# Chapter 5

# The Precise Engineering of Expression Vectors Using High-Throughput In-Fusion™ PCR Cloning

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## Summary

In this chapter, protocols for the construction of expression vectors using In-Fusion™ PCR cloning are presented. The method enables vector and insert DNA sequences to be seamlessly joined in a ligation-independent reaction. This property of the In-Fusion process has been exploited in the design of a suite of multi-host compatible vectors for the expression of proteins with precisely engineered His-tags. Vector preparation, PCR amplification of the sequence to be cloned and the procedure for inserting the PCR product into the vector by In-Fusion™ are described.

**Key words:** In-Fusion; PCR cloning; High throughput

## 5.1. Introduction

The first step in the high-throughput production of proteins is the construction of vectors for the expression of the target proteins. Conventionally, this involves the manipulation of DNA using restriction enzymes and DNA ligase to combine sequences for expression into the appropriate plasmid vector. The process is relatively time consuming, for example, involving gel electrophoresis to purify the component DNA fragments and is limited by the availability of unique restriction enzyme sites for cloning. To overcome these limitations and hence increase the efficiency of vector construction for high-throughput applications, a number of ligation-independent methods have been developed, for example Gateway™ cloning based on λ phage site-specific recombination *(1, 2)* and Ligation-Independent PCR cloning (LIC) which involves hybridization of single-stranded ends produced

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by treatment of both linearized vector and insert with T4 polymerase *(3, 4)*. Although highly efficient, both these methods do not allow the precise fusion of vector and insert sequences. In the case of the Gateway™ system, the addition of the *att* recombination sites to the 5′ and 3′ ends of the cloned sequence means that extra amino acids are incorporated into the expressed protein. In the LIC method the sequences flanking the insert can only be composed of three of the four bases, since the fourth base is required as a 'lock' for stopping, at a specified point, the single-strand production by the 3′ to 5′ processing activity of T4 polymerase. Recently, an alternative method has been developed and commercialized by Clontech as In-Fusion™ PCR cloning (http://bioinfo.clontech.com/infusion/). The system is based on an enzyme with proof-reading exonuclease activity that catalyses the joining of DNA duplexes via exposure of complementary single-stranded sequences. Consequently, vectors and inserts can be precisely joined in an entirely sequence-independent manner. Further, it has been shown that the In-Fusion reaction is efficient over a wide insert DNA concentration range and suitable for the cloning of large PCR products (3–11 kb) *(5)*. We have combined In-Fusion™ PCR cloning with customized multi-promoter plasmids to produce a suite of expression vectors (pOPIN series) that enable the precise engineering of ($His_6$-) tagged constructs with no unwanted vector sequences added to the expressed protein. The use of a multiple host-enabled vector permits rapid screening for expression in both *E. coli* and eukaryotic hosts (HEK293T cells and insect cells, e.g. *Sf*9 cells) *(6)*. In this chapter, the protocols for using In-Fusion™ PCR cloning as part of an HTP protein production pipeline will be described.

## 5.2. Materials

### 5.2.1. Primers and Vectors

1. The 3′ and 5′ regions of homology required for In-Fusion™ cloning are generated by adding approximately 15-bp extensions to both forward and reverse PCR primers. The sequences of these extensions should match precisely the 5′ and 3′ ends of the recipient vector exposed by linearization of the vector at the position into which the PCR product is to be inserted. Typically, oligonucleotide primers are approximately 35-bp long (including the extension and gene-specific region), and purification of the primers is not necessary.
2. Any vector can be used with the In-Fusion™ cloning method. A unique restriction enzyme site(s) is required at the point of cloning for linearization of the vector by enzymatic cleavage. Alternatively the need for a restriction site(s) can be avoided

by producing the linearized vector by inverse PCR, though depending upon the size of the starting vector this risks introducing unwanted PCR errors into the vector backbone *(7)*. The pOPIN suite of vectors developed in the OPPF are designed so that linearization requires cutting with two restriction enzymes releasing a beta-galactosidase expression cassette (**Fig. 5.1**). Therefore blue-white selection is used to screen out colonies transformed with non-linearized vector following the In-Fusion™ cloning reaction (*see* **Section 5.3.4**). The pOPIN vectors and the extensions required for In-Fusion™ cloning into them are listed in **Table 5.1**. The vectors are available from the corresponding author on request.

