OptiPrep™ Application Sheet V14

Purification of Group II (ss)DNA viruses: *Parvoviridae*: recombinant adeno-associated virus (rAAV) and avian AAV

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- This Application Sheet describes two methods for purifying rAAV with pre-formed gradients.
- Zolotukhin et al [1] used a discontinuous gradient (Section 2); this method has also been used for avian AAV [2].
- Hermens et al [3] used a continuous gradient (Section 3).
- The Zolotukhin et al method [1] has also been used for the purification of viruses of the *Parvovirinae* subfamily [4], see **Application Sheet V16** for more details.
- To access other Application Sheets referred to in the text return to the Virus Index; key Ctrl "F" and type the V-Number in the Find Box
- There are two important Axis-Shield Mini-Reviews: to access return to the initial list of Folders and select "**Mini-Reviews**".
  1. **MV01** "Purification of viral vectors using OptiPrep™": summary of the basic methodologies
  2. **MV02** "Recombinant-AAV bibliography": a list of over 1000 references

1. Background

Viral vectors that are of potential use in gene therapy would clearly benefit from isolation methods which are both effective and cause little or no damage to the viral particles. Density gradient centrifugation has always played an important part in the concentration and purification of virus particles but the gradient media that have been used most prominently, sucrose and CsCl, pose a number of problems. Both media are highly hyperosmotic at the densities used to band viruses (sucrose solutions are also very viscous) and generally have to be removed either by pelleting the virus or by dialysis, prior to further processing or analysis. CsCl also leads to poor recoveries and low infectivity of rAAV isolates.

Because of the very low water activity of CsCl solutions, viruses tend to have significantly higher density in this medium compared to media such as sucrose or any of the iodinated density gradient media, although the magnitude of this difference varies from virus to virus. Many viruses in CsCl have a density of approx 1.34 g/ml, in iodixanol the density range is generally 1.16-1.22 g/ml, although some viruses may be as low as 1.14 g/ml or as high as 1.24 g/ml. rAAV falls into the latter category.

OptiPrep™ is widely regarded as the gradient medium of choice for rAAV purification. Compared to CsCl gradients:

- Recovery of virus from the gradient is at least ten times greater
- Particle:infectivity titer is up to 100x lower
- Infectivity measurements and many add-on techniques can be carried out without the need to dialyze the medium.

2. Discontinuous gradient

The method is adapted from Zolotukhin et al [1].

To prepare Solution B: add the following to 100 ml of 10xPBS:

$MgCl_2 \bullet 6H_2O$	0.20 g
KCl	0.19 g

To prepare Solution D:
Add 11.68 g NaCl to 10 ml of Solution B and make up to 100 ml.

2a. Solutions required

A. OptiPrep™

B. 10xPhosphate-buffered saline containing 10 mM $MgCl_2$ and 25 mM KCl (10xPBS-MK)

C. Phosphate-buffered saline containing 1 mM $MgCl_2$ and 2.5 mM KCl (PBS-MK)

D. 2 M NaCl in PBS-MK

E. Working solution of 54% (w/v) iodixanol in PBS-MK: mix 9 vol of OptiPrep with 1 vol of Solution B.

2b. Ultracentrifuge rotor requirements
Fixed-angle rotor with approx 39 ml sealed tubes capable of approx 350,000 *g* (e.g. Beckman 70Ti or Sorvall T865; see Section 4, Note 1).

2c. Protocol
1. Prepare the following gradient solutions (see Section 4, Notes 2 and 3):
   15% (w/v) iodixanol containing 1 M NaCl in PBS-MK: 1.5 vol. of Solution E + 2.7 vol. of Solution D + 1.2 vol of Solution C.
   25% (w/v) iodixanol in PBS-MK: 2.5 vol. of Solution E + 2.9 vol. of Solution C
   40% (w/v) iodixanol in PBS-MK: 4.0 vol. of Solution E + 1.4 vol. of Solution C.

2. Clarify the cell lysate clarified by centrifugation at 4000 *g* for 20 min.

3. Underlayer 10-15 ml of clarified lysate with 9 ml of 15% iodixanol; 6 ml of 25% iodixanol, 5 ml of 40% iodixanol and 5 ml of the 54% iodixanol working solution (see Note 3). Use a long metal cannula (0.8 mm i.d.) attached to a syringe or via tubing to a peristaltic pump to load the tube (see Section 4, Note 4).

4. Centrifuge at 350,000 g_{av} for 1 h at 18°C. Use a slow acceleration and deceleration programme (up to and below 2000 rpm) if this facility is available on the centrifuge, or turn off the brake below 2000 rpm during deceleration.

5. Either collect the whole gradient (Figure 1) in 1-2 ml fractions dense end first or use a syringe inserted just below the 40%/54% interface to aspirate no more than 2-3 ml of the 40% layer (see Section 4, Notes 5-8).

