Systems Bioengineering Laboratory

Lentivirus Production and Infection

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Materials

- 293T cells
- Mirus TransIT®-Lenti Transfection Reagent (Mirus 6603 or other size variants, see link for detailed protocol TransIT-Lenti Transfection Reagent | High Titer Lentivirus Production (mirusbio.com)
- pMD2.G plasmid (Envelope vector, Addgene, plasmid# 12259)
- psPAX2 plasmid (packaging vector, Addgene, plasmid# 12260)
- Lentiviral transfer vectors
- DMEM Dulbecco's modified eagle medium (1×Gibco, Cat#11965-092)
- Opti-MEM reduced serum medium (1×Gibco, Cat# 31985-070)
- 1.5 ml sterile Eppendorf tubes (Eppendorf AG, Cat# 022431021)
- 50 ml conical centrifuge tubes (Thermo Scientific, Cat# 339653)
- 15 ml conical centrifuge tubes (Thermo Scientific, Cat# 339650)
- 75 cm^2 nunc easyflask (Thermo Scientific, Cat# 156499)
- Sartorius Vivaspin 20, 100,000 MWCO PES spin filter (VS2041)
- Warm 0.05% trypsin-EDTA (1×, REF# 25300-054)
- Fetal bovine serum (Gibco, Cat# A3160602)
- Disposable plastic pipette (5ml,10ml, Thermo Scientific, Cat# 170355, 170356)
- Non-Filter pipet tips (10ul, 200ul,1000ul, Fisher Scientific Cat# 02-707-442,413,402)
- Polybrene (Hexadimethrine bromide,10mg/ml, Sigma, CAS# 28728-55-4)
- 12ml syringe (Covidien, REF# 8881512878)
- 0.45 μm syringe-driven filter (Millex, REF# SLHA033SS)
- 5ml polystyrene round-bottom tube with cell-strainer cap (Falcon, REF# 352235)
- 6-well plate (Thermo Scientific, Cat# 140675)
- 24-well plate (Thermo Scientific, Cat# 142475)
- 10ul, 200ul, 1ml sterile pipet tips (Fisher Scientific, Cat# 02707470, 02707413, 02707402)
- Bleach
- Puromycin (Sigma Aldrich #P7255-25MG)
- Hygromycin B (Sigma Aldrich #10843555001)
- G418 (Sigma Aldrich #4727878001)
- Blasticidin S (Fisher #MT30100RB)

Equipment

- 5% CO_2 incubator
- Biosafety hood
- Bench top centrifuge: Beckman Coulter, Allegra X-30R centrifuge
- Inverted phase contrast microscope

Methods

For the entire protocol, use well-established aseptic techniques in a BSL-2+ tissue culture hood. Make sure that cells are actively dividing prior to seeding. Perform lentivirus packaging using antibiotic free media.

Day 0: Seed HEK293T cells in a T-75cm^2 flask 5x10^6. Cells should have enough room for active cell division.

Day 1: 1. Warm the Opti-MEM media and the TransIT-Lenti reagent. Be sure to vortex the Transit reagent gently before using. Prepare DNA stocks according to the table below (values obtained from Mirus 6603 documentation. Maintain a 1:1 (wt:wt) ratio with the packaging DNA premix solution to transfer gene.

Packaging DNA premix solution	**Amount per T75 flask 7.5 ug of packaging DNA 4:1 pspax2:pMD2.G**
psPAX2 (gag, pol)	6000 ng
pMD2.G (vsvg env)	1500 ng

Transfer gene	**Amount per T75 flask**
Transfer Plasmid*	7500 ng
OptiPro SFM to total volume	1500 µL

2. Combine the packaging DNA premix solution to the transfer gene solution in 1500uL of Opti-MEM media and mix gently.
3. Add 45uL of TransIT reagent dropwise to the diluted DNA mixture. Pipet gently and incubate for 10 minutes at room temperature.
4. Distribute the complexes drop-wise to the T75 flask and gently rock the vessel to mix.
5. Incubate at 37°C in 5% CO_2 for 48 hours. Do not change the media, as this will negatively affect lentivirus titer.

