# TrueORF™ cDNA Clones and PrecisionShuttle™ Vector System

## Application Guide

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## *Package Contents and Storage Conditions*

The following components are included:

- One (1) vial containing the cDNA clone as 10 ug lyophilized plasmid DNA.
- Forward (VP1.5) and reverse (XL39) DNA vector sequencing primers; dried onto the bottom of screw cap tubes.

Store at room temperature. Once DNA is re-suspended in water or TE, store at -20ºC.
Certificate of Analysis
Application Guide

*The cDNA clone is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.*

## *Related, Optional Reagents*

Restriction enzymes and buffers
    Sgf I or ASIS I from NEB
    Mlu I from NEB
Nuclease free water
T4 DNA ligase and buffer
Competent *E. coli* cells
LB agar + ampicillin plates
LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH and autoclave)
Ampicillin
Kanamycin
LB agar + kanamycin plates
DNA purification reagents

## *Related Products*

| | |
|---|---|
| HuSH™ shRNA Plasmids | http://www.origene.com/rna/ |
| Validated Antibodies | http://www.origene.com/antibody/ |
| Functional Proteins | http://www.origene.com/protein/ |
| Transfection Reagents | http://www.origene.com/cdna/turbofectin.mspx |
| GFC-Transfection Arrays | http://www.origene.com/cdna/gfc-array/default.mspx |

## *Available Entry & Destination Vectors*

| SKU | PrecisionShuttle Vectors | Drug Selection | Cell Selection | Expression | Description |
|---|---|---|---|---|---|
| PS100001 | pCMV6-Entry | Kanamycin | Neomycin | Mammalian | C-terminal Myc and Flag® tag |
| PS100002 | pCMV6-AC-His | Ampicillin | Neomycin | Mammalian | C-terminal His tag |
| PS100003 | pCMV6-AC-Myc | Ampicillin | Neomycin | Mammalian | C-terminal Myc tag |
| PS100004 | pCMV6-AC-HA | Ampicillin | Neomycin | Mammalian | C-terminal HA tag |
| PS100005 | pCMV6-AC-Flag | Ampicillin | Neomycin | Mammalian | C-terminal Flag® tag |
| PS100006 | pCMV6-AC-Myc-His | Ampicillin | Neomycin | Mammalian | C-terminal Myc and His tag |
| PS100007 | pCMV6-AC-Myc-Flag | Ampicillin | Neomycin | Mammalian | C-terminal Myc and Flag® tag |
| PS100008 | pCMV6-AC-HA-His | Ampicillin | Neomycin | Mammalian | C-terminal HA and His tag |
| PS100009 | pCMV6-AC-Flag-His | Ampicillin | Neomycin | Mammalian | C-terminal Flag® and His tag |
| PS100010 | pCMV6-AC-GFP | Ampicillin | Neomycin | Mammalian | C-terminal GFP expression cassette |
| PS100011 | pCMV6-AN-His | Ampicillin | Neomycin | Mammalian | N-terminal His tag |
| PS100012 | pCMV6-AN-Myc | Ampicillin | Neomycin | Mammalian | N-terminal Myc tag |
| PS100013 | pCMV6-AN-HA | Ampicillin | Neomycin | Mammalian | N-terminal HA tag |
| PS100014 | pCMV6-AN-Flag | Ampicillin | Neomycin | Mammalian | N-terminal Flag® tag |
| PS100015 | pCMV6-AN-His-Myc | Ampicillin | Neomycin | Mammalian | N-terminal His and Myc tag |
| PS100016 | pCMV6-AN-Myc-Flag | Ampicillin | Neomycin | Mammalian | N-terminal Myc and Flag® tag |
| PS100017 | pCMV6-AN-His-HA | Ampicillin | Neomycin | Mammalian | N-terminal His and HA tag |
| PS100018 | pCMV6-AN-His-Flag | Ampicillin | Neomycin | Mammalian | N-terminal His and Flag® tag |
| PS100019 | pCMV6-AN-GFP | Ampicillin | Neomycin | Mammalian | N-terminal GFP tag |
| PS100020 | pCMV6-A | Ampicillin | Neomycin | Mammalian | Not tagged |

Flag® is a registered trademark of Sigma-Aldrich

**NOTE: FOR RESEARCH PURPOSES ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USAGE.**

All vectors are described in detail on OriGene's website at
http://www.origene.com/cdna/trueorf/trueorf.mspx

## *Description*

The TrueORF is an open reading frame (ORF) fused to a C-terminus Myc and Flag® tag. The TrueORF vector system is designed to express the ORF of a gene with one or more epitope tags or with a fluorescent marker. The tags or marker can be used to localize the expressed protein in the cell, to study protein-protein interactions, or to purify proteins using affinity columns and/or antibodies against the epitope tags (Figure 1).

