StrataClone Mammalian Expression Vector Systems

INSTRUCTION MANUAL

Catalog #240228 (Untagged Vector System)
#240229 (N-terminal FLAG Vector System)
#240230 (C-terminal FLAG Vector System)
#240231 (N-terminal c-Myc Vector System)
#240232 (C-terminal c-Myc Vector System)

Revision B.0

For Research Use Only. Not for use in diagnostic procedures.

240228-12

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StrataClone Mammalian Expression Vector Systems

CONTENTS

StrataClone Mammalian Expression Vector Systems

MATERIALS PROVIDED

Catalog # 240228 Materials Provided	Quantity[a]
StrataClone pCMV-SC Vector Mix	21 reactions (1 µl each)
StrataClone Blunt Cloning Buffer	63 µl
StrataClone Cam Control Insert (5 ng/µl)	50 ng
StrataClone SoloPack Competent Cells	21 transformations (50 µl each)
pUC18 Control Plasmid (0.1 ng/µl in TE buffer)	10 µl

[a] Kit provides enough reagents for 20 experimental cloning reactions plus one Control Insert cloning reaction.

Catalog # 240229 Materials Provided	Quantity[a]
StrataClone N-Terminal FLAG Vector Mix	21 reactions (1 µl each)
StrataClone Blunt Cloning Buffer	63 µl
StrataClone Cam Control Insert (5 ng/µl)	50 ng
StrataClone SoloPack Competent Cells	21 transformations (50 µl each)
pUC18 Control Plasmid (0.1 ng/µl in TE buffer)	10 µl

[a] Kit provides enough reagents for 20 experimental cloning reactions plus one Control Insert cloning reaction.

Catalog # 240230 Materials Provided	Quantity[a]
StrataClone C-Terminal FLAG Vector Mix	21 reactions (1 µl each)
StrataClone Blunt Cloning Buffer	63 µl
StrataClone Cam Control Insert (5 ng/µl)	50 ng
StrataClone SoloPack Competent Cells	21 transformations (50 µl each)
pUC18 Control Plasmid (0.1 ng/µl in TE buffer)	10 µl

[a] Kit provides enough reagents for 20 experimental cloning reactions plus one Control Insert cloning reaction.

Catalog # 240231 Materials Provided	Quantity[a]
StrataClone N-Terminal c-Myc Vector Mix	21 reactions (1 µl each)
StrataClone Blunt Cloning Buffer	63 µl
StrataClone Cam Control Insert (5 ng/µl)	50 ng
StrataClone SoloPack Competent Cells	21 transformations (50 µl each)
pUC18 Control Plasmid (0.1 ng/µl in TE buffer)	10 µl

[a] Kit provides enough reagents for 20 experimental cloning reactions plus one Control Insert cloning reaction.

Catalog # 240232 Materials Provided	Quantity[a]
StrataClone C-Terminal c-Myc Vector Mix	21 reactions (1 µl each)
StrataClone Blunt Cloning Buffer	63 µl
StrataClone Cam Control Insert (5 ng/µl)	50 ng
StrataClone SoloPack Competent Cells	21 transformations (50 µl each)
pUC18 Control Plasmid (0.1 ng/µl in TE buffer)	10 µl

[a] Kit provides enough reagents for 20 experimental cloning reactions plus one Control Insert cloning reaction.

Revision B.0

STORAGE CONDITIONS

StrataClone SoloPack Competent Cells and pUC18 Control Plasmid: –80°C
All Other Components: –20°C

Note *The StrataClone SoloPack competent cells are sensitive to variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency.*

ADDITIONAL MATERIALS REQUIRED

Proofreading DNA polymerase or a polymerase blend recommended for blunt PCR cloning
Thermocycler
LB–kanamycin agar plates[§]
LB–chloramphenicol and LB–ampicillin agar plates[§] (for control reactions only)
LB medium[§]

NOTICES TO PURCHASER

Limited Label License for StrataClone Mammalian Expression Vector Systems

US Patent No. 7,109,178 and patents pending.

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Notices continue on the following page

[§]See *Preparation of Media and Reagents*.

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INTRODUCTION

The StrataClone Mammalian Expression Vectors are designed for high-level expression of epitope-tagged proteins in mammalian cells. Use of StrataClone technology* for the cloning steps allows rapid 5-minute cloning of blunt-ended PCR products, produced using high-fidelity PCR enzymes, into the epitope tagging/expression vectors. The StrataClone cloning method is summarized in Figure 1 and discussed in greater detail below.

Epitope Tagging Options

The epitope tagging technique involves fusion of a protein of interest to a peptide epitope that is recognized by a readily available antibody. With this technique, expression of the fusion protein is monitored using a tag-specific antibody, allowing a new protein to be studied without generating a new antibody specific to the protein of interest. Epitope tagging can be used to localize gene products in living cells, identify associated proteins, track the movement of fusion proteins within the cell, or characterize new proteins by immunoprecipitation.

The StrataClone Mammalian Expression Vectors contain sequences encoding three copies of either the FLAG or the c-myc epitope at either the N or C terminus. These specific epitope tags are small, highly immunoreactive, and are not likely to interfere with the function of the target protein. The synthetic FLAG epitope is composed of eight amino acid residues (DYKDDDDK).[1] The c-myc epitope is derived from the human c-myc gene and contains ten amino acid residues (EQKLISEEDL).[2] Tagged constructs generated in the StrataClone Mammalian Expression Vectors can be transfected into mammalian cells and the fusion protein can be easily characterized using commercially available antibodies. The presence of three copies of the epitope in each vector enhances detection of the fusion protein in downstream applications. An expression vector that lacks epitope tagging sequences is also available to allow expression of untagged protein or fusion proteins with a custom tag encoded by the PCR insert.

In addition to the epitope tag sequences, the StrataClone Mammalian Expression Vectors contain features for expression of fusion proteins in eukaryotic cells. The cytomegalovirus (CMV) promoter allows constitutive expression of the cloned DNA in a wide variety of mammalian cell lines. The vectors contain a neomycin-resistance gene under control of a mammalian promoter for selection in mammalian cells.

*US Patent No. 7,109,178 and patents pending.

