OPPF-UK Standard Protocols:

Cloning and Expression Screening

Last Updated 26 July 2012

Louise Bird

louise@strubi.ox.ac.uk

OPPF-UK

OXFORD PROTEIN PRODUCTION FACILITY - UK

Table of Contents

1. Primer Design - Information

Primers are designed for In-Fusion™ cloning, depending on the fusion tag required in the resulting protein, using the following primer extensions:

Vector	Fusion Tag	Parent Vector/ Antibiotic resistance	Forward Primer Extension	Reverse Primer Extension
pOPINA	…KHHHHHH tag	pET28a/Kanamycin	AGGAGATATACCATG	GTGGTGGTGGTGTTT
pOPINB	MGSSHHHHHHSSGLEVLFQ⓫GP … tag	pET28a/Kanamycin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPINE	…KHHHHHH tag	pTriEx2/Ampicillin	AGGAGATATACCATG†	GTGATGGTGATGTTT†
pOPINF	MAHHHHHHSSGLEVLFQ⓫GP… tag	pTriEx2/Ampicillin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPINJ	MAHHHHHHSSG-*GST*-LEVLFQ⓫GP… tag	pTriEx2/Ampicillin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPING	MGILPSPGMPALLSLVSLLSVLL MGCVA⓬ETG… cleavable secretion leader and …KHHHHHH tags	pTriEx2/Ampicillin	GCGTAGCTGAAACCGGC	GTGATGGTGATGTTT
pOPINH	MGILPSPGMPALLSLVSLLSVLLMGCV A⓬ETMAHHHHHHSSGLEVLFQ⓫GP …… cleavable secretion leader and cleavable N-his tag	pTriEx2/Ampicillin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPINI	MAHHHHHHSSG… tag	pTriEx2/Ampicillin	ATCATCACAGCAGCGGC	ATGGTCTAGAAAGCTTTA
pOPINK	MAHHHHHHSSG-*GST*-LEVLFQ⓫GP… tag	pET28a/Kanamycin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPINM	MAHHHHHHSSG-*MBP*-LEVLFQ⓫GP… tag	pTriEx2/Ampicillin	AAGTTCTGTTTCAGGGCCCG‡	ATGGTCTAGAAAGCTTTA‡
pOPINS	MGSSHHHHHH-*SUMO*⓬… tag	pET28a/Kanamycin	GCGAACAGATCGGTGGT	ATGGTCTAGAAAGCTTTA

Table 1 Details of commonly used pOPIN vectors.

Where ⓫ represents the 3C protease cleavage site and ⓬ represents the cleavage sites for either signal peptidase or the specific SUMO protease. Consult the pOPIN Guide for more details on fusion tags and vectors.

Generally the primers are designed with a Tm ≥ than 68°C on the target gene and are ordered in 96-well format such that the forward primer plate co-ordinates match the reverse primer plate co-ordinates for each planned construct. If possible the oligo supplier should normalize the oligo concentrations or oligo amount/well for you, most suppliers will do the normalization step at no extra cost. Normalization to 100µM (10nmoles/well resuspended in 100ul of buffer) is convenient and a simple 1 to 10 dilution is required to prepare them for HTP PCR.

Highly purified (e.g. PAGE purified) oligos are not necessary for In-Fusion™ expression cloning due to the short primer lengths (normally less than 45 bases).

When using plasmids as templates it is advisable to prepare fresh aliquots for HTP PCR and cloning. If only a small number (24 or less) templates are required for the whole plate, then these may be prepared on standard Qiagen spin columns and aliquotted into the appropriate positions (i.e. those corresponding to the forward and reverse primer plate positions) of a template plate. For larger numbers of template it is recommended that the plasmids are transformed, grown and mini-prepped in the appropriate 96-well format to eliminate later pipetting errors.

2. Setting up HTP PCR Reactions

Make up the 100µM master stocks of primers, we use EB (10mM Tris pH8.0) or nuclease free water. Dilute the master stocks 1 to 10 with EB (10mM Tris pH8.0) or nuclease free water to make the working stocks we do this in a green PCR plate to aid identification when the plate is stored in the freezer. (*N.B. do not use buffers containing chelating agents such as EDTA as these will inhibit the Mg^{2+}-dependent activity of the DNA polymerases to be used).*

Making a 'master mix' of all the common reagents is convenient and reduces the possibility of pipetting errors.

