Improve lentiviral production using Lipofectamine 3000 reagent

Invitrogen™ Lipofectamine™ 3000 Transfection Reagent is a highly efficient, cost-effective tool for lentiviral production (Figure 1). This versatile reagent enables high viral titers even with genes that are large or difficult to package. Here, we demonstrate the effectiveness of Lipofectamine 3000 reagent for the production of high-quality, high-titer lentivirus while minimizing time and reagent usage. A workflow is provided in Figure 2, followed by a more detailed protocol.

Advantages of lentiviral production using Lipofectamine 3000 reagent:

- **Superior performance** — higher unconcentrated viral titers than leading reagents (Figure 3).
- **Larger gene sizes** — high viral titers even with genes that are large or difficult to package.
- **Simpler** — no additional reagents required for boosting lentiviral production (e.g., chloroquine phosphate, sodium butyrate, caffeine).
- **Faster** — time to virus collection is 24 hours less than other reagents, and reverse transfection time is almost 2 days less than other reagents with comparable viral titers.
- **Easier** — eliminates the need for coating of plates or flasks.

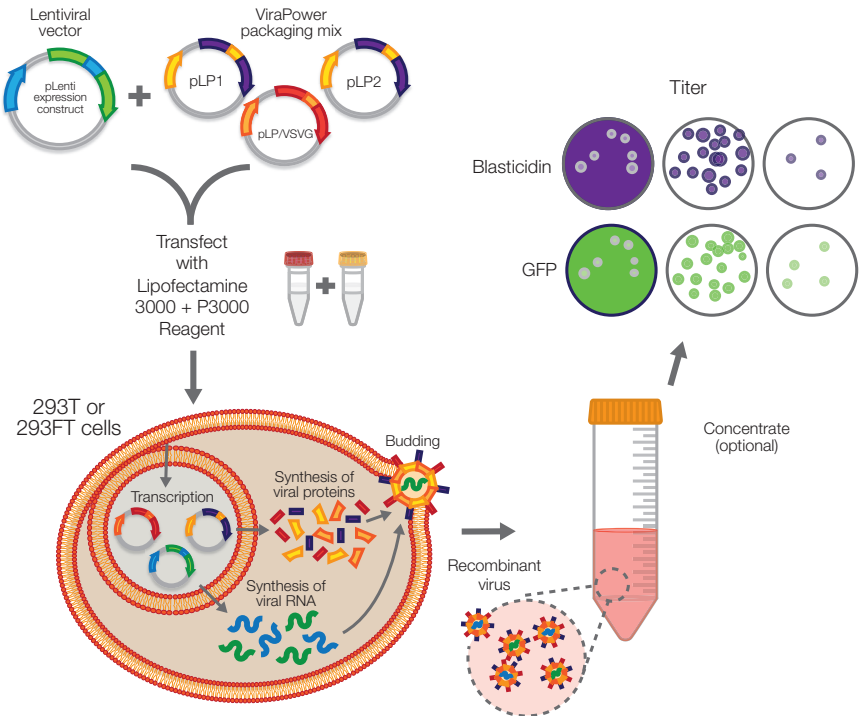

Figure 1. Lentiviral production using Lipofectamine 3000 reagent. A lentiviral construct containing the gene of interest along with lentiviral packaging mix is cotransfected into 293T or 293FT cells using Lipofectamine 3000 reagent. Following incubation of cells, supernatant containing lentivirus is harvested and cellular debris is removed by centrifugation. Depending on the lentiviral construct, viral titer is determined either by blasticidin selection or analyzing the percentage of GFP-positive cells.

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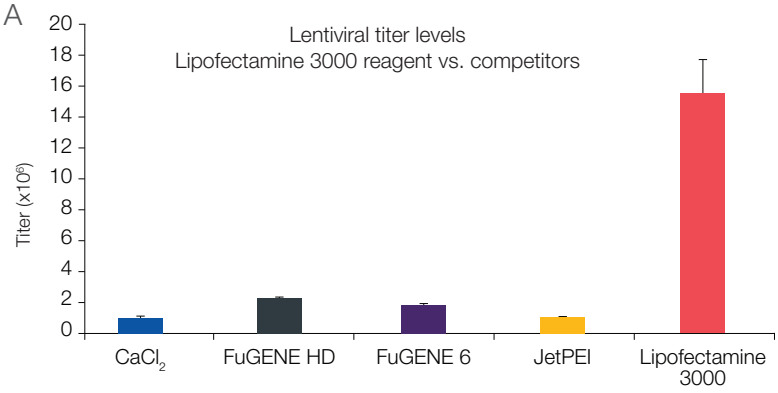
Figure 2. Lentiviral production workflow using Lipofectamine 3000 reagent.

