**Generation of stable cell lines using retroviral system**

Thanh Ngoc Nguyen (Laboratory of Michael Lazarou, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) (nguyen.tha@wehi.edu.au)

**Buffers and reagents:**

* + Growth media: DMEM with 10% FBS, 4.5 g/l Glucose (Sigma, G8769), 1x GlutaMAXTM (ThermoFisher, 35050061), 1x MEM NEAA (ThermoFisher, 11140-050), 25 mM HEPES (1688449)
	+ Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen, A12621)
	+ Opti-MEM™ I Reduced Serum Medium, no phenol red (Gibco, 11058021)
	+ Millex-HV Syringe Filter Unit, 0.45 µm (Millipore, SLHVM33RS)
	+ Polybrene (4 mg/mL)

**Attention:**

* The HEK293T cells detach very easily, be extra gentle when changing the media.
* All viral waste must be bleached and left under UV light for at least 30’ after viral work in TC hoods before disposal.

**Procedures:**

***Day 1***

1. Seed NIH HEK293T cells into a 6-well plate (900k cells/well if set up in the morning, 950k cells/well if set up in the afternoon). Set up 1 well for each construct you wish to generate a virus harvest for, can be scaled up according to your need.

***Day 2: The following protocol is designed for one well of the 6-well plate***

1. Transfect cells with viral and helper vectors using lipofectamine LTX. Combine the following in a 1.5 mL tube:

1.5 µg viral vector construct (pBMN, pBABE or pMX)

1.0 µg well gag-pol vector

0.5 µg well VSV-G vector

500 µL Opti-MEM (RT)

1. Add 3 µL of Plus reagent and mix well. Incubate at RT for 5 min.
2. Add 9 µl of Lipofectamin LTX (1:3 ratio of Plus:LTX is standard in the lab but can be adjusted for your own protocol) and vortex for 15 seconds. Incubate at RT for 20 min.
3. Once the 20 min incubation starts, replace the media in each well with 1ml DMEM/10% FBS media.
4. When the 20 min incubation finishes, add the optimum/liposome mix to the well. Do it gently on the side of the well.

***Day 3:***

* + - 1. In the morning, remove the old media from the HEK293T cells which may contain viruses at this stage) into a beaker of beach and add 1ml of fresh growth media. The next day, viruses can be harvested for infection.
			2. Seed the target cells (about 100k-120k cells) into a 6-well plate if intending to do infection with fresh viruses.

***Day 4:***

1. In the late afternoon, collect viral supernatant from HEK293Ts, spin down at max speed for 5 min to pellet debris and filter through 0.45µm syringe filters. Viral particles can freshly be used for infection on the cells plated out on day 3 (see below) or can be frozen at -80 °C for future use.
2. For second harvest, add 1.5 mL fresh growth media back to HEK293T cells for 2 days and harvest again (on Day 6).
3. For infection, harvested viruses are topped up with fresh growth media to make up a total of 2 ml.
4. Aspirate the media from the target cells.
5. Add the 2ml of virus-containing media (from step 3) to the target cells. Add polybrene to a final concentration of 8 µg/mL to the well and mix well.

***Days 5 and 6:***

1. The viruses can be removed from the cells into a beaker of bleach after 24 h (Day 5) or 48 h (Day 6) and fresh media can be added to the wells.
2. All waste must be treated as viral waste for at least 3 media changes over 3 days post-infection.