Protein Production UK

Protocol for expression vector construction by In-Fusion™

1. Primer design

Primers are designed for Infusion™ cloning depending on the fusion tag required in the resulting protein. Generally, the primers are designed with a $T_m \geq 68°C$ 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 (5nmoles/well resuspended in 50µl of buffer) is convenient and a simple 1 to 10 dilution is then required to prepare them for high throughput (HTP) PCR.

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

2. PCR amplification of target DNA

1. Set up the following reaction mixture:
    - 1.0 µl forward primer (10pmoles/µl)
    - 1.0 µl reverse primer (10pmoles/µl)
    - 12.5µl Phusion flash PCR master mix (2x)
    - 1µl DNA template (10ng/µl)
    - 22 µl nuclease free water

2. Set up the thermocycler as below and run PCR:

Step	Temperature	Duration
Step 1	98 °C	30 s
Step 2	98 °C	10 s
Step 3	55 °C	30 s
Step 4	72 °C	15 s
	Cycle steps 2-4 30x	
Step 6	72 °C	5 min
Step 7	12 °C	Infinite hold

(15s at 72 C up to 1kb insert increase by 15s/kb i.e. 30s for 2 kb insert, 1 min for 4 kb insert)

3. Transfer 5 µl PCR sample to a new purple PCR plate and add 5 µL 2x DNA gel loading buffer to each well using a 20µl multichannel pipette (MCP) and fresh tips per column. Run alongside a well of a suitably sized DNA ladder on a 1 % (w/v) agarose gel containing 1x SYBR™ Safe DNA gel stain. Electrophorese in 1xTBE buffer at 80 V for 40 min.

3. 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, are 'smeared' or larger than about 4kb, then gel purification and extraction is advisable.

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

1. 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).

2. Make up a master mix to aliquot 5µl per well of 5 units DpnI (0.5ul 10U/µl) in 1X cutting buffer (e.g., NEBuffer 4 or CutSmart buffer from New England Biolabs).

3. Add 5µl with a repeat pipettor to each reaction.

4. Incubate at 37°C for 30-60 minutes prior to running the AMPure purification procedure.

5. 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 purified.

6. Mix the AMPure XP and PCR reaction thoroughly by pipette mixing 10 times using a fresh set of tips for each column. The colour of the mixture should appear homogenous after mixing. Let the mixed samples incubate for 3-5 minutes at room temperature for maximum recovery.

7. 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.

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

9. With the reaction plate still situated on a magnet dispense 200 µl of 70% ethanol to each well of the reaction plate (no need to mix) 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 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.

10. The plate should be left to air-dry for 10 minutes at RT to allow complete evaporation of residual ethanol.

11. Remove plate from magnet and add 30 µ 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.

12. Place the plate back on the magnet and use a 200 or 300µl MCP, and a fresh set of tips for each column, to transfer the 30µl supernatant from each well to a fresh **purple** PCR Plate. (We use a purple PCR plate to aid identification when the plate is stored in the freezer.) There will probably be a small amount of bead ‘carry-over’, but we have seen no inhibition of the Infusion™ reactions by the AMPure XP beads.

13. Transfer 5 µl purified PCR product to a new purple PCR plate and add 5 µL 2x DNA gel loading buffer to each well using a 20µl multichannel pipette (MCP) and fresh tips per column. Run alongside a well of a suitably sized DNA ladder on a 1 % (w/v) agarose gel containing 1x SYBR™ Safe DNA gel stain. Electrophorese in 1xTBE buffer at 80 V for 40 min to check purification has worked.

4. In-Fusion Cloning (CloneXpressII)

1. The optimal amount of vector for the recombination with ClonExpress II is 0.03 pmol, while the optimal amount of insert is 0.06 pmol (molar ratio of vector to insertion is 1:2), as roughly calculated as follows:

    a. The optimal mass of vector = [0.02 × number of base pairs] ng (0.03 pmol)
    b. The optimal mass of insert = [0.04 × number of base pairs] ng (0.06 pmol)

2. Set up the following reaction mix:

    1 µl 25ng/µl linearised vector
    X µl PCR product
    2 µl 5X CE II buffer
    0.5 µl Quick-fusion enzyme (Exnase)
    6-X µl of nuclease free water

3. Incubate at 42°C for 30 minutes using a thermocycler.

4. Stop reaction with the addition of 40µl TE (10 mM Tris-HCl, pH8.0, 1mM EDTA) using a 200 or 300µl MCP. Transform into Stellar cells immediately.

5. Transfer 5µl of the diluted In-Fusion reaction per 25µl of stellar competent cells in racked mini tubes (Axygen # MTS-11-C-R).

