



PCR-Script Cam Cloning Kit

Instruction Manual

Catalog # 211192 (25 reactions)

Revision B

Research Use Only. Not for Use in Diagnostic Procedures.

211192-12



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PCR-Script Cam Cloning Kit

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PCR-Script Cam Cloning Kit

MATERIALS PROVIDED

Materials provided	Concentration	Quantity ^a
Cloned <i>Pfu</i> DNA polymerase ^b (Stratagene Catalog #600153)	0.5 U/μl	25 μl
dNTP mix ^c	10 mM (2.5 mM each)	25 μl
Polishing buffer	10×	250 μl
pPCR-Script Cam SK(+) cloning vector (predigested)	10 ng/μl	250 ng
PCR-Script reaction buffer	10×	250 μl
rATP	10 mM	250 μl
Control PCR insert (994 bp) (ampicillin-resistance gene)	100 ng/μl	4 μl
<i>Srf</i> I restriction enzyme (Stratagene Catalog #501064)	5 U/μl	125 U
T4 DNA ligase (Stratagene Catalog #600011)	4 U/μl	100 U
XL10-Gold Kan ultracompetent cells ^{d,e} (Stratagene Catalog #200317)	—	5 × 0.2 ml
pUC18 control plasmid	0.1 ng/μl	10 μl
XL10-Gold β-mercaptoethanol mix (β-ME)	—	50 μl
StrataPrep PCR Purification Kit (Stratagene Catalog #400771)		
DNA-binding solution ^f	—	2.5 ml
PCR wash buffer (5×) ^f	5×	5 ml
Microspin cups ^g	—	25
Receptacle tubes (2 ml)	—	25

^a The PCR-Script Cam cloning kit contains enough reagents for 25 reactions.

^b **The cloned *Pfu* DNA polymerase provided with this kit is formulated specifically for polishing; successful PCR results will not be achieved using this formulation.**

^c Store long-term at –80°C or at –20°C for up to 3 months.

^d The XL10-Gold Kan ultracompetent cells must be stored at the bottom of a –80°C freezer immediately on receipt. The ultracompetent cells are very sensitive to small variations in temperature. Transferring tubes from one freezer to another may result in a loss of efficiency.

^e Genotype: Tet^R Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI*^qZΔ*M15* Tn10 (Tet^R) Tn5 (Kan^R) Amy].

^f Contains enough reagents for 25 PCR purifications.

^g The capacity of the microspin cup is ~0.8 ml.

STORAGE CONDITIONS

Ultracompetent Cells: Store immediately at –80°C

10 mM dNTP Mix: –20°C (up to 3 months) or –80°C (long-term storage)

StrataPrep PCR Purification Kit: Room Temperature

All Other Reagents: –20°C

ADDITIONAL MATERIALS REQUIRED

Equipment

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)

Water baths (37°C, 42°C, and 72°C)

Microcentrifuge

Microcentrifuge tubes

Chemicals

X-gal

IPTG

Ethanol (100%)

INTRODUCTION

The Stratagene PCR-Script Cam cloning kit is identical to the ampicillin-resistant PCR-Script Amp cloning kit in all aspects except for the vector provided with the kit contains the chloramphenicol resistance gene. This chloramphenicol-resistance feature of the cloning vector prevents the recovery of the parental vector when subcloning polymerase chain reaction (PCR) products derived from ampicillin-resistant plasmids.¹

As with the ampicillin-resistant PCR-Script Amp cloning kit, the PCR-Script Cam cloning kit is a PCR cloning method that can be performed in 1 hour without adding bases to the primers.² The kit permits the efficient cloning of PCR fragments with a high yield and a low rate of false positives. PCR products are incubated with the predigested pPCR-Script Cam SK(+) cloning vector, *Srf* I and T4 DNA ligase (see Figure 1). Using the restriction enzyme in the ligation reaction maintains a high-steady-state concentration of digested vector DNA and allows the use of nonphosphorylated, unmodified PCR primers. The ligation efficiency of blunt-ended DNA fragments is increased by the simultaneous, opposite reactions of the *Srf* I restriction enzyme and T4 DNA ligase on nonrecombinant vector DNA.³ *Srf* I is a novel rare-cleavage restriction enzyme that recognizes the oligonucleotide sequence 5'-GCCCIGGGC-3'.⁴

