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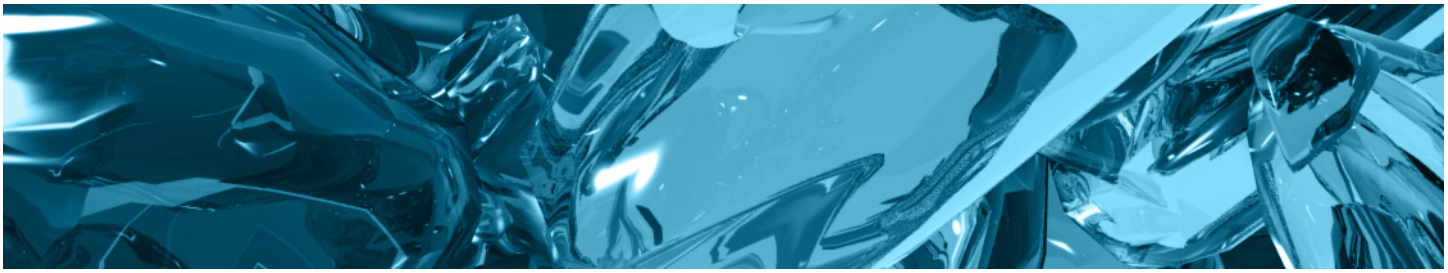

Promega

I. Introduction

The cloning of genes, gene fragments and other DNA sequences is a fundamental part of molecular biology. To study the function of a particular DNA sequence, you must be able to manipulate that sequence. There are two main ways to achieve this: the polymerase chain reaction (PCR) and the more traditional use of restriction enzymes and modifying enzymes to "cut and paste" the desired DNA fragments into cloning vectors, which can then be replicated using live cells, most commonly *E. coli*. The use of PCR has an advantage in that it gives you the option to re-amplify the target DNA each time your DNA supplies dwindle without ligation into a vector or transformation into *E. coli*. Alternatively, PCR products can be ligated into a suitable vector, which can then be transformed into and replicated by *E. coli*. This chapter covers the basics of cloning using PCR and restriction enzymes, including DNA cleanup prior to ligation, ligation, transformation and screening to identify recombinant clones.

The PCR process is a useful tool to quickly and easily amplify the desired sequences. With the successful sequencing of whole and partial genomes of organisms across all biological kingdoms, DNA cloning by PCR is an easily attainable option. Public DNA databases allow researchers to design primers to amplify their DNA fragment of interest directly from the genomic DNA of the desired organism. With the simple addition of a reverse transcription step prior to PCR, RNA sequences can be converted to cDNA, which can then be cloned into a suitable vector. For additional information about amplification of DNA and RNA sequences using PCR, see the PCR Amplification chapter of the *Protocols and Applications Guide*.

PCR products generated using a nonproofreading DNA polymerase such as *Taq* DNA polymerase, which lacks 3′→5′ exonuclease activity, have a single template-independent nucleotide at the 3′ end of each DNA strand (Clark, 1988; Newton and Graham, 1994). This single-nucleotide overhang, which is most commonly an A residue, allows hybridization with and cloning into T vectors, which have a complementary 3′ single T overhang. PCR products generated using a proofreading DNA polymerase, such as *Pfu* DNA polymerase, have blunt ends and must be cloned into a blunt-ended vector or need a single 3′ A overhang added to ligate into a T vector (Knoche and Kephart, 1999).

If PCR amplification of the desired DNA fragment is not possible or desirable, restriction enzyme digestion of the target DNA can be employed. The desired fragment may need to be separated from other DNA fragments in the reaction, so the size of the desired DNA fragment should be known. Once isolated, the fragment is cloned into a vector with compatible ends. If the vector ends are capable of religating (e.g., the vector has blunt ends or is cut with a single restriction enzyme), the vector is often treated with alkaline phosphatase to discourage recircularization and maximize ligation between the insert and vector.

Following transformation into *E. coli*, the resulting bacterial colonies are screened by PCR for the correct recombinant vector using primers to amplify the insert. Alternatively, the recombinant vector can be identified by performing a restriction enzyme digestion to determine the presence of the correct insert. Screening is often simplified by using vectors that contain an antibiotic-resistance gene, so cells containing the vector will survive on medium supplemented with the appropriate antibiotic. Screening can be further simplified by choosing a vector and *E. coli* strain that are compatible with blue/white screening, which takes advantage of intracistronic α-complementation to regenerate β-galactosidase activity. Many *E. coli* strains used for cloning and propagation of plasmids contain a chromosomal deletion of the *lac* operon but carry an F′ episome that provides the remaining coding sequence of the *lac*Z gene. The functional *lac*Z gene product, β-galactosidase, is produced when the *lac*Z coding information missing on the F′ episome is provided by the plasmid. This activity is detected by plating bacteria transformed by plasmids on plates containing isopropyl β-D-thiogalactopyranoside (IPTG; an inducer of the *lac* promoter) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; a dye that produces a blue color when hydrolyzed by β-galactosidase). When the reading frame of the α peptide is disrupted by insertion of a foreign DNA fragment or deletion of vector sequences, α-complementation does not occur, and the bacterial colonies remain white or occasionally light blue.

II. Promega Products for Cloning

A. Thermostable DNA Polymerases

The use of amplification enzymes is the first step in cloning by PCR. Most people use PCR for cloning, taking advantage of the single nucleotide A overhang left after amplification with a nonproofreading DNA polymerase to ligate the amplimer to a vector containing T overhangs. However, products will be blunt-ended if the DNA polymerase has 3′→5′ exonuclease activity, also known as proofreading activity. Alternatively, PCR primers can add sequences for restriction enzyme sites, and the resulting products can be digested and ligated into a vector with compatible ends. Promega provides several thermostable DNA polymerases. These include the GoTaq® Amplification Family, *Tfl* DNA Polymerase and proofreading polymerases. A detailed list of the various enzymes for use in PCR can be found in the *Protocol and Applications Guide* chapter on PCR Amplification, in the section "Thermostable DNA Polymerases". The GoTaq® Amplification Family of products is highlighted in the following section.

GoTaq® Amplification Family

GoTaq® DNA Polymerase is available in various formulations to suit your needs: the standard GoTaq® DNA Polymerase, which is supplied with a 1X reaction buffer that contains 1.5mM $MgCl_2$; GoTaq® Flexi DNA Polymerase, which allows a range of $MgCl_2$ to be used for

PCR; GoTaq® Green Master Mix, which is a premixed, ready-to-use solution containing GoTaq® DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentrations for efficient amplification of DNA templates by PCR; GoTaq® Hot Start Polymerase, which includes a proprietary antibody that blocks polymerase activity until the initial denaturation step; and GoTaq® Long PCR Master Mix, which offers efficient amplification of long templates (e.g., human genomic DNA up to 30kb). All GoTaq® PCR Core Systems offer complete solutions with polymerase and nucleotides; the GoTaq® PCR Core System II also includes a positive control. GoTaq® products contain *Taq* DNA polymerase in a proprietary formulation that offers enhanced amplification over conventional *Taq* DNA polymerase. Each member of the GoTaq® family has a reaction buffer that contains two dyes (a blue dye and a yellow dye) that separate during electrophoresis to show migration progress as well as a compound that increases sample density. Samples can be loaded directly onto gels without the need to add a separate loading dye. If the dyes interfere with your downstream applications, GoTaq® DNA Polymerases are supplied with a 5X Colorless Reaction Buffer. Alternatively, the PCR Master Mix offers a ready-to-use formulation without any dyes. Reaction products generated with these systems contain A overhangs and are ready for T-vector cloning. Alternatively, the PCR product can be digested directly with a restriction enzyme that is active in the PCR buffer and cloned into standard cloning vectors (see Technical Manual #TM367 for a protocol).

Additional Resources for GoTaq® DNA Polymerase

Promega Publications

Introducing GoTaq® DNA Polymerase: Improved amplification with a choice of buffers

Citations

Ning, B. *et al.* (2011) 5-Aza-2'-deoxycytidine activates iron uptake and heme biosynthesis by increasing c-myc nuclear localization and binding to the e-boxes of transferrin receptor 1 (*TfR1*) and ferrochelatase (*Fech*) genes. *J. Biol. Chem.* **286**, 37196–206.

The authors performed real-time PCR using SYBR® Green and 1.25 units of GoTaq® DNA Polymerase to amplify 20ng of cDNA generated from total RNA extracted from murine erythroid leukemia (MEL) cells and mouse erythroid burst-forming units (BFU-Es) in a total reaction volume of 25µl.

PubMed Number: 21903580

Vucurovic, K. *et al.* (2010) Serotonin 3A receptor subtype as an early and protracted marker of cortical interneuron subpopulations. *Cereb. Cortex* **20**, 2333–47.

After reverse transcription, PCR was performed to simultaneously detect mRNAs encoding two isoforms of glutamic acid decarboxylase, three calcium-binding proteins, three neuropeptides, two transcription factors and reelin, a protein thought to be involved in neuronal migration and morphology. Two rounds of PCR using nested primers were required to detect these mRNAs. PCRs were performed using GoTaq® DNA Polymerase. Amplified products were visualized by agarose gel electrophoresis, using the 100bp DNA Ladder as a size standard.

PubMed Number: 20083553

Additional Resources for GoTaq® Flexi DNA Polymerase

Promega Publications

GoTaq® Flexi DNA Polymerase: Robust performance with magnesium optimization

Citations

Westphal, A. *et al.* (2011) General suppression of *Escherichia coli* O157:H7 in sand-based dairy livestock bedding. *Appl. Environ. Microbiol.* **77**, 2113–21.

DNA was extracted from bedding material and the 16S rRNA genes amplified in a 25µl reaction using 1.5 units GoTaq® Flexi DNA Polymerase with 1.8mM $MgCl_2$. The PCR products then were cloned into the pGEM®-T Easy Vector.

PubMed Number: 21257815

Additional Resources for GoTaq® Green Master Mix

Promega Publications

Activity of Promega restriction enzymes in GoTaq® Green Master Mix and PCR Master Mix

Analyses of gene disruption by whole-cell PCR using the GoTaq® Green Master Mix

Recombinant clone screening using the GoTaq® Hot Start Green Master Mix

Citations

Crawford, M.A. *et al.* (2011) Identification of the bacterial protein FtsX as a unique target of chemokine-mediated antimicrobial activity against *Bacillus anthracis*. *Proc. Natl. Acad. Sci. USA.* **108**, 17159–64.

To identify transposon insertion sites, bacterial genomic DNA was isolated, digested and ligated with a partially double-stranded Y-linker. An initial 20µl amplification for 20 cycles using GoTaq® Green Master Mix enriched ssDNA fragments. A second PCR amplified dsDNA, adding more GoTaq® Green Master Mix for a final reaction volume of 100µl with 25 cycles. The amplimers were analyzed by sequencing.

PubMed Number: 21949405

Additional Resources for PCR Master Mix

Promega Publications

Activity of Promega restriction enzymes in GoTaq® Green Master Mix and PCR Master Mix

Performance advantages designed into Promega's PCR Master Mix

Additional Resources for GoTaq® Core PCR Systems

Technical Bulletins and Manuals

TB254 *GoTaq® PCR Core Systems Technical Bulletin*

Citations

Fuehrer, H.P. *et al.* (2011) Novel nested direct PCR technique for malaria diagnosis using filter paper samples. *J. Clin. Microbiol.* **49**, 1628–30.

