Protocol for Lentivial Vector (LV) Production (2nd Generation Packaging)

Lenti virus can be packaged by any transfection reagent. In this protocol, we describe two methods: a calcium phosphate precipitation method and an iMFectin Poly DNA method. The iMFectin method requires a small amount of plasmid DNAs and is simple/efficient. Our preferred method is iMFectin.

Preparation of ATCC 293T cells (ATCC CRL-3216):

Complete Medium:

- Dulbecco's Modified Eagle's Medium(DMEM) high glucose (GenDepot, 500 ml, cat # CM001-050), 10% fetal bovine serum (FBS, Sigma) heat inactivated at 56°C for 30 min, 1 x antibiotic/antimycotic (GenDepot, cat # CA002-010).
- Warm up media to 37°C in a water-bath.

Splitting Cells

A. Citric Saline Method

REAGENTS
10 x Citric Saline: 1.35 M KCl (Sigma #P9542, FW 74.55, 100.6 g/L), 0.15M sodium citrate (Sigma #S 4641, FW 294.1, 44.15 g/L), Autoclave and store at 4°C.

1. Aspirate culture medium.

2. Overlay 0.4 ml (10-cm dish) or 1 ml (15-cm dish) of 1 x citric saline (135 mM KCl, 15 mM sodium citrate).

3. Wait for 2-3 min and hit the dish to detach cells completely.

B. Trypsin Method

1. Aspirate culture medium.

2. Rinse with 10 ml (15-cm dish) or 5 ml (10-cm dish) PBS and aspirate PBS.

3. Overlay 1 ml (15-cm dish), or 0.4 ml (10-cm dish) 0.25% trypsin warmed up in 37°C water bath, and rock the dish to evenly distribute trypsin.

4. Incubate in CO_2 incubator for 1 min, or until cells are rounded and start to float.

5. Hit the dish to detach cells completely.

6. Add 11 ml complete medium for 15-cm dish for 1:6 split, or 5.6 ml for 10-cm dish.

Monday

Split confluent ATCC-293T cells 1:5, or 293T (originating from the Salk Institute) 1:6 into 15-cm, or 10-cm dish.

Note: ATCC-293T cells grow slowly compared with 293T cells originating from the Salk Institute; however, there is no significant difference in vector production.

Tuesday

A. iMFectin Transfection Method

REAGENT
iMFectin Poly DNA Transfection Reagent (GenDepot cat# I7200, available through Thermo Fisher Scientific)
Packaging plasmids
psPAX2 (Addgene #12260)
pMD2.G (Addgene #12259)

- Cells should be ~80% confluent (up to 90% is OK).
- Aspirate media and feed with fresh 9 ml of DMEM/5% FBS for 15-cm dish (4.5 ml of media for 10-cm dish) 30-60 min before transfection.

Preparation of transfection cocktail without iMFectin

1. Add DNA (total 10 µg of DNA/15-cm dish, or 5 µg of DNA/10-cm dish) to a plain DMEM high glucose media and mix by vortexing for 10 sec. Spin down.

ID			
15-cm dish	1 x 15-cm dish	10-cm dish	1 x 10-cm dish
DMEM	µl		µl
Transfer vector (5 µg)	µl	Transfer vector (2.5 µg)	µl
psPAX2 (3.5 µg)	µl	psPAX2 (1.75 µg)	µl
pMD2.G (1.5 µg)	µl	pMD2.G (0.75 µg)	µl
Total DNA	10 µg		5 µg
Total volume	500 µl		250 µl

2. In another tube, dilute 30 µl of iMFection with 470 µl of plain DMEM for 15-cm dish (15 µl/10-cm dish with 235 µl of plain DMEM for 10-cm dish). Vortex for 10 seconds and spin down.

3. Add diluted iMFection reagent to the DNA solution (not in the reverse order), vortex for 10 sec and spin down.

4. Incubate for 15 min at room temperature, no longer than 30 min.

5. After 4 hours, add 22 ml/15-cm dish of fresh medium (DMEM/5% FBS), or 5 ml for 10-cm dish.

Day 3 (Wednesday)

Day 4 (Thursday)

Note: Do not incubate for 3 days as it is difficult to filter the media using a syringe filter. Check fluorescence if the vector contains a fluorescence reporter (transfection efficiency should be higher than 90%). If you want to collect media 3 days after transfection, collect the media 2 days after transfection, feed with fresh media and then collect the second media next day.

1. Collect medium and centrifuge at 3,000 rpm for 10 min at room temperature.

2. Filter the clear supernatant through 0.45 μm filter (Millipore Filter #SCHV U01 RE) into a new 50-ml or 15-ml corning tube.

Concentrating vectors by ultracentrifugation

15-cm dish Protocol

REAGENT
20% Sucrose/DMEM
Dissolve 20 g of UltraPure sucrose, 100 mM NaCl in plain DMEM and sterilize by a 0.22 μm filter. This solution can be stored at 4°C for at least 6 months.

Sterilize centrifuge tubes (Beckman Ultraclear 1 x 31/2 in, 14 x 95mm, 38.5 mL capacity, #344058) with 70% ethanol.