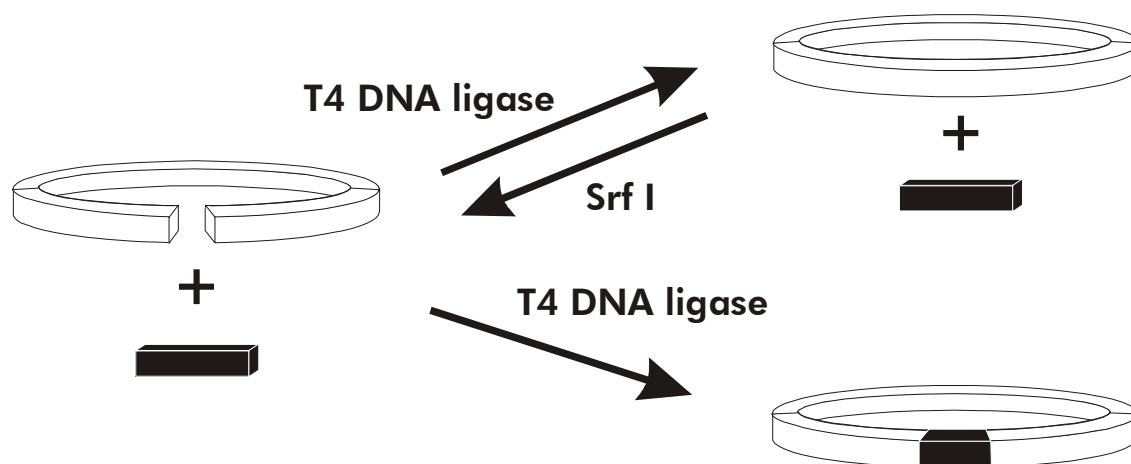


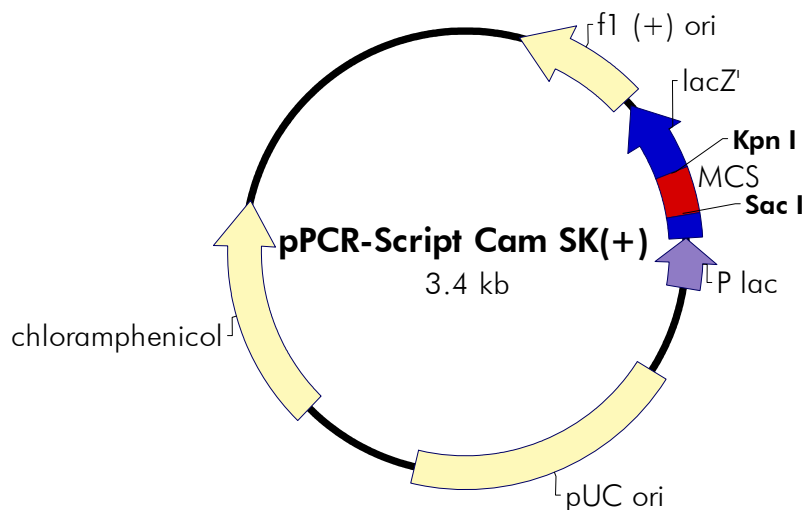
FIGURE 1 The PCR-Script Cam cloning method. An aliquot of a PCR product is added to 10 μ l of a ligation reaction containing PCR-Script 1 \times reaction buffer, 0.5 mM rATP and 10 ng of *Srf* I-digested pPCR-Script Cam SK(+) cloning vector. The enzymes *Srf* I and T4 DNA ligase are added. The reaction is allowed to proceed at room temperature for 1 hour before heat treating at 65°C for 10 minutes. A 2- μ l aliquot of the reaction is then used to transform 40 μ l of XL10-Gold Kan ultracompetent cells.