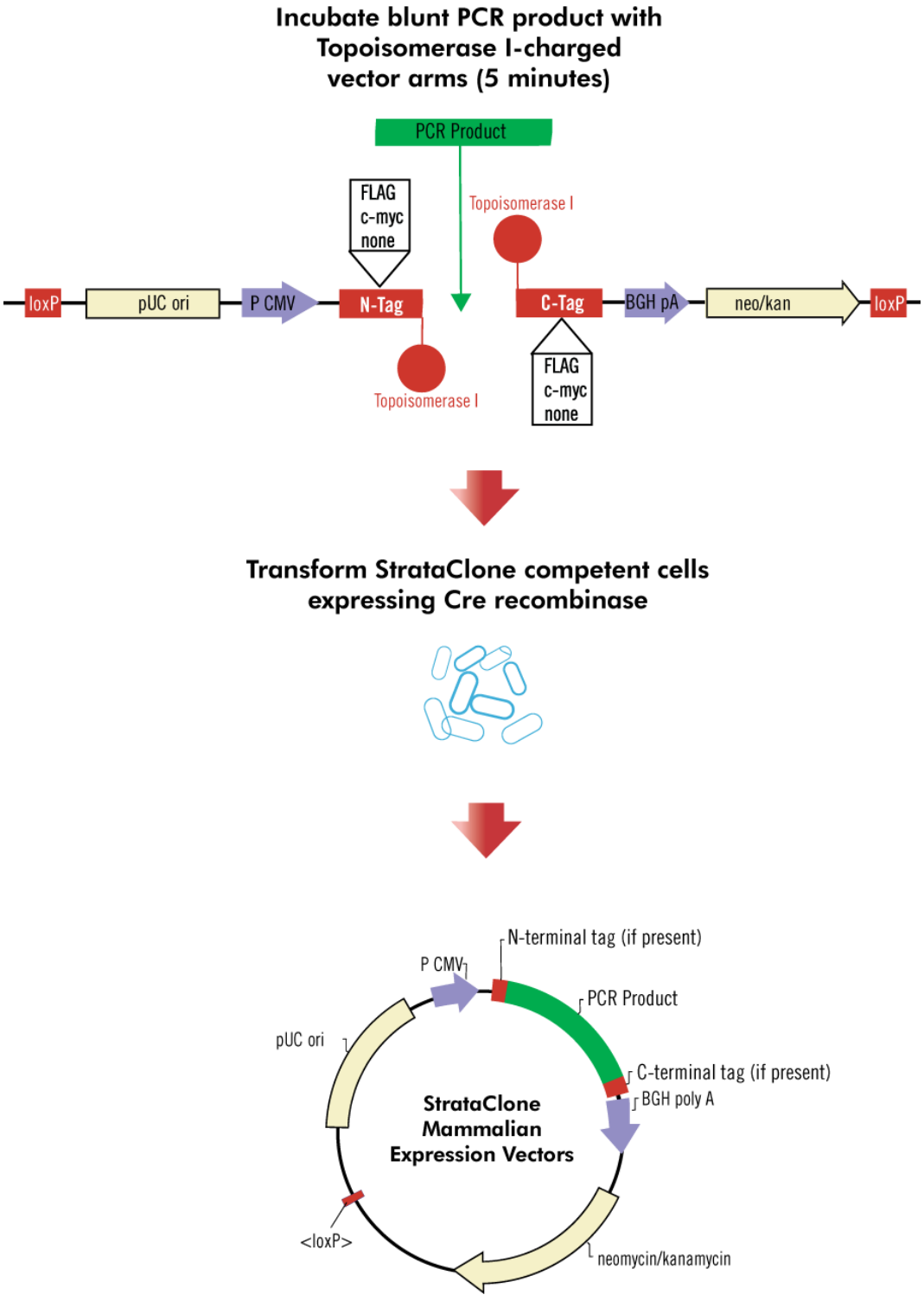


Figure 1 Overview of the StrataClone mammalian expression cloning method. Five varieties of vector arms are available, with either an N-terminal tag (FLAG or c-myc), a C-terminal tag (FLAG or c-myc), or no tag.

Overview of StrataClone Blunt PCR Cloning Technology

Using the method summarized in Figure 1, StrataClone blunt PCR cloning technology exploits the combined activities of topoisomerase I from *Vaccinia* virus and Cre recombinase from bacteriophage P1. *In vivo*, DNA topoisomerase I assists in DNA replication by relaxing and rejoining DNA strands. Topoisomerase I cleaves the phosphodiester backbone of a DNA strand after the sequence 5´-CCCTT, forming a covalent DNA–enzyme intermediate which conserves bond energy to be used for religating the cleaved DNA back to the original strand. Once the covalent DNA–enzyme intermediate is formed, the religation reaction can also occur with a heterologous DNA acceptor.[3] The Cre recombinase enzyme catalyzes recombination between two *loxP* recognition sequences.[4]

Each StrataClone PCR cloning vector mix contains two blunt-ended DNA arms. Both arms are charged with topoisomerase I on one end and contain a *loxP* recognition sequence on the other end. Blunt-ended PCR products, produced by proofreading PCR enzymes, are efficiently ligated to these vector arms in a 5-minute ligation reaction by topoisomerase I-mediated strand ligation.

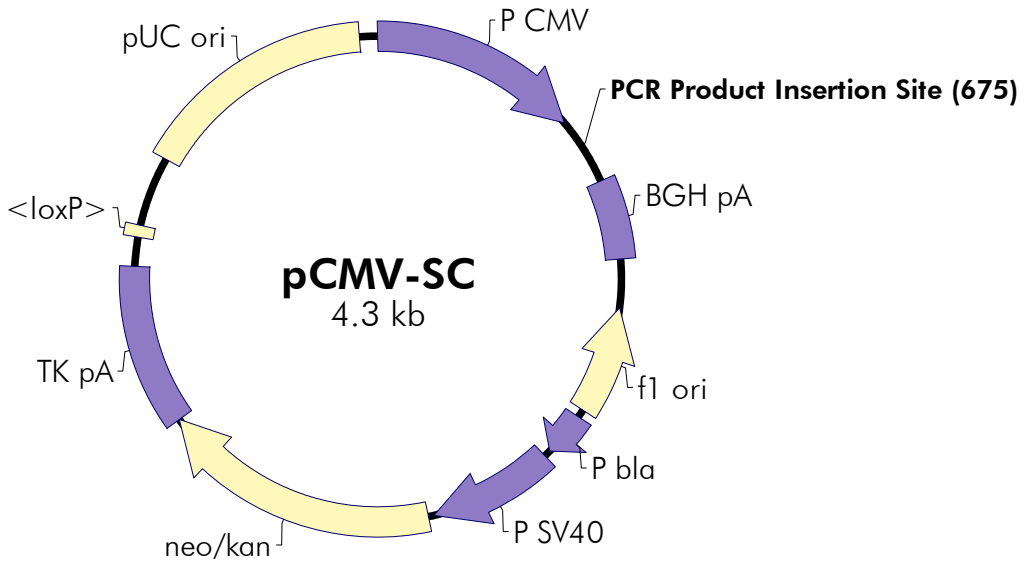
The resulting linear molecule (vector arm^{ori}–PCR product–vector arm$^{neo/kan}$) is then transformed, with no clean-up steps required, into a competent cell line engineered to transiently express Cre recombinase. Cre-mediated recombination between the vector *loxP* sites creates a circular DNA molecule that is proficient for replication in *E. coli* cells growing on media containing kanamycin. Maps for the circular plasmid products of recombination are shown in Figures 2–6 (see table below). The circular maps provided are for reference only; actual circular DNA plasmids will contain user-specific PCR product inserts.

