Lymphocytic Choriomeningitis Virus (LCMV): Propagation, Quantitation, and Storage

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ABSTRACT

Lymphocytic choriomeningitis virus (LCMV) is an enveloped, ambisense RNA virus and the prototypic virus of the arenavirus group. It can cause viral meningitis and other ailments in humans, but its natural host is the mouse. The LCMV/mouse model has been useful for examining mechanisms of viral persistence and the basic concepts of virus-induced immunity and immunopathology. This unit discusses strain differences and biosafety containment issues for LCMV. Recommendations are made for techniques for propagating LCMV to high titers to quantify it by plaque assay and PCR techniques and to preserve its infectivity by appropriate storage. *Curr. Protoc. Microbiol.* 8:15A.1.1-15A.1.11. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is the prototype virus of the arenavirus group. It is an enveloped, ambisense RNA virus containing two RNA segments: L, which encodes an RNA-dependent RNA polymerase and a zinc finger binding Z protein; and S, which encodes a nucleoprotein (NP) and a glycoprotein precursor (GP0) that is cleaved into two subunits, GP1 and GP2 (Welsh, 2000). LCMV causes a persistent infection in its natural host, the mouse, but it is capable of infecting a wide range of animals, including humans. LCMV is easy to isolate from the wild, and many strains have been isolated.

Most studies in scientific laboratories have focused on derivatives of three isolates originating in the early 1930s: the Armstrong strain, isolated from a monkey undergoing a lymphocytic choriomeningitis (hence the name); the Traub strain, isolated from a laboratory colony of persistently infected mice; and the WE strain, isolated from a human after exposure to persistently infected mice. The Armstrong strain is sometimes referred to as "neurotropic," whereas the Traub and WE strains are sometimes referred to as "viscerotropic." The neurotropic designation is a confusing misnomer because each of these strains can grow well in the brain. However, high levels of viral replication in the viscera seem to either distract or clonally exhaust T cells, preventing a strong T cell–dependent meningitis and encephalitis from occurring. LCMV has been an important model for studying T cell–dependent pathology in the brain.

Many variants of these strains also exist. Notably, the clone 13 derivative of the Armstrong strain and the "docile" derivative of the WE strain seem to replicate better in mice than their respective parent strains and are more likely to cause the clonal exhaustion of T cells by high antigen load (Moskophidis et al., 1993; Zajac et al., 1998). Escape variants bearing mutations in T–cell or antibody epitopes have been generated (Lewicki et al., 1995; Ciurea et al., 2000). Recently, reverse genetic techniques for LCMV have been developed, making it possible to do sophisticated molecular studies and to generate recombinants between LCMV and other viruses (Lee and de la Torre, 2002).

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LCMV is considered an Old World arenavirus and may have originated in Africa. It is closely related to Lassa virus, which causes severe and potentially lethal infections of humans in West Africa. LCMV has disseminated throughout the world in its *Mus musculus* host.

CAUTION: Prior to undertaking any experiments outlined in this unit, the researcher should read and understand the information presented in the Safety Considerations section.

CAUTION: Established strains of LCMV are considered Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: New human or field isolates of LCMV are considered Biosafety Level 3 (BSL-3) pathogens. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

SAFETY CONSIDERATIONS

LCMV can cause persistent or acute infections in animal colonies and is a threat to rodents and primates in veterinary facilities. Therefore, it is best to keep LCMV-infected animals well separated from other animals. Most unexpected infections in animal facilities with LCMV have come when the source of LCMV was unknown, e.g., from an unknowingly contaminated cell line or animal that entered the facility. Usually an LCMV-infected animal colony can be safely maintained with appropriate containment, e.g., a biocontainment suite under negative pressure with a two-door autoclave, allowing one to autoclave the bedding material on its way out of the isolated area. It is important to have a facility with two or more exit doors so that animals can never escape.

