

Culturing, Storage, and Quantification of Rotaviruses

UNIT 15C.3

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ABSTRACT

Group A rotaviruses (RVs) infect the young of numerous animal species and cause acute gastroenteritis. Cultivation of animal and human RVs in cells requires proteolytic activation of the viral attachment protein using trypsin. Continuous cell lines, such as rhesus monkey kidney cells, as well as primary monkey kidney cells, are routinely used for the growth and characterization of RVs. Isolation and cultivation of human RVs from clinical fecal specimens is difficult and adaptation to growth in vitro requires multiple rounds of passage in primary cells. Following growth, RV stocks can be purified by centrifugation, if required, and quantified using plaque assay or fluorescence focus assay. This unit describes easily applicable procedures for the culturing, storage, and quantification of RVs. *Curr. Protoc. Microbiol.* 15:15C.3.1-15C.3.24. © 2009 by John Wiley & Sons, Inc.

Keywords: rotavirus • RV • infection • cell culture • purification • plaque assay • fecal specimen • MA104 cells • AGMK cells • trypsin

INTRODUCTION

Group A rotaviruses (RVs) are intestinal pathogens that infect the young of virtually every avian and mammalian species (Estes, 2001). RVs replicate in intestinal tract enterocytes, leading to acute gastroenteritis and viral shedding into the stool. The successful cultivation of both animal and human RVs has been achieved through the use of primary and transformed monkey kidney cells and by proteolytic activation of the virus with trypsin prior to infection (Wyatt et al., 1980, 1983; Sato et al., 1981; Hasegawa et al., 1982; Ward et al., 1984, 1991). However, it is important to note that different RV strains vary in their capacity to grow in culture. In particular, animal strains that are used as laboratory models, such as RRV, SA11-4F, UK, and OSU, grow quite well in continuous cell lines, reaching titers of 10^7 to 10^8 plaque-forming units (PFU) per milliliter of medium. In contrast, culture-adapted human RVs have average titers 1- to 3-logs less than most animal strains. Without a doubt, propagation of human RVs from clinical fecal specimens is very inefficient and does not work for every sample. Growth of RVs from feces requires several blind passages in primary cells prior to growth in continuous cell lines. Once adapted to cell culture, clinical isolates are usually capable of growing as well as the prototypic culture-adapted human RV strains Wa, DS-1, ST3, and P.

This unit covers several techniques for propagating animal and human RVs in cell culture from existing stocks (see Basic Protocol 1 and Alternate Protocol 1) or from clinical fecal specimens (see Alternate Protocol 2). Following propagation, RVs can be concentrated (see Basic Protocol 2) and infectious virus particles can be purified by centrifugation in cesium chloride gradients (see Alternate Protocol 3). Methods of virus quantification are also described, including plaque assay (see Basic Protocol 3) and fluorescence focus assay (see Alternate Protocol 4). Additionally, plaque isolation techniques are outlined, allowing for the generation of clonal RV stocks or the analysis of single virus isolates (see Basic Protocol 4). RVs are very stable and are capable of being stored under a variety of conditions.

Animal RNA
Viruses

15C.3.1

Supplement 15

CAUTION: The protocols presented in this unit are for use with contemporary animal and human RV strains. RVs are classified as Risk Group 2 (RG2) agents and must be handled under Biosafety Level 2 (BSL-2) conditions. Primary cell cultures and continuous cell lines should be maintained, infected, and harvested using BSL-2 practices in a class II biological safety cabinet. Similarly, all work with clinical fecal specimens should be conducted under BSL-2 conditions in a class II biological safety cabinet. For additional information, refer to the *Biosafety in Microbiological and Biomedical Laboratories* manual published by the Center for Disease Control and Prevention and National Institutes of Health.

NOTE: The propagation and quantification of RV is generally performed in rhesus monkey kidney (MA104) cells. Two types of media are commonly used for the culture of MA104 cells, Medium 199 or Eagle's Minimal Essential Medium (EMEM). There does not appear to be any benefit or detriment to using either type of medium, and the type used is usually based on an individual laboratory's preference. More specific information of each medium can be found in the Reagents and Solutions section.

BASIC PROTOCOL 1

PROPAGATION OF ROTAVIRUS IN MA104 CELL CULTURE FROM VIRUS STOCKS

Rhesus monkey kidney (MA104) cells (ATTC #CRL-2378.1) are widely used for the growth and characterization of both animal and human culture-adapted RVs. Yet, depending upon the particular strain, RVs are capable of growing in other types of continuous cell lines in addition to MA104 (Table 15C.3.1; Estes et al., 1979; Ciarlet et al., 2002). If other cell types are used, infection is done in a manner identical to that described below with the exception of substituting the appropriate medium. Use the specific cell culture medium and conditions suggested by the supplier for culturing the cells prior to infection.

The authors usually work with the animal RV strain SA11-4F, which grows very well and, like many other RVs, causes cytopathic effect (CPE) in cultured cells. At a high multiplicity of infection ($\text{MOI} \geq 3$), CPE is visible as cell rounding, detachment, and lysis, beginning at 8 to 10 hr post-infection (p.i.). At very low MOI, CPE may not be visible for several days. For RV propagation, infections are commonly performed at low MOI to reduce the generation of mutations and gene rearrangements and to achieve maximal yields of infectious virus. The protocol described below is for the propagation of existing culture-adapted animal or human RV stocks. Refer to Alternate Protocols 1 and 2 for adaptation of RVs to MA104 cells or for preparation of a virus stock from a clinical fecal specimen. The amount of reagents recommended for this procedure is for a 150-cm² flask; volumes should be adjusted accordingly for other culture vessel sizes.

Materials

- MA104 cells confluent in a 150-cm² flask ($\sim 1.5 \times 10^7$ cells; see the Support Protocol)
- 1 × serum-free Medium 199 (see recipe)
- RV stock vial (10^5 to 10^8 PFU/ml; see Basic Protocol 5)
- 2 mg/ml trypsin in PBS (see recipe)
- 1 × serum-free Medium 199 (see recipe) with 0.5 μg/ml trypsin (see recipe)
- 1 × complete Medium 199 (see recipe)
- 15- and 50-ml conical tubes
- Vortex (optional)
- 37°C water bath
- Platform rocker

Inverted light microscope
–80°C freezer
Table-top centrifuge

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator.

1. Remove growth medium from a confluent monolayer of MA104 cells maintained in a 150-cm² flask. Wash the monolayer three times, each time using 10 ml of prewarmed, serum-free Medium 199. Take care not to add medium directly onto the monolayer as this may disrupt the cells.

Fetal bovine serum (FBS), a component in complete Medium 199, inhibits RV activation and must be removed for efficient infection of the cells. Cells can also be incubated several hours to overnight in one change of serum-free medium.

2. Thaw a stock vial of RV at room temperature. Transfer the volume equivalent of an MOI ≤0.1 of RV stock into a 15-ml tube.

Calculation of the PFU needed for a MOI ≤0.1 requires knowledge of the number of cells in the culture flask. A confluent 150-cm² flask contains ~1.5 × 10⁷ MA104 cells. Use the following equation to determine the PFU:

No. cells × MOI = PFU needed

1.5 × 10⁷ × 0.1 = 1.5 × 10⁶ PFU

If the virus titer is 10⁶ PFU/ml, then ≤1.5 ml would be required for an MOI ≤0.1.

3. To activate the virus, add trypsin to a final concentration of 10 µg/ml. Vortex briefly or swirl to mix. Incubate the mixture for 1 hr in a 37°C water bath.

Trypsin cleaves the RV attachment protein, allowing for entry of the virus into the host cell. The enzyme should be thawed on ice and not subjected to multiple rounds of freeze-thaw.

4. Following activation, dilute the inoculum by adding serum-free Medium 199 to reach a final trypsin concentration of <2 µg/ml. For a 150-cm² flask, the inoculum volume should be at least 3 ml.

5. Remove the serum-free Medium 199 wash from the flask. Add the inoculum and incubate the flask for 1 hr at 37°C on a platform rocker to ensure equal coverage of the monolayer with the inoculum.

In the absence of a platform rocker, gently rock the flask every 10 to 15 min by hand.

Incubation of cells >1 hr or in >2 µg/ml of trypsin may cause the cell monolayer to slough off. Cells should be visualized under an inverted light microscope to ensure the monolayer is intact following virus adsorption.

6. Following adsorption, remove the virus inoculum and wash the monolayer once using 10 ml of prewarmed, serum-free Medium 199. Remove the wash and add 20 ml of prewarmed, serum-free Medium 199 with 0.5 µg/ml trypsin.

The presence of trypsin in the culture medium allows for efficient virus propagation. However, to generate completely non-activated RV stocks, replace the inoculum with complete Medium 199 containing trypsin inhibitors. In this case, cells should be infected at an MOI ≥3.

7. Maintain the cells at 37°C for 3 to 7 days or until CPE has progressed to the point where the cell monolayer is fully disrupted due to lysis. Transfer the flask to –80°C.

