Chapter 3

Isolation of Herpes Simplex Virus Nucleocapsid DNA

Moriah Szpara

Abstract

As an inanimate virus, herpes simplex virus type 1 (HSV-1) necessarily encodes all of its functions in its DNA. Isolation of pure viral DNA allows multiple downstream applications, including the creation of recombinant HSV strains, cloning of selected regions, and sequencing of viral DNA. The term nucleocapsid refers to the combination of the viral genome with the enclosing capsid; these viral genomes are necessarily linear and have been packaged for egress, even if they are not yet released from the cell. In contrast, viral DNA that is not associated with capsids may include episomal or concatenated forms and may have modifications such as histones that are added within cells. During this protocol, the viral capsid protects the HSV genome from reagents that strip away and destroy most cellular contaminants. This procedure describes the isolation of viral nucleocapsids and their subsequent dissolution to purify clean, linear HSV DNA.

Key words Nucleocapsid DNA, Genomes, Centrifugation, Nonionic detergents, Freon®

1 Introduction

The isolation of DNA from herpes simplex virus type 1 (HSV-1) or HSV-2 allows multiple applications in molecular biology, including the creation of recombinant HSV strains, cloning of selected regions, and sequencing of viral DNA. Viral DNA can be isolated directly from infected cells, from purified secreted virions, or from a mixture of the two. The method described here focuses on isolation of genomic viral DNA from nucleocapsids, that is, from the genome-length linear DNA that is packaged inside of C-type capsids [1]. The approach uses the proteinaceous capsid to biochemical advantage, first by lysing all cellular membranes to set the capsids free and second by pelleting the heavy capsids through density gradients that float all other cellular and viral components. Video documentation of this protocol can be found here [2].

This specific protocol was developed initially for use with HSV-1 [3, 4] but works equally well for other herpesviruses [2, 5, 6]; similar extractions are used to isolate genetic material from a broad range of viruses [7–10]. The procedure begins with

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the basic cell and virology techniques of culturing cells to propagate and quantify viral stocks and then to stage a synchronously timed infection at a high multiplicity of infection (MOI). This synchronous infection can be harvested at a point when most capsids have encapsidated DNA but are still intracellular. This allows the harvest of a large number of nucleocapsids in a relatively small volume. An alternate approach to this technique would be to harvest only extracellular virions and then proceed with the same extraction methods. This requires a much greater input volume of extracellular medium for the same DNA yield, but otherwise the procedures are identical. The use of Freon® to disrupt cellular membranes frees capsids from the nucleus and/or subsequent steps of envelopment or egress. The nucleocapsids are collected by pelleting through a density gradient that captures all other components at lower density levels. The conveniently encapsidated DNA can then be set free by breaking open the capsids with a combination of mild detergent and proteinase action. Once the capsids are dissolved, standard phenol–chloroform extractions are used to isolate the viral DNA away from capsid proteins and any remaining contaminants. DNA precipitation recovers the viral genomes from solution, usually at a high concentration that is optimal for restriction digests, cloning, PCR, or deep sequencing. Typically the proportion of viral genomic DNA, relative to host cell DNA, exceeds 80 %.

2 Materials

1. LCM buffer plain (20 ml volume): 16.7 ml water, 2.5 ml 1 M KCl, 600 µl 1 M Tris, pH 7.4, 100 µl 1 M $MgCl_2$, 20 µl 0.5 M EDTA. Mix these components, then add 100 µl IGEPAL (NP40), and rock at 4 °C overnight (*see* **Note 1**). Add 8.6 µl β-mercaptoethanol immediately before use, and invert to mix well.
2. LCM + 5 % glycerol (20 ml volume): 15.7 ml water, 1 ml glycerol, 2.5 ml 1 M KCl, 600 µl 1 M Tris, pH 7.4, 100 µl 1 M $MgCl_2$, 20 µl 0.5 M EDTA. Mix these components, then add 100 µl IGEPAL (NP40), and rock at 4 °C overnight (*see* **Note 1**). Add 8.6 µl β-mercaptoethanol immediately before use, and invert to mix well.
3. LCM + 45 % glycerol (20 ml volume): 7.7 ml water, 9 ml glycerol, 2.5 ml 1 M KCl, 600 µl 1 M Tris, pH 7.4, 100 µl 1 M $MgCl_2$, 20 µl 0.5 M EDTA. Mix these components, then add 100 µl IGEPAL (NP40), and rock at 4 °C overnight (*see* **Note 1**). Add 8.6 µl β-mercaptoethanol immediately before use, and invert to mix well.
4. TNE: 0.1 M NaCl, 50 mM Tris, pH 7.5, 10 mM EDTA. For 50 ml volume, mix 41.5 ml H_2O, 5 ml 1 M NaCl, 2.5 ml 1 M Tris–HCl, pH 7.5, and 1 ml 0.5 M EDTA.