LCMV is also a human pathogen, and in some areas ~5% of the human population is seropositive (Welsh, 2000). The agent can cause a variety of syndromes, from malaise to meningitis or encephalitis. Death from LCMV infection is exceedingly rare, and patients nearly always recover without sequellae. The recommended biosafety level for LCMV strains has been ambiguously listed by the NIH/CDC as BSL-2 or BSL-3. At one time the neurotropic strains were considered BSL-3, presumably because they were thought to be a greater hazard to humans. That, however, was an unfortunate characterization because the neurotropic strains are no more neurotropic to humans and certainly no more infectious in humans than the viscerotropic strains. They simply cause meningitis and encephalitis more easily in the mouse because they grow relatively poorly throughout the host, and the T cells are more free to attack the brain. The neurotropic Armstrong strain is now considered a BSL-2 agent, along with the viscerotropic agents.

There are no reliable quantitative data on the relative threats of the different strains to humans. Anecdotally, the authors have heard of more infections in laboratory personnel of strain WE origin than of strain Armstrong origin, although it is possible that the clone 13 variant of Armstrong may be more virulent, as it has caused laboratory infections. The NIH/CDC manual Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th edition now recommends BSL-2 for most procedures with most strains, but it recommends BSL-3 practices for new human or field isolates or for procedures that would result in high-titer aerosols.

PROPAGATION OF LCMV

The LCMV α-dystroglycan receptor is a ubiquitous protein expressed in many cell types and in different species, and the virus can consequently grow in a wide variety of cell types from many species, including mouse, hamster, monkey, and human (Cao et al., 1998). The best yields are from fibroblast or epithelial cell lines because it grows poorly in lymphocytes. Very good yields, 2–3 $\times$ 10^8 plaque forming units (pfu) per ml, can be obtained in cultures of baby hamster kidney cells, specifically BHK21, which do not shed any endogenous retrovirus (like many mouse cell lines) that may contaminate the end product. Good titers can be obtained at 48 hr by inoculating monolayers (or suspension cultures, an option with BHK21/13s cells) with a multiplicity of infection (MOI) of 0.03 to 0.1 pfu/cell or at 72 hr with an MOI of 0.003 to 0.01 pfu/cell. The advantage of a low-multiplicity infection is that it reduces the problem of viral interference and gives rise to high-titer stocks. For optimal yields these cells should be in a highly active metabolic state, and the monolayer should be ~50% to 75% confluent at the time of infection.

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

Materials

Baby hamster kidney (BHK) cells, lines 21 or 21/13s
BHK propagation medium
Lymphocytic choriomeningitis virus (LCMV; MOI of 0.03 to 0.1 pfu/cell for harvesting after 48 hr *or* MOI of 0.003 to 0.01 pfu/cell for harvesting after 72 hr)
75-cm^2 (T-75) or 150-cm^2 (T-150) tissue culture flasks, or tissue culture roller bottles
Plastic centrifuge tubes of appropriate size
Refrigerated centrifuge, 4°C

Procedure

1. Select an appropriate size plastic tissue culture vessel for propagation, depending on the volume to be harvested.

   These may be T75 or T150 flasks, or roller bottles for larger scale propagation.

2. Seed flasks with BHK cells in BHK propagation medium. Use 30 to 40 ml/T-75 flask, 60 ml/T-150 flask, or 150 ml/roller bottle and incubate.

   BHK cells divide quickly and can undergo up to two divisions a day at 37°C, 5% CO_2.

   The specific cell density to initiate the cultures should probably be empirically derived, due to differences in the rates of divisions of different cell preparations.

3. When vessels are ~50% confluent, decant the culture fluid and infect with virus in a limited volume (3 ml/T-75, 6 ml/T-150, 25 ml/roller bottle). Occasionally tilt flasks during the infection period and have roller bottle turning at about 1.5 rpm.

4. After 1 to 1.5 hr, add BHK propagation medium to previous levels and incubate 2 to 3 days (depending on MOI).

   LCMV does not cause substantial lysis of cells visible as cytopathic effect in these cultures, but there will likely be a reduction in cell density compared to uninfected control cultures.

5. Harvest cells after 48 hr (MOI of 0.03 to 0.1 pfu/cell) or 72 hr (MOI of 0.003 to 0.01 pfu/cell) by decanting the culture fluid into plastic centrifuge tubes and centrifuging the cells away from the virus-containing culture fluid 10 min at 350 $\times$ g, 4°C.

   Although a substantial amount of virus can be cell-associated, the intentional disruption of cells is not needed and, in fact, may impair purification efforts.

