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Quantitative analysis of HSV gene expression during lytic infection

Anne-Marie W. Turner^{*}, Jesse H. Arbuckle, and Thomas M. Kristie

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Herpes Simplex Virus (HSV) is a human pathogen that establishes latency and undergoes periodic reactivation, resulting in chronic recurrent lytic infection. HSV lytic infection is characterized by an organized cascade of three gene classes, however successful transcription and expression of the first, the immediate early class, is critical to the overall success of viral infection. This initial event of lytic infection is also highly dependent on host cell factors. This unit uses RNA interference and small molecule inhibitors to examine the role of host and viral proteins in HSV lytic infection. Methods detailing isolation of viral and host RNA and genomic DNA, followed by quantitative real-time PCR, allow characterization of impacts on viral transcription and replication respectively. Western blot can be used to confirm quantitative PCR results. This combination of protocols represents a starting point for researchers interested in virus-host interactions during HSV lytic infection.

Keywords

Herpes Simplex Virus; lytic infection; RNAi; small molecule inhibitors; qPCR; western blot

INTRODUCTION

The name ‘herpes’ is synonymous in the general population with cold sores and/or the sexually transmitted disease that results in genital lesions. The causative agents of these diseases, Herpes Simplex Virus 1 (HSV-1) and Herpes Simplex Virus 2 (HSV-2), are but two of the viruses within the large *Herpesviridae* family that also includes the viruses responsible for diverse pathologies including chickenpox (Varicella Zoster Virus) and infectious mononucleosis (Epstein Barr Virus). For HSV, in addition to oral and genital lesions, the viruses also are responsible for more significant disease such as herpes keratitis (infection of the eye), the leading cause of viral mediated blindness in the developed world.

^{*}anne-marie.turner@nih.gov.

INTERNET RESOURCES

YouTube Video Series “Real Time Quantitative PCR Analysis” by americanbiotech (<http://www.youtube.com/playlist?list=PL2C46600596D9A876>)

Hunt, Margaret. Real Time PCR. Microbiology and Immunology On-line, University of South Carolina School of Medicine. <http://pathmicro.med.sc.edu/pcr/realtime-home.htm>.

Harvard PrimerBank - <http://pga.mgh.harvard.edu/primerbank/>

Both HSV-1 and HSV-2 undergo primary lytic infection in epithelial cells that results in clinical lesions. However, a consequence of primary infection is the secondary infection of sensory neuronal cells and the establishment of viral latency in the nerve cell bodies of the ganglia. This leads to life-long, persistent pools of quiescent or latent virus that can reactivate to produce recurrent disease. While there are effective therapies for managing recurrence, there is no cure. As such, studies into the molecular biology of HSV and of viral interactions with the host continue with an agenda to produce an effective vaccine or to identify an Achilles heel that can be exploited to more effectively suppress chronic infection.

HSV-1 lytic infection is characterized by an organized cascade of three gene classes, α (immediate early, IE), β (early, E) and γ (late, L) genes (See Background section for additional information on these gene classes). While the efficient expression of all three classes is required for production of progeny virus, both early and late gene expression are dependent on the expression of the viral immediate early proteins. In contrast, the expression of the IE genes, which represents the critical initiation stage of infection, is highly dependent on host cell factors (DNA binding transcription factors and coactivators). As such, inhibition of immediate early transcription is a major aim of research efforts working to identify novel therapeutics that target components of this regulatory paradigm.

The initiation of HSV infection and the expression of the viral IE genes is a complex interplay between viral and host cell transcriptional activators, coactivators, and chromatin modulation machinery [reviewed in (Kristie et al., 2010)]. Transcription factors and specific coactivator complexes assemble IE enhancer complexes that drive high-level transcription of IE genes. However, the virus is also subject to control that is based upon modulation of the viral chromatin structure. In contrast to some classes of smaller DNA viruses, the HSV genome enters the cell as naked DNA (Pignatti and Cassai, 1980). However it is quickly assembled into a nucleosome structure. At this stage, viral gene expression becomes highly dependent upon the histone code and the large family of host proteins involved in chromatin assembly, modification, and remodeling [reviewed in (Conn and Schang, 2013)].

Strikingly, it has been shown that the initial chromatin structure of the viral genome is heterochromatic (repressive) in nature and is characterized by the presence of repressive histone marks such as histone 3 lysine 9 trimethylation (H3K9me3) and histone 3 lysine 27 trimethylation (H3K27me3) (Liang et al., 2009; Narayanan et al., 2007). During lytic infection, these repressive marks are rapidly erased and replaced with activating histone marks such as histone 3 lysine 4 trimethylation (H3K4me3) (Huang et al., 2006; Narayanan et al., 2007), resulting in a transcriptionally active euchromatic state. This modulation of the viral chromatin structure determines immediate early gene expression and the initiation of the viral gene cascade. The significance of studies investigating the factors that control viral IE expression is evident as they have demonstrated that inhibiting the function of host proteins involved in this chromatin transition (heterochromatic to euchromatic) can severely impact viral IE transcription (Liang et al., 2013b; Liang et al., 2009; Oh et al., 2014). These studies have provided the basis for the identification of novel small-molecule inhibitors of these required chromatin modulation enzymes that have the potential to inhibit lytic infection and recurrence from latency (Liang et al., 2013b; Liang et al., 2009).

Here, basic methods are presented to examine HSV gene expression during a lytic infection or after reactivation from latency [see UNIT 14E.6 (Arbuckle et al., 2014) for more information]. Initial protocols detail how to inhibit host or viral proteins of interest using RNA interference (Basic Protocol 1) or small molecule inhibitors (Basic Protocol 2). After infection by HSV-1 (Basic Protocol 3), viral RNA (Basic Protocol 4) or DNA (Basic Protocol 6) is isolated and the impacts on transcription and replication are assessed by quantitative real-time PCR (qPCR) (Basic Protocol 7). These results can be confirmed by assaying the levels of viral proteins by western blot (Basic Protocol 8). This combination of protocols is a starting point to examine the role of viral and host cell proteins in the progression of lytic HSV infection.

CAUTION: Human herpes virus is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See UNIT 1A.1 (Burnett et al., 2009) and other pertinent resources (APPENDIX 1B: Resources for International Biosafety Guidelines and Regulations) for more information.

NOTE: All solutions and equipment coming into contact with living cells must be sterile. Aseptic technique should be used accordingly.

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

BASIC PROTOCOL 1

TRANSFECTION OF siRNAS

The discovery of RNA interference (RNAi) has resulted in an exceptionally powerful tool – the ability to target and decrease expression of a single, specific gene. Prior to the discovery of small interfering RNAs (siRNAs), loss of function studies in cells were generally performed by the laborious generation of knockout mice. Similarly for viral genes, the production of recombinant genomes was required. The study and commercialization of RNAi as a tool now allows scientists to decrease expression of a target gene within a short time frame. While RNAi is transient and rarely eliminates gene expression, it has proved to be an effective tool to examine host and viral proteins involved in viral infection.

Like other viruses, HSV-1 is dependent on many classes of host cell proteins to effectively replicate. The use of siRNAs is an effective first step to target the expression of a host protein hypothesized to be involved in the viral replication cycle. Following siRNA transfection and viral infection, the levels of HSV-1 transcription (RNA) and genome replication (DNA) can be assessed throughout different stages of the lytic infection to determine the impact of the targeted protein.

The following protocol describes the transfection of siRNAs into the MRC-5 cell line, a non-transformed lung fibroblast line susceptible to HSV-1 infection.

Materials—Cells (MRC-5, ATCC CCL-171)

DMEM/10% FBS (See Recipe)

Gene-targeting siRNA (Dharmacon ON-TARGETplus Smartpools)

Non-targeting siRNA control (Dharmacon ON-TARGETplus Non-targeting Pool, Catalog #D-001810-10-05)

Opti-MEM (Life Technologies, Catalog #31985-062)

HiPerFect Transfection Reagent (Qiagen, Catalog #301704)

1. 24 hours prior to transfection, plate desired number of MRC-5 cells in a 6-well plate (see Support Protocol 1).

A 6-well plate is suggested for MRC-5 cells in order to isolate a sufficient amount of RNA in Basic Protocol 4. Always plate $n+1$ wells, where n = number of experimental samples. The extra well (+1) will be used as a 'counting well' during HSV infection.

Alternately, cells may be plated immediately prior to transfection at a higher cell density of 4×10^6 /well of a 6-well dish. This is generally called the 'fast-forward' protocol and works effectively for transfection of MRC-5 cells.

2. For a 6-well plate, transfect siRNAs at a final concentration of 20nM per well. Prepare the transfection mix by diluting the desired amount of siRNA into 100uL Opti-MEM for each well.

The 20nM concentration represents a starting point using MRC-5 cells and Dharmacon siRNA Smartpools. Optimal knockdown conditions can vary by cell type, type of siRNA (pool versus individual siRNAs), transfection reagent, and transfection conditions. The optimal siRNA concentration for efficient knockdown should be determined empirically by the investigator.

