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D-Lab Lentivirus Production Protocol

This protocol is for the generation of high titer lentivirus for *in vivo* injections.

***Supplies**:*

- 293FT Cells (Invitrogen: R700-07)
- T-225 tissue culture flasks (Nunc: 159934)
- T-75 tissue culture flasks (Nunc: 156499)
- 500 cm^2 tissue culture plates (Nunc: 166508)
- Ultracentrifuge tubes (Beckman Culture: 344058)
- DMEM (Cambrex: 12-604Q)
- UltraCULTURE (Cambrex: 12-725F)
- Penicillin/Steptomycin w/ L-Glutamine (Cambrex: 17-718R)
- Sodium Pyruvate Solution (Cambrex: 13-115E)
- Sodium Bicarbonate Solution (Cambrex: 17-613E)
- Phosphate Buffered Saline w/o Ca^{2+} and Mg^{2+} (Cambrex: 17-516F)
- Defined Fetal Bovine Serum (HyClone: SH30070.03)
- Sodium Butyrate (Sigma: 19364-1G)
- HEPES (Sigma: 54457-50G-F)
- Sodium Phosphate Dibasic (Sigma: 71636-250G)
- Sodium Chloride (Sigma: 71376-1KG)
- Hexadimethrine bromide (Sigma: 107689-10G)
- Sodium Hydroxide (Sigma: 71689-500G)
- Distilled H_2O (Quality Biological: 118-162-101)
- Calcium Chloride 2 M solution (Quality Biological: 351-130-061)
- 0.45 um Low-protein binding filter flask (Millipore: SCHVU02RE)

Solutions:

D10 Cell Maintenance Media (per 500 mL)
- 500 mL DMEM
- 50 mL FBS (10% w/v)
- 5 mL Pen/Strep/L-Glu (1% w/v)
- 5 mL Sodium Pyruvate (1% w/v)
- 5 mL Sodium Bicarbonate (1% w/v)

Virus Production Media (per 500 mL)
- 500 mL UltraCULTURE
- 5 mL Pen/Strep/L-Glu (1% w/v)
- 5 mL Sodium Pyruvate (1% w/v)
- 5 mL Sodium Bicarbonate (1% w/v)

20% Sucrose Solution (per 50 mL)
- 10 g sucrose
- Bring the volume to 50 mL using PBS w/o Ca^{2+} or Mg^{2+}
- Filter with 0.22 um filter

2X HBS Buffer (per 500 mL)
- Add to 450 mL of distilled H_2O
    - 5.96 g HEPES (50 mM, MW 238.3)
    - 0.106 g Na_2HPO_4 (1.5 mM, MW 141.96)
    - 8.18 g NaCl (280 mM, MW 58.44)

  Note: the pH should be around 5.9 at this point.
- Titrate with NaOH to 7.05 (use 5 M first then switch to 1 M)
- Fill the volume to 500 mL

- Filter with 0.22 um filter
- This solution is stable at room temperature for 6 months

Protocol:

Note: It is important to use low passage 293FT cells for the production of viruses. To make sure the cell is always in the fastest growth phase, never let the cells grow to 100% confluence.

Day 0:

- Split 4 T-225 flasks of 95% confluent 293FT cells into 4 500 cm^2 plates. For each plate, use 100 mL of D10 media.

- Rock the plate gently to evenly distribute the cells.

- Incubate the plates at 37^{o}C overnight. The cells should reach 90% confluence in 24 hours.

Day 1:

- Prewarm 330 mL of D10 to room temperature.

- In a 50 mL conical tube, prepare the following mixture:
    - 550 ug of lentivirus plasmid (e.g. pLECYT, pFCK-hChR2-mCherry)
    - 550 ug of pCMVdeltaR8.74 (contains GAG, POL)
    - 360 ug of pMD2.G (contains VSVg)
        - At this point mix thoroughly
    - Add 4.55 mL of 2 M $CaCl_2$ solution
    - Bring the volume to 19 mL total with distilled H_2O
    - Mix thoroughly.

- Add 19 mL of 2X HBS to the DNA/$CaCl_2$ mix.
    - Mix thoroughly and quickly. Then pour directly into 330 mL of prewarmed D10

- Remove the old media from the plates. Add 90 mL of the D10-containing transfection mix to each plate
    - Be careful to not tilt the plate too much. Cells may detach easily.

- Put the plates back into the incubator

Day 2:

- Pewarm D10 media to room temperature

- 15 to 16 hours after initial transfection, remove the transfection media from the plates and wash each plate with 50 mL of fresh D10. Then add 90 mL of fresh D10 to each plate

- Put cells back into incubator for 8 hours

8 hours later

- Prewarm 200 mL of Virus Production Media

- 24 hours post transfection, replace the old media with 50 mL of Virus Production Media containing 5 mM Sodium Butyrate

- Put cells back into the incubator. Cells are very easy to detach at this time so be very gentle.

Day 3:

- Sterilize 6 ultracentrifuge tubes by spraying with EtOH and the let them air dry in the tissue culture hood.
- 48 hours post transfection, collect the virus containing supernatant into four 50 mL conical tubes and centrifuge for 5 minutes at 1000 rpm.
- Prefilter a low-protein binding 0.45 um filter flask with 30 mL of D10 media.
- Filter the virus-containing supernatant through the 0.45 um filter flask.
- Divide the filtered virus-containing supernant among the six centrifuge tubes.
- To the bottom of each centrifuge tube, add 2 mL of 20% Sucrose Solution.
- Centrifuge in a Beckman SW-28 rotor for 2 hours at 25,000 rpm, 4^{o}C.
- Gently carry the centrifuge tubes back to the tissue culture hood and pour out the supernatant. There should be a tiny semi-transparent pellet at the bottom of each centrifuge tube, looks like a contact lens.
- Dry the side of each tube with Kimwipe.
- Add 100 ul of cold PBS to the first tube and resuspend the pellet by swirling and gentle pipetting. Do NOT pipet too much because it will degrade the virus.
- Transfer the media from the first centrifuge tube into the next to resuspend the second pellet. Repeat for the 4 additional tubes.
- After resuspending all 6 centrifuge tubes, pipet the virus solution into an Eppendorf tube and spin at 7k for 5 minutes. This step is used to remove the unsuspended virus debris.
- Aliquot the supernatant and store at -80^{o}C.