Catalog #	Expressed Protein Tag	Reference Circular Plasmid Map
240228	None (untagged)	pCMV-SC (Figure 2)
240229	N-terminal FLAG	pCMV-SC-NF (Figure 3)
240230	C-terminal FLAG	pCMV-SC-CF (Figure 4)
240231	N-terminal c-Myc	pCMV-SC-NM (Figure 5)
240232	C-terminal c-Myc	pCMV-SC-CM (Figure 6)

StrataClone SoloPack Competent Cells

The provided StrataClone SoloPack competent cells express Cre recombinase, in order to circularize the linear DNA molecules produced by topoisomerase I-mediated ligation. The cells are provided in a convenient single-tube transformation format. The StrataClone SoloPack competent cells are optimized for high efficiency transformation and recovery of high-quality recombinant DNA. The cells are endonuclease (*end*A), and recombination (*rec*A) deficient, and are restriction-minus. The cells lack the tonA receptor, conferring resistance to T1, T5, and ϕ80 bacteriophage infection, and lack the F´ episome. StrataClone SoloPack competent cells are resistant to streptomycin.

StrataClone Mammalian Expression Vector pCMV-SC



pCMV-SC Expression Vector PCR Product Insertion Site Region (sequence shown 620–776)

T3 promoter — Sac I — EcoR I (non-unique) — EcoR I (non-unique)

AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACTGTGGAATTCGCCCTT PCR Product AAGGGCGAATTCCACATTGGG...

Xho I — Apa I — T7 promoter

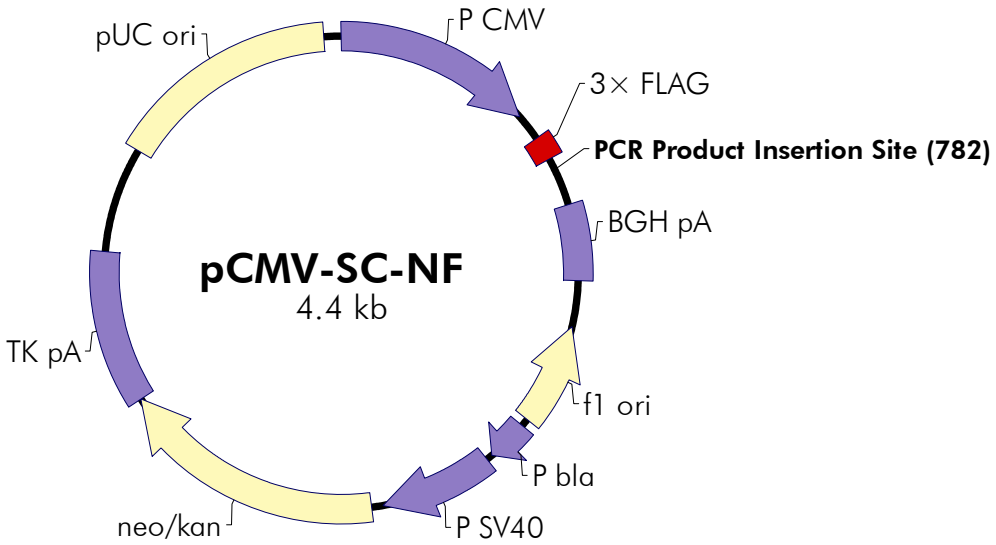
...CTCGAGGGGGGGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC

MULTIPLE STOP CODONS

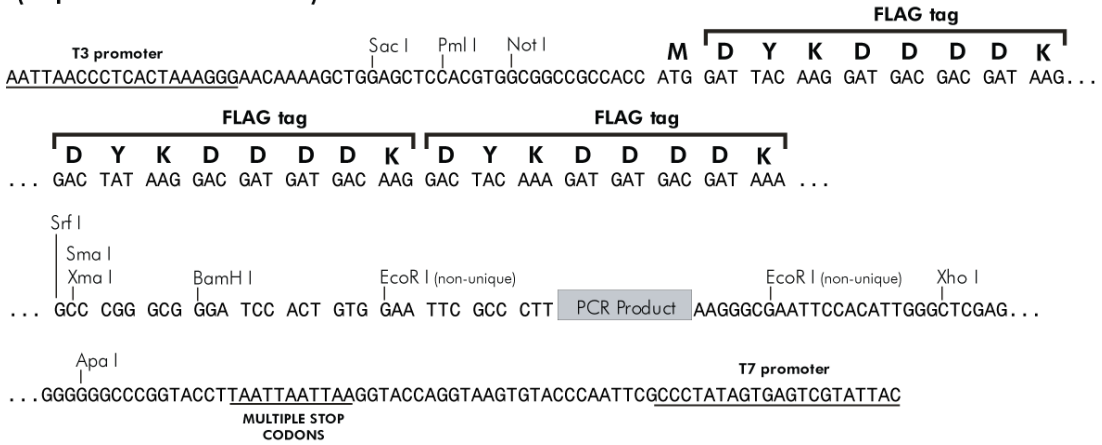
Feature	Nucleotide Position
CMV promoter	1–596
PCR product insertion site	675
BGH poly A signal	791–1017
f1 origin of ss-DNA replication	1156–1462
Neomycin/kanamycin resistance expression sequences (P *bla* through HSV-TK poly A signal)	1487–3254
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	3338–3371
pUC origin of replication	3572–4239

FIGURE 2 StrataClone blunt PCR cloning vector pCMV-SC. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

StrataClone Mammalian Expression Vector pCMV-SC-NF



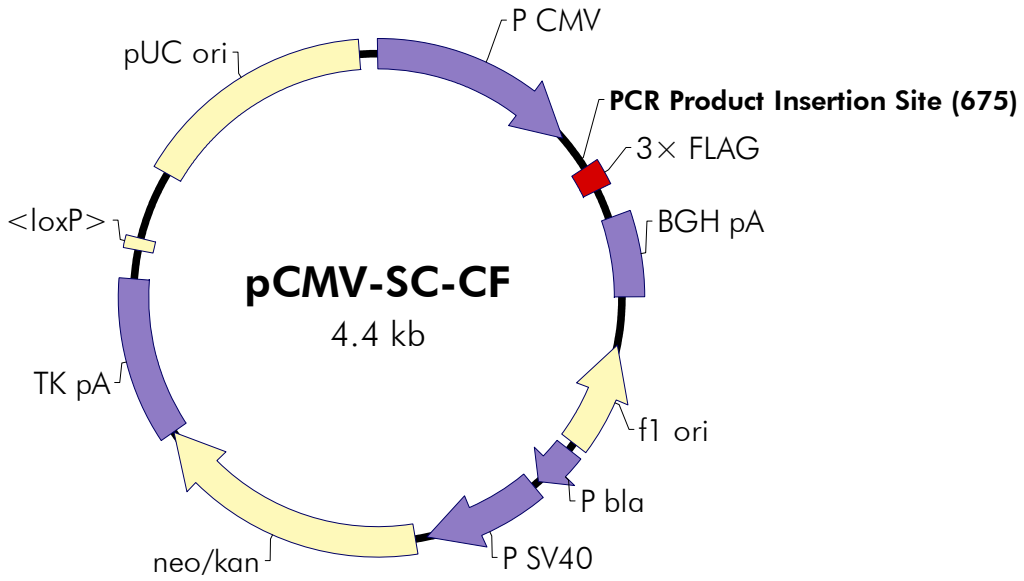
pCMV-SC-NF Expression Vector PCR Product Insertion Site Region (sequence shown 620–883)



