Using In-Fusion® Technology for High-Throughput Precision Cloning of Constructs Expressing Signaling Pathway Protein Domains


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Obtaining recombinant proteins in a soluble form suitable for crystallization remains a bottleneck for high-throughput (HTP) structural biology. The Oxford Protein Production Facility has created the pOPIN vector suite for use with Clontech’s In-Fusion cloning system. This custom vector suite in combination with the In-Fusion enzyme delivers a versatile and precise one-step cloning process (i.e., no unwanted residues in the translated protein), that can be readily adapted to high-throughput structural biology studies. Incorporating the In-Fusion system into our HTP pipeline enabled parallel vector construction, expression screening, and purification, accelerating the protein production process.

Introduction

The Oxford Protein Production Facility (OPPF), a structural proteomics facility associated with the Oxford Module Consortium (OMC), is focused on generating high-quality structural data for proteins of biomedical relevance. Examples of such proteins are those from viral and bacterial human pathogens as well as other proteins associated with human disease. As such, the OPPF target list contains many proteins that can be problematic to express and crystallize. We have recently developed a high-throughput pipeline for the parallel cloning and expression of multiple constructs. At the outset, we identified the following criteria necessary for cloning and vector construction:

1. Amenable to HTP: A single PCR product may be inserted into multiple vectors containing different fusion partners.
3. Seamless: Capable of precise engineering of constructs, with no additional vector or restriction site-derived amino acids added to the expressed protein.
4. Versatile: Sequence-independent insertion.
5. No optimization required: Efficient over a wide insert size and concentration range, i.e., no normalization of PCR product concentration is necessary.

6. Host-independent: Constructs should be capable of expressing proteins from multiple hosts.

7. Capable of HTP purification: Expressed proteins must all be fused to a common affinity purification “tag,” which may be removed, if desired, by enzymatic digestion prior to crystallization.

The limitations of existing cloning systems led us to the development of an HTP cloning and expression pipeline that utilizes the unique features of the In-Fusion enzyme to satisfy all of these requirements. We have incorporated In-Fusion into a workflow suitable for cloning, expression and purification of diverse sequences from a variety of sources and a large number of projects, including the expression of cell signaling domains described in this article. In Figure 1, the In-Fusion reaction, pOPINF was prepared by digestion with KpnI and HindIII. The use of the 3C protease site as a 5’ In-Fusion site can generate a “seamless” fusion protein. After 3C protease cleavage of the fusion protein, a glycine and proline residue remain on the N-terminus of the digested protein.

Conclusion of the In-Fusion Ready pOPIN Vector Suite

We first produced a versatile suite of vectors for the expression of proteins or protein domains that incorporate tags for purification purposes, and protease cleavage sites for the removal of these tags (e.g., the 3C protease recognition site; 2) as the regions of homology are the property of their respective owners. Certain trademarks may not be registered in all jurisdictions.©2011 Clontech Laboratories, Inc.

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These vectors allow proteins to be expressed with either 5’ or 3’ tags, including different soluble expression tags, or tags for purification. The cleavage sites enable the tags to be removed prior to crystallization, which is important for resolving the native protein structure. Encoding cleavage sites into the 15 bp of homology allows for easy production of proteins that lack or contain minimal extraneous recombination sites, or vector-derived amino acids.

In addition, the vectors described here utilize multiple promoter systems such that a single construct may be used for protein expression in E. coli, mammalian or insect hosts. We have also included a lacZ insert between the linearization sites to distinguish inefficiently linearized vector from recombinant constructs by standard blue/white screening. The vectors have been named pOPIN vectors (plasmids for OPPF In-Fusion; 1). Further details are provided on the OPPF website at www.oppf.ox.ac.uk

The three-promoter vector pTriEx2 (Novagen) was used as the basis for construction of the pOPIN series of expression vectors (1). Editor’s Note: Although they are not a part of the OPPF workflow presented in this article, Clontech offers several In-Fusion Ready pre-linearized vectors that can be used in similar protocols for expression in mammalian systems (fluorescent vectors), bacterial systems (pEcoli vectors), or insect host cells (pBacPAK vectors). For more information, visit the Clontech website at www.clontech.com

**In-Fusion Cloning**

First, we designed the appropriate PCR primers sharing 15 bp of homology with the sites of linearization on the pOPIN vectors to enable successful In-Fusion cloning for expression of the desired polyhistidine-tagged proteins (see Table I for primer sequence details). The appropriate primer pairs were subsequently arranged in batches of 96. The DNA templates for amplification were also grown and prepared in a 96-well format such that the position of the template corresponded to the appropriate primer pair position. PCR reactions were performed in a 96-reaction format with each of the primer/template combinations using a high-fidelity enzyme (Novagen). The resulting PCR products were purified by a standard bind-elute method and analyzed by agarose gel electrophoresis (Novagen). The resulting PCR products were checked for efficiency by linearization of the pOPIN vectors (right panels/sequence). The majority of steps in the cloning and expression process have been fully automated across two of our robotic platforms (1). Further details are provided on the OPPF website at www.oppf.ox.ac.uk

96-well plate and incubated for 30 min. For an overview of the OPPF Cloning and Expression Screening Workflow, see Figure 2.