1. Transfer 20 ml LV containing medium.

2. Underlay 6 ml of 20% sucrose/DMEM.

3. Top off to within 2-3 mm with remaining medium and plain DMEM.

4. Centrifuge in SW32 Ti. Max break at 25,000 rpm at 4°C for 2 hrs (can go up to 4 hrs).

5. Aspirate down to yellow pellet (bleach pipette before disposal).
- Add 0.2 ml of DMEM/centrifuge tube and tap the bottom several times.
- Wait a few minutes (~5-10 min)

6. Transfer pellet to cryo vials.
- Scrape bottom of tube with pipette.
- Pipette up and down to release pellet from bottom.
-Combine all tubes if multiple dishes were transfected for a single vector and mix.

7. Aliquot 10 µl for titration and freeze remaining virus in aliquot of 50 μl at -80°C.

Note: For 10-cm dish, use SW 40Ti (12.5 ml capacity, 25,000 rpm, 120 min, at 4°C).

B. Calcium Phosphate Precipitation Method

REAGENTS
2x HEPES-buffered saline, pH7.1 (HBS) solution

Materials
1. HEPES, $C_8H_{18}N_2O_4S$=238.31
2. NaCl=58.44
3. NaOH, FW 40.0. 1N NaOH, dissolve 2 g NaOH in 50 ml of water (double distilled water or sterile water).

Note: 1. pH may be increased by 0.1-0.3 by filtration.
2. The pH influences transfection efficiency. Prepare 3-4 buffer with a pH higher, or lower than 7.1 (7.05-7.15), test by transfection (you can use any lenti transfer vector containing a fluorescence reporter such as pCDH-EF1-copGFP-T2A-Puro, Addgene #72263) and pick the best one.

2xHebs (This is for a total volume of 200 ml).
1. Mix together:
   2.00 g HEPES
   3.39 g NaCl
   And adjust to 190 ml with water (pH~5.19)
2. Adjust the pH to 7.1 with 1 N NaOH (~2.56 ml).
3. Adjust the volume to 200 ml with water.
4. Sterilize by filtration with a 0.22 μm disposable filter.

70 mM Na_2HPO_4
1. Dissolve 5.01 g $Na_2HPO_4.12H_2O$ (MW=368.14) and make up to 200 ml.
2. Sterilize by filtration with a 0.22 μm filter.

2xHBS (This is the reagent that you need).
Mix the following two solutions together and store at room temperature (good for at least 6 months).
500 ml 2xHebs, pH 7.1
10 ml 70 mM Na_2HPO_4

2.5 M $CaCl_2$ $CaCl_2.2H_2O$, FW 147.0
Dissolve 73.5 g $CaCl_2.2H_2O$ and adjust volume to 200 ml.
Store at -20°C. This solution does not freeze at -20°C.

Testing 2xHBS on 293T cells.

293T cells are seeded in a 24-well plate (0.5 ml/well) the day before transfection (1:5-1:6 depending on confluence and growth conditions). Cells should be 70-80% confluent at transfection.

(a) DNA $CaCl_2$ mixture

Total 21.25 μl made in a 1.5 ml micro-centrifuge tube.

	ID	DNA (2.5 μg) (μl)	2.5 M $CaCl_2$ (μl)	H_2O (μl)	Total (21.25 μl)
1	pCDH-EF1-copGFP-T2A-Puro	10 μl (0.1 μg/μl)			
2					
3					
4					
5					
6					

(b) 2 x HBS
1. Pipette 21.25 μl of 2 x HBS into separate tubes.

2. Add "A" to "B" dropwise while vortexing (set at slow speed).

3. Spin down and incubate at room temperature for 20-30 min.

4. Overlay A + B mixture (42.5 μL) directly on top of cells.

5. Gently mix the plate up-down and right-left to evenly distribute the DNA solution over the entire plate. Do not swirl.

6. Place a 24-well plate in a CO_2 incubator.

7. After 6 hours, remove medium by aspiration and add fresh 0.5 ml of DMEM/5% FBS.

8. Take a fluorescence image at 24 and 48 hrs.

Transfection

Follow the protocol for splitting cells described in iMFectin transfection.

Tuesday

Make a DNA mixture in a 15 ml tube (for 15-cm dish), or 1.5 ml tube (for 10-cm dish).

ID			
15-cm dish		10-cm dish	
H_2O	μl	H_2O	μl
Transfer vector (60 μg)	μl	Transfer vector (20 μg)	μl
psPAX2 (45 μg)	μl	psPAX2 (15 μg)	μl
pMD2.G envelop plasmid (18 μg)	μl	pMD2.G (6 μg)	μl
2.5M $CaCl_2$	150 μl	2.5M $CaCl_2$	50 μl
Total Volume	1.5 ml	Total volume	0.5 ml

1. Add the DNA mixture (this is solution A) to a 15-mL tube containing 1.5 ml (15-cm dish) of 2 x HBS (Solution B) dropwise while gently vortexing the tube.

For 10-cm dish, add the DNA mixture to a 15-mL tube containing 0.5 ml of 2 x HBS.

2. Let the reaction incubate at room temp for 10-15-min.

3. Add 1.0 ml of transfection mixture (3 ml for 15-cm dish) to each plate.

4. Gently mix the plate up-down and right-left to evenly distribute the cocktail over the entire plate. Do not swirl.

5. Place plates in a 37°C, 5% CO_2 incubator.

6. After 6 hours, remove the medium with DNA precipitate by aspiration, and add 32 ml/15-cm dish of fresh medium (DMEM/5% FBS), or 12 ml/10-cm dish.

Follow the protocol described for iMFectin.