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## PRODUCTION OF HIGH-TITER HIV-1-BASED VECTOR STOCKS BY TRANSIENT TRANSFECTION OF 293T CELLS

The VSV G-pseudotyped vector is the best choice for most gene-delivery experiments, both in vitro and in vivo. This protocol describes production of the VSV G vectors.

### *Materials*

293T/17 cells (ATCC cat. no. SD-3515)  
Dulbecco's modified Eagle medium/10% FBS (DMEM-10)  
0.05% trypsin/EDTA (e.g., Invitrogen)  
Plasmids (available from Addgene, <http://www.addgene.org>):  
    pMD2G (encoding the VSV G envelope protein)  
    pRRLSIN.cPPT.PGK-GFP.WPRE (third-generation transfer vector, abbreviated "pRRL")  
    pCMVR8.74 (encoding HIV-1 Gag, Pol, Tat and Rev proteins)  
TE buffer, pH 8.0  
Buffered water (see recipe)  
2.5 M CaCl<sub>2</sub> (see recipe)  
2× HeBS (see recipe or use the commercial kit CalPhos(TM) Mammalian Transfection/Clonetch : 631312)  
Phosphate-buffered saline, 37°C  
75% (v/v) ethanol in spray bottle  
Episerf Medium (Invitrogen 10732022) optional, if subsequent experiments require absence of serum)  
Phosphate-buffered saline containing Ca<sup>2+</sup> and Mg<sup>2+</sup> (e.g., Invitrogen; optional, if subsequent experiments require absence of protein)  
15-cm tissue culture dishes (Falcon)  
15- and 50-ml conical centrifuge tubes, sterile  
0.45 µm pore size Stericup 500ml HV sterile/ MILIPORE : SCHVU05RE  
0.22 µm pore size filters units 250ml PES sterile/TPP : TPP-99250  
38,5-ml 25x89mm Beckman ultraclear ultracentrifuge tubes (Beckman Coulter 344058)  
Ultracentrifuge with SW 28 rotor (Beckman Coulter) or equivalent

**CAUTION:** P2 practices require that open tubes always be handled in the laminar flow hood. Tubes can be taken out of the laminar flow only when they are closed, and that they be sprayed with 75% ethanol. All solid waste and plasticware must be discarded in a trash bin in the laminar flow hoods and all liquids must be aspirated into a liquid waste bottle containing fresh concentrated bleach. Refill the liquid waste bottle with fresh bleach when the color of the liquid is no longer yellow. When full, bags are closed inside the laminar flow hood, then autoclaved. When full, and at least 10 min after neutralization with fresh bleach, the liquid waste bottle can be emptied into a regular sink. In case of a major spill of vector-containing liquid, absorb liquid with paper towels and neutralize with fresh concentrated bleach prior to disposal. In case there is a leak in the SW 28 buckets, remove the tubes in the hood, fill the buckets with 75% ethanol, and invert them several times. Leave under the hood for ≥20 min. Discard the 75% ethanol under the hood..

*NOTE:* All solutions and equipment coming into contact with living cells must be sterile, and proper aseptic technique should be used accordingly.

*NOTE:* All culture incubations should be performed in a humidified, 37°C, 5% CO<sub>2</sub> incubator unless otherwise specified.

### ***Prepare 293T cells for transfection***

1. Maintain 293T/17 cells in DMEM-10 medium, in 15-cm tissue culture dishes. Split at a ratio of 1/10 using trypsin/EDTA, twice a week (e.g., every Monday and Thursday or Tuesday and Friday).

*Do not use 293T cells that are in culture for more than 30 passages.*

2. Cells can be seed Friday for Monday, which means 3 days before the transfection, by plating  $2.5 \cdot 10^6$  cells / 15cm dish. This allows using only few amounts of cells and results in tightly adherent cells.

Alternatively seed  $11 \cdot 10^6$  293T cells per dish the day before the transfection.

Ideally cells should be ~80% confluent on the day of transfection

### ***Cotransfect plasmids encoding vector components***

3. Two hours before transfection, replace the medium with 22.5 ml of fresh DMEM-10 medium preheated at 37°C and 0.45µm filtrated.
4. Adjust the DNA concentration of all plasmids: pMD2G, pRRL, pCMVR8.74 to 1 mg/ml in TE buffer, pH 8.0.
5. For five 15-cm plates to be transfected, prepare the following transfection mix in a sterile 50-ml conical tube

*We found that mixes for 5 plates gives best transfection efficiency. If more plates are to be transfected, we recommend making several 5-plate mixes.*

112,5µg transfer vector plasmid (with your gene of interest)

39,5µg pMD2G

73µg pCMVR8.74

6. Add in this order: 3,3ml of TE 0,1X and 1,75ml water
7. Add 565µl CaCl<sub>2</sub> 2,5M and mix well by pipetting.
8. Add 5,7ml of 2x HeBS dropwise under agitation of the 50ml tube by vortexing

*Vigorous vortexing will ensure the formation of a fine precipitate optimal for transfection.*
9. Leave the precipitate at room temperature at least for 5 min but do not exceed 30 min.
10. Add to the cell dropwise 2.25 ml/dish of precipitate from step 9. Mix by gentle swirling until the medium has recovered a uniformly red color. Incubate overnight.
11. Early the next morning, aspirate the medium, and slowly add 14 ml of fresh DMEM-10, prewarmed to 37°C. Incubate 8 hr.