The authors developed a direct-amplification, nested PCR protocol to amplify *Plasmodium* DNA from S&S 903 filter paper punches containing whole blood. The GoTaq® PCR Core System amplified 5µl of template (extracted from paper punches and whole blood in parallel) in the second nested reaction using 2mM $MgCl_2$ and 1 unit of GoTaq® DNA polymerase in a total reaction volume of 50µl.

PubMed Number: 21270224

Additional Resources for GoTaq® Hot Start Polymerase

Technical Bulletins and Manuals

9PIM500 *GoTaq® Hot Start Polymerase Product Information*

Citations

Li, Z. *et al.* (2011) The barley *amo*1 locus is tightly linked to the starch synthase *IIIa* gene and negatively regulates expression of granule-bound starch synthetic genes. *J. Exp. Bot.* **62**, 5217–31.

To examine the mutations in class II and class III starch synthases (ssIIa and ssIIIa, respectively), genomic DNA from young barley leaves was extracted and 50ng amplified in a 20µl reaction that included 1.5U of GoTaq® Hot Start Polymerase, 1.5mM $MgCl_2$ and the additives DMSO and betaine. After 35 cycles, the PCR products were digested overnight using EcoRI and separated on 2% agarose gels.

PubMed Number: 21813797

Additional Resources for GoTaq® Long PCR Master Mix

Technical Bulletins and Manuals

TM359 *GoTaq® Long PCR Master Mix Technical Manual*

Promega Publications

GoTaq® Long PCR Master Mix for reliable amplification of long PCR targets

B. T-Cloning Vectors

T vectors are a specific type of cloning vector that get their name from the T overhangs added to a linearized plasmid. These vectors take advantage of the A overhangs on PCR products after amplification with *Taq* DNA polymerase by providing compatible ends for ligation (Mezei and Storts, 1994; Robles and Doers, 1994). There are three different T-cloning vectors from Promega: Two are basic cloning vectors, and the third is a mammalian expression vector.

pGEM®-T and pGEM®-T Easy Vector Systems

The pGEM®-T (Cat.# A3600, A3610) and pGEM®-T Easy Vector Systems (Cat.# A1360, A1380) are convenient systems for cloning PCR products. The vectors are prepared by cutting with a restriction endonuclease to leave a blunt end and adding a 3′terminal thymidine to both ends (Figures 13.1 and 13.2). These single 3′ T overhangs at the insertion site greatly improve ligation efficiency of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products with 5′ A overhangs.

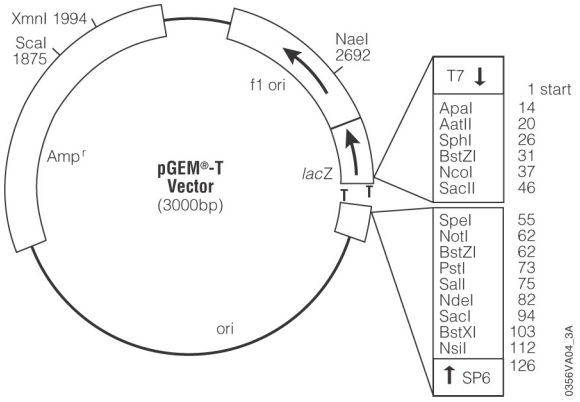

Figure 13.1. pGEM®-T Vector circle map.

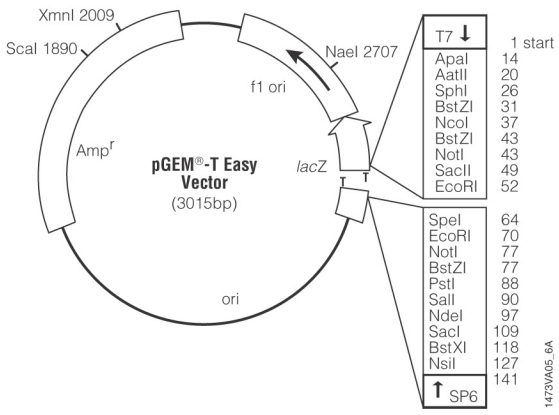

Figure 13.2. pGEM®-T Easy Vector circle map.

The high-copy-number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the coding region for the α-peptide of β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates containing

X-Gal (Cat.# V3941) and IPTG (Cat.# V3955). Both the pGEM®-T and pGEM®-T Easy Vectors contain numerous restriction sites within the multiple cloning region. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, thus providing three single-enzyme digestions for release of the insert. The pGEM®-T Vector cloning region is flanked by recognition sites for the enzyme BstZI. Alternatively, a double digestion may be used to release the insert from either vector.

The pGEM®-T and pGEM®-T Easy Vectors also contain the origin of replication of filamentous phage f1 for the preparation of single-stranded DNA (ssDNA). Both pGEM®-T vector systems include a 2X Rapid Ligation Buffer for ligation of PCR products, which requires only a 1-hour incubation at room temperature. The incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4°C will produce the maximum number of transformants.

Inserts of several kilobases have been successfully cloned into the pGEM®-T and pGEM®-T Easy Vectors (D'Avino *et al.* 2004). However, as the insert gets larger, the ratio of vector to insert may need to be optimized further to maximize ligation efficiency (see Ligation and Transformation in the section "Vector:Insert Ratio").

One of the disadvantages of PCR cloning into a T vector is that the insert can be cloned in either direction. Analysis of recombinant vectors by PCR or restriction enzyme digestion can be used to determine not only the success of cloning but also the insert orientation. To verify the direction of the insert, amplify recombinant plasmids using one of the gene-specific PCR primers and onel of the phage promoter primers that are present on the pGEM®-T Vector (Knoche and Kephart, 1999). The correct orientation is important for transcription or translation or both.

Additional Resources for the pGEM®-T and pGEM®-T Easy Vector Systems

Technical Bulletins and Manuals

TM042 *pGEM®-T and pGEM®-T Easy Vector Systems Technical Bulletin*

Promega Publications

pGEM®-T Easy Vector System is an easy tool for preparing gel shift probes

Cloning blunt-end *Pfu* DNA Polymerase-generated PCR fragments into pGEM®-T Vector Systems

Stability of pGEM®-T Vectors

Online Tools

pGEM®-T and pGEM®-T Easy Vector sequences (select the Specifications tab)

Citations

Maruyama, A. *et al.* (2011) The novel Nrf2-interacting factor KAP1 regulates susceptibility to oxidative stress by promoting the Nrf2-mediated cytoprotective response. *Biochem. J.* **436**, 387–97.

A mouse KAP1 expression plasmid was constructed by amplifying the KAP1 cDNA in three fragments from RNA isolated from NIH3T3 cells. Each of the fragments were cloned into the pGEM®-T Easy Vector. The three recombinant vectors were digested with restriction enzymes (HindIII and BamHI; BamHI; BamHI and XbaI) and the resulting fragments were ligated together and subcloned into an expression vector.

PubMed Number: 21382013

Aquilini, E. *et al.* (2010) Functional identification of the *Proteus mirabilis* core lipopolysaccharide biosynthesis genes *J. Bacteriol.* **192**, 4413–24.

To identify the core lipopolysaccharides (LPS) biosynthesis genes in *Proteus mirabilis*, 11 genes from *P. mirabilis* strain R110 and one from strain 51/57 were amplified from chromosomal DNA, cloned into the pGEM®-T Vector and transformed into DH5α competent cells. Once the cloned genes were confirmed, each recombinant plasmid was transformed into *Klebsiella pneumoniae* core LPS mutants to see if any of the *P. mirabilis* genes complemented the mutants.

PubMed Number: 20622068

pTARGET™ Mammalian Expression Vector System

The pTARGET™ Mammalian Expression Vector System (Cat.# A1410) is a convenient system to clone PCR products and express cloned PCR products in mammalian cells. As with the pGEM®-T and pGEM®-T Easy Vector Systems, the pTARGET™ Vector is supplied already linearized with single T overhangs (Figure 13.3). These single 3′ T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid. The pTARGET™ Vector also contains a modified version of the coding sequence of the α peptide of β-galactosidase, which allows recombinants to be selected using blue/white screening.

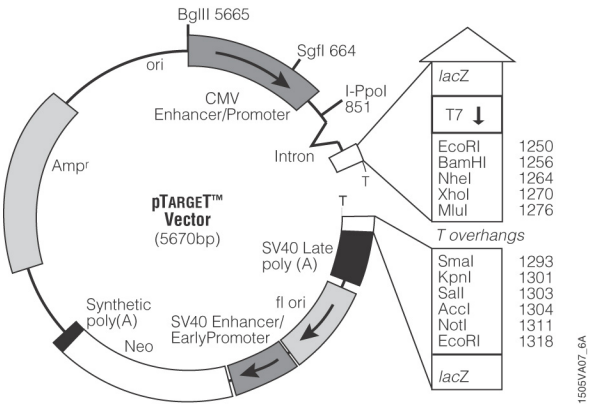

Figure 13.3. pTARGET™ Vector circle map.

The pTARGET™ Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. This vector also contains the neomycin phosphotransferase gene, a selectable marker for

mammalian cells. The pTARGET™ Vector can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G-418. Like the pGEM®-T or pGEM®-T Easy Vectors, inserts of several kilobases can be cloned in and expressed from the pTARGET™ Vector (Sakakida *et al.* 2005; Le Gall *et al.* 2003).

Additional Resources for the pTARGET™ Mammalian Expression Vector System

Technical Bulletins and Manuals

TM044 *pTARGET™ Mammalian Expression Vector System Technical Manual*

Promega Publications

Technically speaking: T-vector cloning

pTARGET™ Vector: A new mammalian expression T-vector

Online Tools

pTARGET™ Mammalian Expression Vector sequence (select the Specifications tab)

Citations

Dastidar, S.G., Landrieu, P.M. and D'Mello, S.R. (2011) FoxG1 promotes the survival of postmitotic neurons. *J. Neurosci.* **31**, 402–13.

Four FoxG1 deletion mutants were generated by PCR, and with an added C-terminal Flag tag, cloned into the pTARGET™ Mammalian Expression Vector. The mutant constructs were transfected into neuronal cells and neuronal survival assessed.

PubMed Number: 21228151

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**, 9414–2.

Four varicella-zoster virus (VZV) major structural glycoproteins open reading frames (ORFs) were amplified from cultured cells infected with laboratory strain VZV-32 and ligated into the pTARGET™ Mammalian Expression Vector. The recombinant vectors were grown, purified and transfected into HeLa cells at a concentration of 0.5 µg/ml. After a six-hour incubation, the medium was changed and the cells observed under confocal fluorescence microscopy up to 24 hours later for autophagosome formation.