8. Freeze and thaw the infected cells three times while in the flask. Transfer the cell lysate and culture supernatant into a 50-ml conical tube.

Multiple rounds of freezing and thawing release cellular-associated RV particles into the supernatant. Loosen the cap on the culture flask to prevent it from cracking during the freeze-thaw process.

9. Remove large cellular debris from the lysate by low-speed centrifugation (5 to 10 min at $300 \times g$, 4°C). Transfer the clarified virus (~ 20 ml) into a new 50-ml conical tube.

The clarified RV stock can now be titered, aliquoted, and stored (see Basic Protocols 3 and 5) or can be used for additional rounds of amplification.

If additional amplification is required, dilute the clarified virus stock 1:2 in serum-free Medium 199 with $0.5 \mu\text{g/ml}$ of trypsin, and then add directly onto a fresh monolayer of washed MA104 cells.

ALTERNATE PROTOCOL 1

PROPAGATION OF ROTAVIRUS IN PRIMARY AGMK ROLLER CELL CULTURE FROM VIRUS STOCKS

Some RV strains, in particular human isolates, are refractory to growth in MA104 cells, but grow in primary African green monkey kidney (AGMK) cells. Following several passages in AGMK cells, RVs characteristically grow more readily in culture and can then efficiently infect continuous cells (see Basic Protocol 1). The authors use roller cultures of AGMK cells in round-bottom glass tubes (Diagnostic HYBRIDS). African green monkeys are often infected with simian virus 5 (SV5) and simian virus 40 (SV40), which have the capacity to transform cultured cells. To prevent transformation, the AGMK cells are maintained in the presence of antisera against SV5 and SV40. The protocol described below is for the propagation of existing animal or human RV stocks. Refer to Alternate Protocol 2 for preparation of a RV stock from a clinical fecal specimen.

Additional Materials (also see Basic Protocol 1)

AGMK cells in a round-bottom glass tube (Diagnostic HYBRIDS, cat. no. 46-0600A)

Roller drum for 37°C incubator

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C , 5% CO_2 incubator.

Table 15C.3.1 Additional Cell Types Commonly Used in Rotavirus Experiments

Cell type	Origin	Tissue	ATCC #
BGM	Buffalo green monkey	Kidney	N/A
BSC-1	African green monkey	Kidney	CCL-26
Caco-2	Human	Colon adenocarcinoma	HTB-37
COS-7	African green monkey	Kidney	CRL-1651
CV-1	African green monkey	Kidney	CCL-70
FRhL-2	Rhesus monkey	Lung	CL-160
HT-29	Human	Colon adenocarcinoma	HTB-38
LLC-MK ₂	Rhesus monkey	Kidney	CCL-7
Vero	African green monkey	Kidney	CCL-81

1. Remove growth medium from primary AGMK cells that have been maintained for <4 days in a glass roller tube. Wash the monolayer three times, each time using 2 ml of prewarmed, serum-free Medium 199. Take care not to add medium directly onto the monolayer on the sides of the tube as this may disrupt the cells.

FBS, a component in AGMK growth medium, inhibits RV activation and must be removed for efficient infection of the cells. Cells can also be incubated several hours to overnight in one change of serum-free medium.

2. Thaw a stock vial of RV at room temperature. Transfer the volume equivalent of an $MOI \leq 0.1$ of RV stock into a 15-ml conical tube.

Calculation of the PFU needed for a $MOI \leq 0.1$ requires knowledge of the number of cells in the culture flask. A confluent roller tube contains $\sim 1.5 \times 10^6$ AGMK cells. Use the following equation to determine the PFU:

$$\text{No. cells} \times \text{MOI} = \text{PFU needed}$$

$$1.5 \times 10^6 \times 0.1 = 1.5 \times 10^5 \text{ PFU}$$

If the virus titer is 10^6 PFU/ml, then $\leq 150 \mu\text{l}$ would be required for an $MOI \leq 0.1$.

3. To activate the virus, add trypsin to a final concentration of 10 $\mu\text{g/ml}$. Vortex briefly or swirl to mix. Incubate the mixture 1 hr in a 37°C water bath.

Trypsin cleaves the RV attachment protein, allowing for entry of the virus into the host cell. The enzyme should be thawed on ice and not subjected to multiple rounds of freeze-thaw.

4. Following activation, dilute the inoculum by adding serum-free Medium 199 to reach a final trypsin concentration of <2 $\mu\text{g/ml}$. The inoculum volume should be at least 0.5 ml.

5. Remove the serum-free Medium 199 wash from the roller tube. Add the inoculum and incubate the tube 1 hr at 37°C in a roller drum to ensure equal coverage of the monolayer with the inoculum.

Incubation of cells >1 hr or in >2 $\mu\text{g/ml}$ of trypsin may cause the cell monolayer to slough off. Cells should be visualized under an inverted light microscope to ensure the monolayer is intact following virus adsorption.

6. Following adsorption, remove the virus inoculum and wash the monolayer once using 2 ml of prewarmed, serum-free Medium 199. Remove the wash and add 2 ml of prewarmed, serum-free Medium 199 with 0.5 $\mu\text{g/ml}$ of trypsin.

The presence of trypsin in the culture medium allows for efficient virus propagation. However, to generate completely non-activated RV stocks, replace the inoculum with complete Medium 199 containing trypsin inhibitors. In this case, cells should be infected at an $MOI \geq 3$.

7. Maintain the cells with constant rotation in a roller drum at 37°C for 3 to 7 days or until CPE is visible, and the cell monolayer is disrupted due to lysis. Transfer the tube to -80°C.

If no CPE is detected during the first infection, the clarified supernatant should be blindly passaged every 7 days up to ten times or until CPE is visible.

8. Freeze and thaw the infected cells three times while in the tube. Transfer the cell lysate and culture supernatant into a 15-ml conical tube.

Multiple rounds of freezing and thawing releases cellular-associated RV particles into the supernatant.

**ALTERNATE
PROTOCOL 2**

9. Remove large cellular debris from the lysate by low-speed centrifugation (5 to 10 min at $300 \times g$, 4°C). Transfer the clarified virus (~ 2 ml) into a new 15-ml conical tube.

The clarified virus stock can now be titered, aliquoted, and stored (see Basic Protocols 3 and 5) or can be used for additional rounds of amplification.

If additional amplification is required, dilute the clarified virus stock 1:2 in serum-free Medium 199 with $0.5 \mu\text{g/ml}$ trypsin, and then add directly onto a fresh monolayer of washed AGMK cells. In general, 2 to 3 passages of RV in primary AGMK cells are sufficient for increasing growth efficiency in continuous MA104 cells.

**PROPAGATION OF ROTAVIRUS IN CELL CULTURE FROM CLINICAL
FECAL SPECIMENS**

Cultivation of RV from clinical fecal specimens is achieved through the use of primary AGMK cells. Bacterial contamination can be reduced by clarifying the specimen using centrifugation and by maintaining antibiotics in the culture throughout the course of infection. Following several passages of the RV clinical sample in AGMK cells, the virus is usually capable of being propagated in continuous cells. However, cultivation of RV from feces is not always possible for reasons that are not fully understood. Additionally, CPE may not be visible in the AGMK cells following infection using clinical specimens. Several blind passages, in which no CPE is detectable, may be required. Alternatively, the feces sample itself may be cytotoxic and dilution may be required prior to infection.

Additional Materials (also see Basic Protocol 1)

Clinical fecal sample

AGMK cells in a round-bottom glass tube (Diagnostic HYBRIDS, cat. no. 46-0600A)

1.5-ml microcentrifuge tubes

Roller drum for 37°C incubator

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work and all handling of clinical specimens should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C , 5% CO_2 incubator.

1. In a 1.5-ml microcentrifuge tube, prepare 1 ml of a 15% (w/v) homogenate of feces using serum-free Medium 199. Clarify the homogenate by centrifuging 15 min at $10,000 \times g$, 4°C . Transfer the supernatant into a new 1.5-ml tube.
2. To activate the virus, add trypsin to the clarified preparation to a final concentration of $10 \mu\text{g/ml}$. Vortex briefly or swirl to mix. Incubate the mixture 1 hr in a 37°C water bath.

Trypsin cleaves the RV attachment protein, allowing for entry of the virus into the host cell. The enzyme should be thawed on ice and not subjected to multiple rounds of freeze-thaw.

3. Following activation, dilute the inoculum by adding serum-free Medium 199 to reach a final trypsin concentration of $<2 \mu\text{g/ml}$. The inoculum volume should be at least 0.5 ml.
4. Remove growth medium from primary AGMK cells that have been maintained for <4 days in a glass roller tube. Wash the monolayer three times, each time using 2 ml of prewarmed, serum-free Medium 199. Take care not to add medium directly onto the monolayer on the sides of the tube as this may disrupt the cells.