1. Prepare a Master Mix on ice according to the total number of reactions required:

Number of (50µl) Reactions	1	25	50	100
2 X KOD Hot Start Buffer (µL)	25	625	1250	2500
dNTP mix (2mM)	10	250	500	1000
KOD Hot Start (1 U/µl)	1	25	50	100
Sterile Water (µL)	6	150	300	600
Total Volume (µL)	42	1050	2100	4200

Table 2 KOD Xtreme™ Hot Start DNA Polymerase (Novagen 71975-3) Master Mix.

   Mix thoroughly before dispensing.

2. Set up the reactions (on ice) in the PCR plate as follows:
   1. Using the Multi-Dispense Pipettors with a 1ml syringe tip dispense 42µl of this Master Mix into each well of the PCR plate.
   2. Using a 10 or 20µl Multi-Channel Pipettes transfer (in columns) 3µls of diluted (10µM) forward primer to the appropriate wells of the PCR plate.
   3. Using a 10 or 20µl Multi-Channel Pipettes transfer (in columns) 3µls of diluted (10µM) reverse primer to the appropriate wells of the PCR plate.
   4. Using the 10 or 20µl Multi-Channel Pipettes transfer (in columns) 2µls of template plasmid (~10-20ng/ul) to the appropriate wells of the PCR plate. *N.B. If it is one template in all the plate add the DNA to the master-mix, similarly if there are two make up two master mixes one for each template*.
   5. Seal the plates with a clear adhesive film or foil seal and load into the Veriti PCR machine (ABI).
3. Perform the thermal cycling using these parameters:

   94°C 2 minutes

   98°C 10 seconds

60°C 30 seconds

68°C x minutes (base this time on the largest PCR product expected and 1min/kbp)

Go to Step 2 29 times

68°C 2 minutes

4°C Hold

4. If another round of PCR is needed then we use Phusion Flash Master Mix (F-548L, Thermoscientific).

Number of (50µl) Reactions	1	25	50	100
2 X Phusion Flash Master Mix (µL)	25	625	1250	2500
Sterile Water (µL)	17	425	850	1700
Total Volume (µL)	42	1050	2100	4200

Table 3 Phuson Flash (Thermoscientific F-548L) Master Mix.

The reactions are set up as in point 2 above. The thermal cycling parameters are as follows:

98°C 10 seconds

98°C 0 or 1 second

60°C 5 seconds

72°C x minutes (base this time on the largest PCR product expected and 15 sec/kbp)

Go to Step 2 29 times

72°C 2 minutes

4°C Hold

3. Analysis of PCR products

1. When thermal cycling is complete, use a 20µl MCP to add 2.0 µl of 5x DNA Loading buffer to each well of a new PCR plate or the spare wells of your existing plate.
2. Using a 10 or 20µl MCP add 5µl aliquots of each product to the dye and load onto a 1.25% TBE Agarose gel a column at a time. Mix each column of samples with a single aspirate/dispense of the MCP before transferring to the gel.
3. Run the gel at 100V for ~30 minutes or until the Bromophenol Blue from one lane nears the loading wells of the next column.
4. Image the gel.

Information:

1. DNA Loading Buffer: 0.25%w/v Bromophenol Blue in 30%v/v Glycerol.
2. The Electro-Fast *Stretch* 108 systems (ABGene AB-0708) are relatively inexpensive and although they have a short run length they do allow the conservation of the 8X12 well format of the reaction plate. The total gel volume of each gel is 100ml.
3. A 1.5% TBE agarose gel with 5µl of Hyperladder I (BioLine BIO-33025/BIO-33026) as reference, is good for most products but if a large proportion of the plate contains products that are of less than 500b.p. then 1.6-2% may be more appropriate, with Hyperladder II (BioLine BIO-33039/BIO-33040) as reference.
4. The use of SYBRSafe stain (InVitrogen S33110/S33100 depending on size) in the gel in place of Ethidium Bromide is also recommended - this can be viewed with normal UV illumination/EtBr camera filters. If you are gel-purifying your PCR products then a blue light illuminator is highly recommended to avoid UV damage.

4. Purification of PCR products by AMPure XP Magnetic Bead Purification

If PCR products are of good quality (i.e. few multiple bands and 'smeared' products) across the plate then you may use the simple AMPure magnetic-bead based purification step. If a significant number of the products contain multiple bands or are 'smeared' then gel purification and extraction is advisable.

Information:

*N.B If this purification protocol is used and the PCR template has the same antibiotic resistance as your target pOPIN vector you **must** DpnI treat your PCR reaction.*

The DpnI enzyme has specificity for methylated DNA and will digest your template without digesting your PCR product. The DpnI enzyme is active in most PCR reaction buffers and can therefore simply be added (0.5µl/5 Units from most suppliers).

