Transfection Protocol for 293T Cells

(Modified by Aaron Robida 9/22/04)

Transfection

1) Thaw out 293T cells (usually p3-5) from liquid nitrogen and thaw at 37°C for 1-2 minutes. Add the cells to 9 ml of growth media (10%FBS/1XPS) and plate into a 10 cm dish.
2) The next day check to ensure that the cells have sat down and refeed with 10 ml of fresh growth media.
   (use cells for 4-6 weeks which corresponds to 10-15 passages)
3) Two days prior to transfection, split the cells 1:2 and plate into 10 cm dishes (we usually obtain 15-30 million cells per plate)
4) Split the 293T cells 12-16hr before the desired transfection start time and plate at $3X10^6$ million cells into 10 cm dishes that are coated with 1 ug/cm^2 of poly-d-lysine. Be careful to ensure that cells do not clump in the 10 cm dish.
   WARNING: DO NOT "BLAST" MEDIA ONTO CELLS EVEN THOUGH THEY ARE ON COATED PLATES
5) Aspirate media from each 10 cm plate and refeed with 8 ml DMEM + 10% FBS + 1XPS containing 8 ul of 25 mM chloroquine. Incubate plates for 1 hour prior to DNA addition.
6) For each 10 cm plate add sterile TC water + DNA (sterile) = 438 µl in a 12x75 mm polypropylene tube (Fisherbrand).
7) Add 62 µl of ice cold 2 M $CaCl_2$ followed quickly by 500 µl of 2X HBS (warmed to 37°C). Mix the solution for 30 seconds by vortexing (setting 2-3) while gently bubbling with a 2 ml fisher pipet.
8) Incubate the sample at RT for 30 min prior to addition to the 10 cm plate.
9) Incubate the plates for 10-12 hr at 37°C.
10) Aspirate off media, rinse twice with 3 ml of 2X HBSS (warmed to 37°C), and refeed with 5 ml of growth media.
11) Collect virus 48 hr after start of the transfection.

Reagent List

Chloroquine (cholorquine diphosphate MW = 515.9)

- Dissolve in 1XPBS to make a 25 mM final concentration (this is 1000X stock)
- 128.7 mg in 10 ml
- Filer sterilize in hood with a 0.22 µcron filter, aliquot in 500 µl tubes and store at -20°C

$CaCl_2$ Calcium chloride dihydrate ($CaCl_2$ H_20 MW = 147.0)

- Dissolve 29.4 g $CaCl_2$ in 80 ml H_20, then add H_20 to a total volume of 100 ml
- Filer sterilize in hood with a 0.22 µcron filter, aliquot in 1.5 ml tubes and store at -20°C

2X HBS
HBS is 50 mM HEPES, 10 mM KCl, 12 mM glucose, 280 mM NaCl, and 1.5 mM Na_2HPO_4.

Prepare 0.5 M Potassium Chloride (KCl MW = 74.55)

- Dissolve 1.863 g KCl in 30 ml H_20, then add H_20 to a total volume of 50 ml.

Prepare 0.1 M dibasic sodium phosphate (Na_2HPO_4 MW = 142.0)

- Dissolve 0.710 g Na_2HPO_4 in 30 ml H_20, then add H_20 to total volume of 50 ml.

These reagents can be stored at RT for several years

Dissolve in 80 ml H_20
- 1.192 g HEPES
- 0.216 g glucose
- 1.636 g NaCl

Add
- 2 ml 0.5 M KCl
- 1.5 ml 0.1 M Na_2HPO_4

Calibrate pH meter (until you have a stable reading – **VERY CRITICAL**)

Adjust pH to 6.90 using freshly prepared 1 N and 0.1 N NaOH. Then add H_20 until volume is 100 ml. Remove 20 ml to a fresh tube, and adjust pH to 6.95 using 0.1 N NaOH (I usually add 5 µl at a time). Remove another 20 ml aliquot and adjust pH to 7.0. Repeat this process every 0.05 pH units until reaching pH 7.1. Filer sterilize in hood with a 0.22 µm filter, and aliquot in 2-3 ml aliquots and store at -20°C.

Each time you make a fresh batch of HBS you need to test the transfection efficiency with each different pH. I usually throw out all conditions where the transfection efficiency is < 50%.

Sterile DNA Preparation

1. Aliquot desired DNA volume into a 1.5 ml centrifuge tube
2. DNA precipitation is done by adding 0.1 volume of 3M sodium acetate to DNA (invert 2-3 times to mix).
3. Add 2 volumes of 100% ice cold ethanol, and mix gently.
4. Place the sample at -20°C for at last 30 min (it can be placed O/N).
5. Centrifuge sample at 16,000 x g for 30 minutes at 4°C.
6. Carefully remove supernatant, and wash pellet with 1 ml of ice cold 70% EtOH.
7. Centrifuge sample for 15 min at 16,000 x g.
8. Remove supernatant and air dry pellet in the hood.
9. Dissolve DNA in sterile 10 mM Tris HCl at a concentration of > 0.5 ug/µl (assume 50% recovery).
10. Determine DNA concentration by UV absorbance. I usually run DNA out on a gel to ensure that the concentration is correct and that the DNA is supercoiled.

Transduction Protocol

1) Plate target cells at appropriate density in 6 well plates coated with 1ug/cm^2 of poly-d-lysine (for 293T cells I use 4.0 X 10^5) the day prior to transduction.
2) Aspirate wells and replace with fresh media (2 ml for each well). Add 2 ul of 6 mg/ml of polybrene to each well. Mix briefly by rocking back and forth a few times.
3) Add between 0.5 and 5 µl of concentrated virus to each well and incubate at 37°C/5%CO_2 for 10 minutes.
4) Spin at 1500 x g in centrifuge for 1 hr at 32°C.
5) Aspirate media and refeed with 2 ml of fresh growth media.
6) Incubate cells for 48 hr, and then determine % positive cells using desired method.