StrataClone PCR Cloning Kit

INSTRUCTION MANUAL

Catalog #240205

Revision C.0

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240205-12

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StrataClone PCR Cloning Kit

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StrataClone PCR Cloning Kit

MATERIALS PROVIDED

Materials Provided	Quantity[a]
StrataClone Vector Mix amp/kan	21 reactions (1 µl each)
StrataClone Cloning Buffer	63 µl
StrataClone 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.

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

Taq DNA polymerase or a polymerase blend recommended for PCR cloning
Thermocycler
LB–ampicillin or LB–kanamycin agar plates[§]
LB medium[§]
5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal)

NOTICE TO PURCHASER

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

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

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INTRODUCTION

The StrataClone PCR Cloning Kit§ allows high-efficiency, 5-minute cloning of PCR products, using the efficient DNA rejoining activity of DNA topoisomerase I and the DNA recombination activity of Cre recombinase.

Overview of StrataClone PCR Cloning Technology

Using the method summarized in Figure 1, StrataClone 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 PCR cloning vector mix contains two DNA arms, each charged with topoisomerase I on one end and containing a *loxP* recognition sequence on the other end. The topoisomerase-charged ends have a modified uridine (U*) overhang. *Taq*-amplified PCR products, which contain 3´-adenosine overhangs, are efficiently ligated to these vector arms in a 5-minute ligation reaction, through A-U* base-pairing followed by topoisomerase I-mediated strand ligation.

The resulting linear molecule (vector arm^{ori}–PCR product–vector arm$^{amp/kan}$) 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 (pSC-A-amp/kan, see Figure 2) that is proficient for replication in cells growing on media containing ampicillin or kanamycin. The resulting pSC-A-amp/kan vector product includes a *lacZ´* α-complementation cassette for blue-white screening.