In addition to cells transfected with the gene-targeting siRNA, cells transfected with a non-targeting siRNA control must also be included. Control siRNAs are generally designed to target non-native genes such as luciferase or GFP and provide an effective control for the impact of transfection on the cell. RNA/DNA isolated from control cells will be used downstream to analyze qPCR results.

3. Add 12uL HiPerFect transfection reagent to the diluted siRNA and mix gently.

The volumes of Opti-MEM and HiPerFect are provided for 1 well of a 6-well dish. Amounts can be scaled for different sized tissue culture vessels. Refer to the Qiagen HiPerFect transfection manual for additional information.

4. Allow the transfection mix to incubate at room temperature for 5-10 minutes.
5. Add the transfection mix drop-wise to cells and swirl gently to ensure even distribution. Place 6-well plate in 37°C incubator.

6. 24 hours post transfection, replace the culture media with fresh DMEM/10%FBS.
7. Incubate the cells for an additional 24-48 hours under normal growth conditions and then proceed to HSV absorption and infection (Basic Protocol 3).

Peak knockdown by siRNA targeting generally occurs 48-72 hours post transfection. However, this can vary depending on stability of the target protein and/or the efficacy of the siRNA. The optimal incubation time for siRNAs should be determined by the investigator (also see **Critical Parameters and Troubleshooting** section for additional information).

BASIC PROTOCOL 2

SMALL MOLECULE INHIBITORS

As an alternative to RNAi, the use of small molecule inhibitors or activators represents another method to probe the functions of host proteins. However, in contrast to RNAi, which can be designed to target any protein of interest, the use of small molecules is obviously limited to availability and whether the inhibitors/activators have been characterized. Nonetheless, small molecule inhibitors can be used to confirm RNAi results.

As an example, Tranylcypromine (TCP) is a monoamine oxidase inhibitor that inhibits the enzymatic activity of the histone demethylase LSD1, a protein responsible for removal of histone H3-lysine 4 and lysine 9 methylation. During HSV infection, TCP-mediated inhibition of LSD1 activity results in the accumulation of repressive H3K9 methylation on the viral genome and a block in HSV-1 IE gene expression (Liang et al., 2013a; Liang et al., 2009). As described below, TCP treatment (or the use of other defined inhibitors) of infected MRC-5 cells can be used as a positive control when testing novel small molecule inhibitors.

Materials—Cells of Interest (MRC-5, ATCC CCL-171)

DMEM/10% FBS (See Recipe)

0.5M stock of TCP in DMSO (Sigma-Aldrich, Catalog # P8511)

Vehicle control (solvent used for suspending inhibitor, in this case DMSO)

1. 48 hours prior to treatment, plate desired number of MRC-5 cells (see Support Protocol 1). Cells should be 70-80% confluent at time of drug treatment.

Always plate $n+1$ wells, where n = number of experimental samples. The extra well (+1) will be used as a 'counting well' during HSV infection.
2. For one well of a 6-well dish, make 2mL DMEM/10%FBS containing TCP at a final concentration of 1.5-2.5mM. For one control well, make 2mL DMEM/10%FBS containing the equivalent amount of DMSO.

DMSO serves as a negative control.
3. Remove the media from wells and replace it with media containing TCP or DMSO.

4. Incubate the cells under normal conditions for 4 hours and proceed to HSV absorption and infection (Basic Protocol 3).

Note: When testing a new small molecule inhibitor, multiple time points (such as 4 and 24 hour pretreatments) and multiple concentrations (based on characterizations of IC_{50} or K_D) should initially be tested to determine impact on HSV-1 and optimal drug treatment conditions. Toxicity may arise from longer treatment and/or higher concentrations. Always examine treated wells and record any observations such as cell death or changes in cell morphology. MTT cell proliferation assays (Life Technologies, Catalog # V13154) can serve as initial monitors of compound mediated toxicity.

SUPPORT PROTOCOL 1

CELL PASSAGE AND PLATING OF MRC-5

MRC-5 cells are adherent, passage-limited, human lung fibroblast cells with a normal diploid XY karyotype. The combination of susceptibility to HSV-1 and normal karyotype make MRC-5 cells ideal for approximating conditions that resemble a natural *in vivo* infection. The use of transformed cell lines should be avoided as numerous differences have been observed between components required for infection in these cell lines versus non-transformed cells.

While MRC-5 cells have many advantages, these same characteristics make MRC-5 cells more susceptible to “non-optimal” culture conditions. Additionally, the cells can be maintained for a limited number of passages, thus requiring appropriate planning of experiments as well as maintenance of rotating stocks. Outlined here is a recommended protocol for the thawing, passage, and plating of MRC-5.

Materials—MRC-5 cells (ATCC CCL-171)

Water bath at 37°C

Sterile 15mL conical tubes

DMEM/10% FBS (see Recipe)

Tissue culture dishes/vented flasks

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

0.25% trypsin-EDTA, phenol red (Life Technologies, Catalog #25200-056)

Cell counter or hemocytometer

Trypan Blue (Optional)

Thawing and Passage of MRC-5 cells

- 1) Thaw one vial of MRC-5 cells (from ATCC) by gently swirling the tube in a 37°C water bath until just thawed. Transfer the contents of the thawed vial to a 15mL conical tube containing 9mL DMEM/10% FBS.
- 2) Spin cells at 1,000xg for 5 minutes to pellet cells and remove the media by aspiration. Resuspend the cell pellet in 1mL of DMEM/10% FBS and transfer cells to a vented T-25 flask containing 3mL DMEM/10% FBS.
- 3) A T-25 flask of MRC-5 should reach confluency within 2 to 3 days. To transfer the cells to a larger flask, first remove the media by aspiration and gently wash the cell monolayer with 3mL DPBS. Remove the DPBS by aspiration.
- 4) Add 0.5mL 0.25% trypsin-EDTA to the flask and rock gently to disperse the solution across the cell monolayer. Incubate the flask at 37°C until the cells just begin to detach.

Incubate for the least amount of time required to dissociate the cells to prevent damage to the cells. Do not incubate for more than 5 minutes.

- 5) Add 4mL of DMEM/10%FBS to the flask and pipet cells gently to obtain a single cell suspension. Add the cell suspension to a 15mL conical tube containing 6mL of DMEM/10%FBS. Gently invert to mix and pellet the cells at 1,000xg for 5 minutes.
- 6) Remove the media by aspiration and resuspend the cell pellet in 5mL DMEM/10% FBS. Transfer the entire suspension to a vented T-75 flask containing 10mL DMEM/10%FBS. Incubate the flask for 3 to 4 days under standard conditions.
- 7) A confluent T-75 flask of MRC-5 cells should next be transferred to a T-175 flask. Follow the standard procedure outlined above. For a T-75 flask, use 5mL DPBS to wash the cells and 1 ml trypsin-EDTA to dissociate the monolayer. Add 9mL DMEM/10%FBS to wash the cells from the flask and transfer the suspension to a 15 ml conical tube. Pellet the cells as described above.
- 8) Resuspend the cell pellet in 10mL DMEM/10%FBS and add the entire suspension to a T-175 flask containing an additional 15mL DMEM/10%FBS. Incubate the flask for 3-4 days under standard conditions.
- 9) A confluent T-175 flask of MRC5 cells can be split 1:3 or 1:4 and the resulting flasks of cells should reach confluency in 3-4 days. Under these conditions, MRC-5 cells can be kept in culture approximately 4 to 5 weeks before the growth and condition of the cells begins to deteriorate.

Frozen cell stocks should be made from cells arising from the first split of a confluent T-175cm flask. Freeze cells at $1-2 \times 10^6$ cells/vial in DMEM/10%FBS/10%DMSO and thaw according to the same protocol.

MRC-5 cells are passage-limited. Appropriate planning is required to complete experiments before cells begin to deteriorate. New vials should be thawed prior to this point to allow for the expansion of the new cells to meet experimental needs. It is also highly recommended that new vials of early-passage cells be purchased from ATCC before the laboratory stocks have been exhausted.

Plating of MRC-5 cells

- 10) Remove media from the stock flask of MRC-5 cells, wash, and dissociate the cells as described above.
- 11) Following centrifugation, resuspend the cells in 8mL of DMEM/10%FBS and pipet cells gently to ensure a single cell suspension.
- 12) Count the cells using an automated cell counter or hemocytometer and determine the number of cells per mL of media.

Some automated cell counters may require the addition of trypan blue. Follow standard operating procedures for your specific equipment. Trypan blue should be added when using a hemocytometer.

One T-175cm flask of confluent MRC-5 cells should yield $\sim 6-8 \times 10^6$ cells.

- 13) Plate 3×10^5 cells in 2mL of DMEM/10% FBS per well of a 6-well dish. For one 6-well dish, 1.8×10^6 total cells suspended in 12mL DMEM/10%FBS will be required.

Adjust this protocol as needed. The protocol may be scaled for plates/dishes of different sizes. The amount of cells and media required can be calculated based on the area of the plate/dish relative to a 6-well dish.

