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Lentivirus production & concentration V.2

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Basic techniques

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We use this protocol and it's working

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Abstract

Lentivirus production protocol based on official protocol for Lipofectamine 3000 and TransIT-Lenti

Attachments

Lipofectamine3000-Le...

1MB

Materials

Lipofectamine 3000 **Thermo Fisher Scientific Catalog #**L3000015

Protocol materials

GlutaMAX™ Supplement **Thermo Fisher Catalog #**35050038 Step 7

Sodium Pyruvate (100 mM) **Thermo Fisher Catalog #**11360070 Step 7

Fetal Bovine Serum, qualified, One Shot™ format, Brazil **Thermo Fisher Catalog #**A3160801 Step 7

Opti-MEM™ I Reduced Serum Medium **Thermo Fisher Catalog #**31985047 Step 7

Lipofectamine 3000 **Thermo Fisher Scientific Catalog #**L3000015 Materials

LentiFuge **Cellecta Catalog #**LFVC1 Step 17

Before start

Prepare culture media for HEK293T:

DMEM, low glucose, GlutaMAX™ Supplement, pyruvate **Thermo Fisher Catalog #**21885025 supplemented with 10% Fetal Bovine Serum, qualified, One Shot™ format, Brazil **Thermo Fisher Catalog #**A3160801

HEK293T seeding density titration

1d

1 Detach, count & seed HEK293T cells into T75 flasks at: 1h

1. 4 x 10E6 cells
2. 6 x 10E6 cells
3. 8 x 10E6 cells
4. 10 x 10E6 cells

Note

>28 x 10E6 cells required in total; recommend detaching cells from 1x T175 at 70-80% confluence.

2 Incubate plates at 37 °C incubator (5% CO2, humidified) for up to 24:00:00 1d

Observe confluence of cells under a microscope at 18 - 24 hours after plating.

The optimal plating density for transfection yield 80-90% confluence 18 - 24 hours after plating.

Lentivirus production (Lipofectamine 3000)

3 Detach, count & seed HEK293T cells at a density that yields 80-90% confluence 18 - 24 hours after plating 1d

Incubate at 37 °C incubator (5% CO2, humidified) for up to 24:00:00

Note

Lentivirus packaging medium (step 7 below) can be used when plating cells & during transfection the next day, but DMEM+10% FBS works well too

4 When HEK293T cells are at 80-90% confluence, transfect cells with lentiviral transfer and packaging plasmids using lipofectamine 3000

Culture plate	Scaling factor	Growth media (ml)	Lipofectamine mix		DNA mix		
			Opti-MEM (ml)	Lipofectamine 3000 (ul)	Opti-MEM (ml)	DNA (ug)	P3000 (ul)
6-well	1	2	0.25	7	0.25	3	6
100mm	6	10	1.5	42	1.5	18	36
T75	8	15	2.0	56	2.0	24	48
150mm	16	30	4.0	112	4.0	48	96
T175	18	35	4.5	126	4.5	54	108

Table 1.

Note

If transfecting multple vessels, make a master mix of 1.1x the total volume required

Source document: Lipofectamine3000-LentiVirus-AppNo…

4.1 Calculate the amount of each plasmid required

Example using lentiCas9-Blast, psPAX2 and pMD2.G:

	A	B	C	D	E	F	G	H
				Mass of plasmids per vessel (ug)				
	Plasmid	Size (bp)	Equimolar ratio (ug)	6-well	10cm	T75	150mm	T175
	lentiCas9-Blast	12859	2.209	1.313	7.88	10.50	21.00	23.63
	psPAX2	10709	1.839	1.093	6.56	8.75	17.49	19.68
	pMD2.G	5822	1.000	0.594	3.57	4.75	9.51	10.70
	Total			3	18	24	48	54

Note

Optimisation of plasmid ratios may be required for each transfer plasmid; an equimolar ratio is a good starting point. Zhang Lab uses a 4:3:2 transfer : psPAX2 : pMD2.G ratio that approximates an equimolar ratio for lentiCas9-blast, lentiGuide-Puro & lentciCRISPR v2 plasmids. With lentiCas9-blast, 4:2:1, 9:8:1, 7.5:1.5:4 ratios all result in similar titres (+/- 20% of 4:3:2), around 1-2e6 TU/ml.

4.2 Make up DNA mix by diluting transfer and packaging plasmids into Opti-MEM, then adding P3000 reagent (2ul / 1ug DNA) 3s

Mix well by vortexing 00:00:03

4.3 Make up lipofectamine mix by diluting Lipofectamine 3000 (7ul / 3ug DNA) into Opti-MEM (volume specified in Table 1) 3s

Mix well by vortexing 00:00:03

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

5 Combine both lipofectamine and DNA mixes and incubate for 00:20:00 20m

Room temperature to allow DNA-lipid complexes to form

Remove 50% volume of media from HEK293T culture vessels intended for transfection

	A	B	C	D	E
	Volume of media to remove per vessel (ml)				
	6-well	100mm dish	T75	150mm dish	T175
	1	5	7.5	15	17.5

6 Gently add DNA-lipid complexes to cells 6h

	A	B	C	D	E
	Volume of DNA-lipid complex to add per vessel (ml)				
	6-well	10cm dish	T75	150mm dish	T175
	0.5	3	4	8	9

