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Lentivirus Production V.1

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We use this protocol and it's working

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Abstract

This protocol is for Lentivirus production. To see the full abstract and additional resources, visit the **Addgene protocol page**.

Sample Data

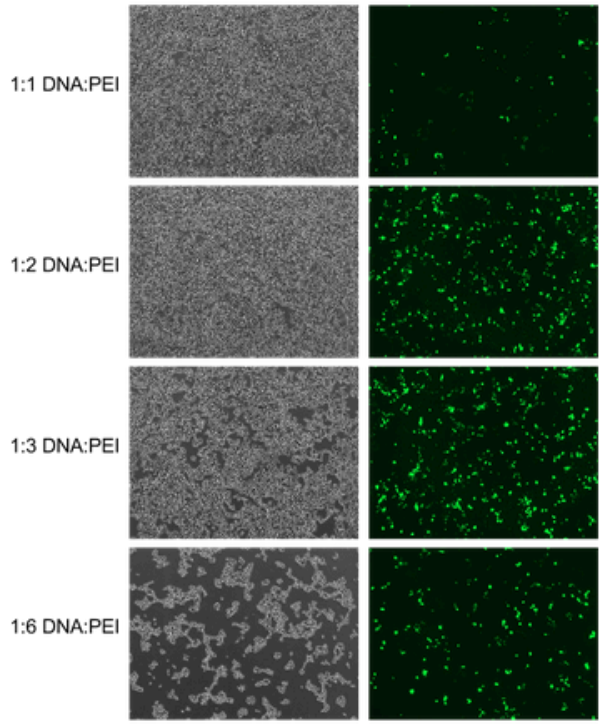

Figure 1: Lenti-X 293T cells were transfected with the GFP-expression plasmid **pRosetta** using µg total DNA to µg PEI ratios of 1:1, 1:2, 1:3 and 1:6. The 1:2 and 1:3 total DNA:PEI µg ratios provided high transfection efficiencies as measured by the highest proportion of GFP positive cells without limiting cell growth. Left panels: bright field images; right panels: GFP channel images.

Guidelines

Workflow Timeline

Day 0: Seed 293T packaging cells

Day 1 (pm): Transfect packaging cells

Day 2 (am): 18 hours post transfection. Remove media, replace with fresh media

Day 3-4 (am): Harvest virus

Materials

Reagents

- DMEM high glucose
- L-alanyl-L-glutamine (or alternative stable glutamine)
- Heat-inactivated FBS
- Low serum medium such as Opti-MEM or Opti-Pro SFM
- Chloroquine diphosphate, 25 µM
- PEI, 1 mg/mL
- Microcentrifuge tubes
- 10 cm tissue culture dishes
- Pipettes
- Pipette tips
- Hydrochloric acid
- Sodium hydroxide
- 0.22 µm polyethersulfone (PES) filter
- 0.45 µm PES filter
- Syringes for filtering

Equipment

- Biosafety cabinet
- Pipetman
- Pipettors
- Incubator
- pH meter
- Stir plate
- Magenetic Stir Bar

Reagent Preparation

1. DMEM Complete: 10% v/v FBS and 4 mM L-alanyl-L-glutamine

- To a 500 mL bottle of DMEM high glucose, add 55 mL of heat inactivated FBS and 11 mL of 200 mM L-alanyl-L-glutamine. Store at 4 °C.

Note

Pro-Tips
- Different brands and lots of FBS can promote or inhibit transfection.
- Test a variety of brands and lots of FBS to find one suitable with your protocols. FBS can be purchased already head inactivated or it can be inactivated in the lab by heating to 56 °C for 00:30:00

2. 25 mM chloroquine diphosphate
- Dissolve 0.129 g of chloroquine diphosphate salt into 10 mL of sterile water.
- Filter sterilize through a 0.22 um filter.
- Aliquot 50 µL - 100 µL and store at -20 °C .
- Aliquots can be thawed and stored at 4 °C prior to use. Thawed aliquots should be discarded after 1-2 months.

3. 1 mg/mL polyethylenimine, linear MW 25,000 Da (PEI)
- Dissolve 100 mg of powder into 100 mL of deionized water.
- While stirring, slowly add hydrochloric acid until the solution clears.
- Check the pH of the solution
- Use hydrochloric acid or sodium hydroxide to adjust the pH to 7.0. Typically the solution will be basic and will need adjustment with hydrochloric acid first.

Note

Pro-Tip
The pH of this solution will drift pretty rapidly upon addition of acid or base. Add only a few drops at a time, allow them to mix and recheck the pH to prevent over or undershooting the desired pH.