- 14) Incubate the dishes at normal growth conditions. The cells will be ready for use 24 hours after plating.

BASIC PROTOCOL 3

HSV ABSORPTION AND INFECTION

Methods used for HSV absorption and infection have been empirically determined by numerous laboratories over many years. In protocols passed down through generations of HSV research laboratories, absorption of the virus to cells is performed in a small volume of media containing no or very low (1-2%) sera for short periods of time (1 hour). The low volume helps to concentrate viral particles and enhance binding to the target cell while low sera reduces the possibility of inactivation of the virus by antibodies, complement, and other inhibitory factors. Provided here is the standard protocol for HSV-1 absorption and infection.

Materials—Cell of interest

Small molecule inhibitor and vehicle control (optional)

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

0.25% trypsin-EDTA, phenol red (Life Technologies, Catalog #25200-056)

DMEM/10%FBS (see Recipe)

37°C Incubator equipped with rocking platform

Infection media (see Recipe)

HSV-1 virus stock [see UNIT 14E.6 (Arbuckle et al., 2014) for more information]

1. Remove media from the extra well plated for counting cells. Wash and dissociate the cells as described in Support Protocol 1 using 200 μ L trypsin-EDTA. Add 800 μ L DMEM/10% FBS, bringing the volume in the well to 1mL. Pipet cells gently to ensure a single cell suspension.
2. Count cells using a hemocytometer or automated cell counter. Determine the total number of cells in the well.
3. Determine the amount of virus needed to infect the experimental wells using the following formulas:

$$(\# \text{ cells})(\text{desired MOI}) = \text{total PFU needed}$$

$$(\text{Total PFU})(1/\text{Titer in PFU}/\mu\text{L}) (n + 1) = \mu\text{L virus stock needed, where } n = \text{number of experimental wells.}$$

Always calculate the amount required including one extra experimental well ($n + 1$) to account for pipetting error and to ensure enough viral working stock for the experiment.

4. Calculate the amount of media needed. For a 6-well dish, the infection is performed in 400 μ L infection media/well. To determine the amount of infection media needed, using the following formula:

$$(n + 1)(400\mu\text{L}) = \mu\text{L infection media needed, where } n = \text{number of experimental wells.}$$

5. Add the amount of virus calculated in step 3 to the media calculated in step 4 and mix gently by inversion.
6. Add 400 μ L of the virus/media to each experimental well.
7. Place the dish in a 37°C/5%CO₂ incubator equipped with a rocking platform. Rock cells at 10rpm for 1 hour to allow absorption of the virus into the cells.

The rocker should be set to rock at the slowest possible rate that maintains an even, moist distribution over the entire well. Alternatively, if a rocker is not available during the viral adsorption stage, plates may be gently rocked every 10-15 minutes by hand to ensure equal distribution of the viral inoculum across the monolayer.

If a more synchronous infection is desired or required, the absorption can be done at room temperature or 4°C. At 4°C, virus will absorb to the cells but will not efficiently penetrate until the temperature is raised by the addition of pre-warmed media. However, if absorptions are done at 4°C, the media must be modified to maintain the appropriate pH during the absorption period.

8. After the 1 hour absorption, remove the virus/media and replace with 2mL fresh pre-warmed DMEM/10% FBS per well.

9. Return cells to 37°C/5%CO₂ incubator and allow virus infection to progress for the desired amount of time.

To examine immediate early gene activation of HSV-1, we recommend an incubation time of 1-3 hours post absorption. Early genes can be readily observed at 4-5 hours and late genes at 6-8 hours. These recommendations are based on an infection of 1.0 to 5.0 PFU/cell.

NOTE: For experiments using small molecule inhibitors, the compounds may be added to the wells prior to viral infection for predetermined times as described in Basic Protocol 2. Compounds may be maintained during viral absorption and post infection, dependent on the parameters of the experiment.

BASIC PROTOCOL 4

RNA ISOLATION USING COMMERCIAL RNA ISOLATION KITS

Analysis of mRNA levels by quantitative real-time polymerase chain reaction (qPCR) is an excellent method to determine the efficacy of siRNA knockdown and the impact on HSV-1 transcription. This method can also be used to analyze the transcriptional patterns during reactivation of latent HSV in mouse model systems [see UNIT 14E.6 (Arbuckle et al., 2014) for more information].

RNA isolation kits using silica-based columns are ubiquitous and sufficient for most purposes. Any commercial kit for total RNA isolation can be used according to the manufacturer's protocol. It is recommended that the selected kit contain a filter column to aid in sample homogenization and also contain an on-column DNase I digestion step.

Materials—HSV-1 infected cells/tissue of interest and controls

Dulbecco's Phosphate Buffered Saline w/o Mg²⁺ or Ca²⁺ (DPBS)

0.1mL micro-sized glass dounce tissue homogenizer (Wheaton, Catalog # 357844)

Total RNA isolation Kit

Isolate II RNA mini kit (Bioline, Catalog # BIO-52072)

RNeasy Mini Kit (Qiagen, Catalog # 74104)

Reducing agent (typically beta-mercaptoethanol)

70% Ethanol

Nuclease-free water

Nuclease-free 1.5mL tubes

As there are numerous column-based RNA isolation kits from various vendors (some examples are provided above), a specific protocol for isolation will not be provided. It is

recommended that the manufacturer's protocol be followed for the specific kit chosen by the end user. The authors make the following recommendations:

1. Choose an RNA isolation kit that incorporates a pre-column filter to aid in homogenization and an on-column DNase I digest. If a DNase digest is not included, see Support Protocol 2.
2. If isolating RNA from latent or reactivated mouse trigeminal ganglia [see UNIT 14E.6 (Arbuckle et al., 2014) for more information], use 4-6 trigeminal ganglia per column. Homogenize the trigeminal ganglia in the provided lysis buffer using a 0.1mL glass dounce homogenizer.
3. When working with MRC-5 cells in a 6-well dish format, elute the final RNA in 40uL nuclease-free water. This will concentrate the isolated RNA and aid in the generation of cDNA (Basic Protocol 5).
4. Store RNA at -80°C to prevent degradation. Proceed with all analysis of RNA as quickly as possible after isolation to ensure the most accurate results.

ALTERNATE PROTOCOL 1

RNA ISOLATION BY TRIzol EXTRACTION

TRIzol-based RNA isolation uses an acidic phenol-guanidinium thiocyanate solution to first lyse cells, denature proteins, and extract nucleic acids. The addition of chloroform allows the separation of organic and aqueous layers and results in the partition of RNA to the aqueous layer for recovery while relegating DNA to the organic layer. While TRIzol is a brand name (Life Technologies), numerous companies sell TRIzol-like reagents that have the same underlying principles and methods.

TRIzol-based RNA isolation can provide two advantages over silica-based column kits: (i) it is more cost-effective than column-based isolation; and (ii) it allows the recovery of the small RNA fractions that are typically lost when using column-based kits. As such, RNA isolated using this protocol can be used to examine changes in smaller RNA species such as HSV-1 or host microRNAs.

The end-use and results for RNA isolation may vary by the user. Therefore, both column-based (Basic Protocol 4) and TRIzol-based isolation protocols are provided. This specific protocol has been adapted for use with phase lock tubes. Phase lock tubes contain a gel that separates the organic and aqueous layers after centrifugation, allowing easier recovery of the aqueous layer and thus improving total RNA isolation.

Materials—Phase Lock Gel Heavy 2mL Tubes (5 PRIME, Catalog #2302830)

HSV-1 infected cells/tissue of interest

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

TRIzol (Life Technologies, Catalog #15596-026)

BioMasher II Closed System Disposable Homogenizer (Kimble Chase, Catalog #749625-0030)

Chloroform (Sigma Aldrich, Catalog #496189)

Nuclease-free 1.5mL tubes

Isopropanol

70% ethanol

Nuclease-free water

Note: The use of this protocol with phase lock tubes does not allow for recovery of DNA or protein and should be used exclusively for RNA extraction.

CAUTION: This protocol involves work with TRIzol, which contains phenol and guanidine isothiocyanate, and chloroform. Wear appropriate personal protective equipment when working with these chemicals. Fumes from TRIzol and chloroform are hazardous. All steps involving TRIzol and chloroform should be performed in a chemical fume hood. Dispose of TRIzol/chloroform waste as per your institute's protocols.

Sample Lysis

- 1) Prepare 2mL heavy gel Phase Lock tubes by centrifuging gel at 12,000xg for 1 minute.
- 2) Lyse samples in TRIzol:
 - a. For cell monolayers:
 - i. Remove media from treated cells and wash quickly with DPBS. Remove DPBS.
 - ii. 1mL TRIzol reagent is recommended per 10cm² of cell surface area. For 1 well of a 6-well plate with a surface area of 9.62cm², add 1mL TRIzol directly to the well. Scale the volume appropriately for other sized plates.
 - iii. Pipet the cells up and down and transfer the lysate to a 2mL heavy gel Phase Lock tube.
 - iv. Allow the lysate to incubate for 5 minutes at room temperature to fully lyse and homogenize any protein complexes.
 - b. For tissue:
 - i. Add 250µL TRIzol to a Biomasher II tube. Add the tissue (maximum of 25mg or 4-6 mouse trigeminal ganglia) directly to the TRIzol. Use the included pestle to homogenize the sample thoroughly. Retain the pestle and allow the sample to incubate at room temperature for 2-3 minutes.