### 5.2.2. Enzymes and Buffers

1. In-Fusion™ enzyme is available lyophilized with the buffer components in microtube format (8 or 96) which conveniently can be stored at room temperature. The enzyme and buffers can also be purchased separately in liquid form (Clontech, Oxford, UK).
2. There are a number of high-fidelity PCR polymerases which are suitable for amplification of DNAs, for example, the KOD Hi-Fi™ and KOD Hotstart™ polymerases (Novagen, Nottingham, UK). Most manufacturers supply the reaction buffer including dNTPs with the enzymes.

### 5.2.3. Gel Electrophoresis and Purification of DNA

1. Tris borate EDTA (TBE) running buffer (×10): 108 g Tris base, 55 g Boric acid, 9.3 g EDTA dissolved in 1 L water; store at room temperature.
2. DNA gel loading dye: 0.25% (w/v) Bromophenol Blue in 30% (v/v) glycerol/TE: store at room temperature.
3. Visualization of DNA: SYBRSafe™ (InVitrogen, Paisley, UK).
4. AMPure magnetic beads and SPRIPlate 96R magnet (Agencourt Biosciences, Beverley, MA, USA).
5. QIAquick and QIAprep kits (Qiagen, Crawley, UK); Wizard® kit (Promega, Madison Wisconsin, USA).
6. QIAVac 96 vacuum manifold (or similar).
7. GS96 medium (Qbiogene, Morgan Irvine, CA, USA).
8. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and Isopropyl-β-D-thio-galactopyranoside (IPTG), (Melford Laboratories Ltd, Ipswich, UK).

### 5.2.4. Cloning-Grade E. Coli

Chemically competent cells with an efficiency of at least $10^8$ cfu/μg circular plasmid DNA are required for In-Fusion™ cloning, for example, TAM1 cells from ActivMotif (Rixensart, Belgium) OmniMax2 cells (InVitrogen, Paisley, UK) and Fusion-Blue™ Competent Cells (Clontech).