Timeline			Steps	Procedure details (scaling optimization)			
				Component	6-well plate	10cm plate	T175 flask
Day 0	1		Seed 293T or 293FT cells to be 95-99% confluent at transfection	Volume of packaging medium	2mL	12mL	37mL
				293T or 293FT cells/well	1.2 x 10^6	7.0 x 10^6	22.1 x 10^6
Day 1	2	Vortex 2-3 sec	Prepare Tube A: Dilute Lipofectamine 3000 reagent in Opti-MEM™ I medium — mix well	Opti-MEM I for complexing	250µL	1.5mL	4.6mL
				Lipofectamine 3000 Reagent	7µL	41µL	129µL
	3		Prepare Tube B: Dilute ViraPower™ Lentiviral Packaging Mix and pLenti expression vector in Opti-MEM I medium, then add P3000 Enhancer Reagent and mix well	Opti-MEM I for complexing	250 µL	1.5mL	4.6mL
				ViraPower packaging mix	2.25µg	13.0µg	41.4µg
				pLenti expression vector	0.75µg	4.3µg	13.8µg
				P3000 reagent	6µL	35µL	111µL
	4		Add Tube A to Tube B (1:1 ratio) and mix well	Tube A volume	250µL	1.5mL	4.6mL
				Tube B volume	250µL	1.5mL	4.6mL
	5	10-20 min	Incubate	Incubate for 10–20 minutes at room temperature			
	6		Remove 50% volume of media from well	Volume of medium removed	1mL	6mL	18.5mL
	7		Add DNA-lipid complexes to cells	Complex volume (per well)	500µL	3mL	9.2mL
	8	6 hours	Incubate for 6 hours	Incubate for 6 hours at 37°C and 5% CO_2			
	9		Remove and replace packaging medium, then incubate overnight	Volume of packaging medium	2mL	12mL	37mL
Day 2	10		1st collection of viral supernatant	24 hours posttransfection, harvest entire volume of cell supernatant; store at 4°C			
	11		Replace with pre-warmed packaging medium	Volume of replacement packaging medium	2mL	12mL	37mL
	12	Overnight	Incubate	Incubate overnight at 37°C and 5% CO_2			
Day 3	13		Collect 2nd collection of viral supernatant and combine with 1st collection	52 hours posttransfection, harvest entire volume of cell supernatant			
				Total collected volume	4mL	24mL	74mL
	14	Centrifuge	Centrifuge viral supernatant at 2,000 rpm				
	15		Filter supernatant through a 45µm pore size filter				
	16		Titer, aliquot and store virus at -80°C				

Complete culturing medium: 500mL Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, pyruvate with 10% Gibco™ FBS.

Lentivirus packaging medium: 500mL Gibco™ Opti-MEM™ I Reduced Serum Medium, GlutaMAX™ Supplement with 1mM Gibco™ Sodium Pyruvate and 5% Gibco FBS.

A



B

Production cell line	Insert gene size	Comparative fold increase in viral titer			
		Lipofectamine 3000 vs. FuGENE™ HD	Lipofectamine 3000 vs. $CaCl_2$	Lipofectamine 3000 vs. FuGENE™ 6	Lipofectamine 3000 vs. JetPEI™ reagent
293FT	0.7kb	3x	26x	9x	4x
	3kb	8x	16x	9x	17x
293T	0.7kb	4x	10x	5x	1x
	3kb	4x	10x	4x	4x