Feature	Nucleotide Position
CMV promoter	1–596
3× FLAG	678–749
PCR product insertion site	782
BGH poly A signal	898–1124
f1 origin of ss-DNA replication	1263–1569
Neomycin/kanamycin resistance expression sequences (P *bla* through HSV-TK poly A signal)	1594–3361
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	3445–3478
pUC origin of replication	3679–4346

FIGURE 3 StrataClone blunt PCR cloning vector pCMV-SC-NF. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

StrataClone Mammalian Expression Vector pCMV-SC-CF



pCMV-SC-CF Expression Vector PCR Product Insertion Site Region (sequence shown 620–847)

T3 promoter — Sac I — EcoR I (non-unique) — EcoR I (non-unique) — Xho I

```
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACTGTGGAATTCGCCCTT PCR Product AAG GGC GAA TTC CAC ATT GGG CTC GAG...
```

FLAG tag — FLAG tag — FLAG tag

```
  D  Y  K  D  D  D  D  K   D  Y  K  D  D  D  D  K   D  Y  K  D  D  D  D  K
...GAT TAC AAG GAT GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT GAC AAG GAC TAC AAA GAT GAT GAC GAT AAA...
```

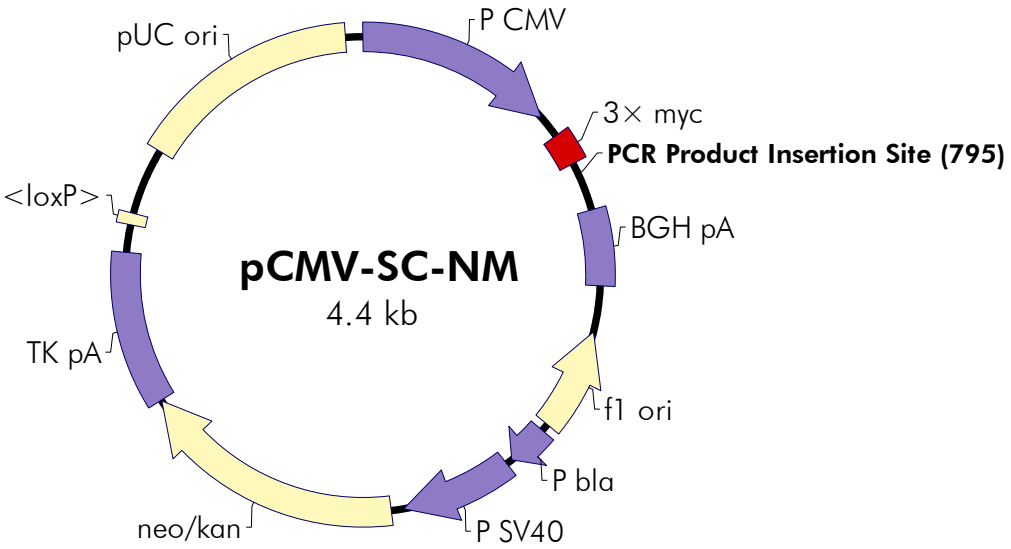
Apa I — T7 promoter

```
...TAG GGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
   STOP
```

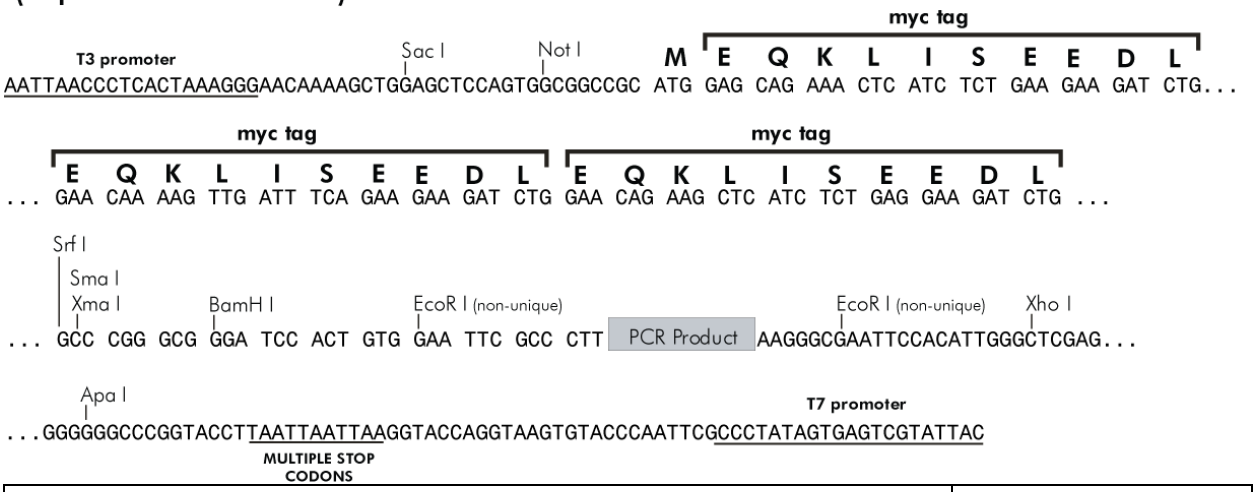
Feature	Nucleotide Position
CMV promoter	1–596
PCR product insertion site	675
3× FLAG	703–774
BGH poly A signal	862–1088
f1 origin of ss-DNA replication	1227–1533
Neomycin/kanamycin resistance expression sequences (P *bla* through HSV-TK poly A signal)	1558–3325
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	3409–3442
pUC origin of replication	3643–4310

FIGURE 4 StrataClone blunt PCR cloning vector pCMV-SC-CF. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

