StrataClone Ultra™ Blunt PCR Cloning Kit

INSTRUCTION MANUAL

Catalog #240218

Revision #046001

For In Vitro Use Only

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StrataClone Ultra™ Blunt PCR Cloning Kit

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StrataClone Ultra™ Blunt PCR Cloning Kit

MATERIALS PROVIDED

Materials Provided	Quantity[a]
PfuUltra™ II Fusion HS DNA Polymerase	40 PCR reactions
10× *PfuUltra*™ II Reaction Buffer	1 ml
StrataClone™ Blunt Vector Mix	21 cloning reactions (1 µl each)
StrataClone™ Blunt Cloning Buffer	63 µl
StrataClone™ Blunt 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] Catalog #240218 provides enough reagents for 40 × 50 µl PCR reactions and for 20 experimental cloning reactions plus one Control Insert cloning reaction.

STORAGE CONDITIONS

StrataClone™ SoloPack® Competent Cells and pUC18 Control Plasmid: –80°C
All Other Components: Prior to first use, store at –80°C. After thawing, store at –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

Thermocycler
LB–ampicillin agar plates[§]
SOC medium[§]
5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal)

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

Revision #046001

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Patents pending.

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INTRODUCTION

The StrataClone Ultra™ Blunt PCR Cloning Kit* couples highest accuracy PCR amplification with easy, robust topoisomerase-based PCR cloning. The kit includes *PfuUltra*™ II Fusion HS DNA Polymerase** which provides highest-fidelity PCR and excellent reliability while dramatically reducing overall PCR extension times. The blunt-end cloning vector mix uses our StrataClone DNA topoisomerase I technology with simple primer design, no PCR clean-up, and an easy three-step process.

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.[1] The Cre recombinase enzyme catalyzes recombination between two *loxP* recognition sequences.[2]

The StrataClone blunt PCR cloning vector mix contains two blunt-ended DNA arms, each charged with topoisomerase I on one end and containing 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^{amp}) 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 (pSC-B, see Figure 2) that is proficient for replication in cells growing on media containing ampicillin. The resulting pSC-B vector product includes a *lacZ´* α-complementation cassette for blue-white screening.

* Patents pending.