Day 3: Collection

1. Collect the lentivirus supernatant and centrifuge for 5 minutes at 500xG. Filter the supernatant using a 0.45um PVDF filter. Supernatant can be flash frozen at this point or concentrated using a Sartorius Vivaspin 20. Alliquote the virus at 100ul/tube. Remember to place all vessels and plastics that came into contact with virus supernatant in a 50% bleach solution for at least 8 hours.
2. If performing transduction, the same day (recommended), set aside enough viral supernatant and freeze the rest and store in -80°C and proceed to the transduction step.

Transduction

1. Prepare cells of interest by trypsinization, followed by resuspension in complete media. Seed cells at a density of 100K/ well.
2. If the viral supernatant is frozen, rapidly thaw lentivirus in the *37°C* water bath followed by brief centrifugation. To each well of a 6 well plate add 100uL of concentrated lentivirus supernatant. Swirl the plate and place in a tissue culture incubator for 24-48 hours. For difficult to transfect lines, higher volumes of lentiviral supernatant can be used. Addition of polybrene to each well (final concentration of 10ug/mL) 6hrs-overnight can improve transduction efficiency.

Note: *Polybrene is prepared at a concentration of 1mg/ml in pure water and filter-sterilized or autoclaved. Store in aliquots at -20°C.*

Day 5-6: Media exchange: 24-48 hours post-transduction, exchange the media with complete culture media. If appropriate, cells can also be passaged. Discarded media should be placed in a solution of 50% bleach for at least 8 hours.

Day 7: Selection: 48 hours post-media exchange, cells can be selected using antibiotic containing media or flow sorted (a minimum of $1x10^6$ cells are recommended). It is highly recommended that cells be cryopreserved prior to selection or flow sorting:

Selection with antibiotic (~ 1 week):

1. Identify the selection marker in your plasmid to ensure the correct antibiotic. Depending on the cell type, an antibiotic kill curve in naive cells should be performed to determine the minimum concentration of antibiotics required to kill non transduced cells. See table below for suggested concentrations of antibiotics. **Please review the instructions on Establishing Antibiotic Selection Concentration**

Selection Antibiotic	Suggested Working Concentrations
G418	0-2.0 mg/ml
Hygromycin B	0-500 µg/ml
Puromycin	0-10 µg/ml
Blasticidin S	0-10 µg/ml

2. Observe the wells and passage as necessary. Be sure to maintain cells in antibiotic containing media.
3. Expand and cryopreserve cells when necessary.

Selection using FACS

1. Passage the cells and change the media as necessary until there are at least $1x10^6$ cells.
2. Trypsinize cells and resuspend in FACS sorting buffer (1xPBS+1%FBS).
3. Pass the cell suspension through a 5ml polystyrene round-bottom cell-strainer tube. In the meantime, prepare collecting tubes (15 ml conical centrifuge tube+1ml culture media).
4. Transport the cells on ice to the flow sorter. At the sorter, be sure to use the correct spectral filter sets. It is helpful to include unlabeled cells as part of your sorting panel to establish the negative sort population. If you are sorting cells that contain multiple fluorophores, be sure to include single color controls to generate a FACS compensation panel. After sorting cells, culture in 6-well plates and passage as necessary.

Establishing antibiotic selection concentration

Using a 24-well plate to titrate the concentration of antibiotic required for cell selection. Acquire the antibiotic that matches the selection marker of the plasmid. Some examples of antibiotics are as follows.

- Puromycin (Sigma Aldrich #P7255-25MG)
- Hygromycin B (Sigma Aldrich #10843555001)
- G418 (Sigma Aldrich #4727878001)
- Blasticidin S (Fisher #MT30100RB)

Day 0: Plate 20,000 naïve 293T cells/well or your cell line of interest in 0.5 ml of complete medium DMEM with 10% FBS and 1% P/S in 24-well plates.

Day 1: Prepare serial dilutions of the antibiotic of interest in complete media. Exchange the media present in the well for the antibiotic containing media.

Day 2-n: Each day observe the wells and passage as necessary. Be sure to re-seed cells in antibiotic containing media. Take note of the wells and corresponding concentration of antibiotic in which no cells remain as this is the recommended antibiotic selection concentration.