There may be many BHK cells in the culture fluid, and a second and similar centrifugation might be required to clear the culture fluid.

6. Keeping the virus cold at all times after the harvest, dispense the culture fluid into aliquots and store indefinitely at −70°C.

BASIC PROTOCOL 2

QUANTITATION OF LCMV INFECTIOUS UNITS BY PLAQUE ASSAY

LCMV has been assayed by many different techniques. Originally it was measured in a lethal dose assay in mice inoculated intracerebrally with dilutions of virus. This assay was very sensitive, as less than one pfu of virus could kill an intracerebrally inoculated mouse, but the assay is very expensive, time consuming (6 to 8 days), and causes needless suffering of mice. The most commonly used technique of the past 30 years has been a plaque assay on Vero cell monolayers.

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

Materials

Vero cells (African green monkey kidney cells; ATCC)
Eagle's MEM with 10% (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum (FBS)
Lymphocytic choriomeningitis virus (LCMV) sample to be tested
Vero cell propagation medium (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2× LCMV plaque assay medium (see recipe)
1% (w/v) Seakem agarose-ME (Lonza), recently boiled (in a microwave)
1% (w/v) neutral red (aqueous solution); store up to several months at 4°C

6-well petri plates
96-well microtiter plates
Platform rocker

Prepare cells and virus

1. Seed ~5×10^5 Vero cells onto six-well petri plates in 4 ml/well Eagle's MEM with 10% FBS and incubate.
2. When the monolayers are ~80% confluent in 1 to 2 days (depending on how fast the cells are growing), decant the medium and replace with 1 ml fresh medium
3. Prepare a series of 10-fold dilutions of the test virus sample in Vero cell propagation medium

   For large scale titrations this can be done in 96-well microtiter plates by serially transferring 20 μl of inoculum into wells containing 180 μl medium, changing pipet tips with each transfer.

Infect cells

4. When dilutions are complete, add 100 μl to the Vero cell monolayers, starting with the most dilute sample and using the same pipet for increasing concentrations.
5. Incubate plates 60 to 90 min, with gentle rocking every 20 to 30 min.
6. *Optional:* For best accuracy, after this adsorption/penetration period, remove the medium and wash the monolayers with PBS.

   This will synchronize the infection and will result in more homogenous plaque sizes. However, this is an additional step that increases the possibility of contamination and may not be necessary for most experiments.

Visualize plaques

7. Prepare an agarose overlay by combining equal volumes of 2× LCMV plaque assay medium with recently boiled 1% agarose-ME solution in water that has cooled in a 42°C water bath.

   This will be about 10 min after the boiling step. The agarose solution can also just be left on the bench top and judged satisfactory to use by it not being uncomfortable to touch by one's inner wrist.

8. To each monolayer add ~4 ml agarose overlay. Allow medium to gel for 15 min.

9. Incubate the plates 4 days.

10. Stain the plates with 1.5 ml of a 1:10,000 dilution of neutral red (from a 1% aqueous solution) made up in 1:1 2× LCMV plaque assay medium/1% agarose and incubate overnight.

    This is the same medium as that used for the overlay (step 7), but with neutral red added.

    Neutral red is self-sterilizing, and a 1% solution can be made up in double-distilled water and be satisfactorily kept in the refrigerator for several months.

    If the neutral red starts crystallizing into clumps, avoid adding any clumps onto the plates because they may kill the cells.

    Plaques should be visible the next day.

Analyze results

11. To calculate a titer in plaque-forming units (pfu)/ml, select a petri well with a sufficient number of plaques to give a reliable count and not too many plaques (where they would be superimposed on each other).

    For example, choose plates with ~20 to 70 plaques.

12. Calculate the titer in pfu/ml using the following formula:

    titer (pfu/ml) = (plaque number)/(volume plated in ml × dilution factor of the virus preparation).

    For example, if 50 plaques were found at a 10^5 dilution of an inoculum of 0.1 ml, then the pfu/ml = $50/(0.1 \times 10^{-5}) = 5 \times 10^7$ pfu/ml.

