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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 basic concepts of virus-induced immunity and immunopathology. Here we discuss strain differences and biosafety containment issues for LCMV. Recommendations are made for techniques to propagate LCMV to high titers, to quantify it by plaque assay and PCR techniques, and to preserve its infectivity by appropriate storage.

Keywords

BHK cell; defective-interfering (DI) virus; lymphocytic choriomeningitis virus; PCR; plaque; plaque-forming unit (PFU); vero cell

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 was isolated from a monkey undergoing a lymphocytic choriomeningitis (hence the name). The Traub strain was isolated from a laboratory colony of persistently infected mice. The WE strain was 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, as 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. 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 infection of humans in West Africa. It has disseminated throughout the world in its *Mus musculus* host.

STRATEGIC PLANNING

Safety

LCMV can cause persistent or acute infections in animal colonies and is a threat to rodents and to 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, such as 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, such as a biocontainment suite under negative pressure and with a 2 door autoclave allowing one to autoclave the bedding material out. It is important to have a facility with two or more doors so that animals can never escape. LCMV is also a human pathogen, and in some areas about 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 sequelae. The recommended Biosafety level for LCMV strains has been ambiguously listed by the NIH/CDC as 2 or 3. At one time the “neurotropic” strains were considered BSL3, 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 is not reliable quantitative data on the relative threats of the different strains to humans, but, anecdotally, I have heard of more laboratory infections of strain WE origin than of 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) 4th Edition now recommends BSL2 for most procedures with most strains, but it recommends BSL3 practices for new human or field isolates or for procedures that would result in high titer aerosols.

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.

BASIC PROTOCOL 1 PROPAGATION OF LCMV—The LCMV alpha-dystroglycan receptor is a ubiquitous protein, and the virus can grow in a wide variety of cell types from many species (Cao et al, 1998). The best yields are from fibroblast or epithelia cell lines, as 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 BHK 21-13s cells) with a multiplicity of infection (MOI) of 0.03-0.1 PFU/cell or at 72 hrs with an MOI of 0.003-.01 PFU/cell. For optimal yields these cells should be in a highly active metabolic state, and the monolayer should be about 50-75% confluent at the time of infection.

Materials:

Baby hamster kidney (BHK) cells, lines 21 or 21/13s
BHK propagation media
T75 or T150 tissue culture flasks
Tissue culture roller bottles

Procedure:

1. Select an appropriately sized tissue culture plastic vessel for propagation, depending on the volume you wish to harvest. These may be T75 or T150 flasks, or roller bottles for larger scale propagation.
2. Seed flasks with BHK cells in BHK media. Use 30-40 ml/T75, 60 ml/T150 and 150 ml/roller bottle. BHK cells divide quickly and can undergo up to two divisions a day at 37°C in 5% CO₂.
3. When vessels are about 50% confluent, decant the culture fluid and infect with virus in limited volume (3 ml/T75, 6 ml/&150, 25 ml/roller bottle). Occasionally tilt flasks during infection period and have roller bottle turning at about 1.5 RPM.
4. After 1-1 1/2 hrs, refill the vessels to previous levels and incubate for 2-3 days.
5. Harvest after 48 or 72 hours by decanting culture fluid and pelleting cells away from the virus-containing culture fluid by centrifuging at 1200 RPM for 10 minutes at 4° C. 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 virus cold at all times after the harvest, the culture fluid can then be aliquoted and frozen down 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 assayed by 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 i.c.-inoculated mouse, but the assay is very expensive, time consuming (6-8 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.

Materials:

Vero cells (African green monkey kidney cells)
6-well Petri plates
96-well microtiter plates
Vero cell propagation medium (see recipe)
LCMV plaque assay medium (see recipe)

Above diluted 1:1 with recently boiled (in a microwave) 1% Seakem agarose-ME

Procedure:

1. Seed vero cells onto six-well Petri plates.
2. When the monolayers are ~80% confluent decant the medium (Eagle's MEM with 10% fetal bovine serum) and replace with 1 ml fresh medium
3. Prepare a series of 10-fold dilutions of the test virus sample For large scale titrations this can be done in 96-well microtiter plates by serially transferring 20 μ l of inoculum into wells containing 180 μ l medium, changing pipet tips with each transfer.
4. When dilutions are complete, add 100 μ l samples to the vero cell monolayers, starting with the most dilute sample and, using the same pipet, increasing in concentration.
5. Incubate plates 60-90 minutes in a humidified 37 C 5% carbon dioxide incubator, with an occasional rocking every 20-30.
6. 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.
7. Prepare agarose overlay by combining equal volumes of 2x 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 minutes 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 minutes and then place in the incubator.
9. Incubate plates for 4 days at 37°C in 5% CO₂.
10. Stain with 1.5 ml of a 1:10,000 dilution of neutral red (from a 1% aqueous solution) made up in 1:1 2x plaque assay medium and 1% agarose; 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, as they may kill the cells.
11. Incubate overnight at 37°C in 5% CO₂.