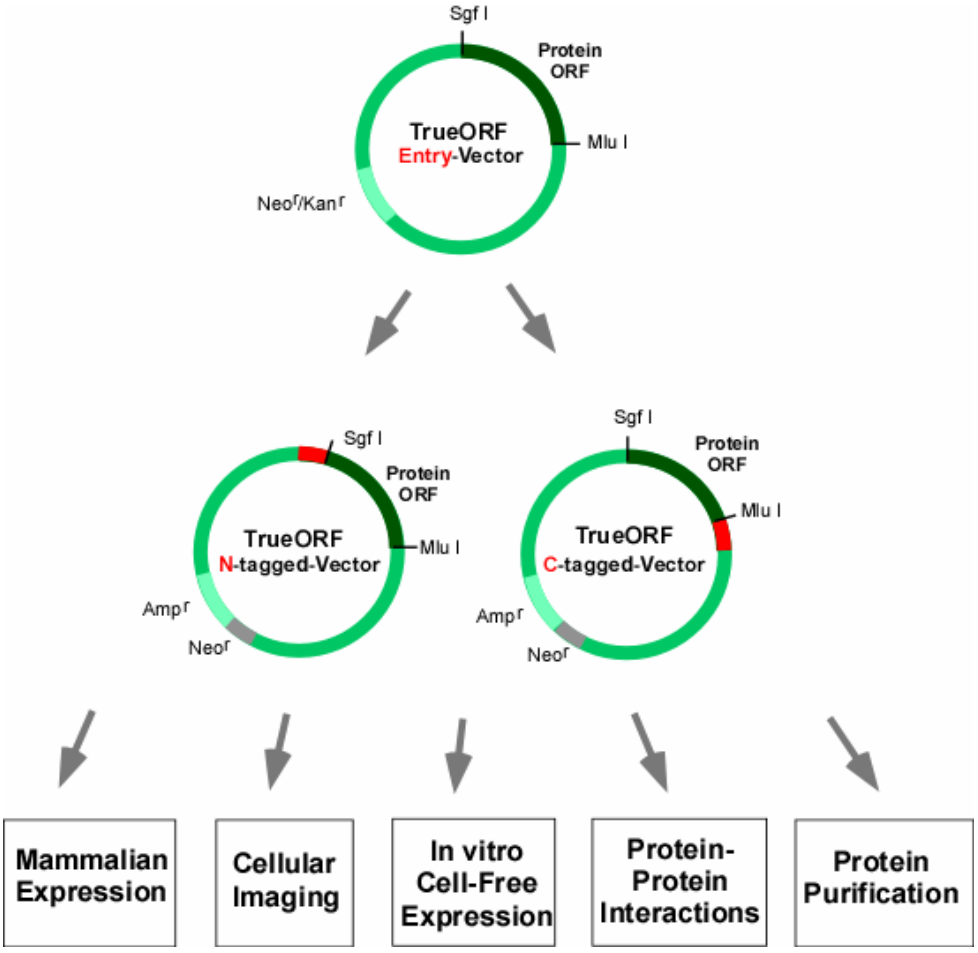



**Figure 1.** Potential applications of the PrecisionShuttle system for protein analysis. The Entry Vector (also called the TrueORF) contains the ORF of the gene of interest and can be directly used in your experiments of protein function analysis. The protein-coding region in the entry vector can also be easily shuffled to PrecisionShuttle Destination Vectors, which contain epitope tags or fluorescent marker.

All of the plasmids in the PrecisionShuttle™ vector system allow high-level target gene expression in mammalian cells or via *in vitro* translation in a cell-free system. The plasmids contain the promoter and enhancers of the human cytomegalovirus (CMV) immediate early gene to drive mammalian gene expression, and the T7 promoter for *in vitro* transcription/translation. An optimal Kozak consensus sequence is included in the plasmid to enhance mammalian cell expression.

All TrueORF entry clones are purified from a single colony and are shipped as a 10 ug quantity. Researchers can immediately use this DNA in their experiments by transfecting the provided purified DNA into target cells. Alternatively, the cDNA can be transferred into a destination

vector of choice, available with a variety of types and locations of fusion tags. All TrueORF destination vectors contain the same multiple cloning sites as the entry vector but a different antibiotic screening marker so that customers can easily transfer the ORF of interest into the destination vector of choice.

The PrecisionShuttle vector system employs a basic "cut and paste" molecular cloning method (Figure 2). It is faster, cheaper, more reliable and flexible than a recombination strategy, and no intellectual license is required for either academic or commercial users. The transfer of the ORF from the entry clone to any destination vector is a rapid process. Digestion, ligation and transformation takes as little as 3 hours (see protocol on page 12) since the entry vector and destination vector use different antibiotic selection markers. Unlike the recombination-based systems in which the entry clone is only a preliminary product, OriGene's Entry Vector contains C-terminal Myc and Flag® tags, and can be used directly for many applications including 1) tagged protein expression (C-terminal Myc/Flag in mammalian cells and 2) tagged protein expression in a cell-free system using the T7 promoter.