FBS, a component in AGMK growth medium, inhibits RV activation and must be removed for efficient infection of the cells. Cells can also be incubated several hours to overnight in one change of serum-free medium.

5. Remove the serum-free Medium 199 wash from the roller tube. Add the inoculum and incubate the tube 1 hr at 37°C in a roller drum to ensure equal coverage of the monolayer with the inoculum.

Incubation of cells >1 hr or in >2 µg/ml of trypsin may cause the cell monolayer to slough off. Cells should be visualized under an inverted light microscope to ensure the monolayer is intact following virus adsorption.

Additionally, the fecal sample may be cytotoxic and as a result, the cells may detach and lyse following this adsorption step. In this case, the inoculum should be diluted 1:10 to 1:100 using prewarmed serum-free Medium 199 with 0.5 µg/ml of trypsin.

6. Following adsorption, remove the virus inoculum and wash the monolayer once using 2 ml of prewarmed, serum-free Medium 199. Remove the wash and add 2 ml of prewarmed, serum-free Medium 199 with 0.5 µg/ml of trypsin.
7. Maintain the cells with constant rotation in a roller drum at 37°C for 3 to 7 days or until CPE is visible, and the cell monolayer is disrupted due to lysis. Transfer the tube to –80°C.

If no CPE is detected during the first infection, the virus should be blindly passaged every 7 days up to ten times or until CPE is visible.

8. Freeze and thaw the infected cells three times while in the tube. Transfer the cell lysate and culture supernatant into a 15-ml conical tube.

Multiple rounds of freezing and thawing releases cellular-associated RV particles into the supernatant.

9. Remove large cellular debris from the lysate by low-speed centrifugation (5 to 10 min at 300 × g, 4°C). Transfer the clarified virus (~2 ml) into a new 15-ml conical tube.

The clarified virus stock can now be titered, aliquoted, and stored (see Basic Protocols 3 and 5) or can be used for additional rounds of amplification.

If additional amplification is required, dilute the clarified virus stock 1:2 in serum-free Medium 199 containing 0.5 µg/ml trypsin, and then add directly onto a fresh monolayer of washed AGMK cells. In general, 2 to 3 passages of RV in primary AGMK cells are sufficient for increasing growth efficiency in continuous MA104 cells.

SEMI-PURIFICATION OF ROTAVIRUSES USING ULTRACENTRIFUGATION

The quantity of RV recovered from the clarified cell supernatant is sufficient for most experimental procedures. However, some experiments may require the concentration or semi-purification of RV stocks, in particular for very low titer human RV stocks. Such concentration is achieved by pelleting the virus particles through a 35% sucrose cushion using ultracentrifugation. For RV stocks amenable to biochemical and structural assays, the virus can be further purified using cesium chloride gradients (see Alternate Protocol 3). RV particles should be maintained in buffer containing calcium (such as TNC buffer), which is essential for the stability of outer capsid. Likewise, buffers containing calcium-chelation agents (such as EDTA) should be avoided.

Materials

- 240 ml of clarified RV stock (from twelve 150-cm² flasks of infected MA104 cells; see Basic Protocol 1)
- 35% (w/v) sucrose in TNC buffer (see recipe)
- TNC buffer (see recipe)

BASIC PROTOCOL 2

Animal RNA Viruses

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Ultra-Clear 25 × 89-mm ultracentrifuge tubes (Beckman)
2-ml and 50-ml pipets
Beckman ultracentrifuge with an SW32Ti rotor (or equivalent)
15-ml conical tube
Sonicator

NOTE: If preparing a sterile RV stock, perform the purification protocol in a class II biological safety cabinet and all equipment and solutions should be sterile. If preparing a nonsterile RV stock, perform the purification protocol on the laboratory bench.

1. Aliquot 35 ml of clarified RV stock into each of six Ultra-Clear 25 × 89-mm ultracentrifuge tubes.

Leave ~1 in. of space from the top of the tube to provide room for the volume expansion that occurs when underlying the sucrose solution.

2. Using a 2-ml pipet, carefully underlay 2 ml of 35% sucrose below the 35 ml of RV stock. Gently fill the remainder of the tube with clarified RV stock. Balance the tubes by weight.

Because sucrose is denser than the virus, an interface should be visible near the bottom of the tube. Care should be taken to avoid disrupting this interface prior to centrifugation.

3. Centrifuge 1.5 hr at $100,000 \times g$, 4°C using an SW32Ti rotor to pellet virus particles.
4. Pour the supernatant into an appropriate waste container and resuspend each pellet in 1 ml of TNC buffer, making 6 ml total volume.
5. Transfer the 6 ml of resuspended pellets into a clean 15-ml conical tube. Sonicate briefly using a microtip probe to create a homogeneous suspension of particles.

The virus should be kept on ice during sonication to avoid protein denaturation. In the absence of a sonicator, a homogeneous suspension can be created by passing the material through a 25-G needle several times.

This concentrated, semi-purified RV stock can be titered or used directly for biological assays. Semi-purified virus can be stored at 4°C in TNC buffer for up to a year with minimal loss in titer.

ALTERNATE PROTOCOL 3

PURIFICATION OF ROTAVIRUS USING CESIUM CHLORIDE GRADIENTS

Further purification of RV particles using cesium chloride (CsCl) gradients is required for some biochemical or structural assays. The starting material for this purification protocol is nonclarified, freeze-thawed RV-infected cell lysate (see Basic Protocol 1). Cellular lipids are removed from the preparation using trichlorotrifluoroethane (Freon), prior to banding virion particles in CsCl. As noted in Basic Protocol 2, RV particles should be maintained in buffer containing calcium (such as TNC buffer), and calcium-chelation agents should be avoided.

Materials

240 ml of RV-infected cell lysate (from twelve 150-cm² flasks of infected MA104 cells; see Basic Protocol 1)
TNC buffer (see recipe)
Trichlorotrifluoroethane (Freon)
Cesium chloride (CsCl), Ultra-Pure, Optical Grade
Ultra-Clear 25 × 89-mm ultracentrifuge tubes (Beckman)
Beckman ultracentrifuge with SW32Ti and SW55Ti rotors (or equivalent)
Sorvall Omni-mixer or vortex
50-ml conical tubes

Benchtop centrifuge
Sonicator
Refractometer
Ultra-Clear 13 × 51-mm ultracentrifuge tubes (Beckman)
Inverted light source
1.5-ml microcentrifuge tubes

NOTE: If preparing a sterile RV stock, perform the purification protocol in a class II biological safety cabinet and all equipment and solutions should be sterile. If preparing a non-sterile RV stock, perform the purification protocol on the laboratory bench.

CAUTION: This protocol requires the use of Freon, which is a colorless liquid that has known toxic properties and is an ozone-depleting substance. Freon should be handled in a chemical fume hood and according to the Materials Safety and Data Sheet (MSDS) provided by the manufacturer. In addition, Freon should be disposed of appropriately.

Pellet the virus

1. Aliquot 40 ml of RV cell lysate into each of six Ultra-Clear 25 × 89-mm ultracentrifuge tubes. Balance the tubes by weight.
2. Centrifuge 1.5 hr at $100,000 \times g$, 4°C using an SW32Ti rotor to pellet virus particles and cellular debris.
3. Pour the supernatant into an appropriate waste container and resuspend each pellet in 10 ml of TNC buffer, making 60 ml total volume.

The pellet may be difficult to resuspend. The authors use a 25-ml pipet for several rounds, followed by a 10-ml pipet for several rounds. Brief sonication can be used to achieve a more homogeneous mixture. The virus should be kept on ice during sonication to avoid protein denaturation.

Purify the RV particles

4. Combine the 60 ml pellet suspension with an equal volume of Freon in the Sorvall mixing vessel. Emulsify the mixture while on ice using a Sorvall Omni-mixer 3 min at speed 7.

In the absence of a Sorvall Omni-mixer, the Freon/virus mixture can be vortexed in a 50-ml conical tube three times for 3 min each until emulsified, with 1-min incubations on ice between each interval.

5. Transfer the emulsion into two 50-ml conical tubes. Balance the tubes by weight. Separate the organic and aqueous phases by centrifuging the emulsion 10 min at $4,100 \times g$, 4°C.
6. Transfer 30 ml of the upper, aqueous phase into each of two Ultra-Clear 25 × 89-mm centrifuge tubes. Fill the remainder of the tubes with TNC buffer. Balance the tubes by weight.
7. Centrifuge 1.5 hr at $100,000 \times g$, 4°C using an SW32Ti rotor to pellet virus particles.
8. Pour the supernatant into an appropriate waste container and resuspend each pellet in 4 ml of TNC buffer, making 8 ml total volume.
9. Transfer the 8 ml pellet suspension into a clean 50-ml conical tube. Sonicate briefly using a microtip probe to create a homogeneous suspension of particles.

The virus should be kept on ice during sonication to avoid protein denaturation.