[Optional] DpnI treatment procedure:

1. Make up a master mix in 1X cutting buffer.
2. Add 5µl (containing 5 units DpnI) with a repeat pipettor to each reaction.
3. Incubate at 37°C for 30-60 minutes prior to running the AMPure purification procedure.

AMPure purification procedure:

1. Gently shake the AMPure XP bottle (Agencourt/Beckman Product Numbers A63881 or A63882 depending on size) to resuspend any magnetic particles that may have settled. Using a 200 or 300µl MCP and reservoir pipette 90µl AMPure into each reaction in the PCR plate to be cleaned.
2. Mix the AMPure XP and PCR reaction thoroughly by pipette mixing 10 times using a fresh set of MCP tips for each column. The color of the mixture should appear homogenous after mixing. Let the mixed samples incubate for 3-5 minutes at room temperature for maximum recovery.

   This step binds PCR products 100bp and larger to the magnetic beads.
3. Place the reaction plate onto a SPRIPlate 96R magnet (Beckman Product Number 000219) OR Bilatest M96 Small Volume (Bilatec Product Number 209606) for around 5 minutes to separate beads from solution. Wait for the solution to clear before proceeding to the next step.

   This step separates the bead-bound PCR products, 100bp and larger, from un-bound PCR reagents and primers.

4. With the reaction plate still situated on a magnet use a 200 or 300µl MCP and a fresh set of MCP tips for each column to aspirate the cleared solution from the reaction plate and discard. Do not disturb the separated magnetic beads.

5. With the reaction plate still situated on a magnet dispense 200 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes. Do not disturb the separated magnetic beads. On the second discard be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants. It can be helpful at this point to use a fresh set of tips for each column to remove the residual ethanol.

6. The plate should be left to air-dry for 10-20 minutes on a bench top to allow complete evaporation of residual ethanol.

7. Add 30 µL of elution buffer (EB 10mM Tris pH 8.0) to each well of the reaction plate and pipette mix 10 times. Elution is quite rapid and it is not necessary for the beads to go back into solution for complete elution to occur.

8. With the reaction plate still situated on a magnet use a 200 or 300µl MCP, and a fresh set of MCP tips for each column, to transfer the 30µl supernatant from each well to a fresh **purple** PCR Plate. There will probably be a small amount of bead 'carry-over' but we have seen no inhibition of the In-Fusion™ reactions by the AMPure XP beads.

9. In a separate PCR plate mix 5 µL of purified PCR product with 2µLof DNA loading buffer and run on a gel as before.

5. In-Fusion Reactions and HTP Transformation into Cloning-Grade *E. coli*

1. Using a 10µl Gilson transfer 1µl (100ng) of the appropriate linearised pOPIN to each well of a PCR plate.
2. Using a 10µl or 20µl MCP Take x µl of purified insert (should equate to 10-250ng on average e.g. use only one volume per plate) and add to the appropriate wells of the PCR plate. Add 9-x µl of water to each well and transfer the whole 10µl to a dry-down In-Fusion™ plate. Mix contents briefly by pipetting up and down - take care: the lyophilized enzyme/buffer pellet may not re-suspend instantly and can stick to the pipette tips. Transfer the reactions to the PCR plate using 10µl or 20µl MCP and seal plate with a foil seal (PCR).*

   This is because the Infusion plate does not fit very well into the PCR machine.
3. React for 30 minutes at 42°C in the Veriti thermocycler.

   10-15 minutes into this reaction thaw the competent cells on ice. The OmniMaxII competent *E. coli* (Invitogen) are sold aliquotted in loose tubes racked in 96-well (Matrix) format and stored at -80°C until required, aliquot size is 50µl/tube.
4. When the In-Fusion reaction is complete transfer the In-Fusion reactions to ice and dilute each reaction **IMMEDIATELY** by the addition of 40µl of TE (use the 200 or 300µl MCP). Transform **IMMEDIATELY** into OmniMaxII cells.
5. Using a 10 or 20µl MCP transfer 3-5ul of the diluted In-Fusion reaction per aliquot of competent cells.

   5µl of the diluted reaction should give many tens of colonies per well of a 24-well plate.
6. Incubate the cells on ice for 30 minutes.
7. Heat-shock the cells for 30 seconds at 42°C in the water bath.
8. Return the cells to ice for 2 minutes.
9. Using a 1200µl MCP add 300µl of Power Broth(PB)/SOC (with no antibiotic) per tube of cells. The use of PB/SOC here allows the cells to recover without shaking and this enables a concentrated aliquot of cells to be pipetted from the bottom of the tube for plating.
10. Transfer to 37°C incubator and incubate for 1 hour.