- ii. Add an additional 250 μ L TRIzol to the sample and repeat the homogenization with the retained pestle.

The maximum volume for the Biomasher II system is 500 μ L. Exercise care with the second homogenization to avoid splashing the TRIzol reagent.

- iii. Centrifuge the sample at 12,000 $\times$ g for 5 minutes at 4°C to pellet any remaining debris. Transfer the supernatant to a 2mL heavy gel phase lock tube.

If more than 25mg of tissue was processed, samples can be combined at this step for a **maximum** volume of 1mL TRIzol per phase lock tube. Alternately, the samples may also be pooled at **Step 5**.

Isolation of RNA

- 3) Add 0.2mL chloroform per 1mL TRIzol to the Phase Lock tube.
- 4) Invert the tube until the two phase layers are well mixed. Do not vortex. Incubate at room temperature for 2-3 minutes.
- 5) Centrifuge the sample at 12,000 $\times$ g for 10 minutes at 4°C.
- 6) Recover the RNA-containing aqueous layer located above the phase lock gel and transfer to a clean 1.5 mL tube. Use of the phase lock tubes will allow near 100% recovery of the aqueous layer.

Precipitation of RNA

- 7) Add an equal volume of 100% isopropanol to the recovered aqueous layer.
- 8) Mix well by inverting and incubate at room temperature for 5 minutes.
- 9) Centrifuge the sample at 12,000 $\times$ g for 10 minutes at 4°C.
- 10) Remove the supernatant, being careful not to disturb the RNA pellet.
- 11) Wash the RNA pellet with 1mL 70% ethanol. Centrifuge the sample at 12,000 $\times$ g for 5 minutes and discard the wash. Repeat the wash.
- 12) After the final wash, remove as much liquid from the RNA pellet as possible and briefly air-dry the pellet.

Do not overdry the pellet as this will result in difficulty in resuspension.

- 13) Resuspend the RNA pellet in 50 μ L nuclease-free water.

RNA isolated using TRIzol should be DNase treated (see support protocol 2) prior to determination of concentration.

Store RNA at -80°C to prevent degradation. Proceed with all analysis of RNA as quickly as possible after isolation to ensure the most accurate results.

SUPPORT PROTOCOL 2

DNASE TREATMENT OF TRIZOL ISOLATED RNA

Any RNA to be used for qPCR should be DNA-free as amplification of contaminating DNA can lead to erroneous results. Many commercially available RNA isolation kits incorporate an on-column DNase treatment step which effectively removes remaining DNA. However, contaminating DNA can exist in TRIzol-isolated RNA or RNA from kits that do not incorporate a DNase treatment. This protocol can be used for any RNA isolations that do not contain DNase treatment steps and is adapted from the protocol provided by Life Technologies for the Turbo DNA-free kit.

Materials—TRIzol isolated experimental RNA

DNase, RNase-free (Life Technologies Turbo DNA-free Kit, Catalog #AM1907)

Water bath, 37°C

Nuclease-free 1.5 mL tubes

DNase Treatment

1. Add 0.1x volume of the 10x TURBO DNase reaction buffer to the isolated RNA sample and mix.
2. Add 1uL TURBO DNase to the RNA sample and mix gently by pipetting.
3. Incubate sample at 37°C for 30 minutes.
4. Add 0.1x volume of the TURBO inactivation reagent and mix by flicking the tube. Incubate with the inactivation reagent at room temperature for 5 minutes.

Flick tube periodically to resuspend inactivation reagent as it will settle quickly.

5. Spin the sample at 13,000xg in a microfuge for 1 minute.
6. Transfer the supernatant to a clean 1.5mL tube taking care to avoid transferring the inactivation reagent.

The inactivation reagent is very sensitive to pipetting force and motion. Do not expect to recover all of the RNA-containing supernatant.

7. The recovered supernatant contains DNA-free RNA that can be used for analysis of RNA transcript levels.

BASIC PROTOCOL 5

GENERATION OF cDNA FOR QUANTITATIVE REAL-TIME PCR

The advent of qPCR has fundamentally changed the way changes in gene expression are evaluated. However, a DNA template is required for amplification. Many biotech companies have commercialized genetically engineered, optimized viral reverse transcriptase enzymes that can generate complementary DNA (cDNA) from RNA.

Reverse transcriptase PCR kits convert RNA to cDNA in a streamlined process (< 1 hour). Described here is an effective one-step kit from Thermo Scientific, the Maxima First Strand cDNA synthesis kit. A mastermix contains the reaction buffer, dNTPs, and primers (both random hexamers and oligo-dT) that are added to the isolated template RNA. A genetically engineered reverse transcriptase and RNase inhibitor enzyme mix is then added and the reaction is cycled through a thermocycler, quickly generating high quality cDNA.

Materials—DNase-treated experimental RNA

Spectrophotometer

Thin walled 0.2mL PCR tubes with lids

Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Catalog #K1642)

Nuclease-free water

Thermal cycler

Generation of cDNA

1. Determine the concentration of isolated RNA by spectrophotometry.

The quality of the RNA samples may also be determined when required using an Agilent Bioanalyzer and the appropriate RNA analysis kit.

2. For each RNA sample, make the following cDNA reaction in a thin-wall 0.2mL PCR tube using the Maxima First Strand cDNA synthesis kit:

4 μ L Maxima 5X Reaction Mix

2 μ L Maxima Enzyme Mix

1pg-5 μ g Template RNA (*Recommended: 1 μ g RNA*)

Bring the reaction up to 20 μ L final volume using nuclease-free water

3. Make one additional cDNA reaction using RNA from control samples. This will be used to make a standard curve for qPCR.
4. Ensure all of the components are well mixed but do not vortex. Pulse centrifuge if needed.
5. Incubate the samples in a thermocycler using the following program:
 - 10 minutes at 25°C
 - 30 minutes at 50°C
 - 5 minutes at 85°C
 - Hold at 4°C

6. cDNA can be stored at 4°C for 1-2 weeks. For long-term, store the cDNA at -80°C. cDNA should be used for qPCR as soon as possible for the most accurate analyses. Do not freeze/thaw the cDNA reactions repeatedly; rather aliquot the reactions if needed.

BASIC PROTOCOL 6

ISOLATION OF VIRAL DNA USING PHENOL/CHLOROFORM EXTRACTION

The removal of DNA-bound proteins is an essential component of any DNA isolation and is critical to obtain reproducible qPCR results. Viral DNA replication is linked to the expression of early genes. As such, siRNA or small molecule treatments which impact viral DNA replication can be observed readily by 4-6 hours post infection.

The protocol outlined here uses an SDS-based lysis buffer containing proteinase K. After a 12 hour incubation, an additional 'spike' of proteinase K is added during an RNase digestion step. Two sequential phenol/chloroform extractions using phase lock tubes will ensure a clean isolation of total DNA (cell and viral genomic DNA).

CAUTION: This protocol involves work with a phenol:chloroform:isoamylalcohol solution. Wear appropriate personal protective equipment when working with these chemicals. Fumes from phenol/chloroform are hazardous. All steps involving phenol/chloroform should be performed in a chemical fume hood. Dispose of phenol/chloroform waste per your institute's hazardous waste protocols.

Materials—HSV-1 infected cells/tissue of interest

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

Cell scraper (Sarstedt, Catalog #83.1830)

Nuclease-free 1.5mL tubes

0.1mL micro-sized glass dounce tissue homogenizer (Wheaton, Catalog #357844)

2X DNA isolation buffer (see recipe)

Thermomixer

20mg/mL RNase A (Life Technologies, Catalog #12091-021)

20mg/mL Proteinase K solution (Roche Applied Science, Catalog #03115887001)

Phase Lock Gel Heavy 2mL Tubes (5 PRIME, Catalog #2302830)

Phenol/chloroform/isoamyl alcohol (25:24:1, v/v) (Roche Applied Science, Catalog #03117979001)

End-over-end rotator

3M sodium acetate pH 5.2

Isopropanol

70% ethanol

10mM Tris pH 8.0

Initial Processing of Samples

1) Collection and lysis of samples:

a. For cell monolayers in a 6-well dish:

- i. Wash cells in DPBS. Remove DPBS and add 0.5mL DPBS.
- ii. Scrape cells in each well using the appropriate sized cell scraper. Collect the cells in a clean 1.5mL tube.

If a smaller well size is used, trypsin can be used to detach and collect cells. Be sure to deactivate trypsin with complete media and wash cells with DPBS before proceeding.

