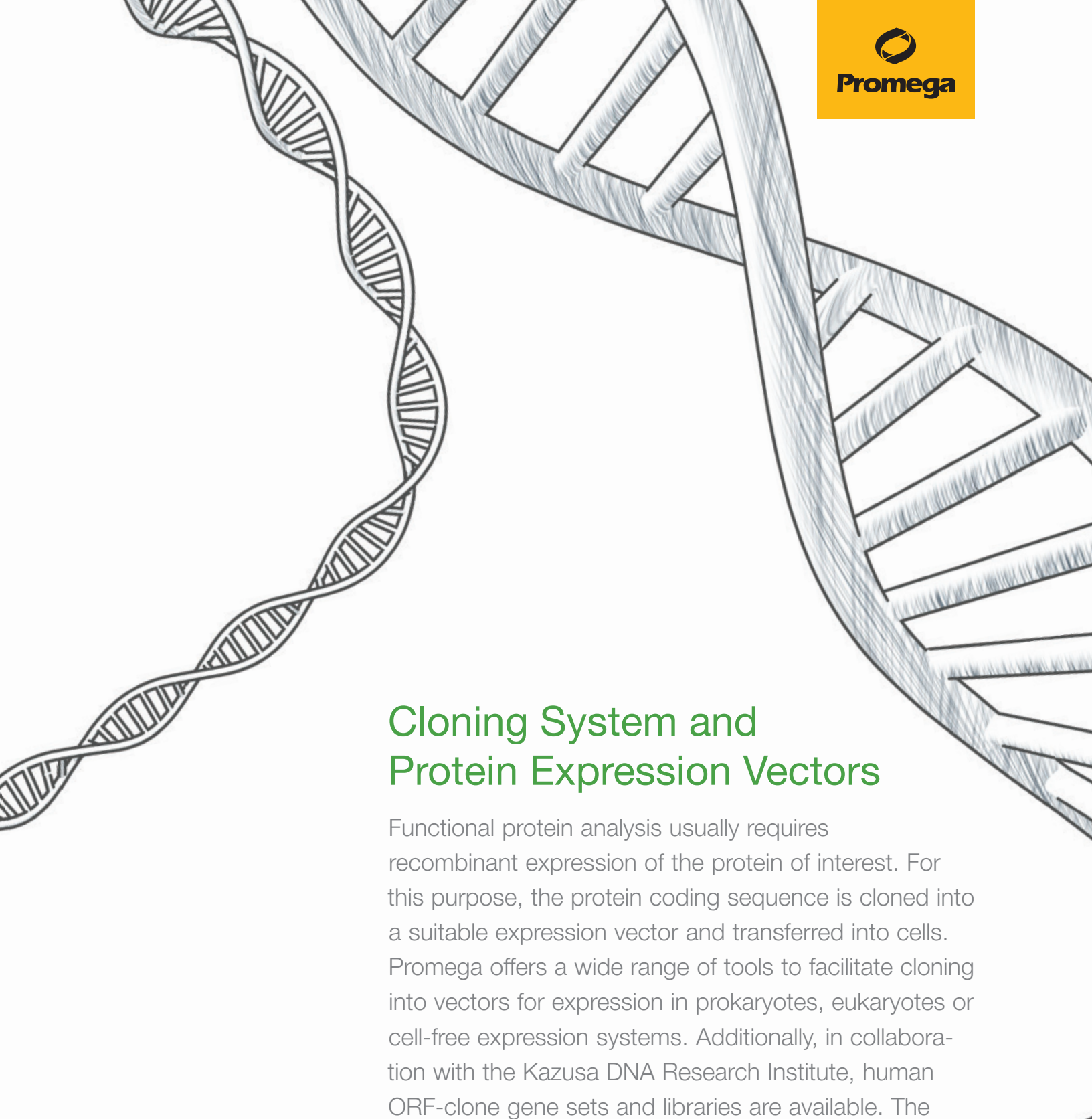
1 Cloning System and Protein Expression Vectors



Cloning System and Protein Expression Vectors

Functional protein analysis usually requires recombinant expression of the protein of interest. For this purpose, the protein coding sequence is cloned into a suitable expression vector and transferred into cells. Promega offers a wide range of tools to facilitate cloning into vectors for expression in prokaryotes, eukaryotes or cell-free expression systems. Additionally, in collaboration with the Kazusa DNA Research Institute, human ORF-clone gene sets and libraries are available. The ORF-clones in the library are extensively validated and are available as native ORFs and N-terminal HaloTag® fusions (HaloTag® ORF-clones). Furthermore, specialized expression vectors as well as competent cells for vector propagation can be found in this chapter.

