Establishment of Stable Cell Lines Using Pseudotyped Retroviral Vectors

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GENERAL NOTES

1) All experiments involved in the use of retrovirus should strictly follow Biosafety Level-2 guidelines. Please read the **MOLab Biosafety Manual** prior to your work: http://www.boneandcancer.org/UCMOLab%20Biosafety%20Manual_3-24-05.pdf
2) The retroviral vectors used in our lab are derived from the Murine Stem Cell Virus (MSCV) vectors modified by MOLab and designed as **pSEB-xxx** or **pSEH-xxx** etc.

I. CONSTRUCTION AND PURIFICATION OF RETROVIRAL PLASMIDS

1) Subclone the gene(s) or siRNA fragments of your interest into **pSEB-xxx**, or **pSEH-xxx** vectors, etc. (see: http://www.boneandcancer.org/ucmolab.html).
2) Purify the following plasmids using the conventional alkaline lysis miniprep protocol:

   a. your **retroviral vectors**, e.g., **pSEB-xxx** (Amp resistance);
   b. the packaging **pCL-Ampho** (Amp resistance);
   c. the VSVG pseudotyping plasmid **pCMV-VSVG** (Amp resistance);
   d. the transfection tracking vector **pMPB-BiFP1** (Spn resistance)

   [**NOTE**: You will roughly need **one miniprep DNA** of **pSEB-xxx** and **pCL-Ampho** to transfect **one** 100mm dish of 293 cells].
3) Completely remove RNA from the miniprep DNA either by RNase A digestion or by using **Mag-Bind Beads** (see a separate lab protocol; *this is the preferred method to remove RNA*). RNA removal should be always followed with **PC-8 extraction** and ethanol precipitation.
4) Dissolve each miniprep DNA in 20µl sterile ddH_2O (e.g., DNA concentration @ ~0.5µg/µl). Check 1-2 µls on agarose gel. (***NOTE**: Do not simply rely on any readings from NanoDrop*).

II. GENERATION OF RETROVIRUS (RV) SUPERNATANTS

[***NOTE**: As the RV titers drop significantly over the time when kept at 4°C or -20°C, you should **time well** with your RV packaging and target cell plating*].

1) Plate healthy HEK293 or 293PA cells in 100mm dish (usually one 100mm dish is more sufficient for making 1~3 stable lines) in the morning. Initial cell density should be **30-40%**.
2) Prepare transfection mix in a 1.5-ml Eppendorf tube while the 293 cells are attaching the dish (usually 2~4h after plating):

   20.0 µl of **Retroviral vector** plasmid (~0.5µg/µl)
   20.0 µl of **pCL-Ampho** packaging plasmid (~0.5µg/µl)
   2~4 µl of **pCMV-VSVG** pseudotyping plasmid (~0.5µg/µl)
   2.0 µl of **pMPB-BiFP1** plasmid (~0.5µg/µl)
   50.0 µl of **PEI** (3.0 µg/µl)
   400.0 µl **serum-free DMEM**

Mix well and incubate for 5-10min at room temperature in the hood

3) Carefully but quickly remove complete DMEM from the dish plated with 293 cells [***Optional****: wash cells carefully with 3ml* ***serum-free DMEM***]. Then add 5ml **serum-free DMEM**.
4) Add the DNA-PEI mix carefully, and gently shake the dish to mix the medium well.
5) At **2-4** hours after adding the DNA-PEI mix, aspirate medium out; add 10 ml **complete DMEM** medium (***NOTE****: If there are too many floaters, do not change the medium. Instead, just add 10ml complete DMEM; change the medium next morning*.)
6) At 16h~24h after transfection, check GFP signal under a fluorescence microscope. The GFP+ cells should be >50%, otherwise your transfection efficiency is too low and should consider set up a new/backup transfection.
7) Collect the retrovirus supernatant at **36h, 48h,** and **60h** [*NOTE: you may collect up to 72h*] (store them at 4°C). The total volume of the pooled retrovirus supernatant should be ~30ml.
8) Remove cell debris by low speed centrifugation for 5 min at room temperature.
9) Filter the pooled retrovirus supernatant through **0.45μm PES syringe filters**. The filtered RV supernatant is ready to infect your target cells for making stable lines (see below).

III. ESTABLISHMENT OF STABLE CELL LINES USING RETROVIRUS

1) Plate your **target cells** in a **T-25 cell culture flask** at subconfluency (i.e., ~30%) **2-6h prior to** your **last collection** of RV supernatant.

   [*NOTE: Do not seed your target cells at high density as you want to maximize the # RV/cell to achieve a high expression level; also, RV integration needs cell division*].
2) Prepare **Polybrene**-containing retrovirus supernatant by adding 150-200μl of 2mg/ml Polybrene (from Sigma-Aldrich) to ~30ml RV supernatant.
3) Aspirate the culture medium out of the **T-25 flask** of your target cells, and add **10-15 ml** of the filtered **Polybrene**-containing retrovirus supernatant. Return the **T-25 flask** to the 37°C CO_2 incubator.
4) At **6~8h** (or overnight) after the infection, aspirate the RV-containing medium and add 10ml complete DMEM to the **T-25 flask**. Incubate at 37°C CO_2 for additional 12-24h.
5) [*Optional: you can do another round of infection by removing the complete DMEM, and add 10-15ml Polybrene-containing RV supernatant. Return the cells to the 37°C CO_2 incubator for 4-6h*]
6) At ~36h after the RV infection, replate the infected cells to a 100mm cell culture dish, and start the antibiotic selection by consulting with the following website for drug concentrations:
   http://www.boneandcancer.org/MOLab%20protocols%20since%2011-2005/E22%20Antibiotics%20Selection%20Table_02-02-06.htm

   [***NOTE****: If your drug selection leads to* ***>70% cell death****, it usually indicates either your RV titer is too low, or your target cells are difficult to be infected. In either case, you should consider doing another round of RV packaging/infection, and see you can get a better luck*].
7) In most cases, **one round selection** should be sufficient for **BSD**-selected stable lines while 1-2 rounds of selection may be required for hygromycin, G418, puromycin or zeocin-selected stable lines.

IV. OTHER CONSIDERATIONS

1) **Use of Retrovirus packaging lines**: It has been reported that retrovirus production can be facilitated by using packaging lines, such as the Phoenix and PT67 packaging lines or 293PA. We have tested the Phoenix packaging line. Although it works reasonably well initially, it gradually loses the packaging efficiency over passages. Since HEK 293 cells (or most 293 derivative lines) are highly transfectable, we prefer co-transfecting the retroviral vectors with the pCL-Ampho packaging plasmid to ensure the production of retrovirus with constant high titers.

2) **Testing the presence of replication-competent retrovirus**: Although this possibility is very low, one should test the potential presence of replication-competent retrovirus on a regular basis. This can be done by infecting HEK 293 cells with the viral supernatant, followed by collecting the supernatant from the infected 293 cells to infect a second cell line in the presence of antibiotic selection. No stable clones should be obtained. Otherwise, it indicates the possible presence of replication-competent retrovirus in the original supernatant, which should NOT be used for any experiments.

IMPORTANT REFERENCE

Liao J. et al., Characterization of retroviral infectivity and superinfection resistance during retrovirus-mediated transduction of mammalian cells. ***Gene Therapy*** *24(6):333-341. doi: 10.1038/gt.2017.24*