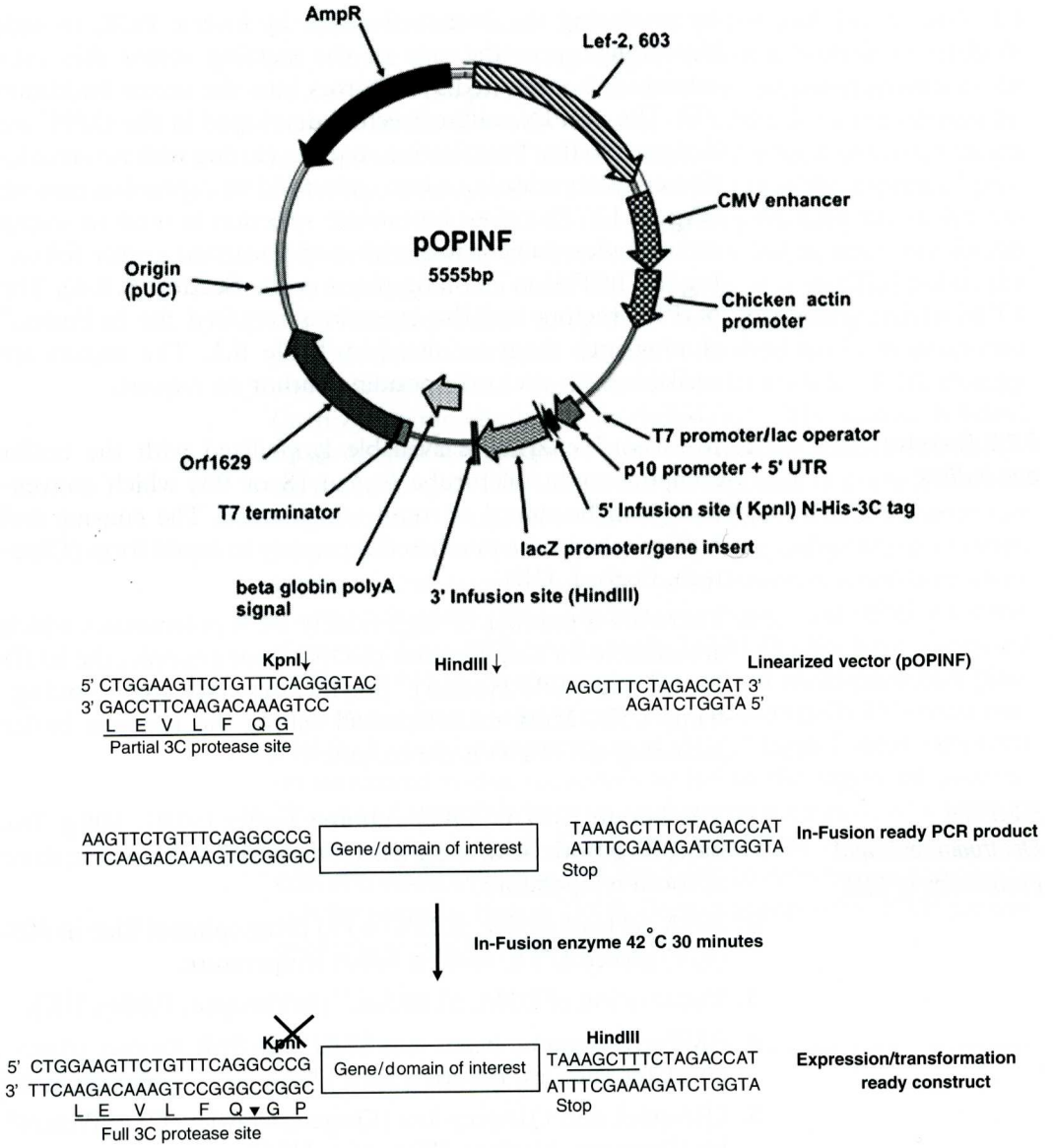


Fig. 5.1. Schematic representation of the pOPINF vector showing key features of the plasmid (a) and details of the In-Fusion cloning region (b) (a) vector map showing key features of pOPINF as follows: pUC origin for high-copy replication in E. coli and ampicillin-resistance marker (AmpR), T7 promoter/lac operator for high-level transcription of insert in E. coli containing the λ (DE3) prophage and T7 transcription terminator, CMV Enhancer and Chicken β-Actin Promoter for transcription of gene insert in mammalian cell lines, a β-globin polyA signal is included to enhance transcript stability, the 'flanking' baculoviral ORFs Lef-2603 and 1629 for recombination into the baculovirus genome and the p10 promoter/5′UTR for expression in insect cell lines, N-His-3C tag for simple affinity purification of gene product from any host cell. The positions of the 5′(KpnI) and 3′(HindIII) In-Fusion sites are indicated. (b) Schematic of an In-Fusion reaction into pOPINF. The two 'free' ends of the linearized vector (top) are shown with a partial 3C Protease cleavage site as the 5′ In-Fusion site and the standard 3′ In-Fusion site. The PCR-amplified insert (centre) flanked by the In-Fusion extensions. During the reaction the In-Fusion enzyme removes the 3′ overhang left by the KpnI digest of the vector, the 5′ overhang generated by the HindIII digestion of the vector is not removed by the In-Fusion enzyme. The transformation-ready reaction product (lower) is shown with the re-constituted 3C Protease cleavage site and translation stop codon now flanking the inserted gene in the circularized plasmid. N.B. the In-Fusion enzyme has no ligase activity but generates single strands on both PCR product and vector to the extent of the homology between the two.

**Table 5.1**
**Summary of In-Fusion™ site sequences and characteristics of the pOPIN vectors where ❶ represents the 3C protease cleavage site and ❶ represents the cleavage sites for either eukaryotic signal peptidase or the specific SUMO protease. Underlined sequences represent sites where translation initiation and stop codons are present in In-Fusion primer extensions. Vectors marked with dagger symbols use the same primer extensions.**