PubMed Number: 21752906

C. Flexi® Cloning Systems

The Flexi® Vector Systems (Cat.# C8640, C8820, C9320) are based on a simple, yet powerful, directional cloning method for protein-coding sequences. First, a PCR product is generated using primers designed with two rare-cutting restriction enzymes, SgfI and PmeI. After restriction enzyme digestion, the insert is ligated in a single orientation. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a positive selection for successful ligation of the insert. The two restriction enzymes provide a rapid, efficient and high-fidelity way to transfer protein-coding regions between a variety of Flexi® Vectors without the need to resequence while maintaining the reading frame (see Figure 13.4 for a system overview and Figure 13.5 for a list of example vectors). Find a current list of available vectors at: www.promega.com. To design PCR primers appropriate for your insert and with SgfI and PmeI restriction sites, visit the Flexi® Vector Primer Design Tool.

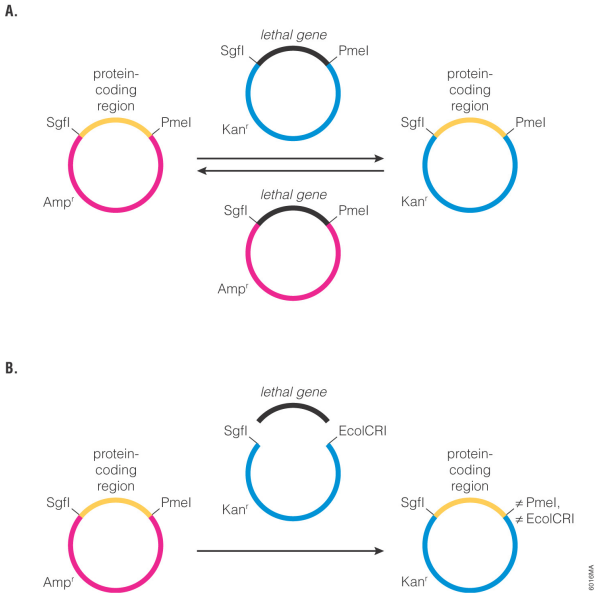

Figure 13.4. Transferring protein-coding regions in the Flexi® Vector Systems. Panel A. The Flexi® Vector Systems employ a flexible, directional cloning method to create plasmids to express protein-coding regions with or without peptide fusion tags. The features necessary for expression and the options for protein fusion tags are carried on the vector backbone, and the 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. Transfer between Flexi® Vectors for expression of native or N-terminal-tagged fusion proteins is reversible (i.e., it is a two-way exchange). **Panel B.** C-terminal Flexi® Vectors contain SgfI and EcoICRI sites and 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., it is a one-way exchange).

Flexi® Vector Name[1]	Expression Application	Promoter for Expression			Peptide Fusion Tag	
		E. coli	Mammalian Cells	in vitro Transcription/ Translation	N-terminus	C-terminus
pF1A	Inducible expression of native protein	T7		T7		
pF1K						
pFN2A	Solubilization and purification via an N-terminal GST tag	T7		T7	GST	
pFN2K						
pF3A WG	In vitro wheat germ expression of native protein			T7, SP6		
pF3K WG						
pF4A CMV	Constitutive expression of native protein		CMV	T7		
pF4K CMV						
pF5A CMV-neo	Constitutive expression of native protein with selection for stable transfectants		CMV	T7		
pF5K CMV-neo						
pFN6A (HQ)	Protein purification via a metal affinity resin	T7		T7	HQHQHQ	
pFN6K (HQ)						
pFC7A (HQ)	Protein purification via a metal affinity resin	T7		T7		HQHQHQ
pFC7K (HQ)						
pFC8A	Protein labeling, cell imaging and surface immobilization		CMV	T7		HaloTag™
pFC8K						
pF9A CMV *hRluc*-neo	Constitutive expression of native protein with selection and reporter screening for stable transfectants		CMV	T7		
pFN10A (ACT)	Mammalian in vivo protein:protein interaction		CMV	T7	HSV VP16 activation domain	
pFN11A (BIND)	Mammalian in vivo protein:protein interaction		CMV	T7	GAL4 DNA-binding domain	

5347MC

[1]The "pF" indicates that the vector is a Flexi® Vector. The letter after "pF" indicates the position of any expression tags (e.g., "N" for an N-terminal expression tag; "C" for a C-terminal expression tag). The number associated with the vector specifies the type of expression and application. The letters "A" and "K" designate the bacterial drug selection for the vector (A = ampicillin and K = kanamycin).

Figure 13.5. A selection of available Flexi® Vectors.

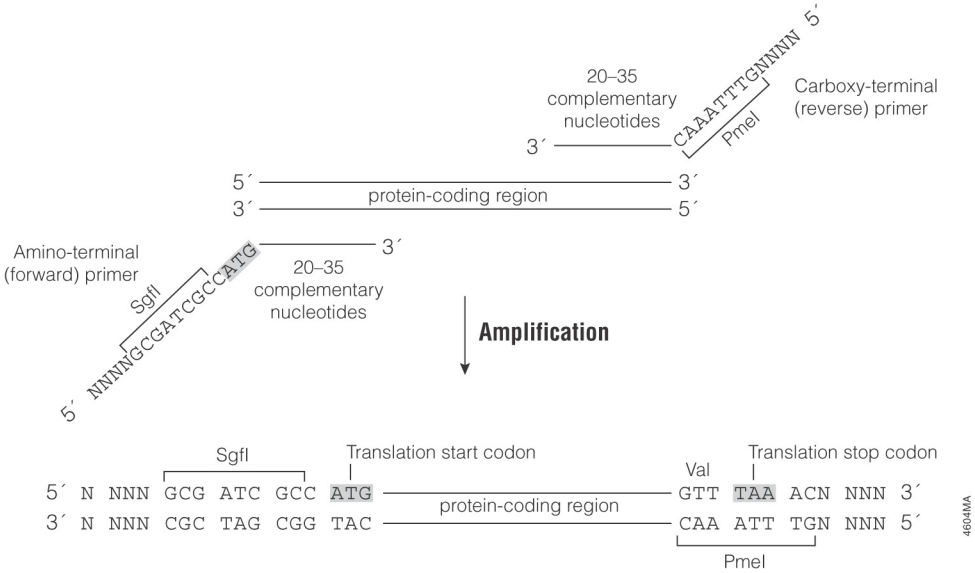

Figure 13.6. PCR primer design. The PmeI site appends a single valine codon at the 3′ end of the protein-coding region and allows either termination or readthrough to append a carboxy-terminal peptide, depending on the vector backbone.

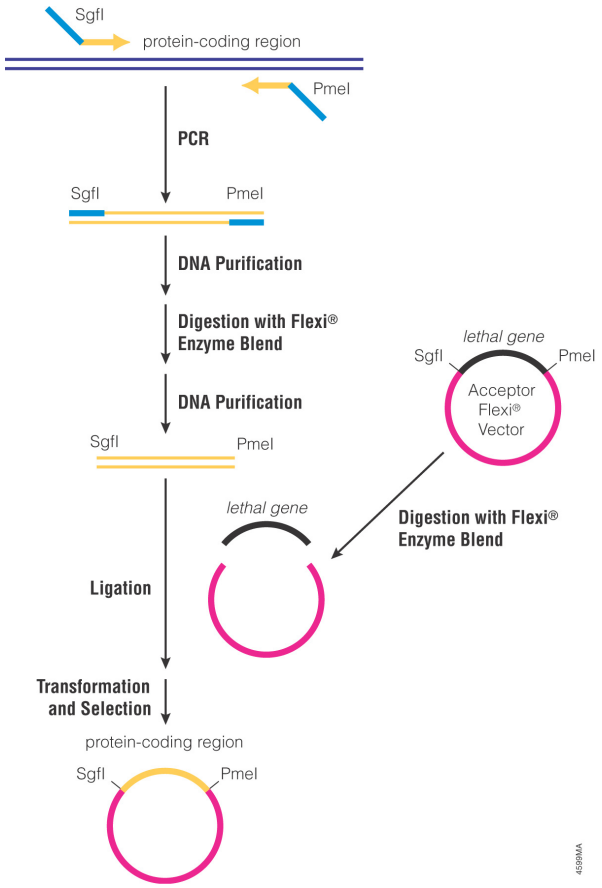

Figure 13.7. Cloning a protein-coding region into the Flexi® Vectors. PCR primers are designed to append SgfI and PmeI sites onto the protein-coding region. After amplification, the PCR product is purified to remove the DNA polymerase and primers and digested with SgfI and PmeI. The DNA is purified again to remove the small oligonucleotides released by the restriction enzymes. The digested PCR product is ligated into an acceptor Flexi® Vector that has been digested with SgfI and PmeI. Following transformation, the cells are selected with the appropriate antibiotic for the particular Flexi® Vector used.

Unlike site-specific recombination vector systems, the Flexi® Vector Systems do not require appending multiple amino acids to the amino or carboxy termini of the protein of interest (Figure 13.6). In addition, the systems do not require an archival entry vector, and most applications allow direct entry into the vector suited to the experimental design (e.g., mammalian expression or N-terminal, glutathione-S-transferase (GST) fusion vectors). For instance, you might clone your PCR product into the pFN2A (GST) Flexi® Vector to express your GST-tagged protein in *E. coli* for purification. However, an easy transfer of your insert after SgfI/PmeI digest followed by ligation into the pF4K CMV Flexi® Vector will allow you to transfect the same protein-coding region into a mammalian cell and determine its expression level.

Any Flexi® Vector can act as an acceptor of a protein-coding region flanked by SgfI and PmeI sites (Figure 13.7). The SgfI site is upstream of the start codon of the protein-coding region, and depending upon the Flexi® Vector used for cloning, this allows expression of a native (untagged) protein or an amino (N)-terminal-tagged protein by readthrough of the SgfI site. The PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the carboxy (C)-terminus of the protein (Figure 13.6).

The C-terminal Flexi® Vectors allow expression of C-terminal-tagged proteins. While these vectors can accept protein-coding regions flanked by SgfI and PmeI, they lack a PmeI site and contain a different blunt-ended site, EcoICRI. Inserts cloned using these sites cannot be removed from the C-terminal Flexi® Vectors and transferred to other Flexi® Vectors (Figure 13.4, Panel B).

Additional Resources for the Flexi® Vector Systems

Technical Bulletins and Manuals

TM254 *Flexi® Vector Systems Technical Manual*

Promega Publications

Clone and express protein-coding regions using the Flexi® Vector Systems

The Flexi® Vector Systems: The easy way to clone

Metal affinity tag for protein expression and purification using the Flexi® Vectors

Online Tools

Flexi® Vector Systems Animation

Flexi® Vector Primer Design Tool

Citations

Kuhn, P. *et al.* (2006) Automethylation of CARM1 allows coupling of transcription and mRNA splicing. *Nucleic Acids Res.* **39**, 2717–2.

Full-length mouse coactivator-associated arginine methyltransferase 1 (CARM1) was amplified and cloned into the pFC14K HaloTag® CMV Flexi® Vector. An R551K mutation was created in the same vector. The HaloTag® constructs were transfected into HEK293T cells, the CARM1 proteins affinity purified using HaloLink™ Resin and the CARM1 cleaved from the C-terminal HaloTag® using TEV protease. The purified CARM1 then was analyzed by mass spectrometry.