** U.S. Patent Nos. 5,545,552, 5,866,395, 5,948,663, 6,183,997, 6,444,428, 6,489,150, 6,734,293 and patents pending.

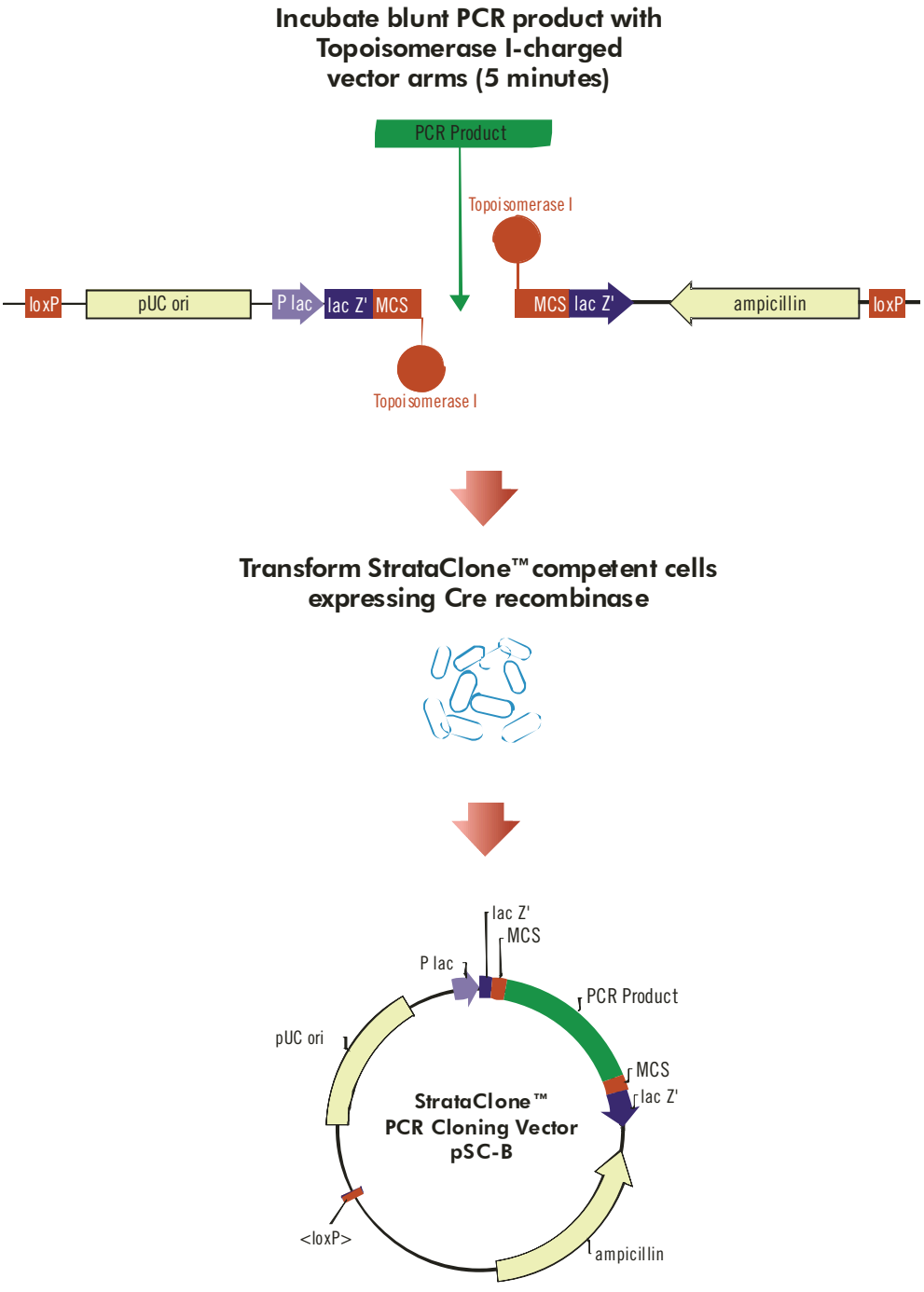


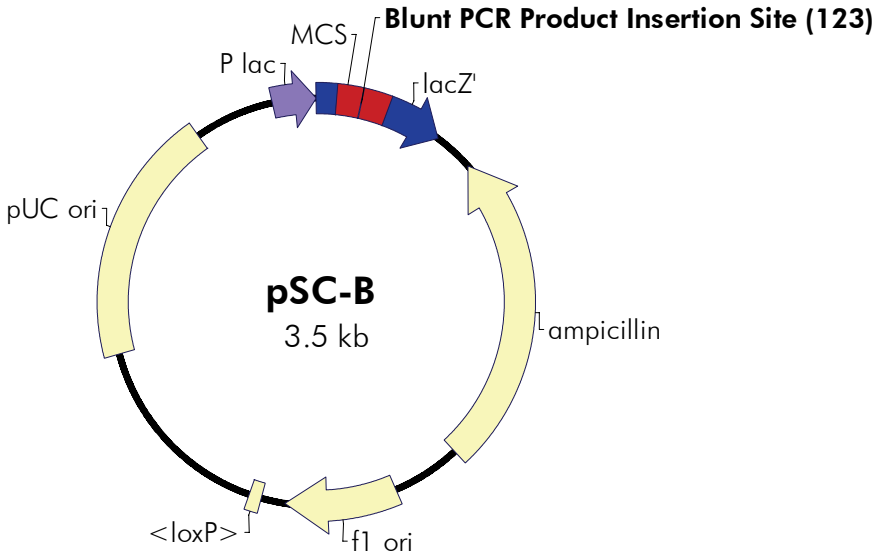
Figure 1 Overview of the StrataClone™ blunt PCR cloning method.