StrataClone Mammalian Expression Vector pCMV-SC-NM



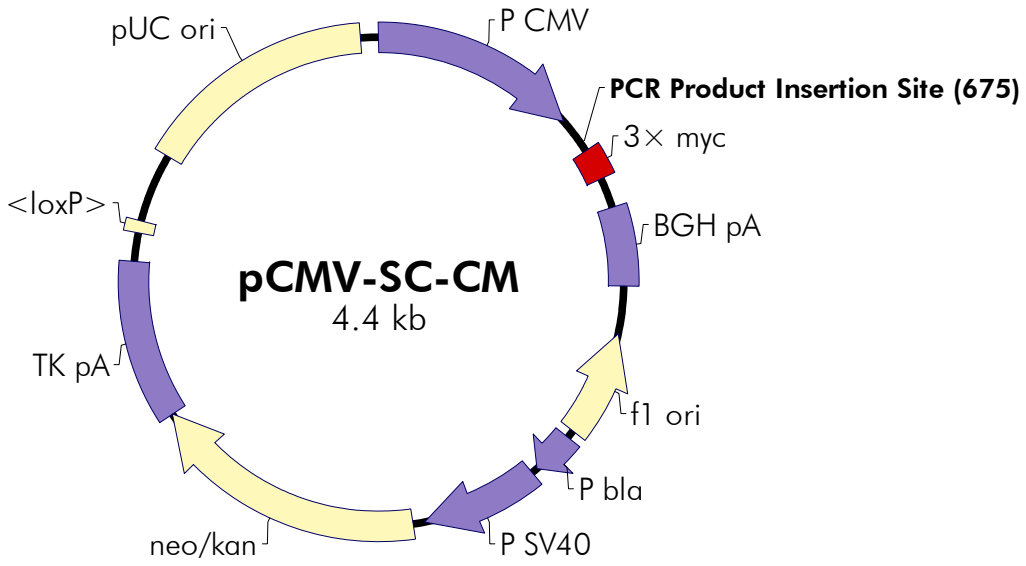
pCMV-SC-NM Expression Vector PCR Product Insertion Site Region (sequence shown 620–896)



Feature	Nucleotide Position
CMV promoter	1–596
3× c-myc	673–762
PCR product insertion site	795
BGH poly A signal	911–1137
f1 origin of ss-DNA replication	1276–1582
Neomycin/kanamycin resistance expression sequences (P *bla* through HSV-TK poly A signal)	1607–3374
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	3458–3491
pUC origin of replication	3692–4359

FIGURE 5 StrataClone blunt PCR cloning vector pCMV-SC-NM. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

StrataClone Mammalian Expression Vector pCMV-SC-CM



pCMV-SC-CM Expression Vector PCR Product Insertion Site Region (sequence shown 620–866)

T3 promoter — Sac I — EcoR I (non-unique) — EcoR I (non-unique) — Xho I

```
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACTGTGGAATTCGCCCTT PCR Product AAG GGC GAA TTC CAC ATT GGG CTC GAG...
```

myc tag — myc tag

```
...GAG CAG AAA CTC ATC TCT GAA GAA GAT CTG GAA CAA AAG TTG ATT TCA GAA GAA GAT CTG GAA CAG AAG CTC ATC...
 E   Q   K   L   I   S   E   E   D   L   E   Q   K   L   I   S   E   E   D   L   E   Q   K   L   I
```

myc tag — Apa I — T7 promoter

```
...TCT GAG GAA GAT CTG TAA GGGCCCGGTACCTTAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
 S   E   E   D   L  STOP
```

Feature	Nucleotide Position
CMV promoter	1–596
PCR product insertion site	675
3× c-myc	703–792
BGH poly A signal	881–1107
f1 origin of ss-DNA replication	1246–1552
Neomycin/kanamycin resistance expression sequences (P *bla* through HSV-TK poly A signal)	1577–3344
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	3428–3461
pUC origin of replication	3662–4329

FIGURE 6 StrataClone blunt PCR cloning vector pCMV-SC-CM. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at *www.stratagene.com*.

PREPROTOCOL CONSIDERATIONS

PCR Primer Design

No specific primers are required for cloning into the StrataClone mammalian expression vectors. The following primer design considerations should be followed, however, for optimal expression and cloning efficiency.

N-Terminal Tagging Vectors (pCMV-SC-NF or pCMV-NM)

- **Optimized 5' terminal sequence:** The nucleotide composition of the 5´-end of the primers influences the cloning efficiency. We have observed improved cloning efficiencies for PCR products containing the sequence 5´-GG. Where possible, consider designing PCR primers to begin with the sequence 5´-GG.
- **Stop codons:** Design the PCR insert to include a stop codon. The sequence **TAG**GCC is recommended to provide an efficient translational stop and to support optimal cloning efficiency. While stop codons are present downstream of the PCR product insertion site (in all three frames), translation termination using the vector-derived stop codons results in the addition of at least 14 exogenous amino acids to the C terminus of the expressed protein.
- **Translation initiation codon and Kozak signal:** The N-terminal tagging vector arms contain an efficient Kozak sequence and start codon upstream of the epitope tag sequence. It is advisable to omit the ATG start codon from the PCR product insert. We have observed that including an insert-encoded start codon can result in some translation initiation at the downstream ATG, producing a mixture of tagged and untagged protein. Substitution of the start codon with a glycine codon (GGN) is recommended for optimal cloning efficiency.
- **Exclusion of topoisomerase binding sites:** Avoid including the sequences C/TCCTT or AAGGG/A in the PCR primers. The presence of one of these sequences in the primer creates a topoisomerase I-binding site (CCCTT, or TCCTT) in the PCR product.
- **Primer phosphorylation**: Do not phosphorylate the 5´-ends of PCR primers. Topoisomerase I strictly requires a 5´-hydroxyl group as a substrate for the DNA strand-joining reaction.

C-Terminal Tagging Vectors (pCMV-SC-CF or pCMV-CM)

- **Optimized 5' terminal sequence:** The nucleotide composition of the 5´-end of the primers influences the cloning efficiency. Where possible, consider initiating PCR primers with the sequence 5´-GG. We have observed improved cloning efficiencies for PCR products containing the sequence 5´-GG.

- **Stop codons:** Design the insert to exclude any in-frame stop codons to allow expression of the C-terminal epitope tag. For optimal cloning efficiency, consider including alanine codon GCC or serine codon TCC as the C-terminal residue of the PCR insert.
- **Translation initiation codon and Kozak signal:** Design the insert to contain a Kozak sequence and a translation initiation codon (ATG). The sequence G**CCGCC**ATG is recommended and has been tested for cloning and expression efficiency.
- **Exclusion of topoisomerase binding sites:** Avoid including the sequences C/TCCTT or AAGGG/A in the PCR primers. The presence of one of these sequences in the primer creates a topoisomerase I-binding site (CCCTT, or TCCTT) in the PCR product.
- **Primer phosphorylation**: Do not phosphorylate the 5´-ends of PCR primers. Topoisomerase I strictly requires a 5´-hydroxyl group as a substrate for the DNA strand-joining reaction.