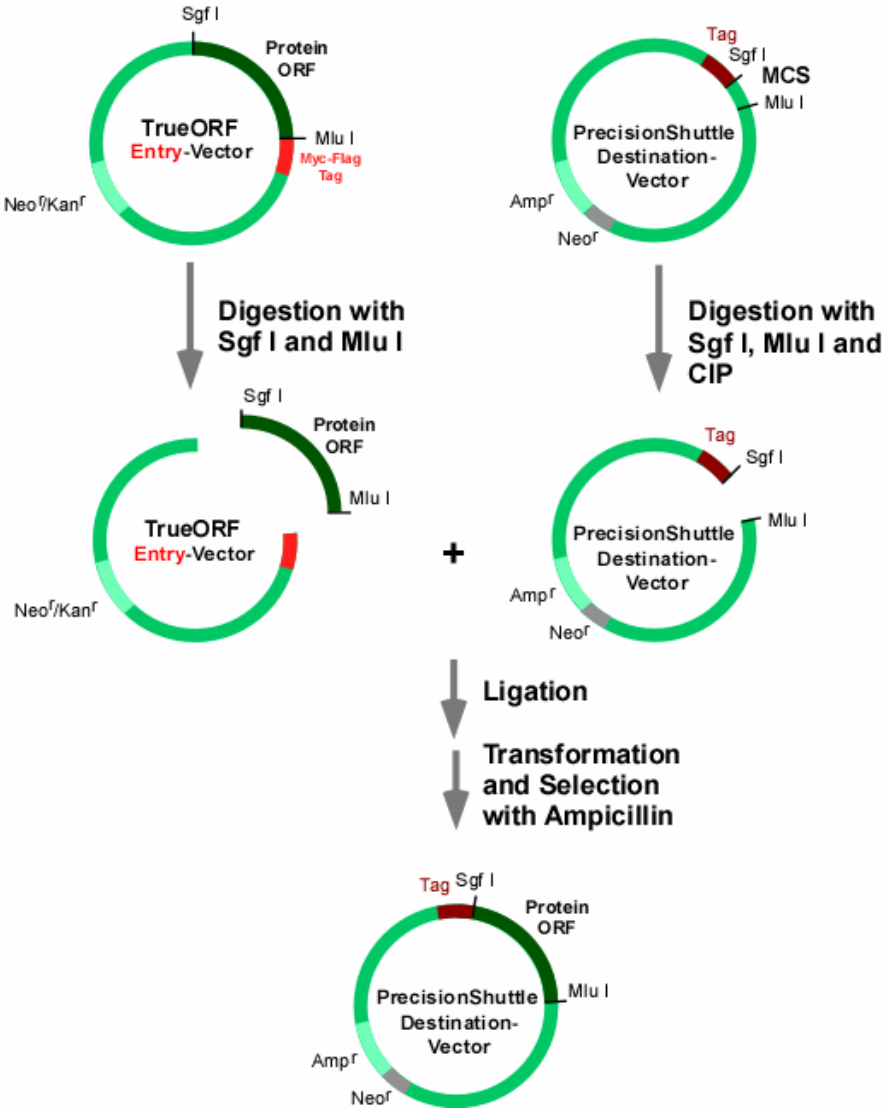



**Figure 2.** Schematic of the PrecisionShuttle subcloning procedure. The entry and destination vectors are digested with Sgf I and Mlu I or other specified enzymes, which rarely cut in mammalian coding sequences. After a ligation reaction, the resulting clones are grown on ampicillin-containing medium to select for successful subcloning of the ORF into the destination vector.

Two rare-cutting restriction enzymes are utilized in a simple yet powerful directional cloning method for transferring an ORF between vectors. Because of the correspondence between the multiple cloning sites (MCS) of the TrueORF entry and destination vectors, users have a precise, rapid and high-fidelity method for transferring an ORF between a variety of vectors. Most subcloning from the entry to a destination vector involves *Sgf* I (present in 0.37% of the human ORF) and *Mlu* I (4%). In the very unusual case when *Sgf* I and *Mlu* I sites are inside the ORF, the TrueORF vector MCS provides other rare restriction sites, such as Asc I, Rsr II, and Not I so that any ORF can be shuttled from the entry vector to a destination vector by using some combination of these five rare restriction enzymes. Unlike site-specific recombination vector systems, the TrueORF Clone System does not append multiple amino acids to the amino or carboxy terminus of the protein of interest. The subcloning strategy maintains insert orientation and reading frame, eliminating the need to resequence the insert after each transfer. Because the entry and destination vectors have different antibiotic resistance genes, selection after subcloning is a very simple process.

While the PrecisionShuttle vector system can be used for any cDNA, we have developed this system to take advantage of the largest collection of human full-length cDNA clones available at OriGene. Every human cDNA clone is offered in the entry vector as a TrueORF clone, and the customer can easily transfer this ORF into any destination vector. A special effort has been incorporated into the synthesis of these TrueORF clones to minimize the generation of mutations. By using a large quantity of high-quality cDNA template, a minimum of PCR cycles and a polymerase with the highest fidelity (one mutation in 40,000,000 bp), the number of mutations is very limited. No mutations were identified during the initial cloning of over 200 ORF cDNAs (ranging from 500-6000bp) into the entry vector after full-length sequencing of each of these clones. The MCS of the PrecisionShuttle vectors was engineered to be compatible with most other commercially available vector systems including Gateway vectors (Invitrogen), PET vectors (Novagen) and Flexi vectors (Promega). In this sense, the TrueORF vector system is truly universal.

The PrecisionShuttle vector system was not only developed for individual researchers to work on one or two genes at a time, but also for high throughput applications that require thousands of ORFs in a standard vector. Unlike recombination-based systems, this digestion-based system requires only nanograms of vector for successful ORF transfer. A specific destination vector can be predigested and then mixed with the digested TrueORF clones, then ligated and transformed into competent *E. coli* cells. The whole process can be readily adapted to a 384-well format. With the availability of over 32,000 unique full-length human cDNA clones, OriGene is in an enviable position to develop and support such high throughput applications.

The PrecisionShuttle entry and destination vectors contain the neomycin phosphotransferase gene under the dual control of the β-lactamase promoter and the SV40 promoter. Expression of the neomycin phosphotransferase gene in mammalian cells allows stable cell selection with a neomycin analog such as G418, whereas in bacteria the gene confers resistance to kanamycin selection.

The development of the PrecisionShuttle vector system has gone through a rigorous quality control (QC) process. Both the entry vector and the destination vectors have been validated for transient and stable mammalian cell transfections using a T-GFP marker (data not shown). The N-terminal and C-terminal fusion tags have been validated by Western blot analysis (shown in Figure 3).

