VVC - 3nd generation VSV.G pseudotyped lentiviral packaging protocol

Seed 2.5 x 10^6 low passage (less than P20) 293T cells per 15cm dish in 15 ml DMEM with 10% serum and 1% Pen/Strep. For a standard prep of 12 dishes you will need to start with 3-4 dishes. Grow until 90% confluent and then split 1:3 – 1:4 to give twelve 15cm dishes. Allow cells to grown until 70% confluent (~11 x10^6, approx 24 hours)

PEI transfection

2 hours prior to transfection, remove medium and replace with 15ml fresh pre-warmed growth medium (GM).

Prepare your DNA mix as follows. Amounts are given for a 1x15 cm dish. Scale up as appropriate for the number of dishes you have.

Vector	amount (ug)	x12	Size (kb)	
Transfer plasmid	2	24	~8-12	*encodes vector genome to be packaged*
Packaging plasmids				
pMDLg/pRRE	7.5	90	8.8	*expresses HIV-1 GAG/POL*
pRSV/REV	7.5	90	4.2	*expresses HIV-1 REV*
pMD2.G	5	60	5.8	*expresses VSV glycoprotein*

Per plate - add DNA mix to 500uL of pre-warmed Optimem II medium. Add 88uL PEI (1ug/ul in 1xPBS pH4.5) to the mixture (4:1 v/w ratio of PEI:DNA). Vortex briefly (10 sec) and leave for 5–10 min at room temp.

Add the transfection complex drop-wise to each 15cm plate, swirl briefly to mix and incubate for 8 hrs in 10% CO2, 35^{O}C. Replace medium with 10 ml of fresh growth medium containing 25mM HEPES and 10% serum (**all medium from this point onwards should be 25mM HEPES buffered**). Incubate as above until 48 hours post-transfection.

For viral preps where *in vivo* toxicity might be a problem, lower the serum concentration to 3% in the collection medium.

Virus collection.

1. Remove medium from each dish and pool. Filter through a 0.22µm low protein binding/fast flow filter unit and store @ 4 ^{O}C.

2. Add 5 ml fresh GM and incubate overnight as above (60-72 hours post transfection).

3. Collect 2nd lot of medium from each dish as in step 1, and pool with previous harvest.

4. Remove plasmid carry-over by digestion with DNASE-I (1mg/ml stock). Use 1 µg per ml of viral supernatant. Also add 1 µl of 1M $MgCl_2$ per ml of supernatant and incubate at RT for 30 min followed by 4 ^{O}C for 2-4 hours.

5. Filtered supernatants can be used directly on cultured cells, or can be aliquoted and stored at -80^{O}C.

Avoid repeated freeze/thawing as this significantly reduces functional titer

If required, vector supernatants can be concentrated and purified as described below: (**Modified from Tiscornia, Singer & Verma Nature Protocols. 2006**).

Virus concentration and purification

1. Concentrate the viral particles by ultracentrifugation. Centrifuge the supernatant at 70,000 x *g* for 2 h at 12°C. We use Konical tubes with a Beckman SW28 rotor (capacity for six tubes) at 19,400 rpm for 2 h at 12°C. Fill the tubes with 30 ml of supernatant. The total volume of supernatant from a 12 × 15 cm dish preparation is **180 ml = one SW28 rotor.**

2. Pour off the supernatant and allow the remaining liquid to drain by resting the inverted tubes on paper towels. Siphon off remaining droplets using an aspirator in order to remove all liquid from the pellet. The pellet should be barely visible as a small translucent spot.

3. Resuspend the viral pellets in 100 µl of sterile 1× HBSS. Avoid frothing. Rinse the tubes with 100 µl of 1× HBSS. Pool both volumes to obtain 200 µl final volume.

4. Transfer to a screw-cap microfuge tube, wrap in parafilm and vortex at low speed for 15–30 min. The resuspended viral preparation will look anywhere from clear to slightly milky.

5. Clear the suspension by spinning for 10 s on a tabletop microcentrifuge. Transfer the supernatant to a fresh screw-cap microfuge tube.

6. Create a sucrose cushion by layering the 200 µl of viral preparation (from Step 5) on 1.5 ml of sterile 20% sucrose (in 1xHBSS). We use Beckman round bottom tubes. Add 1× HBSS (~3.5 ml) to the tubes as needed to fill and balance them before centrifugation. Centrifuge tubes at 50,000 x *g* for 2 h at 12°C. We use a Beckman SW55 swinging bucket rotor at 21,000 rpm for 2 h at 12°C.

7. Pour off the supernatant, allow liquid to drain by resting the tube inverted on a paper towel and siphon off all remaining droplets with an aspirator.

8. Resuspend the clear, translucent pellet in 100 µl of 1× HBSS. Rinse the tube with an additional 100 µl of 1× HBSS. Transfer to a screw-cap microfuge tube, wrap in parafilm and shake on a low speed vortex for 15–30 min. The suspension will range from clear to

slightly milky; clear by spinning for 10 s on a tabletop microcentrifuge. Transfer the supernatant to a fresh microfuge tube and make 10 µl aliquots. Snap freeze in LN or a dry ice/ethanol bath and store at -80°C.

To Make the PEI solution

pH 1xPBS to 4.5 using HCl. Add 50 mg linear PEI (Polysciences #23966-2) to 50ml 1xPBS pH4.5. Place in a 75^{O}C waterbath and vortex every 10 min until completely dissolved. Cool to room temp and filter sterilize through a 0.22µm syringe filter. Can be stored at room temp or aliquoted and stored at 4^{O}C.