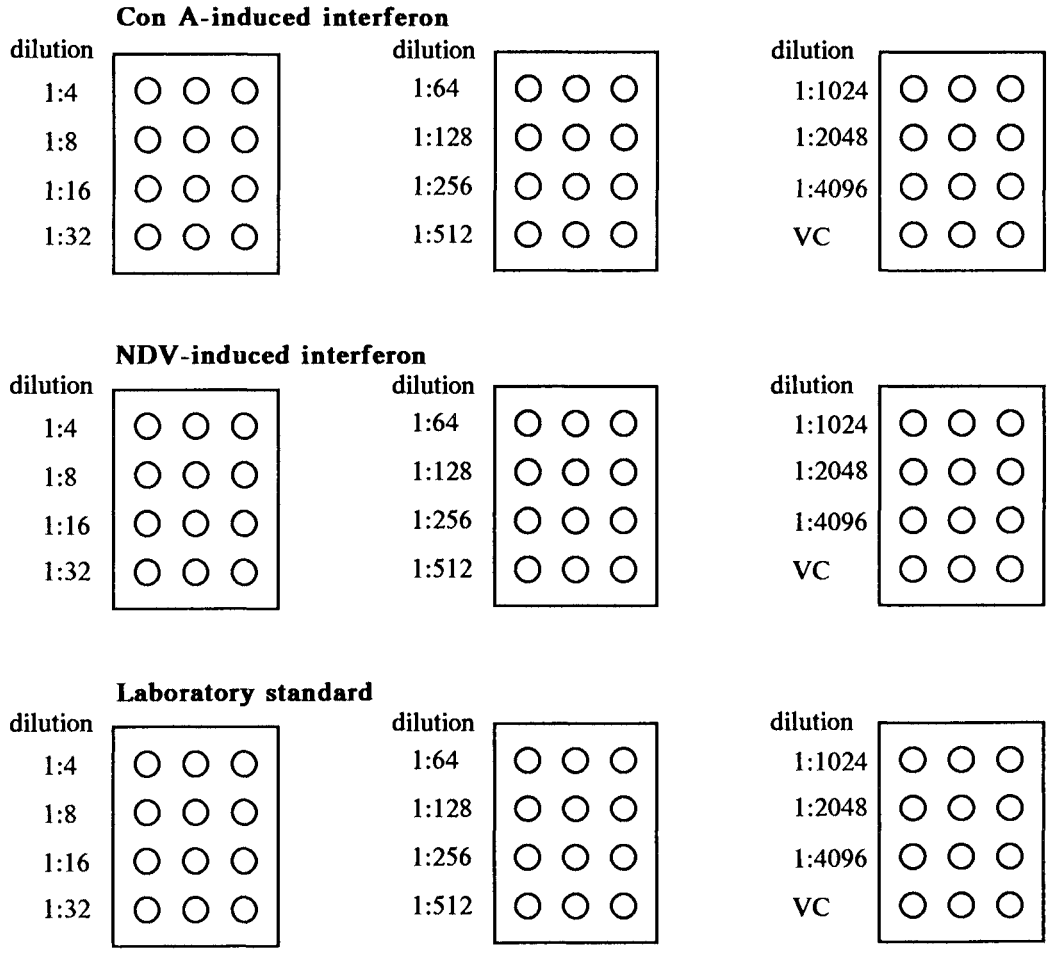


**Figure 33.1** Diagram of the plating of the interferon dilutions for the plaque-reduction bioassay.

## Day 3

8. Make dilutions of EMC virus in GLB.
9. Wash $L_{929}$ cells with HBSS.
10. Add 0.1 ml of virus diluted to contain 20 plaques per well to triplicate wells. Calculate this from the titer obtained in Chapter 27.

11. Adsorb virus by incubation at 37°C in 5% $CO_2$ for 30 minutes. Tilt plate every 10 minutes to keep cell monolayer moist and to distribute the virus.
12. Wash the cells with 1 ml of HBSS.
12. Decant HBSS and add 2 ml methyl cellulose overlay medium per well.
13. Incubate at 37°C in 5% $CO_2$ for 20-22 hours.

**Day 4**

14. Add 10% formalin to fix monolayer (2 ml per well) for 30 minutes.
15. Remove overlay and wash with tap $H_2O$.
16. Stain monolayer with 1% crystal violet for 30 minutes.
17. Wash cells with tap water until excess crystal violet has been removed.
18. Invert plates over absorbent paper to dry.
19. Count and record plaques.
20. Calculate the titer of each interferon sample as the 50% plaque-depressing dose ($PDD_{50}$) according to the formula developed by Langford et al. (1981):

$$PDD_{50} = DL + [(P_{50} - PL)\ (DH - DL) / (PH - PL)]$$

Where:

**DL** is the reciprocal of the lower dilution bracketing the 50% endpoint,

**DH** is the reciprocal of the higher dilution bracketing the 50% endpoint,

$\mathbf{P_{50}}$ is the number of plaques at the 50% endpoint,

**PL** is the number of plaques at the lower dilution bracketing the 50% endpoint,

**PH** is the number of plaques at the higher dilution bracketing the 50% endpoint

In this assay, the highest dilution of sample that inhibits 50 % of the virus plaques, is said to contain 1 interferon unit. The interferon titer of the preparation is the reciprocal of the highest dilution resulting in 50% plaque reduction, per unit volume.

## DISCUSSION QUESTIONS

1. Discuss the important properties of the plaque-reduction interferon bioassay that directly affect the sensitivity of this method.
2. Discuss important properties for selection of a challenge virus for use in the plaque reduction interferon bioassay.
3. Discuss important properties for selection of an indicator cell for use in the plaque-reduction interferon bioassay.

## FURTHER READING

Langford, M.P., D.A. Weigent, G.J. Stanton and S. Baron. 1981. Virus plaque-reduction assay for interferon: Microplaque and regular macroplaque reduction assays In *Methods in Enzymology*, Volume 78, Part A, "Interferons" S. Pestka (ed.), Academic Press, New York, NY, pp. 339-346.

# 34

# CYTOPATHIC EFFECT INHIBITION BIOASSAY

## INTRODUCTION

The biological activity of interferon may be quantified by any assay measuring the result of a virus-cell interaction. In the inhibition of CPE interferon bioassay, dilutions of interferon are added to confluent monolayers of indicator cells and incubated for 20-24 hours in order that an antiviral state may be induced in these cells. Interferon itself has no antiviral activity. A dilution of virus that causes complete cytopathic effect is then added to cell monolayers. Cells not protected by interferon will demonstrate complete destruction of the cell monolayer while those cells treated with interferon will have a reduced cytopathic effect that is proportional to the amount of interferon used in the overnight incubation. This assay depends on the quantal nature of the cytopathic effect assay. The CPE-inhibition interferon bioassay is a sensitive and reproducible bioassay.

## MATERIALS AND PREPARATION

- $L_{929}$ cells
- EMC virus passaged in BHK-21 cells
- Laboratory standard interferon preparation with known interferon titer, and interferon samples from chapters 6 & 32
- E-MEM 10% FBS
- E-MEM 2% FBS
- GLB
- Trypsin-EDTA (1X): Gibco #610-5300, 0.05% trypsin and 0.02% EDTA
- Trypan blue stain (0.4%): Gibco # 630-5250
- HBSS
- HBSS without calcium and magnesium
- Crystal violet: 1% in 70% methanol
- 96-well flat bottom tissue cultures plates (1 per 2 students)
- 96-well round bottom plates (1 per 2 students)

- Multiwell pipetor and sterile towels
- Biohazard Hood: Virus assay conducted under biohazard hood

## EXPERIMENTAL PROTOCOL

### Day 1

1. Wash $L_{929}$ cell monolayers using HBSS *without* calcium and magnesium; add trypsin-EDTA (1X) to cell monolayer; remove after 30 seconds and add HBSS *with* calcium and magnesium.
2. Prepare a single cell suspension of $L_{929}$ cells in E-MEM 10% FBS and determine the viable cell count using trypan blue.
3. Prepare 5-6 ml of 5 x $10^5$ cells/ml in E-MEM 10% FBS and dispense 100 µl/well in 4 (12-well) rows of a 96-well sterile tissue culture plate. Only half a plate will be needed to assay the 2 interferon samples and the interferon standard.
4. Incubate at 37°C in 5% humidified $CO_2$ for 18-24 hours

### Day 2

5. Sterilize a 96-well round-bottom plate by placing under UV germicidal lamp (GE Germicidal Lamp #G8T5) at a distance of about 15 cm for 15 minutes.
6. Prepare a 1:4 dilution (0.1 ml sample + 0.3 ml E-MEM 2% FBS) of the interferon standard and each interferon sample. Add 130 µl of E-MEM 2% FBS to 4 rows (E-H) of the 96-well round-bottom plate. Add 130 µl of the diluted interferon standard in the first well (H-1) and perform serial 2-fold dilutions through well H-12, discarding the last 130 µl in the decontamination pan. Do the same for the other two interferon samples in rows G & F. Do not add any interferon to row E. See figure 34.1.
7. Decant media from cells in 96-well plate into a decontamination pan and blot plate by placing the surface against a sterile towel.
8. Transfer 100 µl from each interferon sample well of the round-bottom plate to its corresponding well in the 96-well flat-bottom plate containing cells. If available, use the multiwell pipetor to transfer 1 row at a time from the round-bottom plate to the plate with the cells.
9. Incubate at 37°C in humidified 5% $CO_2$ for 18-24 hours.

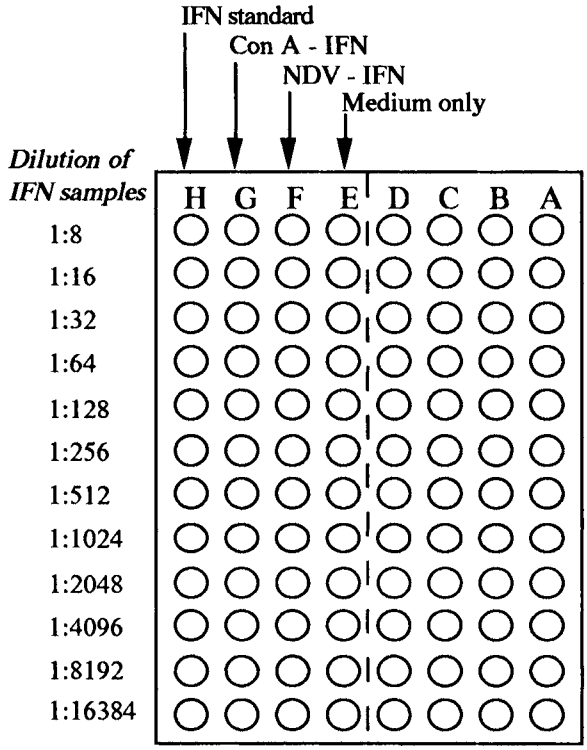


**Figure 34.1** Diagram of the dilution sequence of the interferon (IFN) samples in the 96-well round-bottom plate (step #6).

## Day 3

10. Wash cells with HBSS.
11. Add 50 µl of challenge virus per well (use MOI=1) to all wells except the cell control wells. Cell controls get 50 µl E-MEM 2 % FBS (see figure 34.2).
12. Adsorb challenge virus for 30 minutes at 37°C.
13. Add 100 µl E-MEM 2 % FBS to all wells.
14. Incubate at 37°C in humidified 5% $CO_2$ until complete CPE is observed in virus controls.

## Day 4

15. Decant media into a decontamination pan and stain cells with crystal violet for 30 minutes.
16. Wash cells with tap water to remove excess crystal violet. Invert plates to dry at room temperature.

17. Record the interferon titer, visually, as the reciprocal of the last interferon dilution which results in 50% or greater inhibition of CPE, as compared to controls.

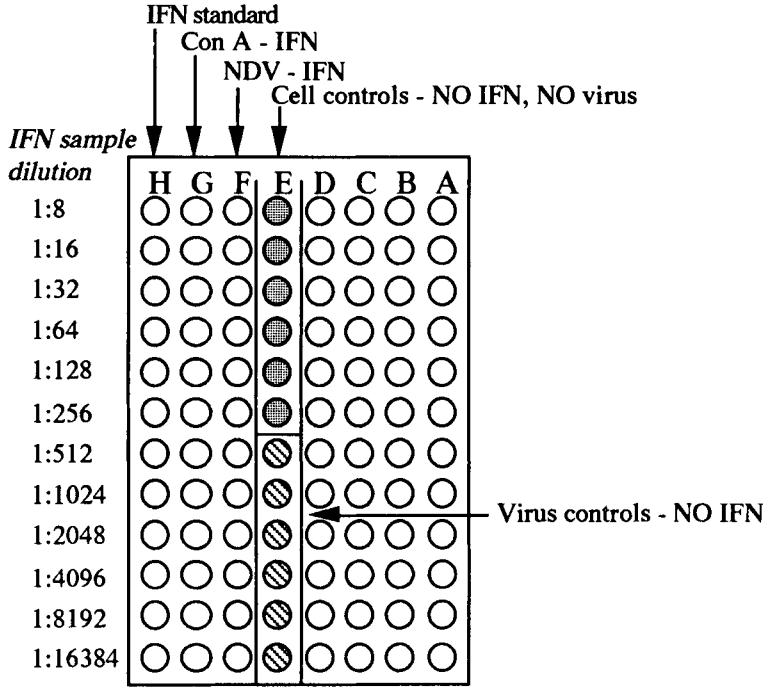


**Figure 34.2** Diagram of the 96-well flat-bottom plate with the cells, interferon sample dilutions, cell controls, and virus controls for the CPE-reduction bioassay (steps #8-13).

## DISCUSSION QUESTIONS

1. Discuss the important properties of the CPE-inhibition interferon bioassay that directly affect the sensitivity of this method.
2. Discuss important properties for selection of a challenge virus and indicator cells for use in the CPE-inhibition interferon bioassay.
3. How would you modify the experimental protocol to assay a direct-acting antiviral agent?

## FURTHER READING

Armstrong, J.A. 1981. Cytopathic effect inhibition assay for interferon: Microculture plate assay. In *Methods in Enzymology*, Volume 78, Part A, "Interferons" S. Pestka (ed.), Academic Press, New York, NY, pp. 381-387.

# 35

# HEMAGGLUTININ (HA) YIELD - REDUCTION BIOASSAY

## INTRODUCTION

Interferon activity may be quantified by measuring the reduction in virus yield of cells treated with interferon. This type of interferon assay, like other interferon assays, consists of a period where interferon is incubated with cells to induce the antiviral state and a period where the cell's antiviral state is quantified by the addition of a challenge virus. However, yield-reduction interferon assays have an additional step: quantification of the inhibition of virus yield. The reduction in viral yield step may utilize various properties of the challenge virus including: cytopathic effect, plaque assay, as well as hemagglutination and neuraminidase measurements for viruses such as influenza virus. This additional assay step makes this type of interferon assay more expensive and more time-consuming. Yield-reduction assays are preferred in order to investigate the kinetic changes in antiviral activity after addition of interferon and the effect of these changes on the viral growth cycle. Other advantages and disadvantages of these assays have been discussed by Weigent et al. (1981). Advantages include (a) the effects of endogenous interferon induced in the assay system is minimized due to the high MOI, (b) decay of antiviral activity during replication of challenge virus is minimal, and (c) a high degree of accuracy may be achieved by utilizing a virus plaque assay to quantify decrease in viral yield. Disadvantages include (a) some viruses are less sensitive to interferon at high MOI and (b) the additional step for titration of virus yield is more expensive, more time-consuming and labor intensive.

## MATERIALS AND PREPARATION

- $L_{929}$ cells
- EMC virus passaged in BHK-21 cells
- Laboratory standard interferon preparation with known interferon titer
- E-MEM 5% FBS
- E-MEM 2% FBS

- GLB
- Trypsin-EDTA (1X): Gibco #610-5300, 0.05% trypsin and 0.02% EDTA
- Trypan blue stain (0.4%): Gibco # 630-5250
- HBSS
- HBSS without calcium and magnesium
- sterile plastic dilution tubes
- 96-well flat bottom tissue culture plates
- Stock EMC-HA Buffer (0.05 M $H_3BO_3$ - 0.12 M KCl buffer): $H_3BO_3$ (boric acid) 3.09 g/L and KCl 8.95 g/L in glass-distilled water: Adjust pH to 7.0 with 1 N NaOH and store at room temperature. EMC-HA Buffer with 0.1% BSA is prepared using stock EMC-HA buffer plus stock BSA solution (35% BSA w/v).
- Sheep red blood cells (SRBC)
- Biohazard Hood: Virus assay conducted under biohazard hood

## EXPERIMENTAL PROTOCOL

### Day 1

1. Wash $L_{929}$ cell monolayers using HBSS *without* calcium and magnesium; add trypsin-EDTA (1X) to cell monolayer; remove after 30 seconds and add HBSS *with* calcium and magnesium.
2. Prepare a single cell suspension of $L_{929}$ cells in E-MEM 10% FBS and determine the viable cell count using trypan blue.
3. Adjust cell number to 5 x $10^5$ cells/ml in E-MEM 10% FBS and dispense 100 µl/well of a 96-well sterile flat bottom tissue culture plate.
4. Incubate at 37°C in 5% humidified $CO_2$ for 18-24 hours.

### Day 2

5. Prepare serial 10-fold (0.1 + 0.9 ml) dilutions ($10^0$-$10^{-4}$)of the interferon samples and the interferon standard in E-MEM 2% FBS in sterile dilution tubes.
6. Decant media from cells in 96-well plate into a decontamination pan and blot plate by placing the surface against a sterile towel.
7. Transfer 100 µl of each interferon sample dilution in triplicates to the 96-well flat-bottom plate containing cells. See figure 35.1.
8. Incubate at 37°C in humidified 5% $CO_2$ for 18-24 hours.

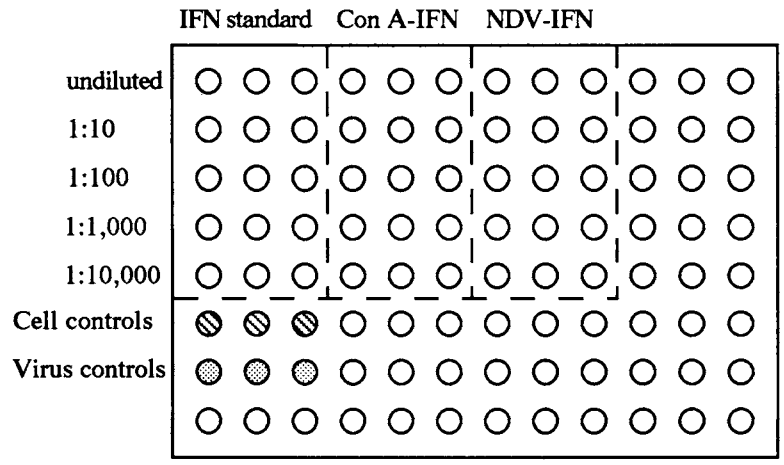


**Figure 35.1** Diagram of step #7 of the Experimental Protocol - Addition of diluted interferon samples to cells in the 96-well plate.

## Day 3

9. Wash cells with HBSS.
10. Add 50 µl of challenge virus per well (use MOI=10). Cell controls get 50 µl E-MEM 2% FBS.
11. Adsorb challenge virus for 30 minutes at 37°C.
12. Wash cells twice with HBSS and add 100 µl E-MEM 2% FBS per well.
13. Incubate at 37°C in humidified 5% $CO_2$ for 24 hours.