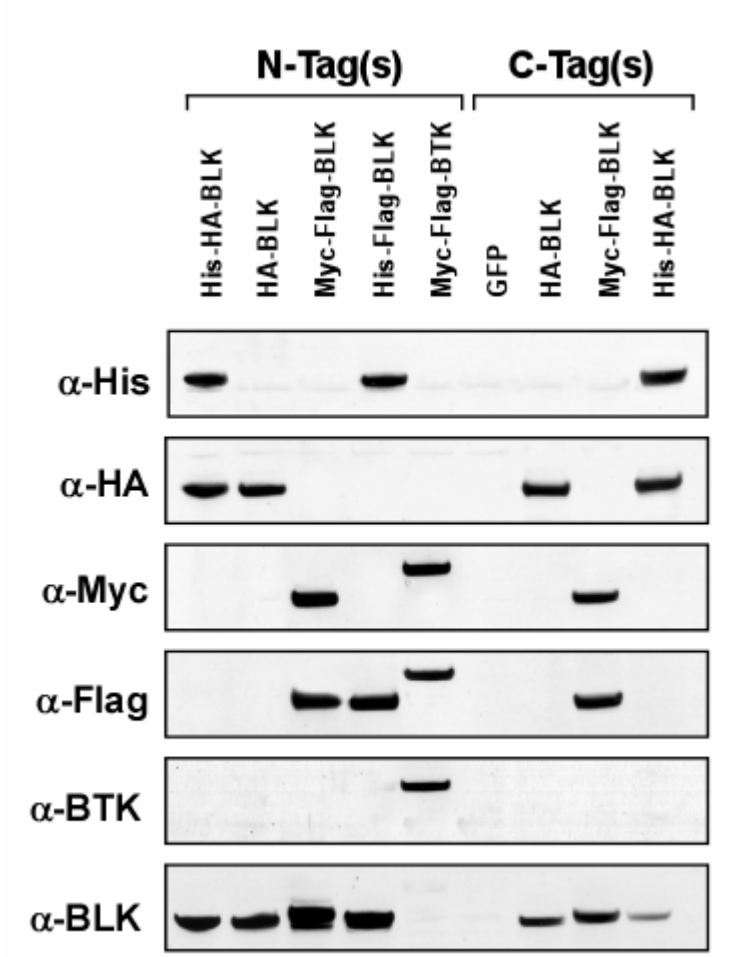



**Figure 3.** Western blot analysis of proteins expressed from N-terminally and C-terminally tagged PrecisionShuttle vectors. Each lane of the blot contains the whole cell lysate from an overexpression experiment using a PrecisionShuttle vector. BLK represents human B lymphoid tyrosine kinase (NM_001715); BTK represents human Bruton agammaglobulinemia tyrosine kinase (NM_000061). These two cDNAs were cloned into the destination vectors identified at the top of the blot. T-GFP represents one empty destination vector, pCMV6-AC-GFP, used for cloning C-terminal T-GFP fusion proteins. Specific antibodies against BLK and BTK detected the same size proteins as antibodies against the N-terminal and C-terminal tags.

## *PrecisionShuttle Vector Details*

### TrueORF Entry Vector (pCMV6-Entry)

OriGene's TrueORF clones contain the fully sequenced ORFs of the gene of interest in pCMV6-Entry. This construct can be directly used for transient or stable transfection of cultured mammalian cells or for *in vitro* protein expression. It also can act as a donor of the protein-coding region using the *Sgf* I and *Mlu* I sites. The different antibiotic resistance genes carried on the TrueORF Vectors (kanamycin resistance in the entry vector and ampicillin resistance in the destination vectors) facilitate the selection of successfully transferred protein-coding regions from entry to destination vectors. The entry vector also contains C-terminal tags of Myc and Flag tag and the neomycin resistance gene.

### N-terminally tagged PrecisionShuttle destination vectors (pCMV6-AN)

These vectors are designed for expressing N-terminally tagged proteins in mammalian cells, or using *in vitro* protein expression systems. The plasmids allow protein expression via human cytomegalovirus (CMV) intermediate-early enhancer/promoter in mammalian cells and via the T7 RNA polymerase promoter in cell free systems. The vectors contain the ampicillin resistance gene for selection in *E. coli* and the neomycin resistance gene enables stable cell selection in mammalian cells. Available tags include Myc, HIS, HA, Flag, and T-GFP.

### C-terminally tagged PrecisionShuttle destination vectors (pCMV6-AC)

These vectors are designed for expressing C-terminally tagged proteins in mammalian cells, or using *in vitro* protein expression systems. The plasmids allow protein expression via human cytomegalovirus (CMV) intermediate-early enhancer/promoter in mammalian cells and via the T7 RNA polymerase promoter in cell free systems. The vectors contain the ampicillin resistance gene for selection in *E. coli* and the neomycin resistance gene enables stable cell selection in mammalian cells. Available tags include Myc, HIS, HA, Flag, and T-GFP.

