Ripcord

Alternative Protocol: Removal of Capsids Containing Cellular DNA Fragments

Chris Buck and Cindy Thompson

Laboratory of Cellular Oncology, NCI

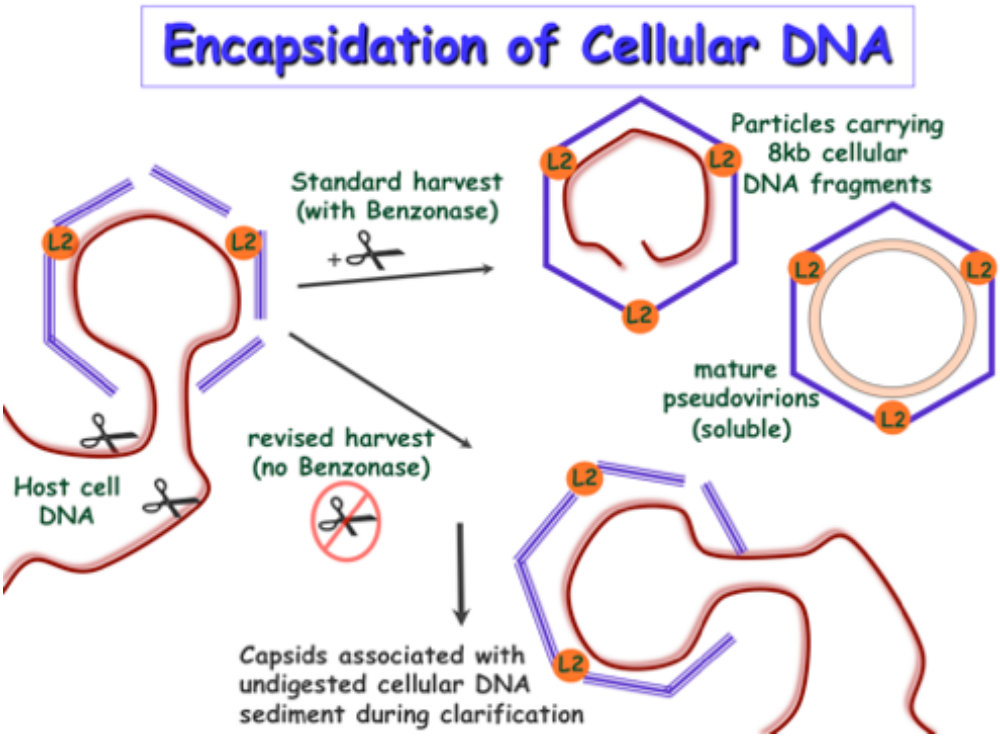
(rev. June 2015)

Note: A more detailed version of this protocol is available from *Current Protocols in Cell Biology*.

Purpose: In addition to encapsidating reporter plasmids, HPV16 and HPV18 proto-capsids also associate with 8kb loops of cellular DNA in the nucleus [1]. If DNases are added to the cell lysate during pseudovirus harvest (e.g., Benzonase and Plasmid Safe, as directed in the standard pseudovirus production protocol), the DNA loops are trimmed, resulting in capsids carrying 8kb linear fragments of cellular DNA. In standard pseudovirus preps, “cold” capsids containing cellular DNA are at least 10-fold more abundant than capsids containing the reporter plasmid.

The purpose of this alternative protocol is to remove capsids associated with cellular DNA from the pseudovirus preparation. The result is an improved particle to infectivity ratio and improved safety, since it is less likely that random cellular DNA fragments will be delivered to target cells.

Theory: pseudovirus purification procedures were originally worked out using BPV1 which, unlike HPVs 16 and 18, tends not to encapsidate much cellular DNA. The purification procedures were also developed prior to any understanding of capsid maturation. Early purification experiments suggested that addition of high salt to the lysate was required to solublize the pseudovirus enough for it to remain in the supernatant during clarification of the lysate. Although salt is needed to solublize immature capsids, it turns out that mature capsids are soluble enough to remain in the supernatant of clarified cell lysates, even under physiologic salt conditions. This allows clarification of the matured cell lysate without the addition of high salt. Since most of the cellular DNA remains trapped in the pellet after clarification in physiologic salt, this has the effect of removing most capsids associated with cellular DNA.



Incorporating 0.8M salt into the Optiprep gradient helps solublize the residual amounts of cellular DNA that remain in the clarified supe. The hydrated cellular DNA is too low-density to penetrate the deeper layers of the Optiprep gradient.

Although the revised harvest protocol works reliably in the hands of experienced users, it is definitely trickier than the standard harvest method. For users who are just starting out, it might be best to initially learn by performing the standard harvest method and just accept the price of some "cold" capsids containing cellular DNA.