PubMed Number: 21138967

Markandeya, Y.S. *et al.* (2011) Caveolin-3 regulates protein kinase A modulation of the $Ca_V3.2$ (α1H) T-type Ca^{2+} channels. *J. Biol. Chem.* **286**, 2433–44.

Full-length and trucated aveolae containing scaffolding protein caveolin-3 (Cav-3) were fused to gluathione-S-transferase (GST) by PCR, Pme1 and Sgf1 digestion and ligation in the pFN2A (GST) Flexi® Vector. After confirming the Cav-3-GST fusion constructs, the vectors were transformed into *E. coli* strain BL21(DE3) and protein expression induced by IPTG and purified using

MagneGST™ Glutathione Particles. After elution, the Cav-3 proteins were analyzed using Western blotting.
PubMed Number: 21084288

D. Modifying and Restriction Enzymes

Promega offers a vast array of both modifying enzymes (e.g., ligase or phosphatase) and restriction endonucleases for use in cloning. This section is an overview of the products available from Promega to enhance your cloning results and highlights the enzymes that may be most useful to you. For example, ligase is a key enzyme in cloning as this enzyme joins the vector and insert to create a circular recombinant plasmid. Restriction enzymes (REs) are used to cut a vector and PCR product, or other type of insert, to generate compatible ends for ligation. REs also can be used to evaluate ligation success by screening the recombinant plasmid for the correct restriction sites. To explore strategies for subcloning, visit the Subcloning Notebook.

DNA Ligase

DNA ligase catalyzes the joining of two strands of DNA using the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides in either a sticky-ended or blunt-ended configuration (Engler and Richardson, 1982). This allows the "pasting" together of inserts and receptive vectors (e.g., A-tailed product into T vectors).

T4 DNA Ligase (Cat.# M1801, M1804, M1794) can join DNA strands together and has been shown to catalyze the joining of RNA to a DNA or RNA strand in a duplex molecule. However, DNA ligase will not join single-stranded nucleic acids (Engler and Richardson, 1982).

Additional Resources for T4 DNA Ligase

Technical Bulletins and Manuals

9PIM180 *T4 DNA Ligase Promega Product Information*

The **LigaFast™ Rapid DNA Ligation System** (Cat.# M8221, M8225) is designed for efficient ligation of sticky-ended DNA inserts into plasmid vectors in just 5 minutes (blunt-ended inserts in as little as 15 minutes). Rapid ligation is based on the combination of T4 DNA Ligase with a unique 2X Rapid Ligation Buffer. The LigaFast™ System eliminates any further purification prior to transformation of ligated DNA. The specially formulated 2X Rapid Ligation Buffer requires no additional ATP or Mg^{2+}.

Additional Resources for the LigaFast™ Rapid DNA Ligation System

Technical Bulletins and Manuals

9PIM822 *LigaFast™ Rapid DNA Ligation System Promega Product Information*

Promega Publications

Cloning differential display-PCR products with pGEM®-T Easy Vector System

Technically speaking: Subcloning plasmid DNA constructs

Rapid ligation for the pGEM®-T and pGEM®-T Easy Vector Systems

Alkaline Phosphatases

Alkaline phosphatases catalyze dephosphorylation of 5′ phosphates from DNA. These enzymes are used to prevent recircularization and religation of linearized vector DNA by removing 5′-phosphate groups from both termini and also may be used to dephosphorylate 5′ phosphorylated ends of DNA for subsequent labeling with [^{32}P]ATP and T4 Polynucleotide Kinase. Unit usage guidelines are usually included with the alkaline phosphatase (e.g., 0.01 units per picomole of ends). For assistance in calculating picomoles of vector or insert ends for dephosphorylation, visit the BioMath Calculators.

TSAP Thermosensitive Alkaline Phosphatase (Cat.# M9910) catalyzes the removal of 5′-phosphate groups from DNA and is effective on 3′ overhangs, 5′ overhangs and blunt ends. TSAP is active in all Promega restriction enzyme buffers, a convenience that allows a single, streamlined restriction enzyme digestion-dephosphorylation step. TSAP also is inactivated effectively and irreversibly by heating at 74°C for 15 minutes. Therefore, a DNA cleanup step is not required before ligation.

Additional Resources for TSAP Thermosensitive Alkaline Phosphatase

Technical Bulletins and Manuals

9PIM991 *TSAP Thermosensitive Alkaline Phosphatase Promega Product Information*

Promega Publications

TSAP Thermosensitive Alkaline Phosphatase activity in restriction enzyme buffers from New England Biolabs

TSAP: A new thermosensitive alkaline phosphatase

Alkaline Phosphatase, Calf Intestinal (CIAP; Cat.# M1821, M2825), catalyzes the hydrolysis of 5′-phosphate groups from DNA, RNA and ribo- and deoxyribonucleoside triphosphates. This enzyme is not inactivated by heat but can be denatured and removed by phenol extraction. CIAP is active on 5′ overhangs and 5′ recessed and blunt ends (Sambrook *et al.* 1989; Seeburg *et al.* 1977; Ullrich *et al.* 1977; Meyerowitz *et al.* 1980; Grosveld *et al.* 1981).

Additional Resources for Alkaline Phosphatase, Calf Intestinal

Technical Bulletins and Manuals

9PIM182 *Alkaline Phosphatase, Calf Intestinal Promega Product Information*

Promega Publications

Technically speaking: Subcloning plasmid DNA constructs

Restriction Enzymes

Restriction enzymes, also referred to as restriction endonucleases, are enzymes that recognize short, specific (often palindromic) DNA sequences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to the recognition sequences. Most restriction enzymes will not cut DNA that is methylated on one or both strands of their recognition site, although some require substrate methylation. A complete listing of restriction enzymes available from Promega can be found on the web.

Additional Resources for Restriction Enzymes

Technical Bulletins and Manuals

TM367 *Assembly of Restriction Enzyme Digestions Technical Manual*

Promega Publications

Rapid DNA digestion using Promega restriction enzymes

Activity of Promega restriction enzymes in GoTaq® Green and PCR Master Mixes

Work smarter using isoschizomers and neoschizomers

Online Tools

Restriction Enzyme Resource Guide

Citations

Zhang, Y. *et al.* (2011) The multidrug efflux pump MdtEF protects against nitrosative damage during the anaerobic respiration in *Escherichia coli*. *J. Biol. Chem.* **286**, 26576–84.

The −338 to +39-bp region of *tnaC* was amplified from MG1655 genomic DNA using primers incorporating NotI and HindIII restriction sites at the 5′ and 3′ends of the amplimer, respectively. After digestion with NotI and HindIII, the PCR product was gel purified and ligated into a plasmid digested with the same restriction enzymes so that the *lacZ* gene in the plasmid is under control of the *tnaC* promoter. Positive clones were confirmed by colony PCR and DNA sequencing.

PubMed Number: 21642439

Datta, M. and Bhattacharyya, N.P. (2011) Regulation of RE1 protein silencing transcription factor (REST) expression by HIP1 protein interactor (HIPPI). *J. Biol. Chem.* **286**, 33759–69.

The upstream promoter region of the mouse REST gene (position −4773 to −4216) was amplified, digested with BglII and KpnI and cloned into the same restriction sites of the pGL3 Basic Vector. Five hundred nanograms of the luciferase reporter construct was transfected into cells, and after 24 hours, the cells lysed and the luciferase measured using the Luciferase Reporter Assay.

PubMed Number: 21832040

E. Competent Cells

Transforming a newly constructed plasmid into competent *E. coli* cells is the primary method to propagate and select the clone or clones of interest. Competent bacterial cells are receptive to importing foreign DNA and replicating it. High-quality competent *E. coli* is an integral part of a successful cloning protocol.

JM109 Competent Cells

JM109 Competent Cells (Cat.# L2001) are prepared according to a modified procedure of Hanahan, 1985. These cells are transformed with plasmid DNA via the heat-shock method. JM109 cells (Yanisch-Perron *et al.* 1985) are an ideal host for many molecular biology applications and can be used for α-complementation of β-galactosidase for blue/white screening.

Additional Resources for JM109 Competent Cells

Technical Bulletins and Manuals

TB095 *E. coli Competent Cells Technical Bulletin*

Promega Publications

What are the effects of the bacterial DNA restriction-modification systems on cloning and manipulations of DNA in *E. coli*?

Citations

Shao, W. *et al.* (2011) Characterization of a novel beta-xylosidase, XylC, from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl. Environ. Microbiol.* **77**, 719–26.

Recombinant xylosidase was expressed from the pHsh-*xylCI* vector in *Escherichia coli* JM109 cells and the protein purified for analysis of its characteristics, including molecular mass and pI.

PubMed Number: 21131522

Vosler, P.S. *et al.* (2011) Ischemia-induced calpain activation causes eukaryotic (translation) initiation factor 4G1 (eIF4GI) degradation, protein synthesis inhibition, and neuronal death. *Proc. Natl. Acad. Sci. USA* **108**, 18102–7.

Hemagglutinin-tagged (HA-), human full-length eIF4G1 was subcloned into a lentiviral transfer vector containing the ubiquitin promoter using the restriction enzymes HindIII and XhoI. A control insert containing enhanced green fluorescent protein (EGFP) also was cloned into the lentiviral transfer vector. Both recombinant plasmids were transformed into JM109 *E. coli* cells, grown and harvested before being cotransfected with a packaging construct and envelope vector to generate an infectious enveloped lentivirus.

PubMed Number: 22006312

Single Step (KRX) Competent Cells

Single Step (KRX) Competent Cells (Cat.# L3001, L3002) are not only highly competent and compatible with blue/white screening but can be used for tightly controlled protein expression. KRX incorporates a chromosomal copy of the T7 RNA polymerase gene driven by a rhamnose promoter (*rha*BAD). T7 RNA polymerase-based systems (Studier and Moffat, 1986) are some of the most widely used protein expression systems by virtue of its well-defined promoter, which is completely independent of *E. coli* RNA polymerase promoters, and the rapid elongation rate of T7 RNA polymerase, about five times that of *E. coli* RNA polymerases. The *rha*BAD promoter is subject to catabolite repression by glucose, is activated by

addition of rhamnose to the medium, and provides precise control of T7 RNA polymerase abundance and thereby precise control of recombinant protein production.

Additional Resources for Single Step (KRX) Competent Cells

Technical Bulletins and Manuals

TB352 *Single Step (KRX) Competent Cells Technical Bulletin*

Promega Publications

^{15}N protein labeling using *Escherichia coli* strain KRX

Compatibility of Single Step (KRX) Competent Cells with the MagneGST™ Pull-Down System

The Single Step (KRX) Competent Cells: Efficient cloning and high protein yields

Citations

Semenova, E. *et al.* (2011) Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc. Natl. Acad. Sci. USA* **108**, 10098–103.

KRX *E. coli* cells carrying three plasmids that expressed Cascade, Cas3 and J3 pre-crRNA were grown in LB medium with antibiotics until reaching an O.D.$_{600}$ of ~0.3. Then the cells were induced with 1mM IPTG and 0.2% L-arabinose and grown for 45 minutes. The cells were washed twice with ice-cold water to render them electrocompetent, then transformed with a random mutant library of 350bp λ phage fragments cloned into pUC19. Colonies that escaped the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas system were grown and the plasmids sequenced to identify point mutations that allowed escape.

