OPPF-UK Standard Protocols:

Mammalian Expression

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OPPF-UK

OXFORD PROTEIN PRODUCTION FACILITY - UK

Table of Contents

1. Materials

DMEM	Sigma
Non-essential amino acids	
Glutamine	
Foetal calf serum	
Trypsin solution	
T75	Corning
T175 flasks	Corning
Bambanker	Merck
24-well plates	Greiner
Vector	pTriEx or pTT-based vector (e.g. pOPINF, pOPING, pOPINTTG)
PCR plate	
GeneJuice	Merck
Roller bottles	Greiner CellMaster
Megaprep kit	Invitrogen HiPure PureLink

Media Preparation

For each 500 ml bottle of DMEM, add 5 ml of non-essential amino acids and 5 ml of glutamine. Then add Foetal calf serum (FCS) to the required concentration.

2. Cell Maintenance

Passaging cells – T75 to T75

1. When flask is 70-90% confluent split cells
2. Remove old media.
3. Add 6ml Trypsin solution and wait 5mins until cells have dissociated.
4. Add 6ml DMEM with 10% FCS. Resuspend well by pipetting without aerating too much.
5. Put 3ml cell solution into a new flask and add 22ml DMEM with 10% FCS. A 1:4 split.
6. Put 1.5ml of cell solution into a new flask and add 24ml DMEM with 10% FCS. A 1:8 split which is used as backup.
7. (Can split further 1:20 or 1:30 depending how fast cells are growing.)

Passaging cells – T175 to T175

1. Remove old media.
2. Add 10ml Trypsin solution and wait 5mins until cells have dissociated.
3. Add 10ml DMEM with 10% FCS. Resuspend well by pipetting without aerating too much.
4. Put 5ml of cell solution into a new flask and add 40ml DMEM with 10% FCS. A 1:4 split.

Expanding cells – T75 to T175

1. Remove old media.
2. Add 6ml Trypsin solution and wait 5mins until cells have dissociated.
3. Add 6ml DMEM with 10% FCS. Resuspend well by pipetting without aerating too much.
4. Put 5ml of cell solution into a T175 flask and add 40ml DMEM with 10% FCS.

Freezing cells

1. Trypsinize the cells from the flask as above.
2. Spin the cells at 100g for 5-10 minutes and remove the supernatant.
3. Resuspend the cells in the volume of Bambanker or 90% FBS/10%DMSO mix, to give 3-4*10^6 cells/ml.
4. Add 1.5 ml of suspension to each cryovial.
5. Either freeze in a Mr. Frosty container (Nalgene) or place straight into -80°C but this does kill more cells (slower freezing, moving from 4°C to -20°C to -80°C over a few hours or days is better.)
6. Either keep in the -80°C freezer or transfer to liquid nitrogen for long term storage

Thawing cells

1. Prepare 25 ml DMEM with 10% FCS in a T75 flask.
2. Rapidly thaw a vial of cells in a water bath at 37°C
3. Add the full contents of the vial to the prepared flask
4. Incubate at 37°C in a 5% CO_2/95% air environment.
5. When the cells are 70-90% confluent, passage the cells.

3. 24-Well Transient Expression Screen

Expression screen - Preparation

1. Pipette 1 ml of cells (at 1.5-2*10^5 cells/ml) into each well of 24-well plates.
2. Incubate at 37°C overnight in a 5% CO_2/95% air environment (cells should be ~70% confluent next day for transfections).

Expression screen - Manual protocol

1. One column at a time, carefully aspirate the media from the cell layer in the 24-well plate and discard. Then add 1 ml DMEM with 2% FCS before proceeding onto the next column.
2. Set up transfection reactions in a v-bottomed 96-well PCR plate. Aliquot ~1 µg of plasmid DNA per well and mix with 60 µl serum-free DMEM and 2 µl of 1.33 mg/ml GeneJuice. Mix thoroughly and incubate for 10 min at room temperature. (NB. A master mix of DMEM and Genejuice can be prepared instead of adding separately.)
3. Add the DNA/GeneJuice cocktail to the cells and incubate at 37°C in a 5% CO_2/95% air environment for 3 days.

Expression screen – Robot protocol using the Tecan Evo75 or similar

1. Pipette 10 µl of DNA at 0.2 µg/µl into each well of a 96 well PCR plate.
2. Follow the steps within the robot software. The Tecan robot essentially follows the same protocol as above.
3. Incubate the cells at 37°C in a 5% CO_2/95% air environment for 3 days.