- Allow the solution to mix for 00:10:00 and then recheck the pH to ensure that it has not drifted.
- Filter the solution through a 0.22 um membrane.
- Aliquot 500 µL - 1000 µL into sterile tubes.
- Store the tubes at -80 °C .
- After thawing the solution can be stored at 4 °C for up to 2 months. After 2 months, discard the tube and thaw a new working stock.
- The optimal mass DNA:mass PEI ratio will need to be empirically determined for each new batch of 1 mg/mL PEI and for each cell line.

Troubleshooting

Safety warnings

See SDS (Safety Data Sheet) for safety warnings and hazards.

Before start

Considerations Before You Start

- The health of the packaging cell line is critical for obtaining high viral titer.
- 293T cells should be split 3 times a week:
  - Monday: Plate 1×10^6 cells in a T75 flask in 15 mL DMEM complete.
  - Wednesday: Plate 1×10^6 cells in a T75 flask in 15 mL DMEM complete.
  - Friday: Plate 8×10^5 cells in a T75 flask in 15 mL DMEM complete.
- Do not add pen-strep to the media.
- Use cells that are below passage 15 for viral production.

Seeding cells

1 Seed 293T packaging cells at 3.8×10^6 cells per plate in DMEM complete in 10 cm tissue culture plates.

2 Incubate the cells at 37 °C, 5% CO_2 for ~ 20:00:00.

Transfection

3 Gently aspirate media, add 10 mL fresh DMEM complete containing 25 micromolar (µM) cloroquine diphosphate and incubate ~ 05:00:00.

> Note
>
> For 10 mL of DMEM complete, add 10 µL of 25 millimolar (mM) chloroquine diphosphate.

4 Prepare a mixture of the 3 transfection plasmids:

	Reagent	Amount per 10 cm dish *
	psPAX2	1.3 pmol
	pMD2.G	0.72 pmol
	Transfer Plasmid*	1.64 pmol
	OptiPro SFM to total volume	500 µL

*Plasmid concentrations and ratios should be optimized for each transfer plasmid.
psPAX2, **pMD2.G**

Note

Pro-Tip
Endotoxins can inhibit transfection, therefore, plasmid DNA purification should include an endotoxin removal step. For high quality plasmid DNA, the plasimd should also be propagated in an endonuclease negative *E. coli* strain such as NEB stable.

5 Dilute the above 500 µL mixture into 500 µL PEI-OptiPro SFM with enough PEI such that the ratio of µg DNA:µg PEI is 1:3 (1000 µL total per 10 cm dish).

Using transfer plasmid **pHAGE TRE dCas9-KRAB** (total ug of plasmid DNA - 27.8 µg), this would be 83.4 µL of 1 mg/mL PEI in 416.6 µL of OptiPro SFM per 10 cm dish.

Note

Pro-Tip
There can be batch to batch variation when making the PEI working stock, therefore the ratio of µg DNA:µg PEI needs to be empirically determined. Once a batch of PEI is prepared, transfect cells with a fluorescent plasmid using a variety of ratios. Check the cells 1-2 days after transfection to determine what ratio gives the highest percentage of GFP positive cells.

5.1

	Ratio of DNA:PEI	Amount of DNA (µg)	Volume of 1 mg/mL PEI (µL)
	1:1	18.9	18.9
	1:2	18.9	37.8
	1:3	18.9	56.7
	1:4	18.9	75.6
	1:5	18.9	94.5
	1:6	18.9	113.4

Refer to this table for a possible range of ratios to test

6 Gently add the diluted PEI to the diluted DNA. Add the diluted PEI dropwise while gently flicking the diluted DNA tube. Incubate the mixture for 15-30 min at room temperature.

6.1 Gently add the diluted PEI to the diluted DNA. Add the diluted PEI dropwise while gently flicking the diluted DNA tube.

6.2 Incubate the mixture 00:15:00 - 00:20:00 at Room temperature .

7 Carefully transfer the transfection mix to the Lenti-X 293T packaging cells.

Note

Add the transfection mix dropwise being careful not to dislodge the cells.

8 Incubate the cells for 18:00:00 , or until the following morning.

9 The following morning, carefully aspirate the media. Replace the media with 15 mL of DMEM complete.

10 Incubate the cells.

Harvest Virus

11 Virus can be harvested at 48, 72, and 96 hours post transfection in individual harvests or a combined harvest where all the individual harvests are pooled.

Note

If pooling harvests, transfer the harvested media to a polypropylene storage tube and store at 4 °C between harvest.

12 Centrifuge the viral supernatant at ~ 500 x g for 00:05:00 to pellet any packaging cells that were collected during harvesting.

13 Filter supernatant through a 0.45 µm PES filter.

14 The viral supernatant can be stored at 4 °C for several hours but should be aliquotted and snap frozen in liquid nitrogen and stored at -80 °C as soon as possible to avoid loss of titer.