MUSC
MEDICAL UNIVERSITY
of SOUTH CAROLINA

Ferreira Lab
86 Jonathan Lucas Street
HO606 (Lab)
MSC635/HO612h (Office)
Charleston, SC 29425-5090
Tel 843 792 0614
www.ferreiralab.com

Second Generation Lentiviral Production Protocol

October 2021 - Capers Zimmerman

Materials:

Cells (in this particular example, Human embryonic kidney HEK293T cells and Jurkat cells)
Cell Counting Slides (Bio-Rad. Cat. No. 1450011)
Chloroquine diphosphate salt, 98% (VWR, Cat. No. BT214870-25G)
DMEM and RPMI complete media (see Ferreira Lab **Making Media/Sterile Technique Protocol**)
DMi1 Inverted Microscope for Cell Culture (Leica Microsystems, Cat. No. 11526220)
DPBS (ThermoFisher, Cat. No. 14190250)
Drummond Original Pipet-Aid Pipette Controller (VWR, Cat. No. 53498-104)
Magnetic Stirrer (Faithful, Model: SH-2)
MillexGP Filter Unit, 0.22 µm (Millipore Sigma, Cat. No. SLGP0333RS)
Nunclon Delta Surface Sterile 96-well Plate (ThermoFisher, Cat. No. 163320)
PEI Prime linear polyethylenimine (Millipore Sigma, Cat. No. 919012-500MG)
Plasmid DNA (packaging plasmids and transfer plasmid/plasmid of interest)
Plastic waste container, tri-corner beaker (VWR, Cat. No. BTC0800)
P20 micropipette (SCILOGEX, Cat. No. 713121209999)
Spinbar Magnetic Stir Bar, PTFE-Coated (Millipore Sigma, Cat. No. Z126942-1EA)
Sterile 50 ml beaker (VWR. Cat. No. 10754-946)
Sterile 150 ml beaker (VWR, Cat. No. 10754-950)
Sterile 15 ml Falcon tubes (VWR, Cat. No. 89039-664)
Sterile 50 ml Falcon tubes (VWR, Cat. No. 89039-656)
Sterile Nuclease-free Water, molecular grade (NEB, Cat. No. B1500L)
Sterile 10 ml Plastic Syringe (ThermoFisher, Cat. No. 14955453)
Sterile P20 Pipette Tips without filter (Corning, Cat. No. 4121)
Sterile 2 ml serological pipettes (VWR, Cat. No. 76093-882)
Sterile 5 ml serological pipettes (VWR, Cat. No. 89130-896)
Sterile 10 ml serological pipettes (VWR, Cat. No. 89130-898)
Sterile 10 cm Tissue Culture Dishes (CytoOne, Cat. No. CC7682-3394)
TC20 Auto Cell Counter (Bio-Rad, Cat. No. 1450102)
Trypan Blue Dye (Bio-Rad, Cat. No. 1450013)
Trypsin-EDTA (0.25%), phenol red (ThermoFisher, Cat. No. 25200056)

ViralBoost Reagent 500x, (Alstem, VWR Cat. No. MSPP-VB100)
ZOE Fluorescent Cell Imager (Bio-Rad, Cat. No. 1450031)

Experimental Setup:

- This protocol requires HEK293T cells to be seeded at 3.8 x 10^6 cells/plate. Your cells should be expanded ahead of time and then split into the desired number of plates, which will depend on the total number of viable cells counted and the number of different viruses you aim to produce
    - Ideally, the timeline of this transfection protocol should proceed as follows:

Monday	**Tuesday**	**Wednesday (24 hrs)**	**Thursday (48 hrs)**	**Friday (72 hrs)**
Seed HEK293T cells at 3.8 x 10^6 cells/plate for transfection following day	Transfect cells (add chloroquine to medium, incubate 5 hours, add DNA/PEI complexes)	Change medium, add ViralBoost	*Collect viral medium, add fresh medium + ViralBoost	Collect viral medium, transduce Jurkat cells for virus titration

*peak viral production should occur at 48 hrs

- Prepare stock solutions of chloroquine diphosphate (CHL) and polyethylenimine (PEI) for transfection
    - Chloroquine diphosphate (25 mM → 1000x stock, will be used at 25 µM)
        - Dissolve 0.129g of chloroquine diphosphate salt in 10 ml of sterile nuclease-free water under the tissue culture (TC) hood
        - Swirl the solution around in a 50 ml autoclaved beaker to make sure the CHL is fully dissolved
        - Prepare and label a sterile 15 ml Falcon tube that will serve as a collection tube for the filtered CHL solution
        - Grab a sterile 10 ml plastic syringe and a sterile 0.22 µm filter. Attach the filter to the bottom of the syringe and remove the plunger, placing it upright such that the tip of the plunger remains sterile
        - Transfer the CHL solution from the beaker to the syringe, replace the plunger, and push down to filter the solution into the 15 ml Falcon tube. The CHL solution is now sterilized
        - Use sterile PCR tubes to make aliquots of 50-100 µl each for freezing (store in -20°C freezer)
    - PEI (1 mg/ml)
        - Dissolve 0.1g of PEI in 90 ml of ultrapure water (via Milli-Q Water Purification System) in a 150 ml autoclaved beaker and stir using a magnetic stir bar/stir plate