C

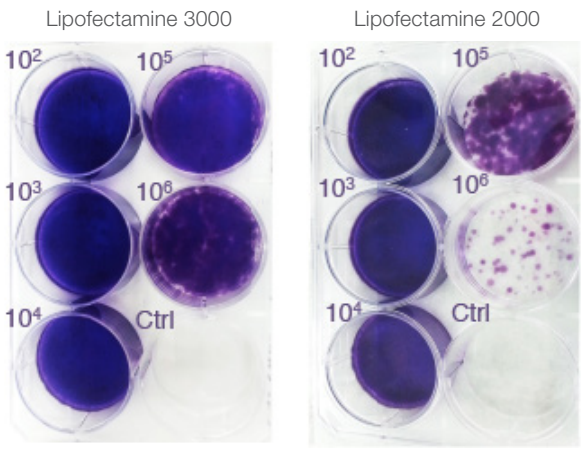


Figure 3. Lipofectamine 3000 reagent performs significantly better than leading competitor transfection reagents for lentiviral production. (A) Titers obtained using Lipofectamine 3000 reagent compared to commonly used reagents in 293FT cells using a 3kb insert gene. **(B)** Summary of fold increases in titer levels using two different cell lines and insert sizes. **(C)** Serial dilution and Blasticidin selection of concentrated virus demonstrates the high titer levels achieved using Lipofectamine 3000 reagent.

Detailed protocol for lentiviral production using Lipofectamine 3000 reagent

Materials

Reagent	Cat. No.
DMEM, high glucose, GlutaMAX Supplement, pyruvate	10569010
Fetal Bovine Serum, certified, US origin	16000044
Geneticin Selective Antibiotic (50mg/mL)	10131035
Opti-MEM I Reduced Serum Medium, GlutaMAX Supplement	51985034
Opti-MEM I Reduced Serum Medium	31985062
Sodium Pyruvate (100mM)	11360070
ViraPower Lentiviral Packaging Mix	K497500
Lipofectamine 3000 Transfection Reagent	L3000015
Vivid Colors pLenti6.3/V5-GW/EmGFP Expression Control Vector	V37006

Note: All virus preparation and work should be in accordance with your institution's guidelines for Biosafety Level 2 (BL-2) protocols.

Media preparation

Complete culturing medium

To prepare complete culturing medium for 293T and 293FT cells, aseptically mix the following components:

- 450mL DMEM, high glucose, GlutaMAX Supplement, pyruvate.
- 50mL Fetal Bovine Serum, certified, US origin.
- If using 293FT cells, additionally include 500µg/mL Geneticin™ Selective Antibiotic.

Lentivirus packaging medium

To prepare lentivirus packaging medium, aseptically mix the following components:

- 474mL of Opti-MEM I Reduced Serum Medium, GlutaMAX Supplement.
- 25mL Fetal Bovine Serum, certified, US origin.
- 1mL Sodium Pyruvate (100mM).

Forward transfection (6-well plate)

Instructions below are tailored to the 6-well plate format. For scaling up transfections, see Table 1.

Day 1 (afternoon): seeding of cells

1. Seed 293T or 293FT cells onto 6-well culture plates at 70-80% confluence. For a 6-well culture plate, plate cells at ~1.2 x 10^6 cells per well in 2mL of lentivirus packaging medium.
2. Incubate cells overnight at 37°C, 5% CO_2.

Day 2 (morning): transfection

At time of transfection, cell density should be 95-99% confluent.

1. Bring Opti-MEM I Reduced Serum Medium to room temperature and prepare Tube A and Tube B as described in the following table:

Component	Volume
Tube A	
Opti-MEM I Reduced Serum Medium	250µL
Lipofectamine 3000 Transfection Reagent	7µL
Tube B	
Opti-MEM I Reduced Serum Medium	250µL
P3000 Enhancer Reagent	6µL
ViraPower Lentiviral Packaging Mix (1µg/µL)	2.25µL
pLenti expression vector	0.75µg

Note: Lipofectamine 3000 reagent diluted in Opti-MEM medium should be used within 15 minutes of dilution. Longer times can result in a loss of transfection efficiency.

