

OptiPrep™ Application Sheet V27

Purification of Group V ((-)ss) RNA viruses: *Rhabdoviridae*: Rabies virus (Lyssavirus) and vesicular stomatitis virus

- ◆ OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- ◆ Whether the method described in this Application Sheet can be applied to other members of the *Rhabdoviridae* can only be determined experimentally
- ◆ **To access other Application Sheets** referred to in the text: return to the **2020Virapp** file and select the appropriate **V number**.

1. Background

In all comparative studies between CsCl and iodixanol, it has been shown that recovery of virus infectivity is much higher and the particle:infectivity ratio much lower when viruses are purified in iodixanol. Although sucrose is generally less deleterious to viral infectivity than CsCl, it can nevertheless also have serious effects on certain important aspects of viral function; in particular the loss of surface glycoproteins from retroviruses has been noted [1]. This may be related to its viscosity, which is much higher than that of iodixanol. Like CsCl, sucrose must be dialyzed before infectivity can be measured. In contrast, many add-on techniques can be performed and cells infected with virus, without dialysis of iodixanol.

2. Rabies virus (*Lyssavirus*)

Rabies virus has been purified in 20-40% iodixanol gradients [2], following concentration by sedimentation through a low-density barrier on to a high-density cushion. Although in the original method this double barrier comprised 20% and 60% sucrose, this has been replaced by a 12%/50% iodixanol double barrier in this OptiPrep™ Application Sheet. In an earlier publication Finke and Conzelmann [3] cited the use of a 10-40% iodixanol gradient, which was also described in ref 4. Klingen et al [5] described the use of a 10-35% iodixanol gradient. Use of density barrier concentration steps introduces the problem of subsequent layering of the collected virus on top of the iodixanol gradient. The density of the recovered liquid must obviously be below that of the top of the iodixanol gradient. If one of the newer alternative iodixanol gradients (10-40% or 10-35%) is chosen then the two-layer gradient described for concentrating the virus may have to be eliminated as in ref 4. Klingen et al [5] used a size exclusion column to concentrate the virus.

2a. Solutions required

- OptiPrep™
- OptiPrep™ diluent: 0.15 M NaCl, 6.0 mM EDTA, 0.3 M Tris-HCl, pH 7.4
- Suspension medium: 0.15 M NaCl, 1.0 mM EDTA, 50 mM Tris-HCl, pH 7.4
- Iodixanol (50% w/v) working solution: mix 5 vol. of OptiPrep™ with 1 vol. of Solution B (see Section 2d, Note 1)

Keep the following stock solutions at 4°C:

1 M Tris (free base),	12.1 g per 100 ml
100 mM EDTANa ₂ •2H ₂ O	3.72 g per 100 ml
1 M NaCl	5.84 g per 100 ml

Solution B: To 40 ml water add 30 ml, 15 ml and 6.0 ml respectively of Tris, NaCl and EDTA stock solutions; adjust to pH 7.4 with 5 M HCl; make up to 100 ml.

Solution C: To 50 ml water, add 5.0 ml, 15 ml and 1.0 ml respectively each of Tris, NaCl and EDTA stock solutions; adjust to pH 7.4 with 1 M HCl; make up to 100 ml.

2b. Ultracentrifuge rotor requirements

Virus concentration: Swinging-bucket rotor e.g. Beckman SW28 or SW28.1 (see Section 2d, Note 2)

Virus purification: Swinging-bucket rotor e.g. Beckman SW28.1 (see Section 2d, Note 3)

2c. Protocol (adapted from refs 2 and 4)