- Depending on what the starting pH is measured to be, HCl or NaOH will need to be added to adjust the pH to 7.0 (measure using pH strips or pH meter)
    - If the pH is acidic, continuously add *small volumes of NaOH (1 M) until the solution has reached a pH ~7
    - If the pH is basic, continuously add *small volumes of HCl until the solution has reached a pH ~7
    - *Adding small volumes of either HCl/NaOH is recommended since the pH of this solution will drift rapidly upon addition of acid or base. Add only a few drops at a time, continue stirring the solution, and then re-measure the pH
- Once the pH is measured to be ~7, prepare and label two 50 ml Falcon tubes to filter the sterilized PEI solution into. Allow the solution to continue stirring for 10 mins, then recheck the pH to make sure it hasn't drifted. If the pH is maintained at ~7, you may proceed to the next step
- Assemble a new sterile 10 ml syringe/0.22 µm filter and transfer 10 ml of the PEI solution into the syringe. Filter the solution through to one of the 50 ml Falcon tubes. Remove the plunger without allowing the tip to touch the surroundings, transfer another 10 ml of the PEI solution into the syringe, and filter it through. Repeat this procedure until 50 ml (5 transfers) of the PEI solution has been filtered into one of the 50 ml Falcon tubes. Then begin filtering into the other 50 ml Falcon tube. Continue this process until the entire 100 ml of PEI solution has been sterilized and stored in two 50 ml Falcon tubes
- Use sterile PCR tubes to make aliquots for freezing (stored at -80°C)

- Test both the PEI solution at different DNA:PEI ratios (1:1, 1:2, 1:3, etc. where 1 µg DNA = 1 µg PEI = 1 µl PEI) and the CHL solution on a small amount of HEK293T cells split from a maintenance plate into a 12-well plate (0.3 x 10^6 cells/well) and a small amount of DNA (1 µg) to ensure that they resulted in sufficient transfection prior to aliquoting/freezing the solution. For our lab, we found a 1:2 DNA:PEI ratio works best.
    - PEI and CHL solutions were stored in the fridge while HEK293T cell transfection efficiency was tested. After 24 hrs, exhausted media from each well was aspirated and replaced with fresh medium with 25 µM CHL. Incubated the cells with CHL at 37°C for 5 hours → then added DNA/PEI complexes in varying ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6) to determine protocol for highest transfection efficiency. Following day, observed and quantified number of transfected cells (in our case, assessed under the microscope by mCherry expression) in each well corresponding to a different DNA:PEI ratio

Procedures:

1. On Day 0, HEK293T cells should be counted and split for transfection according to the Ferreira Lab **Adherent Cell Culture Protocol**
2. On Day 1, aspirate exhausted medium and add *fresh DMEM10 medium with 25 µM CHL

a. *Replace old medium with fresh medium to discard dead cells floating around in the medium, since we do not want to transfect dead cells)
b. If you have multiple plates to transfect, use a 50 ml Falcon tube to prepare enough DMEM10 medium with 25 µM CHL for all of the plates of cells you will be transfecting (if just transfecting one plate of cells, a 15 ml Falcon tube will suffice)
    i. Each plate of cells will need 10 ml of medium + CHL (1:1000) e.g. for 2 plates requiring 20 ml total of DMEM complete, 20 µl of CHL will be added to the medium (allot 2 ml extra of DMEM complete and 2 µl extra of CHL to account for pipetting errors—> 22 ml DMEM complete + 22 µl CHL stock prepared in one 50 ml Falcon tube)
    ii. Be sure to thaw a CHL aliquot from the -20°C freezer ahead of time (we prepared 50 µl aliquots)
c. First, add the necessary amount of DMEM complete to an appropriately sized Falcon tube. Then add the necessary amount of CHL and invert the Falcon tube a couple of times to mix
d. Using a sterile unfiltered P20 pipette tip , aspirate the exhausted medium from each plate (use of one tip ok since all same cell line) → remember to work quickly after completing this step since your cells will dry out if left without medium for too long
e. If working with less than 3 plates, one sterile 25 ml serological pipette can be used to distribute 10 ml of medium with 25 µM CHL to each plate (otherwise use as few serological pipettes as possible to distribute the 10 ml to each plate)
f. Incubate the plates at 37°C for ~5 hours
g. Return the medium to the fridge and wipe down the hood with 70% ethanol (EtOH)
h. After 5 hours, obtain your DNA plasmids from the -20°C freezer, as well as a PEI aliquot from the -80°C freezer (we prepared 50 µl aliquots), and thaw them on ice
i. While waiting for these aliquots to thaw, calculate the volume of each that you will need to add to the cells. Example below:

Plasmid	***Amount of DNA to Add**	**Concentration**	****Volume to Add**
Packaging plasmid (e.g. pCMV-dR8.91)	6.66 µg → 6660 ng	313.7 ng/µl	21 µl
Envelope plasmid (e.g. pMD2.G)	0.84 µg → 840 ng	192.5 ng/µl	5 µl
Plasmid(s) of Interest: e.g. CAR 1 e.g. CAR 2	10 µg → 10,000 ng	 762.6 ng/µl 2805 ng/µl	 13 µl 4 µl

*These values will vary depending on the specific plasmid DNA you're working with → refer to published protocols to determine how many nanograms of each plasmid to add for sufficient transfection

**Rounding these values to the nearest whole number in either direction is acceptable, as long as it amounts to <10% difference

j. Now calculate the volume of PEI you will need to add to each tube containing different constructs in order to form a 1:2 DNA:PEI complex ratio in each 1.5 ml eppendorf tube
   i. If each tube will contain 17.5 µg of DNA total (10 µg plasmid of interest + 6.66 µg packaging plasmid + 0.84 µg envelope plasmid = 17.5 µg of DNA total/tube), then to form a 1:2 DNA:PEI ratio add → 17.5 x 2 = 35 µl of PEI
   ii. Through titration and data analysis using ImageJ, the Ferreira Lab determined adding PEI to the DNA in a ratio of 1:2 produced the highest transfection efficiency

k. Dilute the DNA plasmids and the PEI in serum-free medium in separate tubes
   i. Obtain a rack and enough sterile 1.5 ml eppendorf tubes, such that there is one tube for every plasmid of interest you plan to transfect the cells with. Once you've gathered these tubes for the DNA, grab the same number of tubes such that each DNA tube has a corresponding tube for the PEI dilution that will later be added dropwise to the diluted DNA plasmids
   ii. Label each of the DNA tubes with the specific construct name
   iii. Open all of the tubes (in this case, there will be 4) without touching the inside of the caps
   iv. Add 500 µl of serum-free medium (SFM), e.g. Opti-MEM or DMEM, to each eppendorf tube using a sterile 2 ml serological pipette
   v. Use a micropipette to add the calculated volume of PEI to each PEI tube containing 500 µl SFM
   vi. Use a micropipette to add the calculated volumes of the envelope and packaging plasmids to each labeled DNA tube (all tubes that will get DNA, no matter the plasmid of interest, will get the same packaging/envelope plasmids in the same amounts - However, tips do need to be changed in between tubes so as to not contaminate the stock vials of packaging/envelope plasmid DNA)
   vii. Now use a micropipette to add the calculated volumes of your plasmid(s) of interest to their respective DNA tubes
   viii. Close all of the tubes and gently flick to mix

l. Briefly spin all of the tubes down to make sure all of the contents are in the bottom of the tube and not in the cap

m. Use a P1000 micropipette to add the entire volume (535 µl in this example) of each PEI dilution to each corresponding DNA tube dropwise
   i. Add the PEI slowly, then gently flick the diluted DNA tube to mix
   ii. Do NOT vortex the tubes to mix (prevents shearing the plasmid DNA)

n. Close all of the tubes and gently flick them again. At this point, you should be left with only the DNA tubes (in this case, 2 tubes). Incubate for 15 mins at room temperature (this allows times for the DNA/PEI complexes to form)
    i. During this incubation period, return the SFM to the fridge and the DNA plasmids to the -20°C freezer
o. After 15 mins, retrieve your plate(s) of HEK293T to be transfected from the incubator (1 plate/construct → in this case, we will need 2 plates). Label each plate with the construct you will be transfecting the cells with
p. Take up the entire volume of DNA/PEI (~1 ml) with a P1000 micropipette and add it dropwise to its corresponding plate of HEK293T cells, covering the surface of the plate Repeat for each construct.
q. Place the transfected cell plate(s) back in the incubator overnight

