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Protocol: Lentivirus production in flasks

This protocol describes the production of lentivirus stocks of plasmids such as Cas9, pXPR (sgRNA), pLKO (shRNA) or pLX (ORF) in T25, T75 or T175 flasks. The viral harvest media contains a low percentage of BSA to further stabilize the viral particles when frozen. A Monday – Thursday schedule is noted, but the days of the week can be adapted so long as the timing between each step remains the same.

Materials and reagents needed:

- 293T cells
- 293T maintenance media: DMEM + 10% hiFBS
    - Dulbecco's Modification of Eagle's Medium (Mediatech Cat. #10-013-CV)
    - Heat-Inactivated Fetal Bovine Serum (Sigma Cat. #F2442)
- Pipettes
- PBS and Trypsin
- Cell counter
- T25 (VWR# 29185-300), T75 (VWR # BD353136), or T175 flasks (VWR #29185-308)
- Reduced serum medium Opti-MEM (Thermo Fisher Cat. #31985070)
- Packaging Plasmids: available from gpp-reagents@broadinstitute.org or order from Addgene (VSV-G plasmid #12259, psPAX2 plasmid #12260) then amplify with GenScript
- Transfer vector plasmid DNA
- LT-1 transfection reagent (Mirus Cat. #MIR 2305)
- Sterile microcentrifuge tubes and 5 and/or 15 mL conicals
- Viral harvest media: DMEM + 10% FBS + 1% BSA + 1% Pen/Strep
    - Dulbecco's Modification of Eagle's Medium (Mediatech Cat. #10-013-CV)
    - Fetal Bovine Serum (Sigma Cat. #F4135)
    - Microbiology-grade Bovine Serum Albumin (VWR Cat. #14230-738)
    - Penicillin Strepomycin (VWR Cat. #45000-652)
- 50 or 250 mL conical(s)
- 0.45 um PVDF vacuum filter (such as Millipore #S2HVU05RE)
- Optional: 500 mL or 1 L sterile bottles (VWR #82051-492, #82051-594)

Part 1: 293T cell maintenance/expansion (1 – 2 weeks before transfection)

A: Maintain a stock of 293T cells:

- Doubling time is ~21 hours
- Visually inspect the cell confluency under the microscope at every passage to ensure it always remains below 80%. If ever over 80-90%, thaw a new vial.

B: Passage cells on Mondays, Wednesdays and Fridays:

- Seed cells according to the table below in 293T maintenance media:

Day of week:	**T25**	**T75**	**T175**
Monday, Wednesday	7.1x10e5 in 5 mL	2.1x10e6 in 20 mL	5x10e6 in 40 mL
Friday	3.5x10e5 in 5 mL	1.05x10e6 in 20 mL	2.5x10e6 in 40 mL

C: Expand 293Ts the Friday before transfection:

1. Calculate the required number of flasks for the following week's transfection based on the volume of lentivirus needed:

Flask Size:	**Virus Harvest Volume:**
T25	10 mL
T75, high-titer vectors (i.e. pXPR_003, pLKO, pLX304)	30 mL
T75, low-titer vectors (i.e. pXPR_023, Cas9, pLX317)	20 mL
T175, high-titer vectors (i.e. pXPR_003, pLKO, pLX304)	60 mL
T175, low-titer vectors (i.e. pXPR_023, Cas9, pLX317)	40 mL

2. Seed half the required number of flasks needed for the following week's transfection (i.e. 10x T175s for a 20x T175-transfection) with the number of cells indicated in Part 1, Step B for a Friday.

Part 2: Seeding Cells for Transfection

D: Seed 293Ts (Monday 1 PM):

1. Work in small batches, 3-4 flasks at a time, so that the cells do not get over-trypsinized.
2. Aspirate media, then gently wash with PBS and aspirate. If the cells are starting to lift off without trypsinization, the PBS wash will appear cloudy.

The cells could be unhealthy and should not be used for virus production.

3. Add just enough trypsin to cover the surface of the flask and rock back and forth. Allow the cells to fully trypsinize in the hood (no need to return back to the incubator), typically less than 1 min.
4. Quench the trypsin with 293T maintenance media and collect the cell suspension into a conical.
5. Wash the flask with 293T maintenance media and collect the wash into same conical.
6. Thoroughly mix the cell suspension and count.
7. Seed the cells according to the table below in 293T maintenance media (seeding an additional flask or two as a backup is often beneficial). Incubate overnight at 37^{o}C, 5% CO_2.