StrataClone™ SoloPack® Competent Cells

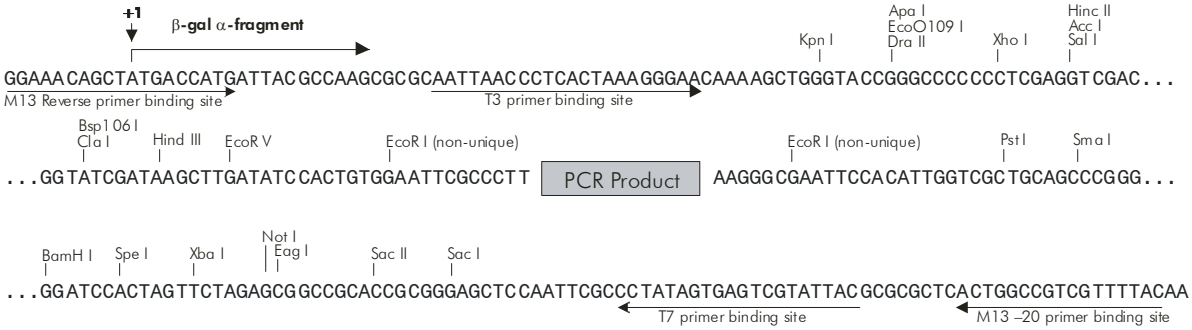
The provided StrataClone SoloPack® competent cells express Cre recombinase, in order to circularize the linear DNA molecules produced by topoisomerase I-mediated ligation. The cells are provided in a convenient single-tube transformation format. This host strain (containing the *lacZ*Δ*M15* mutation) supports blue-white screening with plasmid pSC-B, containing the *lacZ´* α-complementation cassette (see Figure 2). It is **not** necessary to induce *lacZ´* expression with IPTG when performing blue-white screening with this strain.

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.

Vector Map for the StrataClone™ Blunt PCR Cloning Vector pSC-B



pSC-B Blunt PCR Cloning Vector PCR Product Insertion Site Region (sequence shown 3460–3469, 1–252)



Feature	Nucleotide Position
β-galactosidase α-fragment coding sequence (lacZ′)	1–354
Multiple cloning site (MCS)	57–197
PCR product insertion site	123
ampicillin resistance (*bla*) ORF	465–1322
f1 origin of ss-DNA replication	1514–1820
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	1887–1920
pUC origin of replication	2461–3128
lac promoter	3350–3469

FIGURE 2 StrataClone™ blunt PCR cloning vector pSC-B. 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 the StrataClone Ultra blunt PCR cloning system. Cloning efficiency is optimized, however, by implementing the following primer design considerations:

- 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.
- 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. Stratagene has observed improved cloning efficiencies for PCR products containing the sequence 5´-GG… Avoid including a C residue at position +2 of the PCR primer. Stratagene has observed reduced cloning efficiencies for PCR products containing the sequence 5´-NC…
- 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 Reaction Optimization Parameters for *PfuUltra*™ II Fusion HS DNA Polymerase

A basic PCR protocol is provided in the *Protocols* section. This protocol may need to be optimized for your specific target. See *Table I*, below, for optimization guidelines.

TABLE I: OPTIMIZATION PARAMETERS (FOR 50-µL REACTION VOLUME)