Flexi® Cloning System

Flexi® Vector Systems are directional cloning systems that provide a method for transferring protein-coding sequences between different expression vectors without the need to resequence.

Description

Flexi® Vector Systems provide an efficient and high-fidelity method for transferring protein-encoding DNA into vectors capable of expressing native (non-tagged) protein or protein with an amino- (N-) or carboxy- (C-) terminal tag in bacterial, mammalian or cell-free expression systems. Once your protein-coding region is cloned into a Flexi® Vector, you can easily shuttle it into other Flexi® Vectors with different configurations without the need for resequencing **(Figure 1.1)**.

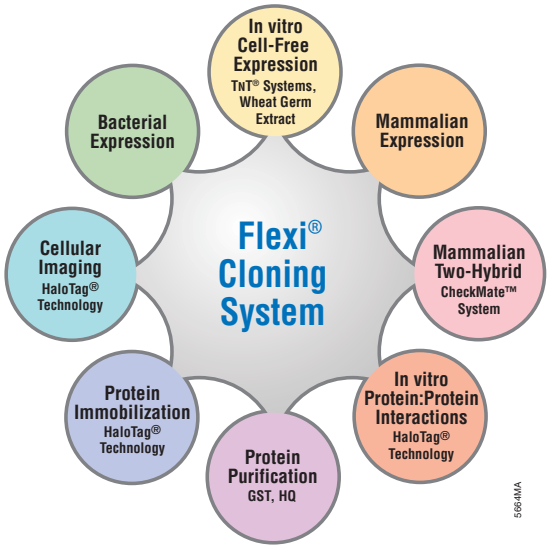


Figure 1.1. The Flexi® Vector Systems allow easy and efficient transfer of a protein-coding region between a wide variety of expression vectors without the need to resequence.

Principle

The Flexi® Vector System uses two rare-cutting restriction enzymes, SgfI and PmeI (both 8-cutters) in a simple, directional cloning method for protein-coding sequences. The desired protein-coding region is amplified by PCR before being cloned into one of the Flexi® Vectors **(Figure 1.2)**. An easy tool is available at the Promega website for primer design and to scan the nucleic acid sequence of the protein of interest sequence for SgfI and PmeI sites.

Flexi® Systems allow direct insertion into the type of vector suited to the experimental design. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a negative selection marker for successful ligation of the PCR-amplified insert. To transfer the protein-coding region from one Flexi® Vector (donor) to another Flexi® Vector (acceptor) choose an appropriate acceptor vector with the desired expression and tag options **(Figure 1.3)**. The donor and acceptor vectors are digested with the Flexi® Enzyme Blend (SgfI and PmeI) prior to ligation of the insert, transformation and selection of cells. The PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the C-terminus of the protein.

C-terminal Flexi® Vectors allow expression of C-terminal-tagged proteins. While these vectors can act as acceptors of a protein-coding region flanked by SgfI and PmeI sites, they lack a PmeI site and contain a different blunt-end site, EcoICRI **(Figure 1.3, Panel B)**. When the blunt PmeI and EcoICRI ends are joined, the stop codon is not recreated, allowing readthrough into the C-terminal peptide sequence. However, this joined sequence cannot be cut by either PmeI or EcoICRI, so the protein-coding region cannot be removed from the C-terminal Flexi® Vectors and transferred to other Flexi® Vectors. In other words, transfer into C-terminal Flexi® Vectors is not reversible (i.e., it is a one-way exchange). By cloning the PCR fragment first into a native or N-terminal Flexi® Vector, the ability to transfer to any other Flexi® Vector is preserved **(Figure 1.3, Panel A)**.

