Amphotrophic virus preparation in 293T cells by calcium phosphate Entered by Kevin Janes
Janes Lab Protocols 2/19/09

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1. Plate 293T cells at 400,000 cells/well on 6-well plates for 24 hr.
   - *Cells should be ~30% confluent on the day of transfection*
   - *Do not package virus with 293T cells that have been cultured for more than two months since thawing from liquid N_2; we have found that packaging efficiency drops dramatically after extended culturing*
2. One hour before transfection, aspirate medium and refeed cells with 2 ml growth medium (DMEM + 10% FBS + pen/strep)
   - *Do not wash cells with PBS, as this will cause most of the cells to detach*
   - *Refeeding before transfection increases transfection efficiency by ~2-fold*
3. For amphotrophic retroviruses, prepare the following calcium phosphate solution for each well of the 6-well plate:
   1.25 μg retroviral vector (pBabe, pLNCX, etc.)
   1.25 μg pCL ampho (retroviral packaging vector)
   10 μl 2.5 M $CaCl_2$
   Volume to 100 μl with 0.1× TE (pH 7.6)
   - *This "double transfection" protocol prepares much higher titer retroviruses than GPG, Phoenix, etc. cells that stably express the packaging genes*
   - *We generally transfect two well per retroviral construct to have enough virus for triple infection if needed (above solution can be scaled up without problems)*

   For amphotrophic lentiviruses, prepare the following calcium phosphate solution:
   1.25 μg lentiviral vector (pLKO.1, etc.)
   0.75 μg psPAX2 (lentiviral gag-pol packaging vector)
   0.5 μg pMD.2G (VSV-G envelope protein)
   10 μl 2.5 M $CaCl_2$
   Volume to 100 μl with 0.1× TE (pH 7.6)
   - *Using the psPAX2-pMD.2G packaging vectors appears to improve virus titers by ~2-fold compared to D8.2-pCMV-VSVG packaging vectors*

   For lentiviral reporters, prepare the following calcium phosphate solution:
   0.25 μg pTRF.1 lentiviral reporter vector
   1.75 μg pFIV-34N (lentiviral gag-pol packaging vector)
   0.5 μg pVSV-G envelope vector
   10 μl 2.5 M $CaCl_2$
   Volume to 100 μl with 0.1× TE (pH 7.6)
   - *pFIV-34N and pVSV-G were cloned from the pPACKF1 plasmid mix from Systems Biosciences #LV100A-1*
4. To each 100 μl of 2× DNA-$CaCl_2$ mixture, add 100 μl of 2× HEPES-buffered saline at room temperature, and mix by pipetting
5. Let the precipitate stand for 1 min, add the entire 200 μl dropwise to the well of a 6-well plate, and gently agitate the plate to mix the precipitate with the medium
   - *Prepare the precipitates individually, because transfection efficiency is reported to drop quickly after 1 min*
   - *Removing the medium and adding the precipitate directly to the 293T cells does not increase transfection efficiency in my hands*
   - *Calcium phosphate precipitates will appear as small black speckles on the inverted microscope; it is good to confirm their presence to ensure that the precipitation was effective*
6. Incubate the precipitates with the cells for 4–6 hrs at 37°C, then aspirate the medium and carefully replace with 1 ml of growth medium

- *The purpose of this medium replacement is to refeed the 293T cells, not remove the precipitates*

7. Collect supernatant at 48 hr for retroviruses and 24+48 hr for lentiviruses
8. Pass virus solutions through a 0.45 μm filter and store at 4°C for short-term storage (days) or –80°C for long-term storage
    - *For low m.o.i. infections, we have found that freshly prepared virus gives the highest titers*

Buffer recipes

- **2.5 M $CaCl_2$**
  36.76 g $CaCl_2$
  Volume to 100 ml in H_2O
  Sterilize by autoclaving and store at room temperature

- **1× TE (pH 7.6)**
  158 mg Tris-Cl
  29 mg EDTA
  Volume to 90 ml and pH to 7.6 with NaOH
  Volume to 100 ml in H_2O and dilute to 0.1× in H_2O
  Sterilize by autoclaving
  Store at room temperature

- **2× HEPES-buffered saline**
  800 mg NaCl
  27 mg $Na_2HPO_4 \bullet 2H_2O$
  1.2 g HEPES
  Volume to 90 ml in H_2O and pH to 7.05 with NaOH (0.5 N)
  Volume to 100 ml in H_2O.
  Sterilize by passing through a 0.45 μm filter
  - *HEPES solutions cannot be autoclaved*

  Store 1 ml and 5 ml aliquots at –20°C