10. Dissolve 4 g of CsCl in the virus suspension to achieve a density of 1.37 g/cm^3 , as determined by refractometry.

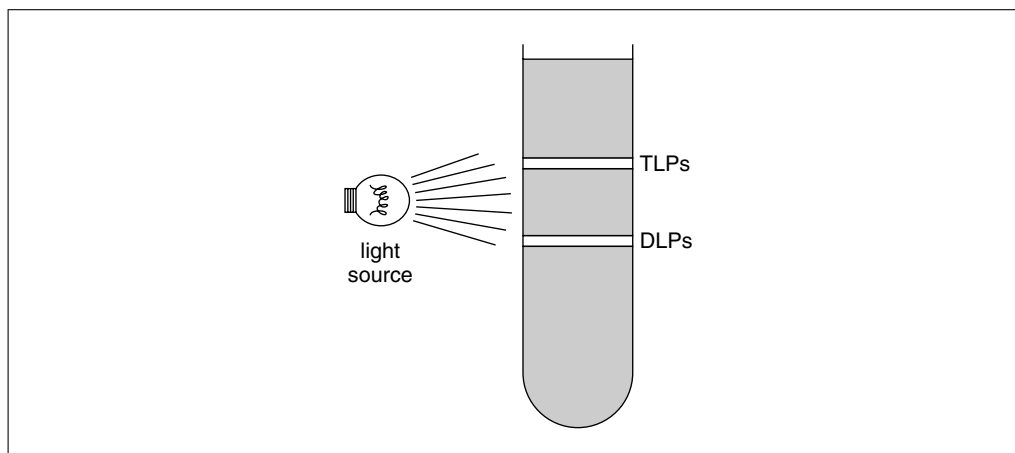


Figure 15C.3.1 Cartoon of two types of RV particles in a CsCl gradient. Following centrifugation of RV in a CsCl gradient, two distinct, cloudy, whitish-colored bands will be visible. Shining a light on the gradient will facilitate visualization of the bands. The upper band consists of infectious, triple-layered particles (TLPs; density $\sim 1.36 \text{ g/cm}^3$) and the lower band consists of non-infectious, double-layered particles (DLPs; density $\sim 1.38 \text{ g/cm}^3$). Harvest the upper band as a source of purified RV.

Precise determination of the density by refractometry is critical for proper separation and purification of virus particles using CsCl.

11. Transfer 5 ml each of CsCl/virus solution into two Ultra-Clear 13 \times 51-mm ultracentrifuge tubes. Balance the tubes by weight.
12. Centrifuge 18 hr at $110,000 \times g$, 12°C using an SW55Ti rotor to create gradients and band virus particles.

Use the slowest deceleration profile (i.e., no brake) to prevent mixing of the gradients.

13. Inspect the gradients in a darkened room with an inverted light source. Two cloudy, whitish-colored bands should be visible (see Figure 15C.3.1).

The upper band consists of infectious, triple-layered RV virions, while the lower band consists of non-infectious, double-layered particles.

14. Using a pipet, carefully remove 100 to 500 μl fractions from the gradient. Save all fractions in separate 1.5-ml microcentrifuge tubes at 4°C . Remove the upper, virus band in the smallest possible volume to achieve a maximum concentration.

If necessary, the upper band containing infectious RV particles can be repurified on another CsCl gradient to remove traces of double-layered particles. In that case, dilute the material from the upper band in a CsCl/TNC solution that has a density of 1.37 g/cm^3 and centrifuge 18 hr at $110,000 \times g$, 12°C , using an SW55Ti rotor.

15. Dialyze the virus sample exhaustively against several changes of TNC buffer. Purified virus can be titered and stored up to a year at 4°C with minimal loss in titer.

Following dialysis, the sample should also be analyzed by SDS-PAGE to verify recovery, purity, and relative concentration of RV particles. Collected fractions can be discarded once virus sample is obtained.

BASIC PROTOCOL 3

Culturing, Storage, and Quantification of Rotaviruses

15C.3.10

QUANTIFICATION OF ROTAVIRUS BY PLAQUE ASSAY

The plaque assay method is one of two common methods for quantifying RV. This assay is used to determine the viral titer, in plaque-forming units per milliliter of virus (PFU/ml), and is based on the CPE caused by RV in cultured cells. A single cell initially infected by RV spreads only to adjacent cells due to an overlay of 0.6% (w/v) agarose in $1 \times$ EMEM. As the infection progresses, cell detachment and lysis causes the formation of localized

clearings, or plaques. RV strains that grow well in cell culture will form visible plaques within a few days, while those strains that do not grow as well can take up to a week for plaques to become visible. Neutral red is used to visualize the plaques in this assay. Living cells take up the neutral red stain, whereas the lysed cells of the plaques do not, leaving the plaques visible as a clear area surrounded by the remaining cell monolayer. In some cases, plaques can be so small as to be difficult to see with neutral red staining. If this occurs, cells can be fixed with formaldehyde and stained with crystal violet to enhance the contrast between plaques and uninfected cells.

Plaque assays for RV are performed in MA104 cells in 6-well tissue culture plates. The cells must form tightly packed monolayers in order to see well-defined plaques. The cells are sensitive to drying, so when removing medium from wells at any step, work quickly to prevent cells from drying out. This protocol uses an inoculating volume of 1 ml of virus per well. Generally, if experimental infections require a larger inoculating volume, then this is appropriate to use. However, if experimental infections use a small inoculating volume, then plaque assays should be performed similarly. Inoculating volumes as low as 100 μ l per well of a 6-well plate can be used, as long as care is taken to prevent the monolayer from drying out. Although the methods as written here use Medium 199 for growth of the cells and dilution of the virus, EMEM can be used as an equivalent substitute.

The authors generally use one 6-well plate per dilution series. A dilution series ranging from 10^{-3} to 10^{-8} is usually sufficient to see individual plaques, though a lower or higher range may be required. Plaque assays are commonly performed in duplicate, along with an uninfected control in duplicate. This would require four 6-well plates of MA104 cells to titer a single RV stock.

Materials

- MA104 cells confluent in a 150-cm² flask ($\sim 1.5 \times 10^7$ cells)
- 1 \times complete Medium 199 (see recipe)
- RV stock (unknown titer; see Basic Protocols 1 and 2)
- 2 mg/ml trypsin in PBS (see recipe)
- 1 \times serum-free Medium 199 (see recipe)
- 2 \times serum-free EMEM (see recipe)
- 1.2% (w/v) agarose (see recipe)
- 0.33% (w/v) neutral red (contains 3.3 g neutral red/liter PBS, sterile filtered, cell culture grade; store at 4°C for up to 1 year; Sigma-Aldrich, cat. no. N2889)
- 6-well tissue culture plates
- 37° and 55°C water baths
- 15- and 50-ml conical tubes
- Vortex
- 10-, 200-, and 1000- μ l pipets and tips
- 5-, 10-, and 25-ml pipets
- Rocking platform
- Light box

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator, unless otherwise specified.

Prepare MA104 cells

1. Determine the number of plaque assays to be performed. Seed MA104 cells into 6-well plates at a density of 3.0×10^5 cells per well, and incubate for 3 to 5 days.

One confluent 150-cm² flask of MA104 cells contains $\sim 1.5 \times 10^7$ cells. For simplified plating, one flask will be sufficient to seed eight 6-well plates. For example, the trypsinized cells from one flask can be resuspended in 150 ml of complete Medium 199, and 3 ml of that cell suspension is plated per well.

If cells are confluent before you are ready to use them, they may be incubated overnight at 30°C.

Activate virus and prepare dilutions

2. Thaw the vial of RV to be assayed at room temperature.
3. Activate the virus by mixing 400 μ l of RV with 2 μ l of trypsin (2 mg/ml stock) for a final trypsin concentration of 10 μ g/ml.
4. Incubate the virus 1 hr in a 37°C water bath.