Parameter	Vector or Genomic DNA Targets ≤10 kb	Vector or Genomic DNA Targets >10 kb	cDNA Targets
Extension time	15 seconds for targets ≤1 kb; 15 seconds per kb for targets >1 kb	30 seconds per kb	30 seconds for targets ≤1 kb; 30 seconds per kb for targets >1 kb
PfuUltra™ II fusion HS DNA polymerase	1 µl	1 µl	1 µl
Input template	100 ng genomic DNA; 5–30 ng vector DNA	200–250 ng genomic DNA; 5–30 ng vector DNA	1–2 µl c DNA from RT-PCR reaction (50–500 ng starting total RNA template)
Primers (each)	0.2 µM each primer	0.4 µM each primer	0.2 µM each primer
dNTP concentration	250 µM each dNTP (1 mM total)	500 µM each dNTP (2 mM total)	250 µM each dNTP (1 mM total)
Final reaction buffer concentration	1.0×	1.0×	1.0×
Denaturing temperature	95°C	92°C	95°C
Extension temperature	72°C	68°C	72°C

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 ampicillin resistance gene, it is important to avoid carry-over 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

PfuUltra™ II fusion HS DNA polymerase is optimized for the production of a wide range of target lengths, including long PCR products. The StrataClone blunt PCR cloning kit has been used to clone PCR products up to 9 kb in length.

The 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 white 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. See *Table I: Optimization Parameters* for more information.

- 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 transforming the maximum volume of cloning reaction (2 µl) and by spreading larger volumes of the transformation mixture.

PROTOCOLS

Preparing the PCR Product

1. Prepare the PCR reaction mixture by adding the components listed below *in order* into sterile thin-walled PCR tubes while mixing gently. The table provides an example reaction mixture for the amplification of a typical single-copy chromosomal target (≤10 kb). (See *Table I: Optimization Parameters* for advice on optimizing PCR for cDNA targets or longer targets.) The amounts listed are for one reaction and must be adjusted for multiple samples.

Reaction Mixture for a Typical Single-Copy Chromosomal Locus PCR Amplification (≤10 kb)

Component	Amount per reaction
Distilled water (dH_2O)	40.5 µl
10× *PfuUltra*™ II reaction buffer[a]	5.0 µl
dNTP mix (25 mM each dNTP)	0.5 µl
Template DNA (100 ng/µl)[b]	1.0 µl
Primer #1 (10 µM)	1.0 µl
Primer #2 (10 µM)	1.0 µl
PfuUltra™ II fusion HS DNA polymerase	1.0 µl
Total reaction volume	50 µl

[a] The 10× buffer provides a final 1× Mg^{2+} concentration of 2 mM.

[b] The amount of template DNA required varies depending on the type of DNA being amplified. Generally 100 ng of genomic DNA is recommended. Less template DNA (5–30 ng) should be used for amplification of lambda or plasmid PCR targets. Use 1–2 µl of cDNA prepared from an RT-PCR reaction containing 50–500 ng total RNA, and adjust the amount of dH_2O accordingly.

2. Perform PCR using optimized cycling conditions. Suggested cycling parameters for using single-block temperature cyclers (Table II) and Stratagene's RoboCycler® temperature cyclers (Table III) are indicated on the following page. The PCR cycling parameters for single block temperature cyclers have been tested on the following instruments: the MJ Research® DNA Engine® PTC-200, the Applied Biosystems® GeneAmp® PCR system 9700, the Applied Biosystems GeneAmp PCR system 9600, and Stratagene's Mx3000P® QPCR system. Optimized cycling parameters are not necessarily transferable between thermal cyclers.

TABLE II

PCR Cycling Programs for Single-Block Temperature Cyclers [a]

A. Targets <10 kb (Vector or Genomic DNA)

Segment	Number of cycles	Temperature	Duration
1	1	95°C[b]	2 minutes
2	30	95°C	20 seconds
		Primer T_m – 5°C[c]	20 seconds
		72°C	15 seconds for targets ≤1 kb 15 seconds per kb for targets >1 kb
3	1	72°C	3 minutes

B. Targets >10 kb (Vector or Genomic DNA)

Segment	Number of cycles	Temperature	Duration
1	1	92°C	2 minutes
2	30	92°C	10 seconds
		Primer T_m – 5°C[c]	20 seconds
		68°C	30 seconds per kb
3	1	68°C	5 minutes

C. cDNA Targets

Segment	Number of cycles	Temperature	Duration
1	1	95°C[b]	1 minute
2	40	95°C	20 seconds
		Primer T_m – 5°C[c]	20 seconds
		72°C	30 seconds for targets ≤1 kb 30 seconds per kb for targets >1 kb
3	1	72°C	3 minutes

[a] Thin-walled PCR tubes are highly recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance.