QUANTITATION OF LCMV mRNA BY QUANTITATIVE POLYMERASE CHAIN REACTION (qRT-PCR)

BASIC PROTOCOL 3

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) technique can be used to accurately measure relative levels of an RNA product. In the case of LCMV, qRT-PCR can be used to measure the amount of LCMV mRNA present in a tissue or cell sample. This can be useful for detecting residual gene expression in vivo when pfu can no longer be found or for detecting viral message from samples treated to remove infectivity. This first requires isolation of RNA from a target cell population followed by reverse transcriptase cDNA synthesis. The cDNA generated is then amplified using a quantitative PCR protocol that allows relative measurement of product generated during the reaction.

This protocol describes two methods by which cDNA may be quantified. The first requires addition of a fluorescent nucleic acid gel stain to the reaction mixture (SYBR Green I). SYBR Green I is a nucleic acid stain that binds to the minor groove of double-stranded DNA. The fluorescence emission of SYBR Green I is increased when bound to double-stranded DNA. After each cycle, a camera captures the amount of fluorescence emitted, and, as the amount of DNA product increases, so does the fluorescence emission. At

the end of the reaction, the qPCR machine brings the mixture to a temperature that is suitably below the expected T_m of the resultant product generated. The machine then increases the temperature of the reaction by half-degree intervals with a fluorescence capture after each half-degree increase. This will result in a melt curve that can then be used to confirm the T_m of the resulting product. This final step helps to ensure that a single expected product is generated during the reaction.

The other method used to quantify DNA product takes advantage of the 5′-exonuclease activity of *Taq* polymerase and fluorescent resonant energy transfer (FRET). A single-strand oligo is designed to anneal to a sequence within the product generated during the PCR reaction. This oligo has been labeled with two fluorochromes, typically a higher-energy fluorochrome designated the "reporter" at the 5′ end, and a lower-energy fluorochrome designated the "quencher" at the 3′ end. The oligo is designed to have a higher T_m than the primers, as the oligo must be 100% hybridized to the PCR product for the assay to be accurate. While the fluorochromes are within close proximity, no fluorescence is observed, as the fluorescence of the reporter is quenched by the 3′ quencher; but as *Taq* DNA polymerase degrades the oligo, the reporter and quencher are separated, and fluorescence emission is observed.

In this protocol two quantitative PCR reactions are performed, one using LCMV primers and an LCMV-specific oligo, and one using beta-actin (normally produced by these cells) and SYBR Green I. The beta-actin qRT-PCR is done to control for variations that may occur during the RNA isolation or reverse transcription steps. Both reactions are performed using the same cDNA sample. Quantification during the qRT-PCR reaction is only possible with use of a dilution series of a standard to compare the relative amount of product generated during the reaction. In other words, qRT-PCR cannot be used to directly quantify RNA levels, but is only useful as a method of comparison by way of quantification against a standard. In the following protocol, a qRT-PCR is described that measures relative LCMV RNA levels using the oligo method for LCMV, as initially reported by Roberts et al. (2004). This is then compared to a beta-actin qRT-PCR using primers initially reported by Miller et al. (2004) using SYBR Green.

Materials

Test sample (cells infected with LCMV)
SuperScript First-Strand Synthesis for RT-PCR (Invitrogen)
Double-distilled (dd) H_2O
500 mM Tris buffer
5 μg/μl bovine serum albumin (BSA)
30 mM $MgCl_2$
2.5 mM (each) dNTPs
SYBR Green I nucleic acid stain, 10,000× concentration (Molecular Probes)
10 μM LCMV GP forward primer: 5′-TGC CTG ACC AAA TGG ATG ATT-3′
10 μM LCMV GP reverse primer: 5′-CTG CTG TGT TCC CGA AAC ACT-3′
10 μM beta-actin forward primer: 5′-CGA GGC CCA GAG CAA GAG AG-3′
10 μM beta-actin reverse primer: 5′-CGG TTG GCC TTA GGG TTC AG-3′
1 μM fluorescein (Bio-Rad)
10 μM LCMV *Taq* Man MGB oligo: 6FAM-TTG CTG CAG AGC TT MGBNFQ (Applied Biosystems)
5 U/μl *Taq* DNA polymerase (Promega)

2-ml Phase Lock Gel tubes, heavy (Eppendorf)
iCycler iQ PCR plates, 96 well (Bio-Rad)
iCycler iQ Optical Tape (Bio-Rad)
iCycler iQ real-time PCR detection system (Bio-Rad)