- iii. Centrifuge the cells at $5,000 \times g$ for 2 minutes. Remove the supernatant from the cell pellet.

The cell pellets can be frozen on dry ice and stored at -80°C for later DNA recovery.

- iv. Resuspend the cells in 150 μL DPBS. Add 150 μL 2X DNA extraction buffer and pipet gently to lyse the cells. Proceed to Step 2.

b. For tissue:

- i. Add 150 μL DPBS to the sample (maximum of 30mg of tissue or 4-6 mouse trigeminal ganglia) in a 0.1mL glass dounce homogenizer and homogenize thoroughly until the sample is uniform.

As little as one ganglion may be processed for DNA extraction. See UNIT 14E.6 (Arbuckle et al., 2014) for more information.

- ii. Transfer the homogenized tissue to a clean 1.5mL tube and add 150 μL 2X DNA extraction buffer. Pipet gently to mix and lyse the cells. Proceed to Step 2.

2) Incubate the samples overnight in a thermomixer at 56°C set to shake at 900rpm.

Sample volume loss can occur overnight. Use 1.5mL tubes with a strong seal to prevent evaporation. Aluminum foil can also be placed over samples in the thermomixer to retain heat and prevent loss.

Recovery of DNA

- 3) To each sample, add 5 μ L of a 20mg/mL RNase A solution and an additional 20 μ g proteinase K. Return the samples to the thermomixer for 1 hour.
- 4) Prepare 2mL heavy gel Phase Lock tubes by centrifuging the tubes at 12,000 $\times$ g for 1 minute.
- 5) Transfer the lysate to a 2mL heavy gel Phase Lock tube and add 1 volume phenol/chloroform/isoamyl alcohol. Rotate the tubes in an end-over-end motion for 10 minutes at room temperature.
- 6) Centrifuge at 12,000 $\times$ g for 5 minutes at room temperature.
- 7) Perform a second extraction by adding 1 volume phenol/chloroform/isoamyl alcohol to the Phase Lock tubes. Rotate tubes in an end-over-end motion for 10 minutes at room temperature.
- 8) Centrifuge at 12,000 $\times$ g for 5 minutes at room temperature.
- 9) Recover the aqueous layer (DNA) and transfer to a clean 1.5mL tube.

Precipitation of DNA

- 10) Precipitate the DNA by adding sodium acetate to a final concentration of 0.3M and 1 volume 100% isopropanol. Mix by gently inverting. The genomic DNA should be visible as fine threads.
- 11) Pellet the precipitated DNA by centrifugation at 13,000 $\times$ g for 10 minutes at 4°C.
- 12) Discard the supernatant and wash the pellet in 1mL 70% ethanol. Spin at 13,000 $\times$ g for 5 minutes at 4°C. Repeat the wash.
- 13) After the final wash, remove as much wash buffer as possible and allow the pellet to air dry briefly. Resuspend the DNA pellet in 50-100 μ L 10mM Tris pH 8.
- 14) Incubate the DNA at 50°C/900rpm in a thermomixer for 30 minutes to 1 hour to promote complete resuspension. Determine the DNA concentration by spectrophotometry.

ALTERNATE PROTOCOL 2

ISOLATION OF VIRAL DNA USING COMMERCIAL DNA ISOLATION KITS

Similar to RNA isolation kits, genomic DNA isolation kits using columns are available. If time is a constraint, DNA isolation via these kits can be completed in a shorter period than the previously presented protocol (Basic Protocol 5).

Note: The authors prefer the use of Basic Protocol 5 for DNA isolation. If the investigator chooses to utilize a commercially available kit, the results should be compared to the Basic Protocol 5 to determine the level of reproducibility of each method.

Materials—HSV-1 infected cells/tissue of interest

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

Cell scraper (Sarstedt, Catalog #83.1830)

0.1mL micro-sized glass dounce tissue homogenizer (Wheaton, Catalog #357844)

Nuclease-free 1.5mL tubes

DNA Isolation Kit

ZR Genomic DNA-Tissue Miniprep (Zymo Research, Catalog #D3050)

QIAamp DNA Mini Kit (Qiagen, Catalog #51304)

10mM Tris pH 8

As there are numerous column-based DNA isolation kits from various vendors (some examples are provided above), a specific protocol for isolation will not be provided. It is recommended that the manufacturer's protocol be followed for the specific kit chosen by the end user. The authors make the following recommendations:

1. If isolating DNA from latent or reactivated trigeminal ganglia, as few as 1 ganglion per column may be used. Homogenize the trigeminal ganglia using a 0.1mL glass dounce homogenizer. See UNIT 14E.6 (Arbuckle et al., 2014) for more information.
2. Most kits contain a Proteinase K digestion step. This step can be extended to an overnight digestion to improve DNA yields, especially from tissue.
3. Elute final DNA in 10mM Tris pH 8 and avoid kit-specific elution buffers that may contain EDTA.

BASIC PROTOCOL 7

ANALYSIS OF VIRAL RNA/DNA BY QUANTITATIVE REAL-TIME PCR

Before the development of qPCR, mRNA expression was assessed via northern blots. Today, qPCR uses fluorescent intercalating dyes or probes to directly correlate the level of fluorescence to the amount of PCR product present in the reaction at a given time. This method can be used to determine an absolute quantification of template present or quantification relative to a control. A basic method is presented for relative quantification of HSV-1 cDNA or DNA relative to host genomic control genes.

Materials—cDNA generated from experimental RNA or isolated DNA

Nuclease-free 1.5mL tubes

Universal Sybr Green Mastermix (Roche Applied Sciences, Catalog #04673484001)

Gene-specific primers (See Table 1)

96-well PCR plate

PCR plate sealing film

Quantitative real-time PCR Instrument

1. Prepare experimental samples:

- a. Dilute the cDNA samples 1:6 with nuclease-free water (i.e., add 100uL water to a 20uL reaction.

Dilution of the cDNA reactions allows numerous primer pairs to be run from one sample. Dilution results in less than a 3 CT shift in qPCR. If the desired target is anticipated to be in low abundance, do not dilute the cDNA reaction.

- b. Prepare an aliquot of the isolated DNA at a concentration of 1-10ng/uL.

While this range is ideal for genomic DNA, the dilution may need to be adjusted depending upon the level of viral DNA present.

2. Generate a standard curve for the samples being analyzed:

- a. Dilute the extra cDNA reaction (Basic Protocol 4, Step 3) 1:5 with nuclease free water (i.e., Add 80uL nuclease free water to the 20uL cDNA reaction). From this stock, prepare three serial 5-fold dilutions. From the stock tube, transfer 20uL cDNA to a new tube and dilute with 80uL water for a 5-fold dilution. Repeat to generate 25-fold and 125-fold dilutions.
- b. For DNA, generate a 25ng/uL stock from DNA isolated from infected cells and prepare three serial 5-fold dilutions (5ng/uL, 1ng/uL, 0.2ng/uL). Alternately, two separate standard curves can be made and used depending on the primer set; one from uninfected genomic DNA and one from purified viral DNA.

Some investigators use a standard curve produced from a known quantity of purified viral DNA. The viral DNA is diluted into a set quantity of cell genomic DNA (i.e. 1 ug).

3. Prepare a mastermix containing Sybr qPCR mix and appropriate primers using the following reagents for each 20uL reaction:

10uL 2X Universal SYBR Green Master
0.5uL 10uM Forward Primer
0.5uL 10uM Reverse Primer
7uL water, nuclease-free

To generate a mastermix, multiply the volume for all components by the number of qPCR reactions that will be run for each primer set. Experimental samples and standard curves are run in triplicate for each primer set.

For a list of recommended qPCR primers for HSV-1 genes and control genes, see Table 1.

4. Pipet 18uL of the qPCR mastermix per well of a 96-well plate.
5. Pipet 2uL of the experimental cDNA or DNA and of the standard curve per well to bring the reaction volume to 20uL.
6. Seal the plate (as appropriate for the specific plate and instrument) and run qPCR plate per the instrument protocol for the SYBR Green reagent being used.
7. qPCR results can be analyzed for the relative expression of HSV-1 to control genes. This will not provide absolute quantification.

For help in analyzing qPCR results and understanding the difference between relative and absolute quantification, the following resources are highly recommended:

YouTube Video Series “Real Time Quantitative PCR Analysis” by americanbiotech (<http://www.youtube.com/playlist?list=PL2C46600596D9A876>).

Hunt, Margaret. Real Time PCR. Microbiology and Immunology On-line, University of South Carolina School of Medicine. <http://pathmicro.med.sc.edu/pcr/realtime-home.htm>.