## Day 4

14. Freeze-thaw cells twice to release intracellular and cell-associated virus.
15. Pool replicate wells and assay for hemagglutination titer.
16. Add 100 µl EMC-HA buffer to each well of two 96-well round bottom plates.
17. Add 100 µl of sample dilution to be assayed for HA to well #1.
18. Perform 2-fold serial dilutions with a Titertek automatic pipetor (12 wells). Do the same for each sample dilution and the virus control. See figure 35.2
19. Add 100 µl/well SRBC (0.5% in EMC-HA buffer) to all the wells.

20. Incubate at room temperature for 1 hour. Read and record HA results.
21. Calculate HYR $_{0.5 \log 10}$ titer using semilogarithmic paper. The interferon titer is defined as the reciprocal of the interferon dilution that results in a 0.5 $\log_{10}$ reduction in viral yield.

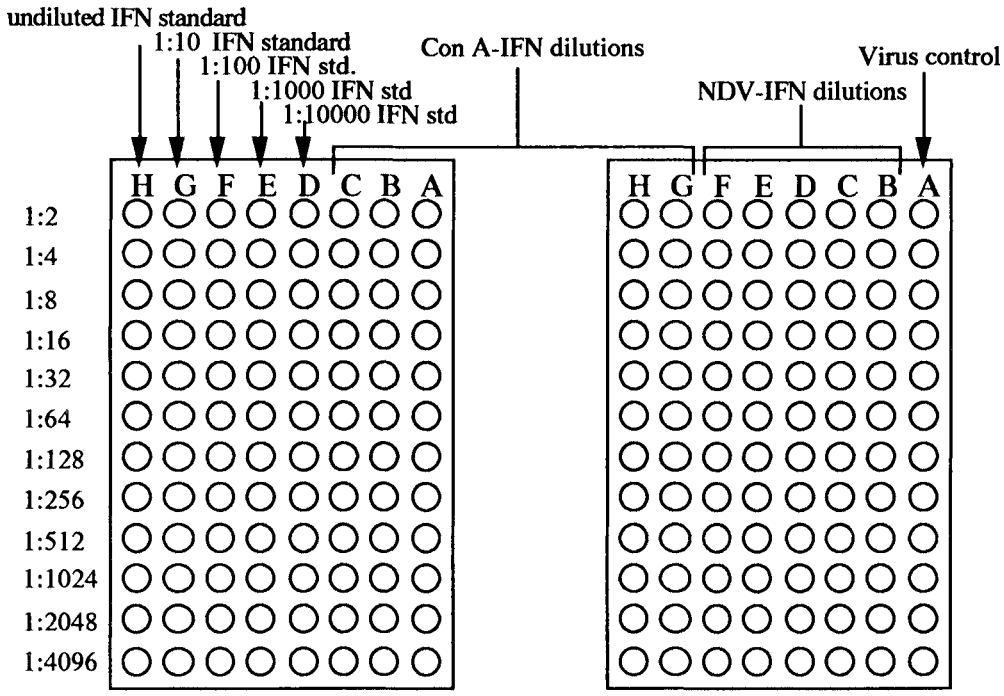


**Figure 35.2** Diagram of the hemagglutination assay for the interferon samples (steps #16-19).

## Calculation of Titer

See table 35.1 and figure 35.3 for sample data and calculation of the hemagglutination-yield reduction titer.

**Table 35.1**
Sample Data for the Hemagglutination-Yield Reduction Interferon Bioasssay

| IFN dilution | # cups positive HA | $Log_2$ | $Log_{10}$ | $\Delta Log_{10}$ |
|---|---|---|---|---|
| $10^0$ | 0 | 0 | 0.0 | 3.0 |
| $10^{-1}$ | 0 | 0 | 0.0 | 3.0 |
| $10^{-2}$ | 5 | 5 | 1.5 | 1.5 |
| $10^{-3}$ | 10 | 10 | 3.0 | 0.0 |
| $10^{-4}$ | 10 | 10 | 3.0 | 0.0 |
| Virus Control | 10 | 10 | 3.0 | |

$Log_{10} = (log_2)\ (0.3)$
$\Delta$ Log10 = (Virus Control $log_{10}$) - (Sample $log_{10}$)

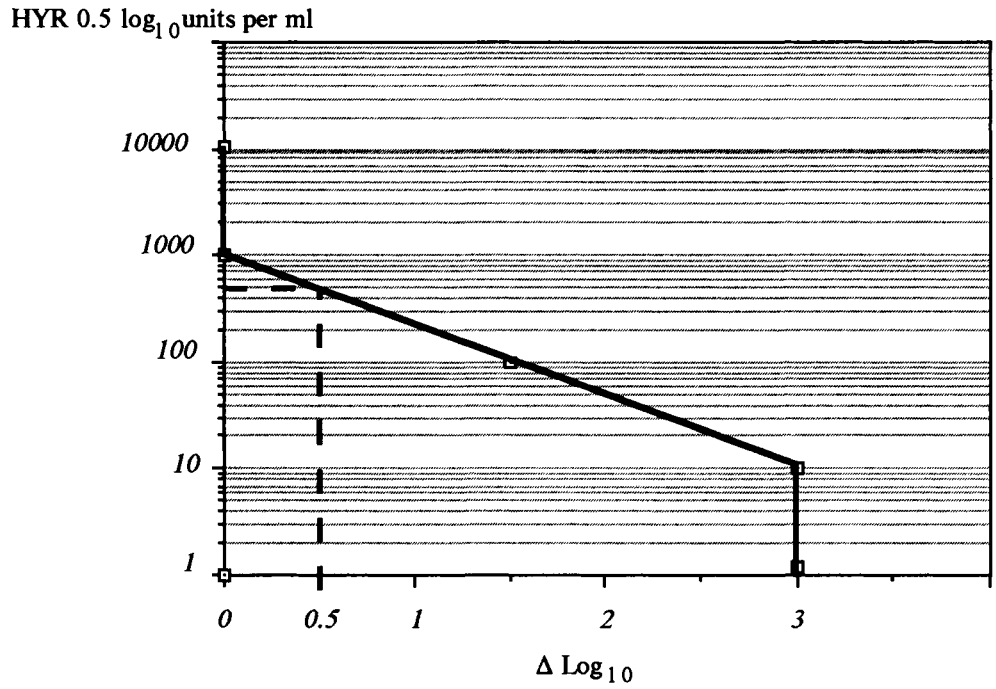


Figure 35.3 Determination of the HYR 0.5 $\log_{10}$ interferon titer using the sample data from Table 35.1 and plotting these values on semilogarithmic graph paper.

Titers may also be calculated by utilizing semilogarithmic graph paper and plotting the reciprocal of the interferon dilution on the ordinate and the $\log_2$ reduction in hemagglutinin yield on the abscissa. The interferon titer is defined as the reciprocal of the interferon dilution that results in a 0.5 $\log_{10}$ reduction in viral yield. This corresponds to a 1.66 $\log_2$ units of hemagglutinin (Jameson and Grossberg, 1981).

## DISCUSSION QUESTIONS

1. Discuss the effect of virus MOI on the following types of interferon bioassay: (a) plaque-reduction interferon bioassay, (b) CPE reduction bioassay, and (c) yield-reduction bioassay.
2. Discuss the advantages and disadvantages of the following types of interferon bioassay: (a) plaque-reduction interferon bioassay, (b) CPE reduction bioassay, and (c) yield-reduction bioassay on the assay of potential chemotherapeutic antiviral agents.
3. Discuss the meaning of "interferon titer".

## FURTHER READING

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# 36

# RADIOIMMUNOPRECIPITATION

## INTRODUCTION

The radioimmunoprecipitation (RIP) assay and Western blots have become important analytical tools for biologists and chemists. The power of this technique revolves around its selectivity. RIP assays are routinely used to detect and quantify specific antigens within a preparation containing many contaminating proteins. The key to this selectivity lies in the specificity of the antigen-antibody interaction and the ability to easily purify an antigen-antibody complex from contaminating proteins. Unlike Western blots where proteins are normally denatured before antibodies are added, RIP assays are generally used to examine antibody interactions using proteins in their native configuration. Because RIP assays can detect as little as 100 pg of radiolabeled protein, RIP assays are routinely used for the detection of viral proteins, characterization of monoclonal and polyclonal antibody preparations, and for determining the specificity of the immune response to viral antigens.

RIP assays are composed of five major steps: (a) metabolic labelling of the cells expressing the target antigens, (b) lysis of the cells, (c) formation of specific immune complexes, (d) collection and purification of the immune complexes, and (e) analysis of the radiolabeled proteins in the immunoprecipitate. In the procedure used in this chapter, cells are infected with EMC virus and the virus is allowed to replicate in the presence of $^{35}$S-methionine. Because methionine is an essential amino acid for mammalian cells, any newly synthesized methionine-containing proteins will have the radioactive tracer incorporated into them. The cells are lysed at various intervals after infection and the antibody preparation is allowed to react with the lysate. The antibody is collected and purified by the addition of protein A sepharose. The protein A portion of this matrix selectively binds to the Fc portion of the antibody while the sepharose allows the antigen-antibody complexes to be removed from contaminating proteins by centrifugation. The target antigens are released from the sepharose by boiling in Laemmli sample buffer and the proteins are separated by polyacrylamide gel electrophoresis. Newly synthesized (radioactive) proteins are detectable using X-ray film.

The isotope most commonly used to radiolabel proteins is $^{35}$S which has a half-life of 87.1 days and emits a weak beta radiation. Because weak beta emitters are difficult to detect with normal X-ray film,

fluorescent chemicals or scintillants are usually incorporated into the gel after the electrophoresis is complete. These chemicals enhance the autoradiographic signal since they will emit a large number of photons whenever a single quantum of radiation is encountered. In this manner, fluorography can increase the sensitivity of $^{14}C$ and $^{35}S$ detection about tenfold and permits the detection of tritium which would otherwise be invisible in conventional autoradiography. Although salicylate is used in this experiment, a number of excellent enhancing fluors are commercially available and can be used in place of the salicylate.

## MATERIALS AND PREPARATION

- $L_{929}$ cells
- EMC virus
- GLB
- E-MEM-2%FBS
- E-MEM without serum
- Hanks' Balanced Salt Solution (HBSS)
- $^{35}S$-methionine; New England Nuclear # NEG-009H
- microcentrifuge; Brinkman, Eppendorf; Fisher Scientific # 5415C
- microfuge tube; Fisher # 05-407-10
- Lysis Buffer (See Appendix)
- Normal Rabbit Serum (or pre-immune serum)
- Protein-A Sepharose CL-4B; Pharmacia # 17-0963-03; Sigma P-3391
- Rabbit anti-EMC Serum (See Appendix)
- TNE-Gelatin Buffer (See Appendix)
- 1X PAGE Sample Buffer (See Appendix)
- $^{14}C$-labelled molecular weight markers; New England Nuclear # NEC-751
- Electrophoresis apparatus; Mini-Protean II Dual Slab Cell, Biorad # 165-1802-1804
- Gel dryer; Hoeffer Scientific # SE-540
- Destain solution (See Appendix)
- 1.0 M sodium salicylate, pH 6.0; Aldrich # 24,135-0
- SDS-polyacrylamide gels
- 3MM paper; Whatman 3MM chromatography paper # 3030-917

- "cool" radioactive ink; add $^{35}$S-methionine to black artist's ink to achieve approximately 500 cpm.
- Saran wrap
- X-ray film cassette
- Kodak SB X-ray film; Eastman Kodak #SB
- Cassette-Lightning Plus intensifying screen combination; Sigma E-5138

## EXPERIMENTAL PROTOCOL

### Radiolabeling EMC Virus Proteins

1. Seed six 25 cm$^2$ cell culture flasks with $L_{929}$ cells.
2. When the cells are confluent, infect three (3) flasks with 1 ml of a $10^{-3}$ dilution of stock EMC virus.
3. Perform a mock infection on the remaining flasks (i.e., use GLB without virus).
4. Feed all of the flasks with E-MEM-2% FBS and incubate the flasks for 4 hours at 37°C in humidified (95% RH) 5% $CO_2$ atmosphere.
5. After 4 hours, decant the media from the flasks, wash the monolayers once with 15 ml of warm (37°C) Hanks' Balanced Salt Solution (HBSS).
6. Add 5 ml of warm HBSS to each flask and incubate for 30 minutes at 37°C to deplete the intracellular methionine pool.
7. Add 1200 microcuries of $^{35}$S-methionine to 12 ml of warm E-MEM without serum.
8. Decant the HBSS and add 2.0 ml of the $^{35}$S-methionine medium to each flask. Rock the flasks to evenly distribute the medium.
9. Incubate the flasks for 1, 6, and 11 hours and at each time point prepare cell lysates from one infected and one uninfected flask.

### Cell Lysis

1. Carefully remove the supernatant fluids from the flasks.
2. Using a rubber policeman, scrape the cells from the flask into 1.0 ml of cold HBSS.

3. Transfer the cell suspension to a microfuge tube and pellet the cells in a microcentrifuge (12,000 x g) at 2-8°C for 20 seconds.
4. Add 1.0 ml of cold (0-4°C) Lysis Buffer and vortex vigorously to resuspend the cell pellet.
5. Incubate the tubes on ice for 20 minutes, vortexing once during that time.
6. Pellet the cell debris by centrifugation at 12,000 x g for 20 seconds at 2-8°C.
7. Carefully transfer the supernatant fluid to a clean microfuge tube.
8. Freeze the cell lysates at -70°C until all samples are collected.

### Immunoprecipitation

1. Thaw the samples and preclear them by adding:

   - 10 µl Normal Rabbit Serum (or pre-immune serum)
   - 25 µl Swollen Protein A Sepharose in 1X Lysis Buffer

2. Incubate for 30 minutes at 2-8°C with constant rocking.
3. Centrifuge at 12,000 x g for 20 seconds as above.
4. Carefully transfer the supernatant fluids to another microfuge tube.
5. Add:

   - 10 µl Rabbit anti-EMC Serum
   - 25 µl Swollen Protein A Sepharose in 1X Lysis Buffer

6. Incubate for 30 minutes at 2-8°C with constant rocking.
7. Centrifuge at 12,000 x g for 20 seconds as above.
8. Wash the gel three times with 1.0 ml of cold 1X Lysis Buffer.
9. Wash the gel three more times with cold TNE-Gelatin Buffer.
10. Resuspend the gel in 100 microliters of 1X PAGE Sample Buffer (see Chapter 37, Western blot or Appendix).
11. Denature the proteins by placing the tubes in a boiling water bath for 3 minutes.
12. Remove the protein A sepharose by centrifuging the tubes for 20 seconds at 12,000 x g in a microfuge.
13. Analyze the specimens using polyacrylamide gel electrophoresis (See Chapter 37, Western blot) through a 15% acrylamide gel and

fluorography. In this procedure, $^{14}C$-labelled molecular weight markers must be used in place of the pre-stained markers.

## Fluorography

1. Remove the gel from the electrophoresis apparatus and fix for 30 minutes at room temperature in destain solution as described in chapter 37, Western Blotting.
2. Soak the gel in a large volume of deionized water for 30 minutes.
3. Transfer the gel to another pan containing 1.0 M sodium salicylate, pH 6.0. Soak the gel for 30 minutes to allow the scintillant to completely penetrate the gel.

**NOTE:** Salicylate is readily absorbed through the skin. Wear gloves whenever handling gels in salicylate solutions.

4. SDS-polyacrylamide gels containing material labeled with $^{35}S$-methionine must be dried before autoradiographic images can be obtained. Therefore, the gel must be dried on a piece of 3MM paper as described by the manufacturer of the gel dryer.
5. Once the gel is dried, tape onto an old, developed radiographic film of an appropriate size and mark the edges of the gel with a "cool" radioactive ink. Cover the gel and film support with Saran wrap when the ink is dry.
6. Place the gel in an X-ray film cassette, gel side up.
7. In a darkroom, place a piece of preflashed Kodak SB X-ray film on the gel, flashed side up. Lay a Lightning Plus intensifying screen on top of the film and assemble the cassette.
8. Place the cassette in a -70°C freezer for 8-12 hours.
9. Remove the cassette from the freezer and allow the cassette to equilibrate to room temperature. Disassemble the cassette in the darkroom and develop the X-ray film. If the exposure is not suitable, place another film in the cassette and repeat the exposure, adjusting the time as needed. The proper exposure time must be empirically determined. In general, exposure times average between 8 and 80 hours.

## Evaluation of Results

1. Carefully measure the distance between the top of the gel and the middle of each band.

2. Plot the molecular weights of the standards (in Kilodaltons) versus the distance migrated to construct a standard curve.
3. Use the standard curve to determine the relative molecular weight of each of the bands on the gel.

## DISCUSSION QUESTIONS

1. How do the experimental results compare with the expected molecular weights for EMC virus proteins?
2. Would you expect the radioimmunoprecipitation data toagree with the Western blot data? Explain.

## FURTHER READING

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# 37

# WESTERN BLOT ANALYSIS OF EMC VIRUS PROTEINS

## INTRODUCTION

Since Towbin, et. al., first described the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose paper, Western blots have become one of the most common analytical tools for the detection of viral proteins, characterization of monoclonal and polyclonal antibody preparations, and in determining the specificity of the immune response to viral antigens. In this experiment, EMC virus proteins will be separated by polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose paper, and the individual proteins will be detected immunologically using rabbit antiserum to EMC virus.

## MATERIALS AND PREPARATION

- $L_{929}$ cells
- EMC virus
- GLB
- HBSS
- Sample Buffer (See Appendix)
- Electrophoresis apparatus; Biorad Trans-Blot Cell
- 2 - 125 ml sidearm flasks
- (3)-5 cc syringes with (2)-27 gauge needles, and (1)-18 gauge needle
- disposable gloves
- Power supply; Hoeffer Scientific #PS 500X
- Sucrose; Sigma # 9378
- 15% acrylamide resolving gel
- 10% ammonium persulfate; Fisher # BP179-25
- TEMED (N,N,N',N'-tetramethylethylenediamine); Sigma # T-7024
- (2) 4-liter sidearm suction flasks
- 20X transfer buffer (See Appendix)
- 5% acrylamide stacking gel

- Running buffer (See Appendix)
- Prestained molecular weight markers; Biorad # 161-0305
- Hamilton syringe; Sigma S-3767
- Coomassie Blue Staining solution (See Appendix)
- Destaining Solution (See Appendix)
- Transblot sponges
- 8.5" x 13" x 2" plastic pan
- 3 MM paper; Whatman Chromatography Paper # 3030-917
- Nitrocellulose (Millipore # HAHY 000 10, 0.45 μm)
- Western transfer chamber and cassettes
- TSA/BSA Blocking Buffer (see Appendix)
- rabbit antiserum to EMC virus (see Appendix)
- PBS-T (See Appendix)
- Horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed # 62-6122)
- 0.22 μm filter
- Conjugate Buffer (See Appendix)
- Chromogen Substrate (See Appendix)
- Saran wrap
- ice buckets and decontamination pans
- boiling water bath

## EXPERIMENTAL PROTOCOL

### EMC Virus Proteins Sample Preparation

1. Seed ten 75 $cm^2$ cell culture flasks with $L_{929}$ cells.
2. When the cells are confluent, infect five (5) flasks with 1 ml of a $10^{-3}$ dilution of stock EMC virus.
3. Perform mock infections on the remaining five flasks (i.e., use GLB without virus).
4. Incubate the flasks for 16 hours at 37°C in a humidified, (95% RH) 5% $CO_2$ atmosphere.
5. Decant the supernatant fluids from the monolayers, pooling the infected and uninfected supernatant fluids in separate tubes. Hold the supernatant fluids on ice until they can be prepared for centrifugation.