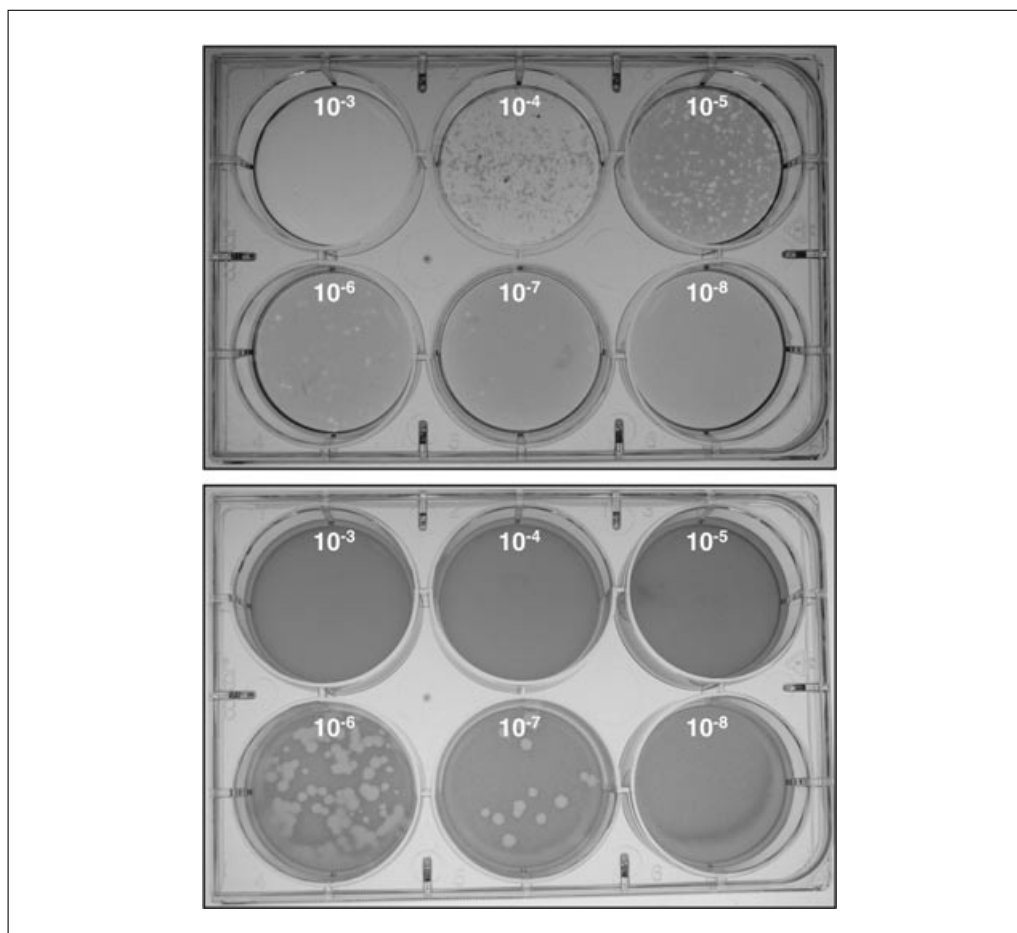


Figure 15C.3.2 Example of RV plaque assay. These images depict the results of a plaque assay using the animal RV strain SA11-4F, which grows well in cell culture. At 48 hr post-infection, the cells were stained with neutral red. The image on the top was taken 4 hr after staining. The image on the bottom is a plate that was incubated an additional 16 hr after staining. The sizes of the plaques for this virus grew substantially during the overnight incubation, and are considered large at 3 days post-infection. Many strains of rotavirus will make plaques that take additional days to form and are smaller in size. To calculate the titer for this plaque assay, the plate on the bottom was used, and the titer was calculated as: 15 plaques $\times 1/(10^{-7}) \times 1/(1 \text{ ml}) = 1.5 \times 10^8$ PFU/ml.

5. Prepare dilution tubes by adding 2.7 ml of serum-free Medium 199 into each 15-ml conical tube. One tube is needed for each dilution, usually requiring eight tubes/virus for an endpoint of 10^{-8} .
6. Prepare serial ten-fold dilutions of the virus by adding 300 μ l of the trypsin-activated RV into the first tube (10^{-1}), bringing the final volume to 3 ml. Vortex to mix and then transfer 300 μ l of the 10^{-1} dilution into the 10^{-2} tube. Continue to prepare serial ten-fold dilutions by adding 300 μ l of each dilution into the next tube until the desired endpoint is reached (usually to 10^{-8}). Change pipet tips between each dilution to ensure virus is not carried over.

Only 300 μ l of the activated RV is used in dilutions, though 400 μ l was activated to account for error in pipetting.

Infect cells

7. Wash MA104 cells in 6-well plates three times, each time using 2 ml/well of prewarmed, serum-free Medium 199.

FBS, a component in MA104 growth medium, inhibits RV activation and must be removed for efficient infection of the cells. Cells can also be incubated several hours to overnight in one change of serum-free medium.

8. Remove the wash and add 1 ml of virus dilution (usually 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8}) to each of two wells. Add virus from most to least dilute, which allows the same pipet tip to be used.
9. Incubate the plates 1 hr at 37°C on a rocking platform to allow the virus to adsorb.

Alternatively, if a rocking platform is not available, plates may be rocked by hand every 10 to 15 min of the incubation period.

10. Remove the inoculum and wash the cells once using 2 ml prewarmed, serum-free Medium 199.

Assay for plaques

11. Prepare a 1:1 mixture of 1.2% (w/v) agarose and serum-free 2 \times EMEM containing 0.5 μ g/ml trypsin as follows:

- a. Warm 2 \times EMEM to 37°C.
- b. Melt presterilized 1.2% (w/v) agarose by microwaving, and allow agarose to cool in a 55°C water bath.
- c. Combine equal volumes of the prewarmed 2 \times EMEM and 1.2% (w/v) agarose, followed by addition of trypsin to a final concentration of 0.5 μ g/ml.

Be sure the agarose/medium mixture is less than 42°C to avoid heat inactivation of the trypsin.

- d. Swirl the agarose/medium overlay several times to ensure equal mixing.

12. Remove the wash medium and gently overlay the cells in each well with 3 ml of the agarose/medium mixture. Immediately swirl the plate one time to incorporate any residual wash medium into the overlay.

Do not swirl the plate once the agarose begins to solidify, as this may damage the cell monolayer.

13. Once the agarose has solidified, transfer the plates to a 37°C incubator for 3 to 4 days or until plaques are visible.

The authors have found that inverting the plates helps prevent the agarose from drying out during the incubation period.

At this stage, prior to staining, the plaques will appear whitish or opaque against the rest of the monolayer when held up to light.

14. Prepare a 1:1 mixture of 1.2% (w/v) agarose and serum-free 2× EMEM containing 50 µg/ml neutral red as follows:
 - a. Warm 2× EMEM to 37°C.
 - b. Melt presterilized 1.2% (w/v) agarose by microwaving, and allow to cool in a 55°C water bath.
 - c. Combine equal volumes of the prewarmed 2× EMEM containing 50 µg/ml neutral red and 1.2% (w/v) agarose.
 - d. Swirl the agarose/medium overlay several times to ensure equal mixing.
 - e. Add 2 ml per well of stain overlay on top of the first agarose/medium overlay. Cover the 6-well plates to protect from light and allow agarose to harden.
15. Once the neutral red overlay has solidified, cover the plates to protect them from light and incubate at 37°C until plaques can be seen (~4 to 24 hr; see Fig. 15C.3.2).
16. Count individual plaques using a light box. Calculate the titer of the virus as follows:
$$\text{No. plaques} \times 1/(\text{dilution factor}) \times 1/(\text{ml of inoculum}) = \text{PFU/ml}$$

For instance, if the 10^{-7} dilution yielded 30 plaques, then the virus titer would be:

$$30 \times 1/(10^{-7}) \times 1/(1 \text{ ml}) = 3.0 \times 10^8 \text{ PFU/ml}$$

ALTERNATE PROTOCOL 4

QUANTIFICATION OF ROTAVIRUS BY FLUORESCENCE FOCUS ASSAY

Quantifying RVs by fluorescence focus assay is a more rapid approach than the plaque assay. This assay detects fluorescence foci through the use of an antibody that reacts with RV-infected cells. Most investigators use antibodies that were made in or have been well characterized in their laboratories. However, there are also commercially available RV antibodies. Polyclonal antibodies generated against intact virions will usually cross-react with several RV strains and provide a strong fluorescence signal. Monoclonal antibodies offer antigenic specificity, but may only recognize some strains of RV. Thus, if using a monoclonal antibody, its antigenicity must cross-react with the strain of RV being tested. A common antibody to use would be directed against the viral capsid protein VP6 of group A RVs (see Commentary). As with all antibodies, the primary and secondary antibodies must be titrated prior to use to determine the optimal working dilution.

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator.

Additional Materials (also see Basic Protocol 3)

- 1× Phosphate-buffered saline (PBS), pH 7.4 (APPENDIX 2A)
- 100% methanol
- Anti-RV antibody (e.g., Santa Cruz Biotechnology, cat. no. SC-101363)
- 3% bovine serum albumin (BSA) in PBS
- FITC-conjugated secondary antibody (can be purchased from Invitrogen and Sigma-Aldrich)
- 96-well tissue culture plates
- Multichannel pipettor

1.5-ml microcentrifuge tubes

Inverted fluorescence microscope with fluorescein isothiocyanate (FITC) filter

Prepare MA104 cells

1. Seed MA104 cells into 96-well plates at a density of 6.4×10^3 cells per well, and incubate for 3 to 5 days.

One confluent 150-cm² flask of MA104 cells contains approximately 1.5×10^7 cells. For simplified plating, the trypsinized cells from one flask can be resuspended in 10 ml of complete medium, and 500 μ l of the cell suspension is diluted in 20 ml of complete medium. Then, 200 μ l of the diluted suspension is plated per well.

2. Using a multichannel pipettor, wash the MA104 cells in 96-well plates two times, each time using 200 μ l/well of prewarmed, serum-free Medium 199.