[b] Denaturing temperatures above 95°C are recommended only for GC-rich templates.

[c] The annealing temperature may require optimization. Typically annealing temperatures will range between 55° and 72°C.

TABLE III

PCR Cycling Program for Stratagene's RoboCycler® Temperature Cycler[a]

Segment	Number of cycles	Temperature	Duration
1	1	95°C	1 minute
2	30	95°C	30 seconds
		Primer T_m – 5°C[b]	30 seconds
		72°C[c]	40 seconds for targets ≤1 kb 40 seconds plus 20 seconds per kb for targets >1 kb[c]
3	1	72°C	5 minutes

[a] Thin-walled PCR tubes are highly recommended. These PCR tubes are optimized to ensure more efficient heat transfer and to maximize thermal-cycling performance.

[b] The annealing temperature may require optimization. Typical annealing temperatures will range between 55° and 72°C.

[c] For genomic and vector targets >10 kb, use an extension time of 30 seconds per kb and use an extension temperature of 68°C.

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

   Note *If the PCR template is a plasmid encoding the ampicillin resistance gene, the plasmid DNA must be eliminated prior to the cloning reaction by* Dpn I *digestion or by gel purification of the PCR product. See* Preprotocol Considerations *for more information.*

4. 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 PCR Product into the StrataClone™ Blunt Vector Arms

1. 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™ Control Insert
   1 µl StrataClone™ Blunt Vector Mix

2. 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

1. 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 of competent cells.*

2. Add 1 µl of the cloning reaction mixture to the tube of thawed StrataClone SoloPack competent cells. Mix gently (do not mix by repeated pipetting).

   Notes *For large PCR products, up to 2 µl of the cloning reaction mixture may be added to the transformation reaction.*

   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.

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

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

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

6. Add 250 µl of pre-warmed SOC medium to the transformation reaction mixture. Allow the competent cells to recover for 1 hour at 37°C with agitation. (Lay the tube of cells on the shaker horizontally for better aeration.)

7. During the outgrowth period, prepare LB–ampicillin plates[§] for blue-white color screening by spreading 40 µl of 2% X-gal[§] on each plate.

8. Plate 5 µl and 100 µl of the transformation mixture on the LB–ampicillin–X-gal plates. Incubate the plates overnight at 37°C.

   Notes *For the Control Insert cloning reaction, plate 25 µl of the transformation mixture.*

   For the pUC18 control transformation, plate 30 µl of the transformation mixture.

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

Analyzing the Transformants

1. Pick white or light blue colonies for plasmid DNA analysis. Do not pick dark blue colonies.

   Notes *Colonies harboring plasmids containing typical PCR product inserts are expected to be white. After prolonged incubation, some of the insert-containing colonies may appear light blue.*

   If the insert contains an in-frame start codon proximal to a ribosome binding site, a functional LacZ´ α-fragment fusion protein may be produced. This typically results in blue or light blue colonies for one insert orientation. If large numbers of blue colonies are obtained, analyze the DNA from a selection of these colonies for the presence of the insert.

2. Prepare miniprep DNA from the selected colonies using standard protocols. Perform restriction digestion analysis of the miniprep DNA to identify colonies harboring the desired clone. The PCR product insertion site is flanked by *Eco*R I sites for convenient identification of insert-containing plasmids. 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 one or a small number of sites in the vector DNA.