| Vector | Fusion tag | Parent vector/antibiotic resistance | Restriction sites for linearization of the vector | Forward primer extension | Reverse primer extension | Approximate increase in size of PCR product with T7 primer (bp) |
|---|---|---|---|---|---|---|
| pOPINA | ...KHHHHHH tag | pET28a/Kanamycin | NcoI and DraI | AGGAGATATACCATG | GTGGTGGTGGT-GTTT | 110 |
| pOPINB | MGSSHHHHHHSSGLEVL-FQ❶GP ... tag | pET28a/Kanamycin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 130 |
| pOPINC | ...KHHHHHH tag | pTriEx4/Ampicillin | NcoI and PmeI | AGGAGATATACCATG† | GTGATGGTGAT-GTTT† | 200 |
| pOPIND | MAHHHHHHSSGLEVL-FQ❶GP... tag | pTriEx4/Ampicillin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 225 |
| pOPINE | ...KHHHHHH tag | pTriEx2/Ampicillin | NcoI and PmeI | AGGAGATATACCATG‡ | GTGATGGTGAT-GTTT† | 170 |
| pOPINF | MAHHHHHHSSGLEVL-FQ❶GP... tag | pTriEx2/Ampicillin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 225 |
| pOPING | MGILPSPGMPALLSLVSLLSVLL MGCVA❶ETG... cleavable secretion leader and.KHHH-HHH tags | pTriEx2/Ampicillin | KpnI and PmeI | GCGTAGCTGAAACCGGC | GTGATGGTGAT-GTTT | 260 |
| pOPINH | MGILPSPGMPALLSLVSLLSVLL MGCVA❶ETMAHHHHHHS SGLEVLFQ❶GP..... cleavable secretion leader and cleavable N-his tag | pTriEx2/Ampicillin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA | 315 |
| pOPINI | MAHHHHHHSSG... tag | pTriEx2/Ampicillin | KpnI and HindIII | ACCATCACAGCAGCGGC | ATGGTCTA-GAAAGCTTTA | 200 |

(continued)

**Table 5.1**
(continued)

| Vector | Fusion tag | Parent vector/antibiotic resistance | Restriction sites for linearization of the vector | Forward primer extension | Reverse primer extension | Approximate increase in size of PCR product with T7 primer (bp) |
|---|---|---|---|---|---|---|
| pOPINJ | MAHHHHHHSSG-*GST*-LEVLFQ❶GP… tag | pTriEx2/Ampicillin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 890 |
| pOPINK | MAHHHHHHSSG-*GST*-LEVLFQ❶GP… tag | pET28a/Kanamycin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 790 |
| pOPINM | MAHHHHHHSSG-*MBP*-LEVLFQ❶GP… tag | pTriEx2/Ampicillin | KpnI and HindIII | AAGTTCTGTTTCAG-GGCCCG‡ | ATGGTCTA-GAAAGCTTTA‡ | 1,330 |
| pOPINS | MGSSHHHHHH-*SUMO*❶… tag | pET28a/Kanamycin | KpnI and HindIII | GCGAACAGATCGGTGGT | ATGGTCTA-GAAAGCTTTA | 400 |

#### 5.2.5. Plastic-Ware in Multi-Well Format

1. Thermo-Fast 96 v-well skirted PCR plates sealed with a clear film (e.g. ABGene, Epsom, UK).
2. 96 deep-well plate sealed with gas-permeable film (e.g. ABGene).
3. 96 v-well microtitre plates (e.g. Greiner Bio-One, Frickenhausen, Germany) and foil seals (e.g. ABGene).
4. 24-Well tissue culture plates with lids (e.g. Corning, Lowell, MA, USA).

## 5.3. Methods

#### 5.3.1. Preparation of Vector

1. Digest vector DNA prepared using QIAPrep (Qiagen) spin column (or similar DNA purification method) with appropriate restriction enzyme(s) using standard conditions. For example, incubate 5 µg pOPINF plasmid DNA with 50 Units KpnI and 50 Units of HindIII, in a total reaction volume of 100 µl for 2 h at 37°C.
2. Purify linearized vector by preparative gel electrophoresis (*see* **Section 5.3.3.2**) or by a spin column (e.g. QIAquick PCR purification kit). If the plasmid DNA has been prepared without the use of an alcohol precipitation step the spin column method is sufficient, otherwise gel purification is recommended.

#### 5.3.2. HTP PCR Amplification

1. Dilute the primers (100-µM stocks) 1–10 with either sterile UHQ water or a buffer such as EB (10 mM Tris–HCl, pH 8.0) prior to use (*see* **Note 1**).
2. The standard amplification reaction for In-Fusion™ cloning comprises 30 pmol each primer (final concentration of 0.6 µM each), 50–100 ng of template, dNTPs (final concentration of 200 µM each), 1 mM $MgCl_2$ (final concentration) and 1 U of KOD HiFi™ polymerase in a final volume of 50 µl (*see* **Note 2**).
3. Perform the thermal cycling in a 96 v-well PCR microtitre plate using the following parameters (*see* **Note 3**):

   Step 1: 94°C 2 min (not necessary for KOD HiFi)

   Step 2: 94°C 30 s

   Step 3: 60°C 30 s

   Step 4: 68°C 2 min

   Step 5: Go to **step 2** and repeat cycle 29 times

   Step 6: 72°C 2 min

   Step 7: 4°C Hold

- When thermal cycling is complete add 10 µl of DNA gel loading buffer to each well of the PCR plate. Analyse 6 µl aliquots of each product (after mixing by pipette) on a 1.25% TBE agarose gel.