Gently rock culture vessel back-and-forth and from side-to-side to evenly distribute
Incubate at 37 °C incubator (5% CO2, humidified) for 06:00:00

7 Prepare Lentivirus packaging medium (Opti-MEM + 1x GlutaMAX + 1mM sodium pyruvate + 5% FBS)

Supplement 500 mL
Opti-MEM™ I Reduced Serum Medium **Thermo Fisher Catalog #**31985047 with:

- 5 mL GlutaMAX™ Supplement **Thermo Fisher Catalog #**35050038
- 5 mL Sodium Pyruvate (100 mM) **Thermo Fisher Catalog #**11360070
- 25 mL
  Fetal Bovine Serum, qualified, One Shot™ format, Brazil **Thermo Fisher Catalog #**A3160801

8 5-6 hours after transfection, replace media with pre-warmed packaging medium

Incubate plates at 37 °C incubator (5% CO2, humidified)

Note

Consider supplementation with [M] 1 millimolar (mM) sodium butyrate or [M] 2 millimolar (mM) caffeine to improve titer.

Supplements will be present in viral supernatant if not concentrated

9 24 hours post-transfection, collect supernatant from culture vessels and store at 4 °C *
Replace media with pre-warmed packaging medium
Incubate plates at 37 °C incubator (5% CO2, humidified) for up to 28:00:00

10 48-52 hours post-transfection, collect supernatant from culture vessels and combine with supernatant harvested the day before. 5m
Centrifuge at 300 x g, Room temperature, 00:05:00 to separate any detached cells, and collect the supernatant.

11 Filter the supernatant through a 0.45µm PES filter to remove any remaining cellular debris

Note

Smaller filters (0.22µm) improve purity but can lower the viral titer.

12 Prepare single-use aliquots in cryovials and snap freeze virus in a dry ice-ethanol bath, then store at -80°C.

Alternatively, proceed with virus concentration or infection of cells.

Note

It is recommended to avoid subjecting lentiviral preparations to multiple freeze-thaw cycles, since each cycle can result in a 10%–20% loss in functional titers.

Lentivirus production (TransIT-Lenti)

13 Detach, count & seed HEK293T cells at a density that yields 80-90% confluence 18 - 24 hours after plating 1d

Incubate at 37 °C incubator (5% CO2, humidified) for up to 24:00:00

14 Change media for HEK293T cells

15 When HEK293T cells are at 80-90% confluence, transfect cells with lentiviral transfer and packaging plasmids using TransIT-Lenti

Culture vessel	Scaling factor	Growth media (ml)	Opti-MEM (ml)	DNA (ug)	TransIT-Lenti (ul)
6-well	1.0	2	0.2	2	6
10cm	6.0	10	1.2	12	36
T75	7.5	15	1.5	15	45
150mm	15.0	30	3.0	30	90
T175	17.5	35	3.5	35	105

Note

If transfecting multple vessels, make a master mix of 1.1x the total volume required

15.1 Calculate the amount of each plasmid required

Example using lentiCas9-Blast, psPAX2 and pMD2.G:

	A	B	C	D	E	F	G	H
				Mass of plasmids per vessel (ug)				
	Plasmid	Size (bp)	Equimolar ratio (ug)	6-well	10cm	T75	150mm	T175
	lentiCas9-Blast	12859	2.209	0.875	5.25	6.56	13.13	15.31
	psPAX2	10709	1.839	0.729	4.37	5.47	10.93	12.75
	pMD2.G	5822	1.000	0.396	2.38	2.97	5.94	6.93
	Total			2	12	15	30	35

Note

Optimisation of plasmid ratios is required for each transfer plasmid but an equimolar ratio is a good starting point, Zhang Lab uses a 4:3:2 ratio that approximates an equimolar ratio for lentiCas9-blast, lentiGuide-Puro & lentciCRISPR v2 plasmids.

15.2 Warm TransIT-Lenti reagent to room temperature and vortex 00:00:03 3s

15.3 Combine plasmids in quantities determined in step 14.1 10m
Add Opti-MEM (Volume specified in Table 2), mix by pipetting
Add TransIT reagent (3ul / 1ug DNA), mix by pipetting

Incubate at Room temperature 00:10:00 to allow transfection complexes to form

Note

Precipitation may be observed when excess DNA is used during complex formation. This may
negatively impact transfection efficiency.

15.4 Add transfection copmlexes drop-wise to different areas of vessel
Gently agitate culture vessel back-and-forth and from side-to-side to evenly distribute

2d

Incubate at 37 °C incubator (5% CO2, humidified) for 48:00:00

16 Harvest virus 48 hours post-transfection by following steps 10 - 12.

Lentivirus concentration

1h

17

1h

Add 1 µL LentiFuge **Cellecta Catalog #**LFVC1 per
1 mL lentiviral supernatant
Incubate at 4 °C 01:00:00

18 Centrifuge at 12000 rpm, 4°C, 01:00:00

1h

Note

Pellets generated from small supernatant volumes may be difficult to see. It is advised to mark the centrifuge tube or bottle with a marker at the site where you expect the virus pellet to be.

19 Resuspend the pelleted lentiviral preparation in 1/100 of original volume using sterile phosphate buffered saline (PBS).

20 Prepare single-use aliquots and snap freeze virus in dry ice, then store at -80°C.