JIN LAB

High titer lentivirus production

2023.8.7 / Zhilin Wang / v1

NOTES

This protocol is adopted from "An Improved Protocol for the Production of Lentiviral Vectors" (https://doi.org/10.1016/j.xpro.2020.100152). Please contact the Jin Lab with further questions.

METHODS

Reagent Preparation

1. Prepare fresh 2x BBS and sucrose solution.

   Following: https://www.aatbio.com/resources/buffer-preparations-and-recipes/bes-buffered-saline-2x-ph-6-95

2XBBS		final concentration
BES(B9879-100g)	5.35 g	0.05 M
NaCl(S9888-1kg)	8 g	0.28 M
Na2HPO4(S0876-100g)	0.1 g	0.0015M
H2O	400 ml	
adjust pH to 6.95 with NaOH		
add water to 500 ml	filter with 0.22 um	

2. Prepare fresh sucrose gradient solutions.

20% sucrose		30 % sucrose		60% sucrose		70% sucrose	
sucrose (84097-5kg)	10 g	sucrose	15 g	sucrose	30g	sucrose	35 g
	5 ml 10x PBS		50 ml DMEM		50ml DMEM		5 ml 10x PBS
Fill to 50 ml:		*add DMEM to 50 ml*		*add DMEM to 50 ml*		*add DMEM to 50 ml*	

3. Prepare 0.2% gelatin solution.

0.2% gelatin	
gelatin (G1890-100G)	1 g
Add PBS	up to 500mL
	Heat to dissolve and filter with 0.22 um filter

Thaw the cells (~15 min)

1. To start a new HEK293T cell culture: Warm 10 ml of medium containing 10% FBS. Thaw the cells (<15 passages) in 37°C water bath for no more than 1 min and add the cell suspension to 10 ml of warm medium. Centrifuge at 300g for

5 min. Resuspend in 25 ml medium and seed low passage HEK293T (<15 passage) cells into a 150 mm tissue culture plate. Grow cells at 37°C with 5% CO_2 in a standard tissue culture incubator. Once cells reach 90%–95% confluent growth, prepare and collect cells for reseeding.

Passage the cells (~30 min)

2. To enhance cell adherence to the tissue culture plate, pre-coat tissue culture plates by adding 5 mL of 0.2% gelatin per plate. Spread the gelatin evenly across the surface of the plate, incubate at 37°C for 10 min, and then aspirate the liquid.
3. Aspirate media from the confluent 150 mm tissue culture plate and gently rinse the cells with ~3.0 mL sterile 1× PBS.
4. Incubate cells with 2.0 mL of dissociation reagent (e.g., Trypsin-EDTA) for 5 min until cells have detached from each other and from the bottom of the plate.
5. Add 8 mL of media prepared with 10% FBS to inactivate the dissociation reagent.
6. Triturate the cells against the bottom of the plate 10–15 times to create single cell suspension with a cell density of approximately 1×10^7 cells/mL.
7. Reseed HEK293T cells into 150 mm plates for production.
8. Add 22.5 mL of warm (37°C) FBS containing media to each 150 mm plate.
9. Seed 2.5 mL of cells from step 2 into each plate (total ~2×10^7 cells/plate).
10. Incubate the plates 12–18 h or until 70%–80% confluence is reached.

Transfection (~45 min)

11. Once the HEK293T cells in the 150 mm tissue culture plates have reached 70%–80% confluence, they are ready for transfection.

    Aspirate the old media from the 150 mm plates and gently replace with 20 mL of freshly prepared media **without** FBS by pipetting the media against the sidewall of the plate. The lack of serum stunts cell growth and enhances uptake of the transfection mixture.

    Prepare transfection mixture in a 15-mL conical tube following the volumes for each reagent listed in table:

	Amount (ug)	Concentration (ug/ul)	# of Reactions	Volume (ul)
shuttle vector (your own plasmid)	37.5	0.362	1	103.6
MDLG/RRE	25	2.377	1	10.5
MD2.G	12.5	2.343	1	5.35
RSV-REV	6.25	0.4	1	15.65
1M $CaCl_2$ (BIOSCIENCES, Cat.#R040)	312.5		1	312.5
H2O				802.4
Total				*1250 uL*
2. While vortexing, add 2xBBS (1250 ul) dropwise to plasmid/$CaCl_2$ mixture				
3. Incubate at RT for 30 min				

    *The transfection mixture must be clear prior to its addition onto the cells to ensure **smaller size** of $CaPO_4$ precipitates, which is crucial for transfection efficiency. (smaller size is easy for cell to uptake)*

12. Following 30 min incubation, immediately add 2.5 ml transfection mixture dropwise to 150 mm tissue culture plate.

13. Swirl plates gently and incubate for 1 h.
14. Check size of $CaPO_4$ precipitates in transfected cells under light microscope. You should see many CaPO4 precipitates attached to the cells' surface under a light microscope.

16. Add 2.5 mL pure FBS to the plate and incubate 12–18 h. The final concentration is 10%.

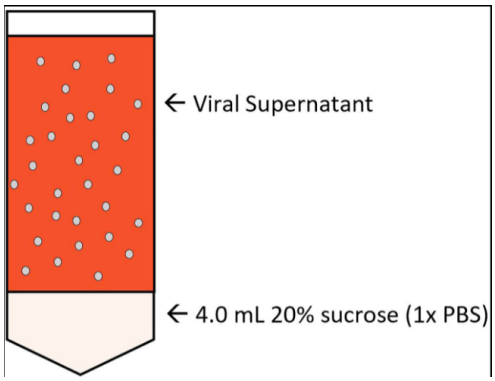
17. 12–18 h post-transfection, replace media with fresh 10% FBS containing media to remove $CaCl_2$ and 2× BBS solution. Observe cells to ensure that they are near 100% confluency with little to no cell death. Gently aspirate old media. You can optionally wash the cells with warm (37C) 1X DPBS.

