Protocol for production, concentration, and titration of B19G- and EnvA-
pseudotyped rabies virus

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General notes:
- Bleach any waste products that touch virus before discarding.
- Refer to A Note to Investigators for a materials description and suggestions for getting started.
- Refer to basic tissue culture protocols for information on handling cells.

Expansion of SAD-ΔG-GFP

Purpose: to generate large quantities of B19G-pseudotyped rabies virus for long-term storage, direct use, or for initial steps in the production of EnvA-pseudotyped rabies virus

Day –n: Prepare necessary cells
- Thaw out 1 vial of BHK-B19G cells and plate onto 1x10cm dish.

Day -2: Expand cells for subsequent rabies virus infection
- Split BHK-B19G cells 1:6 onto 2x15cm dishes.

Day 0: Infect with rabies virus and further expand cells
- Add 19ml reduced serum medium to 50ml conical tube.
- Thaw out 1 vial of SAD-ΔG-GFP rabies virus and add to tube with 19ml medium.
- Aspirate medium from 1x15cm BHK-B19G dish and apply virus medium.
- Split other BHK-B19G plate 1:6 onto 6x15cm dishes.

Day 2: Gather supernatant from virus-infected plate and vacuum filter using Steriflip 50ml tube (Millipore #SCGP00525).
- Bleach and discard the virus plate.
- Aspirate medium from 5x15cm BHK-B19G plates, add 16ml reduced serum medium to each plate, then apply 4ml of filtered viral supernatant to each plate.
- Split the last BHK-B19G plate 1:10 onto 1x15cm plate (for backup).

Day 4: 1st harvest of virus
- Harvest supernatant from virus-infected plates and filter through 0.22µm filter.
- Store filtered viral supernatant at 4°C.
- Apply 20ml fresh reduced serum medium to virus-infected plates.

Day 5: 2nd harvest of virus + aliquotting and/or virus concentration
- Harvest supernatant from virus-infected plates and filter through 0.22µm filter.
- Bleach and discard plates.
- Split backup BHK-B19G plate 1:12 onto 1 15cm plate.
- Make 1ml aliquots of supernatant and freeze at -80°C for future virus production. If you wish to use this virus directly for in vivo injections, go to the Purification and concentration of rabies virus section.
Production of (EnvA)SAD-ΔG-GFP

Purpose: To generate concentrated EnvA-pseudotyped rabies virus for specific infection of TVA-expressing cells in mammalian nervous tissue

Follow “Expansion of SAD-ΔG-GFP” protocol up through Day 2, with the following additions:

Day –n: Prepare EnvA-expressing cells
• Thaw out 1 vial of BHK-EnvARGCD cells and plate onto 1x10cm dish.

Day -2: Maintain cell line for later use
• Split BHK-EnvARGCD cells 1:6 onto 1x15cm dish.

Day 0: Maintain cell line for later use
• Split BHK-EnvARGCD cells 1:6 onto 1x15cm dish.

Day 2: Expand EnvA-expressing cells for subsequent viral pseudotyping
• Split BHK-EnvARGCD cells 1:6 onto 6x15cm dishes.

Day 4: Pseudotype rabies virus by infecting EnvA cells and destroying residual B19 glycoprotein
• Harvest supernatant from virus-infected BHK-B19G plates and filter through 0.22µm filter.
• Bleach and discard infected BHK-B19G plates.
• Aspirate medium from 5x15cm BHK-EnvARGCD plates, and then apply 18ml filtered viral supernatant directly to each plate.
• 2 hours later: Aspirate medium from infected BHK-EnvARGCD plates, rinse plates once with D-PBS (without calcium), and then apply 5ml of 0.05% trypsin to each plate.
• Coat cells well with trypsin solution to destroy residual B19 glycoprotein, wait approximately 30 seconds, and then aspirate trypsin.
• Agitate and replate cells 1:1 in regular serum medium onto new 15cm dishes.
• 4 more hours later: Repeat trypsinizing steps to eliminate any remaining B19 glycoprotein. This step is essential for the production of well-pseudotyped virus!
• Agitate and replate cells 1:1 in reduced serum medium onto new 15cm dishes.
• Split remaining plates of BHK-B19G and BHK-EnvARGCD cells 1:10 onto 1x15cm plate each as backup.

Day 6: 1st harvest of EnvA-pseudotyped virus
• Harvest supernatant from virus-infected plates and filter through 0.22µm filter.
• Store filtered viral supernatant at 4°C.
• Apply 20ml fresh reduced serum medium to virus-infected plates.

Day 7: 2nd harvest + concentration of EnvA-pseudotyped virus
• Harvest supernatant from virus-infected plates and filter through 0.22µm filter.
• Bleach and discard plates.
• Split backup BHK-B19G and BHK-EnvARGCD plates 1:12 onto 1x15cm plate each.
• Go to Purification and concentration of rabies virus section
Purification and concentration of rabies virus

Purpose: To generate aliquots of high-titer rabies virus suitable for in vivo injection from unconcentrated rabies virus-laden supernatant

Notes:
- These steps are virtually identical to the purification steps described in Tiscornia et al., Nature Protocols 1, 241-245 (2006).
- There should be ~180ml filtered viral supernatant from the two collection steps.