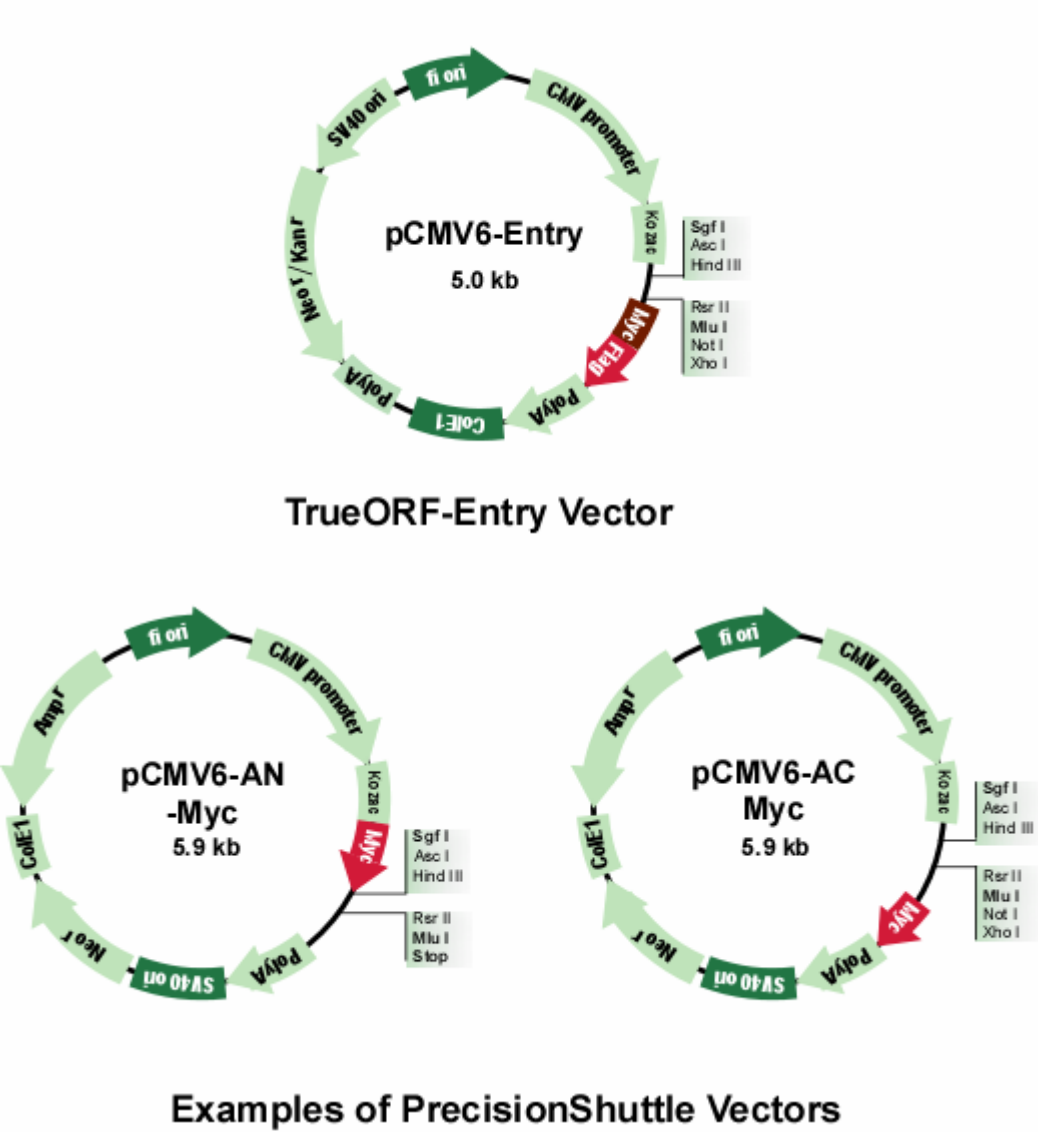
### Untagged PrecisionShuttle vector (pCMV6-A)

This vector is designed for expression of the native (untagged) protein in mammalian cells, or using *in vitro* protein expression systems. The vector contains the same promoters and selection markers (ampicillin and neomycin) as the N- and C-terminally tagged destination vectors. The encoding protein contains 2 additional amino acids at the C-terminus

**Table 1. Features of PrecisionShuttle vectors**

| PrecisionShuttle Vector | SKU | Drug Selection | Peptide Fusion Tag or Marker | |
|---|---|---|---|---|
| | | | N-terminus | C-Terminus |
| pCMV6 Entry Vector (C-terminal Myc-Flag tag) | PS100001 | Kanamycin | - | Myc-Flag |
| pCMV6-AC-His | PS100002 | Ampicillin | - | His |
| pCMV6-AC-Myc | PS100003 | Ampicillin | - | Myc |
| pCMV6-AC-HA | PS100004 | Ampicillin | - | HA |
| pCMV6-AC-Flag | PS100005 | Ampicillin | - | Flag |
| pCMV6-AC-Myc-His | PS100006 | Ampicillin | - | Myc-His |
| pCMV6-AC-Myc-Flag | PS100007 | Ampicillin | - | Myc-Flag |
| pCMV6-AC-HA-His | PS100008 | Ampicillin | - | HA-His |
| pCMV6-AC-Flag-His | PS100009 | Ampicillin | - | Flag-His |
| pCMV6-AC-GFP | PS100010 | Ampicillin | - | GFP |
| pCMV6-AN-His | PS100011 | Ampicillin | His | - |
| pCMV6-AN-Myc | PS100012 | Ampicillin | Myc | - |
| pCMV6-AN-HA | PS100013 | Ampicillin | HA | - |
| pCMV6-AN-Flag | PS100014 | Ampicillin | Flag | - |
| pCMV6-AN-His-Myc | PS100015 | Ampicillin | His-Myc | - |
| pCMV6-AN-Myc-Flag | PS100016 | Ampicillin | Myc-Flag | - |
| pCMV6-AN-His-HA | PS100017 | Ampicillin | His-HA | - |
| pCMV6-AN-His-Flag | PS100018 | Ampicillin | His-Flag | - |
| pCMV6-AN-GFP | PS100019 | Ampicillin | GFP | - |
| pCMV6-A | PS100020 | Ampicillin | - | - |

**PrecisionShuttle Vector Maps**




**Multiple cloning sites in the PrecisionShuttle Entry Vector**

Kozac Consensus

*EcoR I* *BamH I* *Kpn I* RBS *Sgf I* *Asc I*

```
CTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCGGCGCGCCAGATCT
```