Tagless Vector

- **Optimized 5' terminal sequence:** The nucleotide composition of the 5´-end of the primers influences the cloning efficiency. We have observed improved cloning efficiencies for PCR products containing the sequence 5´-GG. Where possible, consider designing PCR primers to begin with the sequence 5´-GG.
- **Stop codons:** Design the PCR insert to include a stop codon. The sequence **TAG**GCC is recommended to provide an efficient translational stop and to support optimal cloning efficiency. While stop codons are present downstream of the PCR product insertion site (in all three frames), translation termination using the vector-derived stop codons results in the addition of at least 14 exogenous amino acids to the C terminus of the expressed protein.
- **Translation initiation codon and Kozak signal:** Design the insert to contain a Kozak sequence and a translation initiation codon (ATG). The sequence G**CCGCC**ATG is recommended and has been tested for cloning and expression efficiency.
- **Exclusion of topoisomerase binding sites:** Avoid including the sequences C/TCCTT or AAGGG/A in the PCR primers. The presence of one of these sequences in the primer creates a topoisomerase I-binding site (CCCTT, or TCCTT) in the PCR product.
- **Primer phosphorylation**: Do not phosphorylate the 5´-ends of PCR primers. Topoisomerase I strictly requires a 5´-hydroxyl group as a substrate for the DNA strand-joining reaction.

PCR Enzyme Selection

The StrataClone mammalian expression vector arms are designed for the cloning of blunt PCR products amplified by proofreading DNA polymerases. PCR enzyme recommendations for specific applications are listed in the table below. PCR products produced by most other proofreading PCR enzymes, including *Pfu* DNA polymerase, *PfuUltra* DNA polymerase, and *PfuTurbo* DNA polymerase, are also compatible with the vector arms.

Note *Do not use* Taq *DNA polymerase, or enzyme blends containing predominantly* Taq *DNA polymerase, to amplify fragments for cloning into the StrataClone mammalian expression vector arms.* Taq *DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-ends of PCR products. These single-stranded A-overhangs are not compatible with the vector arms. PCR products synthesized by proofreading DNA polymerases (such as* Pfu *DNA polymerase), do not contain 3´-A overhangs. If PCR was performed using* Taq *DNA polymerase, or blends that produce 3´-A overhangs, polish the PCR product ends using* Pfu *DNA polymerase prior to the cloning reaction.*

Proofreading Enzymes Recommended for Specific Applications

PCR Enzyme	Features/Applications	Stratagene Catalog #
PfuUltra® II HS DNA Polymerase	Highest-fidelity PCR with improved processivity for long targets	600670
Herculase® II Fusion DNA Polymerase	High yields and success with challenging targets	600675

Using Plasmid DNA as PCR Template

Genomic DNA, plasmid DNA, or cDNA may be used as template for PCR amplification prior to cloning. When the template is a plasmid that encodes the kanamycin resistance gene, it is important to avoid carryover of this plasmid into the transformation reaction. Either of two strategies may be used to remove the plasmid DNA after the PCR reaction is complete:

- Treat the PCR reaction products with restriction enzyme *Dpn* I, which cleaves the methylated plasmid DNA but does not cleave the unmethylated PCR product. To use this approach, add *Dpn* I enzyme to the completed PCR reaction, and incubate the mixture at 37°C for 1 hour. (No buffer modifications are required.) Heat-inactivate the *Dpn* I by incubating the mixture at 80°C for 20 minutes.

- Gel-purify the PCR product of interest. See *Appendix I* for a protocol.

Cloning Long PCR Products

Cloning efficiency varies significantly according to the size and sequence of the PCR product. When cloning long PCR products, it is especially important to analyze the PCR products on a gel prior to performing the ligation reaction. If gel analysis reveals inefficient production of the desired PCR product or reveals the presence of non-specific products, it is generally advantageous to gel-purify the PCR product of interest. This reduces the number of colonies containing inserts other than the desired PCR product. A gel-purification protocol is provided in *Appendix I*.

In addition to gel purification, the following minor protocol modifications can facilitate the recovery of clones containing long (>3 kb) PCR product inserts.

- When performing PCR, implement protocol modifications appropriate for long PCR products, including longer extension times. Consider using a PCR enzyme optimized for the production of long PCR products, such as *PfuUltra* II HS DNA polymerase.
- If gel purification is not performed, add 2 µl of the **undiluted** PCR reaction to the cloning reaction, in order to increase the molar ratio of insert: vector arms.
- Recovery of inefficiently-cloned long inserts may be facilitated by spreading the entire volume of the transformation mixture.

STRATACLONE MAMMALIAN EXPRESSION VECTOR PCR CLONING PROTOCOL

Preparing the PCR Product

1. Prepare insert DNA by PCR using a proofreading DNA polymerase.

   Note *Do not use* Taq *DNA polymerase, which adds 3´-adenine residues to the PCR product. If PCR was performed using* Taq *DNA polymerase, polish the insert ends prior to use in the cloning reaction.*

2. Analyze an aliquot of the PCR reaction on an agarose gel to verify production of the expected fragment.

3. If the fragment to be cloned is <3 kb and gel analysis confirms robust, specific amplification, prepare a 1:10 dilution of the PCR reaction in dH_20. For larger or poorly amplified fragments, omit the dilution step.

   Note *If multiple PCR products are observed on the gel, or when cloning very large PCR products, gel isolate the desired PCR product prior to performing the ligation reaction. See* Appendix I *for a gel-isolation protocol. For a gel-isolated PCR product recovered in 50 µl, add 2 µl (undiluted) of the purified PCR product to the ligation reaction below.*

Ligating the Insert

4. Prepare the ligation reaction mixture by combining (in order) the following components:

   3 µl StrataClone Blunt Cloning Buffer
   2 µl of PCR product (5–50 ng, typically a 1:10 dilution of a robust PCR reaction) or 2 µl of StrataClone Cam Control Insert
   1 µl StrataClone Vector Mix

5. Mix gently by repeated pipetting, and then incubate the ligation reaction at room temperature for 5 minutes. When the incubation is complete, place the reaction on ice.

   Note *The cloning reaction may be stored at –20°C for later processing.*

Transforming the Competent Cells

6. Thaw one tube of StrataClone SoloPack competent cells on ice for each ligation reaction.

   Note *It is critical to use the provided StrataClone SoloPack competent cells, expressing Cre recombinase, for this protocol. Do not substitute with another strain.*

7. Add 2 µl of the cloning reaction mixture to the tube of thawed competent cells. Mix gently (do not mix by repeated pipetting).

   Note *If desired, test the transformation efficiency of the competent cells by transforming a separate tube of competent cells with 10 pg of the pUC18 control DNA. Prior to use, dilute the pUC18 DNA provided 1:10 in dH_20, and then add 1 µl of the dilution to the tube of competent cells.*

8. Incubate the transformation mixture on ice for 20 minutes. During the incubation period, pre-warm LB medium[§] to 42°C.

9. Heat-shock the transformation mixture at 42°C for 45 seconds.

10. Incubate the transformation mixture on ice for 2 minutes.

11. Add 250 µl of pre-warmed LB medium to the transformation reaction mixture. Allow the competent cells to recover for at least 90 minutes at 37°C with agitation. (Lay the tube of cells on the shaker horizontally for better aeration.)