18. Add 25 mL prepared media **with** FBS, and continue incubating for an additional 48 h. (Also, you can collect supernatant every 24 hours)

Ultracentrifugation for Concentrating Viral Particles (*in vitro)* (~2-3 hour)

19. 48 h after replacing the media, the virus is ready to be harvested. Without disturbing the transfected cells, carefully collect the supernatant from the 150 mm tissue culture plate into a new, sterile 50-mL conical tube on ice. Centrifuge the 50-mL conical tube at 400–450 × *g* for 5 min using a tabletop centrifuge to separate any detached cells from the supernatant. Filter the supernatant through a 0.45 µm vacuum filter unit (PES) to remove large cellular debris.

20. Add 2 mL of 20% sucrose dissolved in 1× PBS to the bottom of the conical ultracentrifuge tube. Without disturbing the sucrose, carefully load the filtered supernatant on top of the sucrose layer dropwise as seen in this schematic:



21. Spin down the tubes in the ultracentrifuge for 2 h at 25,000 rpm at 4°C

21. Once the spin is complete, gently extract the conical ultracentrifuge tubes and isolate the pellet.

22. Dump all the media into 10% bleach. Be careful not to disturb the pellet.

23. Use a sterile paper towel to carefully wipe the sides of the tubes, without touching the pellet.

24. Resuspend the pellet with 15 ul cold 1× PBS solution, put on ice overnight, aliquote in a protein lo-bind tube.

Alternative Ultracentrifugation Approach Using an Amicon tube (*in vivo)* (~4 hour)

25. Create a sucrose gradient in the conical ultracentrifuge tube in the order shown below. Use the syringe to dispense each sucrose solution, taking care to not disrupt each layer.

26. Add dropwise 0.5 mL 70% sucrose.

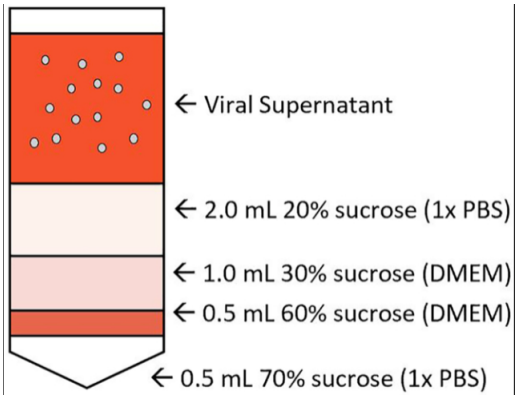
27. Add dropwise 0.5 mL 60% sucrose.

28. Add dropwise 1 mL 30% sucrose.

28. Add dropwise 2 mL 20% sucrose.

29. Being careful not to disturb the sucrose gradient, load the filtered supernatant **on top** of the sucrose gradient, dropwise. Spin down the tubes in the ultracentrifuge for 3 h at 30000 rpm at 4°C.

30. Collect 30%, 60%, and 70% sucrose layer.



31. Add sterile cold 1× PBS solution to the 15-mL amicron tube, add sucrose layer containing lentivirus. Centrifuge at 3000g, 4°C, ~ 30 min, wash twice, aliquote the virus and store at -80°C.

Quantify the lentivirus with qPCR (LV900 abm kit, ~1 hour)

32. Add 1 ul of lentivirus to 9 ul of lysis buffer. Keep it at RT for 3 min. Prepare RT-PCR mixture using the following quantities. The below calculation is for 1 sample with 4 standards, please scale up as needed.

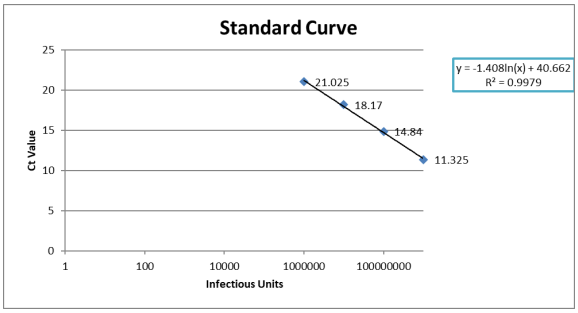
2x qPCR	10 ul		
primer mix	2 ul		
sample	2 ul		
H2O	6 ul		
	temp	*time*	*cycle*
RT	42 C	20 min	1
	95 C	10 min	1
	95 C	15 S	30
	60 C	1 min	30

Titer calculation

33. Use this sample sheet to calculate the titer

1. Plot standard curve

Standard curve				
Ct Duplicate 1	Ct Duplicate 2	Ct Average	Dilution	Titer (IU/ml)
11.34	11.31	11.325	1/100	1000000000
14.85	14.83	14.84	1/1000	100000000
18.24	18.1	18.17	1/10000	10000000
20.9	21.15	21.025	1/100000	1000000



2. Calculate the titer

Ct Duplicate 1	Ct Duplicate 2	Ct Average	Dilution	Titer (IU/ml)
17.08	17.23	17.155	NA	1.77E7

3. Calculate the sample titer (x 10 if the sample was lysed with 10x dilution as this protocol). Typically, the raw supernatant from the transfection should be E6-E7 IU/mL range, and the post ultracentrifugation should lead to titers at E8-E9 IU/mL range.