### 5.3.3. Purification of PCR Products

Providing the PCR products are of good quality (i.e. few multiple bands and 'smeared' products) then the AMPure™ magnetic beads can be used for purification avoiding the need to run preparative agarose gels (**Section 5.3.3.1**). However, if a significant number of the products contain multiple bands, then gel purification and extraction is advisable. The PCR products may be separated by agarose gel electrophoresis on any suitable gel apparatus. A minimum run length of 30 mm is recommended, and the process is simplified if the gel wells are spaced such that they can be used in conjunction with a multi-channel pipette. The wells must also accommodate a sample volume of at least 50 µl/well. The use of SYBRSafe stain in the gel and a Blue Light illuminator (e.g. Clare Chemicals DR88 Dark Reader) is recommended to prevent UV damage to the PCR products during visualization and excision. DNA is extracted using a modified QIAquick® 96 PCR (**Section 5.3.3.2**).

#### 5.3.3.1. Ampure Magnetic Bead

1. If required add approximately 5U DpnI enzyme to each PCR and incubate at 37°C for 1 h prior to the clean-up step (see Note 4).
2. Add 90 µl of the AMPure™ resin to each 50 µl PCR mix thoroughly by pipette mixing 10 times or orbital shaking for 30 s. This step binds PCR products 100 bp and larger to the magnetic beads. The colour of the mixture should appear homogenous after mixing. Incubate the mixed samples for 3–5 min at room temperature to ensure maximum binding of PCR products to the resin.
3. Place the reaction plate onto a SPRIPlate 96R magnet for 5–10 min to separate beads from solution. The separation time is dependent on the volume of the reaction. Wait for the solution to clear before proceeding to the next step.
4. Aspirate the cleared solution from the reaction plate and discard. This step must be performed while the reaction plate is located on the SPRIPlate 96 magnet and can be carried out with any suitable multi-channel pipette. Do not disturb the ring of separated magnetic beads.
5. Dispense 200 µL of (freshly prepared) 70% ethanol to each well of the reaction plate and incubate for 30 s at room temperature. Remove the ethanol by aspiration and discard. Repeat wash step a further two times. It is important to perform these steps with the reaction plate situated on a SPRIPlate 96R. Do not disturb the separated magnetic beads.

Be sure to remove all the ethanol from the bottom of the well as it may contain residual contaminants. The ethanol can also be discarded by inverting the plate to decant off the liquid, but this must be done while the plate is situated on the SPRIPlate 96R.

6. Air dry the plate for 10–20 min on a bench to allow complete evaporation of residual ethanol. If the samples are to be used immediately, proceed to step 7 for elution. If the samples are not to be used immediately, the dried plate may be sealed and stored at 4 or -20°C.
7. To elute the DNA from the resin, add 40 µL of elution buffer (EB: 10 mM Tris–HCl, pH 8.0) to each well of the reaction plate, seal and either vortex for 30 s or pipette mix 10 times (see Note 5).

*5.3.3.2. Gel Purification and Extraction of PCR Products*

1. Excise the electrophoretically separated PCR products from the agarose gel and place each gel slice into an individual well of a 96 deep-well block. Trim the slices if necessary so that there is no more than 400 mg of agarose in each well. Add 3 volumes of Buffer QG (QIAquick kit) to 1 volume of gel (i.e. 100 µl/100 mg) to each well and cover the block with a plate seal.
2. Incubate the block at 50°C for 10 min (or until gel is completely dissolved) in an oven or shallow water bath. Mix by inverting the block every 2–3 min during the incubation to help dissolve the gel.
3. Assemble the QIAvac 96 vacuum manifold (Fig. 5.2) as follows:

   – Place waste tray inside the QIAvac base, and put the top plate squarely over the base.

   – Position the QIAquick™ 96 plate securely into the QIAvac top plate.
4. Attach the QIAvac 96 to a vacuum source (15.20 in. of Hg or the equivalent).
5. Once the gel slices have dissolved completely, remove the seal from the block and check that the colour of the mixture in each well is yellow (similar to Buffer QG without dissolved agarose). If, after solubilization of the agarose, the binding mixture appears orange or violet add 10 µl of 3 M sodium acetate, pH 5.0, to the respective samples, seal the block and mix by inversion.
6. Add 1 gel volume of isopropanol to each of the samples, for example, if the agarose gel slice weighs 200 mg, add 200 µl isopropanol (*see* **Note 6**). Cover the block with a plate seal and mix by inverting 6–8 times.

