

LENTIVIRUS PRODUCTION FOR HIGH-TITER CELL-SPECIFIC LABELING

V2.0

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NOTE: Please note, part numbers for products are liable to change at any time; contact the manufacturer for updates, and please notify the authors of this white paper as well. Feedback always welcome!

0. MATERIALS

Cells:

HEK293FT cells (e.g., Invitrogen R700-07)

Solutions:

D10:

500 mL DMEM (e.g., Cellgro 10-017-CV)

50 mL fetal bovine serum (FBS) (e.g., Hyclone SH30071.03)

5 mL Penicillin/streptomycin (e.g., Cellgro 30-002-CI)

5 mL of Sodium Pyruvate (e.g., Lonza 13-115E)

Mix well; sterile filter with 0.22 micron filter flask (e.g., VWR)

Virus production medium (per 500 mL):

500mL Ultraculture (Lonza 12-725F)

5 mL Penicillin/streptomycin (e.g., Cellgro 30-002-CI)

5 mL of Sodium Pyruvate (e.g., Lonza 13-115E)

5 mL of Sodium Butyrate (100x solution, 0.5M, made from powder (e.g., Sigma # 19364))

Mix well; sterile filter with 0.22 micron filter flask (e.g., VWR)

20% Sucrose solution (per 50 ml)

10 g sucrose (e.g., Sigma)

Bring the volume to 50 ml using PBS

Mix well; sterile filter with 0.22 micron filter flask (e.g., VWR)

Other basic supplies include:

Trypsin-EDTA solution (e.g., Cellgro 25-052-CI)

Fugene 6 (Roche 1 814 443) or Mirus TransIT reagent (MIR 2700)

Ultracentrifuge tubes (e.g., Beckman 344058, or whatever your ultracentrifuge requires)

T175 plate: BD Falcon 353112 (e.g., VWR)

100 mm dish: BD Falcon 353003 (e.g., VWR)

140 mm dish: BD Falcon 353025 (e.g., VWR)

1. CULTURING HEK CELLS

- Use 10 mL of D10 in 10 cm dishes for HEK cell maintenance. Split cells in a 1:10 or 1:20 ratio after ~3 days of growth (they double in population approximately every day). To keep cultures healthy, passage cells within 4 days.
- Use low-passage HEK cells for best virus production results (less than 15 passages).
- Know the standard protocols and methodologies for: cell harvesting, cell plating (e.g., how to rock the plate along multiple axes to insure homogeneous cell plating), culturing (incubator

temperature, etc.), cell washing and medium changing (prewarm all media before use), centrifugation, freezing, thawing, etc. – for virus production to be good, each of these steps has to be streamlined.

- All virus-touching disposables should be bleached when done. Spray down work surfaces with bleach, and then 70% alcohol, after using.
- The following recipes can be scaled up or down, as desired, according to the number of HEK cells wanted (approximated by total plate surface area).

2. VIRUS PRODUCTION

PREPARE HEK CELLS (Day 0):

1. Take HEK293FT cells from three 90% confluent 15 cm dishes, and plate onto four T175 plates. Add 25 mL of D10 to each flask. (Should be almost 100% confluent on the next day.)

TRANSFECTION OF HEK-T CELLS (Day 1):

2. Perform a Fugene 6 transfection within 24 hours of plating (when cells are almost 100% confluent). You can also buy transfection reagents cheaply from Mirus. The following recipe is for 1 T175 flask. For 4 such flasks, multiply the recipe by 4. Ingredients:

DNA mix	22 ug lentiviral gene carrier (e.g., FCK-ChR2-GFP, http://www.addgene.org/pgvec1?f=c&cmd=findpl&identifier=15814) 15 ug pDelta 8.74 (helper plasmid; http://www.addgene.org/pgvec1?vectorid=5682&f=v&cmd=showvecinfo) 5 ug pMD2.G (VSVg, coat protein, from http://www.addgene.org/pgvec1?f=c&cmd=findpl&identifier=12259) 2 ug pAdvantage (Promega) (this is optional: if not using, use 7 ug of VSVg plasmid)
Fugene 6	132 ul
DMEM	Enough to bring up the total volume to 4.5 mL

- a. Place the Fugene into the DMEM without touching the sides of the plastic tube with the pipetter. Mix with light tapping, then let rest for 5 mins at room temperature.
- b. During the 5 min rest, mix DNA (carrier DNA + pDelta + VSVg + pAdvantage) in another tube.
- c. Add DNA mix to the Fugene+DMEM mix while tapping the destination tube lightly, and let the Fugene+DMEM+DNA mixture rest for 20-30 mins at room temperature.
- d. During the 20-30 mins, replace the culture medium in 1 T175 flask with 16 mL of fresh D10.
- e. Then, add the 4.5 mL of Fugene+DMEM+DNA mix to the T175 flask containing fresh medium. Gently rock the flask to distribute evenly.