1. Extract RNA from the test sample using 2-ml Eppendorf Phase Lock Gel tubes according to current manufacturer's protocol.
2. Convert RNA to cDNA using the Invitrogen SuperScript First-Strand Synthesis System for RT-PCR (also see *UNITS 16D.3 & 16F.1*).
3. On ice, prepare the following two PCR reaction mixes (one for LCMV and one for beta-actin):

   54 µl dd H_2O (for LCMV qPCR) *or* 42 µl dd H_2O (for beta-actin qPCR)
   12 µl of 500 mM Tris buffer
   12 µl of 5 µg/µl BSA
   12 µl of 30 mM $MgCl_2$
   12 µl of 2.5 mM dNTPs
   12 µl SYBR Green I, use at 1:1500 dilution (for beta-actin qPCR)
   6 µl of 10 µM forward primer (appropriate for LCMV qPCR *or* beta-actin qPCR)
   6 µl of 10 µM reverse primer (appropriate for LCMV qPCR *or* beta-actin qPCR)
   1.2 µl of 1 µm fluorescein (for beta-actin qPCR; background control)
   10 µl sample cDNA (from step 2)
   1.2 µl of 10 µM LCMV *Taq* Man MGB oligo (for LCMV qPCR)
   1.2 µl of 5 U/µl *Taq* DNA polymerase (added last)

   Where indicated in parentheses, use the appropriate component for each PCR mix. The other components are common to the two mixes.
4. Place two 50-µl aliquots (i.e., duplicates) of the reaction mixture into an iCycler iQ PCR plate and seal with iCycler iQ Optical Tape.
5. Carry out the PCR reactions.

   a. *To measure beta-actin cDNA:* Carry out the following program for beta-actin real-time PCR protocol in an iCycler iQ real-time PCR detection system:

Initial step:	2 min 30 sec	95°C (denaturation)
40 cycles:	30 sec	95°C (denaturation)
	25 sec	62°C (annealing)
	25 sec	72°C (extension)
Final step:	indefinite	72°C (hold).

   b. *To measure LCMV GP cDNA:* Carry out the following program for LCMV real-time PCR protocol in an iCycler iQ real-time PCR detection system:

Initial step:	2 min	50°C
40 cycles:	15 sec	95°C (denaturation)
	1 min	60°C (annealing and extension)
Final step:	indefinite	60°C (hold).

Analyses of data obtained are provided in the Commentary section (e.g., see Critical Parameters, qRT-PCR).

STORAGE OF LCMV

LCMV is a heat-labile, enveloped virus that needs to be kept cold, or it will rapidly lose infectivity. Storage should be at −70°C, preferably in the presence of some protein, such as 10% fetal bovine serum, which will enhance stability. Even with that, there will be an ∼50% loss in titer with each freeze-thaw cycle. Virus purified away from protein contaminants will be extremely unstable with a freeze-thaw, unless the purified virus is at a sufficiently high protein concentration to stabilize itself. When thawing the virus, do so quickly at 37°C and immediately put on ice.

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****.*

BHK cell and virus propagation medium

500 ml Dulbecco's high glucose minimal essential medium (DMEM)
10 ml 200 mM glutamine
50 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
25 ml tryptose phosphate broth (5% final)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
Filter sterilize and store up to 2 weeks at 4°C.

Vero cell propagation medium

500 ml Eagle MEM or MEM-Earles salts
5 ml of 200 mM glutamine
50 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
Filter sterilize and store up to 2 weeks at 4°C.

LCMV plaque assay medium, 2×

250 ml 2× Eagle MEM without phenol red (EMEM, Cambrex Bioscience)
5 ml of 200 mM glutamine
25 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
2.5 ml Fungizone (250 μg/ml amphotericin B)
Filter sterilize and store up to 2 weeks at 4°C.