Flexi® Cloning System

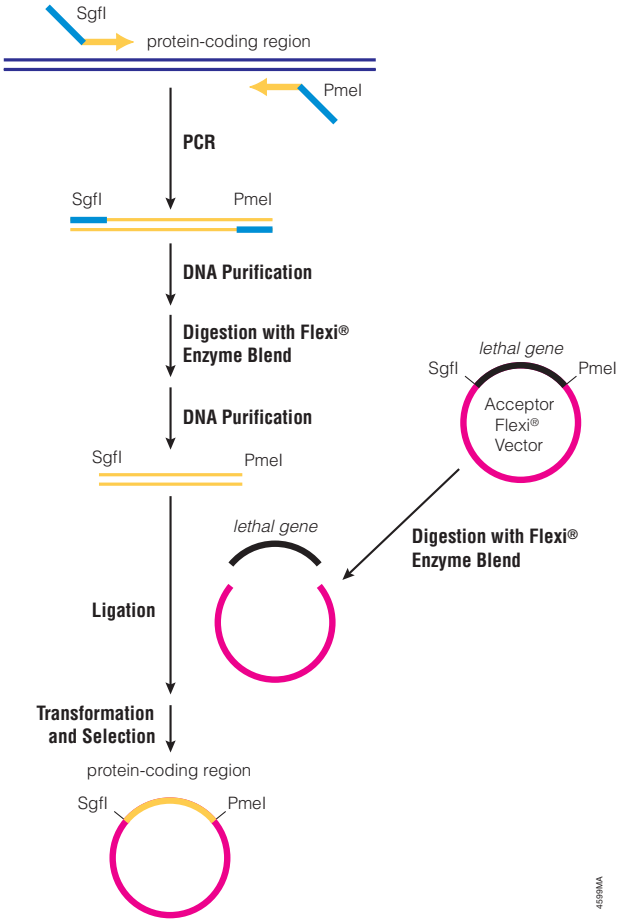
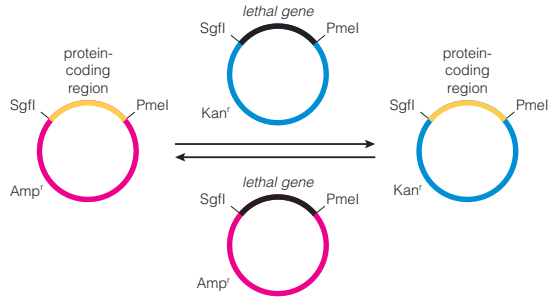


Figure 1.2. Cloning a protein-coding region into a Flexi® Vector. PCR primers are designed to append SgfI and PmeI sites onto the protein-coding region. The digested PCR product is ligated into the acceptor vector that has been digested with SgfI and PmeI. Following transformation, the cells are selected with an antibiotic appropriate Flexi® Vector used.

Getting started with Flexi® Vector Cloning

The Flexi® Vector Cloning System provides an easy way to get started with cloning and expression of genes of interest. For cloning there are many Flexi® Vectors from which to choose **(see Table 9.1)**. However, starting with C-terminal fusion vectors is not recommend since the protein-coding regions cannot be transferred into other vectors **(Figure 1.3, Panel B)**.

A. Transfer of a protein-coding region between N-terminal or native Flexi® Vectors.



B. Transfer of a protein-coding region into C-terminal Flexi® Vectors.

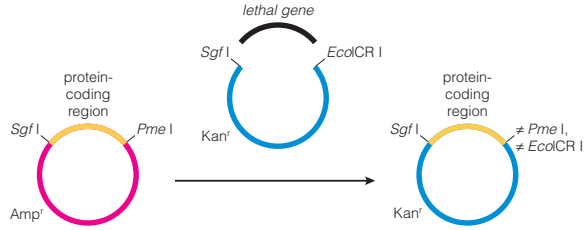


Figure 1.3. Transferring protein-coding regions in the Flexi® Vector Systems. Protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, SgfI and PmeI. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. **Panel A**. Transfer between Flexi® Vectors for expression of native or N-terminal-tagged fusion proteins is reversible (i.e., is a two-way exchange) between native and N-terminal Flexi® Vector. **Panel B.** C-terminal Flexi® Vectors contain SgfI and EcoICRI sites and are designed to allow expression of C-terminal-tagged proteins. Joining PmeI and EcoICRI blunt ends eliminates the stop codon present in the PmeI site and allows readthrough to the C-terminal protein-coding sequences in the C-terminal Flexi® Vectors. Since both restriction sites are destroyed by joining, transfer into C-terminal Flexi® Vectors is not reversible (i.e., is a one-way exchange).