5. Phosphate-buffered saline (PBS): 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.0 (*see* **Note 2**).
6. Freon® (1,1,2-trichloro-1,2,2-trifluoroethane) (*see* **Note 3**).
7. Phenol:chloroform:isoamyl alcohol (25:24:1).
8. Cold ethanol (200 proof).
9. 70 % ethanol.
10. Proteinase K: 1 mg per sample extraction.
11. 10 % SDS.
12. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
13. Polyallomer tubes for ultracentrifugation, 12 ml capacity.
14. Glass Corex®-type centrifuge tubes, 30 ml capacity.
15. Optional: Glycogen or linear polyacrylamide.
16. Optional: 3 M sodium acetate, pH 5.2.
17. Recommended: Phase-lock gel tubes, 15 ml capacity (*see* **Note 4**).
18. Monolayers of confluent host cells in appropriate tissue culture (TC) vessels. Typically, three TC plates (15 cm^2) of confluent cells are sufficient for HSV-1 DNA isolation. It is highly recommended to prepare one extra plate of cells to allow for trypsinization and cell counting. This will allow accurate calculation of a high MOI (*see* **Note 5**).
19. HSV viral stock solution, titered to determine the number of plaque-forming units (pfu) per milliliter.

3 Methods

3.1 Day 1

1. Check that host cell monolayers are confluent. Trypsinize one plate, and count cells using a hemocytometer. Calculate the number of cells per dish, based on dilution used for hemocytometer counting.
2. Infect three dishes of confluent host cells (15 cm^2) at an MOI of 5; e.g., if each dish has 1×10^6 cells, then add 5×10^6 pfu per dish (*see* **Note 6**).

3.2 Day 2

1. Check for uniform cytopathic effect (CPE). Cells should be rounded but not floating (*see* **Note 7**).
2. Aspirate media gently (without dislodging cells) and replace with 2 ml cold PBS per dish (*see* **Note 8**). From this point forward, all solutions and centrifugations should be at 4 °C unless specifically noted otherwise.
3. Scrape cells into suspension, and transfer all cells infected with the same virus strain into one 50 ml conical tube (*see* **Note 9**).

If preparing DNA from multiple strains at the same time, handle and scrape plates in batches related to a single virus to avoid cross-contamination.

4. Spin suspension of scraped cells in PBS for 10 min at 2,000 × *g*. During the centrifugation, begin chilling the ultracentrifuge to 4 °C and also add β-mercaptoethanol to the LCM solution without glycerol.
5. Aspirate supernatant, and resuspend the cell pellet in 5 ml PBS (*see* **Note 10**).
6. Spin suspension of cell pellet in PBS buffer for 10 min at 2,000 × *g*.
7. Aspirate supernatant (*see* **Note 11**).
8. Resuspend the cell pellet in plain 5 ml LCM buffer, making sure that β-mercaptoethanol has already been added (**step 4** above).
9. Carry samples to a fume hood, and then add 1.5 ml Freon® per tube. This extraction will disrupt lipid membranes such as the plasma and nuclear membranes. Vortex hard, and immediately centrifuge for 10 min at 2,000 × *g* (*see* **Note 12**).
10. During centrifugation, add β-mercaptoethanol to the two glycerol-containing LCM solutions. Also prepare two sets of labeled recipient tubes to collect the aqueous top layer after each Freon® extraction.
11. Once the centrifugation step has finished, collect the top layer which contains viral nucleocapsids into the labeled recipient tubes (*see* **Note 13**). Dispose of Freon® in an appropriate hazardous waste stream.
12. Carry samples to a fume hood, and then add 1.5 ml Freon® per tube for a second round of lipid extraction. Vortex hard, and immediately centrifuge for 10 min at 2,000 × *g* (*see* **Note 12**).
13. During the second centrifugation, prepare a sufficient number of step gradients (one per virus strain) in polyallomer tubes suitable for ultracentrifugation. Label each tube, prior to making the step gradients. To make each step gradient, first add 3.0 ml of LCM + 5 % glycerol. Then use a thin pipet or a long-needled syringe to load 2.5 ml of LCM + 45 % glycerol underneath the initial layer.
14. Once the centrifugation step has finished, collect the top layer which contains viral nucleocapsids into the labeled recipient tubes (*see* **Note 13**). Dispose of Freon® in an appropriate hazardous waste stream.
15. Load the aqueous layer containing viral nucleocapsids onto the top of a step gradient. Pipet gently so as not to disrupt the interface between layers.