*Hind III* *Nhe I* *Rsr II* *Mlu I* *Not I* *Xho I* Myc.Tag

```
CAAGCTTAACTAGCTAGCGGACCG ACG CGT ACG CGG CCG CTC GAG CAG AAA CTC ATC TCT GAA GAG
                         T   R   T   R   R   L   E   Q   K   L   I   S   E   E
```

*EcoR V* Flag.Tag *Pme I* *Fse I*

```
GAT CTG GCA AAT GAT ATC CTG GAT TAC AAG GAT GAC GAC GAT AAG GTT TAA ACGGCCGGCCGC
D   L   A   N   D   I   L   D   Y   K   D   D   D   D   K   V  Stop
```

## *Primer Design and PCR Amplification of ORF*

The open reading frame (ORF) of the clone must be PCR amplified in order to append cloning sites to the 5' and 3' ends of the sequence. Add the target sequences of the selected restriction enzymes to the forward and reverse PCR primers; examples are shown below.

Forward primer with Sgf I
5' GAG**GCGATCGC**CNNNNNNNNNNNNNNNNNNNNN 3'
(Ns represent the sequence of the ORF beginning with the start codon, ATG)

Reverse primer with Mlu I
5' GCG**ACGCGT**NNNNNNNNNNNNNNNNNNNNN 3'
(Ns represent the reverse complement of the ORF sequence starting **with the stop codon** for N-terminally tagged or untagged destination vectors. This ensures that the expressed fusion protein will end at the native C-terminal end of the ORF. For C-terminally tagged vectors, the reverse complement of the ORF sequence should start **with the second-to-last codon**, as the stop codon must be removed to generate a fusion protein.

If the recognition sites for Sgf I or Mlu I are present internally in the ORF, another rare cutter such as Asc I, Rsr II or Not I can be used in the cloning strategy. In these cases, the sequences of these alternate restriction sites should be used in place of Sgf I and/or Mlu I (examples below). This same primer design strategy described above should be used for design of other primers. The Ns in the forward primer represent the sequence of the ORF beginning with the start codon, ATG. The Ns in the reverse primers represent the reverse complement of the ORF sequence starting **with the stop codon** for N-terminally tagged or untagged destination vectors, or starting **with the second-to-last codon** for C-terminally tagged vectors.

Forward primer with Asc I:
5' GCC**GGCGCGCC**NNNNNNNNNNNNNNNNNNNNN 3'

Reverse primer with Rsr II:
5' GCGT**CGGACCG**CTNNNNNNNNNNNNNNNNNNNNN 3'

Reverse primer with Not I:
5' GCGAC**GCGGCCGC**TCACGCGTNNNNNNNNNNNNNNNNNNNNN 3'

We recommend using a full-length cDNA clone as the template for ORF cloning. The success rate is rather low when a cDNA pool is used as the template for a PCR cloning reaction. When the GC content of an ORF (or a region of the ORF longer than 100 bp) is above 75%, a special PCR buffer with DMSO or other additive should be used to increase the success rate. The recommended PCR polymerase and buffer are available from New England Biolabs (Phusion™ High-Fidelity PCR Kit, F-553S).

PCR reaction setup:

| Component | Volume |
|---|---|
| 5X PCR buffer | 4 μl |
| dNTPs (2.5 mM each) | 1.6 μl |
| Phusion polymerase (2U/μl) | 0.2 μl |
| Nuclease free water | 11 μl |

| | |
|---|---|
| Forward primer (10 μM) | 0.6 μl |
| Reverse primer (10 μM) | 0.6 μl |
| cDNA template | 2 μl (50-100ng plasmid) |
| Total volume | 20 μl |

All the components should be kept on ice. When setting up multiple reactions, a master mix can be prepared without cDNA template or primers. After aliquotting the master mix, the cDNA template and primers can be added individually to each tube.

PCR cycling conditions:

The optimum Tm for annealing should be 55-60°C. The extension time depends upon the length of the ORF. The following program is generally used for ORF from 500 bp-6000 bp.

| | | |
|---|---|---|
| 95°C | 1 min | |
| 2 cycles of | 95°C | 10 sec |
| | 62°C | 20 sec |
| | 72°C | 4 min |
| 2 cycles of | 95°C | 10 sec |
| | 60°C | 20 sec |
| | 72°C | 4 min |
| 2 cycles of | 95°C | 10 sec |
| | 58°C | 20 sec |
| | 72°C | 4 min |
| 15 cycles of | 95°C | 10 sec |
| | 56°C | 20 sec |
| | 72°C | 4 min |
| | 72°C | 10 min |
| | 4°C | hold |

## *Cloning of ORF into the Entry Vector*

1. Confirm that the size of the amplification product is correct by agarose gel electrophoresis, and purify the remainder of the reaction using a purification column or similar method. Elute the DNA from the purification column in 26 μl of 10 mM Tris buffer. Set up a digestion reaction as described below, substituting other restriction enzymes as appropriate.

| Component | Volume |
|---|---|
| 10X restriction buffer | 3 μl |
| Sgf I (10U/μl) | 0.6 μl |
| Mlu I (10U/μl) | 0.6 μl |
| Purified PCR product | 26 μl |
| Total volume | ~30 μl |

Mix well, and incubate at 37°C for 1 hour.