    Whilst incubating, prepare your LB Agar plates (x2 replicates for each experiment) as follows-you will need 1ml of the agar/antibiotic/X-gal/IPTG mix per well in 24-well plates (e.g. Corning/Costar 3524). Dilute the stocks appropriately as follows: Carbenicillin (Cb) 50mg/ml, 1:1000, or Kanamycin (Kan) 35mg/ml, 1:1000, 20% X-Gal [in DMF], 1:1000 , IPTG 500mM stock; 1:500 in warm agar in 50ml Falcon tubes before pouring.

    Pour the plates using a 50ml pipette and repeat pipettor, add 1ml of your molten LB Agar/well. Tip: to avoid bubbles on your plates never use the last ml of LB Agar from a pipette if you are using a pipette filler.

11. Immediately prior to plating add 20ul of PB/LB to each well of one of the set of plates. Using the Matrix IMPACT pipettors, pipette 30µl and transfer (4 at a time) 25µl of cells/well from the transformation reactions onto the LB Agar plates supplemented with Antibiotic, X-Gal and IPTG and 5µl of cells onto the plates with PB added.
12. Shake the plates vigorously by hand in an orbital motion, and allow at least 10-15 minutes for the plates to dry off before turning.
13. Incubate overnight at 37°C.

* this reaction volume may be reduced by splitting the contents of the of an In-Fusion™ enzyme well into 2 or more wells. Adjust dilution volume in step 4 accordingly. We routinely use reaction volumes of 3.3µl and have used 2.5µL.

6. Colony Picking and HTP Culture

At this point blue colonies should constitute <<10% if the reactions were successful. The blue colonies are derived from inefficiently linearised parental plasmid and are non-'recombinant'. Standard OPPF practice is to pick two white colonies per construct which will normally give us positive clones for ~96% of the PCR products.

Prepare deep-well blocks (BD Falcon 353966, ABgene AB-0932 or similar) by addition of 1.2ml of Power Broth supplemented with the appropriate antibiotic.

1. Using 200/300µl MCP tips pick individual colonies into each well, leaving each tip in the deep-well plate).

   Tip: Leaving the tips in the 'picked' well until the plate is complete is a good memory aid and prevents 'double picking'.

2. Replicate the pattern of your transformation plate to fill the mini-prep plate/s.
3. When plate is complete remove tips 8 at a time, using a MCP for convenience, and seal the plates with gas-permeable adhesive seals (ABgene AB-0718 or similar).
4. Shake the filled plates at 200-225 rpm at 37°C over-night in a normal shaker (NBS Innova; microplate holders for standard NBS Innova shakers are available-M1289-0700 but these may require some further modification) or 600 rpm in the floor standing Vertiga incubaters.

7. HTP Culture Harvesting and Glycerol Stock Preparation

1. Make a glycerol stock of all of the cultures by transferring 100µl from each well to an MTP (e.g. Greiner V-Bottom Microplate -651 261) containing 100µl/well of filter -sterilised LB/30%v/v glycerol, seal this plate and store at -80°C.

   Glycerol stocks are a very convenient and stable archive format and can save time if plasmids require re-prepping at later dates.

2. Replace the gas-permeable seal on each 96-well culture plate with a solid seal (e.g.ABGene AB-0558) and harvest the cells by centrifugation at 5000*g* for 15 minutes (the Beckman JS5.3 rotor for the Beckman *Avanti* centrifuges is ideal for this).

3. Decant the media to waste by inverting the plate over a large beaker and then rest the plate upside down on a wad of absorbent tissue to remove residual media (take care here as the pellets may not be tightly stuck to the blocks).

8. HTP mini plasmid preparation Using Wizard SV96 purification plates

The plasmid mini-preps are performed on the Bio-Robot 8000. N.B. The plates can be used manually on a vacuum manifold according to the manufacturer's protocols.

Plasmids prepared by this method are suitable for use in E.coli transformations, construct verification and are also of sufficient quality for HEK and/or Sf9 transfections.

9. Construct Verification (PCR screening)

Setting up HTP PCR Reactions

1. Master Mixes are prepared in a Universal tube:

	For 1x96
Number of (25µl) Reactions	**100**
10 X KOD Buffer#2 (µl)	**1250**
Sterile Water (µl)	**935**
pOPIN forward primer at 100µM (µl)	**15**
Total Volume (µl)	**2200**

Table 4 Master Mix for construct verification

   Mix thoroughly before dispensing.