Plaques should be visible the next day.

BASIC PROTOCOL 3 QUANTITATION OF LCMV MRNA BY QUANTITATIVE POLYMERASE CHAIN REACTION (qRT PCR)—The quantitative reverse transcriptase polymerase chain reaction (q RT- PCR) technique can be used to accurately measure relative levels of an RNA product. In the case of LCMV, q RT-PCR can be used to measure the amount of LCMV mRNA present in a tissue or cell sample. 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. (1) 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. (2) 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 done, one using LCMV primers and an LCMV specific oligo, and one using beta-actin and SYBR® Green I. The beta-actin q RT-PCR is done to control for variations that may occur during the RNA isolation or reverse transcription steps. Both reactions are done on the same cDNA sample. Quantification during the q RT-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, q RT 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 q RT 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 q RT-PCR using primers initially reported by Miller et al.(2004) using SybrGreen.

Materials:

- iCycler iQ real-time PCR detection system (Bio-Rad)
- iCycler iQ PCR plates, 96 well (Bio-Rad catalog no. 2239441)
- iCycler iQ Optical Tape (Bio-Rad catalog no. 2239444)
- SuperScript™ First Strand Synthesis for RT-PCR (Invitrogen catalog no. 11904-018)
- 2ml Phase Lock Gel tubes, Heavy (Eppendorf catalog no. 955 15 404-5)
- double distilled (dd) H₂O
- 500 mM Tris buffer
- 5 µg/µl bovine serum albumin (BSA)
- 30 mM MgCl₂
- 2.5 mM dNTP's
- 5 U/µl Taq DNA polymerase (Promega catalog no. M1661)
- Beta Actin primer 1 (10 µM): 5'-CGA GGC CCA GAG CAA GAG AG-3'
- Beta Actin primer 2 (10 µM): 5'-CGG TTG GCC TTA GGG TTC AG-3'

LCMV GP forward primer (10 μ M): 5'-TGC CTG ACC AAA TGG ATG ATT-3'

LCMV GP reverse primer (10 μ M): 5'-CTG CTG TGT TCC CGA AAC ACT-3'

LCMV Taq Man MGB oligo (10 μ M): 6FAM-TTG CTG CAG AGC TT MGBNFQ
(Applied Biosystems, catalog no. 4316034)

SYBR® Green I nucleic acid stain, 10,000x concentration (Molecular Probes catalog no. S7585)

Fluorescein (1 μ M) (Bio-Rad catalog no. 170-3730)

Procedure:

1. Extract RNA from the test sample using the 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.
3. On ice, prepare the following reaction
 - 54 μ l dd H₂O (LCMV qPCR) or 42 μ l dd H₂O (beta-actin qPCR)
 - 12 μ l 500 mM Tris buffer
 - 12 μ l 5 μ g/ μ l BSA
 - 12 μ l 30 mM MgCl
 - 12 μ l 2.5 mM dNTP's
 - 12 μ l SYBR® Green I, use at 1:1500 dilution (for beta-actin qPCR)
 - 6 μ l 10 μ M forward primer
 - 6 μ l 10 μ M reverse primer
 - 1.2 μ l 1 μ M Fluorescein (for beta-actin qPCR)
 - 10 μ l sample cDNA
 - 1.2 μ l 10 μ M LCMV Taq Man MGB oligo (for LCMV qPCR)
 - 1.2 μ l 5 U/ μ l Taq DNA polymerase (added last)
4. Place two 50- μ l aliquots (i.e., duplicate) of the reaction mixture into an iCycler iQ PCR plate and seal with iCycler iQ Optical Tape.
5. Perform the following programs in an iCycler iQ real-time PCR detection system:
 - Beta Actin Real Time PCR protocol
 - Initial step: 95°C for 2 min 30s (denature)
 - 40 cycles: 95°C for 30s (denature)
 - 62°C for 25s (anneal)
 - 72°C for 25s (extend)
 - Final step: hold at 72°C
 - LCMV Real Time PCR protocol
 - Initial step: 50°C for 2m
 - 40 cycles: 95°C for 15s (denature)