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

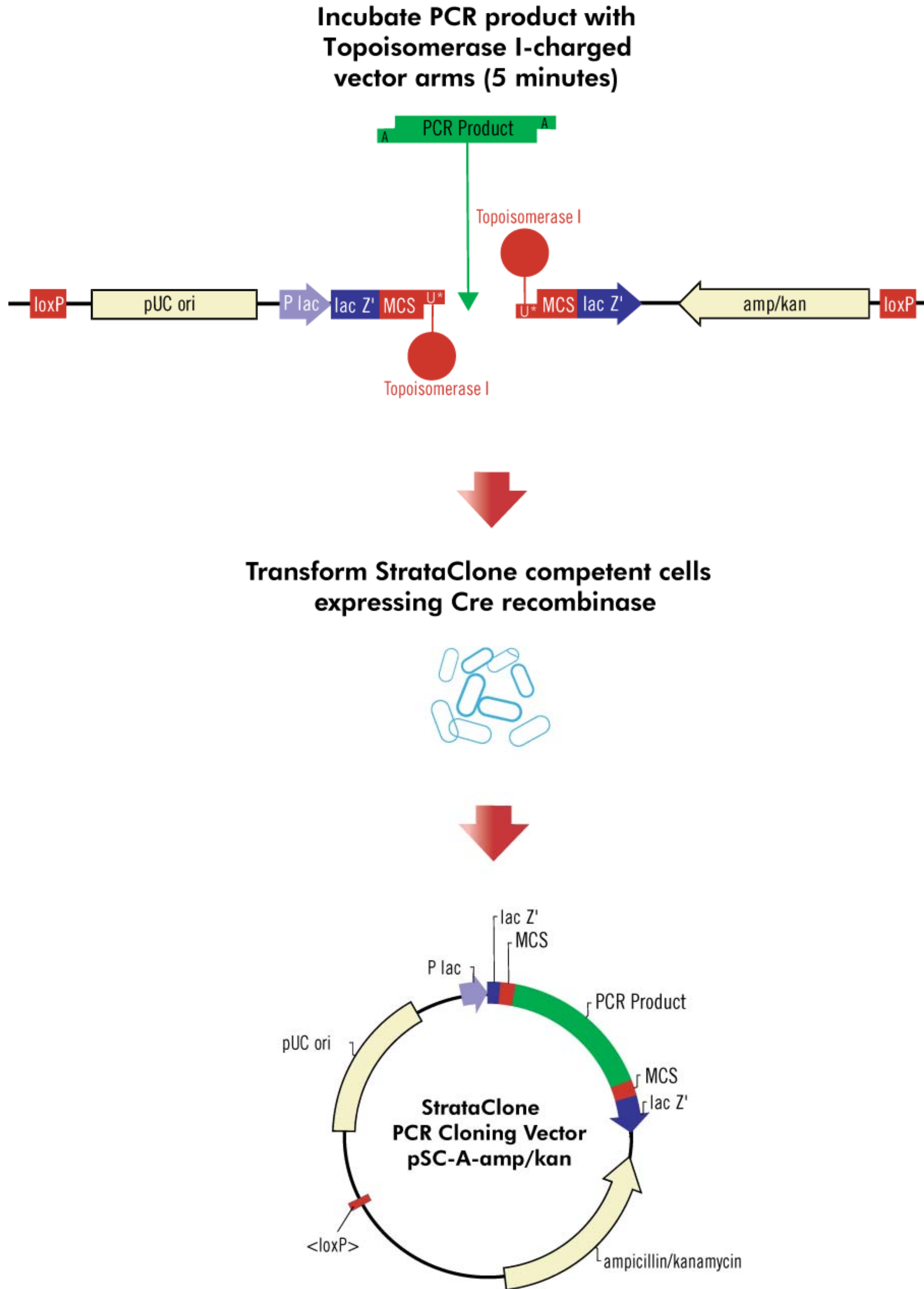


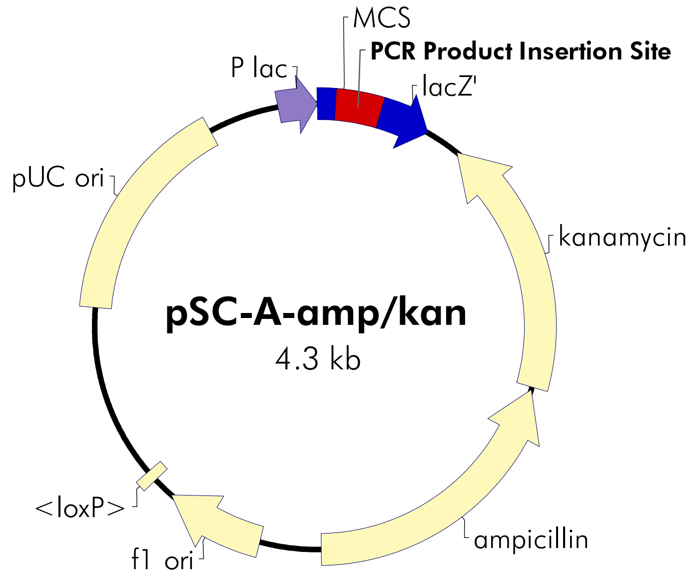
Figure 1 Overview of the StrataClone PCR cloning method.

StrataClone SoloPack Competent Cells

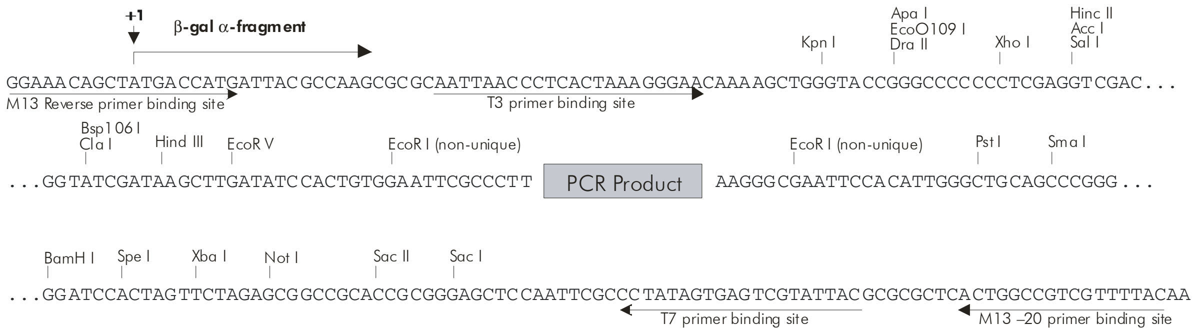
The provided StrataClone SoloPack competent cells express Cre recombinase, in order to circularize the linear DNA molecules produced by topoisomerase I-mediated ligation. The cells are provided in a convenient single-tube transformation format. This host strain (containing the *lacZΔM15* mutation) supports blue-white screening with plasmid pSC-A-amp/kan, 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.

Map for the StrataClone PCR Cloning Vector pSC-A-amp/kan



pSC-A-amp/kan PCR Cloning Vector PCR Product Insertion Site Region (sequence shown 4261–4270, 1–250)



Feature	Nucleotide Position
β-galactosidase α-fragment coding sequence (lacZ´)	1–352
Multiple cloning site (MCS)	57–195
PCR product insertion site	123
Kanamycin resistance ORF	463-1254
ampicillin resistance (*bla*) ORF	1266–2123
f1 origin of ss-DNA replication	2315–2621
<loxP> (mutant *loxP*-derived sequence *lox66/71*; nonfunctional in Cre-mediated recombination)	2688-2721
pUC origin of replication	3262–3929
lac promoter	4151-4270

Figure 2 StrataClone PCR cloning vector pSC-A-amp/kan. 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 Enzyme Selection

The StrataClone PCR cloning system is designed for the cloning of *Taq* DNA polymerase-amplified PCR products. *Taq* DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-end of both strands of the PCR product. These single-stranded A-overhangs are required for base-pairing with the 5´-modified U (U*) overhangs of the StrataClone vector arms. PCR products synthesized by proofreading DNA polymerases (such as *Pfu* DNA polymerase), do not contain 3´-A overhangs.