6. Add 2-3 ml of HBSS to each cell culture flask and carefully scrape the cells from the flask. Use a separate rubber policeman for each flask.
7. Pool the infected cells and the uninfected cells in separate tubes. Store on ice until centrifugation.
8. Centrifuge the supernatant fluids at 600 x g for 15 minutes at 2-8°C to pellet the cells and cell debris.
9. Carefully remove the supernatant fluids from the cells with a pipet and save on ice.
10. Add the cell fraction (step #7) to the tube containing the cell pellet (step #9).
11. Centrifuge as before at 600 x g to pellet the cells.
12. Using a pipet, carefully remove and discard the supernatant fluids.
13. Resuspend the cell pellet in 1.0 ml of Sample Buffer.
14. Place the tube in a boiling water bath for 5 minutes.
15. Vortex the tube vigorously to break up any DNA strands. Failure to vortex vigorously will cause the samples to smear during electrophoresis.
16. Label the tubes as cell pellets and store at -70°C until electrophoresis.
17. Prepare a 30% sucrose solution as follows:

    - 30 grams of sucrose
    - 100 ml of distilled water

18. Place enough 30% sucrose in an ultracentrifuge tube to fill the tube 2-5 cm from the *bottom* of the tube.
19. Carefully overlay the supernatant fluids (step #9) onto the sucrose cushion.
20. Balance the tubes and centrifuge at 100,000 x g for 60 minutes at 2-8°C to pellet the virus.
21. Carefully remove the supernatant fluids and resuspend the virus in a total of 1.0 ml of Sample Buffer.
22. Place the virus suspension in a boiling water bath for 5 minutes.
23. Vortex briefly and store these tubes at -70°C until electrophoresis.

## Setting Up Gels

1. To make the *15% acrylamide resolving gel*, place the following reagents in a 125 ml sidearm suction flask containing a small stir bar.

   ***15% Acrylamide Resolving Gel***

   - 5.0 ml acrylamide/bisacrylamide solution
   - 2.0 ml 1.875 M Tris/HCl, pH 8.8
   - 0.1 ml 0.2 M EDTA
   - 2.0 ml distilled water

2. Place a stopper on the 125 ml sidearm flask, connect the flask to a suction source, and degas with constant stirring for 15 minutes at room temperature.
3. While the resolving gel is degassing:
   a. Set up the electrophoresis apparatus as directed by the manufacturer.
   b. Prepare 1 ml of 10% ammonium persulfate:
      - 100 mg of ammonium persulfate
      - 1.0 ml of distilled water.
4. After degassing for 15 minutes, turn off the suction and carefully remove the suction tubing and the stopper. Continue stirring the acrylamide solution.
5. Quickly add:
   - 5 µl TEMED (N,N,N',N'-tetramethylethylenediamine)
   - 0.1 ml 10% ammonium persulfate
6. Continue stirring for 20 seconds.
7. Draw up 5 ml of the acrylamide solution into a 5 cc syringe.
8. Connect a 27 gauge needle to the syringe and dispense 2 ml of the acrylamide into the space between the first set of glass plates. Repeat the procedure for the second set of plates.
9. Overlay the resolving gels with distilled water.
10. Set a timer for 15 minutes.
11. While the acrylamide is polymerizing, prepare the **Western transfer buffer** as follows:
    a. Add the following reagents to each of two 4-liter sidearm suction flasks containing a magnetic stir bar:

- 62.5 ml 20X transfer buffer
- 2500 ml deionized water

b. Place a stopper on each flask, connect the sidearm flasks to a suction source, and degas with constant stirring for at least 60 minutes at 2-8°C.

12. When the timer goes off, set up the *5% acrylamide stacking gel* by placing the following reagents into a 125 ml sidearm suction flask:

***5% Acrylamide Stacking Gel***

- 1.25 ml acrylamide/bisacrylamide solution
- 0.94 ml 1.0 M Tris/HCl, pH 6.8
- 0.75 ml 0.2 M EDTA
- 5.15 ml distilled water

**NOTE : Acrylamide is a neurotoxin.** Wear gloves when handling acrylamide and wear a mask when weighing powdered acrylamide.

13. Place a stopper on the 125 ml sidearm flask, connect to a suction source, and degas with constant stirring for 15 minutes at room temperature.

14. While the stacking gel is degassing, make up one liter of *running buffer* as follows:

***Running Buffer***

- 3.03 g Tris base (Sigma T-1503)
- 1.0 g sodium dodecyl sulfate (SDS); (Sigma L-4390)
- 14.4 g glycine; (Sigma G-4392)
- 1000 ml deionized water

15. After the stacking gel has degassed for 15 minutes:

a. Remove the water overlay from the resolving gel.

b. Using a wash bottle, gently rinse the top of the resolving gel with distilled water to remove any unpolymerized acrylamide.

c. Dry the sandwich with pieces of filter paper. Be careful not to touch the resolving gel.

16. Turn off the suction and carefully remove the suction tubing and the stopper from the sidearm suction flask. Continue stirring the acrylamide solution.

17. Quickly add:

- 3.8 microliters TEMED
- 75 microliters 10% ammonium persulfate

18. Continue stirring for 20 seconds.
19 Draw up the acrylamide solution into a 5 cc syringe.
20. Connect a 27 gauge needle to the syringe and fill the space between the first set of glass plates with acrylamide. Repeat the procedure for the second set of plates.
21. Quickly insert the 10 well combs into the acrylamide and allow the gel to polymerize for at least 30 minutes.

**Electrophoresis**

1. Remove the samples (Sample Preparation, step #23) from the freezer and quickly thaw in a 37°C water bath.
2. Quickly thaw an aliquot of prestained molecular weight markers that have been prepared in Sample Buffer as directed by the manufacturer.
3. Place the samples in a boiling water bath for 2 minutes.
4. Remove the combs from the acrylamide and assemble the electrophoresis apparatus as directed by the manufacturer.
5. Add running buffer to the upper chamber until the buffer reaches a level halfway between the long and short glass plates.
6. Pour the remainder of the running buffer into the lower chamber until at least 1 cm of the gel is submerged.
7. Using a 5 cc syringe and a bent 18 gauge needle, gently flush the bottom of the gel with running buffer to remove any air bubbles from the bottom of the gel. Air bubbles along the bottom of the gel will cause local distortions in the electrical field and an irregular protein migration pattern.
8. Carefully flush the stacker wells with running buffer to remove any unpolymerized acrylamide.
9. Using a Hamilton syringe, load 10 µl of each sample into a separate well on each gel (see the loading scheme below) as follows:
   a. Insert the syringe through the running buffer and within 1-2 mm of the bottom of the well.
   b. Gently deliver the sample to the bottom of the well.
   c. Carefully remove syringe from the well so that the sample is not splashed into adjacent wells.
10. Loading Scheme:

    Lane 1 - Prestained Markers

Lane 2
Lane 3 - Prestained Markers
Lane 4 - Infected Cell Pellet
Lane 5 - Uninfected Cell Pellet
Lane 6 - Infected Cell Supernatant
Lane 7 - Uninfected Cell Supernatant
Lane 8 - Prestained Markers
Lane 9
Lane 10

11. Once both gels have been loaded, assemble the electrophoresis apparatus and connect the electrical leads to the power supply.
12. Electrophorese at 150 V (constant voltage) for 60 minutes or until the tracking dye is approximately 1 cm from the bottom of the gel.
13. Disassemble the apparatus and stain one gel with Coomassie Blue. The bands in the other gel will be transferred to nitrocellulose and developed using immunological reagents.

### Coomassie Blue Staining

1. Carefully remove the glass plates from the electrophoresis apparatus and remove one of the spacers from the sandwich.
2. Using the spacer, gently and carefully pry open the glass plates so that the gel does not tear.
3. Remove the gel from the plate and place it in a glass dish containing Coomassie Blue Staining solution.
4. Place the dish on a rotating platform and stain for 30 minutes at room temperature.
5. Carefully decant the Coomassie Blue solution and add enough Destain solution to cover the gel. Replace the dish on the rotating platform.
6. Continue to destain the gel with frequent changes of Destain solution until the background diminishes enough to distinguish the bands in the gel (1-3 hours).

### Western Transfer

1. When electrophoresis is completed, disconnect the power leads and disassemble the apparatus.
2. Soak two of the Transblot sponges in a 8.5" x 13" x 2" plastic pan filled about half full of degassed transfer buffer.
3. Set aside the top sponge but keep it wet.
4. Place an open cassette into the pan.

5. Place a sponge on the cassette.
6. Place a piece of wet 3 MM paper on the sponge.
7. Carefully open the gel sandwich and place the gel on the wet 3MM paper.
8. Carefully lay the gel on the wet filter paper. Squeeze out any air bubbles as you go or the transfer will not work.
9. Wet a piece of nitrocellulose (Millipore # HAHY 000 10, 0.45 μm) in transfer buffer. Wet the nitrocellulose by carefully rolling the paper onto the surface of the buffer. If any portion of the nitrocellulose does not wet, discard the sheet and try again. **Be sure to wear gloves when handling nitrocellulose paper.**
10. Carefully place the nitrocellulose paper on the gel and squeeze out any air bubbles. Do not move the paper around too much as you will get bands with smudges.
11. Wet a piece of 3 MM paper and place it on the nitrocellulose paper as before.
12. Place the remaining sponge on the 3 MM paper, close the cassette and lock it.
13. Quickly shift the cassette to the transfer chamber (don't let it dry). The cassette should be placed in the slot closest to the red terminal. *It is very important that the cassette is oriented so that the* ***nitrocellulose is closest to the red*** *terminal and that the* ***gel is closer to the black*** *terminal. If the orientation is reversed, the proteins will not transfer to the nitrocellulose.*
14. Connect the transfer chamber to the power supply and apply 25 volts for 60 minutes.
15. When the transfer is complete, remove the cassette and disassemble. Using a permanent marker or pen, trace the edge of the gel, marking the location of the top and the bottom of the gel.
16. Place the nitrocellulose in a pan containing enough blocking buffer (TSA/BSA) to cover the nitrocellulose.
17. Block the unadsorbed protein binding sites for 2 hours at room temperature. Alternatively, the blot can be blocked overnight at 2-8°C.

### Developing the Blot

1. Add 1.0 ml of rabbit anti-EMC virus serum to 25 ml of PBS-T. Filter the antibody solution through a 0.22 μm filter. This

solution can be reused 5-10 times, however, the solution should be filtered before each use to remove any protein aggregates.

2. Decant the Blocking Buffer from the blot.
3. Add the antiserum to the container.
4. React for 1 hour at room temperature using an orbital shaker.
5. Carefully remove the antiserum and store at -20°C until the next use.
6. Wash the blots three times for 3 minutes each by filling the containers about half full of PBS-T and placing them on an orbital shaker.
7. Add 0.25 ml of horseradish peroxidase-conjugated goat anti-rabbit IgG, 25 ml of Conjugate Buffer and filter through a 0.22 µm filter. This solution can be stored for up to 6 months at 2-8°C and reused 5-10 times. However, the conjugate solution solution should be filtered (0.22 µm) before each use to remove protein aggregates.
8. Decant the final wash solution and add the conjugate. Allow the conjugate to react for 1 hour as before.
9. Carefully remove the conjugate from the blot and store at 2-8°C.
10. Wash the blot 10 times with PBS-T. This step will consume 3-4 liters of PBS-T. Failure to thoroughly wash the blot will result in significant background coloration.
11. Make up fresh chromogen/substrate for each blot as follows:

***Chromogen Substrate***

- 42 mg 4-Chloro-1-naphthol (4C1N); (Sigma C-8890)
- 15 ml methanol
- 10 ml 10X PBS
- 80 ml deionized water
- 0.4 ml 3% hydrogen peroxide (Sigma H-6520)

Dissolve the 4C1N in 15 ml of methanol. Add the 10X PBS and the water. Mix thoroughly and add the hydrogen peroxide. This solution will remain stable for approximately 1 hour at room temperature.

12. Decant the last wash solution from the blot and add the chromogen substrate. Place the pan on an orbital shaker and gently rotate the vessel for approximately 15 minutes. Longer incubations will result in increased signal and increased background. Shorter incubation times may not allow visualization of all of the bands. **Do not use a metal pan.**

13. Decant the chromogen substrate and thoroughly wash the blot with deionized water.
14. Place the blot face down on a piece of Saran wrap. Place two pieces of 3MM paper on the blot and allow the blot to dry overnight in a 37°C dry air incubator. Alternatively, the Saran wrap/blot/3MM paper sandwich can be dried between the pages of a heavy book or catalog.

### Evaluation of Results

1. Carefully measure the distance between the top of the gel and the middle of each band. In addition, measure the distance between the top of the gel and the dye front.
2. Calculate the relative mobility (Rf) of each band as follows:

$$R_f = \textit{distance migrated by protein / distance migrated by dye}$$

3. Plot the molecular weights of the standards (in Kilodaltons) versus the $R_f$ to construct a standard curve.
4. Use the standard curve to determine the relative molecular weight of each of the bands on the gel.

## DISCUSSION QUESTIONS

1. Given the experimental data in Table 37.1, plot the molecular weights and $R_f$ values to obtain a standard curve. How do the experimental results compare with the expected molecular weights?

**Table 37.1.**
Experimental results obtained from PAGE analysis of EMC-infected $L_{929}$ cells.

| Band Visualized | Molecular Weight | Distance Migrated |
|---|---|---|
| Phosphorylase B | 106,000 | 5 |
| Bovine Serum Albumin | 80,000 | 7 |
| Ovalbumin | 49,500 | 12 |
| Carbonic Anhydrase | 32,500 | 18 |
| Soybean Trypsin Inhibitor | 27,500 | 23 |
| Lysozyme | 18,500 | 30 |
| Dye Front | | 40 |
| Band 1 | | 16 |
| Band 2 | | 18.5 |
| Band 3 | | 21 |
| Band 4 | | 25 |
| Band 5 | | 33 |

2. Why do the cell pellets and the cell free supernatant fluids exhibit different banding patterns?

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# 38

# ISOLATION OF VIRAL NUCLEIC ACIDS FROM VIRIONS

## INTRODUCTION

With the advent of modern molecular biology, it has become possible to study the gene structure of viruses in fine detail - down to the level of single base changes which can mark different plaque isolates of the same virus stock. DNA viruses can be used as vectors for the expression of entirely foreign genes from other viruses, bacteria, plants, or animals, by the insertion of the appropriate DNA into the vector virus genome; or site-specific mutagenesis techniques allow the creation of custom-designed variants of viral proteins. In this way genes and proteins can be studied in standardized systems, separated from the complexities of their normal organism, allowing their activities, interactions, and regulation to be more precisely defined.

To understand the genetics of a virus at the molecular level, it is necessary to study the following:

1. Does the virus have DNA or RNA? Is it single-stranded or double-stranded?
2. Does the virus have a linear or closed circular genome? Is it segmented or non-segmented?
3. Identify genes: coding regions, regulatory regions, introns and exons; identify mRNAs.
4. Nucleotide sequence of genes, amino acid sequence of proteins.

In this and the following two chapters, virus-specific nucleic acids which make up the viral genome or viral messenger RNAs (mRNAs) will be isolated from virions (this chapter) or cells (Chapter 39) and analyzed by gel electrophoresis (Chapter 40). These techniques will provide information on the size and number of segments of the viral genome, and the number and size of the various viral mRNAs. Isolation of these RNAs is a first step in the cloning of viral genes, i.e. creation of cDNA copies which are inserted into vectors for their replication or expression.

Precautions need to be emphasized for two aspects of these experiments:

First, the experiments involve work with radioactive compounds. Accordingly they should be performed under the supervision of a

person authorized by the institution to use these compounds, and with the awareness of the institutional office for radiation safety. The experiments make use of tritium, which is not a dangerous radioisotope and does not require extensive shielding or dosimetry precautions. However, tritiated nucleic acids, such as [$^3$H]-uridine used here, can be dangerous if ingested. **Handle materials containing tritium with care and avoid contamination of skin, clothing, or laboratory surfaces with the isotope.** All institutional policies regarding work with tritium must be observed. A properly labelled bag will be provided for disposal of solid materials contaminated with $^3$H (solid waste) and a plastic jar for disposal of liquid $^3$H waste.

Second, RNA is not dangerous but is very labile. Improper technique or use of contaminated reagents or pipets will introduce ribonucleases from the environment into the RNA preparation and destroy the RNA. Use only sterile, previously untouched plastic pipets, pipet tips, tubes, etc.. Methods for diethylpyrocarbonate treatment of glassware and aqueous solutions to eliminate ribonucleases are given in the Appendix.

## MATERIALS AND PREPARATION

### Day 1

- 2 sterile low-speed 50 ml disposable centrifuge tubes/person
- 1 100 ml sterile bottle/person
- (2)-175 cm$^2$ (Falcon) flasks of Vero cells, infected with NDV (3 pfu/cell) and [$^3$H]-U added (5μCi/ml) on previous day by the instructor/person
- 2 rubber policemen or cell scrapers (long handle)/person
- (2)-36 ml centrifuge tubes for Beckman SW28 rotor/person
- 10 or 25 ml pipets
- Ultracentrifuge and rotor, Beckman SW28 or equivalent
- sterile STE, chilled to 4°C (see Appendix)
- ice bucket
- disposable gloves
- plastic jar for liquid $^3$H waste disposal (with Osyl)
- bag for disposal of solid radioactive waste
- absorbent paper to cover benches used for $^3$H work
- balance (double pan)

**Day 2**

- Proteinase K solution (see Appendix)
- SDS solution (see Appendix)
- Phenol solution (see Appendix)
- Chloroform solution (see Appendix)
- sterile polypropylene centrifuge tubes with caps, 5 or 15 ml
- sterile pipets
- sterile DEPC-treated high-speed centrifuge tube, Corex or equivalent
- 100% EtOH
- low-speed centrifuge (e.g. 3,000 x g)
- sterile 3 M NaOAc solution, pH 5.2 (see Appendix)
- 37°C and 56°C water baths
- glass or polypropylene jar for disposal of waste phenol/chloroform

**Day 3**

- high-speed centrifuge, rotor (eg Sorvall HB-4) and adaptors for Corex tubes
- DEPC-treated sterile distilled $H_2O$
- 3 M NaOAc solution, pH 5.2
- sterile 1.5 ml microcentrifuge tubes
- 100% ethanol
- pipet tips and pipetors or equivalent
- UV spectrophotometer (optional)
- plastic disposable semimicro cuvette (optional)
- vial for liquid scintillation counting (optional)
- liquid scintillation fluid (e.g. National Diagnostics Ultrafluor or equivalent) (optional)
- liquid scintillation counter (optional)

## EXPERIMENTAL PROTOCOL

1. Each group will be provided with 2 large (175 $cm^2$) flasks of Vero cells (2 flasks per group to provide enough RNA). The flasks were previously infected with NDV 48 hr earlier, and 5 μCi/ml of [$^3$H] uridine was added to each. Each flask contains 36 ml of maintenance medium.