Protocol:
- Spin viral supernatant at 70,000g for 2hr at 4°C in an ultracentrifuge.
  - Note: we use the Beckman SW28 rotor with conical tubes, and spin at 19,400 RPM. This gives 6x30ml = 180ml total capacity. You can freeze any extra SAD-ΔG-GFP supernatant at -80°C and use for future virus production.
- Gently aspirate supernatant and resuspend each pellet in 100µl Hanks’ Balanced Salt Solution (HBSS). Transfer suspension to a 1.7 ml microcentrifuge tube.
- Rinse conical tubes with 100µl HBSS to recover any remaining rabies virus and transfer suspension to the 1.7ml tube.
- Place 1.5ml of HBSS+20% sucrose in a round-bottom tube suitable for centrifugation in a Beckman SW55 rotor.
- Layer viral suspension on top of the sucrose cushion, and spin at 50,000g for 2 hr at 4°C (21,000 RPM with the SW55 rotor). Since only one tube will have the viral preparation, make sure to include a proper counter-balance!
- Gently aspirate supernatant and resuspend the viral pellet in 100µl HBSS, and save in a 1.7ml tube.
- Rinse bottom of ultracentrifuge tube with 100µl HBSS to maximize viral recovery, and save in the 1.7ml tube.
- Agitate the 1.7ml tube on a vortexer at low speed for ~5 minutes.
- Spin the 1.7 ml tube in a microcentrifuge for 10 seconds at maximum speed to pellet out any remaining insoluble material.
- Make aliquots of the supernatant (5-20µl each) and freeze at -80°C for future use.
- Proceed to Determining virus titer section
**Determining virus titer**

**Purpose:** to determine the infectious concentration of newly-generated rabies virus

**Notes:**
- 293T-TVA800 cells are necessary for the titration of (EnvA)SAD-ΔG-GFP. 293T cells are required for the titration of SAD-ΔG-GFP and serve as a pseudotyping control for (EnvA)SAD-ΔG-GFP.
- Grow any necessary cells in advance so that a plate of cells will be mostly confluent in time for the titration steps.

**Protocol:**
- Thaw out virus aliquot and add 4.44µl virus to a tube containing 495.6µl of cell culture medium, mixing well.
- Transfer 50µl of viral medium to a new tube containing 450µl cell culture medium and mix well.
- Continue making these 10-fold dilutions until you have 5 tubes of serially-diluted virus. Discard 50µl of the most-diluted virus, yielding 5 tubes with 450µl virus each (they will contain 4x10⁰ µl virus, 4x10⁻¹ µl virus…4x10⁻⁴ µl virus).
- **For SAD-ΔG-GFP:** Transfer the viral dilution series to the first five wells of a 24-well plate, and add 450µl of cell culture medium to the sixth well. This sixth well will serve as a blank.
- Trypsinize 293T cells and resuspend in medium, count cells using a hemacytometer, and transfer 10⁵ cells to each of the six wells.
- **For (EnvA)SAD-ΔG-GFP:** Add 550µl of cell culture medium to each tube of virus. Transfer 500µl of virus to each of two wells of a single column of a 24 well plate, producing two rows with a full dilution series of virus (now with 2x10⁰ µl virus, 2x10⁻¹ µl virus, etc). Add 500µl of cell culture medium to the last well of each row, as a blank.
- Trypsinize 293T-TVA800 cells and resuspend in medium. Count cells using a hemacytometer and transfer 10⁵ cells to each of the six wells in the first row.
- Do the same for 293T cells and transfer 10⁵ cells to each of the six wells in the second row. These cells will serve as a control for the quality of pseudotyping.
- The next day, aspirate medium from wells and replace with low serum medium.
- 48 hours post-infection, determine the percentage of fluorescent cells in each well either through FACS or in a cell counting chamber.
- To calculate biological titer, plot the number of fluorescent cells (10⁵ * fraction of fluorescent cells) vs. amount of virus to determine a best-fit slope across the linear range of the dilution series.
- If titer is good (>10⁵ particles/µl is more than sufficient for *in vivo* experiments), discard any remaining backup plates from previous virus production steps.

**Comments:**
- Tiscornia *et al.* recommend performing lentivirus titration in PBS to prevent cell division, but rabies virus-infected cells do not remain healthy under such conditions.
- Cells will continue to divide during the 48 hour period, but any progeny of infected cells should also be fluorescent, keeping the proportion of infected to uninfected cells relatively constant over time. (If anything, this will yield a slightly conservative estimate of virus titer, since infected cells will divide a bit more slowly than uninfected cells).