*Cautious medium replacement is necessary because 293T cells have a high tendency to detach.*

*Check for transfection efficiency under microscope if fluorescent reporter is*

*encoded in the vector plasmid. We recommend always testing the reagents with a test transfection of a GFP encoding lentiviral vector.*

*The transfection can be started late in the afternoon and the medium changed early the next morning. Medium needs to be changed within approximately 16h of addition of the DNA.*

### **Harvest and concentrate vector stocks**

12. Harvest the culture medium from each plate to a 50-ml centrifuge tube. Close the tubes and spray them with 75% ethanol before taking them out of the laminar-flow hood. Store the supernatant at 4°C. Add another 14 ml of fresh 37°C DMEM-10 to the cell monolayer and incubate for another 8-12 hr.

13. At the end of the step 12 incubation, pool the second supernatant with supernatant from step 13. Centrifuge 5 min at  $500 \times g$ , 4°C, to pellet detached cells and debris.

*Collect supernatant for the first time in the evening, around 8 hours after medium replacement. The second harvest can take place early in the next morning around 12h later.*

*Supernatant can be harvested 2 or 3 times, every 8-12 hours. Keep it at 4°C over the collecting period.*

*The cleared supernatants can be kept at 4°C for 5-7 days.*

*Supernatants can be used directly, stored at -80°C as aliquots, or concentrated if needed. Freeze/thaw cycles strongly reduce the titer.*

14. Filter the pooled supernatant using a 0.22- $\mu\text{m}$  filter unit.

15. Slowly pipet the supernatant from step 14 into 38,5-ml Beckman ultraclear tubes. (put at least 32 ml/tube but not more than 35ml. Ultracentrifuge 120 min at  $50,000 \times g$ , 16°C. Gently discard the supernatant by inversion. Let the tube dry inverted.

*Final volume for 5 plates harvested 3 times is around 210 mL which allows to use a 250 mL filter unit and 6 ultraclear tubes filled with 32 mL each.*

*After centrifugation, do not let the pellet dry too much, around 2-3 min is sufficient, then remove the remaining medium on the tube with a paper.*

16. Resuspend the pellet (not always visible) with PBS1X, by pipetting first 10-15 times all around pellet, and then up and down 15 times.

*The vector pellet of one tube can be resuspended in a minimal volume of 30  $\mu\text{l}$ . In this case, a 1000-fold concentration will be obtained.*

*As there is always some medium remaining even after drying, add first only 15 $\mu\text{l}$  of PBS1X per tube. The advantage of this remaining medium is that it brings some proteins important for viral particles stabilization.*

*Practically, had 15 $\mu\text{l}$  to each ultraclear tube and an extra 15 $\mu\text{l}$  to the first one. After resuspension of the first tube, recover 15 $\mu\text{l}$  in a sterile eppendorf and transfer the remaining volume to the second ultraclear tube Proceed sequentially until the last tube.*

*Try to avoid bubbles when resuspending the pellet since their presence will result in decrease of final volume, and hence decrease of yield.*

17. Clear the final concentrate by a brief centrifugation (around 5 seconds) at maximum speed on a bench top centrifuge. Aliquot the supernatant and store at 80°C.

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## REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

### **Buffered water**

50 ml H<sub>2</sub>O

125 µl 1 M HEPES, pH 7.3 (2.5 mM final)

Filter sterilize through 0.22-µm nitrocellulose filter

Store up to 6 months at 4°C

*The authors have observed that the appearance and quality of the DNA/CaCl<sub>2</sub> precipitate (see Basic Protocol) can vary depending upon the batch of distilled water used to dilute the plasmids. To circumvent this problem, it is advisable to buffer this distilled water. A final concentration of 2.5 mM HEPES in the water will help maintaining a proper pH and will not compete for the final pH with the HeBS pH 7.00 (see recipe) added subsequently (i.e., the HeBS, pH 7.00, provides for a final concentration of 25 mM HEPES final, whereas the buffered water, pH 7.3, results in a final concentration of 0.625 mM HEPES).*

### **CaCl<sub>2</sub>, 2.5 M**

9.18 g CaCl<sub>2</sub>•2H<sub>2</sub>O (mol. wt., 147; SigmaUltra)

25 ml H<sub>2</sub>O

Filter sterilize through 0.22-µm nitrocellulose filter

Store at +4°C.

### **HEPES-buffered saline (HeBS), 2×**

Dissolve the following reagents (all SigmaUltra from Sigma) in 800 ml H<sub>2</sub>O:

16.36 g NaCl (mol. wt., 58.44; 0.28 M final)

11.9 g HEPES (mol. wt., 238.3; 0.05 M final)

0.213 g anhydrous Na<sub>2</sub>HPO<sub>4</sub> (mol. wt., 142; 1.5 mM final)

Adjust pH to 7.00 with 10 M NaOH (proper pH is critical)

Add H<sub>2</sub>O to 1000 ml and make final pH adjustment to pH 7.00

Filter sterilize through a 0.22-µm nitrocellulose filter

Store up to 2 years at –70°C in 50-ml aliquots

*Once thawed, the HeBS solution can be kept at 4°C for several weeks without significant change in the transfection efficiency.*

*It is critical that the pH be adjusted accurately; below 6.95, the precipitate will not form; above 7.05, the precipitate will be coarse and the transfection efficiency will be low.*