1. Prepare a 12% (w/v) iodixanol solution by diluting Solution D with Solution C (1.2:3.8 volume ratio).

2. Clarify the cell supernatant by centrifugation at 1500 g for 20 min.
3. Transfer clarified cell supernatants to tubes for the chosen swinging-bucket rotor. Underlayer 8 vol. of supernatant with approx 3.5 vol. of 12% (w/v) iodixanol and 1 vol. of 50% (w/v) iodixanol (**see Section 2d, Notes 4-6**).
4. Centrifuge at 120,000 g for 2 h; allow the rotor to decelerate from 2000 rpm without the brake.
5. Towards the end of this centrifugation prepare two further solutions of iodixanol of 20% and 40% (w/v) by diluting Solution D with Solution C (2:3 and 4:1 volume ratios respectively) and prepare a 13-14 ml continuous gradient from equal volumes of the two iodixanol solutions using a two-chamber gradient maker or Gradient Master™ in 17 ml tubes for the swinging-bucket rotor (**see Section 2d, Notes 7 and 8**).
6. Carefully aspirate the liquid above the rabies virus band above the dense iodixanol layer, leaving approx 1 ml of the upper layer.
7. Using a thin metal cannula or a length of narrow-bore Teflon tubing attached to a 2 ml syringe remove as much as possible of the 50% iodixanol (**see Section 2d, Note 9**).
8. Harvest the rabies virus in the remaining 12% iodixanol, taking as little as possible of any residual 50% iodixanol (**see Section 2d, Note 10**).
9. Dilute the harvested virus with 1-2 vol. of Solution C (if necessary) and layer on top of the 20-40% (w/v) iodixanol gradient to fill the tube and centrifuge at 27,000 rpm (approx 90,000 g_{av}) for 18 h.
10. Collect the gradient by aspiration from the meniscus, upward displacement with a dense medium or tube puncture (**see Section 5, Note 11**) and analyze the fractions. The virus bands in the top third of the gradient. When the 10-40% iodixanol gradient is used in a Beckman SW28 rotor, the first 8 ml of the gradient can be discarded; the virus bands maximally in the 3-6 ml cut of the following gradient [4].

◆ **See Note 12 for a brief summary of more recently published methods**

5. Notes

1. The production of a working solution from OptiPrep™ and Solution B, as described, ensures that the buffer and EDTA concentration is constant throughout the gradient. If Solution B also contains six times the NaCl concentration of Solution C, the NaCl concentration will also be constant but the dense part of the gradient will be very hyperosmotic. For more information on the preparation of density gradient solutions **see Application Sheet V01**.
2. Use whatever rotor is suitable to the volume of clarified cell supernatant (see Step 3).
3. The method can be scaled down to smaller volume tubes as required, or it can be scaled up to the Beckman SW28, as in ref 4.
4. Conical tubes facilitate this process, and Beckman manufacture so-called *k*onical™ tubes for all their swinging-bucket rotors. This two-barrier format achieves both a partial purification and a concentration of the virus. For more information on concentrating virus **see Application Sheet V06**.
5. **For more information on setting up discontinuous gradients see Application Sheet V02.**
6. If the 10-35% iodixanol gradient is chosen for the virus purification, the 12% iodixanol layer might be omitted from the concentration step and the 50% iodixanol layer reduced to 40% iodixanol (see Note 10).
7. For the 10-35% gradient use volume ratios of 1:4 and 3.5:1.5 respectively. For a 10-40% gradient use 1:4 and 4:1 respectively.

8. If a gradient making device is unavailable, then make a discontinuous gradient (5-10% iodixanol steps) and allow the formation of a continuous gradient by diffusion. **For more information about making continuous gradients see Application Sheet V02.**
9. Removing most of the dense cushion is facilitated by the use of Beckman *konical* tubes.
10. The iodixanol concentration of the harvested virus suspension needs to be <20% (w/v) to permit layering on top of the next gradient or <10% if the 10-35% or 10-40% iodixanol gradient is chosen. But note that the harvested virus may be diluted with a small volume of buffer in Step 9. If a suitably low iodixanol concentration cannot be attained then an alternative means of concentration will be required such as the size exclusion column used by Klingen et al [5] or centrifugal ultrafiltration using for example the Centricon PBHK Centrifugal Plus-20 filter unit with an Ultracel PL membrane (100 kDa cut off) as described by Yi et al [6] for the removal of iodixanol from hepatitis C virus preparations. **For more information on concentrating virus see Application Sheet V06.**
11. Once the banding position of the virus has been well established it may be permissible to harvest the virus band with a syringe. **See Application Sheet V04 for gradient harvesting methods.**
12. More recently gradients have been constructed from 1 ml each of 20%, 30%, 40% and 50% (w/v) iodixanol (112,000g for 18h) for banding virus-like particles [7], although in a later publication the time was reduced to 6 h [8].