7. Apply maximum volume of 1 ml sample to the wells of the QIAquick™ 96 Plate and switch on vacuum source. After the samples in all wells have passed through the plate, switch off vacuum source. Repeat if the volume of the sample is greater than 1 ml until all the dissolved gel has been applied to the binding plate.
8. Wash each well of the QIAquick™ 96 plate twice by adding 1 ml of Buffer PE to the well and apply vacuum.
9. After washing apply *maximum* vacuum for an additional 10 min to dry the membrane of the binding plate.
10. Switch off the vacuum source and slowly ventilate the QIAvac 96 manifold. Lift the top plate from the base keeping the QIAquick Plate and the top plate together and vigorously rap the top plate onto a stack of absorbent paper until no further liquid comes out. Blot the nozzles of the QIAquick Plate with clean absorbent paper, reassemble the manifold and apply *maximum* vacuum for an additional 10 min to dry the membrane (*see* **Note 7**).
11. To elute directly into a standard height microtitre plate replace waste tray with the inverted plate holder (provided with QIAvac 96) and place a 96-well plate directly onto the rack. Place the top plate with QIAquick™ 96 back onto the base.
12. To elute, add 50 µl of Buffer EB to the centre of each well of the QIAquick 96 Plate, allow to stand for 1 min, and switch on vacuum source for 5 min. Once finished, switch off vacuum source and ventilate QIAvac 96 slowly (*see* **Note 8**).
13. Take 5-µl samples from the elution plate to check recovery of PCR products by Agarose gel electrophoresis, and either seal the plate for storage at −20°C or use immediately in In-Fusion reactions.

### 5.3.4. In-Fusion Reaction and HTP Transformation of E. Coli

1. Take 10–100 ng of purified insert and 100 ng of linearized and purified vector (these are convenient to prepare and store at 100–200 ng/µl) in a total volume of 10 µl of either EB/H2O (**Note 9**).
2. Add this to a well of the dry-down In-Fusion™ plate. Mix contents briefly by pipetting up and down, taking care that the lyophilized enzyme/buffer pellet is resuspended. Cover the In-Fusion plate with a self-adhesive foil plate seal.
3. Incubate the plate for 30 min at 42°C in either a thermal cycler or water bath.
4. Dilute immediately with 40 µl TE and either transform into E. coli straight away or freeze the reaction for use later. Five microlitres of the diluted reaction should give tens to hundreds of colonies per well of a 24-well plate.

5. Thaw competent *E. coli* on ice, add 50 µl of cells to 5 µl of the diluted In-Fusion reaction and incubate on ice for 30 min.
6. Heat shock the cells for 30 s at 42°C and return the cells to ice for 2 min.
7. Add 450 µl of GS96 media supplemented with glycerol (0.05%, v/v) or Luria Broth (LB) per tube. The use of GS96 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.
8. Transfer to 37°C incubator (shaking is not required if GS96 media is used) and incubate for 1 h.
9. Plate on LB Agar supplemented with the appropriate antibiotic for the vector, X-Gal and IPTG. Plates are prepared by the addition of 1 ml of molten LB agar (plus appropriate supplements) to each well of the 24-well plates. Plate 10 µl of cells/well, shake plates laterally/orbitally by hand to distribute the culture and allow at least 10–15 min for the plates to dry before inverting.
10. Following overnight incubation at 37°C, wells should contain predominantly white colonies. Any blue colonies are derived from inefficiently linearized parental plasmid and are non-recombinant. Picking two colonies should be sufficient to obtain a cloned PCR product (*see* **Note 10**).

### 5.3.5. Colony Picking, Culture, Preparation of Glycerol Stock

1. Prepare 96 deep-well blocks by addition of 1.5 ml of GS96 (plus glycerol) supplemented with the appropriate antibiotic for the pOPIN vector used.
2. Using 200/300-µl pipette tips pick individual white colonies into each well, leaving the tips in the deep well plate to keep track of which wells have been picked into.
3. When picking is complete, remove tips (tips can be removed eight at a time using a multi-channel pipette) and seal plates with gas-permeable adhesive seals.
4. Shake the filled plates at 200–225 rpm at 37°C overnight; microplate holders for standard shakers are available (e.g. single-layer plate holders or plate 'stackers' from New Brunswick Scientific, St. Albans, UK).
5. Make a glycerol stock of all the cultures by transferring 100 µl from each well to a microtitre plate containing 100 µl of filter sterililized LB/30% (v/v) glycerol, seal and store at −80°C (*see* **Note 11**).
6. Replace the gas-permeable seal on each plate with a solid seal and harvest the cells by centrifugation at 5,000 × *g* for 15 min (the Beckman JS5.3 rotor for the Beckman *Avanti* centrifuge is ideal for this).