BASIC PROTOCOL 8

ANALYSIS OF VIRAL PROTEIN EXPRESSION BY WESTERN BLOT

siRNA or small molecule inhibitor treatments which result in observable changes in viral mRNA expression by qPCR should also result in corresponding changes in viral protein levels. These levels can be assessed by protein immunoblot (western blot). To examine viral protein levels, the protein extracts of mock and infected cells are isolated and run on denaturing acrylamide gels to separate proteins by size. Proteins are transferred to a membrane, immobilized, and the membrane is probed using antibodies specific to the protein of interest. Differing levels of viral proteins in treated cells relative to infected control cells can be quantified and used to confirm the qPCR results. If an antibody is available to the protein targeted by siRNAs, western blots should be used to confirm the level of depletion of the target protein. Provided here is a basic western blot protocol and a list of commercially available antibodies to HSV-1 proteins of various gene classes (Table 2).

Materials—HSV-1 infected cells (siRNA/drug treated and corresponding control)

Dulbecco's Phosphate Buffered Saline w/o Mg^{2+} or Ca^{2+} (DPBS)

Cell scraper (Sarstedt, Catalog #83.1830)

Nuclease-free 1.5mL tubes

RIPA lysis buffer (see recipe)

Bradford Dye Reagent Concentrate (BioRad, Catalog #500-0006)

4X Reducing Sample Buffer (see recipe)

Tris/Glycine/SDS Buffer (10X) (BioRad, Catalog #161-0732)

Deionized, filtered water

Criterion Gel Box

Syringe with needle

Criterion Tris-HCl 4-20% Acrylamide Gels (BioRad, Catalog #345-0033)

Precision Plus Protein Standards (BioRad, Catalog #161-0373)

Millipore Immobilon-P PVDF membranes (Millipore, Catalog #IPVH00010)

Whatman 3MM Chr Cellulose Filter Paper

Methanol

Tris Glycine Transfer Buffer (10X) (BioRad, Catalog #161-0743)

Transfer Module

TBS (see recipe)

Carnation Instant Milk Powder (purchased at any grocery store)

TBST (see recipe)

Primary and Secondary Antibodies

Pierce SuperSignal West Dura Substrate (Thermo Scientific, Catalog #34075)

Stripping solution (see recipe)

Sample Preparation

- 1) For a 6-well dish of MRC-5 cells
 - a. Wash the cells in DPBS. Remove the DPBS and add 0.5mL DPBS.
 - b. Scrape the cells in each well using a cell scraper. Collect the cells in a clean 1.5mL tube.
 - c. Pellet the cells at 5,000xg for 2 minutes. Remove the DPBS from the cell pellet.
 - d. For MRC-5 cells, lyse the cell pellet in 30-50 μ L RIPA for 30 minutes on ice.

For westerns, use at least one well from a 6-well dish of MRC-5 cells. Protein yields from this cell type are generally low. Scale up if necessary.

- e. Spin the cell lysates at $13,000 \times g$ for 10 minutes at 4°C . Transfer the cleared supernatant to a clean 1.5mL tube.

- 2) Determine the protein concentration using a standard Bradford assay.

See APPENDIX 3A (Lovrien and Matulis, 2005), Assays for Total Protein, for help in performing a Bradford or an alternate protein concentration assay.

Typical protein yields for one well of a 6-well plate of MRC-5 cells ranges from 3-5mg/mL.

- 3) Prepare the sample gel electrophoresis:

10-25 μg experimental sample protein

5 μL 4X Loading Buffer

Adjust the volume up to 20 μL with sterile water.

- 4) Heat the samples in a 95°C dry block for 5 minutes. Pulse centrifuge the samples if needed to collect any condensate.

Prepare and Load the Protein Gel

- 5) Prepare 1X SDS running buffer by adding 50mL of 10X Tris/Glycine/SDS stock to 450mL water.

- 6) Assemble a gel in the Criterion electrophoresis box and add the appropriate amount of running buffer. Use a syringe fitted with a needle to wash out the wells of the gel with the 1X SDS buffer prior to loading the sample.

- 7) Load the prepared samples on gel. Use prestained protein standards as a ladder (i.e. BioRad Precision Plus; 5 μL /well).

It is recommended the ladder be loaded on the left side of the gel as this will be used as a reference point during the transfer process. Markers should also be loaded in lanes between sets of experimental samples.

- 8) Run the gel at a constant 30mA for approximately 2 hours or until the running dye is near the bottom.

Prepare for Transfer

- 9) Prepare 1X transfer buffer by combining 100mL 10X Tris Glycine Transfer Buffer, 200mL methanol, and 800mL water.

- 10) Cut a PDVF membrane and two sheets of filter paper slightly larger than the gel to be transferred but smaller than the transfer apparatus sponges.

- 11) Soak the membrane in 100% methanol for 15 seconds. Discard the methanol, rinse with deionized water, and add transfer buffer. Equilibrate the membrane in the transfer buffer on a shaking platform for at least 10 minutes or until the gel is ready.

Gel Transfer

12) Carefully remove the gel from the plates. Place the gel in 10-20mL transfer buffer and rock for 5-10 minutes.

The presence of SDS can enhance the transfer of high molecular weight proteins (>200kDa). If desired, low concentrations of SDS (0.03-0.05%) can be added to the transfer buffer.

13) Layer the transfer module in the following order (bottom to top) and ensure no bubbles exist between layers. All materials should be saturated in transfer buffer.

Blotting pad-sponge

Filter paper-1 sheet

Gel

Transfer membrane (PVDF)

Filter paper-1 sheet

Blotting pad-sponge

When layering the gel, flip the gel over the vertical axis so that the ladder is on the left side or else the transfer will be mirrored.

14) Orient the transfer assembly so the gel is closest to the cathode (–) and the PVDF membrane closest to the anode (+). Fill the transfer apparatus to the appropriate level with 1X transfer buffer.

15) Transfer at room temperature at appropriate voltage for the blot module in use. The time and voltage vary significantly depending upon the specific transfer apparatus.

Removing Membrane After Transfer

16) Open the assembly, remove the membrane, and wash by immersion in transfer buffer. Mark the membrane with a pencil to delineate the orientation.

17) Immersing the membrane in 100% methanol for 10 seconds. Remove and place the membrane on filter paper to dry.

Drying of PDVF membranes helps to further immobilize transferred proteins and reduces background.

18) Once dry, cut off any excess membrane. Cut the membrane into strips based on the protein standards and the MW of the protein to be detected. This allows for multiple proteins of different MW to be probed from a single gel blot.

One segment of the blot should be used to probe for a loading control such as TBP, Actin, Tubulin, etc.

Blocking and Antibody Incubations

19) Re-wet the membrane with 100% methanol for 15 seconds. Discard the methanol and wash the membrane with TBS. Block the membrane with TBS+5% milk for 1 hour at room temperature.

Refer to the antibody product sheet for the preferred method of blocking. Milk or 3% BSA in TBS are preferred blocking solutions.

20) Dilute the desired primary antibody in blocking solution. Prepare enough to cover the membrane, which may be probed in a small container or sealed in a “seal-a-meal” bag to conserve antibody.

Recommended dilutions for primary antibodies can often be found on product sheets provided by the company.

21) Incubate primary antibody 2 hours to overnight on a rocking platform.

The standard for immunoblotting with an untested antibody is an overnight incubation at 4°C. However, primary antibodies with high background or strong affinity can be incubated for as little as 2 hours at room temperature. Optimal antibody incubation conditions should be determined empirically by the investigator.

22) After incubation with the primary antibody, move the membrane to a fresh container and wash it with TBST 3 times for 10 minutes each on a rocking platform at room temperature.

23) Dilute the appropriate HRP-conjugated secondary antibody (refer to the manufacturer’s specification sheet) in TBST-5% milk. Incubate the membrane with the solution at room temperature for 1 hour.

24) Wash the membrane with TBST 3 times for 10 minutes each on a rocking platform.

Detection

25) Prepare the Pierce SuperSignal West Dura Substrate by mixing the provided solutions in a 1:1 ratio.

26) Overlay the substrate on the membrane and incubate for 5 minutes. Drip off excess reagent by blotting the edge of the membrane on a paper towel or Whatman paper.

27) Detect chemiluminescence by x-ray film or appropriate imager.

Multiple exposures (film) are often taken as a permanent physical record while imagers are used to appropriately quantitate the signals. Signals are normalized to the level of the loading control protein to account for small variances in the amounts of extracts in a given lane.

Stripping and Reprobing

28) The membrane may be stripped in order to reblot with a different antibody. To do so, wash the membrane twice in water after the initial imaging to remove any detection

reagent and buffered solutions. Incubate the membrane in stripping solution (see recipe) for 30 minutes at room temperature.

29) Wash the membrane twice in TBS.

30) Repeat the protocol beginning with incubation in blocking solution.

REAGENTS AND SOLUTIONS

Unless otherwise noted, stock solutions of many reagents (i.e. Tris-HCl, SDS, NaCl, etc) can be purchased sterile from various companies. Also see APPENDIX 2A (Commonly Used Reagents) for commonly used reagents and recipes.