COMMENTARY

Background Information

Quantifying LCMV

Many techniques for quantifying RNA levels using PCR approaches are currently available, and two such workable techniques are suggested in this unit. It is also possible to use probe sets that allow quantification of two cDNA products within the same tube (different fluorescent outputs, i.e., multiplexing), but this protocol has not been tested within our laboratory at the time of this writing. The primers shown in this unit for the qRT-PCR assay are designed to quantify LCMV GP1-specific mRNA (Roberts et al., 2004). The initial reverse transcriptase reaction uses an oligo(dT) primer that hybridizes to poly-A on the 3′ end of mRNA. This assay is unlikely to detect LCMV virion RNA, because the virion RNA, even though of similar positive polarity to mRNA, is not polyadenylated. An alternative assay could be designed to detect mRNA encoding other LCMV proteins, notably for NP, which is well expressed and not synthesized in synchrony with the GP RNA. If the purpose of the technique is to detect low levels of virus and viral gene expression, the analysis of the NP mRNA may be the most sensitive.

Critical Parameters

Autointerference

Of major importance in the generation of high-titer virus stocks is avoidance of the problem of autointerference (Welsh and Oldstone, 1978). LCMV very rapidly generates defective interfering (DI) viruses, especially late in its infecting cycle, where the ratio of DI to standard viruses increases. These DI viruses will greatly interfere (as much as 1000-fold) with the propagation of standard virus if they are present in the inoculum.

To prevent the accumulation of DI virus in seed stocks, these seed stocks should be initiated at a low multiplicity of infection and harvested just prior to the peak of standard virus synthesis. Serial passage of virus should always be with a diluted inoculum, because otherwise the DI virus proportion will increase. Similarly, for the propagation of high titer virus for purification, the carefully prepared seed stock should also be diluted, to ensure that cells are not initially co-infected with DI and standard virus. High-multiplicity infections (e.g., MOI = 1 to 10) rarely produce high-titer stocks, unless the seed stock is virtually devoid of DI virus, and that is a rarity.

The presence of DI viruses in stocks can often be detected in plaque assays, where, at high concentrations of virus the monolayers may look normal, without detectable cytopathic effect. Under those conditions plaques will be seen on monolayers receiving a more diluted inoculum, where there is a lower likelihood that a cell will be co-infected with a standard and a DI virus. LCMV, under optimal conditions, should grow to titers of $1–2 \times 10^8$ pfu/ml.

High titers of virus can also be obtained by freeze-thawing or sonically disrupting cells because more than half of the infectivity in a culture may be cell-associated, but this virus will be heavily contaminated with cell debris and may not be useful in some types of experiments.

Host cells

BHK cells. For optimal LCMV proliferation and plaque assays, cells must be in a suitable and healthy condition. Our subjective (though not quantitatively assessed) observation is that viral yields are poor if BHK cells are heavily confluent prior to seeding vessels with cells for the production of viral stocks, and that seeding the vessels with cells previously growing in log phase is more suitable. Also, waiting for BHK cell monolayers to be confluent before infection seems to reduce viral yield. BHK cells metabolize media very quickly, so limiting the amount of medium in order to increase the viral concentration may not be wise because it is likely to reduce the numbers of healthy BHK cells and the subsequent production of virus.

Vero cells. For plaque assays it is important to use Vero cells that have been carefully maintained in culture and have not been allowed to overgrow vessels during confluence. Vero cells sometimes alter their growth characteristics and become less useful in plaque assays after continuous passage in culture. Avoid mycoplasma contamination (see *APPENDIX 3B*).

qRT-PCR

The RNA levels of each of the tissue samples in each of the qPCR's are quantified by use of a standard. It is critical that quantification of RNA levels is performed at early cycles during the PCR reaction. At earlier cycles, all PCR reagents are in excess and the reaction occurs in an exponential manner, with a doubling of PCR product occurring after each cycle. As the reaction progresses, reagents become limiting, and different samples will generate different amounts of product at each cycle. The amount of relative LCMV RNA calculated is then divided by the amount of relative beta-actin RNA calculated, and this number will be a relative measure of how much LCMV RNA is present in each sample.

Troubleshooting

Propagation of LCMV

BHK cells do not stick to roller bottles. Slow down the RPM, especially during the initial seeding of the vessels. BHK cells slough off from monolayers into culture fluid. This is common for the BHK21/13s cell line, which can be adapted to suspension culture. Usually this does not pose any problems for generating high viral titers, but they should be cleared from the culture fluid by centrifugation before aliquoting virus.