Ordering Information

Flexi® System, Entry/Transfer (Cat.# C8640)

Carboxy Flexi® System, Transfer (Cat.# C9320)

The Flexi® System, Transfer Kit (Cat.# C8820)

Human ORF-Clone Library

Human protein expression without cloning.

Description and Principle

The Promega Open-Reading-Frame (ORF-) clone library consists of more than 9,000 experimentally validated human clones for native and tagged protein expression. The tagged ORF-clones are fused to HaloTag®, a protein fusion tag that is used in multiple applications such as cellular imaging, protein purification and protein pull-down (see Chapters 4 & 6). HaloTag® ORF-clones (FHC-clones) are provided in the Flexi® Vector pFN21A, suitable for transient protein expression in mammalian cells. In FHC clones HaloTag® is fused to the N-terminus of the ORF sequence and expression of the protein of interest is under the control of a CMV promoter **(Figure 1.4)**. The native expression clones (FXC clones) are provided in Flexi® Vector pF1K **(Figure 1.5)**. All ORF-clones can be easily transferred into other Flexi® Vectors with different features **(see Table 9.1)**. For testing different expression strengths we offer vectors with a modified CMV immediate-early enhancer/promoter for constitutive expression in mammalian cells.

Features and Benefits

Experimentally validated ORF-clone library:

- Sequence validation of both the 5′- and 3′-end sequences by single-pass sequencing.
- Size confirmation through agarose gel electrophoresis.
- Expression validation by SDS-PAGE of expressed HaloTag® fusions in HEK293 cells (only for HaloTag® clones).
- Fluorescent microscopy demonstrating in situ labeling and visualization of HaloTag® fusions with HaloTag® TMR Ligand (only for HaloTag® clones) in HEK293 cells.

How to find and order an ORF-clone

Available ORF-clones can be found by using the online tool *Find My Gene*™ at: **www.promega.com**. *Find My Gene*™ allows the search by ORF-clone Name, Gene Symbol/ID/Name, Accession Number, or by blasting the protein or nucleic acid sequence of interest. Alignments of the sequence of interest with the offered ORF-clones can be performed within the online tool. The ORF-clones can be ordered online, using FAX or email with respective catalog/clone number.

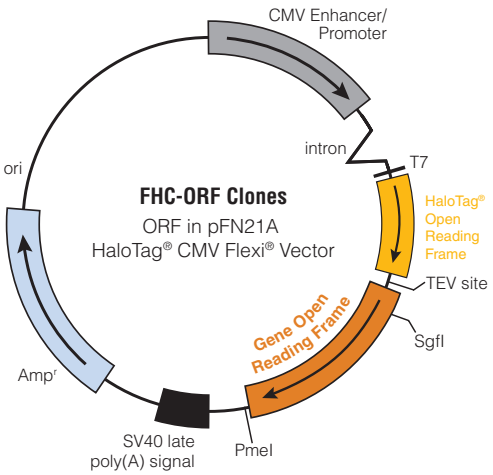


Figure 1.4. Vector map of HaloTag® ORF-clones.

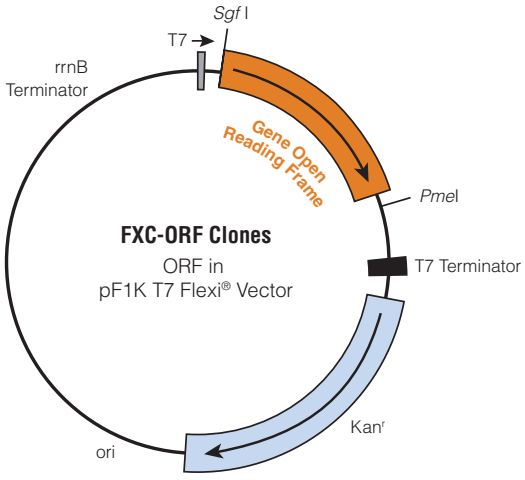


Figure 1.5. Vector map of native ORF-clones.