2. Set up the reactions (on ice) in the skirted PCR plate as follows:
   a. Using the Multi-Dispense Pipettors with a 1ml syringe tip dispense 22µl of this Master Mix into each well of the PCR plate
   b. Using the 10 or 20µl Multi-Channel Pipettes transfer (in columns) 1.5µls of diluted (10µM) reverse primer to the appropriate wells of the PCR plate. Remember that you have to repeat this twice, once for each clone.
   c. Using the 10 or 20µl Multi-Channel Pipettes transfer (in eights/columns) 1.5µls of construct plasmid to the appropriate wells of the PCR plate.
   d. Seal the plates with a clear adhesive film or foil and load into the Veriti thermal cycler.
   e. Perform the thermal cycling using these parameters:

      94°C 2 minutes

      94°C 30 seconds

      60°C 30 seconds

      68°C 2 minutes (1 minute/kbp)

      Go to Step 2 29 times

      68°C 4 minutes

      4°C Hold

3. Analyse a 6.5µl aliquot of PCR product (as for the initial PCR) on an 1.5% Agarose gel. Score your colonies. The best way to load the gel is to add 6.5µl loading buffer to the well, mix and then load 6.5µl.

The pOPIN Forward primer is based on the T7 forward priming sequence and is present in most pOPIN vectors (**gac cga aat taa tac gac tca cta tag gg**).

Note the increase in PCR sizes with this primer (c.f. original gene-specific forward primers)-see table below and simplifies the screening process.

pOPIN Vector	Approximate Increase in PCR Product Size when using the T7 primer (b.p.)
pOPINA	110
pOPINB	130
pOPINE	170
pOPINF	225
pOPING	260
pOPINH	315
pOPINI	200
pOPINJ*	890
pOPINK*	790
pOPINM*	1330
pOPINS	400

Table 5 Approximate increase in PCR product size when priming with the T7 primer compared to the gene-specific primer.

*It may be worth considering a fusion-specific forward primer (i.e. MBP or GST specific primers) for these constructs to reduce the PCR product sizes.

Suitable pOPINJ/pOPINK-specific primer is:

pGEX5-FP: AACGTATTGAAGCTATCCC

Suitable pOPINM-specific primer is:

pMalE: TCAGACTGTCGATGAAGC

10. *E. coli* Expression Strain Transformations

E. coli expression strains are aliquotted in loose tubes racked in 96-well (Matrix) format and stored at -80°C until required, aliquot size is 50µl/tube. Thaw aliquotted *E. coli* on ice after first removing the rack base.

1. Using a 10 or 20µl MCP transfer, 8 at a time, 3µl of mini-prepped expression plasmids to the competent cell aliquots. Incubate on ice for 30 minutes.
2. Heat-shock the cells for 30 seconds at 42°C by placing the whole rack in the 42°C water bath. Make sure that the water level covers the competent cells in the racked tubes.
3. Return the cells to ice for 2 minutes.
4. Using a 1200µl MCP add 300µl of Power Broth (no antibiotic selection!) per tube. The use of Power Broth here allows the cells to recover without shaking and this enables a concentrated aliquot of cells to be pipetted from the bottom of the tube for plating.
5. Transfer to 37°C static incubator and incubate for 1 hour.
6. Prepare your LB Agar plates as follows - you will need 1ml/well for the 24-well plates. Dilute the antibiotic stock 1:1000 in warm agar in 50ml Falcon tubes before pouring.

   We use two strains B834(DE3) and Rosetta(DE3)LysS in order to retain the pRARELysS plasmid Chloramphenicol must be added to the media for the latter.

   Pour the plates as before.
7. Using the Matrix IMPACT pipettors transfer 30µl of cells/well, 4 at a time, from the transformation reactions onto the LB Agar plates supplemented with Antibiotic.
8. Tip the plates to spread the cells, and allow at least 10-15 minutes for the plates to dry off before turning.
9. Incubate overnight at 37°C.