Low viral titers. Viral titers of $<3 \times 10^7$ pfu are suboptimal; this could be caused by not harvesting the culture fluid at the peak of viral production, which may need to be empirically determined by titrating virus at different times after infection. It should be noted that viral titers sharply decline within 12 hr of the peak titer, even though the cells remain with only mild cytopathic effects, such as reduced cell growth. Low viral titers can also be caused by inoculating monolayers with too high a dose

of virus stock containing DI virus (see Critical Parameters, Autointerference). This can be overcome by generating seed stocks from very low dose inocula (MOI = 0.001 to 0.01 pfu/cell) and harvesting just before the peak in titer, because thereafter, DI virus will accumulate. Dilutions of this stock could then be used as inocula for the production of high-titer viral stocks.

Quantitation of LCMV infectious units by plaque assay

Plaques show abnormal morphology. Sometimes LCMV plaques develop concentric rings with a bull's eye effect. These are more readily apparent when incubator CO_2 levels are 5% or higher and less apparent at lower CO_2 concentrations.

Plaques are hard to read. This can happen when cell monolayers or media are suboptimal; sometimes plaques become clearer if the stained plates are left in the incubator for an extra day or two. The contrast in the plaque assay can also be enhanced by adding a 0.5 ml concentrated acetic acid on top of the gel in the plaque assay plate. In 5 to 10 min the staining will be briefly enhanced, and the plaques will need to be counted immediately because the cells will soon die thereafter.

Vero cells slough off monolayer. This can be due to contamination with mycoplasma or to a drift in the nature of Vero cells with continuous passage: use new or earlier passage Vero cells. The effect could also be due to problems with the medium or incubator. If the agarose is repeatedly boiled and used, it will become more concentrated due to evaporation of water, and the more concentrated agarose can be toxic for cells.

qRT-PCR

It is suggested that all reagents purchased for the qRT- PCR are of molecular biology grade, i.e., DNase and RNase free. All molecular biology work should be done using sterile aerosol filter tips because small amounts of a contaminating product are quickly amplified during a PCR. The $MgCl_2$ concentration within the PCR may need to be empirically adjusted. It is known that small variations within the amount of $MgCl_2$ may drastically alter the efficiency of the PCR. The parameters for PCR amplification listed (times and temperatures) may be further optimized because these may vary from machine to machine.

Anticipated Results

Time kinetic studies during the preparation of virus stocks should show a log-phase growth period followed by a peak in viral titer that may plateau for about 12 hr, after which there will be a rapid decline in viral titer, despite the fact that the BHK cell monolayer will remain intact. The peak in titer should be 5–30 $\times$ 10^7 pfu/ml, as measured in the Vero cell plaque assay. When titrating, the plaques should be readily readable on day 5 after infection, which is one day after staining with neutral red. The plaque number should be linear with dilution of inoculum, until the concentration becomes so high that plaques overlap with each other. It will not be uncommon to see little indication of plaques or cytopathic effect on plaque assay monolayers infected with undiluted or 10-fold diluted inocula because there often will be an interference phenomenon (see Critical Parameters, Autointerference).

The qPCR assay should parallel the pfu assay in assessment of viral load by quantifying mRNA for the GP gene. GP mRNA synthesis will slightly precede the increase in viral pfu because it encodes the GP necessary for the virion envelop and infectivity. GP mRNA is rapidly shut down late in the LCMV infection and is associated with an inhibition in viral GP expression on the plasma membrane and reduced release of infectious virus. Its shut down in virus-infected cells will likely be a few hours before the loss of viral titer in the culture fluid, because released virus, which is relatively labile at 37°C, will still take several hours to inactivate.

Time Considerations

Infection of cells with virus can be accomplished within 2 hr. Harvest of virus from culture fluid as well as dispensing into aliquots can be done within 1 hr, including centrifugation steps. Infection of plaque assay plates, incubation, and overlay with medium/agarose can be completed within 2 hr. RNA isolation, if done according to the manufacturer's protocol, takes about 3 hr. Conversion to cDNA takes ~2 to 3 hr, and setup for the PCR takes ~2 to 3 hr, with the actual reaction time varying depending on the setup of the machine (typically 2 to 4 hr).

Literature Cited

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