16. Balance the step gradient-containing tubes to within 0.1 g of each other by adding LCM dropwise to the tops of the tubes as needed (*see* **Note 14**).
17. Spin samples in an ultracentrifuge for 1 h at 77,000 × *g* (e.g., 25,000 rpm in an SW41 rotor on a Beckman ultracentrifuge). It is fine to use the brake at the end of the run.
18. During ultracentrifugation, prepare glass hooks (one per virus) that can be used to collect viral DNA that precipitates visibly out of solution (*see* **step 31**) (*see* **Note 15**).
19. When ultracentrifugation is finished, you may be able to see a thin translucent pellet with a faint spot in the middle, in the bottom of each sample tube. Carefully aspirate all fluid from the tube, including drips along the tube walls. Change the pipet or aspirator tip with each tube to avoid cross-contamination.
20. To each tube, add 0.5 ml TNE at room temperature. Let samples rest for a minimum of 10 min, with fluid covering the translucent pellet, to aid resuspension (*see* **Note 16**).
21. During the pause for resuspension, prepare two phase-lock gel tubes per virus strain. Spin down the gel layer in these tubes, according to the manufacturer's instructions (*see* **Note 17**). Make sure to label tubes in advance, according to virus strain name.
22. Use a small-bore pipet tip (e.g., p200) to mix the pellet into solution (*see* **Note 18**).
23. To each resuspended pellet, add 4.25 ml TNE, 0.25 ml 10 % SDS, and 1 mg of proteinase K. Cover the polyallomer tube with parafilm and begin inverting immediately (*see* **Note 19**). Look for a subtle increase in viscosity. Beware of shearing from this point forward, as viral DNA is no longer protected by a capsid.
24. Add 5 ml phenol/chloroform/isoamyl alcohol to extract the viral DNA. This step clears the DNA of contaminants such as capsid proteins and proteinase K. Immediately begin inverting to generate an emulsion; continue inverting back and forth for 10 s per tube. Transfer the phenol/chloroform solution into the pre-labeled phase-lock gel tubes by pouring (to reduce shear forces generated by pipetting).
25. Centrifuge the phenol/chloroform solution according to the instructions of the phase-lock gel tube manufacturer and tube size (e.g., for Eppendorf phase-lock gel tubes of 15 ml capacity, spin for 10 min at 3,000 × *g* in a tabletop centrifuge).
26. Pour top aqueous layer into the next labeled phase-lock gel tube for each virus. Avoid pipetting to reduce shear forces.
27. Repeat **steps 24–26** for a second round of phenol–chloroform extraction and centrifugation.

28. During second centrifugation, label one glass Corex®-type centrifuge tube (30 ml capacity) for each virus (*see* **Note 20**).
29. Pour top aqueous layer into the appropriately labeled glass centrifuge tube for each virus.
30. If desired, add 100 μg/ml glycogen or 10 μg/ml linear polyacrylamide to the DNA solution to facilitate precipitation (*see* **Note 21**). Cover tube with parafilm and mix by inverting to minimize shear forces.
31. Add 10 ml of cold ethanol. Parafilm the glass centrifuge tube and, with your hand covering the parafilm as added reinforcement, invert slowly to mix. Observe carefully for any signs of visible DNA precipitation in solution. Continue inverting until visible DNA coalesces into a finite number of strands. If there are no signs of visible precipitation, invert back and forth for 1 min, and then continue to **step 33**.
32. If precipitation in solution occurs, submerge a glass hook into the solution (made in **step 18**) and use the hook to catch the precipitated strands. After catching all visible strands, gently dab off any excess droplets of ethanol, put the glass hook tip into a 1.5 ml Eppendorf tube, and leave the tube and pipet to air-dry. Proceed to **step 38**.
33. If precipitation does not occur in solution, remove the parafilm and add 1.8 ml of 3 M sodium acetate (pH 5.2). Add an additional 5 ml ethanol. Chill in –20 °C freezer for 20 min or more.
34. Centrifuge at 12,000 × *g* for 10 min at 4 °C (e.g., 9,100 rpm in a Sorvall SA-600 rotor).
35. Aspirate supernatant carefully. Look for and avoid dislodging pelleted DNA.
36. Rinse DNA pellet by adding 5 ml of 70 % ethanol. Parafilm tube and invert gently; it is fine if pellet stays attached to tube wall. Remove parafilm before centrifuging at 12,000 × *g* for 10 min.
37. Aspirate supernatant carefully. Look for and avoid dislodging pelleted DNA. Leave tube(s) open in a fume hood or other laminar flow hood to facilitate evaporation of the remaining ethanol.
38. Once dry, add an appropriate volume of water or TE to resuspend the DNA from the pellet or the glass hook. A volume of 250 μl works well for the host cell input recommended in Subheading 3.1, **step 2** (*see* **Note 22**).
39. If the DNA is on a glass hook, then after the addition of resuspension volume, you can break the hook into the tube and close the lid (*see* **Note 23**).
40. Allow viral DNA to resuspend at least overnight (at 4 °C). The solution should be mixed gently, e.g., by stirring with a pipet tip, to minimize shearing of the DNA.

