Expression of Recombinant Proteins in human cells (Ponnambalam Lab, Leeds)

1) The easiest system for expression of mammalian proteins is the human embryo kidney cell line 293. The line, HEK-293, also has a derivative called HEK-293T that stably expresses the SV40 T-antigen: this causes the effective replication of episomal plasmids in the line. This would of course be dependent on SV40-derived viral sequences within the plasmid that contribute to T-antigen recognition. These cells produce 95-100% transfection efficiency and this has been checked and verified using GFP constructs.

2) HEK-293 cells can be obtained from commercial companies, ATCC, ECACC and the Ponnambalam lab in Leeds. These cells are very easy to maintain but require standard tissue culture conditions including a Category I tissue culture hood and facility, a 5-10% CO₂ cell culture incubator and standard tissue culture plastic, etc.

3) HEK-293 cells grow very easily in most media; we use High Glucose DMEM (Invitrogen) with 10% FCS, glutamine, non-essential amino acids and penicillin/streptomycin. Grow the cells in 10 cm dishes and split at 1:10 every 2-3 days. The cells are semi-adherent and come off the surface easily; trypsinisation is not needed. We usually aspirate off the medium, rinse the cell gently with PBS/2 mM EDTA and wait a few minutes. Then the cells are rinsed and removed off the surface using 5 mls of fresh media. The cells are then pipetted up and down 15-20x with a 10 ml pipette to generate a single cell suspension. 0.1x vol. of cells is added to a fresh 10 cm dish containing 8-10 mls of fresh media and cells gently distributed evenly over the surface by swirling a few times.

Molecular biology considerations

4) Most generic mammalian expression systems or plasmids function in the HEK-293 system e.g. pTriEx, pCDNA3, pCMV5, pEGFP, Gateway plasmids, etc. We have found efficient expression with pCDNA3 systems and this or pTriEx is a reasonable starting point.

5) Clone your favourite protein (yfp) as a HexaHis-tagged protein into a mammalian expression vector. There is also a system to express a GST-yfp protein; however, cleavage of the GST moiety is probably needed for structural studies. Additionally, an N-terminal GST is probably not effective for secreted proteins or things that are targeted or moved across membrane bilayer systems within the cell. Mammalian GST fusion constructs work best when fused to a cytosolic protein of interest I think but there may be many exceptions.

6) Confirm your plasmids by restriction digestion, sequencing etc. Ensure that there is an effective Kozak sequence (ribosome-binding site) just upstream of the ATG in your plasmid. Many people ignore this (especially when using home-made vectors) and end up with pretty crap expression!

7) Make supercoiled DNA maxipreps using CsCl-based protocols or commercial resin-based kits (Qiagen, etc.). Plan to use 5-25 ug of plasmid DNA per 10 cm dish. Titration of plasmids may be required to work out the best expression per unit DNA added. I usually find that 10 ug of DNA per 10 cm dish gives 100% transfection; however, levels of expressed protein may increase with DNA added.
Transfection

8) On the day of transfection, split each dish 5-fold to approximately 10-20% confluency. That is, after resuspending the cells, redistribute the cells into 5 x 10 cm dishes (or less if needed)
- 1 ml of resuspended cells + 9 mls of fresh medium
- swirl to distribute cells evenly

9) The cells will be transfected after 1-2 hrs in the incubator. Thaw the 2X HBS at room temperature and make sure it is warmed to ~20°C before use.

10) 10-20 mins before transfection (adding the DNA to the cells), prepare the following (for each 10 cm dish)
- prepare sterile 15 ml tissue culture tubes in the lab
- DNA + sterile water to a final volume of 439 ul (5-25 ug DNA)
- mix by gently vortexing
- add 61 ul of 2M Calcium chloride
- mix by vortexing
- add 500 ul of freshly thawed 2X HBS drop-wise with constant vortexing using a P1000 Pipetman; this generates a fine calcium phosphate-DNA ppt

11) Take tubes to tissue culture hood and add DNA drop-wise and in an even manner over the surface of the dish of HEK-293 cells.
**DO NOT SWIRL OR AGITATE THE DISH AT ALL.**

12) Transfer the dish of cells back to the incubator carefully.

13) 20-24 hrs later (next day), carefully aspirate off the medium. Gently add 10 mls of pre-warmed medium to the dish.

14) 20-24 hrs after washing the cells (48 hrs post-transfection) process either the cells, medium or both for expression of recombinant proteins.

*This system can produce 5-50 ug of protein per 10 cm dish. Multiple dishes (10-30) may be needed to generate 1-10 mg of protein for biochemical or structural studies.

Solutions

**2M Calcium Chloride**
Check that pH is near neutral and filter through a 0.2 um filter unit and store in sterile 50 ml tubes at 4°C.

**2X HBS**
16 g NaCl per litre
0.4 g Na₂HPO₄·7H₂O (can use dibasic salt as well; important thing is that final phosphate conc. is 1.5 mM)
13 g Hepes
dd water to 1 litre

pH the solution to 7.0 carefully using a freshly calibrated pH electrode
Filter the solution using a 0.2 um filter unit and store in 5-10 ml tube aliquots at -20°C
Discard each aliquot of 2X HBS after thawing and use in a transfection