PubMed Number: 21646539

Kim, K.K. *et al.* (2011) Fox-3 and PSF interact to activate neural cell-specific alternative splicing. *Nucleic Acids Res.* **39**, 3064–78.

GST–Fox-3 fusion proteins were expressed in KRX *E. coli* cells and captured on glutathione sepharose beads. Myc-tagged polypyrimidine tract binding-associated splicing factor (PSF) and non-POU domain-containing octamer-binding protein (NonO) constructs were transcribed and translated using the TNT® Coupled Reticulocyte Lysate System. The cell-free expressed proteins were mixed with the GST-Fox-3 fusion proteins bound to beads and incubated for 1 hour in a pull-down assay. SDS sample buffer denatured the protein complexes prior to SDS-PAGE separation. These gels were stained with Coomassie® blue or analyzed by immunoblotting.

PubMed Number: 21177649

HB101 Competent Cells

HB101 Competent Cells (Cat.# L2011) are prepared according to a modified procedure of Hanahan, 1985. HB101 cells (Yanisch-Perron *et al.* 1985) are useful for cloning with vectors that do not require α-complementation for blue/white screening.

Additional Resources for HB101 Competent Cells

Technical Bulletins and Manuals

TB095 *E. coli Competent Cells Technical Bulletin*

III. PCR Cloning Protocols

A. Ligation and Transformation

Materials Required:

(see Composition of Solutions section)

- PCR product (has an A overhang; purification is optional) or blunt DNA fragment with an A residue added
- pGEM®-T Easy Vector System (Cat.# A1380) or pGEM®-T Easy Vector System (Cat.# A3610) Both systems include T4 DNA Ligase and chemically competent high-efficiency JM109 cells.
- Nuclease-Free Water (Cat.# P1193)
- **Optional:** 4°C water bath
- LB-Ampicillin plates containing X-Gal and IPTG
- high-efficiency competent cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step KRX Competent Cells (Cat.# L3002)], if needed
- SOC medium
- 42°C water bath
- ice

Vector:Insert Ratio

After the insert DNA is prepared for ligation, estimate the concentration by comparing the staining intensity with that of DNA molecular weight standard of similar size and known concentrations on an ethidium bromide-stained agarose gel. If the vector DNA concentration is unknown, estimate the vector concentration by the same method. Test various vector:insert DNA ratios to determine the optimal ratio for a particular vector and insert. In most cases, a 1:1 or 1:3 molar ratio of vector:insert works well. The following example illustrates the calculation of the amount of insert required at a specific molar ratio of vector:insert.

[(ng of vector × kb size of insert) ÷ kb size of vector] × (molar amount of insert ÷ molar amount of vector) = ng of insert

Example:

How much 500bp insert DNA needs to be added to 100ng of 3.0kb vector in a ligation reaction for a desired vector:insert ratio of 1:3?

[(100ng vector × 0.5kb insert) ÷ 3.0kb vector] × (3 ÷ 1) = 50ng insert

Ligation

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.

2. Set up ligation reactions as described below. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Use 0.5ml tubes known to have low DNA-binding capacity.

Reagents	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Nuclease-Free Water to a final volume of	**10µl**	**10µl**	**10µl**

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature. Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

Transformation

1. Prepare LB/ampicillin/IPTG/X-Gal plates (see Composition of Solutions).

2. Centrifuge the ligation reactions briefly. Add 2µl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice. Prepare a transformation control tube with 0.1ng of an uncut plasmid. pGEM®-T Vectors are **not** suitable for the transformation control as they are linear, not circular.

   Note: In our experience, the use of larger (17 × 100mm) polypropylene tubes (e.g., BD Falcon Cat.# 352059) increases transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing colony number, and should be avoided.

3. Place the high-efficiency JM109 Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by gently flicking the tube.

4. Carefully transfer 50µl of cells to the ligation reaction tubes prepared in Step 2. Use 100µl of cells for the transformation control tube. Gently flick the tubes, and incubate on ice for 20 minutes.

5. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tubes to ice for 2 minutes.

6. Add 950µl of room temperature SOC medium to the ligation reaction transformations and 900µl to the tranformation control tube. Incubate for 1.5 hours at 37°C with shaking (~150rpm).

7. Plate 100µl of each transformation reaction onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC is recommended prior to plating.

8. Incubate plates overnight at 37°C. Select white colonies.

Calculation of Transformation Efficiency

For every transformation with competent cells, we recommend performing a transformation control using a known quantity of a purified, supercoiled plasmid DNA (e.g., pGEM®-3Z Vector, Cat.# P2151). Calculate the transformation efficiency as described below.

transformation efficiency (cfu/µg) = (cfu on control plate ÷ ng of supercoiled vector plated) × (10^3ng/µg) × final dilution factor

cfu = colony forming units

Example:

A 100µl aliquot of competent cells is transformed with 1ng of supercoiled pGEM®-3Z Vector DNA. Ten microliters of the transformation reaction (0.1ng total DNA) is added to 990µl of SOC medium (1:100 dilution). Of that volume (1,000µl), a 100µl aliquot is plated (1:1,000 final dilution), and 100 colonies are obtained on the plate. What is the transformation efficiency?

(100cfu ÷ 0.1ng of supercoiled vector plated) × (10^3ng/µg) × 1,000 = 1 x 10^9 cfu/µg

B. Amplification, Analysis and PCR Cleanup

The following protocol is a general procedure to analyze and purify a PCR fragment. Amplification protocols can be found in the PCR Amplification chapter of the *Protocols and Applications Guide*. Additional information regarding PCR, analysis and product purification can be found in the following resources:

- PCR Amplification chapter of the *Protocols and Applications Guide*
- DNA Purification chapter of the *Protocols and Applications Guide*

Amplification

A basic protocol for amplifying genomic DNA by PCR can be found in the PCR Amplification chapter of the *Protocols and Applications Guide*, in the section "Example of a PCR Protocol".

Analysis

Materials Required:

(see Composition of Solutions section)

- aliquot of amplification reaction (usually 5–10µl)
- **Optional:** Blue/Orange Loading Dye, 6X (Cat.# G1881) if GoTaq® Green Reaction Buffer is not used
- appropriately sized DNA marker
- appropriate agarose gel (typically 0.8–1.2%; see Table 13.1 for guidelines)
- gel running buffer (1X TAE or 0.5X TBE)
- 10mg/ml ethidium bromide

1. Analyze 5–10µl of the amplification reaction using agarose gel electrophoresis. Include at least one lane containing a DNA size marker to determine if the PCR products are of the correct size. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel (50µg/ml final concentration in the agarose).

2. Store reaction products at –20°C until needed.

Table 13.1. Gel Percentages: Resolution of Linear DNA on Agarose Gels.

Recommended % Agarose	Optimum Resolution for Linear DNA
0.5	1,000–30,000bp
0.7	800–12,000bp
1.0	500–10,000bp
1.2	400–7,000bp
1.5	200–3,000bp
2.0	50–2,000bp

If there are primer dimers or at least two PCR products present, the band of interest will need to be excised and purified (see the next section, PCR Cleanup, for more information). To minimize the number of extraneous amplimers, the PCR conditions may need to be optimized. For suggestions on troubleshooting PCR, visit the PCR Amplification chapter of the *Protocols and Applications Guide* .

PCR Cleanup

Once you have determined that the PCR was successful, you can purify the desired product from the rest of the reaction components. This can be accomplished using a number of procedures including direct purification of the product using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281, A9282, A9285) or separating the DNA fragments on an agarose gel. Alternatively, you can use a portion of the amplification reaction directly in a ligation. However, the presence of primer dimers or other amplimers present can cause false-positive reactions or yield an incorrect clone (see Figure 13.8). If the reaction is clean (i.e., a single band is seen on an analytical gel), and the desired product is a minimum of 100bp in size, you can use the Wizard® SV Gel and PCR Clean-Up System to directly purify the PCR product [see the DNA Purification chapter of the *Protocols and Applications Guide* for product protocol].

If there are other bands or a large primer-dimer band present, we recommend gel electrophoresis to separate the products so that the desired band can be excised. The DNA can be recovered by melting the excised agarose and using the Wizard® SV Gel and PCR Clean-Up System.

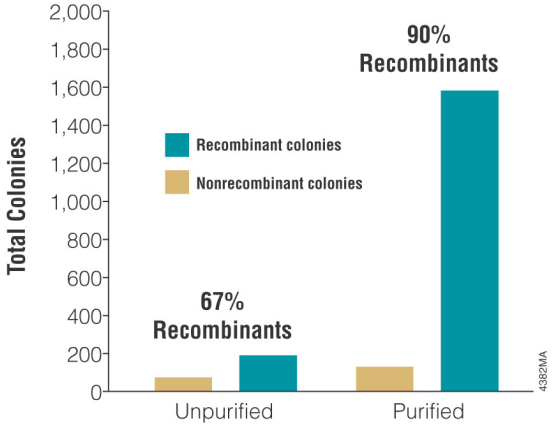

Figure 13.8. Purification of PCR products enhances cloning success. A 500bp PCR product was purified with the Wizard® SV Gel and PCR Clean-Up System and cloned into the pGEM®-T Easy Vector. Both the percent recombinants and total number of colonies increased with a pure PCR product.

***Optional:* A-Tailing Reaction for Blunt-Ended Products:**

If a proofreading DNA polymerase was used for amplification and you want to clone into a T vector, an adenosine residue must be added to the PCR product. This can be accomplished by incubating the DNA fragment with dATP and a nonproofreading DNA polymerase, which will add a single 3′ A residue. Blunt DNA fragments resulting from restriction enzyme digestion also can be cloned into T vector after adding an adenosine residue.

Materials Required:

- blunt-ended product (from PCR or restriction enzyme digestion), purified
- GoTaq® Flexi DNA Polymerase
- 25mM $MgCl_2$
- 5X GoTaq® Colorless or Green Reaction Buffer
- 1mM dATP (Cat.# U1205; diluted 1:100 in nuclease-free water)

1. Set up the following reaction in a thin-walled PCR tube:

Purified DNA fragment	1–4.4µl
5X GoTaq® Reaction Buffer (Colorless or Green)	2µl
1mM dATP (0.2mM final concentration)	2µl
GoTaq® Flexi DNA Polymerase (5u/µl)	1µl
25mM $MgCl_2$ (1.5mM final concentration)	0.6µl
Nuclease-free water to	**10µl**

2. Incubate at 70°C for 15–30 minutes in a water bath or thermal cycler. After the tailing reaction is finished, 1–2µl can be used without further cleanup for ligation with pGEM®-T or pGEM®-T Easy Vector Systems.

C. Screening of Transformants

To determine if the insert was successfully cloned, there are two methods for screening the transformed bacteria: colony PCR or plasmid miniprep followed by restriction enzyme digestion.

Successful cloning of an insert into the pGEM®-T and pGEM®-T Easy Vectors disrupts the coding sequence of the β-galactosidase α peptide. Recombinant clones usually can be identified by blue/white screening on X-Gal/IPTG indicator plates following transformation of competent cells. However, the characteristics of PCR products cloned into these T vectors can significantly affect the ratio of blue:white colonies obtained. Clones that contain PCR products, in most cases, produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lac*Z gene. Such fragments are usually a multiple of 3 base pairs long (including the 3′-A overhangs) and do not contain in-frame stop codons. There have been reports of DNA fragments of up to 2kb that have been cloned in-frame and have produced blue colonies.

Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (e.g., deletions or point mutations) that may result in blue colonies when competent cells are transformed with the fragment inserted into the pGEM®-T or pGEM®-T Easy Vectors.

Screening of recombinant clones using restriction enzymes is more time-consuming than colony PCR and involves isolating the plasmid DNA from liquid cultures of individual *E. coli* colonies, performing the restriction enzyme digestion and determining if the insert is of the correct size. To learn more about screening by restriction enzyme digestion, visit the Subcloning Notebook.

The following protocol is for colony PCR analysis of transformants.

Materials Required:
(see Composition of Solutions section)

- plate of colonies containing the recombinant plasmid
- toothpicks or sterile bacterial loop
- LB Broth (optional)
- upstream screening primer
- downstream screening primer
- GoTaq® Flexi DNA Polymerase
- 5X Green GoTaq® Flexi Buffer
- 25mM $MgCl_2$
- Nuclease-Free Water (Cat.# P1193)
- Nuclease-Free Light Mineral Oil (e.g., Sigma Cat.# M5904 or Promega Cat.# DY1151) if you are using a thermal cycler without a heated lid; do **not** autoclave
- dNTP Mix (10mM of each dNTP; Cat.# U1511, U1515)

1. Pick a well-isolated colony using either a sterile toothpick or a flamed and cooled bacterial loop, and transfer to 50µl of sterile water. Part of the colony may be transferred to LB medium containing the appropriate antibiotic for overnight culture and plasmid miniprep, if desired.
2. Boil for 10 minutes to break open the bacterial cell wall and release the DNA.
3. Centrifuge at 16,000 × *g* for 5 minutes to pellet the cell debris.
4. Use 5µl of the supernatant in a 50µl amplification reaction (see Table 13.2 for a sample reaction).

Table 13.2. Colony PCR using GoTaq® Flexi DNA Polymerase.

Components	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
5X Reaction Buffer	10µl	1X
dNTP mix (10mM of each dNTP)	1µl	0.2mM each
GoTaq® DNA polymerase (5u/µl)	0.25µl	0.025u/µl
25mM $MgCl_2$	3µl	1.5mM
Downstream screening primer	50pmol[1]	1µM
Upstream screening primer	50pmol[1]	1µM
Boiled colony supernatant	5µl	

[1]A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × b; where b is the number of bases in the primer.

5. Amplify the target DNA using cycling conditions appropriate for your screening primers and size of amplimer (see Table 13.3 for suggestions). Place reactions in a thermal cycler that has been preheated to 94°C.

Table 13.3. Suggested Amplification Conditions.

Step	Temperature	Time (minutes)	Cycles
Initial denaturation	94°C	2	1
Denaturation	94°C	0.5–1.0	25–35
Annealing	42–65°C[1]	0.5–1.0	
Extension	72°C	1 minute/ kilobase[2]	
Final extension	72°C	5	1
Soak/Hold	4°C	Indefinite	1

[1]Annealing temperature should be optimized for each primer set based on the primer melting temperature (T_m). To calculate melting temperatures of primers in GoTaq® Reaction Buffer, go to BioMath Calculators.

[2]The extension time should be at least 1 minute per kilobase of target. Typically, amplimers smaller than 1kb use a 1-minute extension.

6. Remove an aliquot of the completed PCR and analyze by agarose gel electrophoresis for the product of appropriate size, which indicates the correct insert is present in the clone.

7. **Recommended:** Culture the appropriate colony or colonies to create a glycerol stock of your recombinant plasmid or plasmids, and purify the plasmids in larger quantities [e.g., PureYield™ Plasmid Systems (Cat.# A2492, A2495)] for downstream applications or further manipulation.

D. Subcloning

Classic subcloning involves restriction digestion of the plasmid of interest to remove the desired DNA fragment followed by ligation into a second vector with compatible ends. PCR can be used for subcloning as well, amplifying the insert from one plasmid and cloning the product into a T vector. Alternatively, the PCR product can be generated using primers with restriction enzyme sites, cut with the appropriate enzymes, then cloned into a vector with compatible ends. Further information on subcloning can be found by visiting the Subcloning Notebook.

IV. Cloning Protocol for the Flexi® Vector Systems

A. PCR Primer Design, Amplification and Cleanup

The desired protein-coding region must be amplified by PCR before being cloned into the Flexi® Vectors (Figures 13.6 and 13.7). The optimal conditions for amplifying the protein-coding region will depend on the DNA template, DNA polymerase, PCR primers and other reaction parameters. We recommend following the protocol provided with the DNA polymerase to generate the PCR product. For protein-coding regions less than 700bp, consider using GoTaq® DNA Polymerase to amplify your protein-coding region. For regions greater than 700bp, we recommend the use of a high-fidelity DNA polymerase, such as *Pfu* DNA polymerase. To facilitate cloning, the PCR primers used to amplify the protein-coding region must append an SgfI site and a PmeI site to the PCR product. To append these sites, incorporate an SgfI site in your amino-terminal PCR primer and a PmeI site in your carboxy-terminal PCR primer. Transfer of protein-coding regions into N-terminal Flexi® Vectors results in translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala. The PmeI site is placed at the carboxy terminus, appending a single valine residue to the last amino acid of the protein-coding region. The valine codon, GTT, is immediately followed by an ochre stop codon, TAA. Primer design guidelines are provided in Technical Manual #TM254 and the Flexi® Vector Primer Design Tool.

To cleanup the PCR product, refer to Amplification, Analysis and PCR Cleanup.

B. Restriction Digest of PCR Product and Acceptor Flexi® Vector

Digestion reactions for the PCR product and the acceptor Flexi® Vector can be performed concurrently.

Note: Do not use C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors for PCR products if you plan to transfer the protein-coding region to a different Flexi® Vector in the future. C-terminal Flexi® Vectors lack PmeI sites and cannot serve as donors for other Flexi® Vectors.

Materials Required:

- Flexi® System, Entry/Transfer (Cat.# C8640)
- chosen acceptor Flexi® Vector
- purified PCR product

1. Thaw the 5X Flexi® Digest Buffer, the acceptor Flexi® Vector and Nuclease- Free Water, and store on ice. Vortex the 5X Flexi® Digest Buffer and the acceptor Flexi® Vector before use.

2. Combine the following reaction components to cut the PCR product with SgfI and PmeI.

Component	Volume
5X Flexi® Digest Buffer	4µl
Purified PCR product (up to 500ng)	Xµl
Flexi® Enzyme Blend (SgfI and PmeI)	4µl
Nuclease-Free Water to a final volume of	**20µl**

3. Combine the following reaction components to cut the acceptor Flexi® Vector with SgfI and PmeI.

Component	Volume
Nuclease-Free Water	12µl
5X Flexi® Digest Buffer	4µl
Acceptor Flexi® Vector (200ng)	2µl
Flexi® Enzyme Blend (SgfI and PmeI)	2µl
Final Volume of	**20µl**

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

4. Incubate both reactions (Steps 2 and 3) at 37°C for 30 minutes.

5. Heat the reaction with the Flexi® Vector (Step 3) at 65°C for 20 minutes to inactivate the restriction enzymes. Store on ice until the PCR product and vector are ligated in Ligation of PCR Product and Acceptor Flexi® Vector.

6. Directly purify the digested PCR product using the Wizard® SV Gel and PCR Cleanup System (Cat.# A9281).

C. Ligation of PCR Product and Acceptor Flexi® Vector

1. Combine the following reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Acceptor Flexi® Vector from Restriction Digest of PCR Product and Acceptor Flexi® Vector, Step 5 (50ng)	2µl
PCR product (approximately 100ng)	Xµl
T4 DNA Ligase (HC; 20u/µl)	1µl
Nuclease-Free Water to a final volume of	**20µl**

2. Incubate at room temperature for 1 hour.

Note: The 2X Flexi® Ligase Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.

D. Screening Clones with SgfI and PmeI

Note: Do not use this protocol to screen for inserts in C-terminal Flexi® Vectors, which have names starting with "pFC", since these clones lack PmeI sites.

Materials Required:
(see Composition of Solutions section)

- 10X Flexi® Enzyme Blend (SgfI and PmeI; Cat.# R1851, R1852)
- Blue/Orange 6X Loading Dye (Cat.# G1881)
- plasmid isolated from colonies

1. Place reaction components and reaction tubes or plates on ice.

2. Prepare a master mix by combining the components listed below. Increase volumes proportionately depending on the number of reactions.

Component	Volume per Reaction
Nuclease-Free Water	10.5µl
5X Flexi® Digest Buffer	4µl
10X Flexi® Enzyme Blend (SgfI and PmeI)	2µl
Final Volume of	**15µl**

3. Add 15µl of master mix to 5µl (200–500ng) of plasmid DNA. Mix thoroughly by pipetting.

4. Incubate for 2 hours at 37°C.

5. Add 5µl of loading dye (Blue/Orange Loading Dye, 6X, Cat.# G1881). Incubate at 65°C for 10 minutes.

6. Load 20µl of the reaction onto a 1% agarose gel and separate fragments by electrophoresis. Visualize the fragments by ethidium bromide staining.

E. Transfer of Protein-Coding Region

Transfer refers to moving your 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 and a different antibiotic resistance marker than the donor because antibiotic selection is the basis for selecting the desired clone (Figures 13.5, 13.9 and 13.10).

There are two basic categories of Flexi® Vectors: those containing SgfI and PmeI sites and expressing either a native (untagged) protein or an N-terminal-tagged protein, and those containing SgfI and EcoICRI sites and expressing a C-terminal-tagged protein. Flexi® Vectors for expressing C-terminal-tagged proteins act only as acceptors, never as donor vectors. To transfer protein-coding regions between Flexi® Vectors expressing native protein or an N-terminal-tagged protein, the donor and acceptor vectors are digested with SgfI and PmeI simultaneously, prior to ligation of the insert, transformation and selection of the cells (Figure 13.9).

To create a C-terminal-tagged protein, the donor plasmid expressing native protein or an N-terminal-tagged protein is digested with SgfI and PmeI. Because EcoICRI cuts frequently in protein-coding regions, the acceptor plasmid containing the C-terminal tag is digested with SgfI and EcoICRI in a separate reaction. The two separate digests are combined for ligation of the insert, transformation and selection of the cells (Figure 13.10).