DMEM/10%FBS

To a bottle of 500mL Dulbecco's Modified Eagle Medium [DMEM with high glucose (4.5g/L) and phenol red], add the following:

50mL heat-inactivated fetal bovine serum

6mL 10,000U/mL Penicillin – 10,000ug/mL Streptomycin solution (Pen/Strep)

6mL 200mM L-glutamine

6mL 100X MEM Non-Essential Amino Acids (MEM-NEAA)

Store at 4°C. Properly stored media maintained under sterile conditions should last 2-4 weeks. However, all media components are labile. Changes in media color, opacity, or cell growth characteristics may result from degraded or contaminated media. Media appearance should be assessed prior to each use.

Infection Media

To a bottle of 500mL Dulbecco's Modified Eagle Medium [DMEM with high glucose (4.5g/L) and phenol red], add the following:

5mL heat-inactivated fetal bovine serum

6mL 10,000U/mL Penicillin – 10,000ug/mL Streptomycin solution (Pen/Strep)

6mL 200mM L-glutamine

Store at 4°C. Properly stored media maintained under sterile conditions should last 2-4 weeks. However, all media components are labile. Changes in media color, opacity, or cell growth characteristics may result from degraded or contaminated media. Media appearance should be assessed prior to each use.

2X DNA Isolation Buffer

Make fresh prior to each use

50mM Tris-HCl, pH 7.5

50mM EDTA, pH 8

300mM NaCl

1% SDS

200µg/mL Proteinase K

RIPA

Make fresh prior to each use.

25mM Tris-HCl, pH 7.5

150mM NaCl

1% NP-40 (or Triton X-100)

1% Sodium Deoxycholate

0.1% SDS

1X Complete protease inhibitor

Complete Mini EDTA-free Protease cocktail tablets (Roche, Cat #11873580001) are dissolved in 2mL sterile water to generate a 25X solution. This solution can be stored at 4°C for a maximum of 2 weeks and should be added fresh to RIPA buffer before use

Reducing Sample Buffer (4x)

Sample buffer excluding the DTT can be made and stored for up to 1 year at room temperature. DTT should be added fresh prior to each use.

8% SDS

40mM Tris pH 6.8

200mM DTT

40% Glycerol

0.1% Bromophenol Blue

TBS

TBS can be stored at room temperature for up to 1 year.

10mM Tris-HCl, pH 7.5

150mM NaCl

TBST

TBST can be stored at room temperature for up to 1 year.

10mM Tris-HCl, pH 7.5

150mM NaCl

0.05% Tween-20

TBS (or TBST) + 5% Milk

TBS/T + 5% milk should be made fresh prior to each use.

100mL TBS/T

5g powdered milk

Stripping Buffer

The stripping buffer should be made fresh prior to each use.

25mM Glycine, pH 2.0

1% SDS

To make a 1M Glycine-HCl stock solution, dissolve 7.507g glycine in 80mL sterile water and pH to 2.0 using concentrated hydrochloric acid. Adjust the volume to 100mL with water. Recheck the pH and adjust as necessary. Filter the solution and store at room temperature for up to 1 year.

COMMENTARY

Background Information

Herpes Simplex Virus 1 (HSV-1) is an alpha-herpesvirus in the *Herpesviridae* family (Fields et al., 2007). The 153kDa linear, double-stranded DNA genome is packaged within an icosahedral capsid and surrounded by a lipid envelope. Between the capsid and lipid envelope is the tegument, a proteinaceous structure containing viral proteins important for the initiation of the lytic infection cycle. Primary infection by HSV may be asymptomatic or associated with minor lesions (oral or genital). Following the initial lytic infection, viral progeny can infect local sensory neurons where it establishes a life-long latent reservoir. Periodic reactivation of latent virus results in recurrent disease that can range from reiteration of limited lesions to more significant pathologies such as viral encephalitis and herpetic keratitis.

HSV lytic infection is characterized by an organized cascade of gene expression. Upon infection, transcription of the viral immediate early genes (IE) is dependent upon a complex interplay of host and viral factors. One essential component, the cellular coactivator HCF-1 coordinates the activity of multiple stimulatory transcription factors and chromatin modulation components that are important for driving the high level expression of viral IE genes. This initial stage of infection is critical as IE protein products drive the next wave of viral gene expression as well as modulate the host cell to promote viral lytic infection. Transcription of the IE genes can be detected using quantitative real-time PCR methods (qPCR) almost immediately after infection. However, peak transcription and detectable levels of viral protein expression (via western blot) occur within 2 to 4 hours post infection.

As noted, activation of the next class of viral genes (Early, E) is dependent on IE protein products and these E proteins can be clearly observed by 4-6 hours post infection. Early gene products are primarily involved in viral DNA replication and nucleotide metabolism. Examples include the viral DNA polymerase (U_L30), the single stranded DNA binding protein (U_L29 or ICP8), and the broad spectrum nucleoside kinase U_L23 (TK).

In the final stage, late gene expression is dependent on both IE protein activators and viral DNA replication. Transcription of late genes can be observed 6-8 hours post infection. Late genes are clearly subdivided into two expression groups, the leaky late (γ_1) and true late (γ_2) genes. While leaky late genes can be observed earlier in infection, expression of true late genes is highly dependent on DNA replication and their expression is blocked in the presence of DNA replication inhibitors. These protein products include the structural, membrane, and tegument proteins involved in the assembly of the progeny viral particles. A full cycle of HSV-1 infection is complete within 12 to 18 hours (dependent upon the cell type) and results in death of the cell. Table 3 contains a list of genes in each kinetic class (Fields et al., 2007).

Latent HSV infection occurs as a result of infection of the axonal termini of sensory neurons that are proximal to the site of primary infection. The infecting virion travels via retrograde transport to the neuronal cell nucleus where the viral DNA circularizes. Lytic genes are not expressed at any significant level during latency and the viral genome exists primarily in a transcriptionally repressed heterochromatic structure. The notable exception to this is the expression of the viral non-coding RNAs (Latency-Associated Transcripts (LATs) and microRNAs. Reactivation of the virus and re-entry into the lytic cycle can be induced by a variety of “stress signals” that can include injury to local tissues, hormonal alterations, or immune suppression. The exact molecular mechanism(s) involved in viral reactivation remain unclear. However, it is likely that signaling pathways leading to the induced coordinated expression of the viral IE genes is key to initiation of reactivation. Several animal models of HSV latency and reactivation are currently in use to study the state of latency and the mechanisms of viral reactivation [see UNIT 14E.6 (Arbuckle et al., 2014) for more information].

The methods provided in this unit represent a biological toolbox that will allow investigators to examine the molecular mechanisms of HSV at any point in the outlined viral replication cycle. Nucleic acid isolations (RNA, Basic Protocol 4 and DNA, Basic Protocol 6) followed by quantitative real-time PCR (Basic Protocol 7) can be used to detect viral RNA transcripts originating from various gene classes (see Table 3) or assay successful replication of the viral DNA genome. These nucleic acid centric methods can be correlated to western blots (Basic Protocol 8) that allow detection of viral proteins. Investigators can adapt these techniques to focus on any time point or gene class of interest during the viral lytic cycle. These same protocols can also be used to examine reactivation from latency in animal models [see UNIT 14E.6 (Arbuckle et al., 2014) for more information]. Furthermore, investigators can target host proteins of interest using siRNA knockdown (Basic Protocol 1) or small molecule inhibitors (Basic Protocol 2) and determine the impact of these treatments on HSV lytic replication. This unit provides a strong foundation for starting HSV-related research.

Critical Parameters and Troubleshooting

siRNA Knockdown—When using siRNAs to target host proteins potentially involved in the viral replication cycle, knockdown efficiency is critical. Ideally, a decrease of >75% in RNA levels of the target gene should be observed. Knockdown at the RNA level should also translate to a decrease in the protein levels of the target gene, which should be confirmed by western blot if antibodies are available. If significant decreases in the target RNA/protein are not observed, the levels of the transfection reagent and siRNA can be optimized to increase knockdown. Protocols to test these conditions are provided in the instruction manuals for the transfection reagent selected. Additionally, the time point post transfection can vary from 48-72 hours depending upon the initial transfection efficiency and the stability of the pool of the target protein. Multiple sequential transfections have also been shown to increase the efficiency of depletion.

When designing siRNAs, it is important to search the literature to determine if another laboratory has successfully utilized a particular siRNA sequence to deplete the target of interest. If purchasing siRNAs, different manufacturers use various proprietary siRNA design programs and as such alternate siRNA sequences may be available if unsuccessful with one source. shRNA mediated depletion is also an alternative when dealing with issues of ineffective depletion.

MRC-5 Cell Culture—MRC-5 cells are sensitive to cell culture conditions. Do not over-split cells (i.e. split more than 1:4) as this can significantly slow cell growth and accelerate senescence. When confluent, MRC-5 cells should appear elongated and line up in row-like formations with little to no space between neighboring cells (Figure 1). MRC-5 cells may also be sensitive to trypsin. If poor doubling times are observed, reduce the trypsin concentration to 0.05% for dissociation and minimize incubation time as much as possible.

HSV Absorption and Infection—HSV-1 absorption is sensitive to infection media conditions and CO₂ levels. If problems arise during HSV-1 infection, check the infection media and the incubator CO₂ levels by fyrite.