FBS, a component in MA104 growth medium, inhibits RV activation and must be removed for efficient infection of the cells. Cells can also be incubated several hours to overnight in one change of serum-free medium.

3. Remove the second wash and add 100 μ l/well of prewarmed, serum-free Medium 199 containing 1 μ g/ml trypsin.

Medium containing trypsin is added to the cells to prevent them from drying out. The activated virus inoculum will be added directly to this medium.

Activate virus and prepare dilutions

4. Thaw the vial of RV to be assayed at room temperature.
5. Activate the virus by mixing 200 μ l of RV with 1 μ l of trypsin (2 mg/ml stock) for a final trypsin concentration of 10 μ g/ml.
6. Incubate the virus 1 hr in a 37°C water bath.
7. Prepare dilution tubes by adding 900 μ l of serum-free Medium 199 into each 1.5-ml microcentrifuge tube. One tube is needed for each dilution, usually requiring eight tubes/virus for an endpoint of 10^{-8} .
8. Prepare serial ten-fold dilutions of the virus by adding 100 μ l of the trypsin-activated RV into the first tube (10^{-1}), bringing the final volume to 1 ml. Vortex to mix and then transfer 100 μ l of the 10^{-1} dilution into the 10^{-2} tube. Continue to prepare serial ten-fold dilutions by adding 100 μ l of each dilution into the next tube until the desired endpoint is reached (usually to 10^{-8}). Change pipet tips between each dilution to ensure virus is not carried over.

Infect cells

9. Add 100 μ l of each dilution of virus (usually 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8}) to each of two wells that already contain 100 μ l of serum-free Medium 199 with 1 μ g/ml trypsin. Add virus from most to least dilute, which allows the same pipet tip to be used.
10. Incubate the plates 1 hr at 37°C.
11. Remove the inoculum and add 200 μ l/well of prewarmed, complete Medium 199. Incubate the infected cells 12 to 18 hr at 37°C.

Complete medium containing FBS is added to prevent the virus from spreading in the culture. A neutralizing antibody can also be added into the medium.

12. Wash the infected MA104 cells once using 200 μ l of PBS. Fix the cells by adding 200 μ l of 100% methanol and incubating for 2 min at room temperature. Wash the fixed cells again using 200 μ l PBS.

Add antibody

13. Remove the PBS and add the anti-RV primary antibody diluted in PBS containing 3% BSA (final volume of 200 μ l). Incubate 30 min at room temperature with gentle rotation.

The anti-RV primary antibody must be titrated prior to use to determine the optimal working dilution.

14. Remove the antibody solution and wash the cells two times, each time using 200 μ l PBS.
15. Remove the PBS and add the secondary antibody conjugate to fluorescein isothiocyanate (FITC) diluted in PBS containing 3% BSA (final volume of 200 μ l). Incubate 30 min at room temperature with gentle rotation.

The secondary antibody must be directed against the species the primary antibody was raised in, and must be titrated prior to use to determine the optimal working dilution, which usually ranges from 1:500 to 1:5000.

16. Remove the secondary antibody solution and wash the cells four times, each time using 200 μ l PBS.
17. Count individual fluorescence foci on an inverted fluorescence microscope with a FITC-compatible filter. Calculate the titer of the virus as follows:

$$\text{No. foci} \times 1/(\text{dilution factor}) \times 1/(\text{ml of inoculum}) = \text{FFU/ml}$$

For instance, if the 10^{-6} dilution yielded 30 foci, then the virus titer would be:

$$30 \times 1/(10^{-6}) \times 1/(0.1 \text{ ml}) = 3 \times 10^8 \text{ FFU/ml}$$

PURIFICATION OF ROTAVIRUS FROM PLAQUES

Plaque purification is a technique used to prepare a clonal RV stock. The protocol below describes how to isolate a single plaque, which can then be amplified in a 25-cm² tissue culture flask. To ensure purity, three sequential rounds of plaque purification are generally performed prior to large-scale propagation (see Basic Protocol 1).

Materials

RV stock to be plaque purified
1 \times serum-free Medium 199 (see recipe)

Marker
1.5 ml microcentrifuge tubes
Pasteur pipets with rubber bulbs

Additional reagents and equipment for preparing a plaque assay (Basic Protocol 3)

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator.

1. Prepare a plaque assay as in Basic Protocol 3 such that several individual wells of each 6-well plate contain 10 to 30 plaques.
2. Identify plaques that are completely separated from one another to ensure that only a single plaque is isolated. On the bottom of the plate, use a marker to draw a circle around the plaques to be picked.

3. Add 500 μ l of serum-free Medium 199 to 1.5-ml microcentrifuge tubes so that each plaque to be picked has its own tube.
4. For each plaque, insert a Pasteur pipet into the agarose above the plaque, staying perpendicular to the agarose surface. Push the pipet tip through the agarose until it contacts the cell monolayer. Using the rubber bulb, gently expel a little air to disrupt the monolayer around the plaque. Slowly suck the infected cell material and the agarose plug up into the pipet. Try to prevent the agarose plug from moving too far up into the pipet.
5. Move the agarose plug to an individual 1.5-ml microcentrifuge tube containing 500 μ l of serum-free Medium 199. Dislodge the agarose into the medium by pipetting up and down.
6. Incubate the tubes containing the picked plaque/medium overnight at 4°C to allow the virus to elute from the agarose. The tubes may be rocked overnight at 4°C to facilitate virus elution. Transfer the tubes to –80°C.
7. Freeze and thaw the tubes two times at –80°C. The supernatant can now be plaque purified again or used to infect MA104 cells in a 25-cm² flask (see Basic Protocol 1).

For sequential rounds of plaque purification, a plaque assay can be performed with medium from which the agarose plug was soaked. A dilution series of 10^{-1} , 10^{-2} , and 10^{-3} will allow for single plaque isolation again. This procedure is usually repeated for the third and final time.

STORAGE OF ROTAVIRUSES

RV stocks should be stored long-term (<1 year) at –70° to –80°C. RV does not require the presence of a cryoprotectant. Avoid repeated freezing and thawing to prevent loss of infectivity by storing the virus in small aliquots of 1 to 5 ml. Semi-purified or purified RV can also be stored in TNC buffer up to 1 year at 4°C with minimal loss of titer.

GROWTH AND MAINTENANCE OF MA104 CELLS

MA104 cells are African green monkey kidney cells and are available from ATCC as MA104 clone 1. Early isolates of MA104 cells were found to be a mixture of African green monkey and Rhesus monkey cells. ATCC isolated a pure MA104 cell population in 2001. If obtaining cells from sources other than ATCC, the history of the cells should be taken into account. Though MA104s can be cultured beyond 80 passages, the authors do not recommend using them for RV growth and quantification beyond 50 passages.

Cell cultures should be maintained in an actively growing state (log phase or exponential growth) to ensure optimum health. Using an inverted phase contrast microscope, check the general appearance of your culture. It is also important to examine the culture with the unaided eye to look for signs of microbial contamination. Your standard protocol routinely used for subculturing cell cultures can generally be used to maintain MA104 cells. The amounts of reagents recommended in this procedure are for a 150-cm² flask; volumes should be adjusted accordingly for other culture vessel sizes.

Materials

- MA104 cells (ATCC #CRL-2378.1)
- 1 \times Phosphate-buffered saline (PBS), pH 7.4 (APPENDIX 2A)
- 0.05% Trypsin/0.1% EDTA solution (or alternative disassociating reagent)
- 1 \times complete Medium 199 (see recipe)
- Trypan blue solution (optional)

**BASIC
PROTOCOL 5**

**SUPPORT
PROTOCOL**

**Animal RNA
Viruses**

15C.3.17

5-, 10-, and 25-ml pipets
Inverted light microscope
150-cm² tissue culture flasks

NOTE: All equipment and solutions coming in contact with cells must be sterile and sterile technique should be used. Cell culture work should be conducted in a class II biological safety cabinet.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO₂ incubator, unless otherwise specified.

1. Using a sterile pipet, remove and discard the culture medium from the flask containing the MA104 cells.
2. Wash the cell monolayer once using 10 ml of prewarmed PBS to remove all traces of FBS.
3. Remove the PBS and wash the cell monolayer once using 5 ml of trypsin-EDTA.
4. Remove the trypsin-EDTA wash. Add 1 ml of trypsin-EDTA and rock the flask by hand to ensure coverage of the cell monolayer. Incubate the flask at 37°C to detach the cells from the culture dish.

Prewarming the trypsin-EDTA solution will decrease the time needed to detach the cells.

5. Look at the cells every 2 to 3 min using an inverted light microscope. Once the cells have rounded up, gentle tapping of the flask should detach them from the plastic surface.
6. Add 9 ml of prewarmed, complete Medium 199 to the flask to inactivate the trypsin-EDTA solution. The detached cells are now resuspended in 10 ml total volume.