2. Purify the digestion reaction using a purification column and elute in 18 μl of 10 mM Tris buffer.

3. Digest pCMV6-Entry with the restriction enzymes corresponding to the sequences added to the ORF. pCMV6-Entry is available from OriGene as 5 μg lyophilized DNA. Resuspend the

lyophilized DNA in 50 μl 10 mM Tris buffer, and incubate for at least 30 min before use. Set up a digestion reaction as described below, substituting other restriction enzymes as appropriate.

| Component | Volume |
|---|---|
| 10X restriction buffer | 3 μl |
| Sgf I (10U/μl) | 0.8 μl |
| Mlu I (10U/μl) | 0.8 μl |
| Nuclease free water | 15.4 μl |
| Vector DNA | 10 μl |
| Total volume | 30 μl |

Incubate at 37°C for 1.5 hr, then add 0.5 μl alkaline phosphatase, and continue the incubation at 37ºC for another 30 min. A two hour digestion is recommended to ensure that the vector is completely digested. Dephosphorylation of the digested vector is essential to eliminate self-ligation.

4. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolating the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 μl of 10 mM Tris buffer.

5. Set up a ligation reaction with the purified vector and insert fragments:

| Component | Volume |
|---|---|
| 10X ligase buffer | 1 μl |
| nuclease free water | 3.5 μl |
| T4 DNA ligase | 0.5 μl |
| Vector fragment | 2 μl |
| PCR product | 3 μl |
| Total volume | 10 μl |

Incubate the ligation reaction at room temperature for 30-60 minutes.

6. Transform 1 μl of the ligation mixture using 20 μl high efficiency competent E. coli cells (ideally $1x10^8$ CFU/ug). Following transformation, resuspend cells in 200 uL LB.

7. Plate the entire transformation reaction on a standard LB-agar plate containing 25 μg/ml kanamycin. Incubate at 37°C overnight.

8. Pick at least 4-8 independent colonies from each ligation. Confirm the insert by restriction digestion and/or vector primer sequencing.

## *Transfer of ORF from TrueORF Entry Vector to destination vectors*

To transfer the protein-coding region from the TrueORF Entry Vector (donor) to a PrecisionShuttle destination vector (recipient), choose the appropriate destination vector with the desired tag options (Table 1). There are three types of PrecisionShuttle destination vectors, each of which is designed to express a native (untagged) protein, an N-terminally tagged protein, or a C-terminally tagged protein. The translation of an N-terminally tagged protein initiates from the ATG of the tag and continues through the ORF of the gene of interest, whereas translation of an untagged or a C-terminally tagged protein initiates from the ATG of the protein of interest. The ORF of a C-terminally tagged protein is followed by either a single

tag, or a double tag including a short spacer (5-6 amino acid residues). The transfer protocol between TrueORF vectors is shown schematically in Figure 2, and is detailed below.*

1. Digest the TrueORF entry clone:

| Component | Volume |
|---|---|
| 10X restriction buffer** | 2 µl |
| Sgf I (10 U/µl) | 0.6 µl |
| Mlu I (10 U/µl) | 0.6 µl |
| nuclease-free water | 13.8 µl |
| TrueORF entry vector (100-200ng) | 3 µl |
| Total volume | 20 µl |

   Incubate at 37°C for 1 hour.

2. Digest the TrueORF destination vector:

| Component | Volume |
|---|---|
| 10X restriction buffer** | 2 µl |
| Sgf I (10 U/µl) | 0.6 µl |
| Mlu I (10 U/µl) | 0.6 µl |
| nuclease-free water | 14.8 µl |
| TrueORF destination vector (200ng) | 2 µl |
| Total volume | 20 µl |

   * For the 4% of the clones that have internal Sgf I or Mlu I sties, please use the appropriate combination of restriction sites as recommended by OriGene
   ** .NEB buffer 3 has been shown to work well with dual digestion of Sgf I and Mlu I.

   Incubate at 37°C for 1 hour. Add 0.4 µl calf intestine phosphatase to the digestion, and continue to incubate at 37°C for an additional 30 minutes.

3. Purify the digestion using a commercial PCR purification column and elute in 20 ul 10 mM Tris.

4. Set up a ligation reaction:

| Component | Volume |
|---|---|
| 10 x T4 DNA ligation buffer | 1 µl |
| T4 DNA Ligase (4U/µl) | 0.75 µl |
| nuclease-free water | 3.25 µl |
| digested DNA from Step 1 | 2 µl |
| digested DNA from Step 2 | 3 µl |
| Total volume | 10 µl |

   Incubate the ligation reaction at room temperature for 1 hour.

5. Transform the ligation reaction into high-efficiency, competent *E. coli* cells (≥ $1\times10^8$ CFU/µg DNA) following the appropriate transformation protocol. Plate the transformants on LB-agar plates supplemented with 100 µg/ml ampicillin.
6. Pick at least four colonies for subsequent DNA purification and screening. Amplify and purify the selected clone(s) by growing overnight in liquid LB-amp media, then isolating the DNA using standard plasmid purification procedures.

7. Confirm the insert by restriction digestion and/or vector primer sequencing using the provided V1.5 for 5' end sequencing and XL39 for 3' end sequencing.

## *Troubleshooting*

For questions not addressed here, please contact OriGene's Technical Support professionals. You may dial 888-267-4436 from any US location, or 301-340-3188 outside the US. E-mail inquiries to techsupport@origene.com are also invited.