2. To prepare lipid-DNA complexes, transfer contents of Tube A to Tube B and mix well.
3. Incubate the complexes for 10-20 minutes at room temperature.
4. Prior to adding complex, remove 1mL of medium from each well, leaving a total of 1mL in each well.
5. Add 500µL of lipid-DNA complex to each well, taking care to dispense liquid against the well wall to avoid disrupting cells. Gently agitate plate to evenly distribute.
6. Incubate plate for 6 hours at 37°C, 5% CO_2.
7. At 6 hours posttransfection, change plate medium from each well. Carefully remove medium that contains lipid-DNA complexes from each well and treat with 10% bleach prior to disposal. Replace with 2mL of pre-warmed lentivirus packaging medium.

Note: Because removed medium contains a small amount of lentivirus, it should be appropriately treated with bleach and discarded as per institutional guidelines.

8. Return plate to incubator, and incubate overnight at 37°C, 5% CO_2.

Day 3 (morning): harvest of first batch of virus

1. At 24 hours posttransfection, collect the 2mL cell supernatant from each well and store in 15mL conical tubes at 4°C.
2. Replace the collected medium with 2mL of pre-warmed lentivirus packaging medium.
3. Incubate the plate overnight at 37°C, 5% CO_2.

Day 4 (mid-afternoon): harvest of second batch of virus

1. Approximately 52 hours posttransfection, collect 2mL of cell supernatant from each well and combine with the first collection for a total of 4mL of collected supernatant.

Note: Prior to disposal, treat all cell culture vessels and tips with 10% bleach.

2. Centrifuge the 4mL of collected supernatant at 2,000 rpm for 10 minutes at room temperature to remove cellular debris. Collect and transfer supernatant, discarding cell pellet.
3. Filter the clarified lentiviral supernatant using a 45µm pore filter to remove any remaining cellular debris.
4. Aliquot virus into cryovials and store at -80°C. Virus can also be concentrated prior to freezing. Limit freezing and thawing of virus aliquots to maintain titer.

Table 1. Scale-up of viral production using forward transfection.

Cell culture vessel	Multiplication factor*	Volume of packaging medium for seeding (mL)	293T or 293FT cell numbers per well†	DNA cotransfection					Volume of lentivirus packaging medium for replacement (mL)	Total volume of harvested lentivirus (mL)
				Volume of Opti-MEM I medium for complexing	Lentivirus packaging (DNA)		P3000 reagent (µL)	Lipofectamine 3000 reagent (µL)		
					ViraPower packaging mix (µg)	pLenti-based vector (µg)				
6-well	1	2	1.2×10^6	2 x 250µL	2.25	0.75	6	7	2	2 x 2
60mm	2.2	4	2.6×10^6	2 x 500µL	5.0	1.7	13	15	4	2 x 4
10cm	5.8	12	7.0×10^6	2 x 1.5mL	13.0	4.3	35	41	12	2 x 12
T75	7.9	16	9.5×10^6	2 x 2mL	17.8	5.9	47	55	16	2 x 16
T175	18.4	37	22.1×10^6	2 x 4.6mL	41.4	13.8	111	129	37	2 x 37

*Multiplication factor is based on 6-well plate growth area.
†Confluency required the day before transfection is ~75-80% and ~95-99% the day of transfection.

Reverse transfection (6-well plate)

Day 1 (morning): complex formation and seeding of cells

1. Prepare cell suspension in pre-warmed lentivirus packaging medium at the appropriate cell concentration (see Table 2).
2. Bring Opti-MEM I Reduced Serum Medium to room temperature and prepare Tube A and Tube B as described in the following table:

Component	Volume
Tube A	
Opti-MEM I Reduced Serum Medium	250µL
Lipofectamine 3000 Transfection Reagent	7µL
Tube B	
Opti-MEM I Reduced Serum Medium	250µL
P3000 Enhancer Reagent	6µL
ViraPower Lentiviral Packaging Mix (1µg/µL)	2.25µL
pLenti expression vector	0.75µg

3. Add contents of Tube A to Tube B, and incubate for 10-20 minutes at room temperature.
4. Add 500µL of lipid-DNA complex directly to the plate well.
5. Seed 293T or 293FT cells onto 6-well culture plates at ~3.6×10^6 cells per well in 1mL of lentivirus packaging medium.

Note: For reverse transfection, seed cells at 3x higher density than for forward transfection.