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**Table I: Summary of In-Fusion Site Sequences and Characteristics of Selected pOPIN Vectors**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Genbank Accession Number</th>
<th>Fusion Tag</th>
<th>Primer Extension</th>
<th>Restriction Enzymes Used for Linearization</th>
</tr>
</thead>
</table>
| pOPIN E | EF372397 | C-terminal ...KH HHHHHH | Forward: AGGAGATATACCATG  
Reverse: GTGATGGTGATGTTT | NcoI and HindIII |
| pOPIN F | EF372398 | N-terminal MAHHHHHHSSINGLEFQ ↓2 GP... | Forward: AAGTTCTGTTTCCAGGCGCCG  
Reverse: ATGGCTCAGAAAGCCTTA | KpnI and HindIII |
| pOPIN J | EF372395 | N-terminal MAHHHHHHSS-GST-LELVFQ ↓2 GP... | Forward: AAGTTCTGTTTCCAGGCGCCG  
Reverse: ATGGCTCAGAAAGCCTTA | KpnI and HindIII |
| pOPIN M | EF372396 | N-terminal MAHHHHHHSS-MBP-LELVFQ ↓2 GP... | Forward: AAGTTCTGTTTCCAGGCGCCG  
Reverse: ATGGCTCAGAAAGCCTTA | KpnI and HindIII |

1. These vectors use the same primer extensions, enabling the same PCR product to be cloned into all marked vectors. Underlined sequences represent translation initiation codons (methionine) or translation stop codons (as appropriate) and may be excluded from the gene-specific primers. The lysine residue at the beginning of the C-terminal polyhistidine-tag is included to act as a “lock” amino acid during removal of the C-terminal polyhistidine-tag by Carboxypeptidase A.

2. ↓ represents the protease cleavage site for 3C protease.

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**Figure 2. OPPF Cloning and Expression Screening Workflow.** Process flowchart from PCR amplification of target sequences to PCR-verification (left panels/sequence) and expression screening in E. coli (right panels/sequence). The majority of steps in the cloning and expression process have been fully automated across two of our robotic platforms (1).
Expression in E. coli

PCR-verified expression constructs were transformed into E. coli in a 96-well format for expression screening where protein expression was auto-induced (3). After harvesting, cell pellets were subjected to lysis and expression screening. The expressed polyhistidine-tagged proteins were extracted and purified by Ni-NTA magnetic beads and analyzed on SDS-PAGE gels (Figure 3).

OPPF In-Fusion Expression Screening Results

Using a combination of the pOPIN vector suite and In-Fusion enzyme, we were able to perform expression screening of all 96 target proteins in a two-week time frame. Our overall cloning efficiency was 94%, with 78% of the proteins expressed as soluble proteins in E. coli (Figure 3). Over a 12 month period, the OPPF used In-Fusion to construct a total of 661 vectors from 703 PCR products, with an overall cloning efficiency of 94%. The cloning efficiency achieved by using the In-Fusion system compares very favorably with data reported by other HTP structural genomics consortia using either the Gateway recombinatorial system (e.g., 79%; 4) or "classical" restriction enzyme and ligation-dependent methods (e.g., 87%; 5).

Editor’s note: Clontech now offers In-Fusion HD EcoDry™ Kits, an improved version of In-Fusion Dry-Down Kits.

Figure 3. Expression Screening of OMC pOPINF constructs (N-His-3C-tagged proteins) in E.coli. A plasmid master plate of 96 OMC pOPINF constructs was assembled by choosing a single PCR-verified clone for each construct, from the four clones that were picked for each construct after transformation of the In-Fusion reactions. This master plate represents (recombinant) coverage of 94% of the targets in the plate and was used for expression-screening in Rosetta pLysS cells (Novagen) using Overnight Express Instant TB Medium (Novagen). The resulting cells were lysed and polyhistidine-tagged soluble protein expression was analyzed by a Ni-NTA assay on a QIAGEN BioRobot 8000 and SDS-PAGE analysis. Soluble expression was observed for 78% of the cloned targets. Each lane, except the marker lanes (M), represents the purified protein expressed from a single polyhistidine-tagged construct. Each gel (Panels A–D) represents three columns of a 96-well block.

Editor’s Note: We understand that the gels reproduced in this article may be difficult to interpret. These gels are an important part of the scientific data and therefore are necessary. We believe that all scientific data should be faithfully reproduced and have chosen to display these gels in their original format.
Conclusion
The In-Fusion enzyme can be used in combination with any 15 bp homology region to enable ligation-independent cloning of PCR products. In-Fusion cloning enabled us to obtain the desired constructs without the addition of extraneous amino acids to the expressed protein. Unlike other systems, In-Fusion allowed us to clone the same insert into several distinct vectors of our own design, eliminating the need for additional steps required to prepare our insert for cloning with manufacturer-specified vectors and enzymes. We have exploited these properties, in combination with multiple promoter vectors, to produce a simple and versatile system that significantly accelerates the cloning process for the expression of recombinant proteins.

Over the past two years the OPPF has used In-Fusion to clone in excess of 1,000 gene sequences from a variety of sources into the pOPIN vectors. These constructs have been used for the expression of proteins not only in *E. coli*, but also in insect and mammalian cells for both structural and functional analyses.

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References