Several Stratagene PCR enzymes have been qualified for use with the StrataClone PCR cloning kit. These include two Stratagene high-fidelity PCR enzyme blends that feature both proofreading and terminal transferase (3´-A addition) activities. See Table 1 for a list of qualified PCR enzymes.

If the PCR product to be cloned is synthesized using a PCR enzyme that has not been qualified for PCR cloning applications (including most proofreading DNA polymerases), use the simple protocol supplied in *Appendix II* to add 3´-A overhangs after PCR amplification.

TABLE I

Enzymes Qualified for use with the StrataClone PCR Cloning Kit

PCR Enzyme	Features/Applications	Stratagene Catalog #
Easy-A High-Fidelity PCR Cloning Enzyme	Highest-fidelity PCR	600400
PicoMaxx High-Fidelity PCR Master Mix	High-fidelity PCR with improved sensitivity and yield	600650
Taq2000 DNA Polymerase	Routine PCR	600195

PCR Primer Design

No specific primers are required for the StrataClone 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.

- Avoid initiating a PCR primer with a 5´-T residue. When T is present at the 5´-end of the primer, the resulting PCR product contains a template-directed 3´-A residue. Non-template A-addition by *Taq* DNA polymerase is least efficient adjacent to a 3´-A residue and is most efficient adjacent to a 3´-C residue.[3]

- 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.

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, plate the transformation on kanamycin plates to eliminate carryover of the template plasmid. If plating the transformation on ampicillin plates is preferred, the resulting ampicillin-resistant colonies may be grown up in liquid overnight cultures containing kanamycin, to ensure that ampicillin resistance is not derived from a carryover template plasmid. Similarly, if the template plasmid confers kanamycin resistance, plate the transformation mixture on ampicillin plates.

Note *Development of kanamycin resistance in transformants requires more time than development of ampicillin resistance. When selecting transformants on kanamycin plates, increasing the duration of the outgrowth period in liquid LB prior to plating may increase the number of transformants obtained.*

Cloning Long PCR Products

The StrataClone PCR cloning kit has been used to clone PCR products up to 9.2 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.
- 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.

PCR Cloning Protocol

Note *StrataClone vector arms purchased prior to 2/1/2008 do not carry the kanamycin resistance marker. If planning to use kanamycin selection, ensure that the cloning reaction is performed using* StrataClone Vector Mix amp/kan *(as listed on the tube label).*

Preparing the PCR Product

1. Prepare insert DNA by PCR using *Taq* DNA polymerase or an enzyme blend qualified for PCR cloning applications.

   Note Taq *DNA polymerase is required for the addition of 3′-adenine residues to the PCR product. If PCR was performed using a proofreading DNA polymerase, see* Appendix II *for a protocol for adding 3′-A overhangs after the PCR reaction is complete.*

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 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 Vector Mix amp/kan

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 1 µl of the cloning reaction mixture to the tube of thawed 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 transformation efficiency of the competent cells by transforming a separate tube of competent cells with 10 pg of 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 1 hour at 37°C with agitation. (Lay the tube of cells on the shaker horizontally for better aeration.)

    Note *When selecting transformants on kanamycin plates, increasing the recovery period to 1.5–2 hours will increase the number of transformants obtained.*

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

13. Plate 5 µl and 100 µl of the transformation mixture on the color-screening plates. Incubate the plates overnight at 37°C.

    Notes *For the Control Insert cloning reaction, plate 10 µl of the transformation mixture on LB–ampicillin plates.*

    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.

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

Analyzing the Transformants

14. 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.

15. 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.*

Expected Results for the Control Insert Transformation

After plating 10 µl of the Control Insert transformation reaction, >100 cfu are expected. Greater than 97% of these colonies should be white on agar plates containing X-gal. Plasmid DNA prepared from >95% of the white colonies should contain the 664-bp Control Insert DNA.

Note *Colonies harboring plasmids containing the Control Insert are white after overnight incubation at 37°C. After prolonged incubation, ~50% of the insert-containing colonies appear light blue. In one orientation, the Control Insert contains an in-frame ATG and ribosome binding site, which allows the synthesis of a* LacZ′ *fusion protein that produces a light blue phenotype after prolonged incubation.*

The presence of the Control Insert is easily verified by digestion of miniprep DNA with *Eco*R I restriction enzyme. DNA fragments expected from *Eco*R I-digestion of plasmids containing the Control Insert are 4252 bp, 557 bp, and 125 bp. Plasmids lacking insert DNA are expected to produce a single detectable *Eco*R I fragment of 4.3 kb.