3. Vesicular stomatitis virus (VSV)

3a. Density separations

VSV (*Vesiculovirus*) has been purified in an iodixanol gradient, using a broadly similar approach to that described above for rabies virus, although the centrifugation conditions were rather different [9, 10]. Solutions for the continuous gradient were prepared in a similar manner except that the solution used for suspending the virus contained 100 mM NaCl, 0.5 mM EDTA and 50 mM Tris-HCl, pH 7.4. Gradient solutions also contained these components at the same concentrations. An approx. 10 ml continuous gradient (15-35% (w/v) iodixanol) was prepared in tubes for a Beckman SW41 rotor and the crude virus suspension layered on top. There were two major differences in the protocol; the gradient was centrifuged at 160,000 *g* for 1.5 h and the initial concentration of the virus was achieved simply by pelleting at 28,000 *g* for 90 min. Otherwise the protocol was in essence, very similar to that described above and the virus banded in the middle of the gradient. This methodology was also used by Kim et al [11] and Beug et al [12] in their studies on Smac-mimetics.

A 5-50% (w/v) iodixanol gradient, centrifuged at 160,000 *g* for 1.5 h was described by Cuevas et al [13] for purification of VSV-mCherry, VSV-GFP and VSV-A3853C. A similar gradient was described in refs 14 and 15 but no other details were provided.

3b. Sedimentation velocity separation

After concentration by sedimentation through a 20% (w/v) sucrose barrier, the pellet was resuspended in HEPES-buffered saline overnight and layered on top of a continuous 7.5-27.5% (w/v) iodixanol gradient [16-18]. The gradients were centrifuged at approx. 85,000 *g*_{av} for 25-30 min. The position of the virus band under these conditions was not given. There is no reason why the sucrose cushion would not be replaced with an iodixanol solution of the same density (approx. 12% iodixanol).

- ◆ A simple centrifugation through a 10% (w/v) iodixanol cushion has been used to concentrate and partially purify the VSV [19, 20]. **For more information on concentrating virus see Application Sheet V06**

4. References

1. Palker, T.J. (1990) *Mapping of epitopes on human T-cell leukemia virus type 1 envelope glycoprotein* In: Human Retrovirology: HTLV (ed. Blattner, W.A.) Raven Press, NY, pp 435-445
2. Finke, S., Brzozka, K. and Conzelmann, K-K. (2004) *Tracking fluorescence-labeled rabies virus: enhanced green fluorescent protein-tagged phosphoprotein P supports virus gene expression and formation of infectious particles* J. Virol., **78**, 12333-12343