Expression screen – Analysis

For secreted products:

1. Pipette the supernatant from the cells either into a 1.5 ml eppendorf tube or a 96 deep-well block.
2. Centrifuge for 15 min at 6000g.
3. Take 20 µl of supernatant and mix with 20 µl of SDS-PAGE sample buffer.
4. Run a Western blot of the products comparing the expression yield with a known positive control. (OPPF routinely use anti-His monoclonal antibody from R+D MAB050 as most of our suite of vectors are His-tagged.)

For intracellular soluble products:

1. After removing the supernatant, freeze the 24-well plate of cells at -80°C for at least 30 mins.
2. Defrost the plates for 10 minutes, then add 150 µl of lysis buffer (NPI-10 + DNase + protease inhibitors) to each well.
3. Pipette the mixture up and down to detach the cells or shake the plate at 300rpm for 30 mins at room temperature.

4. Transfer the solution to a 96-deep well plate. Centrifuge at 6000g for 30 minutes.
5. Take 20 µl of supernatant and mix with 20 µl of SDS-PAGE sample buffer.
6. Run a Western blot of the products comparing the expression yield with a known positive control. (OPPF routinely use anti-His monoclonal antibody from R+D MAB050 as most of our suite of vectors are His-tagged.)

For membrane protein products:

1. After removing the supernatant, freeze the 24-well plate of cells at -80°C for at least 30 mins.
2. Defrost the plates for 10 minutes, then add 150 µl of lysis buffer (NPI-10 + DNase + protease inhibitors) + 1% n-Dodecyl β-D-maltoside (DDM) to each well.
3. Shake the plate on an orbital MTP shaker (~700 rpm for 60 minutes at 4°C)
4. Transfer the solution to a 96-deep well plate. Centrifuge at 6000g for 30 minutes.
5. If GFP fusions are being tested a sample of the cleared lysate can be analysed by in-gel fluorescence.
    a. Transfer 10 µl of the cleared lysate to a microtitre plate and add 10 µl of SDS PAGE gel loading buffer (100 mM Tris, pH 6.8, 4% w/v SDS, 0.2% w/v Bromophenol blue, 10% v/v β-mercaptoethanol, and 20% v/v glycerol). **CAUTION! DO NOT BOIL THE SAMPLE.**
    b. Load 10 µl of this onto SDS PAGE gel(s) and run at 100-120 V (constant voltage) in a cold room (4°C) until the dye front reaches the bottom (2-2.5 hr).
    c. Place the gel onto an imager (e.g. with a blue-light filter to detect the GFP fusion proteins). The exposure time is chosen to ensure that the brightest bands are not saturated.
6. If there is no GFP tag run a Western blot as above – gels can be run as normal (200V at room temperature) but **DO NOT BOIL THE SAMPLE.**

Western blot protocol (or any standard Western blot protocol):

1. Transfer the protein from PAGE gel to a nitrocellulose membrane using iBlot or other blotting device following the Manufacturer's instructions.
2. Take the membrane and block using 5 % milk powder in PBST for 30 minutes at room temperature (can be left overnight preferably at 4°C).
3. Remove the blocking buffer and incubate the membrane in Primary antibody (eg Anti-His from R&D MAB050) using the manufacturer's dilution factor into PBST. Incubate for 1 hour at room temperature.
4. Wash the membrane 3 times using PBST for 5 minutes for each wash.
5. Remove the wash buffer and incubate the membrane in Secondary antibody (If the R&D Anti-His was used then use an Anti-mouse HRP antibody) using the manufacturer's dilution factor into PBST. Incubate for 1 hour at room temperature.
6. Wash the membrane 3 times using PBST for 5 minutes for each wash.
7. Remove the liquid and incubate the membrane in ECL reagent (eg ECL Prime from GE Healthcare) following the manufacturer's instructions. For ECL prime, this is 5 minutes.
8. Visualize the blot using chemiluminescence detection on an imager or use film.
9. NOTE: Blots can be developed with coloured reagents instead of ECL although ECL is more sensitive.