41. From this point on, viral DNA is ready for quantitation and use in downstream applications. It can be stored for a long term at 4 °C or −20 °C (*see* **Note 24**).

4 Notes

1. Make LCM solutions the day before they are needed to allow overnight mixing. When dispensing viscous components such as glycerol and IGEPAL into the lower viscosity destination solution, pipet up and down multiple times to rinse out as much of the viscous material as possible. The glycerol should rinse free of the pipet relatively easily; however, IGEPAL will dispense as a near-solid extrusion into the solution. Gentle rocking action over multiple hours will dissolve the IGEPAL into solution.
2. Use standard PBS as for cell culture. Commercial preparations work fine; pH carefully if preparing from scratch.
3. Though Freon® is no longer used as a standard refrigerant, it is used here in small quantities to provide superb disruption of cellular membranes. Freon® is available from specialty suppliers such as American Refrigerants, Inc. (http://www.order113.com). Alternatives to Freon® have been investigated for other enveloped viruses and may also be applicable for HSV [7].
4. Phase-lock gel tubes are used to separate organic and aqueous layers after phenol/chloroform extraction. These are not absolutely necessary but greatly facilitate the process of collecting the upper aqueous layer with no chance of carryover from organic components. The so-called gel layer has the consistency of silicone grease.
5. The choice of host cells depends on the intended use. Monkey kidney (Vero) cells have been frequently used to grow HSV-1; these are fast growing, pack densely on the dish, support robust viral infection, and typically yield abundant DNA. Human foreskin fibroblasts (primary cells) or human lung fibroblasts (e.g., MRC5 cell line) provide a more appropriate host species for HSV, but these cells grow more slowly, pack less densely on the plate, and may yield less viral DNA. The number of plates can be scaled up to provide more viral DNA from cells that pack less densely. Unless you are working with a cell line that has very predictable and reproducible plating characteristics, it is highly recommended to prepare one extra plate of cells to allow for trypsinization and cell counting before infection. This provides an optimal MOI (*see* **Note 6**).
6. It is important to initiate a synchronous infection to optimize viral yield; using a high MOI helps to ensure this. If the infection is not synchronous, the yield of viral DNA will be

suboptimal. Label all infected plates with the name of the HSV strain being grown to avoid any chance of cross-contamination between strains. Depending on the expected duration of infection (*see* **Note 7**), it may be useful to initiate the infection late in the evening, so that you can begin observing the progression of infection and be ready to harvest early the next day.

7. Different strains of HSV may require more or less time to reach CPE. If you are working with a strain for the first time, check cells frequently (every few hours) to discern the optimal harvest time, which is when cells are rounding up but still attached to the substrate. Prior to the optimal point, cells will not be rounded up yet, but infection can often be detected by noticing subtle changes in cell morphology (e.g., change in nuclear appearance, shift in diffractive properties). Past the optimal point, many cells will be floating and/or cells lysing into the media. At this point, free-floating virions cannot be recovered and viral DNA yield will be lower. However you can attempt to recover floating cells by pelleting them out of the supernatant, using the same centrifugation settings as in Subheading 3.2, **step 4**.
8. From the addition of cold PBS onwards, all solutions and centrifugations should be at 4 °C unless specifically noted otherwise. This limits native enzymatic activity from cells and facilitates successful isolation of viral DNA.
9. If you are handling multiple nucleocapsid preparations on the same day, it is crucial to handle infected plates in batches associated with a single virus, so as not to mix dishes and virus strains. *See* **Note 6** about labeling plates.
10. Take care not to aspirate the cell pellet at this stage. It is not necessary to remove the total volume of PBS from the preceding step; leave some behind in favor of not losing any of the cell pellet. When resuspending in fresh PBS, the cell pellet will be thick and potentially clumpy. It is fine to pipet multiple times to break up the clumps; however, you do not want to be so rough that cells break open and release capsids into the media.
11. If desired, this is an appropriate stopping point. The cell pellet with a thin layer of PBS above (*see* **Note 8**) can be stored at −20 °C for weeks to months. This can be a convenient way to accumulate multiple samples for further processing. When desired, thaw the pellets and proceed with Subheading 3.1, **step 8**.
12. The Freon® and LCM will immediately begin to partition into separate layers after vortexing. If you are handling multiple samples, vortex each one directly after addition of Freon®. Then carry all samples to the centrifuge, and vortex each one again before loading into the centrifuge.