6. Incubate plate for 6 hours at 37°C, 5% CO_2.

Day 1 (afternoon): media change

7. At 6 hours posttransfection, change plate medium from each well. Carefully remove complex-containing medium from each well and treat with 10% bleach prior to disposal. Replace with 2mL of pre-warmed packaging medium.
8. Return plate to incubator, and incubate overnight at 37°C, 5% CO_2.

Day 2 (morning): harvest of first batch of virus

1. At 24 hours posttransfection, collect the 2mL cell supernatant from each well and store in 15mL conical tubes at 4°C.
2. Replace the collected medium with 2mL of pre-warmed packaging medium.
3. Incubate plate overnight at 37°C, 5% CO_2.

Day 3 (mid-afternoon): harvest of second batch of virus

1. Approximately 52 hours posttransfection, collect 2mL of cell supernatant from each well and combine with first collection for a total of 4 mL of collected supernatant.
2. Centrifuge the 4mL of collected supernatant at 2,000rpm for 10 minutes at room temperature to remove cellular debris. Collect and transfer supernatant, discarding cell pellet.
3. Filter the clarified lentiviral supernatant using a 45µm pore filter to remove any remaining cellular debris.
4. Aliquot virus into cryovials and store at -80°C. Virus can also be concentrated prior to freezing. Limit freezing and thawing of virus aliquots to maintain titer. Titers achieved using forward and reverse transfection are compared in Figure 3.

Table 2. Scale-up of viral production using reverse transfection.

Cell culture vessel	Multiplication factor*	Volume of packaging medium for seeding (mL)	293T or 293FT cell numbers per well	DNA cotransfection					Volume of lentivirus packaging medium for replacement (mL)	Total volume of harvested lentivirus (mL)
				Volume of Opti-MEM I medium for complexing	Lentivirus packaging (DNA)		P3000 reagent (µL)	Lipofectamine 3000 reagent (µL)		
					ViraPower packaging mix (µg)	pLenti-based vector (µg)				
6-well	1	1	3.6×10^6	2 x 250µL	2.25	0.75	6	7	2	2 x 2
60mm	2.2	2	7.92×10^6	2 x 500µL	5.0	1.7	13	15	4	2 x 4
10cm	5.8	6	21×10^6	2 x 1.5mL	13.0	4.3	35	41	12	2 x 12
T75	7.9	8	28.4×10^6	2 x 2mL	17.8	5.9	47	55	16	2 x 16
T175	18.4	18	66.2×10^6	2 x 4.6mL	41.4	13.8	111	129	37	2 x 37

*Multiplication factor is based on 6-well plate growth area.