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
Low colony numbers (all insert sizes)	Verify that PCR amplification was performed using *Taq* DNA polymerase, or an enzyme blend qualified for use in PCR cloning applications, to allow efficient A-tailing of the PCR product. If a proofreading DNA polymerase was used, perform the A-tailing procedure outlined in *Appendix II*.
	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 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 a circular plasmid from the vector arms.
	When selecting transformants using kanamycin resistance, allow the cells to recover in liquid LB medium for up to 2 hours prior to plating the transformation mixture on LB-kanamycin plates. See *Preprotocol Considerations* for more information.
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 Control Insert	After prolonged incubation, 50% of Control Insert-transformed colonies are expected to be light blue. In one orientation, the Control Insert contains an in-frame ATG, which allows the synthesis of sufficient amounts of a LacZ′ fusion protein to produce light blue colonies after extended incubation times.

Table continues on the following page

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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.
	Inefficient A-tailing of the PCR product may shift the ratio of blue/white colonies by reducing the cloning efficiency of the PCR product. Verify that PCR amplification was performed using *Taq* DNA polymerase, or an enzyme blend qualified for use in PCR cloning applications. If a proofreading DNA polymerase was used, perform the A-tailing procedure outlined in *Appendix II*.
	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, plate the transformation on kanamycin-containing plates. Conversely, if a plasmid containing the kanamycin resistance gene was used as the PCR template, plate the transformation on ampicillin-containing plates. Alternatively, template plasmid may be removed from the cloning reaction by either gel-purification of the insert of interest or by treating the final PCR product with restriction enzyme *Dpn* I.
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 PCR cloning reaction or that are better tolerated in *E. coli*.
Low ratio of insert-containing vectors to empty vectors	Inefficient A-tailing of the PCR product reduces the cloning efficiency of the PCR product, thereby reducing the ratio of insert-containing plasmids recovered. Verify that PCR amplification was performed using *Taq* DNA polymerase, or an enzyme blend qualified for use in PCR cloning applications. If a proofreading DNA polymerase was used, perform the A-tailing procedure outlined in *Appendix II*.
	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-A-amp-kan	Ampicillin- or kanamycin-resistant plasmids may be carried-over from the PCR reaction. If a plasmid containing the ampicillin resistance gene was used as the PCR template, plate the transformation on kanamycin-containing plates. Conversely, if a plasmid containing the kanamycin resistance gene was used as the PCR template, plate the transformation on ampicillin-containing plates. Alternatively, template plasmid may be removed from the cloning reaction by either 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.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)	LB Broth (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)	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–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–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)	**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

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.[4] 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 *PCR Cloning Protocol*, and add 2 μl of the purified DNA, **undiluted**, to the cloning reaction mixture.

APPENDIX II: POST-PCR 3´-A ADDITION BY *TAQ* DNA POLYMERASE

Taq DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-end of both strands of DNA molecules. When PCR is performed using a proofreading DNA polymerase, such as Stratagene *Pfu* DNA polymerase, adenine residues may be added to the 3´-ends of the DNA molecule using the simple protocol provided below.

This protocol is based on a typical 50-µl PCR reaction volume. If using a different reaction volume, adjust the amount of *Taq* polymerase accordingly.

1. After performing PCR, place the 50-µl PCR reaction mixture on ice.

2. Add 0.7–1 U *Taq* DNA polymerase to the reaction mixture and mix well. (It is not necessary to change the buffer or supply additional dATP.)

3. Incubate the mixture at 72°C for 8–10 minutes. Do not perform thermal cycling.

4. Transfer the reaction mixture to ice. Proceed to the *PCR Cloning Protocol*.

Note *If the PCR product is gel-purified prior to A-addition, use the following protocol modifications: In step 2, bring the DNA solution to 1×* Taq *polymerase buffer conditions and 100 µM dATP, and then add 0.5 U Taq DNA polymerase. In step 3, incubate the reaction mixture at 72°C for 10–15 minutes.*

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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 PCR Cloning Kit

Catalog #240205

QUICK-REFERENCE PROTOCOL

- Prepare insert DNA by PCR using *Taq* DNA polymerase or an enzyme blend qualified for PCR cloning applications.
- 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 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 amp/kan

- 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 LB medium (pre-warmed to 42°C). Allow the cells to recover at 37°C with agitation for at least 1 hour (incubate for 1.5–2 hours before plating on kanamycin plates).
- Plate 5 μl and 100 μl of the transformation mixture on LB–ampicillin or LB-kanamycin 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.