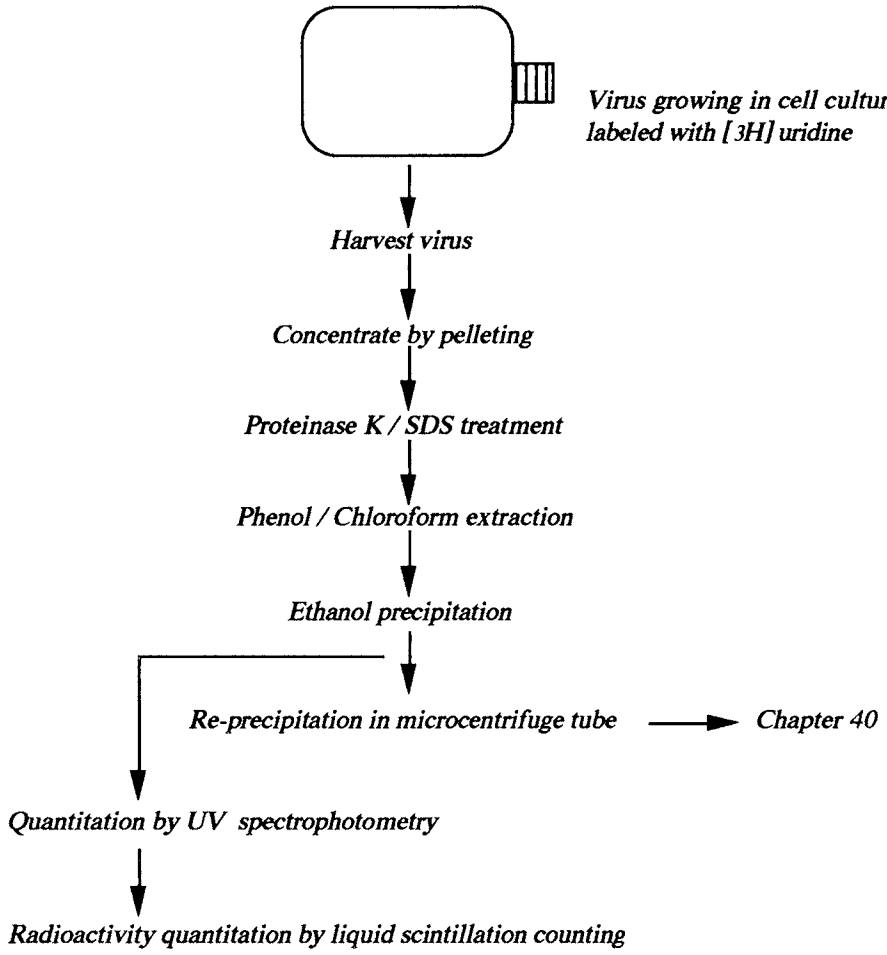


**Figure 38.1** Diagram of the RNA isolation procedure

2. Inspect the flasks under the inverted microscope to verify that the cell monolayer is completely lysed. If desired, a small aliquot of medium can be removed for HA assay. *Wear gloves.*
3. Using rubber policemen, scrape the remains of the cell monolayers and debris loose from the surface and into the medium in each flask.
4. Transfer the entire contents of each flask into a sterile 50 ml centrifuge tube. Put on ice until ready to centrifuge.
5. Centrifuge at 2000 x g and 4°C for 10 minutes, to pellet the cell debris.
6. Without disturbing the pellet, transfer the supernatant (containing virus) to a sterile bottle. Keep on ice. Supernatants from both

flasks may be pooled together. Pellets are to be disinfected by addition of Osyl, and discarded with solid radioactive waste.

*At this point virus particles will be pelleted from the medium by ultracentrifugation. The instructor will demonstrate and supervise the proper use of the ultracentrifuge and rotor. Depending on class size and number of ultracentrifuges and rotors available, it may be necessary to schedule ultracentrifuge runs at different times for different groups (1 run per 3 groups using SW28 rotor). Virus supernatants should be stored at 4°C until the time for ultracentrifugation, then proceed as follows.*

7. Transfer 36 ml of virus-containing supernatant to each of two ultracentrifuge tubes. Place each tube in a rotor bucket so that the two tubes will be on opposite sides of the rotor (e.g. for 6-bucket SW28 rotor, a group would use buckets #1 and 4, *or* #2 and 5, *or* #3 and 6). Balance the buckets by addition of sterile STE so that they are precisely equal in weight. Include each bucket's proper cap on the balance pan with the bucket. After weight adjustment, tighten the cap in place on the bucket. Throughout, be careful not to tip the buckets to avoid spillage of fluid.
8. Under the instructor's supervision, attach the buckets to the rotor at their proper positions. When the rotor is full, the instructor will supervise the loading and operation of the ultracentrifuge. To pellet the virus, spin (at 4°C) at 25,000 rpm (110,000 x g) for 2 hr, or 20,000 rpm (70,000 x g) overnight.
9. At the conclusion of the ultracentrifuge run, remove the buckets from the rotor under the instructor's supervision. Using forceps, pull out the tubes and observe the virus pellets at the bottom. Carefully decant the supernatant into the designated container for liquid radioactive waste without disturbing the pellets.
10. Resuspend the virus pellets by addition of cold sterile STE (0.8 ml per tube) . The pellets will be sticky. Do not touch the pipet tip to the pellet, but instead repeatedly pipet the STE up and down into the pellet until it is dispersed. (It will help if the pellet is left to sit in STE at 4°C for about 1 hour before pipetting up and down). After the 2 pellets are resuspended, pool them together in a sterile polypropylene tube. At this point the virus suspensions may be stored 1-2 days at 4°C.

**Day 2**

11. Add 0.2 ml of 2 mg /ml solution of proteinase K to the virus suspension. Mix gently (hand swirl) and incubate 15 minutes at 37°C. Proteinase K will destroy ribonucleases in the suspension.

12. Add 0.2 ml of 5% sodium dodecyl sulfate (SDS) solution to the virus suspension. Mix gently and incubate 30 minutes at 56°C. SDS will disrupt the virus particles, and is an inhibitor of ribonucleases.
13. Add 2 ml of buffer-saturated phenol to the virus suspension. Mix thoroughly by vortexing or rapid inversion (but avoid prolonged vortexing).

**NOTE**: *phenol is corrosive and will burn skin.* Handle with appropriate precautions. If spilled on skin or clothing immediately wash with running water for 10 minutes.

14. Leave tube upright until liquid has settled away from the cap, then add 2 ml of chloroform solution. (Use glass or polypropylene pipets or tips to dispense chloroform, not polystyrene). Mix thoroughly.

**NOTE**: *chloroform is flammable, highly volatile, a suspected carcinogen, and a skin irritant.* Do not inhale or contact it with skin. Handle with appropriate precautions.

15. Centrifuge at 3,000 x g for 5 minutes to separate the organic and aqueous phases. Transfer the tubes from the centrifuge to the bench without disturbing the phases. Observe the tubes. Cloudy or white material may be visible at the interface between the two phases.
16. Carefully remove the aqueous (top) phase by pipet, without disturbing the interface or the organic phase. Do not collect any cloudy material. Transfer the aqueous phase to a clean sterile centrifuge tube.

    If only a minimal volume (e.g. < 10-15%) of aqueous solution has been lost in this extraction sequence, it is desirable to repeat steps 13-16 to further clean the RNA. But if the volume loss was ≥ 20%, proceed instead to step 17 to avoid further loss.
17. Add 2 ml chloroform solution (no phenol) to the aqueous phase. Mix thoroughly, centrifuge, and collect the aqueous (top) phase as before, transferring it to a sterile 12 ml Corex tube. The final extraction with chloroform alone serves to remove any residual dissolved phenol from the aqueous phase. Used phenol/chloroform should be disposed of in the containers designated by the instructor for this purpose.
18. Determine the volume of the final aqueous suspension. Add to it 1/20 volume of sterile 3 M NaOAc solution and mix.

19. Add 2.5 volumes of 100% ethanol. Seal the tube tightly and mix thoroughly. Incubate the tube at -20°C until the next period (Day 3).

**Day 3**

20. Under the instructor's supervision, centrifuge the tube of RNA at 4°C in a high-speed centrifuge at 10,000 x g for 30 minutes. The centrifuge rotor *must be* properly balanced before starting the motor.
21. Remove the tubes and look for a small white pellet of RNA. Carefully decant or suction out the supernatant without disturbing the pellet.
22. Add to the tube 0.36 ml sterile DEPC-treated $H_2O$ and 40 µl of 3 M NaOAc solution. Resuspend the pellet by pipeting up and down.
23. Once it is completely resuspended, transfer the RNA solution to a sterile, siliconized 1.5 ml microcentrifuge tube (see Step 24 below). Add 1.0 ml of 100% ethanol, mix, and store at -20°C until Chapter 40 (analysis of viral nucleic acids by gel electrophoresis) is performed.

**Quantitation of RNA by optical density measurement. (OPTIONAL)**

24. Before addition of NaOAc or ethanol, remove 80 µl of RNA solution to a separate tube and dilute it to 400 µl with sterile $H_2O$.
25. Transfer this RNA solution to a microcuvette and determine its optical density at 260 nm and 280 nm in a UV spectrophotometer (blanked with water). The expected $OD_{260}$ will probably be between 0.01 and 0.1. Discard the RNA solution after measurement, or alternatively use it for step 28.
26. Calculate the quantity of RNA as follows: $[OD_{260}]$ x 40 µg/ml/OD unit x (400/80 dilution factor) x (360/80 total RNA volume) = _____ µg RNA. This is the total quantity of RNA which was precipitated in the centrifuge tube.
27. Calculate the $OD_{260}/OD_{280}$ ratio. This is an indicator of the purity of the RNA (i.e. the extent of contamination by protein). Ideally this ratio should be about 2. If the ratio is < 1.5, the RNA is still "dirty". If so, and if there is a relatively large amount of RNA (> 10 µg) based on the calculation in step 26 you may wish to pellet and resuspend your remaining RNA and repeat the phenol/chloroform extraction (steps 13-19) to clean it further.

28. (OPTIONAL) Transfer the RNA solution (step 25) from the microcuvette to a liquid scintillation counting vial. Add (for a 20 ml vial) 10 ml of liquid scintillation fluid, and shake vigorously. The instructor will demonstrate the use of the liquid scintillation counter. Using it, obtain counts per minute (cpm) emitted by your sample, correct for counting efficiency as indicated by the instructor to obtain disintegrations per minute (dpm), and calculate the activity (dpm per μg) of your labelled RNA. Dispose of the vial as instructed and in accordance with institutional policy for liquid scintillation vials.

NOTE: *many liquid scintillation cocktails are flammable and skin irritants.* Handle with appropriate precautions.

## DISCUSSION QUESTIONS

1. At the molecular level, how does the viral RNA become "labelled" in this experiment? What else will also be labelled? What is the danger to humans of [$^3$H]U contamination?
2. The virus particle is, in essence, a transport vehicle facilitating the movement of the viral genetic material from one host cell to the next. Can the isolated genetic material itself (in this case, the viral RNA) produce a viral infection? Discuss the requirements for this to occur.
3. Assuming that the viral RNA of NDV contains 15,300 nucleotides, and that one RNA is packaged per virus particle, calculate the number of virus particles required to supply the amount of RNA that you isolated (optional steps 24-27)? Are all these RNA strands identical? Why is this number an underestimate of the total viral RNA produced in these cell cultures?
4. What are sources of ribonucleases in the environment? Since these enzymes are considered ubiquitous, why don't they inactivate all virus particles with RNA genomes? How do steps 11-20 serve to "clean" the RNA?

## FURTHER READING

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# 39

# ISOLATION OF VIRAL NUCLEIC ACIDS FROM INFECTED CELLS

## INTRODUCTION

The essential principle followed here for isolation of viral RNA from cells is the same as for isolation of viral RNA from virions: the material containing the RNA is treated with proteinase K and SDS to degrade proteins and solubilize lipid membranes; then extracted with phenol/chloroform to separate these contaminants from the nucleic acid which localizes in the aqueous fraction; and finally the RNA is precipitated from solution with ethanol. The chief differences between virions and cells, as sources of viral RNA, are that 1) cells are complex bodies containing both nuclear and cytoplasmic compartments as well as other organelles, 2) very little of the nucleic acid in an infected cell is virus-specific, and 3) different classes of viral RNAs will be present in infected cells. In the case of NDV, a paramyxovirus with a genome consisting of a single strand of RNA of negative polarity (i.e. complementary to mRNA-sense), the NDV-infected cells will contain viral genomic RNA, full-length positive polarity RNA complementary to the genomic RNA, and several smaller messenger RNAs. The students will try to separate these forms in this experiment. The method follows that of Maniatis et al. (1982), which is modified from Favaloro et al. (1980). Products will be analyzed by gel electrophoresis in Chapter 40.

As in Chapter 38, use appropriate care and proper technique in the handling (1) of radioisotopes, because of the possibility of contamination; and (2) of RNA, because of its susceptibility to degradation.

## MATERIALS AND PREPARATION

**Day 1, per group:**

- 2 each 175 $cm^2$ (Falcon) tissue culture flasks of Vero cells set up by instructor, infected with NDV (MOI=5) and [$^3$H]U-labelled (20 $\mu$Ci/ml) on the morning of the experiment.
- 2 each rubber policemen or cell scrapers
- sterile PBS, ice-cold

- RNasin ribonuclease inhibitor (Promega Biotec) or equivalent
- 100 mM DTT solution
- Lysis buffer (see Appendix), ice-cold
- Lysis - sucrose buffer (see Appendix), ice-cold
- 1 ml syringe plus 1-1.5 in. needle
- 2 X PK buffer (see Appendix)
- Proteinase K solution (see Appendix)
- 2 each Corex (Corning) or equivalent, 12 ml capacity capped high speed centrifuge tube, sterile, siliconized
- ice bucket
- Phenol solution (see Appendix)
- Chloroform solution (see Appendix)
- 100% ETOH
- 3 M NaOAc solution (see Appendix)
- jar for disposal of liquid $^3H$ waste
- sterile polypropylene tubes, 15 ml
- bag for disposal of solid $^3H$ waste
- sterile pipets
- centrifuge, high speed, with swing-out rotor (e.g. Sorvall HB-4 or equivalent) and adaptors for Corex tubes

**Day 2**

- high speed centrifuge as for Day 1
- 70% ethanol
- sterile DEPC-treated distilled $H_2O$ (see Appendix)
- 2 X loading buffer (see Appendix)
- 1 X loading buffer (see Appendix)
- Eluting buffer (see Appendix)
- disposable small column (e.g. BioRad Econocolumn)
- Oligo-(dT) cellulose (available from Pharmacia, Sigma, others), suspended in eluting buffer
- pipets or pipetors and tips
- 100°C water bath (e.g. beaker of boiling water)
- 100% ethanol
- 3 each sterile siliconized Corex tubes, 12 ml
- 3 M NaOAc solution
- ringstand and clamp
- E. coli tRNA

### Day 3

- high speed centrifuge as for Days 1-2
- 70% ethanol
- sterile DEPC-treated $H_2O$
- sterile siliconized microcentrifuge tubes
- Poly (dT) solution, 0.5 mg/ml (see Appendix)
- 4X RNase H buffer (see Appendix)
- RNase H enzyme (available from BRL, New England Biolabs, others)
- 37°C water bath
- 100°C water bath
- Phenol solution (see Appendix)
- Chloroform solution (see Appendix)
- 3 M NaOAc solution (see Appendix)
- 100% ethanol
- 0.5 M EDTA (see Appendix)
- 5% SDS (see Appendix)
- pipets, pipetors and tips
- Optional:††Liquid scintillation counter, counting fluid, and vial as in Ch. 38.

## EXPERIMENTAL PROTOCOL

1. Each group will be provided with 2 large flasks of Vero cells. The flasks were infected earlier in the day with NDV, and labelled with 20 µCi/ml of [$^3$H] uridine. Observe the required precautions for handling of $^3$H (Ch. 38). Chill the flasks flat on ice for 10 minutes.
2. Remove the supernatants and discard them in the disposal jars for liquid $^3$H waste. Keep the cells cold during the procedure.
3. Wash the cells 3 times with ice-cold PBS. Following the last wash, add 5 ml of ice-cold PBS to each flask and scrape the cells into the PBS using rubber policemen or cell scrapers.
4. Transfer both cell suspensions to a pre-chilled sterile Corex centrifuge tube, and pellet the cells by centrifugation at 2000 x g for 5 minute at 4°C. Discard the flasks as solid $^3$H waste.
5. Discard the supernatant and, within the Corex tube, resuspend the cell pellets in 0.5 ml of cold lysis buffer. Vortex for 10 seconds,

then add 1000 units of RNasin and 10 µl of DTT solution to the suspension. Underlay the suspension with 0.5 ml of cold lysis-sucrose buffer, by carefully injecting the lysis-sucrose buffer beneath the cell suspension using a needle and syringe.

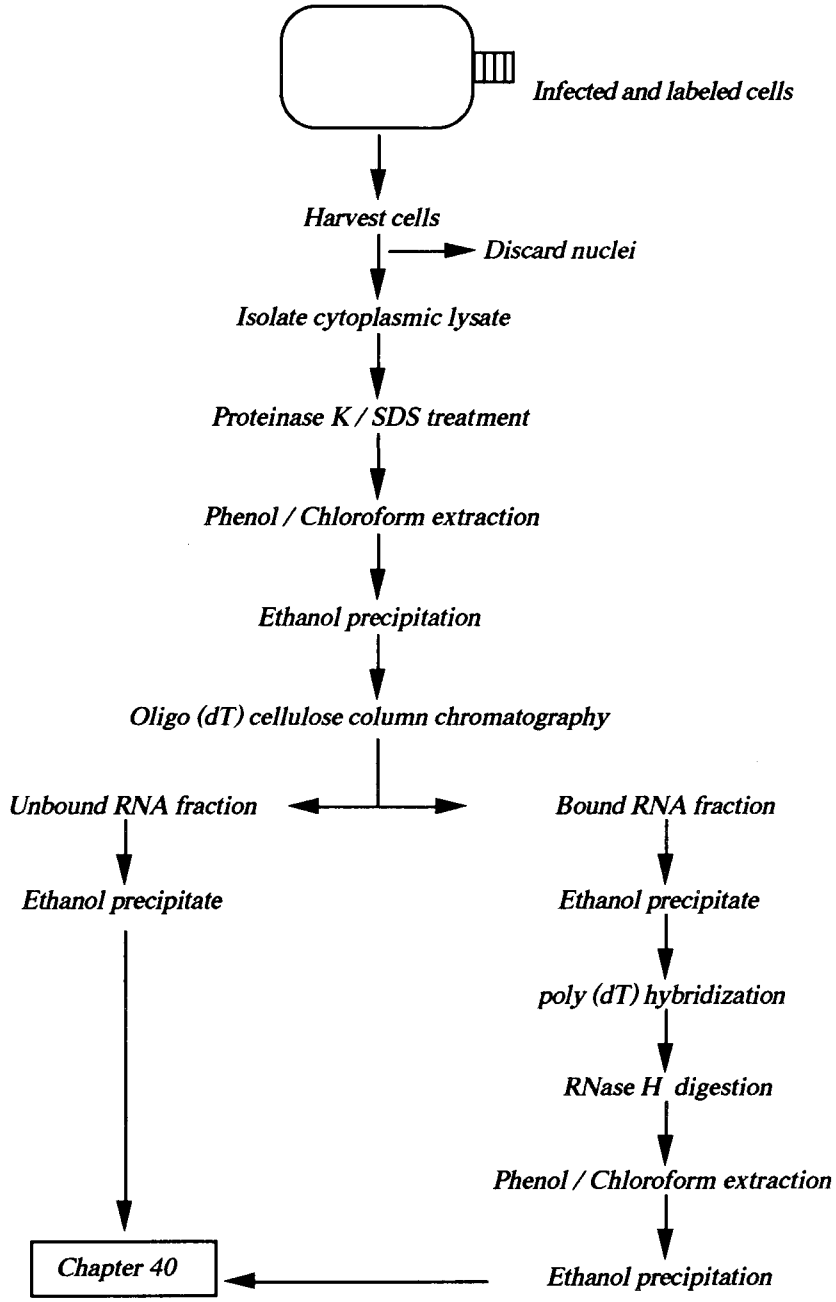


**Figure 39.1** Diagram of the RNA isolation procedure.

6. Without disturbing the two layers, incubate the tube on ice for 5 minutes. Then carefully transfer it to a pre-chilled high speed centrifuge with a swing-out rotor (e.g. Sorvall HB-4) and centrifuge at 4°C at 10,000 x g for 20 minutes.
7. Collect the supernatant, containing the cytoplasmic fraction of the cell lysate, leaving the pellet of cell nuclei undisturbed. (Remove the nuclear pellet later and discard it as solid $^{3}H$ waste).
8. To the cytoplasmic lysate add 1.0 ml of 2 X PK buffer and 0.2 ml of 2 mg/ml Proteinase K solution. Mix by hand swirling and incubate at 37°C for 30 minutes.
9. As described in Chapter 38, (steps 13-19), extract the cytoplasmic lysate with phenol and chloroform, and ethanol-precipitate cytoplasmic RNA, using a fresh Corex tube. Store in ethanol overnight at 20°C.