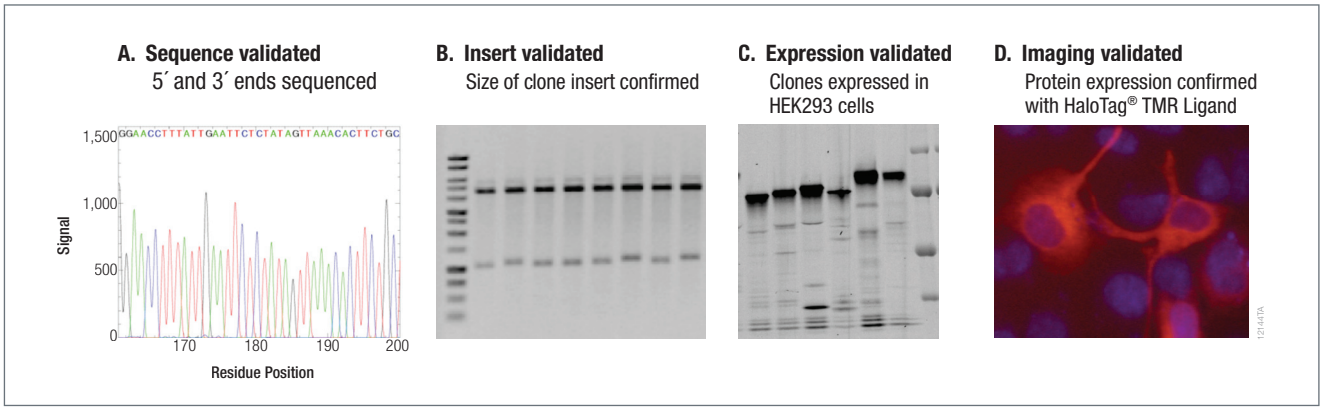


Figure 1.6. Experimental validation of ORF-clones. All ORFs clone are sequence validated (5'- and 3'-ends sequenced; **(Panel A)** and insert validated to confirm the size of the cloned insert **(Panel B)**. The HaloTag® ORFs are also expression validated by SDS-PAGE **(Panel C)** and imaging validated by fluorescent microscopy using HaloTag® TMR Ligand **(Panel D)**.

Table 1.1. Overview of the Human ORF-clone Collection.

Features	HaloTag® Collection	Native Collection
Size of Collection	>9,000	>6,300
Fusion Tag	HaloTag® for protein purification, imaging, pull-downs and NanoBRET™.	Native
Validated Clones		
Sequence Validated	✓ 100% clones	✓ 100 % clones
Insert Validated	✓ 99% clones	✓ 97%
Expression Validated	✓ 99% clones	(–)
Imaging Validated	✓ 78% clones	(–)
Format	DNA	DNA

Additional Information

The ORF-clones are provided in 100ng of purified plasmid DNA in TE buffer. We recommend that you transform your ORF-clone in competent cells, and create a bacterial glycerol stock. Upon request, the generation of other ORF-clones is offered via Promega Custom Ordering (www.promega.com/products/manufacturing-and-custom-capabilities/). The Arabidopsis Biological Resource Center at the Ohio State University distributes HaloTag® ORF-clones from Arabidopsis. For more information visit: www.arabidopsis.org/abrc/halo_tagged_orf_clones.jsp

Regulated Mammalian Expression System

Inducible expression in mammalian cells.

Description

The Regulated Mammalian Expression System features low basal levels, robust and rapid induction, and downregulation of gene expression in mammalian cells. The Regulated Mammalian Expression System is based on a novel on/off switch that relies on the rapid and sensitive modulation by coumermycin-related compounds of a chimeric transactivator protein. The levels of protein expression can be regulated by adjusting the coumermycin concentration. More significantly, this expression can be promptly and effectively switched off by adding novobiocin **(Figure 1.7)**.

Features and Benefits

- **Enhanced Data:** High level of controlled induction combined with low basal protein expression.
- **Regulated Expression:** Dose-response induction of protein expression; rapid and sensitive on/off switch for protein expression.
- **Versatility:** Compatible with other Flexi® Vectors.

References

Zhao, H-F. *et al.* (2003) A coumermycin/novobiocin-regulated gene expression system. *Hum. Gene Ther.* **14**(47), 1619–29.