   Note *Alternatively, positive clones may be identified by PCR analysis of plasmid DNA using the T3/T7 primer pair, or using one primer corresponding to insert sequences and a second primer corresponding to vector MCS sequences.*

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

Expected Results for the Control Insert Transformation

After plating 25 µl of the Control Insert transformation reaction, >100 cfu are expected. Greater than 95% of these colonies should be white on agar plates containing X-gal. Plasmid DNA prepared from >95% of the white colonies should contain the 659-bp Control Insert DNA.

The presence of the Control Insert is easily verified by digestion of miniprep DNA with *Pvu* II restriction enzyme. DNA fragments expected from *Pvu* II-digestion of plasmids containing the Control Insert are 3 kb and 1.1 kb. Plasmids lacking insert DNA are expected to produce *Pvu* II fragments of 3 kb and 0.48 kb.

Note *Analysis for the presence of the Control Insert using* Eco*R I digestion is not recommended because the Control Insert contains an* Eco*R I restriction site.*

Expected Results for the Experimental Insert Transformation

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*.

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. Virtually all of these colonies will be blue on plates containing X-gal, since pUC18 contains the intact *lacZ´* gene cassette.

TROUBLESHOOTING

Observation	Suggestion
Gel analysis reveals low amounts of PCR product	The PCR reaction may need to be optimized for specific targets. See *Table I: Optimization Parameters* in the *Preprotocol Considerations* section for a list of optimization parameters and good starting points for various template types.
	Verify that the correct PCR cycling program was chosen, based on the size and nature of the template DNA and on the type of temperature cycler used.
	Use the recommended amount of DNA template. Excess template can reduce PCR product yield. Use intact and highly purified template.
	Lower the annealing temperature in 5°C increments.
	Use a high-quality dNTP mix to supply a final concentration of ≥250 µM each dNTP.
	Ensure that 10× *PfuUltra*™ II fusion HS DNA polymerase buffer is used.
	Remove extraneous salts from the PCR primers and DNA preparations.
	Use higher denaturing temperatures (98°C) for GC-rich targets ≤10 kb.
	Use the recommended primer concentrations between 0.2 and 0.4 µM.
	Use high-quality primers. Check the melting temperature, purity, GC content, and length of the primers.
	Consider using PCR adjuncts (e.g., 1–2 U of Perfect Match® PCR enhancer).
	Longer denaturation times may damage the DNA template; use the shortest denaturation time compatible with successful PCR on the thermal cycler.
	Use thin-walled PCR tubes. These PCR tubes permit more efficient heat transfer and maximize thermal-cycling performance.
Multiple bands produced from PCR reaction	Increase the annealing temperature in 5°C increments.
	Use Perfect Match® PCR enhancer to improve PCR product specificity.
Artifactual smears produced from PCR reaction	Decrease the amount of *PfuUltra*™ II fusion HS DNA polymerase.
	Reduce the extension time utilized.
Low colony numbers obtained from the cloning procedure (all insert sizes)	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 blunt PCR 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.
	Increase the amount of the cloning reaction mixture added to the transformation reaction to 2 µl.
	Increase the amount of the transformation reaction plated (e.g. plate 100 µl and 200 µl of the transformation reaction mixture).
	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 the pSC-B plasmid from the vector arms.

Table continues on the following page

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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 cloning reaction mixture added to the transformation reaction to 2 µl.
	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, are appropriate for long PCR products.
Greater than expected ratio of blue/white colonies for the experimental insert	If the insert contains an ATG start codon in-frame with the *lacZ´* gene, a functional LacZ´ fusion protein may be produced. This typically results in blue or light blue colonies for one insert orientation and white colonies for the other orientation. Analyze the DNA from some of the blue colonies for the presence of the insert.
	Non-specific PCR products may be preferentially cloned and those that produce an in-frame fusion with LacZ´ may convey a blue phenotype. Spread a greater quantity of the transformation reaction and then select the white colonies, or gel-purify the PCR product of interest prior to performing the cloning reaction (see *Appendix I*).
	The blue phenotype may be caused by transformation of a LacZ´-expressing plasmid carried-over from the PCR reaction. If a plasmid containing the ampicillin resistance gene was used as the PCR template, the template DNA must be removed before performing the cloning procedure. Following the guidelines in *Preprotocol Considerations*, either treat the final PCR product with restriction enzyme *Dpn* I or gel-purify the insert of interest.
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.
	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.
Plasmids recovered from white colonies do not have the expected restriction pattern for pSC-B	The recovered plasmid may have been carried-over from the PCR reaction. If a plasmid containing the amp resistance gene was used as the PCR template, the template DNA must be removed before performing the cloning procedure. Following the guidelines in *Preprotocol Considerations*, either treat the final PCR product with restriction enzyme *Dpn* I or gel-purify the insert of interest.
	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.