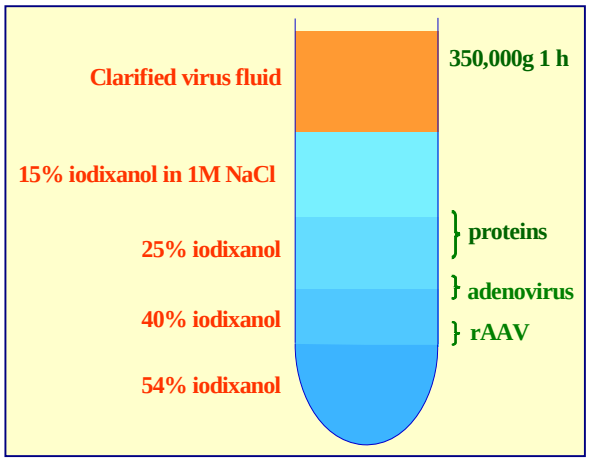

Figure 1 Banding of rAAV in a discontinuous iodixanol gradient [1]

3. Continuous gradient
The method is adapted from Hermens et al [3] who used a preformed continuous gradient in a near-vertical rotor. After release of rAAV from cultured cells by freeze/thawing, the virus particles from the clarified fluid were first concentrated either by ammonium sulfate precipitation or by cellufine sulfate column chromatography [3].

3a. Solutions required
A. OptiPrep™
B. Phosphate-buffered saline

3b. Ultracentrifuge rotor requirements
Near-vertical rotor with approx 5 ml tubes, capable of approx 360,000g (see Section 4, Note 9)

3c. Protocol
1. After concentration by ammonium sulfate precipitation or chromatography, suspend rAAV in phosphate-buffered saline, pH 7.4.

2. Transfer 2.7 ml of rAAV-containing fluid to a suitable tube and underlayer with OptiPrep™ to fill the tube.

3. After sealing the tube, form the gradient in a Gradient Master by rotating at 20 rpm at 80° for 12 min (see Section 4, Note 10)

4. Centrifuge at 71,000 rpm (348,000 g_{av}) for 3 h at 16°C (see Section 4, Note 10).

5. Collect the gradient from the bottom by tube puncture, the rAAV bands close to the bottom of the gradient (see Section 4, Note 11).

4. Notes

1. For smaller volume tubes scale down all volumes proportionally. It may be necessary to increase the centrifugation time proportionally if the rotor cannot achieve 350,000 g_{av}.
2. Phenol red (0.01 µg/ml) may be included in the alternate gradient layers to enhance visual identification of the layers. At 350,000 *g*, the iodixanol itself will sediment and may make the interfaces less obvious.
3. Aggregation of rAAV with proteins in the cell lysate can pose a serious problem to its isolation as the aggregates are heterogeneous and consequently exhibit a broad range of densities. Inclusion of 1 M NaCl in the 15% iodixanol prevents this aggregation and allows the rAAV to be isolated as a single band in the 40% iodixanol layer.
4. Because of the large volumes used in this gradient, the use of a peristaltic pump to introduce the iodixanol solutions makes this task easier. For more information on preparing discontinuous gradients **see Application Sheet V02**.
5. All of the contaminating proteins in the lysate band within the 25% iodixanol layer and more than 99% of any adenovirus contaminant bands at a lower density (<1.22 g/ml.) than the rAAV.
6. Great care needs to be exercised in removing the rAAV band to avoid contamination not only from any adenovirus but also empty capsids which are also band at a lower density.
7. Further purification by ion exchange or heparin affinity chromatography can be carried out directly on the iodixanol-containing fractions.
8. For many applications such as electrophoresis, infection of cultured cells, administration to experimental animals removal of the iodixanol is not a requirement. If it is an absolute requirement to remove (or at least reduce the concentration of) the iodixanol then some form of ultrafiltration is widely regarded as the most effective method: Vivaspin membranes from Sartorius and Centricon Plus 70 centrifugal filters from Millipore, or a PBHK Centrifugal Plus-20 filter unit with an Ultracel PL membrane (100 kDa cut off). Tangential flow filtration is also effective. For more information on this subject see **Application Sheet V05**
9. In ref 2 a NVT90 was used; other vertical and near vertical rotors with similar sedimentation path lengths may be suitable.
10. Because of the high density of rAAV, it is necessary to ensure that the bottom of the gradient is sufficiently dense to avoid the rAAV from reaching the wall of the tube during centrifugation. The gradient that is generated by the Gradient Master will tend to increase sharply in density towards the bottom and this will be enhanced during the subsequent centrifugation.
11. For more information about harvesting gradients see **Application Sheet V04**.

- Lock et al [5] compared the use of iodixanol and CsCl methods for the purification of rAAV and noted the considerable improved transduction (both *in vitro* and importantly *in vivo*) of the iodixanol-purified material. Moreover only the iodixanol gradient separated infectious particles from empty capsids, which the authors deemed “a desirable property for reducing toxicity and unwanted immune responses during preclinical studies”.
- There are many reviews of the rAAV technology; some provide very detailed protocols of the entire procedure, some are concerned with comparisons of the available methodology; these are all listed in **Mini-Review MV02.** Just two of the latest publications are given: refs. 6 and 7. The former provides very detailed methodology for the entire purification procedure the latter compares handling of two different serotypes rAAV2/5 and rAAV2/9 during the continuous collection of rAAV from producer cell medium.

5. References

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