**Day 2**

10. Centrifuge the tube at 10,000 x g, 4°C, for 30 minutes to pellet the RNA. Carefully decant or suction out the supernatant without disturbing the pellet. Gently add 3 ml of cold 70% ethanol to the tube, and swirl gently, without disturbing the pellet, to wash the tube. This wash removes excess NaOAc. Then spin in the centrifuge at 10,000 x g for 10 minutes, and decant or suction off the supernatant.
11. If a vacuum lyophilization device is available, the tubes may be evaporated to dryness. Otherwise, leave for 15 minutes at room temperature with caps loosened to permit residual liquid to evaporate.
12. Resuspend the pellet in 0.75 ml of sterile DEPC-treated distilled water. Store the RNA at 4°C while oligo(dT) cellulose column is prepared.
13. Suspend the disposable column by clamp from a ringstand (see fig. 39.2). Wear gloves to avoid contaminating the body of the column with ribonucleases. Pour the column with oligo(dT) cellulose suspension, provided by the instructor, to a final bed volume of 1 ml.
14. Equilibrate the column by washing with 10 ml of 1 X loading buffer, letting the buffer drip out the bottom into a beaker . Permit all excess buffer to drain out of the column, but do not let the column dry. It is now ready for loading of the sample.

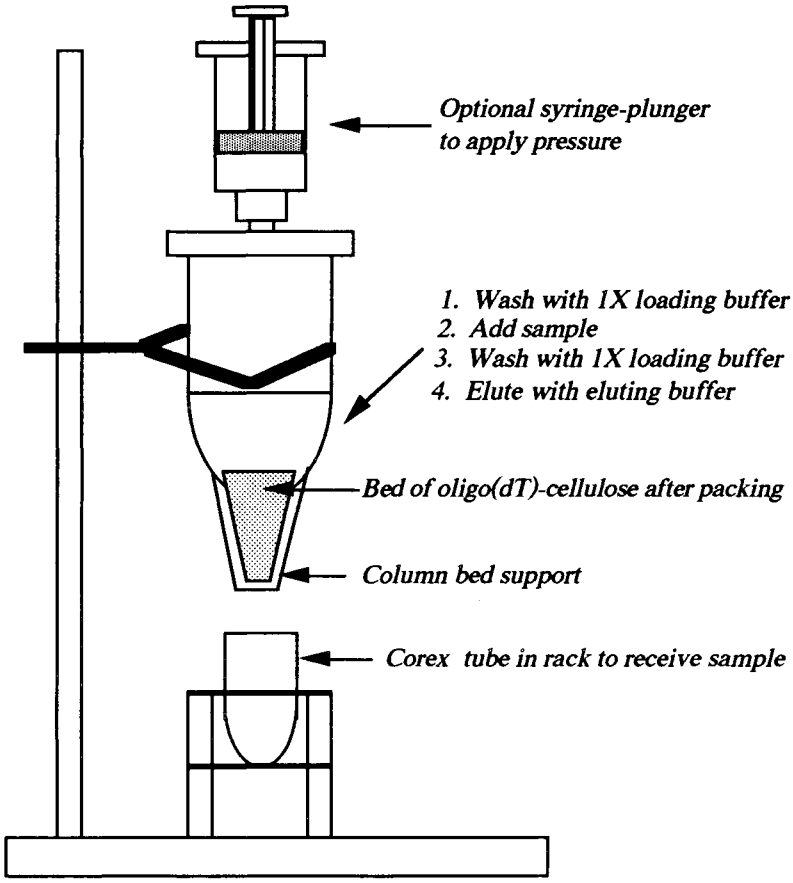


**Figure 39.2** Diagram of the oligo (dT) - cellulose column.

15. Incubate the RNA solution at 100°C for 5 minutes. Then cool to room temperature, and add an equal volume (0.75 ml) of 2 X loading buffer.
16. Load the RNA solution into the column, and collect the runoff into a sterile Corex tube. Return the runoff liquid to the column and pass it through twice more, for a total of 3 passes. Save the final runoff in the Corex tube.
17. Wash the column with 10 ml of loading buffer. The first 1-2 ml of this runoff will contain unbound RNA that was trapped in the column bed, and can be pooled with the runoff from (Step #16). This is the unbound RNA fraction. Add to it 2.5 volumes of 100% ethanol, mix, and store at -20°C until the next day. The remainder of the wash can be discarded.
18. Add 6 ml of eluting buffer to the column to elute the bound RNA. After flow-through, divide this eluate into 2 Corex tubes at 3 ml

per tube, and add to each tube 1/10 volume of 3 M NaOAc and then 2.5 volume of 100% ethanol. Mix and store at -20°C until the next day. The used column should be disposed as solid $^3H$ waste.

**Day 3**

19. Pellet the RNA from the tubes obtained in steps 16 and 18 by centrifugation at 4°C at 10,000 x g for 30 minutes in a high speed centrifuge. Wash the pellets with 70% ethanol, dry, and resuspend each in 100 µl of sterile DEPC-treated $H_2O$. Transfer to sterile microcentrifuge tubes.
20. (Optional) Take a small volume (5 µl) of both the bound and unbound RNA suspensions and determine their radioactivity by liquid scintillation counting as in Ch. 38 (step 28).
21. Store the remainder of the unbound RNA at -20°C for use in Ch. 40.
22. To the bound RNA solution, add the following:

    - 50 µl of poly(dT) solution (0.5 µg/µl),
    - 50 µl of 4 x RNase H buffer.

    Mix and heat to 100°C for 1 minute, then let cool at room temperature for 20 minutes.
23. Add 10 µl of RNase H enzyme solution, containing 0.5 units of enzyme, to the bound RNA/poly(dT). Mix gently by pipeting up and down (do not make bubbles) and incubate 30 minutes at 37°C. RNase H specifically cleaves the RNA strand of DNA:RNA hybrids.
24. Add 4 µl of 0.5 M EDTA and 4 µl of 5% SDS. Extract the solution with phenol and chloroform, as in Ch. 38. Following extraction, add 1/10 volume of 3 M NaOAc and 2.5 volumes of 100% ethanol to precipitate the RNA. Store at -20°C until use for Chapter 40.

## DISCUSSION QUESTIONS

1. What types of NDV virus-specific RNA are distinguished by oligo(dT) - cellulose chromatography? What is the molecular basis for this separation?
2. What is the purpose of the RNase H digestion steps (#22-23)? What products do you expect? RNase H was identified as one component activity of the holoenzyme reverse transcriptase, i.e. RNA-dependent DNA polymerase. If reverse transcriptase had

been used in step #23 instead of RNase H, what products would you expect? If steps #22-23 had been carried out on the unbound RNA (step #17), what products would you expect?

3. Only cytoplasmic nucleic acids were isolated in this experiment. What NDV virus-specific nucleic acids would you expect to find in infected cell nuclei? What if the infecting virus had been vaccinia? SV40? influenza?

## FURTHER READING

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# 40

# ANALYSIS OF VIRAL NUCLEIC ACIDS BY GEL ELECTROPHORESIS

## INTRODUCTION

Characterization of viral nucleic acids can be done at different levels of detail. With modern techniques of molecular biology it is possible to determine the complete nucleotide sequence of a nucleic acid such as viral RNA, which may contain several thousands of nucleotides. However, the most common characterization is done on the basis of nucleic acid size, or length. The length of a gene is a characteristic often sufficient by itself to identify the gene in a particlar experimental context. The principle of size separation is essential to current sequencing technology as well, as it "orders" the many unique cDNA fragments produced in sequencing reactions.

Size separation of nucleic acids is done by electrophoresis through a porous gel of polymerized agarose or polyacrylamide. An electric field is placed across the length of the gel. Nucleic acids, being acidic and thus negatively charged at neutral to basic pH, migrate through the gel in the direction of the positive pole. Longer nucleic acids are more retarded in their migration by the trapping effect of the gel matrix than are the smaller ones. Thus, the smaller nucleic acids migrate faster in a given length of time. A gel of $\geq$ 8% polyacrylamide can resolve fragments which differ in length by only 1 nucleotide, provided the total length of a fragment is no more than a few hundred nucleotides. Agarose gels cannot resolve to single-base specificity, but can be used to resolve fragments of several thousand nucleotides total length.

In this experiment the RNA products from Chapters 38 and 39 will be analyzed by size distribution, so as to determine the relative sizes of virion RNAs, viral mRNAs, and positive-strand template RNAs used as replicative intermediates. An agarose gel system will be used so as to resolve the various-size RNAs on the same gel. RNA bands will be blotted to nylon membranes for autoradiography. This is an essential preliminary step in the process of "Northern blotting", although the hybridization steps of Northern blotting will not be done.

## MATERIALS AND PREPARATION

### Day 1 (see Appendix for all solutions)

- Microcentrifuge
- 70% ethanol (cold)
- sterile DEPC-treated $H_2O$
- gel loading buffer
- 65°C water bath
- sterile microcentrifuge tube
- horizontal gel electrophoresis box and comb
- formaldehyde-agarose gel (prepared by instructor, see Appendix)
- gel running buffer, 10 X and 1 X
- micropipetor (eg Rainin P20, P100 Pipetman)
- RNA molecular weight marker (eg Boehringer Mannheim type 1 or equivalent), 0.3 µg/µl in gel loading buffer

### Day 2

- ruler
- knife, eg carving knife
- glass tray (length ≥ length of gel)
- chromatography paper (eg Whatman 3MM paper)
  - 1 piece cut to gel size in one dimension but extra long in the other dimension, to serve as wick (Step #8)
  - 2 pieces cut to gel size (Step #11)
  - 1 piece larger than gel size (Step #15)
- nylon transfer/hybridization membrane (eg DuPont/NEN Gene-screen Plus, Stratagene Duralon-UV, others)
- transfer buffer
- tray (reservoir for transfer buffer) on which to assemble blot
- paper towels
- Weight (0.5 - 1 kg)

### Day 3

- sterile forceps
- dry-heat oven, 80°C
- autoradiography enhancer spray (eg DuPont/NEN EN$^3$HANCE spray)

- dark room
- X-ray film cassette
- X-ray film (eg DuPont Cronex or equivalent)
- intensifying screen (eg DuPont Cronex Lightning Plus or equivalent)
- Ultralow temperature freezer, -70°C

**Day 4**

- X-ray film processor or developing solutions

## EXPERIMENTAL PROTOCOL

### Day 1

1. Recover the viral RNA from Ch. 38, and bound and unbound RNA from Ch. 39, by centrifugation in a microfuge at 10,000 x g for 30 minutes. Wash the pellets with 70% ethanol, centrifuge at 10,000 x g for 10 minutes, then remove the supernatants and dry the pellets as in Ch. 38.
2. Resuspend the RNA samples in a minimal volume (10-20 µl, as needed) of sterile $H_2O$.[*Optional*: take 1 µl of the bound and unbound intracellular RNA samples and obtain $^3H$ counts as described in Ch. 38.]
3. Mix 6 µl of each RNA sample with 24 µl of gel loading buffer in a sterile microcentrifuge tubes. Incubate at 65°C for 15 min to denature the RNA, then cool on ice.
4. Formaldehyde-agarose gels were prepared in advance by the instructor (see Appendix). The proper way to load samples onto the gel will be demonstrated. Each group will be assigned 4 wells: 3 for the RNA samples and 1 for the molecular weight marker. Wash the assigned wells of the gel by forcefully expelling running buffer into them. Be careful not to damage the well! Load each of your denatured RNA and marker (30 µl) samples onto the gel as directed, and remember which lane contains which sample. Don't disturb the gel once samples have been loaded.
5. When all samples have been loaded by each group using that gel, connect the gel to the power supply. The RNAs should be loaded at the end connected to the black electrode (cathode), so that they migrate in the direction of the red electrode (anode). Turn on the power supply so that it provides constant voltage of 1 volt per cm of gel length, and let run overnight (see fig. 40.1).

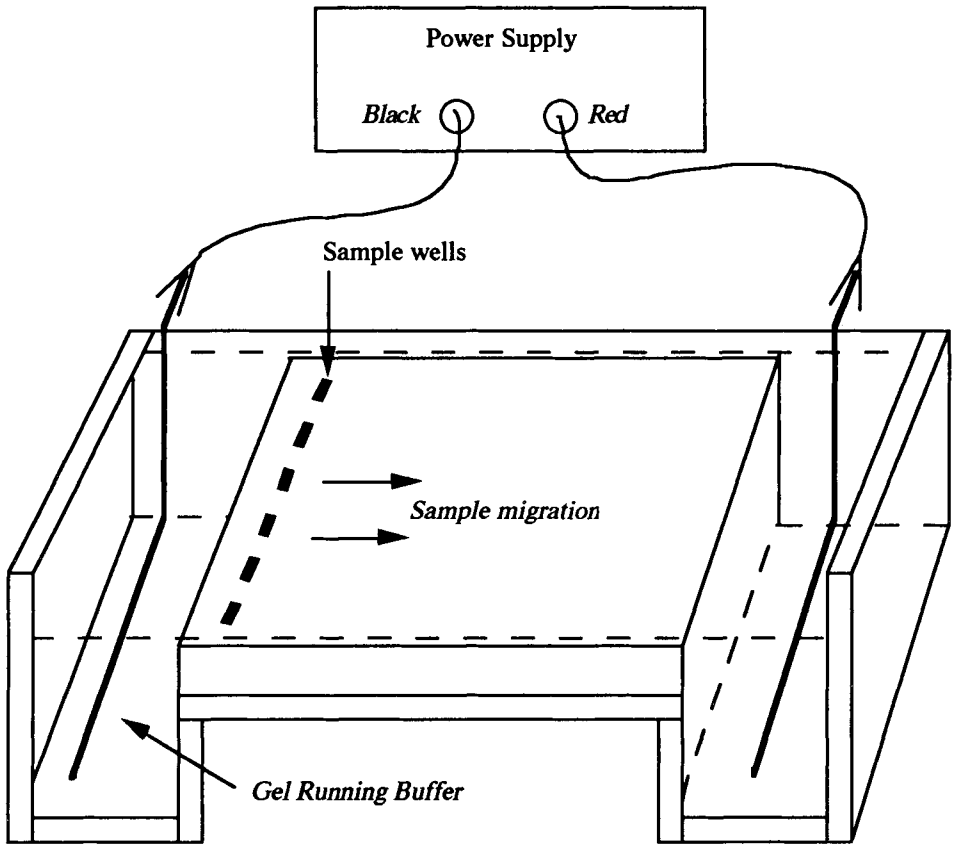


**Figure 40.1** Diagram of the agarose gel electrophoresis.

## Day 2

6. Stop the run when the bromphenol blue dye present in the gel loading buffer has migrated 90% of the gel length. Under the instructor's supervision, expose the gel to UV light (**wear protective goggles or glasses**), and measure the positions of the molecular weight marker bands relative to the origin using a ruler. If sample RNA bands are visible, measure their position also. If equipment is available, the gel may be photographed under UV illumination.
7. Under the instructor's supervision, use a long knife (such as a carving knife) to cut a lengthwise slice of the gel which completely contains your sample lanes. Carefully slide your gel slice onto a glass tray and remove it from the electrophoresis box.
8. Wearing gloves, set up the gel slice for blotting as follows : the wick of chromatography paper, pre-cut to the width of the gel

slice and pre-soaked in transfer buffer, is laid over the glass plate so that its two ends hang free on either side. Position the glass plate so that it sets over a tray containing transfer buffer. The ends of the wick must lay in the buffer (Figure 40.2).

9. The gel slice is carefully slid into place atop the wick. Wet the plate, wick, or gel with transfer buffer for lubrication as necessary. There must be no air bubbles between the wick and the gel.

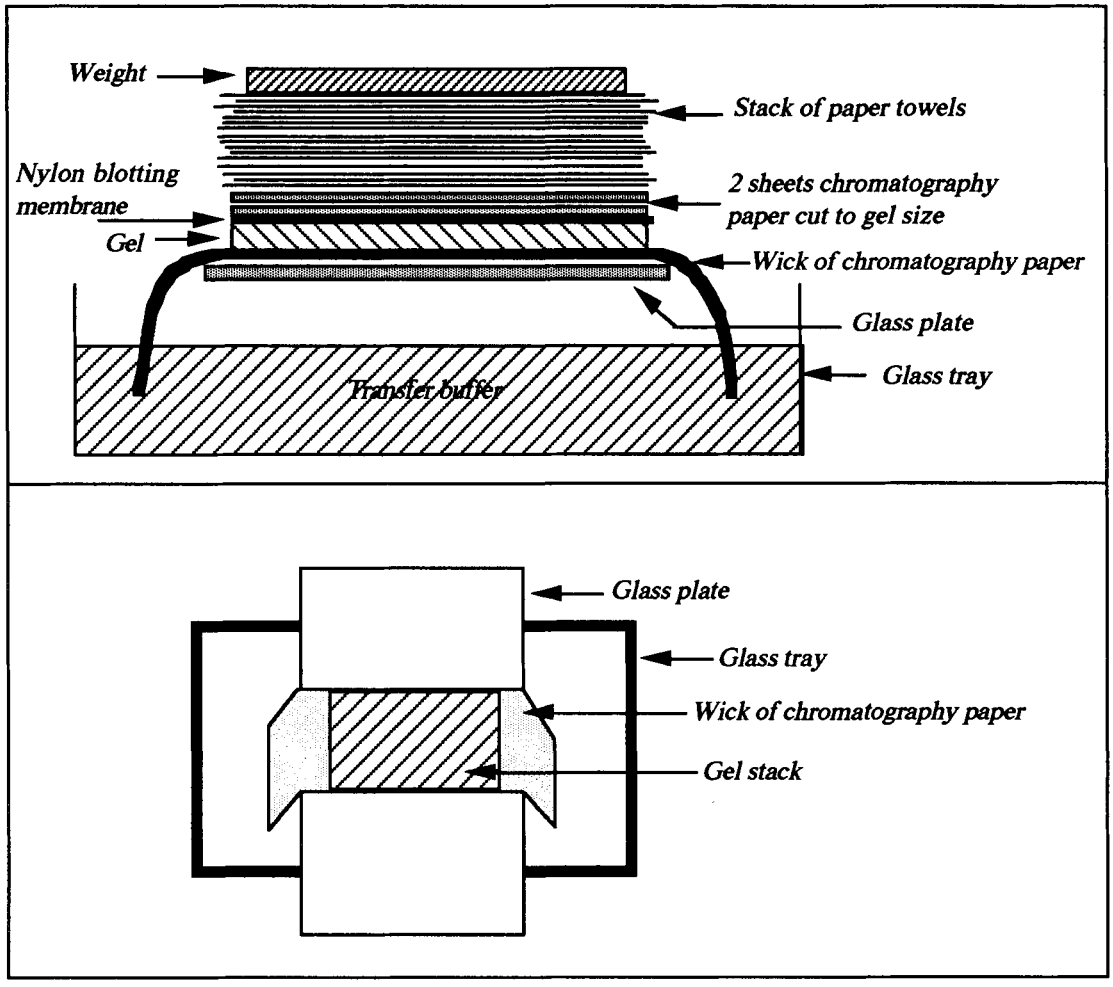


**Figure 40.2** Diagram of blot transfer of RNAs from agarose gel to nylon membrane. The top panel is a side view of the blot transfer of RNA from the agarose gel to the nylon membrane. The bottom panel is a top view.