Vigorous pipetting may be required to detach any remaining cells from the bottom of the culture vessel or to create a single cell suspension.

If desired, trypan blue staining can be performed to determine the viability and number of MA104 cells.

7. For a 1:5 dilution, transfer 2 ml of the cell suspension into a new 150-cm² tissue culture flask. Add 28 ml of prewarmed, complete Medium 199 to bring the volume up to 30 ml. Place the flask in the incubator with the cap loosened. Cells will produce a confluent monolayer in 4 to 7 days.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Agarose, 1.2% (w/v)

Prepare 1.2% (w/v) cell culture grade agarose (SeaPlaque or SeaKem) with cell culture grade water. Autoclave to sterilize. Store up 1 year at room temperature.

Before use, melt presterilized 1.2% (w/v) agarose by microwaving, and allow agarose to cool in a 55°C water bath.

Eagle's Minimal Essential Medium (EMEM), 1×, complete

Supplement 500 ml incomplete 1× EMEM (contains Earle's balanced salts, without L-glutamine; Quality Biological, cat. no. 112-016-101) with:
5 ml of 200 mM L-glutamine stock (2 mM L-glutamine final concentration)

continued

5 ml penicillin/streptomycin stock (100 U/ml penicillin/100 µg/ml streptomycin final concentration; see recipe)
0.5 ml 250 µg/ml amphotericin B stock (0.25 µg/ml amphotericin B final concentration)
57 ml fetal bovine serum (10% FBS; Invitrogen, cat. no. 26140-079)
Store up to 3 months at 4°C

EMEM, 1×, serum-free

Supplement 500 ml incomplete 1× EMEM (contains Earle's balanced salts, without L-glutamine; Quality Biological, cat. no. 112-016-101) with:
5 ml of 200 mM L-glutamine stock (2 mM L-glutamine final concentration)
5 ml penicillin/streptomycin stock (100 U/ml penicillin/100 µg/ml streptomycin final concentration; see recipe)
0.5 ml of 250 µg/ml amphotericin B stock (0.25 µg/ml amphotericin B final concentration)
Store up to 3 months at 4°C

EMEM, 2×, serum-free

Supplement 500 ml incomplete 2× EMEM (contains Earle's balanced salts, without L-glutamine, without phenol red; Quality Biological, cat. no. 115-073-101) with:
10 ml 200 mM L-glutamine stock (4 mM L-glutamine final concentration)
10 ml penicillin/streptomycin stock (200 U/ml penicillin/200 µg/ml streptomycin final concentration; see recipe)
1 ml 250 µg/ml amphotericin B stock (0.5 µg/ml amphotericin B final concentration)
Store up to 3 months at 4°C

Medium 199, 1×, complete

Supplement 500 ml incomplete Medium 199 (contains Earle's salts, 2200 mg/liter sodium bicarbonate, L-glutamine, and 25 mM HEPES buffer; Invitrogen, cat. no. 12340-030) with:
5 ml penicillin/streptomycin stock (100 U/ml penicillin/100 µg/ml streptomycin, final concentration; see recipe)
0.5 ml 250 µg/ml amphotericin B stock (0.25 µg/ml amphotericin B final concentration)
27 ml fetal bovine serum (5% FBS; Invitrogen, cat. no. 26140-079)
Store up to 3 months at 4°C

Medium 199, 1×, serum-free

Supplement 500 ml incomplete Medium 199 (contains Earle's salts, 2200 mg/liter sodium bicarbonate, L-glutamine, and 25 mM HEPES buffer; Invitrogen, cat. no. 12340-030) with:
5 ml penicillin/streptomycin stock (100 U/ml penicillin/100 µg/ml streptomycin final concentration; see recipe)
0.5 ml 250 µg/ml amphotericin B stock (0.25 µg/ml amphotericin B final concentration)
Store up to 3 months at 4°C

Penicillin/streptomycin stock solution

Available commercially; Quality Biological, cat. no. 120-095-061
10,000 U/ml penicillin
10,000 µg/ml streptomycin
Store up to 1 year at −20°C

Sucrose, 35% (w/v), in TNC buffer

Dissolve 35 g sucrose in 50 ml TNC buffer (see recipe)
Bring volume up to 100 ml using TNC buffer (see recipe)
Store up to 1 year at 4°C

TNC buffer (pH 8.0)

To make a 500 ml solution, add:
10 ml of 1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*; 20 mM Tris·Cl final concentration)
10 ml of 5 M NaCl (100 mM NaCl final concentration)
0.5 ml of 1 M CaCl₂ (1 mM CaCl₂ final concentration)
479.5 ml distilled, deionized water
Store up to 1 year at 4°C

Trypsin in PBS, 2 mg/ml

Dilute 0.2 g trypsin porcine pancreatic type IX (Sigma Aldrich) in 100 ml phosphate-buffered saline (PBS; *APPENDIX 2A*) to make a stock solution of 2 mg/ml. Filter sterilize. Aliquot into small volumes (≤ 200 μ l). Store up to 1 year at –20°C. Avoid thawing and re-freezing aliquots more than twice.

COMMENTARY

Background Information

RVs are members of the *Reoviridae* family and are major pathogens associated with acute gastroenteritis in humans and animals. The RV genome consists of eleven segments of double-stranded RNA (dsRNA) contained within a non-enveloped, triple-layered capsid (Pesavento et al., 2006). In general, each gene segment encodes a single polypeptide, allowing RVs to express five or six nonstructural proteins (NSP1-5 and sometimes NSP6) and six capsid proteins (VP1-4, VP6, and VP7). The inner layer of the virion is composed of the core lattice protein (VP2) and encases the viral RNA-dependent RNA polymerase (VP1), the RNA capping enzyme (VP3), and genomic RNA. The intermediate layer is made entirely of VP6, and the outermost layer of the RV particle is composed of capsid proteins VP7 and VP4. The structural proteins within each shell can vary among strains, leading to antigenic differences that can be detected using immunological assays. As such, the reactivity pattern of antibodies against certain rotavirus capsid proteins is the primary method by which viruses in this family are classified (Desselberger et al., 2001; Estes, 2001; Kapikian et al., 2001). Specifically, the serogroups described for RVs (groups A to G) are based on the binding of non-neutralizing monoclonal antibodies to VP6 (Estes, 2001; Kapikian et al., 2001). Though groups A, B, and C have been isolated from humans, group A RVs represent by far the most common cause of infantile gastroenteritis (Parashar et al.,

2003). Group A RVs are further classified into subgroups and serotypes. Subgroups are based on the immunoreactivity pattern of non-neutralizing monoclonal antibodies against VP6 of the same serogroup, while serotypes are based on the reactivity of outer capsid proteins (VP7; G-types and VP4; P-types) to neutralizing antibodies (Kalica et al., 1981; Kapikian et al., 1981; Estes, 2001). Along with the more recently described genotypes, serotypes remain the most common method of classifying group A RVs in epidemiological studies (Desselberger et al., 2001). To date, a total of 14 G-types (19 G-genotypes) and 14 P-types (27 P-genotypes) have been identified in nature (Matthijnssens et al., 2008). In humans, G1P1A[8], G2P1B[4], G3P1A[8], and G4P1A[8] strains are commonly associated with disease (Desselberger et al., 2001). Recently, a classification system has been developed to assign genotypes for each of the eleven RV genes based on nucleotide sequence (Matthijnssens et al., 2008).

Group A RV infections cause severe diarrhea in infants and young children and are responsible for up to 600,000 deaths each year worldwide (Parashar et al., 2003). Although morbidity and mortality are usually associated with the very young, asymptomatic and symptomatic infections of adults can occur (Ramig, 2007). RVs are transmitted via the fecal-oral route and replicate in the mature enterocytes covering the tips of intestinal tract villi (Estes et al., 2001). The incubation period of the virus is 24 to 48 hr, and the

onset of symptoms is abrupt and characterized by watery diarrhea, vomiting, and fever (Kapikian et al., 2001). While the mechanism of RV-induced gastroenteritis is not fully understood, CPE caused by viral replication is thought to play a role. Infected enterocytes are visibly damaged, and defective in digestive and absorptive functions, resulting in an osmotic imbalance and development of diarrhea (Estes et al., 2001). Additionally, several lines of evidence suggest that a RV-encoded non-structural protein (NSP4) has endotoxin-like properties and may contribute to the development of disease (Ball et al., 2005). In developed countries, the acute gastroenteritis is often self-limiting, and with proper rehydration therapy, infected individuals generally make a full recovery (Parashar et al., 2003). However, children in developing countries, who lack access to clean water and hospital care, often die from dehydration (Parashar et al., 2003). Emerging data suggests that RV infection is systemic, where both viral antigens and infectious virus enter into the bloodstream; yet, the contribution of such antigenemia and viremia to RV pathogenesis and spread is unknown (Ramig, 2007). Two live-attenuated vaccines, RotaTeq and Rotarix, are available for use in the United States as well as other countries. Both vaccines have been proven safe and effective in preventing RV disease. RotaTeq is a bovine-derived multivalent vaccine that contains the G1-G4 and P1A[8] components of human RVs. Rotarix is monovalent, attenuated human vaccine with G1P1A[8] specificity (Ward, 2008). Vaccination is considered the best strategy to decrease the disease burden associated with RV infections.