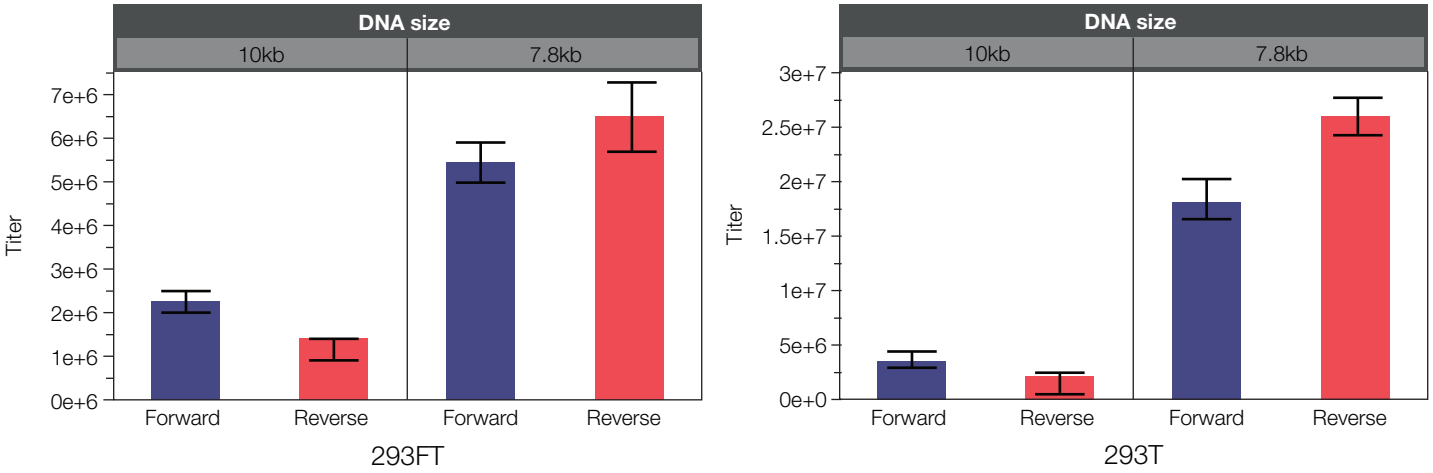


Figure 3. Comparative titers achieved using forward vs. reverse transfection. Titers were compared in 293FT and 293T cells using forward and reverse transfection protocols and 2 different gene sizes. Titer levels were comparable between the two methods.

Measurement of lentiviral titer by GFP selection or Blasticidin selection

Important tips:
Lentivirus must be thawed and remain on ice throughout use. Following thaw, mix virus by gently tapping tube or inverting slowly — do not vortex.

Handling of lentivirus must be performed as per institutional guidelines. All materials should be treated with a 10% bleach solution prior to disposal.

Titering by GFP selection

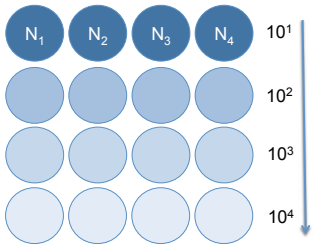
Materials

- Lentivirus.
- HT1080 cell line.
- Culture medium:
  Gibco DMEM, high glucose, GlutaMAX Supplement, pyruvate with 10% FBS.
- Polybrene™ reagent at 10mg/mL (Fisher Scientific™ Cat. No. NCO663391).
- Corning™ 96-well round-bottom plate for dilutions (Fisher Scientific™ Cat. No. 05539200).

Day 1 (morning)

This protocol is tailored for a 96-well plate format for high-throughput flow cytometery analysis.

1. Seed a 96-well plate with HT1080 cells at a density of 7,000 cells/well in 100µL of culture medium.
2. Store in an incubator for 4-5 hours until transduction.
3. Prior to transduction, freshly prepare virus dilutions as follows:
   a. Combine 15mL of fresh culture medium and 12µL of 10mg/mL Polybrene reagent (final concentration of 8mg/mL). Vortex well to combine.



   b. Per viral sample, add 135µL of medium prepared in the previous step to 16 wells of a 96-well round-bottom plate in a 4-well by 4-well pattern (see illustration above).

      Note: n = 4 is recommended for titering.

   c. Add 15µL of lentiviral supernatant to each well in row 1 (total volume 150µL; 1:10 dilution).

   d. Mix well using a pipette.

   e. Perform a serial dilution of row 1 (use a multichannel pipette, if available):

      – Transfer 15µL from row 1 to row 2, mix well (1:100)
      – Transfer 15µL from row 2 to row 3, mix well (1:1,000)
      – Transfer 15µL from row 3 to row 4, mix well (1:10,000)

4. To transduce cells, remove plating medium from HT1080 cells and transfer 100µL of the prepared dilutions to each corresponding well (use a multichannel pipette, if available).
5. Centrifuge the plate at 2,000 rpm for 30 minutes at room temperature.
6. Incubate the cell plate overnight.

Day 2

1. Remove medium containing viral supernatant, and replace with fresh HT1080 culture medium (without Polybrene reagent).
2. Incubate cells for an additional 3 days. Analyze the percentage of GFP-positive cells (flow cytometry analysis is recommended).