10. The nylon blotting membrane, pre-cut to the exact size of the gel slice and pre-soaked according to manufacturer's directions, is laid squarely atop the gel slice. There must be no air bubbles between the gel and the membrane. It is helpful to wet the top of the gel with transfer buffer, then lay down the membrane starting from one end so that its weight squeezes out bubbles. Once it is laid, a plastic pipet can be used as a rolling pin if necessary to squeeze out any remaining bubbles.
11. Lay 2 pieces of chromatography paper (pre-cut to the exact size of the gel and pre-soaked in transfer buffer) atop the membrane, again with care to avoid trapping air bubbles.
12. Lay a 10 cm stack of paper towels atop the chromatography paper. It is helpful if they are pre-cut to size, but if not, it is essential that they do not contact the gel itself, the wick, or the glass plate. Uncut paper towels can be propped up around the gel using plastic pipets or other plastic objects.
13. Lay a heavy weight, 0.5 - 1 kg, such as a book, small brick, or jar atop the stack, propped and balanced as necessary to keep from falling over.
14. Fill the bottom tray with transfer buffer, and let the blotting apparatus set overnight. It will be helpful to occasionally remove the wet paper towels from the bottom of the stack, to facilitate capillary movement of buffer.

**Day 3**

15. At the end of the overnight transfer period, carefully dismantle the set-up. Handling the blot with sterile forceps, lay it on a dry piece of chromatography paper, RNA side up, and transfer it to a dry-heat oven. Bake for 2-4 hours at 80°C to fix the RNA to the blot.
16. Remove the blot from the oven. Lay it on a clean sheet of paper and spray thoroughly with an autoradiography enhancer spray. Let air dry 10 minutes. Spray lightly 3 coats letting the membrane dry between coats and rotating the membrane 90° between coats to obtain a more even coating of the surface.
17. In a dark room, set up the treated blot for autoradiography in an x-ray film cassette, with x-ray film and intensifier screen (eg DuPont Cronex Lightning-Plus or equivalent). Lay the blot in the cassette first, RNA side up; then the film, then the screen, coated side down. Close the cassette so that it is tight and sealed against light. Store at -70°C overnight.

### Day 4

18. Develop the film and look for the appearance of RNA-specific bands. If under- or over-exposed, put a new film in the cassette and expose for a shorter or longer time as needed.
19. Once a suitable autoradiograph has been obtained, the blot should be disposed as solid $^{3}H$ waste.

## DISCUSSION QUESTIONS

1. In this experiment, how would you distinguish virus-specific RNAs from cellular RNAs?
2. Are any cellular RNAs detected in the RNA preparation from virions? If so, why might they have been there?
3. What kinds of virus-specific RNAs did you expect to find in each of the three RNA preparations? What did you actually find?
4. What sizes of virus-specific RNAs did you find? These may be estimated from a standard curve, made by plotting the log [number of bases] of the RNA molecular weight markers versus the log[distance from origin] that they migrated. This curve should be approximately linear. Cellular 28S (approximately 4.7 kb) and 18S (approximately 1.9 kb) ribosomal RNAs may also be used as size markers. What conditions might affect the linearity of a nucleic acid migration curve?
5. What is the Northern blot hybridization technique, and how does it differ from the experiment that you did (Ch. 38-40)? Design a set of Northern blot experiments to probe specifically for each kind of virus-specific RNA.
6. In this gel system, formaldehyde serves to "denature" RNA. What is the difference between a denatured and non-denatured nucleic acid? Why were denaturing conditions used here? What is an example of an experiment where non-denaturing conditions would be preferred?

## FURTHER READING

Kingsbury, D.W. 1990. Paramyxoviridae and their replication. In *Virology* (B. Fields, D. Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, B. Roizman, eds) 2nd edition, Raven Press, New York, pp. 945-962.

Selden, R.F. 1987. Analysis of RNA by Northern hybridization. In *Current Protocols in Molecular Biology* (F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl, eds), Greene Publishing and Wiley Interscience, New York, pp. 4.9.1-4.9.8.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77:5201-5205.

# APPENDIX A - Viruses and Cell Lines

The number of cell lines and viruses used in this manual were kept to a minimum for the sake of continuity and simplicity. Only two viruses and three cell types are used throughout this text.

## *VIRUSES*

**Encephalomyocarditis (EMC) virus**; murine; picornavirus. Available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. Catalog # VR-129B.

**Newcastle Disease virus, (NDV)**; paramyxovirus. Available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. Catalog # VR-108.

## *CELL LINES*

**$L_{929}$ cells**; mouse connective tissue; strain L and clone 929. Available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. ATCC-CCL 1.

**Vero cells**; kidney, from African green monkey; fibroblast-like. Available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. ATCC-CCL 81.

**BHK-21 cells**; baby hamster kidney cells. Available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852. ATCC-CCL 10.

# APPENDIX B - Media and Solutions

### Eagle's Minimum Essential Medium (E-MEM)

This medium may be purchased from a number of suppliers in a liquid or powder form. The liquid is shipped sterile while the powder form must be rehydrated and filter-sterilized prior to use. Penicillin and streptomycin are added to the sterile medium to a final concentration of 100 units penicillin and 100 µg streptomycin per ml of medium.

- E-MEM powder (Gibco# 410-1500)
- Penicillin-Streptomycin solution (Penicillin 10,000 units/ml & Streptomycin 10,000 µg/ml - Gibco# 600-5140)
- glass-distilled water

Place 3960 ml of distilled water and a stirring bar in a 4 liter erlenmeyer flask. Add 40 ml of the penicillin-streptomycin solution, and 38.44 g of the E-MEM powder. Dissolve completely and filter sterilize. Using sterile technique aliquot in sterile bottles.

### Serum

Serum from fetal or adult origin is available from commercial sources. The quantity ordered will vary with the requirements of a given laboratory. Once received from the supplier, the serum should be transferred, using sterile technique, into smaller sterile bottles. These should be heat inactivated in a 56°C water bath for 30 minutes and then kept frozen at -20°C until needed for use.

### 10 % Sodium bicarbonate solution

- 100.0 grams sodium bicarbonate ($NaHCO_3$)
- 1 liter glass distilled water

Dissolve the sodium bicarbonate in enough distilled water to make 1000 ml. Filter sterilize and dispense in sterile bottles. Store at room temperature.

A 7.5% sodium bicarbonate solution is available commercially from Gibco (#670-5080). This can be used instead of the 10% solution described above.

## Thioglycollate medium

- 29.5 grams thioglycollate medium (BBL #11260)
- 1000 ml glass distilled water
- screw capped tubes (16 x 125 mm) or cotton plugged test tubes

Place the thioglycollate medium in a 2 liter erlenmeyer flask. Add the 1000 ml glass distilled water and a stirring bar. Cover loosely with aluminum foil and heat, with stirring, to boiling. Continue to boil for 1 minute to completely dissolve the powder. Dispense 10 ml into each tube and cap loosely. Autoclave for 15 minutes. Cool the tubes, tighten the caps, and store at room temperature.

## 1 N HCL

- concentrated hydrochloric acid (HCL); 12 M, specific gravity 1.18, 36 percent by weight
- glass distilled water

Dilute the 12 N HCL in the glass-distilled water to obtain 1 N HCL.

**NOTE:** Concentrated HCl can cause severe burns. Perform dilution in a fume hood and observe necessary precautions.

## Hanks' Balanced Salt Solution (HBSS)

- 0.35 grams sodium bicarbonate ($NaHCO_3$)
- 1 liter glass distilled water

Solution A

- 1.00 grams D-glucose
- 0.01 grams phenol red
- 0.06 grams potassium phosphate ($KH_2PO_4$)
- 0.09 grams sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)

Solution B

- 0.14 grams calcium chloride, anhydrous ($CaCl_2$)
- 0.40 grams potassium chloride (KCl)
- 8.00 grams sodium chloride (NaCl)
- 0.10 grams magnesium chloride ($MgCl_2 \cdot 6H_2O$)
- 0.09 grams magnesium sulfate ($MgSO_4 \cdot 7H_2O$)

Dissolve the components of solution A in 450 ml of glass distilled water. Do the same for solution B. Mix the 2 solutions, add the sodium bicarbonate, and bring the total volume to 1 liter with glass-distilled water. Make sure that all the components are in solution and filter-sterilize. The final pH should be around 7.4.

This solution is available commercially as a liquid or powder form. The liquid is shipped sterile whereas the powder has to be reconstituted and filter-sterilized.

Store at room temperature.

### HBSS *without* calcium or magnesium

As with the regular HBSS, this formulation is available from a number of vendors in a liquid or powder form.

### 0.5% trypsin in HBSS without calcium or magnesium

- 100 ml trypsin - (a) commercially available as 2.5% (10x) (Gibco #610-5090) sterile solution, or (b) by adding 25 grams trypsin powder (Difco,1:250) per liter of normal (0.85%) saline. This should be filter-sterilized, aliquoted in sterile bottles (in 100 ml quantities), and stored frozen at -20°C.
- 400 ml HBSS *without* calcium or magnesium

Thaw 100 ml of the trypsin. Using sterile technique, add 400 ml of the HBSS without calcium and magnesium. Mix and dispense in small sterile tubes (5.0 ml per tube). Store at -20°C.

### Trypan blue stain (0.4%) for viable counts

- Trypan blue stain - 0.2 grams
- Normal (0.85%) saline - 50 ml

Dissolve the trypan blue in 50 ml normal saline. Filter and aliquot in 5-10 ml quantities. Store at room temperature. This is available commercially as a 0.4% trypan blue solution (Gibco #630-5250).

## RPMI 1640

This medium can be obtained commercially in a liquid or powder form. The liquid form is shipped sterile as a 1x or 10x concentration. The powder form has to be reconstituted using glass distilled water and filter sterilized. This is kept frozen at 4°C.

The medium used for cell culture contains RPMI 1640 to which the following components are added: serum (FBS), 2 mM L-Glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

## $NH_4Cl$ Lysing Buffer

- 0.83 grams $NH_4Cl$
- 0.10 grams $KHCO_3$
- 100 ml glass distilled water

Dissolve the ingredients in the glass distilled water. Filter sterilize and store at 4°C.

## 2-Mercaptoethanol (2-ME)

- 2-mercaptoethanol (2-ME); (Sigma chemical) molarity-14.3 M, density-1.114 g/ml
- glass-distilled water

Prepare a 1 M solution of 2-ME in glass distilled water by adding 0.5 ml of the 2-ME to 6.6 ml glass-distilled water

Dilute 5 ml of the 1 M 2-ME solution in 95 ml of glass-distilled water, to obtain a $5 \times 10^{-2}$ M solution. Filter sterilize and store at 4°C. This is the stock solution. When added to the spleen cell culture it is used at a final concentration of $5 \times 10^{-5}$ M.

**NOTE:** *Concentrated 2-ME is toxic* and has a strong odor. Use a chemical hood and automatic pipetor.

## Glycine-HCl buffer, pH 3.0

*0.2M glycine solution:*

- glycine ($C_2H_5NO_2$), F W 75.07 (Sigma #G 6340)
- glass-distilled water

Add 1.50 g glycine to 100 ml of glass-distilled water. Stir to dissolve.

*pH 3.0 glycine-HCl buffer:*

- 0.2 M glycine solution (see above)
- 0.2 N HCl
- glass-distilled water

Add 25 ml of 0.2 M glycine solution to 5.7 ml of 0.2 N HCl. Bring to a final volume of 100 ml with glass-distilled water. Test the pH.

## Tryptose phosphate broth (TPB)

- 20.0 grams tryptose powder
- 2.0 grams dextrose
- 5.0 grams NaCl
- 2.5 grams $Na_2HPO_4$
- 1 liter glass distilled water

In a 2 liter flask, with gentle heat and stirring, dissolve all ingredients in 900 ml of glass distilled water. When all the components are dissolved bring the volume up to 1000 ml with the glass distilled water. Cover with foil and autoclave. Cool, aliquot into sterile bottles, and store at 4°C.

## Gelatin-lactalbumin hydrolysate (GLB)

- 5.0 grams gelatin (Gibco #M00210B)
- 2.5 grams lactalbumin hydrolysate (Gibco #670-1800)
- 1000 ml HBSS
- 1.0 ml penicillin-streptomycin solution (Gibco #600-5140, penicillin 10,000 units/ml and streptomycin 10,000 µg/ml)
- sterile 7.5% soldium bicarbonate solution

Add the gelatin, lactalbumin hydrolysate, and the HBSS to a 1 liter Erlenmeyer flask. Stir with gentle heat until the gelatin is completely dissolved and the medium is clear. Aliquot 99 ml into each of ten glass bottles and sterilize by autoclaving. Store at room temperature.

*Before use:* Sterilly add 0.1 ml of the penicillin-streptomycin solution per 100 ml of GLB (final concentration of penicillin of 100 units/ml and for streptomycin of 100 µg/ml). Adjust the pH with the sterile sodium bicarbonate solution until the phenol red indicator is a cherry red color. Store at 4°C.

## Collodion

This may be obtained as a liquid from Mallinckrodt. If the liquid solidifies, dissolve the solidified material in 1 part ether and 1 part 95% ethanol until a viscosity similar to that of glycerol is obtained.

## Phosphate Buffered Saline (PBS), Dulbecco's

- NaCl, Sigma #S-9625 8.00 g
- KCl, Sigma #P-4504 0.20 g
- $Na_2HPO_4$, Sigma #S-0876 1.15 g
- $KH_2PO_4$, Sigma #P-5379 0.20 g
- glass-distilled water

Weigh all the components and place in a 1 liter erlenmyer flask. Add 1 liter glass-distilled water and a stirring bar to the flask. Mix until completely dissolved. Check pH (must be 7.2-7.4). Label and store at room temperature. This is not sterile.

## Methyl Cellulose Overlay Medium, 0.5%

- Methyl cellulose; 4,000 centipoise viscosity (Fisher #M-88)
- HBSS
- E-MEM
- Penicillin (10,000 units/ml)/Streptomycin (10,000 µg/ml); Gibco #600-514

1. Add 5.0 g methyl cellulose to a clean 2 liter flask.

2. Add 150 ml boiling HBSS to the 2 liter flask. Swirl to dissolve as much of the methyl cellulose as possible.
3. Add a magnetic stir bar to the flask and autoclave immediately for 20 minutes and 15 p.s.i..
4. Cool the solution to 37°C.
5. The solution is now sterile. Use sterile procedures to add the following components (each warmed to 37°C) to the flask:

| | |
|---|---|
| HBSS | 40 ml |
| E-MEM | 800 ml |
| Pen.-Strep. | 10 ml |

6. Mix thoroughly on a stir plate.
7. Dispense 98 ml per sterile bottle.
8. Cool rapidly at 4°C with intermittent swirling to allow the methyl cellulose to dissolve. Allow 20 minutes for the solution to become clear.

   Note: Methyl cellulose may be stored at 4°C or -20°C. Thaw frozen methyl cellulose at 4 °C. Methyl cellulose hardens at 37°C.
9. Add 2.0 ml fetal bovine serum per 98 ml methyl cellulose before use as an overlay medium for virus plaque experiments.

### 5% Neutral Red Solution

- Neutral red, Sigma #N-6634
- PBS

Prepare a 5% neutral red solution in PBS, filter-sterilize, and store wrapped in foil at room temperature.

### STE Buffer - Ch. 30

- 0.15 M NaCl (Sigma S-9625) 8.77 g
- 10 mM Tris base, pH to 7.4 with HCl (Sigma T-1503) 1.21 g
- 1 mM EDTA,, free acid, anhydrous (Sigma E-6758) 0.38 g
- distilled water 1000 ml

Mix the above components and adjust the pH to 7.4 with HCl.

### 25% Sucrose - Ch. 30

- 25 g sucrose crystal, Baker Analyzed Reagent, J.T. Baker Chemical Co., # 4072-05
- 100 ml STE

Prepare a 25% sucrose solution in STE by combining the above. Autoclave at 121°C for 15 minutes and 15 p.s.i.

### Coating Buffer: carbonate-bicarbonate buffer (pH 9.6) - Ch. 30

- $Na_2CO_3$ 1.59 g
- $NaHCO_3$ 2.93 g
- distilled $H_2O$ 1000 ml

Combine the above and test the pH.

### PBS-Tween 20 (0.05%), pH 7.4 - Ch. 30

- NaCl 8.0 g
- $KH_2PO_4$ 2.9 g
- $Na_2HPO_4 \cdot 12H_2O$ 2.9 g
- KCl 0.2 g
- Tween 20 (Sigma # P-1379) 0.5 ml

Combine the above and test the pH.

### Phosphate-citrate buffer (pH 5.0)

- 0.1 M citric acid (19.2 g per 1000 ml $H_2O$) 24.3 ml
- 0.2 M phosphate (28.4 g $NaHPO_4$ per 1000 ml) 25.7 ml
- $H_2O$ 50.0 ml

Combine the above and test the pH.

### 1 % BSA

- bovine serum albumin 1.0 g
- PBS 99.0 ml

**Substrate (ortho-phenylenediamine) - Ch. 30**

- ortho-phenylenediamine 40.0 mg
- phosphate-citrate buffer 100.0 ml
- 30% $H_2O_2$ 40.0 µl

**NOTE:** *o-phenylenediamine is mutagenic*, and light sensitive. It must be protected from light and made up fresh immediately before use.

**Reaction stopping solution - Ch.30**

Dilute concentrated $H_2SO_4$ with glass-distilled water.to obtain 2.5 M $H_2SO_4$

**NOTE:** Concentrated sulfuric acid can cause severe burns. Use a chemical hood and appropriate precautions.

**2 N perchloric acid - Ch. 32**

- from 60% $HClO_4$ (92.3 g/1000 ml - 9.2 M, Specific gravity - 1.539
- distilled water

Add 43.5 ml 60 % perchloric acid plus 156.5 ml distilled water.

- from 70% $HClO_4$ - Specific gravity - 1.67
- distilled water

Add 34.2 ml of 70 % perchloric acid plus 165.8 ml distilled water.

**5 N KOH - Ch. 32**

- 33 gm of 85% KOH pellets
- glass-distilled water

Add the KOH pellets in a volumetric flask and bring the volume to 100 ml with glass-distilled water.