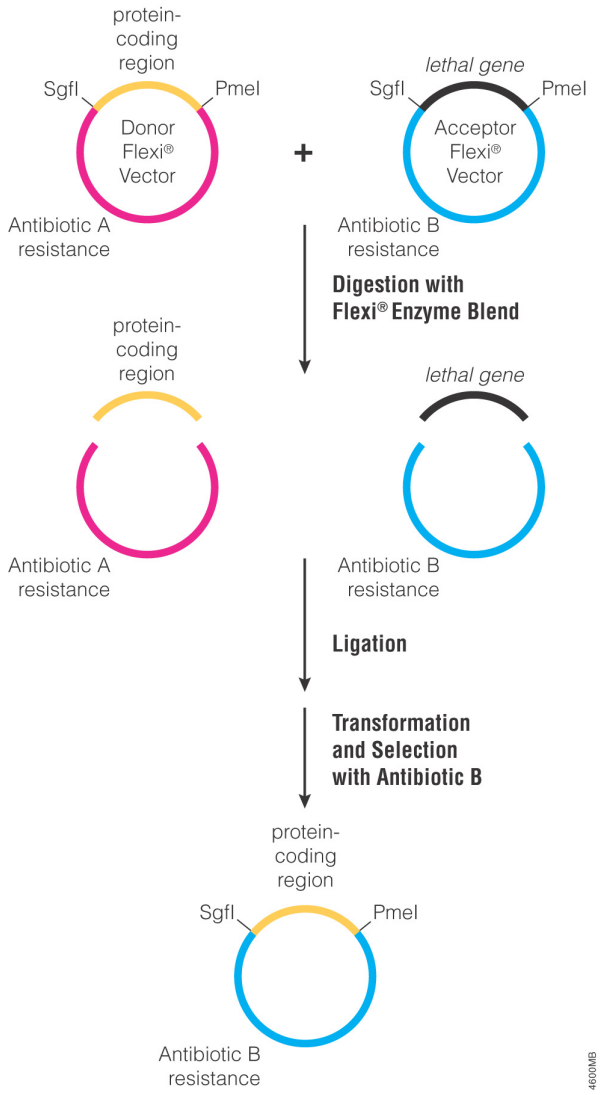

Figure 13.9. Transfer of a protein-coding region between N-terminal or native Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is mixed with an acceptor Flexi® Vector that has a different antibiotic resistance. The two plasmids are digested with SgfI and PmeI, and the mixture is ligated and transformed into *E. coli*. The cells are plated on the appropriate selective medium for the acceptor Flexi® Vector. Transfer of protein-coding regions into N-terminal fusion vectors allow translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala.

Transfer of Protein-Coding Regions Between Flexi® Vectors Expressing Native or N-Terminal Fusion Proteins

Materials Required:

- Flexi® System, Transfer (Cat.# C8820)
- competent *E. coli* cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step (KRX) Competent Cells (Cat.# L3002)]
- LB plates supplemented with the appropriate antibiotic at the appropriate concentration (see Composition of Solutions and Figure 13.5)

1. Use the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA [see the DNA Purification chapter of the *Protocols and Applications Guide*]. Adjust the volume so that the final DNA concentration is 50–100ng/µl.

2. Combine the following reaction components to cut the Flexi® Vectors:

Component	Volume
5X Flexi® Digest Buffer	4µl
Acceptor Flexi® Vector (100ng)	1µl
Donor Flexi® Vector (100ng)	Xµl
Flexi® Enzyme Blend (SgfI and PmeI)	2µl
Nuclease-Free Water to a final volume of	**20µl**

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. Incubate at 37°C for 15–30 minutes.

4. Heat the reaction at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reaction on ice while assembling the ligation reaction in Step 5.

5. Combine the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Digested DNA from Step 4 (100ng total)	10µl
T4 DNA Ligase (HC; 20u/µl)	1µl
Final volume of	**21µl**

6. Incubate at room temperature for 1 hour.

7. Transform the ligation reaction into high-efficiency, *E. coli* competent cells (≥1 × 10^8cfu/µg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for our high-efficiency JM109 Competent Cells is provided in Ligation and Transformation. Selection for transformants should be on LB plates supplemented with 100µg/ml ampicillin for Flexi® Vectors with the letter "A" in the name or 30µg/ml kanamycin for Flexi® Vectors with the letter "K" in the name. See Figure 13.5 for a list of antibiotic-resistance genes carried on the various vectors.

8. Screen at least four colonies for each protein-coding region. Digest the plasmid to ensure that SgfI and PmeI cleave their recognition sites flanking the protein-coding region so that the insert can be cloned into other Flexi® Vectors.

   Screen at least eight colonies for each protein-coding region transferred to the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors. Lower transfer frequencies with these vectors are due to a higher background of plasmid backbone heterodimers between the WG (BYDV) Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites such that plasmid backbone dimers are unstable (Yoshimura *et al.* 1986). The pF3A and pF3K WG (BYDV) Flexi® Vectors lack these common flanking regions due to the inclusion of the BYDV translation-enhancing sequences.

   If you are using the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors, the number of minipreps performed can be reduced by prescreening colonies to identify those harboring plasmid backbone heterodimers. Colonies containing such heterodimers can be identified by their ability to grow on both antibiotics. Pick individual colonies, and restreak on an ampicillin plate and a kanamycin plate or inoculate medium containing ampicillin and medium containing kanamycin. Grow overnight. Colonies containing the clone of interest will grow only in the antibiotic associated with the acceptor plasmid.

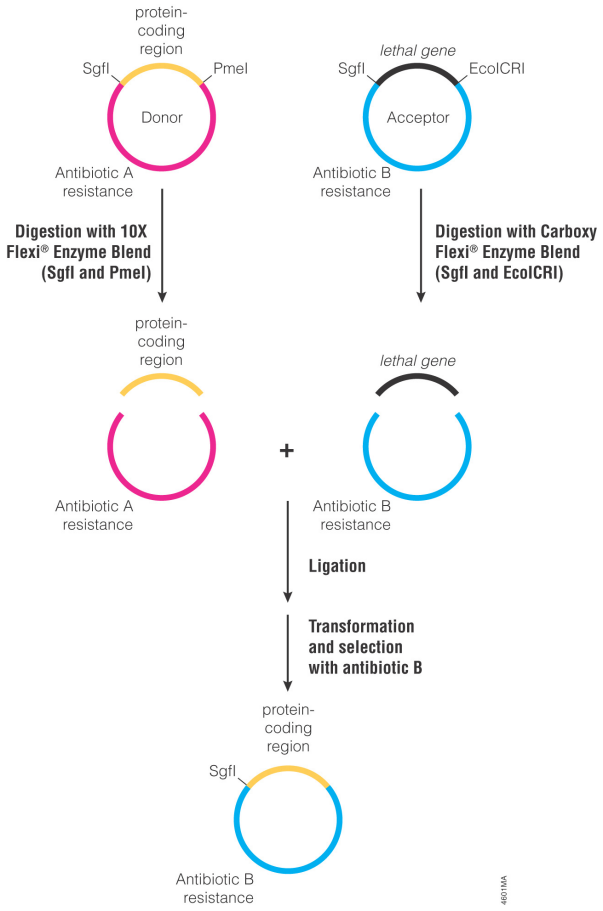

Figure 13.10. Transfer of a protein-coding region into the C-terminal Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is digested with SgfI and PmeI. The acceptor Flexi® Vector, which has a different antibiotic resistance, is digested with SgfI and EcoICRI in a separate reaction. The two digested plasmids are combined, the mixture is ligated and transformed into *E. coli*, and cells are plated on the appropriate selective medium for the acceptor Flexi® Vector. When the blunt ends of PmeI and EcoICRI are joined, an in-frame Ser codon, which is appended to the downstream C-terminal protein-coding region contained on the Flexi® Vector backbone, is created.

Transfer of Protein-Coding Regions from Native or N-Terminal Flexi® Vectors to C-Terminal Flexi® Vectors

Use the C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors but not as donors because they lack PmeI sites.

Materials Required:

- Carboxy Flexi® System, Transfer (Cat.# C9320)
- competent *E. coli* cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step (KRX) Competent Cells (Cat.# L3002)]
- LB plates supplemented with the appropriate antibiotic at the appropriate concentration (see Composition of Solutions and Figure 13.5)

1. Use the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA [see the DNA Purification chapter of the *Protocols and Applications Guide*]. Adjust the volume so that the final DNA concentration is 50–100ng/µl.

2. Combine the following reaction components to cut the donor Flexi® Vector:

Component	Volume
5X Flexi® Digest Buffer	2µl
Donor Flexi® Vector (100ng)	Xµl
Flexi® Enzyme Blend (SgfI and PmeI)	1µl
Nuclease-Free Water to a final volume of	**10µl**

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. In a separate tube, combine the following reaction components to cut the acceptor C-terminal Flexi® Vector:

Component	Volume
Nuclease-Free Water	6µl
5X Flexi® Digest Buffer	2µl
Acceptor C-Terminal Flexi® Vector (100ng)[1]	1µl
Carboxy Flexi® Enzyme Blend (SgfI and EcoICRI)	1µl
Final volume	**10µl**

[1]Acceptor C-terminal Flexi® Vectors will have names starting with "pFC".

4. Incubate both reactions at 37°C for 15–30 minutes.

5. Heat both reactions at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reactions on ice while assembling the ligation reaction in Step 6.

6. Combine the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Digested donor Flexi® Vector prepared in Step 2 (approximately 50ng)	5µl
Digested acceptor C-terminal Flexi® Vector prepared in Step 3 (50ng)	5µl
T4 DNA Ligase (HC; 20u/µl)	1µl
Nuclease-Free Water to a final volume of	**21µl**

7. Incubate at room temperature for 1 hour.

8. Transform the ligation reaction into high-efficiency, *E. coli* competent cells (≥1 × 10^8cfu/µg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for our high-efficiency JM109 Competent Cells is provided in Ligation and Transformation. Selection for transformants should be on LB plates supplemented with 100µg/ml ampicillin for Flexi® Vectors with the letter "A" in the name or 30µg/ml kanamycin for Flexi® Vectors with the letter "K" in the name. See Figure 13.5 for a list of antibiotic-resistance genes carried on the various vectors.

9. Screen at least eight colonies for each protein-coding region. Successful plasmid constructs will not cut with PmeI but will cut with SgfI. Lower transfer frequencies are due to a higher background of plasmid backbone heterodimers between the C-terminal Flexi® Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites such that plasmid backbone dimers are unstable (Yoshimura *et al.* 1986). The C-terminal Flexi® Vectors may lack these common flanking regions due to the inclusion of the protein fusion tag sequence.

V. Supplemental Cloning Techniques

A. Converting a 5´ Overhang to a Blunt End

Materials Required:

- Nuclease-Free Water (Cat.# P1193)
- DNA Polymerase I Large (Klenow) Fragment and 10X Reaction Buffer (Cat.# M2201) or T4 DNA Polymerase (Cat.# M4211)
- T4 DNA Polymerase 10X buffer (optional)
- Bovine Serum Albumin (BSA), Acetylated, 1mg/ml (Cat.# R9461)
- dNTPs, 100mM (Cat.# U1240)

Both Klenow (DNA Polymerase I Large Fragment) and T4 DNA Polymerase can be used to fill 5′-protruding ends with deoxynucleotide triphosphates (dNTPs). Properties of these enzymes are discussed in Anderson *et al*. 1980 and Challberg and Englund, 1980.

Klenow Fragment Method

For optimal activity, use the Klenow 10X Buffer supplied with the enzyme. DNA Polymerase I Large (Klenow) Fragment is also active in many restriction enzyme buffers, and some users may choose to perform the fill-in reaction directly in the restriction buffer.