13. Immediately after centrifugation at 4 °C, the Freon® layer (bottom) will be semisolid. If you work quickly, you can collect the aqueous top layer by pouring. To do this, pre-label recipient tubes for each virus. Have these uncapped and ready by the centrifuge. As soon as centrifugation finishes, remove tubes and pour the top layer of each one into the appropriate recipient tube. Watch for any sliding or motion of the Freon® base layer. The moment that motion is observed, you should cease attempts to pour and switch to collecting the top layer by pipet. Collecting the top layer by pipet is always a safe option; however, it is important to not allow the pipet tip to carry Freon® over into the subsequent tube. Therefore when collecting the top layer by pipet, it is best to leave some volume behind, rather than risk carryover by attempting to collect everything.
14. As with all ultracentrifugation, take care that you select a polyallomer tube of appropriate final volume (~10–12 ml). It is important that the volume of liquid in the tube is close to the top; if not the tube may crumple during ultracentrifugation and the gradient and sample will be lost.
15. Glass hooks provide a sterile, inexpensive, and disposable way to collect DNA out of solution. To create a glass hook, grasp both ends of a glass Pasteur pipet and hold the thinner side over the flame of a Bunsen burner. Pull gently, and as soon as you see the glass start to stretch and give way, release your tension and pull the pipet away from the flame. Before the glass cools, pull the thinner side back at an angle, until it is so thin that it breaks off. The goal is to create a v-shaped hook at the thin end of the pipet, preferably with a sealed-off tip, so that fluid containing viral DNA cannot enter the pipet tip. This process has been previously illustrated [2].
16. If desired, this is an appropriate stopping point. The resuspending pellet may be stored at 4 °C overnight. Continue directly with Subheading 3.2, **step 21**, the following day.
17. It is important to spin the phase-lock gel tubes before use to pellet the gel into the bottom of the tubes and create a flat-topped surface. If you forget this step, the aqueous and organic layers will not separate cleanly, e.g., a portion of the aqueous layer may become trapped under a smear of the grease (gel) layer.
18. Do not worry about shearing during pipetting. At this stage, viral DNA is still encapsidated and thus protected from shearing.
19. Invert gently, because this step causes the viral capsids to break open. From this point forward, shearing of the viral DNA is a concern. Therefore pipetting should be kept to a minimum, and samples should never be vortexed.

20. Using glass centrifuge tubes at this stage allows for the best possible visualization of any DNA "ghosts" that may precipitate out of solution. This process has been previously illustrated [2]. However it is important to use clean glass centrifuge tubes, ideally ones without scratches from prior use and cleaning with brushes. Scratches in the glass create an opportunity, albeit a low one, of cross-contamination between past and present samples.
21. Glycogen or linear polyacrylamide can be used to facilitate nucleic acid precipitation, which is especially useful with a low input of host cells or with virus strains that replicate poorly. We have also used glycogen conjugated with blue dye (Ambion® GlycoBlue™ by Life Technologies™), which makes the precipitated pellet particularly easy to see.
22. If you expect a particularly low yield of viral DNA, this is an opportunity to manipulate the resuspension volume to optimize DNA concentration. For low DNA yields, it may be useful to limit the resuspension volume to obtain a high enough concentration of DNA for downstream application. However you need sufficient volume to rehydrate the DNA and allow it to come fully into solution. For instance, if you have a highly concentrated sample (i.e., one that formed a "ghost" precipitate in solution) and you do not add sufficient resuspension volume, the DNA will not fully enter the solution. In this case, you may observe Schlieren lines from the mixing of regions of differing DNA concentration, whenever you move the glass hook shards around in the tube. Allowing ample time (days or weeks) for resuspension will also improve the DNA concentration.
23. If you break the tip off the glass hook before adding liquid volume for resuspension, there is a higher risk that glass shards may fly out of the tube. This creates hazard for the user and potential loss of sample.
24. Multiple freeze–thaw cycles create a risk of DNA shearing, so 4 °C storage is recommended for most cases.

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