### No colonies or low number of colonies from transformation

| Cause | Remedy |
|---|---|
| The competent cells used in the transformation were not as efficient as necessary. | Obtain a fresh batch of competent cells and ensure that the efficiency is ≥ $1\times10^8$ CFU/μg DNA by performing a separate transformation reaction with a transformation-qualified control (usually a fixed amount of supercoiled plasmid such as pUC19). In some extreme cases, especially for larger inserts (>5 kb), higher efficiency cells or electroporation may be required. Should a gene prove to be toxic to the cells, transforming into strains that reduce the copy number can increase the odds of obtaining colonies (i.e. ABLE-C or ABLE-K strains; Stratagene, La Jolla CA). |
| Too little DNA was used in the transformation reaction. | Add more DNA (but not more than 10% of the volume of competent cells used). |
| The ligation of the ORF donor DNA into the recipient plasmid was not successful. | The ligase enzyme may not work properly. Repeat the reaction with fresh ligase and ligation buffer (which contains the temperature-sensitive component, ATP) or perform troubleshooting as recommended by the manufacturer of the ligase. |
| The wrong antibiotic selection plate was used. | Make sure to use an LB-agar plate containing the correct antibotics (e.g. ampicillin for destination vector and kanamycin for entry vector. |

### Too high self-ligation background (no insert) from destination vector

| Cause | Remedy |
|---|---|
| The destination plasmid was not completely digested. | Allow the digestion reaction to continue for 1-2 hours at 37°C. |
| The dephosphorylation of the destination plasmid was not complete, and the destination vector religated with its own fragment. | Increase the concentration of CIP and/or the length of the dephosphorylation incubation as recommended by the ligase manufacturer. |

## *Frequently Asked Questions*

### Why should I use OriGene's TrueORF clones?

Answer: All TrueORF Clones are derived from OriGene's unique TrueClone Collection, and were isolated from high quality human cDNA libraries made from a variety of tissues. TrueORF Clones provide enough (10 µg) purified DNA to allow customers to directly apply these expression-ready, tagged ORF clones to experiments designed for protein expression, purification, protein-protein interaction and stable clone selection. TrueORF clones also serve as the entry vector for OriGene's PrecisionShuttle system and allow easy construction of variably tagged ORFs. This saves valuable time by eliminating the need for subcloning, verification and amplification. All TrueORF vectors share the same multiple cloning sites (MCS), which are compatible with many other commonly used vector systems, such as Promega's Flexi system, Invitrogen's Gateway system and Novagen's PET system. Customers can easily shuffle the cDNA of a TrueORF clone between multiple TrueORF destination vectors to generate clones with different epitope tags, or transfer the ORF to other expression systems designed for specific experimental purposes.

**What is the difference between OriGene's entry vector and the destination vectors?**
The major differences are the antibiotic selection marker and the epitope tags or markers. The entry vector carries kanamycin resistance, while all destination vectors contain the ampicillin resistance gene. This allows simple screening for successful subcloning products. All of the vectors have a unique combination of N- and C-terminal epitope tags or a fluorescent marker, as described in Table I.

**What restriction enzymes should I use if Sgf I or Mlu I sites are present in my ORF?**
While 96% of all human ORFs can use the Sgf I - Mlu I combination, some ORFs do contain internal Mlu I site(s). Most of those ORFs with an internal Mlu I site can be transferred using another rare cutter (Rsr II), whose restriction site is upstream of Mlu I, or Not I, whose site is immediately downstream of Mlu I. Using one of the four different subcloning combinations, any ORF can be transferred from one vector to another. The recommended subcloning combination for every TrueORF cDNA is listed in the product information on our website.

**Has OriGene fully sequenced all TrueORF clones?**
Answer: Not always. When transferring the cDNA into the TrueORF Entry Vector, OriGene always uses fully sequenced plasmids as templates and Phusion High-Fidelity DNA Polymerase (New England Biolabs), which has a mutation rate less than $4 \times 10^{-7}$. This ensures the highest fidelity of every TrueORF clone. After cloning into the entry vector, each of OriGene's TrueORF clones was sequenced at both the 5' and 3' ends, and the resulting sequence was matched to the corresponding reference sequence. For many ORFs 1 Kb or less in length, the 5' and 3' sequencing reads have covered the full ORF. For longer cDNAs, the ORF was not fully covered by sequencing reads.

**Do TrueORF clones exactly match the reference gene sequence?**
Answer: All TrueORF clones are guaranteed to match the ORF of the corresponding gene sequences as published on OriGene's website. However, some clones may contain nucleotide changes compared to the published reference sequences. This is due to SNPs (single nucleotide polymorphisms) reflecting the unique differences from genes expressed in different tissues and different individuals. Published references may represent a different SNP than the OriGene transcript. Should a specific SNP be required, this can be contracted from OriGene at an additional charge.

**Can I transfer large ORFs using this system?**
Answer: It has been reported that ORFs larger than 4 Kb are unstable in recombination-based systems; conversely, our restriction digest-based vector system has no real size limitation. An ORF up to 18 Kb can be readily transferred from one vector to another.

**What does your disclaimer mean?**
Answer: OriGene's disclaimer for the TrueORF clones reads as follows: "Our molecular clone sequence data has been matched to the accession number below as a point of reference. Note that the complete sequence of our molecular clones may differ from the sequence published for this corresponding accession number, e.g., by representing an alternative RNA splicing form or single nucleotide polymorphism (SNP)."

The NCBI RefSeq human mRNA sequences are continuously being revised, as some may have been derived from aberrantly spliced transcripts or generated by incorrect prediction of intron-exon junctions in silico. These sequences are therefore used only as a "reference" and not as a "standard". OriGene's clones are isolated from full-length cDNA libraries and may differ from the reference sequence for this reason.

**What is the TrueORF Guarantee?**
Answer: OriGene warrants that the product will meet specifications listed. At OriGene's discretion, free replacement of any non-conforming product will be made if OriGene is notified within 30 days of product receipt. If you experience any difficulty with any OriGene product, please contact our Technical Support Staff at 888-267-4436, or 301-340-3188 outside the US.

## *Nucleotide Sequences of PrecisionShuttle vectors*

All sequences are available electronically on the OriGene website at
http://origene.medigent.com/cdna/trueorf/destinationvector.mspx