## 0.1 N HCl - Ch 32

- 0.83 ml of conc. (12 N ) HCl
- glass-distilled water

In a chemical hood, add the concentrated HCl in a volumetric flask and bring the volume to 100 ml with distilled water.

## 1X Lysis Buffer for Immunoprecipitation - Ch. 36

| Final Concentration | Amount Added |
|---|---|
| 50 mM Tris Base; Sigma T-1503 | 0.61 g |
| 0.15 M NaCl; Sigma S-9625 | 0.88 g |
| 1.0 mM EDTA; Sigma E-5134 | 0.03 g |
| 0.5% Sodium Deoxycholate; Sigma D-6750 | 0.50 g |
| 0.1% Sodium Dodecyl Sulfate; Sigma L-4390 | 0.10 g |
| 1.0% Nonidet P-40 (NP-40); Sigma N-3516 | 1.00 ml |
| 0.1% Gelatin*; Sigma G-1890 | 1.00 ml |
| 0.1% Sodium Azide; Sigma S-2002 | 0.10 g |
| 0.1% Trasylol (Aprontinin); Sigma A-6012 | 0.10 ml |
| Glass-distilled Water | 80.00 ml |

1.Stir to dissolve.
2. Adjust pH to 8.0 with 5 N NaOH
3. Adjust the volume to 100 ml with additional distilled water
4. Store at 4°C for up to 6 months.

*Gelatin should be added from a 10% stock solution. This stock solution is made by adding 1 gram of gelatin to 10 ml distilled water. The gelatin is then solubilized by autoclaving for 20 minutes at 15 $lb/in^2$. Prior to addition to the lysing buffer, the gelatin can be liquefied by heating in a water bath or in a microwave oven.

## TNE-Gelatin Buffer - Ch. 36

| Final Concentration | Amount Added |
|---|---|
| 50 mM Tris Base; Sigma T-1503 | 0.61 g |
| 0.15 M NaCl; Sigma S-9625 | 0.88 g |
| 1.0 mM EDTA; Sigma E-5134 | 0.03 g |
| 0.1% Gelatin*; Sigma G-1890 | 1.00 ml |
| 0.1% Sodium Azide; Sigma S-2002 | 0.10 g |
| Distilled Water | 80.00 ml |

1. Stir to dissolve.
2. Adjust pH to 8.0 with 5 N NaOH
3. Adjust the volume to 100 ml with additional distilled water.
4. Store at 4°C for up to 6 months.

*Gelatin should be added from a 10% stock solution. This stock solution is made by adding 1 gram of gelatin to 10 ml distilled water. The gelatin is then solubilized by autoclaving for 20 minutes at 15 $lb/in^2$. Prior to addition to the lysing buffer, the gelatin can be liquefied by heating in a water bath or in a microwave oven.

## 20X Transfer Buffer - Ch. 37

- $Na_2HPO_4$, Anhydrous; (Fisher # 5374-500) 60.35 g
- $Na_2PO_4 \cdot H_2O$; (Fisher 5369-500) 10.33 g
- distilled water 1000 ml

Mix the components. The resulting pH should be 7.5 (nonsterile).

## PBS-T

- 10X PBS 300 ml
- 10% Tween-20 7.5 ml
- distilled water 700 ml

Mix all the components. Make this solution fresh daily.

## 10X PBS

- $Na_2PO_4 \cdot 7H_2O$ 26.8 g
- $NaH_2PO_4$, anhydrous; (Sigma S-9625) 13.8 g
- NaCl 75.9 g
- distilled water 1000 ml

Mix all the components, adjust pH to 6.9 and filter sterilize.

## Acrylamide/bisacrylamide

- acrylamide; (Sigma A-9099) 150.0 g

- N,N'-methylene-bisacrylamide; (Sigma M-2022) 4.0 g
- deionized water 500.0 ml

Mix the components and filter through a 0.45 µm Millipore filter. This can be stored at 4°C for up to 6 months.

**NOTE: Acrylamide is a neurotoxin.** Always wear gloves when handling acrylamide. A mask should also be worn when weighing powdered acrylamide.

## 1.875 M TRIS

- Tris base; (Sigma T-1503) 113.4 g
- deionized water 400 0 ml
- concentrated HCl

Mix the Tris base and the deionized water, and stir to dissolve. Adjust to pH 8.8 with concentrated HCl and then adjust the volume to 500 ml with additional deionized water. Store at 4°C for up to 6 months.

## 1.0 M TRIS

- Tris base; Sigma T-1503) 24.2 g
- deionized water 170.0 ml
- concentrated HCl

Mix the Tris base and the deionized water, and stir to dissolve. Adjust pH to 6.8 with concentrated HCl and then adjust volume to 200 ml with additional deionized water. Store at 4°C for up to 6 months.

## 0.2 M EDTA

- EDTA; (Sigma E-5134) 7.44 g
- deionized water (37°C) 100.00 ml

Stir to dissolve. Warm this solution to 37°C to dissolve the EDTA. Filter through a 0.45 µm membrane and store at 4°C for up to 8 months.

## Sample Buffer (SABU) - Ch. 36-37

| | | |
|---|---|---|
| • 1 M Tris, pH 6.8; (Sigma T1503) | 0.625 | ml |
| • glycerol; (Fisher G-33) | 1.00 | ml |
| • 0.2% bromphenol blue; (Sigma B-7021) | 0.20 | ml |
| • 0.2 M EDTA; (Sigma E-5134) | 150.0 | µl |
| • deionized water | 6.65 | ml |
| • 2-mercaptoethanol; (Sigma M-3148) | 0.40 | ml |
| • 20% (w/v) SDS; (Sigma L-4390) | 1.00 | ml |

## 5% Acrylamide Stacking Gel

- 1.25 ml acrylamide/bisacrylamide solution
- 0.94 ml 1.0 M Tris/HCl, pH 6.8
- 0.75 ml 0.2 M EDTA
- 5.15 ml distilled water

Set up the 5% acrylamide stacking gel by placing the reagents into a 125 ml sidearm suction flask. Place a stopper on the 125 ml sidearm flask, connect to a suction source, and degas with constant stirring for 15 minutes at room temperature.

After the stacking gel has degassed for 15 minutes. Turn off the suction and carefully remove the suction tubing and the stopper from the sidearm suction flask. Continue stirring the acrylamide solution.

Quickly add:

3.8 µl TEMED
75 µl 10% ammonium persulfate

Continue stirring for 20 seconds.

**NOTE: Acrylamide is a neurotoxin.** Always wear gloves when handling acrylamide. A mask should also be worn when weighing powdered acrylamide.

## 15% Acrylamide Resolving Gel

- 5.0 ml acrylamide/bisacrylamide solution
- 2.0 ml 1.875 M Tris/HCl, pH 8.8
- 0.1 ml 0.2 M EDTA
- 2.0 ml distilled water

To make the 15% acrylamide resolving gel, place the above reagents in a 125 ml sidearm suction flask containing a small stir bar. Place a stopper on the flask, connect the flask to a suction source, and degas with constant stirring for 15 minutes at room temperature.

After degassing for 15 minutes, turn off the suction and carefully remove the suction tubing and the stopper. Continue stirring the acrylamide solution.

Quickly add:

5 µl TEMED (N,N,N',N'-tetramethylethylenediamine)
0.1 ml 10% Ammonium persulfate in distilled water

Continue stirring for 20 seconds.

**NOTE: Acrylamide is a neurotoxin.** Wear gloves when handling acrylamide. A mask should be worn when weighing powdered acrylamide.

## Running Buffer - Ch.37

- 3.03 g Tris base (Sigma T-1503)
- 1.0 g sodium dodecyl sulfate (SDS); (Sigma L-4390)
- 14.4 g glycine; (Sigma G-4392)
- 1000 ml deionized water

Combine the reagents and use as indicated in the experimental protocol.

## Coomassie Blue Staining Solution

- 5 ml 1.0% Coomassie Blue R-250 in 95% Ethanol; (Sigma B-0149)
- 20 ml 95% ethanol; (Fisher 9229-1)
- 5 ml glacial acetic acid; (Fisher A38-212)
- 20 ml distilled water

Combine the reagents and use, as indicated, in the experimental protocol.

### Destaining Solution

- 250 ml isopropyl alcohol; (Mallinckrodt UN 1219)
- 70 ml glacial acetic acid; (Fisher A38-212)
- 680 ml deionized water

Combine the reagents and use, as indicated, in the experimental protocol.

### TSA/BSA Blocking Buffer

- 6.6 g Tris HCl; (Sigma T-3253)
- 0.97 g Tris base; (Sigma T-1503)
- 9.0 g NaCl; (Sigma S-9625)
- 10.0 g bovine serum albumin (BSA), essentially fatty acid free; (Sigma A-7030)
- 1.0 g sodium azide; (Sigma S-2002)
- 1000 ml deionized water

Dissolve the reagents in 900 ml of water with constant stirring. Adjust the pH to 7.4 with 1.0 N HCl. Add enough deionized water to bring the final volume to 1.0 liter. Store at 2-8°C for up to 6 months.

### Conjugate Buffer

- 10.0 ml 10X PBS
- 10.0 ml fetal bovine serum; (Hyclone # A-1115L)
- 0.3 ml gentamycin sulfate (10 mg/ml); (Gibco # 600-571OAD)
- 10.0 mg thimerosal; Sigma # T-5125)
- 80 ml deionized water

Adjust pH to 7.4 with acid or base as necessary. Store at 2-8°C for up to 1 year.

### Chromogen Substrate

- 42 mg 4-Chloro-1-naphthol (4C1N); (Sigma C-8890)
- 15 ml methanol

- 10 ml 10X PBS
- 80 ml deionized water
- 0.4 ml 3% hydrogen peroxide; (Sigma H-6520)

Dissolve the 4C1N in 15 ml of methanol. Add the 10X PBS and the water. Mix thoroughly and add the hydrogen peroxide. This solution will remain stable for approximately 1 hour at room temperature.

## Rabbit Antiserum To EMC Virus

### Virus Preparation for Immunization

1. Seed a 150 $cm^2$ tissue culture flask with $L_{929}$ cells.
2. When the cells are confluent, infect them with 1.0 ml of a $10^{-3}$ dilution of stock EMC virus in GLB. After adsorption at 37°C for 1 hour, remove the virus inoculum, and add 20 ml of E-MEM-2% FBS.
3. Incubate at 37°C for 20 hours.
4. Freeze-thaw the flasks three times to release intracellular virus.
5. Centrifuge the cell culture fluids at 600 x g for 15 minutes to remove cell debris.
6. Overlay the cell culture fluids onto a 30% sucrose cushion established in an ultracentrifuge tube.
7. Centrifuge the suspension at 100,000 x g for 2 hours at 4°C to pellet the virus.
8. Resuspend the virus pellet in 1.0 ml of saline.
9. Determine an ultraviolet absorption reading at $A_{260}$ to determine the virus concentration. The ultraviolet absorption spectrum of concentrated EMC virus has a maximum at 259 nm and a minimum at 239 nm (Rueckert and Pallansch, 1981). Utilizing an extinction coefficient of 77 for a 1% solution of virus (Rueckert, 1971), the concentration of virus in milligrams per milliliter is $A_{260}$ divided by 7.7.
10. Store at -70°C until used for immunization.

Rueckert, R.R. and M.A. Pallansch. 1981. "Preparation and characterization of encephalomyocarditis (EMC) virus. In, *Methods in Enzymology*, Volume 78, Part A, "Interferons". S. Pestka, (ed.), Academic Press, New York, pp. 315-325.

Rueckert, R.R. 1971. In, *Comparative Virology.* K. Maramorosch and E. Kurstak, (eds.), Academic Press, New York, pp. 255-306.

### Immunization Schedule

Week 1 - 100 µg EMC Antigen in Complete Freund's Adjuvant injected subcutaneously (S.C.) over the left hindquarter of the rabbit..

Week 2 - 100 µg EMC Antigen in Incomplete Freund's Adjuvant injected S.C. over the left foreleg.

Week 3 - 100 µg EMC Antigen in Incomplete Freund's Adjuvant injected S.C. over the right hindquarter.

Week 4 - 100 µg EMC Antigen in Incomplete Freund's Adjuvant injected S.C. over the right foreleg.

Bleed the rabbit on week 6.

Allow the blood to clot overnight at 2-8°C. Separate the clot from the side of the tube, centrifuge tube at 600 x g, and carefully remove the serum with a pipet. Aliquot and store the serum at -20°C.

## SOLUTIONS FOR CHAPTERS 38-40

### Ribonuclease-free materials and solutions:

For experiments involving RNA, it is essential that care be taken to avoid introduction of ribonucleases into RNA preparations. Ribonucleases will be present in any substance in which living cells have been present, e.g. solutions contaminated with bacteria, or pipet tips that were touched by fingers. Ribonucleases may be remarkably resistant to treatments that inactivate other enzymes, such as boiling or autoclaving. Thus when a reagent solution is prepared, autoclaving may sterilize it and yet fail to inactivate ribonucleases that were introduced in chemicals, water, or spatulas.

To avoid ribonuclease contamination, the following guidelines should be followed:

1. Wear gloves when handling RNA solutions or when handling any object that may come into contact with RNA solutions, e.g. Corex tubes or microcentrifuge tubes.
2. Heat-resistant solids such as spatulas or glassware (but not Corex tubes) may be baked at 250°C for 4 hours to inactivate ribonucleases. Before baking they should be wrapped in paper or otherwise covered in some heat-resistant container, for storage, so that they will remain free from contamination until actually used.

3. It is desirable to purchase separate chemicals specifically for reagents for RNA work. These chemicals should be handled only with sterile baked spatulas and while wearing gloves, so that ribonucleases are not introduced into the chemical bottle itself.
4. Other materials such as Corex tubes should be treated with a solution of 0.1% diethylpyrocarbonate (DEPC) in water for at least 12 hours at 37°C, then emptied, covered, and autoclaved. DEPC is an inhibitor of ribonucleases. After treatment, traces of DEPC must be removed by heating or autoclaving.
5. Aqueous solutions (but not Tris solutions) should also have DEPC added to 0.1% final concentration and incubated for 12 hours before autoclaving. When this is not practical, e.g. when making a small quantity of a buffer, use reagents and sterile distilled water which were themselves DEPC-treated.
6. Sterile disposable plasticware such as pipets, tips, or centrifuge tubes are normally free from ribonucleases and can be used without treatment. Avoid inadvertent contamination.
7. When it is not possible to rid an object of ribonucleases by these methods, an alternative is to include a ribonuclease inhibitor in the RNA solution. Examples of such inhibitors are:
   - human placental ribonuclease inhibitor, e.g. RNasin (Promega Biotec)
   - vanadyl ribonucleoside complex (available from, e.g. BRL)
   - SDS detergent, up to 1% concentration

The following stock solutions will be required in order to make others listed below.

### Ethidium bromide solution (per 1.0 ml)

- 10 mg ethidium bromide
- 1.0 ml sterile DEPC-treated $H_2O$

Mix to dissolve. Store at 4°C in covered tube.

**NOTE:** *ethidium bromide* is a suspected *carcinogen.*

### EDTA, 0.5 M, pH 8.0

- 93.06 g Na2 EDTA•$2H_2O$
- 350 ml distilled $H_2O$

Stir while measuring pH. Add and dissolve, 1 at a time, NaOH pellets. Bring pH to 8.0 (by then all EDTA should be dissolved). Bring to 500 ml final volume with $H_2O$. Autoclave to sterilize. Store at room temperature.

### KCl, 1 M

- 7.46 g KCl
- distilled water

Dissolve and bring to 100 ml with distilled $H_2O$. Autoclave to sterilize. Store at room temperature.

### $MgCl_2$, 0.5 M

- 10.16 g $MgCl_2 \cdot 6H_2O$
- distilled water

Dissolve and bring to 100 ml with distilled $H_2O$. Autoclave to sterilize. Store at room temperature.

### NaCl, 5 M

- 146 g NaCl
- distilled water

Dissolve and bring to 500 ml with distilled $H_2O$ (heating may be necessary). Autoclave to sterilize. Store at room temperature.

### Tris·HCl, 1 M, pH 7.5 and pH 8.0

- 60.6 g Tris base
- DEPC-treated distilled water

Dissolve Tris base in 400 ml DEPC-treated distilled $H_2O$[a]. Adjust pH to 7.5 using conc. HCl[b]. Bring volume to 500 ml. Sterilize by autoclaving. Store at room temperature.

[a] Tris solutions cannot be DEPC-treated to remove ribonucleases.

(b) Tris buffers exhibit very large shifts in $pK_a$ with changing temperature (-0.03 pH units per 1°C). Thus, different Tris solutions should be made and adjusted to required pH at the intended temperature of use (pH 7.5: 4°C, 22°C, 37°C; pH 8.0: 4°C). Addition of HCl will release heat and tend to raise the buffer temperature during adjustment.

## Sodium dodecyl sulfate (SDS), 5% w/v

- 25 g SDS
- distilled water

Dissolve in $H_2O$ and bring to 500 ml final volume. Filter through 0.22 μm filter to sterilize. Store at room temperature.

## Binding buffer, 1 X (per 100 ml) - Ch.38-40

- 10 mM Tris•HCl (pH 7.5 at 22°C) : 1.0 ml of 1 M stock solution
- 0.5 M NaCl : 10 ml of 5 M stock solution
- 1 mM EDTA : 0.2 ml of 0.5 M stock solution
- 0.05% SDS : 1.0 ml of 5% stock solution
- sterile DEPC-treated $H_2O$ : 87.8 ml

## Binding buffer, 2 X (per 50 ml) - Ch. 38-40

- 20 mM Tris•HCl (pH 7.5, 22°C) : 1.0 ml of 1 M stock solution
- 1.0 M NaCl : 10 ml of 5 M stock solution
- 2 mM EDTA : 0.2 ml of 0.5 M stock solution
- 0.1% SDS : 1.0 ml of 5% stock solution
- sterile DEPC-treated $H_2O$ : 37.8 ml

SDS may precipitate in the high-salt solution. If so, heat slightly to dissolve.

## Chloroform solution

- 24 parts chloroform, reagent grade
- 1 part iso-amyl alcohol

Store at room temperature in tightly-capped bottle in fume hood.

### 100 mM DTT solution (per 1.0 ml)

- 15.43 mg dithiothreitol (Cleland's reagent)
- 1.0 ml sterile DEPC-treated $H_2O$

Store at -20°C; discard after 1 month

### Eluting buffer (per 100 ml) - Ch. 38-40

- 10 mM Tris•Cl (pH 7.5, 22°C) : 1.0 ml of 1 M stock solution
- 1 mM EDTA : 0.2 ml of 0.5 M stock solution
- 0.05% SDS : 1.0 ml of 5% stock solution
- sterile DEPC-treated $H_2O$ : 97.8 ml

### 70% Ethanol (for RNA washes)

- 70 ml 100% ethanol
- 30 ml distilled $H_2O$

Combine the reagents and store at -20°C.

### Formaldehyde-agarose gel

The formaldehyde-agarose gel is prepared as follows (for a 20 x 20 cm gel; scale up or down as needed):

**NOTE:** *Formaldehyde is toxic.* Prepare and pour the gel in a fume hood. Thereafter it can be loaded and run on a benchtop, except if persons severely allergic to formaldehyde are involved.