Calculation of titer

1. To calculate the titer in transducing units (TU) per mL, determine the appropriate dilution factor to use based on the percentage of GFP-positive cells. The desired transduction range is 1-20%.
2. Use the following formula:
   Titer = (F × C/V) × D, where
   F = frequency of GFP-positive cells (percent GFP-positive cells/100)
   C = cell number per well at the time of transduction (7,000 cells)
   V = volume of inoculum in mL (0.1mL)
   D = lentivirus dilution factor

Example:

(Based on 96-well protocol)

Lentivirus dilution	GFP-positive cells
10^2	82%
10^3	30%
10^4	4.4%

The lentivirus dilution of 10^4 is chosen for the calculation because the GFP-positive cell value falls within the desired 1-20% range:

F = 4.4/100
C = 7,000 (cell number at time of transduction)
V = 0.1 (100µL of medium)
D = 10^4

Calculation is as follows:

Titer = (0.044 x 7,000/0.1) × 10^4 =
3.08×10^7 TU/mL

Titering by Blasticidin selection

Materials:

- Lentivirus.
- HT1080 cell line.
- Culture medium:
  Gibco DMEM, high glucose, GlutaMAX Supplement, pyruvate with 10% FBS.

- Polybrene reagent at 10mg/mL (Fisher Scientific™ Cat. No. NCO663391).
- Crystal violet (Fisher Scientific™ Cat. No. ICN15251150).
- Selection medium: Culture medium with Gibco™ Blasticidin S HCl (Cat. No. A1113903, 10µg/mL final concentration).

Day 1 (morning)

This protocol is tailored for a 24-well plate format.

1. Seed 24-well plate with HT1080 cells at a density of 42,000 cells per well (30-50% confluent) in 500µL of culture medium.
2. Store in incubator for 4-5 hours until transduction.

Note: Duplicate titering is recommended.

3. Prior to transduction, freshly prepare virus dilutions as follows:
   a. Combine 15mL of fresh culture medium with 12µL of 10mg/mL Polybrene reagent (final concentration of 8mg/mL). Vortex well to combine.
   b. Prepare 5 aliquots of 135µL of medium from step a, and label tubes as 1-5.
   c. Add 15µL of lentivirus to tube 1 and pipette to mix well.

   Perform a serial dilution:
   – Transfer 15µL from tube 1 to tube 2, mix well (1:100).
   – Transfer 15µL from tube 2 to tube 3, mix well (1:1,000).
   – Transfer 15µL from tube 3 to tube 4, mix well (1:10,000).
   – Transfer 15µL from tube 4 to tube 5, mix well (1:100,000).
4. At 4-5 hours post-seeding, aspirate medium from 24-well plate and replace with 450µL of medium prepared in step a. Number wells 1-5 and a control.
5. Transduce HT1080 cells by transferring 50µL of dilution 1 to well 1 and mix well by pipetting.

Note: This step further dilutes your virus by 10-fold, thus well 1 now contains a 10^2 dilution.

6. Repeat Step 5 for remaining prepared dilutions 2-5, transferring dilutions to corresponding numbered wells.
7. For the control, add 50µL of culture medium with Polybrene reagent (from step a) to control well.
8. Gently swirl the plate to mix.
9. Spin plate at 2,000 rpm at room temperature for 30 minutes.
10. Incubate cell plate overnight.

Day 2

1. Change medium in each well and replace with 500µL of culture medium (without polybrene).
2. Incubate cells for an additional 24 hours.

Blasticidin Selection

1. 48 hours posttransduction, remove medium and replace with selection medium (culture medium with Blasticidin S HCl at 10µg/mL final concentration)
2. Change and replace the selection medium every 2 days for 10 days.

Note: The control well should have no surviving cells prior to staining.

Day 12

1. Remove medium and wash each well with 1mL of PBS.
2. Prepare a working solution of 1% crystal violet in a 10% solution of ethanol in water.
3. Stain wells by adding 250µL diluted crystal violet.
4. Incubate plate for 20 minutes at room temperature.
5. Remove and collect staining solution (staining solution can be reused, or appropriately discarded).
6. Wash plate with water several times to reduce background.

Calculation of titer

1. To calculate the viral titer, count the number of colonies stained with crystal violet per well. Use the following formula to calculate the titer (TU/mL) of the viral stock:
   TU/mL = (No. of discrete colonies x dilution factor)/volume of inoculum

Example:
Count of 35 plaques stained in the 1:100,000 dilution well.
Volume of diluted virus: 0.5mL
Titer = $(35 \times 10^5)/0.5 = 7 \times 10^6$ TU/mL

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