7. Decant the media to waste by inverting the plate and then rest the plate upside down on a wad of absorbent tissue to remove residual media (make sure that the pellets are tightly stuck to the blocks). The cell pellets may be stored at −20°C until required or used immediately for extracting plasmid DNA.

### 5.3.6. Plasmid Preparation (Wizard® Protocol)

1. Resuspend each cell pellet by adding 250 µl of Cell Resuspension Solution. This may be done by pipetting with a multi-channel pipette or on a microtitre plate shaker for 30–60 s until a uniform cell suspension is achieved.
2. Add 250 µl of Cell Lysis Solution to each sample. Seal and mix by inversion 3–4 times or 30 s on microtitre plate shaker, incubate for 3 min at room temperature but do not incubate for longer than 5 min (*see* **Note 12**).
3. During the incubation, prepare the vacuum manifold with the Binding plate (unmarked plate) in the QIAvac base (on plate holder) and the Lysate Clearing plate (with blue spot) in the upper plate holder of the manifold (**Fig. 5.2**).
4. Add 350 µl of the Neutralization Solution to each sample. Mixing is not necessary.

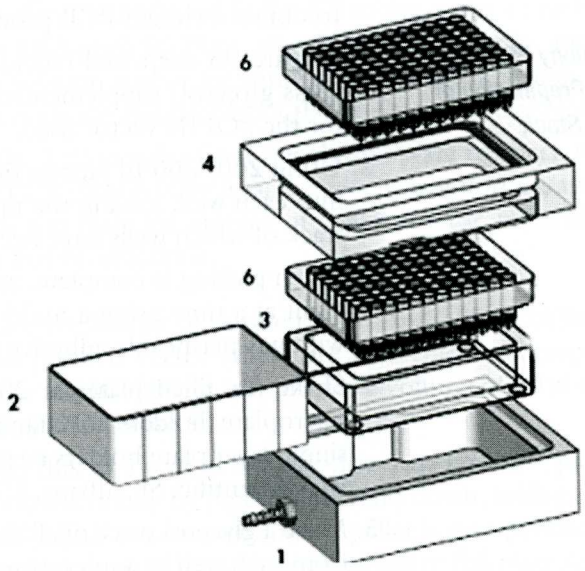


Fig. 5.2. Components of the qiavac 96-exploded view-reproduced from the qiavac handbook, QIAGEN Ltd (UK permission currently being sought from QIAGEN, UK). (1) QIAvac base: holds either the waste tray or the lower plate holder; (2) Waste tray; (3) Lower Plate holder (shown with 96-well plate); (4) QIAvac 96 top plate with aperture for 96-well filter plate; (5) Microtube rack: elution into standard height microtitre plate is described in text. If using racked 1-ml tubes in this format place directly into QIAvac base for elution; (6) 96-well filter plate, e.g. QIAquick™ 96 plate or Wizard® Lysate and Binding plates.