6. Incubate the cells on ice for 30 minutes.

7. Heat-shock the cells for 45 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.

8. Return the cells to ice for 2 minutes.

9. Add 400 µl SOC/LB to each tube using the 200 or 300µl MCP using fresh tips per column. The use of SOC here allows the cells to recover without shaking.

10. Incubate the reactions at 37°C for 60mins (if using Kan resistant vectors, incubate for 90 minutes).

11. Plate 50 µl on to 8.5 cm agar plates containing required antibiotic and precoated with X-gal (40 µl of 20 mg/ml) and IPTG (40 µl of 1M).

12. Incubate overnight at 37°C.

5. Picking colonies

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 practice is to pick two white colonies per construct which will normally give positive clones for ~96% of the PCR products.

1. Prepare 24 well deep-well blocks (BD Falcon 353966, ABgene AB-0932 or similar) by addition of 4ml of LB supplemented with the appropriate antibiotic.

2. Using 10µl tips, pick individual colonies and transfer 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'.***

3. When each well has been innoculated, remove tips 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 overnight 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 incubators.

5. Replace the gas-permeable seal on each 24 or 96-well culture plate with a solid seal (e.g., ABGene AB-0558) and harvest the cells by centrifugation at 6000*g* for 15 minutes.

6. 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).

7. Prepare miniprep DNAs for PCR screening and/or sequencing.

PPUK Vectors available from Addgene (www.addgene.org)

Addgene id	Plasmid	Tag	Vector Type
26040	pOPINVL	Mukappa	Mammalian Expression, Bacterial Expression, Insect Expression
26041	pOPINVH	MuCH1-C-his	Mammalian Expression, Bacterial Expression, Insect Expression
41114	pOPINTF	N-His-Trigger factor-3C	Mammalian Expression, Bacterial Expression, Insect Expression
41117	pOPINHALO7	N-His-Halo7-3C	Mammalian Expression, Bacterial Expression, Insect Expression
41118	pOPINMSYB	N-His-Mysb-3c	Mammalian Expression, Bacterial Expression, Insect Expression
41119	pOPINNUSA	N-His-NusA-3C	Mammalian Expression, Bacterial Expression, Insect Expression
41121	pOPINGS	SS-C-Strep-His	Mammalian Expression, Bacterial Expression, Insect Expression
41123	pOPINJB	N-His-GST/C-BAP tag	Mammalian Expression, Bacterial Expression, Insect Expression
41126	pOPINE-3C-HALO7	C-3C-Halo7-His	Mammalian Expression, Bacterial Expression, Insect Expression
41127	pOPINE-3C-CD4	CD4	Mammalian Expression, Bacterial Expression, Insect Expression
41128	pOPINRSF	N-His-3C	Bacterial Expression
41131	pOPINRSE	C-His-3C	Bacterial Expression
41132	pOPINCDF	N-His-3C	Bacterial Expression
41134	pOPINCDM	N- His-MBP-3C	Bacterial Expression
41135	pOPINCDE	C-His	Bacterial Expression
41136	pOPINMalE	SS-MalE-C-His	Bacterial Expression
41137	pOPINDsbA	SS-DsbA-C-His	Bacterial Expression

41138	pOPINTolB	SS-TolB-C-His	Bacterial Expression
41139	pOPINP	SS-Pel-C-his	Bacterial Expression
41140	pOPINO	OmpA SS	Bacterial Expression
41141	pOPINA	C-His	Bacterial Expression
41142	pOPINB	N-His-3C	Bacterial Expression
41143	pOPINK	GST	Bacterial Expression
53534	pOPINEneo-3C-GFP	C- 3C-GFP-His8	Mammalian Expression, Bacterial Expression, Insect Expression
53536	pOPINEneo	C-His	Mammalian Expression, Bacterial Expression, Insect Expression
53537	pOPINH	N-SS-His-3C	Mammalian Expression, Bacterial Expression, Insect Expression
53541	pOPINN-GFP	N-His-GFP-3C	Mammalian Expression, Bacterial Expression, Insect Expression
41116	pOPINTRX	His-thioredoxin-3C	Mammalian Expression, Bacterial Expression, Insect Expression
26046	pOPING	SS peptide-C-His	Mammalian Expression, Bacterial Expression, Insect Expression
41125	pOPINE-3C-eGFP	C- 3C-GFP-His	Mammalian Expression, Bacterial Expression, Insect Expression
41115	pOPINS3C	N-His-SUMO-3C	Mammalian Expression, Bacterial Expression, Insect Expression
26044	pOPINM	N-His-MBP-3C	Mammalian Expression, Bacterial Expression, Insect Expression
26045	pOPINJ	N- His-GST-3C	Mammalian Expression, Bacterial Expression, Insect Expression
26043	pOPINE	C-His	Mammalian Expression, Bacterial Expression, Insect Expression
26042	pOPINF	N-His-3C	Mammalian Expression, Bacterial Expression, Insect Expression