Description of the Vector

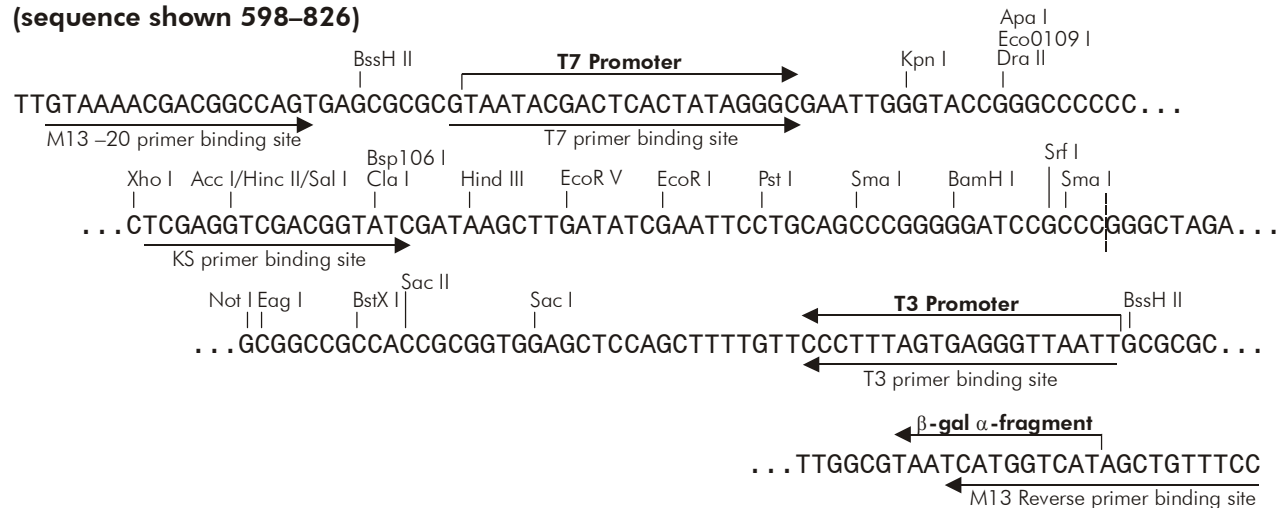
The pPCR-Script Cam SK(+) cloning vector is derived from the pBC SK(+) phagemid. This cloning vector includes a chloramphenicol-resistance gene, a *lac* promoter for gene expression, T3 and T7 RNA polymerase promoters for *in vitro* production of RNA, an f1 intergenic region for single-stranded DNA (ssDNA) rescue, the SK multiple cloning site (MCS), which is modified to include the *Srf* I restriction-endonuclease target sequence (see Figure 2), and five conveniently located sequencing primer sites. Additional information regarding the sequence and restriction sites of the pPCR-Script Cam SK(+) cloning vector is available at www.genomics.agilent.com.

The pPCR-Script Cam SK(+) cloning vector works ideally with blunt-ended PCR products. Use of *Pfu* DNA polymerase, a proofreading enzyme with 3' → 5' exonuclease activity, to generate the inserts ensures the production of high-fidelity, blunt-ended PCR products.⁵ When cloning *Pfu* DNA polymerase-generated PCR products purified with the StrataPrep PCR purification kit into the pPCR-Script Cam SK(+) cloning vector, the percentage of white colonies (i.e., recombinants) determined by blue-white color selection is ≥80%, with ≥90% of the white colonies being true positives (i.e., clones containing the desired insert). Use of *Taq* DNA polymerase or another low-fidelity DNA polymerase to generate the inserts enables efficient cloning into the pPCR-Script Cam SK(+) cloning vector after polishing the ends with the reagents provided in this kit. Regardless of the type of PCR enzyme used to generate the inserts, the pPCR-Script Cam SK(+) cloning vector allows rapid and efficient blunt-ended cloning of PCR products.

pPCR-Script Cam SK(+) Vector Map



pPCR-Script Cam SK(+) Multiple Cloning Site Region (sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication	135–441
β -galactosidase α -fragment coding sequence (<i>lacZ'</i>)	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
<i>lac</i> promoter	817–938
pUC origin of replication	1158–1825
chloramphenicol resistance (<i>bla</i>) ORF	2125–2781

FIGURE 2 Circular map and polylinker sequence of the pPCR-Script Cam SK(+) cloning vector. The pPCR-Script Cam SK(+) cloning vector provides the following features: blue-white color selection, fusion protein expression, T3 and T7 RNA promoters, high-resolution restriction mapping, exonuclease III/mung bean nuclease deletions, single-stranded rescue, and prokaryotic expression. The *Sma* I site within the *Srf* I recognition sequence will not be recreated after cloning. The vector provided has been digested with *Srf* I. The nucleotide sequence is available from www.genomics.agilent.com.