11. Small Scale Expression Screening

Expression screen with IPTG induction

1. Prepare deep-well blocks (BD Falcon 353966 or ABgene AB-0932) by addition of 0.7ml of Power Broth supplemented with and 1µl/ml of the appropriate antibiotic per well (if LysS/pRARE plasmids are to be retained then media must also be supplemented with 34µg/ml Chloramphenicol).
2. Using 200/300µl pipette tips pick individual colonies into each well, when plate is complete remove tips (tips can be removed 8 at a time using a MCP for convenience).
3. Seal plates with gas-permeable adhesive seals (ABgene AB-0718 or similar).
4. Shake the filled plates at 240 rpm (innova) or 600rpm (Vertiga) at 37°C overnight
5. Prepare four 24-well deep well plates per 96 construct experiment plate by addition of 3 ml of Power Broth supplemented with the appropriate antibiotic/s.
6. Dilute the overnight cultures by transferring 150µl (B834) or 250µl (Rosetta) of overnight culture using the Rainin 6 channel expanding p1200 to the 24-well blocks as prepared in step 5.
7. Shake the filled plates at 240 or 600 rpm at 37°C for 3-5 hours (the average O.D. at 595 nm is normally ~0.5 at this point).
8. Cool the cultures by shaking at 240 rpm at 20°C for 20 minutes or place on the bench for a few minutes.
9. Induce by addition of IPTG to final concentration of 1mM (6µl of 500mM IPTG in H_2O) per well using a 20µl MCP with double-spaced tips or alternatively a repeat pipettor.
10. Grow the cultures overnight (~18 hours) by shaking at 200-225 rpm or 600 rpm at 20°C.

Expression screen with Overnight Express™ Instant TB Medium (auto-induction).

1. Prepare deep-well blocks (BD Falcon 353966 or ABgene AB-0932) by addition of 0.7ml of Power Broth supplemented with 1µl/ml of the appropriate antibiotic per well (if LysS/pRARE plasmids are to be retained then media must also be supplemented with 34µg/ml Chloramphenicol).
2. Using 200 or 300µl pipette tips pick individual colonies into each well, when plate is complete remove tips (tips can be removed using MCP for convenience).
3. Seal plates with gas-permeable adhesive seals (ABgene AB-0718 or similar).
4. Shake the filled plates at 200-225 rpm at 37°C overnight.
5. Prepare four 24-deep well plates per 96 constructs by addition of 3 ml of **Overnight Express™ Instant TB Medium** (TBONEX) supplemented with the appropriate antibiotic.

6. Dilute the overnight cultures by transferring 150µl (B834) or 250µl (Rosetta) of overnight culture using the Rainin 6 channel expanding p1200 to the 24-well blocks as prepared in step 5.
7. Shake the filled plates at 200-225 or 600 rpm at 37°C for 3-5 hours (the average O.D. at 595 nm is normally ~0.5 at this point).
8. Reduce the shaker temperature to 25°C and continue shaking at 200-225 or 600 rpm for a further 20-24 hours.

HTP Expression Screen Plate Re-formatting and Harvesting

1. Transfer 1.0 ml of culture from each well into 96-well deep-well blocks (BD Falcon 353966 or ABgene AB-0932) carefully maintaining the plate matrix/layout.

   Do this step in duplicate.
2. Harvest the cells by centrifugation at 6000*g* for 10 minutes (the Beckman JS5.3 rotor for the Beckman *Avanti* centrifuges is ideal for this).
3. Carefully decant the media from the cell pellets by inverting the plates and allowing them to drain for a few minutes on blue towel.
4. Seal the plates (Corning foil seals are preferred as they stick at -80°C) and store at -80°C until required.

N.B. for efficient lysis during the robot screen the cell pellets must be frozen to -80°C for a minimum of 20 minutes before robotic Ni-NTA screening.

The Ni^{2+}-NTA Miniature Expression Screen Protocol

This is available as a standard protocol on the Qiagen BioRobot 8000 and has been adapted for the AVISO Theonyx in the OPPF-UK and must be specifically scripted for all other robotic platforms.

This manual protocol is adapted from that used on the QIAGEN BioRobot 8000 with QIAGEN Magnetic Ni-NTA beads but can be easily performed with the aid of a multi-channel pipettor (MCP) and either a vigorous orbital microplate shaker (minimum 'throw' 2mm-BigBear automation do a good range of these manual or PC/robot-controlled shakers). Bead volumes may require adjustment if those from another supplier are used.

1. Resuspend the cells completely in 210µl of Lysis Buffer (NPI-10-Tween) supplemented with 1mg/ml Lysozyme and either 3 units/ml of Benzonase* (Merck, Germany; purity grade I, ≥ 25 U/µl, Cat. No. 1.01694.0001) or 400 units/ml of DNAse Type I. This can be done by either repeated aspirate/dispense with a suitable multi-channel pipette or on an orbital MTP shaker (~1000 rpm for 30 minutes). Allow 30 minutes for the action of the Lysozyme and DNAse before clearing the lysate by centrifuging the deep-well block

at 5000*g* for 30 minutes at 4°C (the Beckman JS5.3 rotor for the Beckman *Avanti* centrifuges is ideal for this).