60°C for 1m (anneal and extend)

Final step: Hold at 60°C

BASIC PROTOCOL 4 STORAGE OF LCMV—LCMV is a heat-labile, enveloped virus that needs to be kept cold or else 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 about a 50% loss in titer with a freeze-thaw. 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 as to stabilize itself. When thawing out the virus, do so quickly at 37° C and immediately put on ice.

REAGENTS AND SOLUTIONS

BHK cell and virus propagation medium: Dulbecco's high glucose minimal essential medium (DMEM) (500 ml)

10 ml 200 mM glutamine

10% (50 ml) heat-inactivated (56 C, 30 min) fetal bovine serum

5% (25 ml) tryptose phosphate broth

5 ml pen/strep solution (10,000 u/ml penicillin G sodium and 10,000 ug/ml streptomycin sulfate)

Vero cell propagation medium

Eagle MEM or MEM-Earles salts (500 ml)

5 ml 200 mM Glutamine

10% fetal bovine serum (FBS) (heat inactivated)

5 ml pen/strep solution (see above)

LCMV plaque assay medium

250 ml 2X Eagle MEM without phenol red (EMEM, Cambrex Bioscience)

5 ml 200 mM glutamine

25 ml 10% FBS

5 ml pen/step solution (see above)

2.5 ml Fungizone (amphotericin B at 250 µg/ml)

COMMENTARY

Background Information

Protocols 1 and 2: Of major importance in the generation of high titer 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=1-10) 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 and 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, as 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.

Protocol 3: Many techniques for quantifying RNA levels by PCR approaches are currently available, and two such workable techniques are suggested here. 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 here for the q 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.

Critical Parameters

Protocols 1 and 2: 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 also seems to reduce viral yield. BHK cells metabolize media very quickly, so limiting the amount of media in order to increase the viral concentration may not be wise, as it is likely to reduce the numbers of healthy BHK cells and the subsequent production of virus. For plaque assays it is also 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.

Protocol 3: The RNA levels of each of the tissue samples in each of the q PCR'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

Protocols 1 and 2: *BHK cells do not stick to roller bottles*-slow down the RPM, especially during the initial seeding of the vessels. BHK cells sluff off 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 hrs 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; this can be overcome by generating seed stocks from very low dose inocula (MOI=.001-.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. *Plaques show abnormal morphology*-sometimes LCMV plaques develop concentric rings with a “bull’s eye” effect; these are more readily apparent when incubator CO₂ levels are 5% or higher and less apparent at lower CO₂ 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 half ml of concentrated acetic acid on top of the gel in the plaque assay plate; in 5-10 minute the staining will be briefly enhanced, and the plaques will need to be counted immediately, because the cells will soon die thereafter. *Vero cells sluff 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; it could also be due to problems with the media 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.

Protocol 3: It is suggested that all reagents purchased for the q RT- PCR are of molecular biology grade, i.e. DNase and RNase free. All molecular biology work should be done using sterile aerosol filter tips, as small amounts of a contaminating product are quickly amplified during a PCR. The MgCl₂ concentration within the PCR may need to be empirically adjusted, as it is known that small variations within the amount of MgCl₂ may drastically alter the efficiency of the PCR. The parameters for PCR amplification listed (times and temperatures) may be further optimized as 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 hours, 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 from $5-30 \times 10^7$ PFU/ml, as measured in the vero cell plaque assay. The plaques should be readily readable on day 5 after infection, which is and 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 will 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 ten-fold diluted inocula, as there often will be an interference phenomenon. The q PCR 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, as 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 done within 2 hrs. Harvest and aliquoting of virus from culture fluid can be done within 1 hr, including centrifugation steps. Infection of plaque assay plates, incubation, and overlay with medium/agarose can be done within 2 hrs. RNA isolation, if done according to the manufacturer's protocol, takes about 3 hours. Conversion to cDNA takes about 2-3 hours and setup for the PCR takes about 2-3 hours, with the actual reaction time varying depending on the setup of the machine (typically between two and four hours).

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