3. Finke, S. and Conzelmann, K-K. (2003) *Dissociation of rabies virus matrix protein functions in regulation of viral RNA synthesis and virus assembly* J. Virol., **77**, 12704-12082
4. Marschalek A., Drechsel, L. and Conzelmann, K-K. (2012) *The importance of being short: The role of rabies virus phosphoprotein isoforms assessed by differential IRES translation initiation* Eur. J. Cell Biol., **91**, 17–23
5. Klungen, Y., Conzelmann, K-K. and Finke, S. (2008) *Double-labeled rabies virus: live tracking of enveloped virus transport* J. Virol., **82**, 237-245
6. Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T. and Lemon, S.M. (2006) *Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells* Proc. Natl. Acad. Sci. USA, **103**, 2310-2315
7. Fontana, D., Kratje, R., Etcheverrigaray, M. and Prieto, C. (2015) *Immunogenic virus-like particles continuously expressed in mammalian cells as a veterinary rabies vaccine candidate* Vaccine, **33**, 4238–4246
8. Fontana, D., Etcheverrigaray, M., Kratje, R. and Prieto, C. (2016) *Development of rabies virus-like particles for vaccine applications: production, characterization, and protection studies* In Vaccine Design: Methods and Protocols, Vol. 1: Vaccines for Human Diseases, Methods in Molecular Biology, vol. 1403 (ed. Thomas, S.) Springer Science+Business Media New York pp 155-166
9. Diallo, J-S., Vähä-Koskela, M., Le Boeuf, F. and Bell, J. (2011) *Propagation, purification, and in vivo testing of oncolytic vesicular stomatitis virus strains* In Methods Mol. Biol., **797**, Oncolytic Viruses: Methods and Protocols, (ed. Kirn, D.H. et al.), Springer Science+Business Media, pp 127-140
10. Arulanandam, R., Batenchuk, C., Varette, O., Zakaria, C., Garcia, V., Forbes, N.E., Davis, C. Krishnan, R. et al (2015) *Microtubule disruption synergizes with oncolytic virotherapy by inhibiting interferon translation and potentiating bystander killing* Nat. Commun., **6**: 6410
11. Kim, D-S., Dastidar, H., Zhang, C., Zemp, F.J., Lau, K., Ernst, M., Rakic, A., Sikdar, S., Rajwani, J. et al (2017) *Smac mimetics and oncolytic viruses synergize in driving anticancer T-cell responses through complementary mechanisms* Nat. Comm., **8**: 344
12. Beug, S.T., Beauregard, C.E., Healy, C., Sanda, T., St-Jean, M., Chabot, J., Walker, D.E., Mohan, A., Earl, N. et al (2017) *Smac mimetics synergize with immune checkpoint inhibitors to promote tumour immunity against glioblastoma* Nat. Comm., **8**: 14278
13. Cuevas, J.M., Durán-Moreno, M. and Sanjuán, R. (2017) *Multi-virion infectious units arise from free viral particles in an enveloped virus* Nat. Microbiol., **2**: 17078
14. Diallo, J-S., Le Boeuf, F., Lai, F., Cox, J., Vaha-Koskela, M., Abdelbary, H., MacTavish, H., Waite, K., Falls, T., Wang, J., Brown, R., Blanchard, J.E., Brown, E.D., Kirn, D.H., Hiscott, J., Atkins, H. Lichty, B.D. and Bell, J.C. (2010) *A high-throughput pharmacoviral approach identifies novel oncolytic virus sensitizers* Mol. Ther., **18**, 1123-1129
15. Garijo, R., Hernández-Alonso, P., Rivas, C., Diallo, J-S. and Sanjuán, R. (2014) *Experimental evolution of an oncolytic vesicular stomatitis virus with increased selectivity for p53-deficient cells* PLoS One, **9**: e102365
16. Kalvodova, L., Sampaio, J.L., Cordo, S., Ejsing, C.S., Shevchenko, A. and Simons, K. (2009) *The lipidomes of vesicular stomatitis virus, Semliki Forest virus and the host plasma membrane analyzed by quantitative shotgun mass spectrometry* J. Virol., **83**, 7996-8003
17. Hastie, E., Besmer, D.M., Shah, N.R., Murphy, A.M., Moerdyk-Schauwecker, M., Molestina, C., Das Roy, L., Curry, J.M., Mukherjee, P. and Grdzlishvili, V.Z. (2013) *Oncolytic vesicular stomatitis virus in an immunocompetent model of MUC1-positive or MUC1-null pancreatic ductal adenocarcinoma* J. Virol., **87**, 10283–10294.
18. Moerdyk-Schauwecker, M., Hwang, S-I., Grdzlishvili, V.Z. (2014) *Cellular proteins associated with the interior and exterior of vesicular stomatitis virus virions*. PLoS One, **9**: e104688
19. Betancourt, D., Ramos, J.C. and Barber, G.N. (2015) *Retargeting oncolytic vesicular stomatitis virus to human T-cell lymphotropic virus Type 1-associated adult T-cell leukemia* J. Virol., **89**, 11786-11800
20. Betancourt, D., de Queiroz, N.M.G.P., Xia, T., Ahn, J. and Barber, G.N. (2017) *Cutting edge: innate immune augmenting vesicular stomatitis virus expressing Zika virus proteins confers protective immunity* J. Immunol., **198**, 3023–3028

OptiPrep™ Application Sheet V27; 8th edition, February 2020