PREPARATION OF MEDIA AND REAGENTS

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–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)

2% X-Gal (per 10 ml)

0.2 g of 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-Gal)
10 ml of dimethylformamide (DMF)
Store at –20°C
Spread 40 µl per LB-agar plate

SOB Broth (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add the following filer-sterilized supplements prior to use:
10.0 ml of 1 M $MgCl_2$
10.0 ml of 1 M $MgSO_4$

SOC Broth (per 100 ml)

Note *This medium should be prepared immediately before use.*

2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose
SOB medium (autoclaved) to a final volume of 100 ml

APPENDIX I: GEL-ISOLATION OF PCR PRODUCTS

Special Considerations for Long PCR Products

The StrataClone blunt PCR cloning kit has been used to clone PCR products up to 9 kb in length. 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.[3] 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.

REFERENCES

1.Shuman, S. (1994) *J Biol Chem* 269(51):32678-84.
2.Abremski, K., Hoess, R. and Sternberg, N. (1983) *Cell* 32(4):1301-11.
3.Turgut-Balik, D., Celik, V. C., Moreton, K. and Brady, R. L. (2005) *Acta Biol Hung* 56(3-4):389-97.

ENDNOTES

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StrataClone Ultra™ Blunt PCR Cloning Kit

Catalog #240218

QUICK-REFERENCE PROTOCOL

- Prepare insert DNA by PCR using *PfuUltra*™ II Fusion HS DNA Polymerase. Add the components *in order* into sterile thin-walled PCR tubes while mixing gently.

Component	Amount per reaction
Distilled water (dH_2O)	40.5 µl
10× *PfuUltra*™ II reaction buffer	5.0 µl
dNTP mix (25 mM each dNTP)	0.5 µl
Template DNA (100 ng/µl genomic DNA or 5–30 ng/µl lambda or plasmid DNA)	1.0 µl
Primer #1 (10 µM)	1.0 µl
Primer #2 (10 µM)	1.0 µl
PfuUltra™ II fusion HS DNA polymerase	1.0 µl
Total reaction volume	50 µl

- Perform PCR using optimized cycling conditions. Suggested cycling parameters for typical targets using single-block temperature cyclers are shown below. See the *Protocols* section of the manual for PCR cycling parameters optimized for cDNA targets or longer targets.

Amplification of <10 kb Targets using a Single-Block Thermal Cycler

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	20 seconds
		Primer T_m – 5°C	20 seconds
		72°C	15 seconds for targets ≤1 kb 15 seconds per kb for targets >1 kb
3	1	72°C	3 minutes

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

- 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™ Blunt Vector Mix

- Incubate at room temperature for 5 minutes, then place the reaction on ice.

- Add 1 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 SOC medium (pre-warmed to 42°C). Allow the competent cells to recover for 1 hour at 37°C with agitation.
- Plate 5 µl and 100 µl of the transformation mixture on LB–ampicillin plates that have been spread with 40 µl of 2% X-gal.
- Incubate the plates overnight at 37°C.
- Pick white or light blue colonies for plasmid DNA analysis. Do not pick dark blue colonies.
- Prepare miniprep DNA from the selected colonies. Identify plasmids containing the PCR product insert and determine insert orientation by restriction analysis.