Note: although pseudoviruses based on cottontail rabbit papillomavirus (CRPV) can be produced with very high efficiency using the standard harvest protocol, our attempts to harvest CRPV pseudoviruses using the revised harvest protocol below have been very disappointing. BPV1 pseudovirus production using the revised protocol are also somewhat disappointing. These two papillomavirus types (and perhaps others) should be harvested using the standard method.

Protocol:

1) Transfect and harvest producer cells according to the standard protocolup to the point where producer cells have been collected, washed and re-pelleted.

2) Measure the volume of the cell pellet by comparison to fluid in a dummy tube. Partially re-suspend the cell pellet by briefly vortexing or flicking the tube.

3) Add 1.5 pellet volumes of DPBS (no glucose) supplemented with pen-strep-fungizone (Invitrogen) and 9.5 mM $MgCl_2$. For example, for a 100 µl cell pellet, add 150 µl of DPBS, for a total volume of 250µl. It is critically important that the cells be suspended at very high density (>100 million cells/ml) – see notes section of standard protocol for details. Resuspend the cells by gently flicking the tube. Do not resuspend the pellet by triturating (pipetting up and down), as this may result in unwanted shearing of cellular DNA.

4) Add 1/20thvolume of 10% Triton X-100 (i.e., ~0.5% final concentration of Triton for the whole suspension). Use of Brij58 instead of Triton will also work. It is important to use fresh (less than on month old) Brij stock.

5) Add 1/1000thvolume RNase mix (Ambion cat# 2286) (i.e., ~0.1% final). RNase is an important addition because it destroys ribosomes, which are large enough to migrate down Optiprep gradients. DO NOT add Benzonase or Plasmid Safe as per the standard protocol.

6) Add 1/40thvolume of 1M ammonium sulfate adjusted to pH 9 and sterile filtered. See improved maturation protocolfor details.

7) Incubate in a 37ºC bath or non-CO_2incubator overnight. It may be helpful to mix the lysate by gentle inversion once or twice during the first couple hours.

8) After 20-24 hours of maturation, chill the lysate on ice. DO NOT add salt. Adding salt at this step would solublize the cellular DNA. This would ruin the preparation due to extreme viscosity.

9) Clarify the lysate by centrifuging at 5,000 x g for 5 minutes (preferably at 4ºC). There will be only a small amount of supernatant. The pelleted material may look somewhat loose and goopy.

10)Transfer the clarified supernatant into a fresh siliconized tube.

11) Wash the pelleted material (still in the original tube) by adding two pellet volumes of DPBS, resuspending, then re-centrifuging at 5,000 x g for 5 minutes. Combine wash supe with main supe from step 8.

12) Add one pellet volume of DPBS. Vigorously resuspend then freeze the suspension on dry ice. Thaw and clarify 5,000 x g for 5 minutes. Combine supe with the previous two supes.

13)Add one pellet volume of DPBS containing 0.8 M NaCl. There is not enough salt to fully solubilize the cellular DNA, but the somewhat higher salt in this final wash may help extract additional pseudovirions from the pellet.

14)Re-clarify the pooled supernatants 5 minutes at 5,000 x g. Load the clarified material onto an Optiprep gradient according to the Standard Protocol.

15)Screening fractions using PicoGreen reagent (as in the Standard Protocol) can be misleading due poor signal to noise ratio. Optiprep fractions should instead be screened for infectious titer. For example, place 1µl of each fraction onto a 96-well containing 10,000 293TT cells, incubate two days, and use an inverted fluorescent scope to look for GFP-positive cells. Fractions with peak GFP transducing units can then be pooled.

Development notes:

If the clarified lysate is run through Optiprep made with regular DPBS (0.15 M NaCl), overall titer yield is somewhat better (up to two-fold higher titer yield compared to the method above). However, huge amounts of cellular protein and DNA migrate down the gradient. Some of the cellular DNA that migrates down the gradient is still dangling out of capsids, such that if nucleases are added after the Optiprep / 0.15M NaCl gradient, capsids containing 8kb linear DNA fragments are liberated.

Since mature virions are resistant to digestion with trypsin, a brief trypsin digestion of the clarified lysate can improve the purity of the pseudovirus prep. •Transfer the clarified lysate to a fresh tube.

•Add 1/50thvolume of 2.5% trypsin (Invitrogen #15090-046 – store frozen in aliquots at –80ºC). Incubate the lysate at 37ºC for 15 minutes.

•Make a 10 mg/ml stock of soybean trypsin inhibitor (Invitrogen # 17075-029) in DPBS. Sterile filter and freeze in aliquots at –80ºC.

•Add 1/50thvolume of trypsin inhibitor stock to the digested lysate. Chill on ice. Trypsin inhibitor is added due to the theoretical concern that trypsin might degrade mature capsids due to transient distortions induced by ultracentrifugal forces.

1. Buck, C. B., Thompson, C. D., Pang, Y. Y., Lowy, D. R. & Schiller, J. T. (2005). Maturation of papillomavirus capsids. *J Virol* **79**, 2839-46.