4. DNA Megaprep for Largescale Transient Transfections

Invitrogen PureLink HiPure Megaprep

1. Defrost the pellet for DNA (from 1 L *E. coli*).
2. Transfer the pellet to a bottle and add 100 ml Resuspension buffer (R3 + RNaseI). Resuspend the pellet thoroughly.
3. Add 100 ml Lysis buffer (L7). Mix by inverting gently and incubate at room temperature for 5 minutes.
4. Add 100 ml Precipitation buffer (N3). Mix by inverting gently.
5. Load the lysate onto a Lysate filter cartridge screwed onto 500mL Duran bottle. Apply the vacuum until the lysate has passed through the filter.
6. Add 50 ml Wash buffer (W8) to filter. Stir and apply the vacuum as before. Keep the filtered lysate.
7. Screw the DNA binding cartridge onto a clean 1L bottle and add 100 ml Equilibration buffer (EQ1). Apply the vacuum and discard the flow through.
8. Apply the filtered lysate to the DNA binding cartridge and apply the vacuum until all the lysate has passed through the filter.
9. Add 175 ml Wash buffer (W8) to the cartridge and apply the vacuum until all the liquid has passed through the filter.
10. Repeat step 9.
11. Screw the filter onto a clean 250 ml duran bottle and add 50 ml Elution buffer (E4) to the cartridge. Apply a SOFT vacuum until 10-20 ml of liquid has gone into the cartridge. Turn off the vacuum and stand for 1 minute.
12. Apply a full vacuum until all the liquid has passed through the filter.
13. Repeat elution steps 11 and 12 into same bottle.
14. Transfer the liquid into a 500 ml centrifuge bottle and add 70 ml of isopropanol (0.7X volume). Centrifuge at 6000g for 90 minutes.
15. Carefully remove the isopropanol solution from the DNA pellet.
16. Add 13 ml 70-80% ethanol to tube. Resuspend pellet and transfer to 14ml Falcon tube to wash the pellet. Centrifuge at 6000g for 90 minutes.
17. Carefully remove the ethanol and air-dry the pellet for 10-20 minutes.
18. Resuspend the pellet in 0.5 ml TE and transfer to Eppendorf tube. Repeat by washing out Falcon tube with further 0.5ml TE. Quantify and add more TE buffer to make approximately 1 mg/ml DNA solution. Record the concentration and A260/280 ratio for the DNA.

5. Roller Bottle Transient Expression

Roller Bottle Preparation

1. Pipette 20 ml of cells (a whole 175cm^2 flask) into each roller bottle.
2. Add 250 ml of DMEM with 10% FCS per roller bottle.
3. Incubate at 37°C for 3 days in the roller rig.

NB. Need 4 roller bottles for our standard 1L scale up

Transient Transfection

1. Remove the old media from the roller bottles.
2. Add 200 ml of DMEM with 2% FCS and return to the incubator for 10 minutes.
3. In one sterile container (250ml Erlenmyer flask is convenient for this), add 100 ml serum-free DMEM and 2 mg of DNA. Mix thoroughly.
4. In a second sterile container (250ml Erlenmyer flask is convenient for this), add 100 ml serum-free DMEM and 3.5 ml 1mg/ml PEI. Mix thoroughly.
5. Combine the DNA and PEI solutions and incubate at room temperature for 10 minutes AND NO LONGER. Efficiency drops if the complex is left too long.
6. Add 50 ml of transfection cocktail to each of the four roller bottles and return to the incubator.
7. Grow for 5 to 6 days until the media starts to turn orange (after the end of the course).
8. Harvest secreted products by removing the media from the roller bottles and centrifuging at 6000g for 20 minutes.
9. Then filter the supernatant through a 0.45 µm bottle top filter.
10. Store the supernatant at 4°C until purification.

6. Purification of Secreted Products

This protocol is for an Äkta Xpress platform using programmes described in Nettleship *et al. Methods Mol Biol.* **2009**;498:245-63. This article also includes a transcript of the "Mammalian Affinity Gel Filtration" programme.

1. Equilibrate the gel filtration column using the "Gel Filtration Equilibration" programme and the following buffer: 20 mM Tris, pH 7.5, 200 mM NaCl.
2. Follow the method "Mammalian Prepping System" to set up the system.
3. Run the method "Mammalian Affinity Gel Filtration".
4. Leave the protocol to run overnight.
5. Using the trace from the Äkta select the correct fractions containing the protein of interest.
6. Mix 10 µl of protein solution with 10 µl of sample buffer and boil for 3 minutes.
7. Run an SDS-PAGE gel of the samples, staining using Instant Blue.
8. Clean the Äkta by running the programme "Mammalian Cleaning System".
9. Then run the "Gel Filtration Equilibration" programme using water.
10. Concentrate the protein to the required concentration for the next application, e.g. crystallisation.