2. Approximately 5 minutes before the end of the centrifugation run, dispense 20µl of the Ni-NTA magnetic bead suspension (ensure full re-suspension before you commence pipetting!) into each well of a flat-bottomed MTP (not all microtitre plates are magnet-compatible-check before you commence the assay, Greiner 655101 work well).

3. Transfer the supernatant from Step 1 without disturbing the 'Insoluble' pellet to each well of the MTP containing the Ni-NTA magnetic beads. Mix for 30 min at room temperature using either a MTP shaker or, alternatively, vortex at 600 rpm using an adapter for MTPs. The pellets may be re-suspended in 8 M Urea buffered with 100 mM NaH_2PO_4 and 10 mM Tris to pH 8.0 for analysis of the 'insoluble' fraction on SDS-PAGE.

4. Place the 96-well microplate on the 96-Well Magnet (we find the QIAgen magnets Type A or B, Qiagen part no.s 9014061 and 9012916 respectively, work well but there are many other suitable magnets on the market) for 1 min and remove the supernatant carefully from the beads with a MCP.

5. Add 200 µl of Wash Buffer (NPI-20) to each well, remove from magnet and mix on the microplate shaker for 5 minutes.

6. Place on the 96-Well Magnet for 1 min, and remove buffer.

7. Repeat steps 5 and 6.

8. Add 50 µl of Elution Buffer (NPI-250) to each well, mix on the MTP shaker (or vortex) for 1 min, place on the 96-Well Magnet for 1 min, and transfer the supernatant (eluate) to a fresh MTP for analysis on SDS-PAGE. SDS-PAGE sample buffer may be used instead of elution buffer if you require a more concentrated sample for analysis.

Notes: The addition of Tween 20 to the buffers is necessary to enable optimal collection of the magnetic beads on the sides of the MTP wells and also facilitates efficient cell lysis in step 1. Bead volumes etc. are based on the QIAgen protocol and may require adjustment if reagents from another supplier are used.*Benzonase is more stable than DNase I.

Buffers used:

a. Buffer NPI-10-Tween:

   50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1% v/v Tween 20, adjust pH to 8.0 using NaOH and filter before use. Store at 4°C.

b. Buffer NPI-20-Tween (Wash buffer):

   50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% v/v Tween 20, adjust pH to 8.0 using NaOH and filter before use. Store at 4°C.

c. Buffer NPI-250-Tween, 50 ml: (elution buffer):

50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM Imidazole , 0.05% Tween , adjust pH to 8.0 using NaOH and filter before use. Store at 4°C.

d. DNAse I Stock Solutions (40,000 units/ml):

To a 200,000 unit bottle of DNAse I (Sigma D-4527) add 5ml of UHQ Sterile Water. Aliquot into 25µl aliquots, store at -20°C. Add one aliquot to the 25ml of Buffer NPI-10-Tween buffer to be used for lysis.

e. Lysozyme:

Add 25mg of freshly-weighed Lysozyme (Sigma L-6876 or equivalent) to the 25ml of Buffer NPI-10-Tween buffer to be used for lysis.

High Throughput denaturing purification of his-tagged proteins

Materials:

QiaFilter plate
TurboFilter plate
Ni-Sepharose (GE Healthcare)
Ethanol
Triton X-100
TCEP
Vacuum Manifold
Insoluble pellets (left over from native Ni-NTA robot run for instance), frozen.

The materials can be bought in a kit:

Qiagen Ni-NTA Superflow 96 BioRobot Kit (4), 969261

Buffer A: 50mM Tris pH8.5
300mM NaCl
8M urea
30mM imidazole

Buffer B: 50mM Tris pH8.5
8M urea
300mM imidazole

Protocol:

1. Solubilisation of pellets
    a. Defrost pellets at room temp for 30min
    b. Add to the insoluble pellet (left from the ni-nta robot) 500ul of buffer A plus 0.5% Triton X-100 and 1mM TCEP (final concentrations)
    c. Resuspend the cells
    d. Shake for 1h at room temp at 700rpm

e. Centrifuge for 1h at 6500g

2. Preparation of the (yellow) Qiafilter plate
    a. Place the qiafilter plate on top of the vacuum manifold with a waste tray inside
    b. Add 100ul Ni-sepharose to each well
    c. Apply vacuum until the liquid has drawn through
    d. Release vacuum
    e. Add 500ul water
    f. Apply vacuum until all liquid has been pulled through