5. Transfer the bacterial lysates to the Lysate Clearing Plate assembled on the Vacuum Manifold. Allow 1 min for the filtration discs to wet uniformly, and then apply a vacuum to the manifold (15.20 in. of Hg or the equivalent) using a vacuum pump fitted with a control valve. Allow 3–5 min under vacuum for the lysates to pass through the Lysate Clearing Plate.
6. Release the vacuum, discard the Lysate Clearing Plate and move the Binding Plate from the Lower Qiavac plate holder to the QIAvac top plate. Place the white Qiavac waste tray in the lower chamber.
7. Add 500 µl of the Neutralization Solution to each well of the Binding Plate in the QIAvac top plate.
8. Apply a vacuum for 1 min, then turn off the pump. Ensure all the wash solution has passed through the Binding Plate.
9. Add 1.0 ml of Wash Solution containing ethanol to each well of the Binding Plate. Apply a vacuum for 1 min.
10. Turn off the pump and repeat the wash procedure (**step 9**). After the wells have been emptied, continue for an additional 10 min under vacuum to allow the binding matrix to dry.
11. Remove the Binding Plate from the Qiavac and blot by tapping onto a clean paper towel to remove residual ethanol. If you find residual ethanol in your mini-preps then you can augment this drying step by wrapping the plate in tissue and incubating at 37°C for 10–15 min.
12. Place a V-bottomed well microplate, or PCR plate, in the Qiavac base on the inverted plate holder. Return the Binding Plate to the QIAvac top plate, ensuring that the Binding Plate tips are centred over the Elution Plate wells and both plates are in the same orientation.
13. Add 100 µl of Nuclease-Free Water/EB (10 mM Tris–HCl, pH 8.0, to the centre of each well of the Binding Plate and incubate 1 min at room temperature.
14. Apply a vacuum for 2–3 min as previously described. Ensure that the entire elution buffer has passed through the plate.
15. Release the vacuum and remove the Binding Plate and Qiavac Upper plate holder. Carefully remove the Elution Plate from the QIAvac base seal the plate and store at 4°C or –20°C. Eluate volumes may vary but are generally 60–70 µl.

### 5.3.7. Verification of Constructs

1. PCR screen the plasmid mini-preps using the PCR protocol described in **Section 5.3.2** replacing the gene-specific forward primers with a T7 forward primer (5′ TAATACGACTCACTATAGGG 3′). Twenty-five microlitres reactions can be used for screening.
2. Amplify and analyse the products as described in **Section 5.3.2**. The T7 forward primer is present in all the pOPIN vectors; the

increases in PCR size with this primer relative to the original gene-specific forward primer are shown in **Table 5.1**.

3. PCR-verified pOPIN vectors can be used directly for expression screening. The DNA prepared by the procedure described in **Section 5.3.6** is of sufficient quantity and quality for transforming the appropriate *E. coli* expression strains, transiently transfecting mammalian cells (e.g. HEK 293T cells; see Nettleship et al. this volume for protocols) and for constructing recombinant baculoviruses. In this way expression of the cloned gene can be evaluated in multiple hosts in parallel.

## 5.4. Notes

1. 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.
2. Making a 'master mix' of all the common reagents is convenient and reduces the possibility of pipetting errors.
3. We have obtained the best results, in terms of target coverage and product quality with KOD HiFi™ in Buffer 2 with a 60 °C annealing temperature. If reactions produce multiple or smeared bands then consider using KOD Hot Start™ as this may reduce non-specific product formation. These parameters represent good starting conditions for testing and can usually amplify products up to 2 kbp in size. Optimization with specific primer pairs and templates may still be necessary.
4. If the PCR template has the same antibiotic resistance as your target pOPIN vector you must DpnI treat your PCR to digest away any template DNA. The DpnI enzyme is active in most PCR buffers and therefore can simply be added to each reaction. If large numbers of samples are to be processed simultaneously then you may consider making a large 'master mix' of buffer and DpnI such that approximately 5 µl (containing 0.5–1.0 units) of this mix can be added to each reaction.
5. When setting up downstream reactions, pipette the DNA from the plate while it is situated on the SPRIPlate96R. This will prevent bead carry over (however, the beads do not inhibit In-Fusion™ reactions). For long-term freezer storage, transferring AMPure™ purified samples into a new plate away from the magnetic particles is recommended.

6. This step increases the yield of DNA fragments <500 bp and >4 kb; for DNA fragments from 500 bp to 4 kb addition of isopropanol has no effect on the yield.
7. This step removes residual Buffer PE which may be present around the outlet nozzles and collars of the QIAquick Plate. Residual ethanol, from Buffer PE, may inhibit the subsequent In-Fusion reactions.
8. It is important to ensure that the elution buffer is dispensed directly onto the centre of QIAquick membrane for complete elution of bound DNA.
9. The In-Fusion reaction volume may be reduced by splitting the contents of the In-Fusion™ enzyme well into two or more wells. Adjust dilution volume in **step 4** accordingly. Total reaction volumes of 2.5 µl have been reported, but we have only tried down to 5 µl to date.
10. In our experience, picking two clones gives an average cloning efficiency for cloning 96 PCR products in parallel of approximately 90%; picking a further two clones can improve this to approximately 95%.
11. Glycerol stocks are a very convenient and stable archive format and can save time if plasmids require re-prepping at later dates.
12. Over-incubation during the alkaline lysis step can lead to nicking of the plasmid DNA.

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