7. Formation of Stable Cell Lines

This protocol is for production of stable HEK 293S cell lines using G418 selection.

Transfection and Selection

1. Seed HEK 293S cells in DMEM with 10% FCS in a T75 flask so that the following day they are approximately 70 % confluent (i.e. ~ $2x10^5$ cells/ml in 25 ml).
2. Prepare transfection mix by diluting 75 µl of Genejuice in 2 ml serum-free DMEM. Incubate for 5 min at RT and then add 25 µg of DNA (Qiagen midiprep). Incubate for further 20-30 min at RT.
3. Replace media on cells with 50 ml fresh DMEM with 10% FCS and then slowly add transfection mix to the cells. Mix by gentle rocking.
4. Incubate overnight at 37°C in a 5% CO_2/95% air environment.
5. Remove cells by adding 5 ml of trypsin-EDTA and incubating for 5 min at 37°C and then adding 45 ml DMEM with 10% FCS to inhibit the trypsin. Spin down the cells and resuspend in 25 ml fresh DMEM with 10% FCS. Count the cells – there should be approximately 1x 10^^{7} in total vol. of 25 ml (i.e. 400 cells/µl).
6. Dilute the cells 1:5 in DMEM with 10% FCS by adding 10 ml of cell suspension to 40 ml media in a Falcon tube.
7. Dilute these cells again by 1:5 by adding 10 ml of the cell suspension from step 6 to 40 ml of media in a 50 ml Falcon tube (i.e. 1:25 of original cell suspension).
8. Repeat with a third dilution - again by 1:5 by adding 10 ml of the cell suspension from step 7 to 40 ml of media in a 50 ml Falcon tube (i.e. 1:125 of original cell suspension).
9. Plate 100 µl cell suspension per well into 96-well flat-bottomed tissue plate as follows:
    a. Diluted 1:5 plated 100 µl/well into 2 x 96 well plate
    b. Diluted 1:25 plated 100 µl/well into 2 x 96 well plate
    c. Diluted 1:125 plated 100 µl/well into 2 x 96 well plate
10. The following day add G418 to a final concentration of 0.8 mg/ml by adding 100 µl of 1.6 mg/ml to each well of the 96-well plates (**except for well A1 which serves as an untreated reference**).
11. Leave plates to incubate at 37°C in a 5% CO_2/95% air environment.

Picking colonies into 24 well plates

By 10 days after G418 antibiotic selection all non-transfected cells will die off and after approximately another week, discrete colonies should begin to be visible. By approximately three weeks after antibiotic selection, single colonies can begin to be picked into 24 well plates. Only pick from wells with single colonies. Mark these by examining the plates under the microscope.

1. Remove spent media from wells and add 25 µl trypsin-EDTA. Incubate for ~5 min at 37°C, then add 200 µl DMEM with 10% FCS without G418 and pipette into a 24-well plate. Top up the well with 0.5 ml media and incubate at 37°C in a 5% CO_2/95% air environment.
2. Continue picking colonies from 96 into 24 well plates. Since clones grow at different rates aim for a total of approximately 50 colonies picked over a 1-2 week period.

3. When cells in the 24-well plates are confluent (media turns yellow) expand into 6-well plates. Remove media from 24-well plates and save for expression testing - centrifuge at 12,000 rpm for 10 min. Store the supernatant at 4°C for analysis by Western blot.
4. Wash the cells in the 24-well plate with PBS. Detach the cells with 0.2 ml trypsin-EDTA and then added 1 ml media. Transfer to 6-well plate and make up to 3 ml with media.
5. When the 6-well plates are confluent, screen for expression by Western blotting and identify clones for expansion to T25 flasks. It is convenient to accumulate 24 clones for screening, therefore depending upon the rate of cell growth, cells may need to be expanded before screening.
6. Remove the media from the wells of the 6-well plate, wash with 2 ml of PBS and then add 0.5 ml Trypsin-EDTA. Added 2.5 ml media aspirated cells into a T25 and top up with 12 ml media.
7. From confluent T25, freeze down 1 vial of cells and expand selected lines to T75 flask depending upon the screening results. The length of time from transfection to freezing down a selection of cell lines expressing the product is approx. 6 weeks. Aim to freeze down up to 10 cell lines with a view to focusing on 2-3 cell lines at T75 flask stage. At this scale (50 ml of media) protein expression yields from these cell lines can be measured quantitatively and 3-4 vials of cells frozen down.