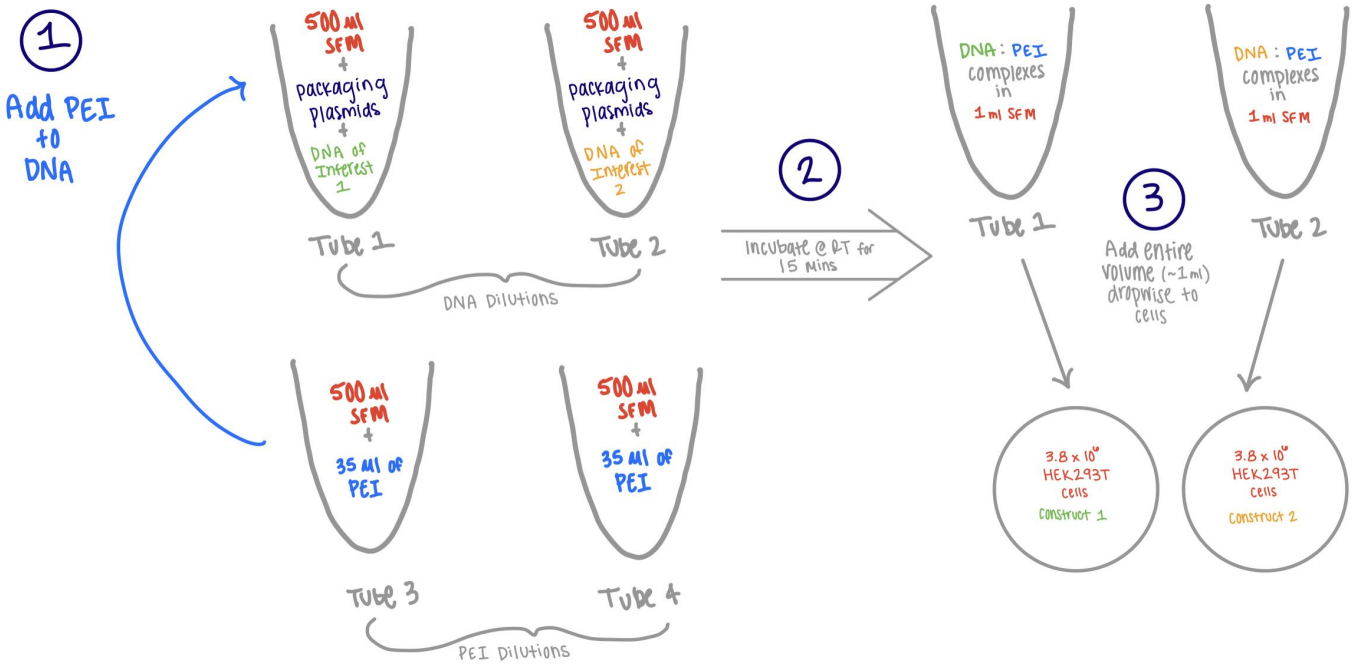

Figure 1. Workflow of DNA/PEI Dilutions for Transfection of HEK293T Cells

3. On Day 2, 24 hours after transfection, aspirate exhausted medium of each plate and replace with 10 ml of fresh DMEM complete with 20 µl ViralBoost to maximize viral production
    a. Virus production typically peaks at 48 hours after transfection
    b. To calculate how much ViralBoost to add to the medium:
        i. First calculate how much medium you will need in total to replace the 10 ml of exhausted medium in each transfected plate (in this case, 2 plates → 20 ml fresh medium needed)

ii. ViralBoost reagent stock is 500x concentrated, so divide the total volume of medium needed in µl by the concentration factor (20,000 µl/500 = 40 µl of ViralBoost to add to 20 ml of medium)
iii. If you have multiple plates that need new medium with ViralBoost, make a stock solution in a sterile 50 ml Falcon tube and then distribute 10 ml to each plate)
iv. Place the plates back in the incubator overnight

4. On Day 3, 48 hours after transfection, the viral medium is collected from each plate in labeled, sterile 50 ml Falcon tubes and replaced with 10 ml of fresh DMEM complete + ViralBoost
    a. Once the viral medium has been collected from each plate, place the Falcon tubes in the fridge to ensure the virus doesn't go bad while we wait to collect the viral medium again after another 24 hours
    b. Add fresh medium + ViralBoost in the same quantities you did the day before
    c. Place the plates back in the incubator overnight
5. On Day 4, 72 hours after transfection, the viral medium is collected from each plate and added to its corresponding 50 ml Falcon tube containing the collection from 48 hours
    a. Once this viral medium is collected, the HEK293T plates can be thrown away.Keep the viral medium in the fridge until ready to titrate
6. Each virus will need to be titrated via flow cytometry prior to experimentation in order to determine the volume of each virus required to transduce e.g. 250,000 T cells, as well as the number of aliquots we are able to freeze at -80°C for long term storage
    a. The Ferreira lab transduces Jurkat cells (10,000 cells/well) grown in RPMI10 complete medium to titrate each virus
    b. Flow cytometry is used to obtain transduction efficiencies (by quantifying the percentage of mCherry-positive cells, for example) in order to calculate the volume of virus needed to transduce 250,000 T cells (a value that is commonly used in several protocols of this lab) - see Ferreira Lab **Lentiviral Transduction Protocol** for more information