Flask size:	**# of 293Ts**	**Volume of media**
T25	2.5x10e6	3.5 mL
T75	7.5x10e6	10.5 mL
T175	1.8x10e7	25 mL

Part 3: Transfection

<u>E: Prepare transfection reagents (Monday PM or Tuesday AM):</u>

1. Calculate the amounts of plasmids needed for the transfection based on the table below:

	Reagent	**Amount needed per flask**	**Amount needed total**
T25 flask	Transfer vector pDNA	1.4 ug	x (# of required flasks)
	psPAX2	6.7 ug	
	VSV-G	0.67 ug	
	Opti-MEM	6.7 uL	
T75 flask	Transfer vector pDNA	4.3 ug	x (# of required flasks)
	psPAX2	20 ug	
	VSV-G	2 ug	
	Opti-MEM	20 uL	
T175 flask	Transfer vector pDNA	10 ug	x (# of required flasks)
	psPAX2	50 ug	
	VSV-G	5 ug	
	Opti-MEM	50 uL	

2. Make a master mix of all four transfection reagents.

3. Divide the master mix total volume by the number of required transfection flasks to calculate the master mix volume per flask. Aliquot this volume into microcentrifuge tube(s), one for each flask.
4. Aliquot the required volume of Opti-MEM into appropriately sized conical(s), one for each flask:
    - T25 – 835 uL, 5 mL conical
    - T75 – 2.5 mL, 15 mL conical
    - T175 – 6 mL, 15 mL conical
5. If preparing the transfection mix tube(s) and Opti-MEM conical(s) the day before, store at 4^{o}C overnight.
6. Before beginning the transfection:
    - Warm Opti-MEM conical(s) in a heat bath until lukewarm.
    - Allow for the LT-1 to come to room temperature.
    - Check a flask of 293Ts for their confluency; they should be around 60-70%.

F: <u>Perform transfection(s) (Tuesday 9 AM, or 20 hr post-293T seeding):</u>

1. Pipette the LT-1 into the Opti-MEM conical (see volumes below). Carefully invert and rotate the conical 5 – 7 times, avoiding bubbles, and let the mixture sit for 3 minutes.
    - T25 – 42 uL
    - T75 – 125 uL
    - T175 – 305 uL
2. Pipette the transfection mix from the microcentrifuge tube into the LT-1+Opti-MEM conical. Carefully invert and rotate the conical 5 – 7 times, avoiding bubbles, and let the mixture sit at room temperature for 27 minutes.
3. Subsequent transfection conicals can be processed in 3-minute intervals to optimize time (see the Virus Production Flow Chart at the end).
4. Change the pipette-aid to slow release if possible.
5. After the 27 min, carefully remove a 293T flask from the incubator.
6. Slowly drip the plasmid+LT-1+Opti-MEM mixture in a zig-zag or circular motion over the surface of the cells. Make sure to cover as much surface area as possible.
7. Keeping the flask parallel to the floor, carefully place it back into the incubator.
8. Note the time of transfection completion in order to change the media 6 hours later.

G: <u>Change media (Tuesday, 6 hr post-transfection):</u>

1. Carefully remove the flask(s) from the incubator and tilt upright.
2. Gently aspirate the media, aiming the aspirating pipette away from the cells.
3. Add viral harvest media by pipetting down the top side of the flask (not onto the cells) with slow dispense speed:

- T25 – 10 mL
- T75, high-titer vectors – 30 mL
- T75, low-titer vectors – 20 mL
- T175, high-titer vectors – 60 mL
- T175, low-titer vectors – 40 mL

4. Gently move the flask(s) back into the incubator for an additional 42 hours.

Part 4: Harvesting and Aliquoting Virus

H: Harvest the virus(es) (Thursday 9 AM, or 42 hr post-media change):

1. Choose appropriately sized aliquots to avoid freeze/thawing the virus and label the tubes.
2. Carefully pipette the viral supernatant from the flask(s) into a conical or split across multiple conicals if necessary.
3. Centrifuge at 230 g for 1 min to pellet any cell debris.
4. Avoiding the cell debris pellet, pipette off the viral supernatant into a 0.45 um PVDF vacuum filter or split across multiple filters if necessary.
5. Prior to aliquoting, the filtered viral supernatant should be homogenized. If necessary, combine the virus from the multiple filters into one vessel, such as a 500 mL or 1 L bottle. If multiple 1 L bottles are needed, very carefully mix the virus by pouring from one bottle to the next several times.
6. Aliquot the virus into the pre-labeled tubes and freeze at -80°C.

Virus Production Flow Chart

Number the Opti-MEM conicals and transfection mix tubes
Add LT-1 to Opti-MEM conical #1, mix gently by inverting 5-7 times
Wait 3 min

Add LT-1 to Opti-MEM conical #2
Immediately after, add transfection mix #1 to LT-1+Opti-MEM conical #1, mix gently by inverting 5-7 times
Wait 3 min

Repeat until all plasmid+LT-1+Opti-MEM mixtures have been made
Wait 27 min from the addition of the first transfection mix

Dropwise add the plasmid+LT-1+Opti-MEM from conical #1 over surface of the cells
Wait 3 min

Continue adding plasmid+LT-1+Opti-MEM mixture to all flasks in numerical order
Wait 6 hours

Change media on cells to viral harvest media
Wait 42 hours.

Harvest and aliquot