CHANGE MEDIUM (Day 2):

3. At 24 hours post-transfection, remove the transfection medium from each flask and replace with 20 mL virus production medium per flask. Handle the plates gently, as the virus production process may cause cells to detach.

COLLECTING VIRUS (Day 3 and Day 4):

4. At 44-48 hours post-transfection, collect virus supernatant from each plate into a 50 ml conical flask and replace with 20 ml fresh virus production medium, if you want to collect more virus (we usually collect a second round, as the cells continue to stay healthy and produce high-quality virus for additional time). Spin the collected supernatant at ~1000 rpm for 5 min in a tabletop centrifuge to pellet cellular debris, then filter the supernatant through a 0.45 um (NOTE: not 0.22 micron!) filter flask, pre-wetted with a small amount of virus production medium to reduce protein binding. You can use a 0.45 um syringe filter for small volumes of virus. With this filtered supernatant, proceed to step **6** for this batch, immediately, for best results; storing this filtered supernatant at 4°C for processing along with the second round of virus collection may result in lower effective titers for the virus obtained during the first round.

5. OPTIONAL: If you added a second round of virus production medium in step **4**, collect the second batch of virus supernatant at 68-72 hours post-transfection, repeating the process described in step **4**, and proceeding to step **6** when you have acquired it. A third round of virus harvesting is usually not warranted for a given set of HEK cells.

6. To ultracentrifuge your coarsely-filtered viral supernatant, to concentrate the virus: transfer the 20 ml of supernatant from each conical flask to ultracentrifuge tubes (sprayed with ethanol in a biosafety cabinet to clean, then air dried). Gently pipette 2 ml of 20% sucrose+PBS solution to the bottom of the supernatant, to make a sucrose cushion, so that light debris will not be collected at the bottom of the ultracentrifuge tube, whereas virus will pellet out. Make sure that each of the tubes (usually 6; if you've been following the above instructions for 4 flasks, you may need two "dummy" tubes to balance the 6-tube rotor) is well balanced to avoid ultracentrifuge malfunction: you should weigh the tubes just before centrifuging, to insure balance, to the balance criterion of your ultracentrifuge – often a fraction of a gram. Also, make sure that each of the tubes is decently full – you may want to increase the volumes of virus produced above, or to augment the volumes with sterile PBS at this point, to make sure your ultracentrifuge tubes do not collapse. Spin in an SW-28 rotor in a pre-chilled Beckman ultracentrifuge at 22,000 rpm at 4°C for 2 hours. Follow all manufacturer instructions closely.

7. Carry the ultracentrifuge tubes gently at all times to prevent spillage, but especially now that your virus has pelleted out. Aspirate supernatant very gently, leaving behind the pellet. Observe the pellet – it may appear to be a thin translucent disc, or a white coating, or even be invisible – at the bottom of the centrifuge tube. Put the centrifuge tube upside down on a kimwipe to dry sides of tubes and use a Pasteur pipet to remove any additional medium on the side of the tubes, to prevent the virus production medium from ending up in your resuspension.

8. Resuspend the pelleted virus in a total of 100 ul cold PBS (assuming you've been following the instructions for four T175 flasks). Add 25 ul cold PBS to each of the 4 centrifuge tubes. Let the PBS sit on the pellet for some time, typically one-two hours at 4°C. Then gently pipette the PBS up and down in each tube, avoiding bubble production, and combine the four resuspensions.

9. Aliquot 2.5 ul-5 ul/tube, or as needed. (We tend to inject on the order of 1 microliter per injection, so aliquotting enough for an entire surgery session is good, to minimize freeze-thaw cycles.) Freeze at -80°C for up to 1 year. During freezing, it is important to use a mammalian cell-freezing box (i.e., which lets temperature drop at ~1°C per minute), for optimal titer preservation.

10. When using: after thawing the virus on ice, centrifuge at 5,000 rpm for 5 minutes, in a refrigerated centrifuge, to pellet out any clumps that may have formed. Keep the virus cold in preparation for surgery (it will last for several hours at 4°C).