1. Mix: 4.2 g agarose (electrophoresis grade)
   35 ml 10 X MOPS running buffer (see Appendix)
   260 ml sterile DEPC-treated $H_2O$
   Boil to dissolve, and cool to 56°C.
2. Add 55 ml of 37% formaldehyde (pH > 4) and 35 µl of ethidium bromide solution. Mix well without forming bubbles for 1 minute.
3. Pour the gel in a horizontal agarose gel electrophoresis box according to manufacturer's directions, with comb in place, and let cool 1 hour at room temperature.

4. Fill the buffer trays of the gel box with 1 X gel running buffer (see Appendix), until the buffer completely covers the gel to a depth of 3 mm.
5. Carefully remove the comb by pulling it straight upward; do not damage any wells. The gel is now ready for use.

### Gel loading buffer (per 10 ml) - Ch. 38-40

- 5.6 ml deionized formamide
- 1.0 ml 10 X MOPS running buffer
- 2.0 ml 37% formaldehyde
- 1.0 ml sterile glycerol
- 10 mg Bromphenol blue
- 10 µl Ethidium bromide solution

Combine the reagents and store at 4°C.

### Lysis buffer (per 50 ml) - Ch. 38-40

- 0.14 M NaCl : 1.4 ml of 5 M stock solution
- 1.5 mM $MgCl_2$ : 0.15 ml of 0.5 M stock solution
- 10 mM Tris•Cl (pH 8.0, 4°C) : 0.5 ml of 1 M stock solution
- 0.5% NP-40 : 0.25 ml of 100% NP-40
- sterile DEPC-treated $H_2O$ : 47.7 ml

Combine the reagents andstore at 4°C.

### 10 X MOPS running buffer (per 1 liter) - Ch. 38-40

- 0.2 M MOPS : 41.86 g free acid
- 0.05 M sodium acetate : 6.8 g of trihydrate
- 0.005 M EDTA : 1.86 g Na2 EDTA•$2H_2O$
- distilled water : 1 liter

Dissolve the first three reagents above in 900 ml $H_2O$. Adjust the pH to 7.0. Bring to 1 liter total volume. DEPC treat. Autoclave to sterilize. Store at room temperature.

## 1 X MOPS running buffer - Ch. 38-40

- 1 part 10 X MOPS running buffer
- 9 parts sterile DEPC-treated $H_2O$

Combine the above.

## Lysis-sucrose buffer - Ch. 38-40

Make solution of 48 g sucrose plus distilled $H_2O$ to 100 ml final volume. Autoclave to sterilize.

per 50 ml of buffer: Combine reagents and store at 4°C.

- 0.14 M NaCl : 1.4 ml of 5 M stock solution
- 1.5 mM $MgCl_2$ : 0.15 ml of 0.5 M stock solution
- 10 mM Tris•HCl (pH 8, 4°C) : 0.5 ml of 1 M stock solution
- 0.5% NP-40 : 0.25 ml of 100% NP-40
- 24% sucrose : 25 ml of 48% sucrose (see above)
- sterile DEPC-treated $H_2O$ : 22.7 ml

## Phenol buffer, saturated

**NOTE**: observe warnings on bottle

1. Melt a bottle of redistilled phenol (available from BRL, Sigma, others) at 65°C.
2. Add an equal volume of $H_2O$, mix, let stand 10 minute for phases to separate.
3. Carefully remove most of the aqueous (top) phase by decanting or aspiration.
4. Repeat steps 2,3 using 50 mM Tris base (6 g Tris in 1000 ml $H_2O$, do not adjust pH).
5. Repeat steps 2,3 using 20 mM Tris•HCl pH 8.0 (20 ml of 1 M Tris stock solution, pH 8.0 at room temp, plus 980 ml $H_2O$). Repeat until pH of aqueous phase (after mixing and separation) is 7.5-8.0.
6. Store in brown or covered bottle at 4°C for 1 month. Discard sooner if phenol turns pink.

### 2 X PK buffer (per 50 ml)

- 0.2 M Tris•HCl pH 7.5 (37°C) : 10 ml of 1 M stock solution
- 25 mM EDTA : 2.5 ml of 0.5 M stock solution
- 0.3 M NaCl : 3.0 ml of 5 M stock solution
- 2% SDS : 20 ml of 5% stock solution
- sterile DEPC-treated $H_2O$ : 14.5 ml

### Poly(dT) solution (per 1 ml)

- 0.5 mg poly(dT) (available from Pharmacia, others)
- 1.0 ml sterile DEPC-treated $H_2O$

Pipet up and down to dissolve. Store at -20°C.

### Proteinase K solution, 2 mg/ml

Dissolve 20 mg Proteinase K (available from BRL, Boehringer Mannheim, Sigma, others) in 10 ml $H_2O$. Aliquot and store at -20°C.

### 4 X RNase H buffer (per 1.0 ml)

- 100 mM Tris•Cl (pH 7.5, 37°C) : 0.1 ml of 1 M stock solution
- 0.4 M KCl : 0.4 ml of 1 M stock solution
- 40 mM $MgCl_2$ : 80 µl of 0.5 M stock solution
- 1 mM DTT : 10 µl of 100 mM stock solution
- sterile DEPC-treated $H_2O$ 0.41 ml

Combine the above and store at 4°C.

### RNase H enzyme dilution buffer (per 1.0 ml)

Prepare the following buffer:

- 4 X RNase H buffer : 0.25 ml
- Sterile DEPC-treated $H_2O$ : 0.25 ml
- Glycerol, sterilized by autoclaving : 0.5 ml

Dilute the enzyme, as-supplied, to a concentration of 0.5 U per 10 µl, using the above buffer as diluent. Store the diluted enzyme at -20°C until use. When brought out for use, keep on ice.

### Sodium acetate (NaOAc), 3 M, pH 5.2

- 204 g sodium acetate•$3H_2O$
- distilled water
- glacial acetic acid

Add the sodium acetate to a flask. Add distilled water to a final volume of 450 ml and dissolve. Adjust pH to 5.2 using glacial acetic acid. Bring to 500 ml volume with distilled water. Autoclave to sterilize. Store at room temperature.

### STE solution - Ch. 38-40

- 10 mM Tris•HCl pH 7.5 ( 4°C) : 10 ml of 1 M stock solution
- 100 mM NaCl : 20 ml of 5 M stock solution
- 1 mM EDTA : 2 ml of 0.5 M stock solution
- sterile DEPC-treated $H_2O$ : 968 ml

Mix the above and store at room temperature.

### Transfer buffer : 10 X SSC (per 1 liter) - Ch. 38-40

- 3 M NaCl 175.5 g NaCl
- 0.3 M sodium citrate 88.2 g of dihydrate
- distilled water 1.0 liter

Add the first two components and bring to 950 ml with $H_2O$. Adjust the pH to 7.0. Bring to 1 liter final volume. DEPC-treat. Autoclave to sterilize. Store at room temperature.

### EMC-HA buffer (0.05 M $H_3BO_3$ - 0.12 M KCl) with 0.1 % BSA

- 3.09 g $H_3BO_3$ (boric acid)
- 8.95 g KCl
- 1 liter glass-distilled water

- 1 N NaOH
- Bovine serum albumin (BSA)

Add 3.09 g boric acid and 8.95 g KCl to a flask and bring the volume to 1 liter in glass-distilled water. Adjust pH to 7.0 with 1 N NaOH. This is a stock EMC-HA buffer solution. Store at room temperature.

The EMC-HA buffer with 0.1% BSA, for use in EMC virus HA assay and hemadsorption, is prepared by adding 0.1 g of BSA per 100 ml of the stock EMC-HA buffer.

## Formalin, 10% in PBS

- formaldehyde, 37% solution (Fisher #F-79)
- PBS

Combine 125 ml of the formaldehyde with 375 ml of PBS. Store at room temperature.

## Crystal violet, 1% in 70% methanol

- crystal violet (Sigma #C-3886)
- methanol (Fisher #A-408)
- Nalgene vacuum filter (Fisher #09-740-25B)
- glass-distilled water

Add 700 ml of methanol and 300 ml glass-distilled water to a 2 liter flask with a stir bar. Add 10 g of crystal violet and stir to dissolve. Filter using a 500 ml Nalgene vacuum-filtering flask with a 0.45 μ filter. Label and store at room temperature.

## Sheep red blood cells (SRBC)

The sheep red blood cells may be purchased from Environmental Diagnostics, as a 50% solution.

# APPENDIX C - Procedures and Calculations

## Preparation of cell culture medium

1. Using the bunsen burner and aseptic technique, transfer the required amount of serum (previously heat-inactivated at 56°C for 30 minutes) to E-MEM containing 100 units/ml of penicillin and 100 µg/ml of streptomycin. The medium should be yellow or light orange.
2. Using a sterile 1.0 ml pipet, add the sterile $NaHCO_3$ solution, in a *dropwise* fashion, until the medium is red - NOT PURPLE. After each addition, mix the contents before reading the color. If the color is purple, backtitrate with the dropwise addition of sterile 1 N HCl.
3. Sterility Test: transfer 0.5 ml of medium to a thioglycollate tube. Label the tube with your name, the date, and the medium being tested. Incubate at 37°C. Discard the negative tubes after 2 weeks. Positive tubes are indicative of a contaminated medium. The contaminated medium should be autoclaved and discarded, and new medium prepared.
5. Label the E-MEM medium with your name and the date and return to the refrigerator.

## Cell counts

1. Thoroughly wash the hemacytometer and cover slip with the 7x solution and rinse with distilled water. Rinse with 95% alcohol and wipe dry with lens paper.
2. Decant the medium from the flask into a decontamination pan.
3. Wash the cells with 5 ml of HBSS. Decant into a decontamination pan.
4. Add 2 ml of medium.
5. Gently scrape the cells. Pipet up and down to obtain a single cell supension.

6. Transfer 0.1 ml of the cell suspension to a dilution tube and add 0.4 ml trypan blue stain (1:5 dilution).
7. Gently swirl the cell suspension and remove a sample with a Pasteur pipet.
8. Removing the bulb and using finger control, place the tip of the Pasteur pipet in the V-shaped loading groove of the hemacytometer chamber. At about a 45° angle and by capillary action, let the cell suspension flow under the coverslip until the chamber is just filled but not overflowing into the overflow trough. Clean the hemacytometer and start over if the chamber is overfilled, or if air bubbles and dirt particles are present.
9. Load both chambers of the hemacytometer and allow the cells to settle for 1 minute before starting to count.
10. Use the low power objective on the microscope to count all the cells (stained and unstained) in each of the five large squares in one of the counting chambers. Do the same for the second chamber. Take the average of these two values to obtain the *total cell count.*

    (a) Count the cells touching the right and bottom lines but do not count the cells touching the top and left lines. Remember that cell clumps are counted as one cell.

    (b) There should be 20-60 cells per large square (100-300 cells per 5 squares). If there are more, further dilute the cell suspension, and clean and reload the hemacytometer.

    (c) The cell counts of the two hemacytometer chambers should be within 10% of each other. If they are not, obtain further counts until the data cluster about a mean.
11. Following the same procedure as in step #10, count all the unstained (viable) cells in the 5 large squares of each chamber. Take the average of these two values to obtain the *viable cell count.*
12. The percent viable cells in the original suspension is determined as follows:

**% viable cells** = *100% x (viable cell count / total cell count )*

13. The number of cells per ml and the number of viable cells per ml, in the original cell suspension, can be calculated in the following manner:

**Total cells/ml** = *(total cell count / 5) x (1 / dilution) x* $10^4$

**Viable cells/ml** = *(viable cell count/5) x (1/dilution) x $10^4$*

NOTE: Trypan blue will bind to serum proteins and it is therefore important to dilute out the growth medium in HBSS prior to adding trypan blue. If there is excessive background staining the dye concentration may have to be increased or the cells centrifuged and resuspended prior to staining.

## Preparation of Cell Suspensions

Once a cell count is performed, the following formula is used to calculate the dilution necessary to obtain a desired volume of cells at a specific concentration (number of cells/ml).

$V_1 \times C_1 = V_2 \times C_2$

where:
$V_1$ is the volume in ml of diluted cell suspension to be made.
$C_1$ is the concentration (number of cells/ml) to be made.
$V_2$ is the volume in ml of the original cell suspension (to be calculated).
$C_2$ is the concentration (number of cells/ml) of the original cell supension.

For example, if you have a cell suspension containing $3 \times 10^6$ cells/ml and you need 15 ml of a $4 \times 10^5$ cells/ml suspension for a given experiment you can calculate the volume of the original cell suspension needed ($V_2$) in the following manner:

$15 \text{ ml} \times (4 \times 10^5 \text{ cells/ml}) = V_2 \times (3 \times 10^6 \text{ cells/ml})$
$V_2 = 2 \text{ ml}$

Thus, 2 ml of the original cell suspension would be added to 13 ml of medium to obtain 15 ml of $4 \times 10^5$ cells/ml.

## Thawing of frozen cells

1. Remove the ampule to be thawed from the -70°C freezer and immediately place in a 37°C water bath.
2. Open the ampule aseptically and transfer the cell suspension to a sterile centrifuge tube.

3. Centrifuge at 600 x g for 10 minutes. Discard the supernatant and resuspend in 10 ml of medium.
4. Transfer 0.1 ml of the cell suspension to a small test tube and add 0.1 ml of trypan blue solution (1:2). Obtain a total and a viable cell count, and calculate the % viability. Compare that figure to the percent viability of the cell culture before freezing.
5. Dispense 5 ml of the cell suspension into each of 2 - 25 $cm^2$ flasks.
6. 24 hours later change the medium and observe the cells.

## Virus Dilutions

Whenever one is performing an assay to determine the titer of a given virus suspension, serial dilutions of the virus stock must be prepared. These serial dilutions are most commonly done by factors of 2, 5, or 10. The smaller the factor, the more precise the titer but when a broad range of dilutions are needed, 10-fold dilutions are most commonly used.

The first thing that needs to be determined is how much volume of each virus dilution will be needed to perform the assay. For each dilution one will need the exact amount used for infection plus the amount to be transferred to perform the next dilution, plus a small additional volume. This additional volume accounts for the volume that is undeliverable by some pipets, since one should not forcefully expel a virus suspension. For example: if 0.1 ml of a given dilution is to be added to each of 5 wells and 0.1 ml is needed for the next dilution, the exact amount needed would be 0.6 ml, and the needed additional volume would be 0.1-0.2 ml. Thus for this given dilution one would then need a minimum of 0.7-0.8 ml of suspension.

Once the dilution scheme has been determined the actual dilutions are performed. By way of example let us use the following dilution sequence:

tube#1 - 0.1 ml of virus + 0.9 ml diluent - ($10^{-1}$)
tube#2 - 0.1 ml of tube#1 + 0.9 ml diluent - ($10^{-2}$)
tube#3 - 0.1 ml of tube#2 + 0.9 ml diluent - ($10^{-3}$)
tube#4 - 0.1 ml of tube#3 + 0.9 ml diluent - ($10^{-4}$)

To do this:

1. Label the tubes and dispense 0.9 ml of diluent with a 1.0 ml pipet.

2. Using a new 1.0 ml pipet, add 0.1 ml of the virus stock suspension to the first tube. The tip of the pipet should touch the side of the tube and the desired amount (0.1 ml in this case) should be allowed to run down the side. This pipet should not otherwise touch any other side of the tube nor the liquid therein, and should be properly discarded after delivering the required volume.
3. Use a *new* pipet to mix the contents of this first tube by drawing up and releasing the virus suspension 6-7 times (or vortex gently).
4. Once mixed, 0.1 ml is withdrawn and transferred to tube #2, again being careful to only touch one side of the tube and to discard the pipet in a decontamination pan.
5. Use a *new* pipet to mix the contents of tube #2 and to transfer 0.1 ml of the mixed suspension to tube #3.

It is very important to use a new pipet for each transfer and to mix thoroughly each virus dilution. A used pipet will have a large number of virus particles on its outer surface. If the pipet is not discarded after each transfer, millions of virus particles will be carried along resulting in a large dilution error. Transfer from an improperly mixed suspension will also lead to dilution errors. Errors made in virus dilutions result in an erroneous titer and a loss of time and supplies.

## Reed and Muench Calculation of the 50% Endpoint:

In Table A.1 the dilution that would correspond to the 50% endpoint lies somewhere between the $10^{-6}$ (66.7% infected) and $10^{-7}$ (14.3% infected) dilutions. The proportionate distance between these two dilutions is calculated in the following manner:

$$\frac{\textit{(\% positive above 50\%} \quad - \quad \textit{50\%}}{\textit{(\% pos. above 50\%) - (\% pos. below 50\%)}} = \textit{proportionate distance}$$

$$\frac{66.7 - 50.0}{66.7 - 14.3} = 0.3 = \text{proportionate distance}$$

The 50% endpoint is now calculated in the following manner:

*(log dilution above 50%)+(proportionate distance x log dilution factor)*

The log of the dilution above 50% is -6, the proportionate distance as calculated previously is 0.3, and the log of the dilution factor is -1 (1:10 dilutions were used).

$$(-6) + (0.3 \times -1.0) = -6.3 = \log ID_{50}$$

$ID_{50} = 10^{-6.3}$. This is the end-point dilution, namely the dilution that will infect 50% of the cultures inoculated (with 0.1 ml). The reciprocal of this number yields the titer in terms of infectious dose per unit volume. Given that the inoculum added to the individual wells was 0.1 ml, the titer of the virus suspension would therefore be:

$$10^{6.3}\ ID_{50}/0.1\ ml = 10 \times 10^{6.3}\ ID_{50}/ml = 10^{7.3}\ ID_{50}/ml$$

**Table A.1**
Sample data used to determine the 50% endpoint using the Reed and Muench method.

| Log of virus dilution | Infected test units | Cumulative infected (A) | Cumulative non-infected (B) | Ratio of A/(A +B) | Percent infected |
|---|---|---|---|---|---|
| - 5 | 5/5 | 9 | 0 | 9/9 | 100 |
| - 6 | 3/5 | 4 | 2 | 4/6 | 66.7 |
| - 7 | 1/5 | 1 | 6 | 1/7 | 14.3 |
| - 8 | 0/5 | 0 | 11 | 0/11 | 0 |

Infected test units would be wells exhibiting obvious CPE in a $TCID_{50}$, dead animals in an $LD_{50}$, or infected eggs in the $EID_{50}$. Five test units were inoculated at each dilution. The cumulative infected column is calculated based on the assumption that the 1 test unit that was infected at a $10^{-7}$ dilution of virus would also have been infected at a $10^{-6}$ dilution. Therefore, at $10^{-6}$ there would be 4 (1 at $10^{-7}$ + 3 at $10^{-6}$) cumulative infected units. Similarly, at $10^{-5}$ the cumulative infected units would be 1 (at $10^{-7}$) + 3 (at $10^{-6}$) + 5 (at $10^{-5}$) = 9. A similar argument is made to calculate the cumulative non-infected column. Here the assumption is that test units which were not infected by a given dilution of virus would also be uninfected by a higher dilution of the virus.

## $PDD_{50}$ Calculation

The $PDD_{50}$ calculation according to the formula developed by Langford et al. (1981) is as follows:

$$PDD_{50} = DL + [(P_{50} - PL)\ (DH - DL) / (PH - PL)]$$

Where:

**DL** is the reciprocal of the lower dilution bracketing the 50% endpoint,

**DH** is the reciprocal of the higher dilution bracketing the 50% endpoint,

$P_{50}$ is the number of plaques at the 50% endpoint,

**PL** is the number of plaques at the lower dilution bracketing the 50% endpoint,

**PH** is the number of plaques at the higher dilution bracketing the 50% endpoint.

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