Principle

The protein coding region of interest is cloned into either the pF12A RM Flexi® Vector or pF12K RM Flexi® Vector, both of which are specially designed for Regulated Mammalian (RM) protein expression. These vectors incorporate regulatory promoter sequences upstream of the protein-coding region and are compatible with the Flexi® Vector System. In transient transfection paradigms, the pF12A or pF12K RM Flexi® Vector containing the protein-coding region of interest is co-transfected into mammalian cells together with the pReg neo Vector. The pReg neo Vector is designed to express a chimeric transactivator protein that interacts with the regulatory promoter region in the pF12A and pF12K RM Flexi® Vectors in a regulated fashion in response to coumermycin and novobiocin. Additionally, the pReg neo Vector encodes a neomycin phosphotransferase gene that allows stable cell selection and generation with the antibiotic G-418.

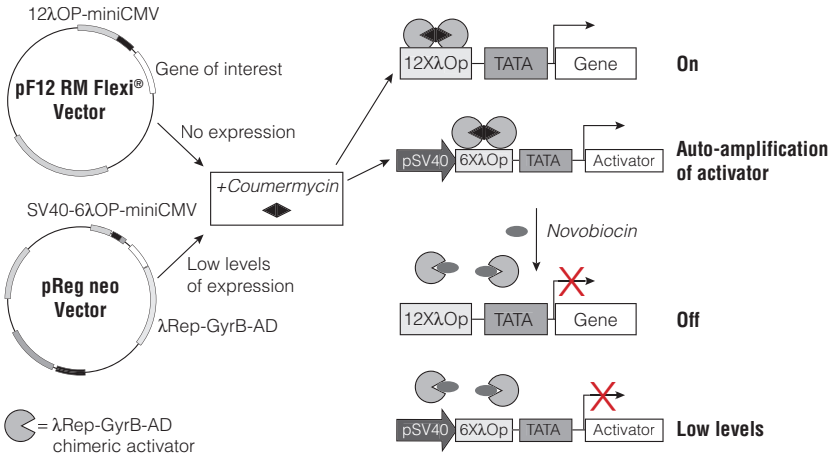


Figure 1.7. Diagram of the coumermycin-regulated mammalian expression system.

Ordering Information

Regulated Mammalian Expression System (Cat.# C9470)

pTARGET™ Mammalian Expression Vector System

PCR products can be cloned directly into the T-overhang of the pTARGET™ Vector and used for protein expression in mammalian cells under a CMV promoter.

Description

The pTARGET™ Mammalian Expression Vector System is a convenient system for cloning PCR products and for expressing cloned PCR products in mammalian cells. The pTARGET™ Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. For cloning of ampllifed PCR products in general, the pGEM®-T Vector and pGEM®-T Easy Vector Systems are recommended.

Principle

The vector is prepared by digestion with EcoRV followed by addition of a 3′-terminal thymidine to each end. These single 3′T-overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid in two ways. First, the overhangs prevent recircularization of the vector; second, they provide a compatible overhang for PCR products, as thermostable polymerases add a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of amplified fragments.

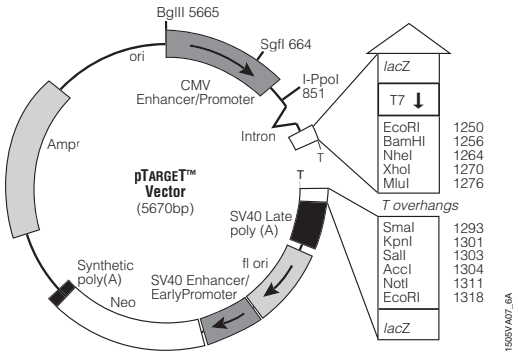


Figure 1.8. pTARGET™ Mammalian Expression Vectors for transient and stable protein expression.

Features and Benefits

- **Simple PCR Cloning:** T-overhangs permit direct ligation of PCR products. Note: If amplifying long fragments, use GoTaq® Long PCR Master Mix, which produces A-overhangs.
- **Strong, Constitutive Expression:** The CMV enhancer/promoter region allows strong, constitutive expression in many cell types.
- **Blue/White Screening:** Easy identification of recombinant clones. A single digest removes the insert DNA.
- **Stable Transfectants:** Select for stable transfectants using the antibiotic G-418.

References

Dastidar, S.G. *et.al.* (2011) FoxG1 promotes the survival of postmitotic neurons. *J. Neurosci.* **31**(2), 402–13.

Carpenter, J.E. *et al.* (2011) Autophagosome formation during varicella-zoster virus infection following endoplasmic reticulum stress and the unfolded protein response. *J. Virol.* **85**(18), 9414–24.