    Note *Increasing the recovery period to 2 hours may increase the number of transformants obtained.*

12. Plate 25 µl and 200 µl of the transformation mixture on LB-kanamycin plates.[§] Incubate the plates overnight at 37°C.

    Notes *For the Cam Control Insert cloning reaction, plate 100 µl of the transformation mixture on LB–kanamycin plates. The resulting colonies will be tested for chloramphenicol resistance in later steps.*

    For the pUC18 control transformation, plate 30 µl of the transformation mixture on LB–ampicillin plates. [§]

    When spreading <50 µl of transformation mixture, pipette the cells into a 50-µl pool of LB medium before spreading.

Analyzing the Expression Plasmid Candidate Transformants

13. Pick several colonies for plasmid DNA analysis and prepare miniprep DNA from the selected colonies using standard protocols.

[§]See *Preparation of Media and Reagents*.

14. Perform restriction digestion analysis of the miniprep DNA to identify colonies harboring the desired clone in the correct orientation. To screen for clones with a specific insert orientation, digest the miniprep DNA with a restriction enzyme with a single cleavage site in the insert DNA and a single or few sites in the vector DNA. See the table below for a partial list of restriction enzymes with a single cleavage site in each of the StrataClone mammalian expression vectors, listed in order of appearance on the circular vector map. See Figures 2–6 for vector-specific unique restriction site information.

Enzymes with Single Restriction Site in the StrataClone Mammalian Expression Vectors			
Nde I	*Bcl* I	*Stu* I	*Fsp* I
*Sna*B I	*Bbs* I	*Cla* I	*Pfl*F I
Sac I	*Mlu* I	*Bsa*B I	*Bst*B I
Xho I	*Dra* III	*Nar* I	*Bsa* I
Apa I	*Sfi* I	*Msc* I	*Apa*L I
Pvu I			

Note *Alternatively, positive clones may be identified by PCR analysis of plasmid DNA using a primer pair in which one primer corresponds to insert sequences and the other primer corresponds to vector sequences. Ensure that the two primers correspond to opposite strands of the DNA when the insert is cloned in the desired orientation.*

Expected Results

The number of colonies obtained and the cloning efficiency depend upon the size, amount, sequence, and purity of the PCR product used for ligation. For typical PCR products, the standard protocol produces hundreds of colonies for analysis. Cloning large or challenging inserts may benefit from some minor protocol alterations discussed in *Preprotocol Considerations* and *Troubleshooting*.

Analyzing Cam Control Insert Transformants to Determine Cloning Efficiency

After plating 100 µl of the Cam Control Insert transformation reaction on kanamycin-containing medium, >100 cfu are expected. Patch at least 20 of the kanamycin-resistant colonies onto LB-chloramphenicol plates, and incubate plates overnight at 37°C. Greater than 90% of the kanamycin-resistant colonies should demonstrate chloramphenicol resistance after patching.

Expected Results for the pUC18 Control Transformation

If transformation of the pUC18 control plasmid was performed, >50 colonies should be observed, indicating a transformation efficiency $>5 \times 10^7$ cfu/µg pUC18 DNA.

TROUBLESHOOTING

Observation	Suggestion
Low colony numbers (all insert sizes)	Verify that PCR amplification was performed using a proofreading DNA polymerase.
	Verify that the PCR primer design considerations outlined in *Preprotocol Considerations* were implemented.
	Verify that the PCR reaction produced a sufficient amount of the PCR product of interest by analyzing an aliquot on an agarose gel.
	Perform a control cloning reaction using the Control Insert provided to verify that all of the kit reagents are working properly.
	Titrate the amount of PCR product added to the cloning reaction. For most inserts <3 kb, using 2 µl of a 1:10 dilution of the PCR reaction will produce plenty of colonies. In some cases, however, adding a greater amount of insert will increase the number of colonies recovered. Conversely, adding an excess of the PCR reaction may inhibit the cloning reaction.
	Perform the transformation control reaction with pUC18 DNA to verify the expected transformation efficiency of the competent cells.
	Verify that the StrataClone SoloPack competent cells (provided with the kit) were used for transformation. Other competent cells lack the Cre recombinase required for production of a circular plasmid from the vector arms.
	During the transformation reaction, allow the cells to recover in liquid LB medium for up to 2 hours prior to plating the transformation mixture on LB-kanamycin plates. (Development of kanamycin resistance in transformants requires more time than development of ampicillin resistance.)
Low colony numbers (large inserts)	Gel-purify the PCR product prior to performing the cloning reaction (see *Appendix I*). Using crystal violet stain to visualize the PCR product may help preserve the integrity of long PCR products during isolation.
	Increase the amount of the transformation reaction plated (e.g. plate 100 µl and 200 µl of the transformation reaction mixture).
	Verify that PCR conditions, including extension time and PCR enzyme selection, are appropriate for long PCR products.
Low recovery of vectors containing the insert of interest	Analyze an aliquot of the PCR reaction on an agarose gel. If a single, discrete band is not observed, gel-purify the PCR product of interest (see *Appendix I*).
	Redesign primers and/or optimize the PCR reaction to maximize the specificity of the PCR amplification for the amplicon of interest. Verify the specific amplification of the product of interest on an agarose gel.
	Non-specific PCR products may be preferentially cloned. The cloning efficiency of PCR products varies greatly according to the size and sequence of the amplicon. For PCR products that are refractory to cloning, it may be necessary to gel-purify the PCR product of interest to remove minor contaminants that are preferentially ligated in the blunt PCR cloning reaction or that are better tolerated in *E. coli*.
Low ratio of insert-containing vectors to empty vectors	Primer sequence composition can affect cloning efficiency. Follow the guidelines in *PCR Primer Design* in the *Preprotocol Considerations* section.
	The insert may be toxic to *E. coli* or contain secondary structures that interfere with cloning.

Table continues on the following page

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Recovered plasmids do not have the expected restriction pattern	Kanamycin-resistant plasmids may be carried-over from the PCR reaction. If a plasmid containing the kanamycin resistance gene was used as the PCR template, remove the plasmid from the cloning reaction either by gel-purification of the insert of interest or by treating the final PCR product with restriction enzyme *Dpn* I.
	Cloning an insert that is toxic to *E. coli* can result in selection for plasmids with large deletions or other mutations that affect the restriction pattern.
Western analysis does not detect fusion protein	Insert is cloned out of frame. Sequence the MCS region of the plasmid to verify the reading frame. Reclone if insert is out of frame.
	Transfer of proteins is poor. Repeat transfer and optimize time of transfer, current and gel concentration and/or use molecular weight markers that cover the range to be transferred.
	Membrane preparation is inadequate. Ensure proper membrane hydration.
	Primary or secondary antibody concentration is too low. Titrate antibody conjugates for optimum concentrations.
	Protein has degraded during storage of the membrane. Use fresh blots.
	Poor isolation of tagged protein. Use a different cell lysis procedure.
	Proteolytic cleavage may have occurred. Include protease inhibitors in the lysis buffer.
Western analysis produces excessive background	Insufficient blocking solution may have been used or the membrane may not have been thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly.
	Too much protein was loaded on the gel. Load less protein.
	Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps when handling membranes.
	The concentration of the anti-FLAG, anti-c-myc, or secondary antibody is too high. Check the concentration of the antibodies and dilute if necessary.