The discovery that RV infectivity is dependent upon proteolytic activation was a major breakthrough in the routine cultivation of these viruses (Graham and Estes, 1980; Estes et al., 1981; Sato et al., 1981). Prior to this discovery, few animal strains had been propagated, and infection of cultured cells using a human RV (strain Wa) first required several passages in gnotobiotic piglets (Wyatt et al., 1980). Early studies utilized a mix of gastrointestinal enzymes to increase RV infectivity. Further studies demonstrated that the enzyme trypsin is solely responsible for cleaving the attachment protein VP4 into products VP8* and VP5*, promoting the entry of the virus into a host cell (Estes et al., 1981; Arias et al., 1996; Dormitzer et al., 2004). The mechanism of RV entry is not fully understood and the search for cellular receptors is continu-

ing. Because non-activated RV is capable of binding to cells, trypsin activation is probably required following the initial viral attachment step (Clark et al., 1981). Studies have shown that RV is capable of entering cells either by directly penetrating the plasma membrane, or by penetrating an endosome following receptor-mediated endocytosis (Jayaram et al., 2004). While the usage of these distinct entry pathways may be cell-type or strain dependent, both require the virus to be trypsin-activated. In addition to VP4 cleavage, efficient RV entry is also contingent upon the integrity of the outer capsid protein VP7, which is stabilized by calcium (Shirley et al., 1981). The concentration of calcium is low (10 to 20 nM) in the intracellular environment, leading to RV uncoating and initiation of the viral replication cycle (Jayaram et al., 2004). Treatment of RV with calcium-chelating agents, such as EDTA, will result in disruption of the VP7 layer and loss of infectivity. It is particularly important when cultivating RV using medium other than those described in this unit that the calcium concentration is taken into account.

Critical Parameters and Troubleshooting

Most difficulties encountered when cultivating RVs result from problems with cell culture. To maintain the health and quality of MA104 cells, optimal growth conditions should be used. Cells should be subcultured when the monolayer is just confluent and diluted 1:5 each passage to ensure log-phase or exponential growth. The optimal monolayer is one in which the cells are just touching, but with no visible spaces in between. Over a number of passages, MA104 cells can lose their susceptibility to RV infection; the authors recommend starting a fresh culture around passage 50. In addition, if a monolayer is subconfluent or overgrown, it is less supportive of infection. For primary AGMK cells, infections should be performed on a healthy cell monolayer that is not more than 4 days old.

Proper trypsin activation is of paramount importance when amplifying or quantifying RV. For best results, the authors recommend using porcine pancreatic trypsin type IX (Sigma Aldrich), which can be prepared as a 2 mg/ml stock solution in PBS and frozen in small aliquots. Trypsin is a proteolytically active enzyme, and is sensitive to changes in temperature. To prevent inactivation, the enzyme should be thawed on ice and not subjected to multiple rounds of freeze-thaw. Because

FBS will inhibit trypsin and may contain RV-neutralizing antibodies, problems with RV infection can arise when cells are not washed sufficiently to remove serum-containing growth medium. To facilitate the complete removal of FBS, MA104 cells can be incubated for several hours to overnight in one change of serum-free medium. Once activated by trypsin, RV infection of cells may proceed. Presence of trypsin in the viral inoculum at a concentration exceeding 2 $\mu\text{g/ml}$ may cause the cell monolayer to slough off. The authors advise diluting virus activated with 10 $\mu\text{g/ml}$ of trypsin to $<2 \mu\text{g/ml}$ to prevent loss of the cell monolayer during infection.

The presence of trypsin in the culture medium at a concentration of 0.5 $\mu\text{g/ml}$ allows for efficient virus propagation. However, to generate a completely non-activated RV stock, replace the inoculum with complete Medium 199 containing trypsin inhibitors (such as aprotinin). In this case, cells should be infected at an $\text{MOI} \geq 3$. Due to the presence of trypsin inhibitors in the resulting clarified

lysate, virus cannot be reactivated by incubation with trypsin. The non-activated RV stock must be semi-purified by pelleting (see Basic Protocol 2) or purified in a cesium chloride gradient (see Alternative Protocol 3) to remove trypsin inhibitors if reactivation is necessary.

When growing RV from clinical specimens, bacteria present in the feces are a significant source of contamination. Such bacterial contamination can be reduced by clarifying the specimen using centrifugation and by maintaining antibiotics in the culture throughout the course of infection. Additionally, the clarified fecal material can be passed through a 0.45- μm filter to remove most bacteria.

The plaque assay can be a difficult technique to master, with many critical steps that could potentially cause the assay to fail. Please refer to Table 15C.3.2 for solutions to frequently encountered problems with the RV plaque assay. Some of the same problems may be encountered in the fluorescence focus assay, but most difficulties are due to the primary antibody used to detect RV antigen. The optimal

Table 15C.3.2 Troubleshooting Guide for the RV Plaque Assay

Problem	Possible cause	Solution
No plaques	Cells dried out	Work quickly at each step when removing medium from wells
	Unhealthy cells	Monolayers should be confluent, but do not allow cells to overgrow
	Agarose overlay too hot, inactivating trypsin or killing cells	Equilibrate molten agarose to 55°C before mixing with 2 \times EMEM
	Entire monolayer of cells lysed	Make more serial dilutions so less virus is present in the inoculum
	Virus too dilute	Make fewer serial dilutions so more virus is present in the inoculum
	Infection failed	Repeat assay in parallel with a positive control
	Plaques too small to be visible with neutral red staining	Stain plaque assays with crystal violet to enhance contrast between plaques and uninfected cells
Clumpy agarose	2 \times EMEM too cold	Warm 2 \times EMEM to 37°C before adding molten agarose
	Agarose too cool	Equilibrate molten agarose to 55°C before mixing with 2 \times EMEM
Contamination	Improperly sterilized reagents	Prepare all reagents fresh, ensuring solutions are autoclaved or filter sterilized; use sterile technique
	Fungal growth	Increase amphotericin B concentration to 2.5 $\mu\text{g/ml}$

antibody concentration must be determined in advance, and it must immuno-react with the strain of RV being tested.

Anticipated Results

Animal RVs that are cell culture-adapted should be expected to grow to titers of $\sim 10^7$ to 10^8 PFU/ml. Cell culture-adapted human RVs grow to titers of $\sim 10^4$ to 10^6 PFU/ml. Human RVs from clinical fecal specimens do not grow efficiently in cell culture, and must be passaged in primary cells prior to growth in continuous cell lines. Once adapted to cell culture, clinical isolates usually grow as well as the prototypic cell culture-adapted human RV strains. It is of note that the RV titer obtained by plaque assay will likely differ from that obtained by the fluorescence focus assay. Usually, one method of quantification is chosen and used for all RVs of interest to maintain consistent results.

Time Considerations

For growth and maintenance of MA104 cells, solutions coming into contact with cells should be warmed to 37°C. This time will vary from 30 min to 2 hr, depending on the size of the solution vessel. The time it takes to physically manipulate the cells from splitting is <30 min. MA104 cells must be plated 3 to 5 days in advance of the infection for RV propagation.

The time necessary for thawing the RV sample will vary, depending on the size of the frozen aliquot. The RV activation step requires 1 hr, followed by an adsorption time of 1 hr. For propagation at a low MOI, infected MA104 cultures can take 3 to 7 days before CPE is visible. Clinical specimens may need to be passaged in primary cells every 7 days up to 10 times (i.e., 70 days) before CPE is seen.

RV purification requires several days, mostly as a result of the centrifugation and dialysis steps. If using the sucrose protocol to semi-purify RV, the spin time is only 1.5 hr; yet, if banding virus in a CsCl gradient, >18 hr is required. The authors recommend dialyzing the purified RV against TNC buffer for 2 to 3 days, changing the buffer at least once a day. Thus, it may take up to a week to go from RV-infected cell lysate to purified RV particles amenable to biochemical or structural studies.

For quantifying RV by plaque assay, MA104 cells must be plated 3 to 5 days in advance of the infection. The RV activation step requires 1 hour, followed by preparing serial dilutions of the virus, which requires

~ 30 min. After the 1-hr adsorption time, the agarose overlay requires 30 min to solidify. Cell-culture adapted animal RVs form plaques 3 to 4 days post-infection, while human RVs may take as long as 7 days for plaques to form. The fluorescence focus assay takes less time than the plaque assay. The infection proceeds for only 12 to 18 hr and the antibody-binding steps can be completed in a single day. Thus, while a plaque assay takes ~ 4 days (from infection to titer determination), the fluorescence focus assay only takes 2 days.

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