1. Optional: Following the restriction enzyme digestion that generated the 5′-protruding ends, purify the DNA using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) or other DNA purification system. Alternatively, the DNA can be extracted with phenol:chloroform, ethanol precipitated and rehydrated for use in the conversion reaction.

2. Proceed with one of the following for the fill-in reaction (total reaction volume can be 10–100µl.):

   For DNA purified over a column: The optimal reaction conditions for filling in are: 1X Klenow Reaction Buffer [50mM Tris- HCl (pH 7.2), 10mM $MgSO_4$, 0.1mM DTT], 40µM of each dNTP, 20µg/ml acetylated BSA and 1 unit of Klenow Fragment per microgram of DNA.

   For digested DNA in restriction enzyme buffer: Klenow Fragment is also partially active in many restriction enzyme buffers (such as our 4-CORE® Buffers), and the fill-in reaction may be performed directly in the restriction enzyme buffer supplemented with 40µM of each dNTP, thereby eliminating the clean-up step. Add 1 unit of Klenow Fragment per microgram of DNA.

   For ethanol-precipitated DNA: Resuspend DNA in Klenow 1X Buffer containing 40µM of each dNTP and 20µg/ml of Acetylated Bovine Serum Albumin (BSA). Add 1 unit of Klenow Fragment per microgram of DNA.

3. Incubate the reaction at room temperature for 10 minutes.

4. Stop the reaction by heating at 75°C for 10 minutes.

T4 DNA Polymerase Method

Prepare the DNA as described for the Klenow Fragment method. Like the Klenow Fragment, T4 DNA Polymerase functions well in many restriction enzyme buffers. Add 5 units of T4 DNA Polymerase per microgram of DNA, 100µM of each dNTP and 0.1mg/ml Acetylated BSA. The recommended reaction buffer for T4 DNA Polymerase is 1X T4 DNA Polymerase Buffer. Incubate the reaction at 37°C for 5 minutes. Stop the reaction by heating at 75°C for 10 minutes or by adding 2µl of 0.5M EDTA.

B. Converting a 3´ Overhang to a Blunt End

Materials Required:

- Nuclease-Free Water (Cat.# P1193)
- Bovine Serum Albumin (BSA), Acetylated, 1mg/ml (Cat.# R9461)
- dNTPs, 100mM (Cat.# U1240)
- T4 DNA Polymerase (Cat.# M4211)
- T4 DNA Polymerase 10X buffer (optional)

Note: T4 DNA Polymerase has a 3′→5′ exonuclease activity that will, in the presence of excess dNTPs, convert a 3′-protruding end to a blunt end (Burd and Wells, 1974).

1. Following the restriction enzyme digestion that generates 3′-protruding ends, leave the DNA in restriction enzyme buffer, exchange the buffer for 1X T4 DNA Polymerase Buffer or gel purify the desired fragment (see the PCR Cleanup section of Amplification, Analysis and PCR Cleanup).

2. Add 5 units of T4 DNA Polymerase per microgram of DNA and 100µM of each dNTP.

3. Incubate at 37°C for 5 minutes.

   Note: At high concentrations of dNTPs (100µM), degradation of the DNA will stop at duplex DNA; however, if the dNTP supply is exhausted, the very active exonuclease activity (200 times more active than that of DNA polymerase I) will degrade the double-stranded DNA (Sambrook *et al.* 1989).

4. Stop the reaction by heating at 75°C for 10 minutes or adding 2µl of 0.5M EDTA.

C. Dephosphorylation of 5´ Ends

If the ends of the prepared vector are identical (e.g., following a single digestion), it is advantageous to treat the vector with TSAP Thermosensitive Alkaline Phosphatase (Cat.# M9910) to remove the phosphate groups from the 5′ ends to prevent self-ligation of the vector (Sambrook *et al*. 1989). For linear vectors with unique 5′ ends, TSAP treatment is not necessary.

Note: Since TSAP is active in all Promega restriction enzyme buffers, the vector DNA easily can be restriction digested and dephosphorylated at the same time. The following protocol reflects this streamlined method. See the *TSAP Thermosensitive Alkaline Phosphatase Product Information* #9PIM991 for alternative protocols.

1. As a general guideline, for reactions containing up to 1µg of DNA, add 15 units of restriction enzyme and the amount of TSAP listed below to the vector DNA in a total reaction volume of 20–50µl. Set up the reaction in the appropriate 1X Promega restriction enzyme reaction buffer.

Reaction Buffer	Amount of TSAP for Reactions Containing ≤1µg DNA
Promega 10X Reaction Buffers A–L (except F)	1µl
Promega 10X Reaction Buffer F	2µl
MULTI-CORE™ 10X Buffer	1µl

2. Incubate the reaction at 37°C for 15 minutes. This is sufficient to digest and dephosphorylate all vector DNA overhang types (3′, 5′ or blunt).

3. Heat-inactivate the TSAP and restriction enzyme by incubating the reaction at 74°C for 15 minutes.

   Note: Not all restriction enzymes can be heat-inactivated. If the restriction enzyme cannot be heat-inactivated, clean up the digest using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281).

4. Briefly centrifuge the reaction, and use approximately 40ng of dephosphorylated vector in a ligation reaction containing DNA insert, 1X Rapid Ligation Buffer and 2µl (6 units) of T4 DNA Ligase (LigaFast™ Rapid DNA Ligation Sytem, Cat.# M8221). Incubate ligation reactions containing vector with 5′ or 3′ overhangs at 25°C for 5 minutes. Incubate ligation reactions containing vector with blunt ends at 25°C for 15 minutes.

   Note: Optimal vector:insert ratios may need to be determined. We recommend using a 1:2 molar ratio of vector to insert DNA as a starting point. See the *LigaFast™ Rapid DNA Ligation System Product Information* #9PIM822 for additional information.

5. Transform the ligated material directly into *E. coli* competent cells following the recommended transformation protocol provided with the cells.

VI. Composition of Solutions

antibiotic stock solutions

100mg/ml ampicillin in deionized water (sterile filtered)
25mg/ml kanamycin; kanamycin sulfate in deionized water (sterile filtered)

Store at –20°C.

Blue/Orange 6X Loading Dye

0.03% bromophenol blue
0.03% xylene cyanol FF
0.4% orange G
15% Ficoll® 400
10mM Tris-HCl (pH 7.5)
50mM EDTA (pH 8.0)

5X Flexi® Digest Buffer

50mM Tris-HCl (pH 7.9 at 37°C)
250mM NaCl
50mM $MgCl_2$
5mM DTT
0.5mg/ml acetylated BSA

2X Flexi® Ligase Buffer

60mM Tris-HCl (pH 7.8 at 25°C)
20mM $MgCl_2$
20mM DTT
2mM ATP

Store in single-use aliquots at –20°C. Avoid multiple freeze-thaw cycles.

IPTG stock solution (0.1M)

1.2g isopropyl β-D-thiogalactopyranoside

Add deionized water to 50ml final volume. Filter sterilize, and store at 4°C.

LB medium

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Add deionized water to approximately 1L. Adjust pH to 7.5 with 10N NaOH, and autoclave. For LB plates, include 15g agar prior to autoclaving.

LB plates with antibiotic

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml or kanamycin to a final concentration of 30µg/ml, as appropriate for the acceptor Flexi® Vector. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as described above, then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, spread 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal over the surface of an LB-ampicillin plate and allow to absorb for 30 minutes at 37°C prior to use.

2M Mg^{2+} stock

20.33g $MgCl_2 \bullet 6H_2O$
24.65g $MgSO_4 \bullet 7H_2O$

Add distilled water to 100ml. Filter sterilize.

SOC medium (100ml)

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg^{2+} stock, filter-sterilized
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml of distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring the volume to 100ml with sterile, distilled water. The final pH should be 7.0.

1X TBE buffer

89mM Tris base
89mM boric acid
2mM EDTA (pH 8.0)

1X TAE buffer

40mM Tris base
5mM sodium acetate
1mM EDTA (pH 8.0)

X-Gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Dissolve in 2ml N,N´-dimethyl-formamide. Cover with aluminum foil, and store at –20°C. Alternatively, use 50mg/ml X-Gal (Cat.# V3941).

VII. References

Anderson, S. *et al.* (1980) A short primer for sequencing DNA cloned in the single-stranded phage vector M13mp2. *Nucleic Acids Res.* **8**, 1731–43.

Burd, J.F. and Wells, R.D. (1974) Synthesis and characterization of the duplex block polymer d($C_{15}A_{15}$)-d($T_{15}G_{15}$). *J. Biol. Chem.* **249**, 7094–101.

Challberg, M.D. and Englund, P.T. (1980) Specific labeling of 3' termini with T4 DNA polymerase. *Methods Enzymol.* **65**, 39–43.

Clark, J.M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* **16**, 9677–86.

D'Avino, P.P. *et al.* (2004) Mutations in *sticky* lead to defective organization of the contractile ring during cytokinesis and are enhanced by *Rho* and suppressed by *Rac*. *J. Cell Biol.* **166**, 61–71.

Engler, M.J. and Richardson, C.C. (1982) In: *The Enzymes*, Boyer, P.D., ed., Academic Press, New York, NY.

Grosveld, F.G. *et al.* (1981) Isolation of β-globin-related genes from a human cosmid library. *Gene* **13**, 227–37.

Hanahan, D. (1985) *DNA Cloning,* Vol. 1, Glover, D., ed., IRL Press, Ltd., 109.

Knoche, K. and Kephart, D. (1999) Cloning blunt-end *Pfu* DNA polymerase-generated PCR fragments into pGEM®-T Vector Systems. *Promega Notes* **71**, 10–4.

Le Gall, S.M. *et al.* (2003) Regulated cell surface pro-EGF ectodomain shedding is a zinc metalloprotease-dependent process. *J. Biol. Chem.* **278**, 45255–68.

Meyerowitz, E.M. *et al.* (1980) A new high-capacity cosmid vector and its use. *Gene* **11**, 271–82.

Mezei, L.M. and Storts, D.R. (1994) Purification of PCR products. In: *PCR Technology: Current Innovations,* Griffin, H.G. and Griffin, A.M., eds., CRC Press, Boca Raton, FL, 21.

Newton, C.R. and Graham, A. (1994) In: *PCR*, BIOS Scientific Publishers, Ltd., Oxford, UK, 13.

Robles, J. and Doers, M. (1994) pGEM®-T Vector Systems troubleshooting guide. *Promega Notes* **45**, 19–20.

Sakakida, Y. *et al.* (2005) Importin α/β mediates nuclear transport of a mammalian circadian clock component, mCRY2, together with mPER2, through a bipartite nuclear localization signal. *J. Biol. Chem.* **280**, 13272–8.

Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Seeburg, P.H. *et al.* (1977) Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature* **270**, 486–94.

Studier, F.W. and Moffat, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–30.

Ullrich, A. *et al.* (1977) Rat insulin genes: Construction of plasmids containing the coding sequences. *Science* **196**, 1313–9.

Yanisch-Perron, C. *et al.* (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–19.

Yoshimura, H. *et al.* (1986) Biological characteristics of palindromic DNA (II). *J. Gen. Appl. Microbiol.* **32**, 393–404.