3. Filtration of the solubilised pellets
    a. Place the yellow qiafilter plate containing the beads inside the manifold
    b. Place a TurboFilter plate on top.
    c. Transfer 450ul of the solubilised pellet to the turbofilter plate, taking care not to disturb the pellet
    d. Overlay each well with 100ul absolute ethanol
    e. Apply vacuum until all liquid has been drawn through
    f. Remove turbofilter plate

4. Ni-NTA step
    a. Place the qiafilter plate on top of the vacuum manifold with a waste tray inside
    b. Incubate for 5-10min
    c. Apply a soft vacuum to slowly draw the liquid through.

        Note: not all wells will empty at the same rate. You can slowly increase the vacuum as you go to draw the slower wells through
    d. Wash twice with 500ul buffer A (i.e. add 500ul, apply full vacuum until drawn through and repeat)
    e. Release vacuum
    f. Gently wrap the qiafilter plate on tissue paper to dry the nozzles
    g. Re-assemble the manifold with the qiafilter plate on top and an elution plate inside
    h. Add 200ul Buffer B to each well
    i. Incubate for 5min
    j. Apply soft vacuum until all liquid has gone through.

Information: For sample loading the *Impact* Equalizer MCPs from Matrix (see http://www.matrixtechcorp.com) are ideal as they allow pipetting between standard 9mm MTP pitch wells and the 4.5mm pitch wells found in most of these gel systems.

12. SDS-PAGE Analysis of Ni^{2+}-NTA purified proteins

1. Mix 10µl of each fraction with 10µl of SDS-PAGE loading buffer (100mM Tris pH6.8, 4% w/v SDS, 0.2% w/v Bromophenol blue and 20% v/v Glycerol) in a 96-well PCR plate.
2. Seal the plate and boil the samples for 3 min.
3. Follow the instructions from Invitrogen for setting up the gel tanks. Load 10µl/ well into the gels (starting in well 2) a column at a time using the 30µl or 125µl Matrix Expanding pipette. Place 5µl of Low M.W. marker in well 1 of each gel and 5µl in well 26 of each gel. Set the Voltage to 200V and run the gel until the blue dye reaches the bottom.
4. To stain the gel, remove it from the plastic casing and place in a plastic box. Cover the gel with Instant Blue (ISB01L Expedon) and leave for 30-60 min.
5. De-stain the gel by removing the stain and covering the gel with water until destained. Repeat if necessary.
6. Photograph the gels and score the expression.

Appendix 1. Preparation of competent *E. coli* - RbCl method

N.B. Volumes given in this protocol are sufficient for one 96-well plate of 50µl cell aliquots bit it is feasible to prep ~1000 aliquots at a time.

1. Grow up overnight culture in 5ml LB (plus appropriate antibiotic for modified strains), shake at 37°C.
2. Dilute overnight culture 1 in 100 (**2.5 ml in 250 ml**).
3. Grow at 37°C (shaken) for 2-3 h, until OD_{560} ~0.25-0.3. (0.08-0.10 for 200µl samples in plate reader)
4. Incubate on ice, 5 min.
5. Spin at **4000g, 5 min 4°C**. Discard supernatant.
6. Resuspend pellet in **50 ml Tfb I** (transformation buffer I), for 250 ml prep. K*eep pipetting to the minimum required for re-suspension*.
7. Incubate on ice, 5 min.
8. Spin at **4000g, 5 min 4°C**. Discard supernatant.
9. Resuspend pellet in **5 ml Tfb II**. *Be **very** gentle and keep pipetting to the minimum required for re-suspension*.
10. Incubate on ice, 15 min.
11. Make aliquots of 50 µl in transformation tubes on dry ice. Store at -80°C.

Tfb I

Compound	Concentration	Per 1litre	Notes
KOAc	30 mM	2.94g	Adjust to pH 5.8 using 0.2M acetic acid. Sterilise by filtration.
RbCl	100 mM	12.10g	
$CaCl_2$	10 mM	1.47g	
$MnCl_2$	50 mM	9.90g	
Hexamine cobalt Cl	3 mM	0.80g	
Glycerol	15%	150ml	

Tfb II

Compound	Concentration	Per 200ml	Notes
MOPS (or PIPES)	10mM	419mg	Adjust to pH 6.5 using KOH. Sterilise by filtration
$CaCl_2$	75mM	2.205g	
RbCl	10mM	2.418g	
Glycerol	15%	30ml	