PREPARATION OF MEDIA AND REAGENTS

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 2.5 ml of 20-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Chloramphenicol Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 3 ml of 10-mg/ml filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

APPENDIX I: GEL-ISOLATION OF PCR PRODUCTS

Special Considerations for Long PCR Products

When cloning long PCR products, it is generally advantageous to gel-purify the insert prior to performing the cloning reaction. Long PCR products have been successfully cloned after gel purification using conventional ethidium bromide staining. In some cases, however, using crystal violet stain to visualize the DNA may help preserve DNA integrity and increase the cloning efficiency.[5] When performing crystal violet staining, use the following modifications to the basic protocol below: Crystal violet should be added to the melted agarose, prepared in 1× TAE buffer, to a final concentration of 1.6 µg/ml. It is not necessary to add crystal violet to the running buffer. Prepare 6× loading buffer containing 30% glycerol, 20 mM EDTA, and 100 µg/ml crystal violet. (Do not use a gel loading buffer containing xylene cyanol or bromophenol blue.) During electrophoresis, the free crystal violet migrates toward the negative electrode, or "up" the gel. Continue electrophoresis until the crystal violet front is about 25% of the way up the gel, or until the DNA-bound crystal violet bands, appearing as thin purple lines, are sufficiently resolved. Crystal violet is less sensitive than ethidium bromide, with a detection limit of ~200 ng/band. If you do not see one or more purple bands migrating toward the positive electrode, insufficient DNA was loaded. It is possible to stain the crystal violet-containing gel with ethidium bromide to visualize less abundant DNA species.

Gel-Isolation Protocol

The following protocol uses the StrataPrep® DNA Gel Extraction Kit (Catalog #400766) for recovery of PCR products from a conventional 1% agarose gel (TAE or TBE). Other gel-isolation protocols may also be used.

1. After performing PCR, electrophorese the entire PCR reaction (typically 50 µl) on a 1% agarose gel (TAE or TBE buffer).

2. For conventional agarose gels (prepared without crystal violet), stain the gel with ethidium bromide and visualize the PCR products under UV-light. For crystal violet-containing gels, the PCR product(s) should appear as a thin purple band, visible under ambient light.

3. Excise the gel segment containing the fragment of interest and place the gel slice(s) in a 1.5-ml microcentrifuge tube. Estimate the total volume of the gel slice(s). (A gel slice with dimensions of 0.8 cm × 0.3 cm × 0.5 cm has a volume of ~0.12 cm^3, or 120 µl, and weighs ~120 mg.)

4. Add 300 µl of DNA extraction buffer for each 100 µl of gel volume or for each 100 mg weight. Heat the mixture at 50°C for at least 10 minutes with occasional mixing. Be sure that the gel is completely dissolved before continuing to the next step.

Note *For gels with an agarose concentration ≥2%, use 600 µl of DNA extraction buffer for each 100 µl of gel slice volume.*

5. Seat a microspin cup, provided with the StrataPrep DNA gel extraction kit, in a 2-ml receptacle tube. Transfer the gel extraction mixture to the spin cup, exercising caution to avoid damaging the fiber matrix.

6. Cap the spin cup, and then spin the tube in a microcentrifuge at maximum speed for 30 seconds.

   Note *The DNA is retained in the fiber matrix of the microspin cup. The binding capacity of the microspin cup is ~10 µg.*

7. Retain the microspin cup, and discard the liquid filtrate in the tube. Replace the microspin cup in the 2-ml receptacle tube

8. Prepare the 1× wash buffer, provided with the StrataPrep DNA gel extraction kit, by adding an equal volume of 100% ethanol to the container of 2× wash buffer. Store the 1× wash buffer at room temperature.

9. Add 750 µl of 1× wash buffer to the microspin cup.

10. Cap the spin cup, and then spin the tube in a microcentrifuge at maximum speed for 30 seconds.

11. Retain the microspin cup, and discard the wash buffer. Place the microspin cup back in the 2-ml receptacle tube.

12. Cap the spin cup, and then spin the tube in a microcentrifuge at maximum speed for 30 seconds. After spinning, verify that all of the wash buffer is removed from the microspin cup.

13. Transfer the microspin cup to a fresh 1.5-ml microcentrifuge tube and discard the 2-ml receptacle tube.

14. Add 50 µl of elution buffer or dH_2O directly onto the fiber matrix in the microspin cup.

15. Incubate the tube at room temperature for 5 minutes.

16. Cap the spin cup, and then spin the tube in a microcentrifuge at maximum speed for 30 seconds.

17. Retain the microcentrifuge tube, containing the purified DNA solution, and discard the microspin cup.

18. Proceed to step 4 of the *Blunt PCR Cloning Protocol*, and add 2 µl of the purified DNA, **undiluted**, to the cloning reaction mixture.

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

An Agilent Technologies Division

StrataClone Mammalian Expression Vector Systems

Catalog #240228, #240229, #240230, #240231, #240232

QUICK-REFERENCE PROTOCOL

- Prepare insert DNA by PCR using a proofreading DNA polymerase.
- Prepare the ligation reaction mixture by combining the following components. Add the components in the order given below and mix gently by repeated pipetting.

  3 µl StrataClone Blunt Cloning Buffer
  2 µl of PCR product (5–50 ng, typically a 1:10 dilution of a robust PCR reaction)
  1 µl StrataClone Vector Mix
- Incubate at room temperature for 5 minutes, then place the reaction on ice.
- Add 2 µl of the cloning reaction mixture to a tube of thawed StrataClone SoloPack Competent Cells. Mix gently (do **not** mix by repeated pipetting).
- Incubate the transformation mixture on ice for 20 minutes.
- Heat-shock the transformation mixture at 42°C for 45 seconds.
- Incubate the transformation mixture on ice for 2 minutes.
- Add 250 µl of LB medium (pre-warmed to 42°C). Allow the cells to recover at 37°C with agitation for 1.5–2 hours.
- Plate 25 µl and 200 µl of the transformation mixture on LB–kanamycin plates.
- Incubate the plates overnight at 37°C.
- Pick several colonies for plasmid DNA analysis, and prepare miniprep DNA from the selected colonies.
- Identify plasmids containing the PCR product insert in the correct orientation by restriction analysis using diagnostic restriction sites or by PCR analysis using a diagnostic primer pair.