The RNAi Consortium *08.27.2007*

Section II: Lentiviral production

Introduction:
This contains the protocol for lentiviral production in 6 cm plate.

Lentiviral production consists of the following steps:

Day 1	Seed 293T cells
Day 2 (pm)	Transfect cells with 3 plasmids
Day 3 (am)	*16 hours post-transfection* – change media; replace with BSA-containing media
Day 4 (am)	*24 hours after media change* – harvest virus (1) ; replace with BSA-containing media
Day 5 (am)	*24 hours after harvest (1)* – harvest virus (2); combine harvest (1) and (2); discard cells

I. Materials
1. Cell line: **293T** packaging cells <*recommended: healthy; avoid confluent culture, and no continuous subculture more than 10 passages*>
2. Growth meidia: DMEM+10% heat-inactivated FBS (Hyclone) + 1X Pen/Strep
3. BSA-containing media: DMEM+10% heat-inactivated FBS (Hyclone) + 1% (W/V) BSA < Bovine Serum Albumin (Sigma, A9430)> + 1X Pen/Strep
4. Plates: 6 cm tissue culture plate
5. Transfection-quality plasmid DNA--
   TRC library plasmid: pLKO.1-shRNA vector
   packaging plasmid: pCMV-ΔR8.91 (containing *gag*, *pol* and *rev* genes)
   envelope plasmid: pMD.G (VSV-G expressing plasmid)
6. Transfection reagent: TransIT LT1 (Mirus Bio.) < alternative: FuGene 6 (Roche) >
7. Sterile Polypropylene storage tube

II. Instructions
1. **Day 1:** Seed 293T cells in 6 cm tissue culture plate. (seeding density: 1.6*10^5 cells/ml; seeding volume: 5 mL) and incubate cells in the incubator (37 °C, 5% CO2) until the following afternoon.
   Note: [1] The cells are seeded in no Pen/Strep containing-growth media.
   [2] Before transferring to a culture incubator, keep cells settled for one hour in hood to reduce uneven distribution.

2. **Day 2:** The cell density should be among 50% to 70% confluence for transfection with TransIT LT1 reagent.
   a. Prepare a mixture of three plasmids:

Reagent	per 6 cm plate
pCMV-ΔR8.91	**2.25 μg**
pMD.G	**0.25 μg**
hairpin-PLKO.1	**2.5 μg**
OPTI-MEM to total volume	**250 μl**

Note: The cell density at transfection, DNA concentration for transfection and reagent to DNA ratio should be optimized according to the different transfection reagent kit.

b. Dilute transfection reagent:

Reagent	per 6 cm plate
TransIT-LT1	**15 μl**
OPTI-MEM to total volume	**250 μl**

Add the TransIT-LT1 reagent dropwise into OPTI-MEM, mix by swirling the tip or gently flicking the tube (do not mix by pipetting), and incubate at RT for 5 min.

c. Add the plasmid mixture dropwise into the diluted transfection reagent and mix by swirling the tip or gently flicking the tube

d. Incubate the transfection mix for 20-30 minutes at room temperature.

e. Carefully transfer the transfection mix to the cells. 293T cells would be sensitive to perturbation, therefore take care not to dislodge the cells from the plate.

f. Incubate cells in the incubator (37 °C, 5% CO_2) for 16 hours.

3. **Day 3**: Change media to remove the transfection reagent and replace with **5 mL** BSA-containing media per plate. Incubate cells for 24 hr (37 °C, 5% CO_2).

4. **Day 4:** Harvest media containing lentivirus at 40 hours post-transfection. Transfer media to a polypropylene storage tube and store at 4 °C. Replace with **5 mL** BSA- containing media for further harvest.

5. **Day 5:** Harvest media containing lentivirus at 64 hr post-transfection and then discard the cells. Pool the viral harvests and spin the media at 1250 rpm for 5 minutes to pellet the cells that were collected during harvest. Transfer the supernatant to a polypropylene storage tube and then aliquot to smaller storage tubes to reduce the numbers of freeze/thaw cycles.
Note: Virus may be stored at 4 °C for a few days but should be stored at -80 °C for long-term storage.

Version Notes:
BSA-containing media for viral harvests: By comparison with growth media, viral harvest media containing 1 g/100 mL supplemental BSA can improve virus yield by ~2 fold.