Ordering Information

pTARGET™ Mammalian Expression Vector (Cat.# A1410)

pGEM®-T Vector (Cat.# A3600)

pGEM®-T Easy Vector Systems (Cat.# A1360, A1380)

JM109 Competent Cells

Competent cells for high-efficiency transformation of vectors for cloning purposes.

Description and Principle

JM109 Competent Cells are derived from an *E.coli* K strain that is *recA*– and *endA*– to minimize recombination and improve the quality of plasmid DNA. In addition, the cells carry the F´ episome, which allows blue/white screening. The Competent Cells are available for convenient transformation in two efficiencies: at greater than 10^8cfu/µg and at greater than 10^7cfu/µg. In addition, single-use sizes are supplied for maximal ease-of-use.

Features and Benefits

- **Convenient:** Ready-to-use; no preparation time necessary, blue/white screening.
- **Reliable:** Transformation efficiencies guaranteed.
- **Safe:** The *recA*– mutation prevents undesirable recombination events, and the *endA*– mutation in JM109 cells prevents carryover nuclease in miniprep DNA.

Additional Information

JM109 Genotype: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk–, mk+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´*tra*D36, *pro*AB, *laq*I^{q}ZΔM15].

Ordering Information

Single-Use JM109 Competent Cells >10^8cfu/µg (Cat.# **L2005**)

JM109 Competent Cells >10^8cfu/µg (Cat.# **L2001**)

JM109 Competent Cells >10^7cfu/µg (Cat.# **L1001**)

Pro 5-alpha Competent Cells

Competent cells for maximal efficiency transformation of vectors for difficult cloning experiments.

Description and Principle

Single-Use Pro 5-alpha Competent Cells are an *E.coli* strain that can be used for the efficient transformation of unmethylated DNA derived from PCR, cDNA and many other sources. The elimination of nonspecific endonuclease I (endA1) enables the highest quality plasmid preparations. The strain is resistant to phage T1 (fhuA2) and suitable for blue/white screening by α-complementation of the β-galactosidase gene.

Features and Benefits

- **Convenient:** Ready-to-use; no preparation time necessary, blue/white screening.
- **Reliable:** Transformation efficiencies guaranteed.
- **Safe:** The *recA*– mutation prevents undesirable recombination events.

Additional Information

Pro 5-alpha Genotype: *fhu*A2, Δ(argF-*lacZ*), U169, *pho*A, *gln*V44, φ 80, Δ(*lacZ*) M15, *gyr*A96, *rec*A1, *rel*A1, *end*A1, *thi*-1, *hsd*R17.

Ordering Information

Single-Use Pro 5-alpha Competent Cells >10^9cfu/µg (Cat.# L1221)

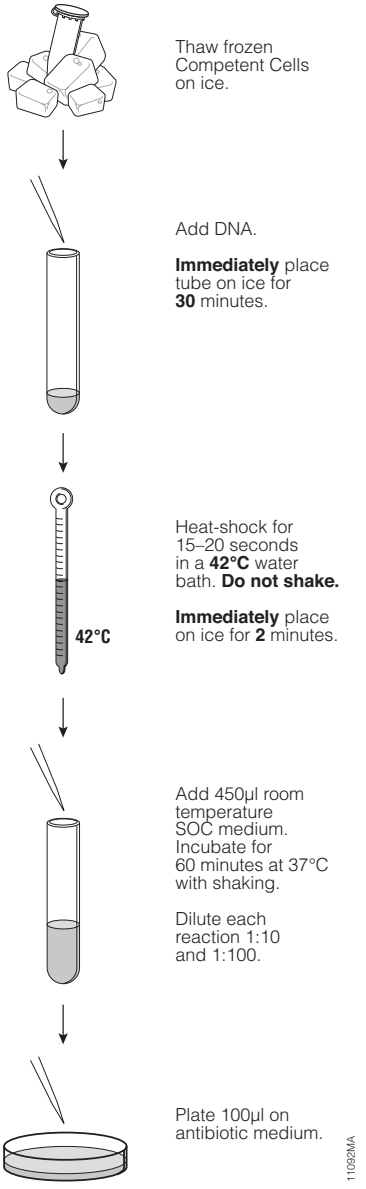


Figure 1.9. Standard transformation protocol using Single-Use Competent Cells.