RNA Isolation by commercial kit/TRIZOL—The largest concern with RNA isolation is the maintenance of an RNase-free environment. Ensure all reagents and consumables used are RNase-free and minimize any sources of contamination. Use molecular grade water that is certified DNase and RNase-free for eluting/resuspending RNA and store RNA at -80°C for long-term storage. When in use, RNA should be kept on ice. If available, an Agilent Bioanalyzer should be used to determine the quality of the isolated RNA.

DNase/cDNA—Both methods presented here are commonly used and straightforward. Should any problems arise, refer to troubleshooting guides provided by the manufacturers.

DNA isolation by Phenol/Chloroform—A common problem with DNA isolation through this method is low DNA yields or poor quality of DNA ($A_{260}/A_{280} < 1.8$). Both of these issues are generally due to inefficient proteinase K digestion. Ensure the proteinase K stock has not lost activity. In addition, ensure the phenol:chloroform:IAA solution is at the proper pH (8.0), as acidic pH solutions are used specifically for RNA isolations and leave

DNA in the organic layer. Finally, phenol:chloroform:IAA solutions with a red tint/coloring indicates phenol oxidation and should be discarded.

DNA Isolation by Commercial Kit—Column-based isolations are convenient, although low or variable yields are often observed. Extension of the proteinase K digestion step found in these kits to an overnight incubation may improve isolation. The manufacturer's protocol book may also be consulted for additional troubleshooting tips.

Analysis by quantitative real-time PCR—Efficient primer sets for qPCR amplification are critical for accurate results. Newly designed primer sets should have efficiencies between 0.9 and 1.1 (or 90-110%). If using a SYBR based qPCR mix, melting curves for primer sets should only display one distinct peak at the appropriate melting point. A good resource for qPCR primer sets for cellular genes is the Harvard PrimerBank (Spandidos et al., 2008; Spandidos et al., 2010; Wang and Seed, 2003).

Another potential issue is the presence of contaminating DNA. When making cDNA, a control reaction (-RT) should be included. This reaction does not contain the reverse transcriptase enzyme. The -RT control should be treated the same as the cDNA samples and analyzed with the same qPCR primers. A signal in this control sample indicates the presence of contaminating DNA (i.e. inefficient genomic DNA removal).

Analysis of viral proteins by western blot—Ensure all reagents are stored properly and use within the recommended storage time. The most variable of parameters in this procedure is the specific conditions required for efficient detection by a select antibody. Variables are the blocking and dilution buffer, the dilution factor, length of time of incubation, and temperature of incubation. If the antibody is from a commercial source, many of these parameters can be found either in the specification sheet or can be found in the literature. However, irrespective of the source, empirical determination of the optimal conditions is often required.

Anticipated Results

The protocols described here will allow the end user to assess levels of HSV RNA, DNA, and protein products throughout the course of lytic infection. Additionally, the methods can be used to efficiently assess the status of the infection in cells depleted for specific components (siRNA depletion) or in cells treated with small molecule inhibitors.

For Basic Protocol 2 (Small Molecule Inhibitors), we specify a protocol using the drug TCP. Treatment with TCP will result in observable decreases in viral IE mRNA levels by qPCR and concomitant decreases in protein expression of these gene products by western blot. This compound as well as the DNA replication inhibitor Acyclovir can be used as controls, depending upon the goals of the study.

Time Considerations

Time considerations vary by protocol and are listed in Table 4.

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Figure 1. MRC-5 Cells

Confluent MRC-5 cells appear elongated and line up in row-like formations with little to no space between neighboring cells.

Table 1

Recommended qPCR Primer Sets

Gene	Class	Primer Sequences (5'-3')	Source
ICP4 (RS1)	Immediate Early	F: GAAGTTGTGGACTGGGAAGG R: GTTGCCGTTTATTGCGTCTT	(Ottosen et al., 2006)
ICP0	Immediate Early	F: CCCACTATCAGGTACACCAGCTT R: CTGCGCTGCGACACCTT	(Liang et al., 2013a)
ICP22 (U _S 1)	Immediate Early	F: TTTGGGGAGTTTGACTGGAC R: CAGACACTTGCGGTCTTCTG	(Ottosen et al., 2006)
ICP27 (U _L 54)	Immediate Early	F: GCATCCTTCGTGTTTGTCAATTCTG R: GCATCTTCTCTCCGACCCCG	(Liang et al., 2009)
ICP8 (U _L 29)	Early	F: GGGCGCAACTTTCGCAATCAATTC R: GGCCGACAGAAACCCGTTGTTAAA	(Arbuckle and Kristie, 2014)
U _L 30	Early	F: TCCAGCGACGTCGAGTTTAACTGT R: ACATGAGCTTGTATGCCGGTAGGT	(Arbuckle and Kristie, 2014)
U _L 23 (TK)	Early	F: GCGTCGGTCACGGCATAAG R: GGGTGAGATATCGGCCGGG	(Kent et al., 2004)
U _L 52	Early	F: GCCCTCCTCACAACTCTCTACTG R: CCGTCCCAATAAAACAAAAGG	(Peng et al., 2010)
gD (U _S 6)	Late	F: GTCAGCGAGGATAACCTGGGG R: GGGAGGGCGTACTTACAGGAGC	(Arbuckle and Kristie, 2014)
gC (U _L 44)	Late	F: TGATTATCGGCGAGGTGAC R: ACAAACTCCACGGGGTTACG	
GAPDH	Control	F: TTCGACAGTCAGCCGCATCTTCTT R: CAGGCGCCCAATACGACCAAATC	(Cliffe and Knipe, 2008)
TBP	Control	F: TGACCCCATCACTCCTGC R: CGTGGTTCGTGGCTCTCTTATC	(Liang et al., 2009)
HPRT	Control	F: ATTGTAATGACCAGTCAACAGGG R: GCATTGTTTTGCCAGTGTCAA	
UbC	Control	F: CGGGATTGGGTCGAGTTCTTG R: CGATGGTGTCACTGGGCTCAAC	

Table 2

Recommended HSV-1 Antibodies

Antibody	Class	Type	Source
ICP4	Immediate Early	Mouse IgG1	Virusys Corporation, H1A021
ICP0	Immediate Early	Mouse IgG1	Virusys Corporation, H1A027
ICP27	Immediate Early	Mouse IgG1	Virusys Corporation, P1113
ICP8	Early	Mouse IgG1	Santa Cruz, sc-53330
VP16	Early	Rabbit IgG Fraction	Sigma Aldrich, V4388
gC	Late	Mouse IgG2	EastCoast Bio, H1A022

Table 3

Viral Genes and Kinetic Classes (Fields et al., 2007)

α (Immediate Early)	β (Early)	γ (Late)		
RS1 (ICP4)	U _L 2	RL1	U _L 22	U _L 47
ICP0	U _L 5	U _L 1	U _L 24	U _L 48
U _S 1 (ICP22)	U _L 8	U _L 3	U _L 25	U _L 49/49.5
U _L 54 (ICP27)	U _L 9	U _L 4	U _L 26/26.5	U _L 51
U _S 1.5	U _L 12	U _L 6	U _L 27	U _L 53
U _S 12 (ICP47)	U _L 23	U _L 7	U _L 28	U _L 55
	U _L 29	U _L 8.5	U _L 31	U _L 56
	U _L 30	U _L 9.5	U _L 32	U _S 2
	U _L 39	U _L 10	U _L 33	U _S 3/3.5
	U _L 40	U _L 11	U _L 34	U _S 4
	U _L 42	U _L 13	U _L 35	U _S 5
	U _L 50	U _L 14	U _L 36	U _S 6
	U _L 52	U _L 15/15.5	U _L 37	U _S 7
		U _L 16	U _L 38	U _S 8
		U _L 17	U _L 41	U _S 8.5
		U _L 18	U _L 43/43.5	U _S 9
		U _L 19	U _L 44	U _S 10
		U _L 20/20.5	U _L 45	U _S 11
		U _L 21	U _L 46	

Table 4

Time Considerations

Protocol	Estimated Time
Basic Protocol 1: siRNA Treatment	3-4 days
Basic Protocol 2: Small Molecule Inhibitors	2-3 days
Support Protocol 1: Cell Plating and Passage	7-8 days to grow MRC-5 cells from frozen stock 1.5 hours for cell plating
Basic Protocol 3: HSV Absorption and Infection	4-10 hours depending on desired time point
Basic Protocol 4: RNA isolation by commercial kit	1.5 hours
Alternate Protocol 1: RNA isolation by Trizol	2 hours
Support Protocol 2: DNase Treatment	1 hour
Basic Protocol 5: Generation of cDNA	1.5 hours
Basic Protocol 6: DNA isolation by phenol/chloroform	2 days
Alternate Protocol 2: DNA isolation by commercial kit	4-6 hours
Basic Protocol 7: Analysis of viral RNA/DNA by qPCR	4 hours
Basic Protocol 8: Analysis of viral proteins by western blot	2 days