Genotype of XL10-Gold Ultracompetent Cells

XL10-Gold Kan ultracompetent cells exhibit increased transformation efficiencies with ligated DNA and large supercoiled DNA molecules.⁶ This is due to the presence of the Hte allele. XL10-Gold Kan cells are tetracycline- and kanamycin-resistant, endonuclease deficient (*endA1*), and recombination deficient (*recA*). The *hsdR* mutation prevents the cleavage of cloned DNA by the *EcoK* (*hsdR*) endonuclease system, and the *recA* mutation helps ensure insert stability. The *endA1* mutation greatly improves the quality of plasmid miniprep DNA. The *mcrA*, *mcrCB* and *mrr* mutations prevent cleavage of cloned DNA that carries cytosine and/or adenine methylation, which is often present in eukaryotic DNA and cDNA.⁷⁻¹¹ The McrA and McrCB systems recognize and restrict methylated cytosine DNA sequences. The Mrr system recognizes and restricts methylated adenine DNA sequences. The Mrr system also restricts methylated cytosine DNA sequences with a specificity differing from that of McrA and McrCB. This activity has been named McrF. This McrF activity against methylated cytosines has been shown to be equal to or greater than the restriction activity of the McrA and McrCB systems. All of these systems (McrA, McrCB, McrF, Mrr and HsdR) have been removed from XL10-Gold. XL10-Gold cells grow faster than XL1 or XL2-Blue cells, resulting in larger colonies.

Purifying PCR Products with the StrataPrep PCR Purification Kit

The StrataPrep PCR purification kit provides a rapid method to separate PCR products from PCR primers, unincorporated nucleotides, buffer components, and enzymes. The method employs a microspin cup that contains a silica-based fiber matrix. In the presence of a chaotropic salt, DNA binds to the fiber matrix.¹² Following PCR amplification, the PCR product is combined with a DNA-binding solution and transferred to a microspin cup that is seated inside a receptacle tube. The PCR product binds to the fiber matrix in the microspin cup. The contaminants are then washed from the microspin cup with a wash buffer. The purified PCR products are eluted from the fiber matrix with a low-ionic-strength buffer and captured in a microcentrifuge tube. Double-stranded DNA ≥ 100 bp is retained. This simple method of DNA purification eliminates tedious manipulation of resins, the toxic phenol–chloroform extraction, and the time-consuming ethanol precipitation used in other DNA purification methods. The result is a highly purified PCR product that is ready for restriction digestion, ligation, and sequencing reactions.

PCR AND CLONING CONSIDERATIONS

- ◆ For optimal fidelity and yield, use *Pfu* DNA polymerase to generate the PCR products. The proofreading exonucleolytic activity of *Pfu* DNA polymerase, which has the lowest error rate of any thermostable DNA polymerase,^{13, 14} contributes to a lower error rate compared to *Taq* DNA polymerase. Many species of DNA polymerases (e.g., *Taq* and Vent_R[®]) exhibit terminal deoxynucleotidyl-transferase (TdT) activity,¹⁵ and the 3'-end nucleotide extension of PCR products by DNA polymerases is both nucleotide- and polymerase-specific. *Pfu* DNA polymerase does not exhibit any terminal transferase activity and therefore creates blunt-ended DNA fragments,¹⁶ which facilitates cloning into the pPCR-Script Cam SK(+) cloning vector.
- ◆ Neither PCR primer may have the sequence 5'-GGGC-3' included at the 5' end. If necessary, add one base to the primer or delete one base.
- ◆ PCR primers can be nonphosphorylated or phosphorylated and used after ethanol precipitation.
- ◆ After thawing, spin down and gently mix the pPCR-Script Cam SK(+) cloning vector and the control PCR insert.
- ◆ Verify the integrity and quality of the PCR products by gel electrophoresis. If multiple PCR products are present, gel isolation of the appropriate fragment is recommended. Determine the quantity of the PCR product to be cloned to ensure that the proper insert-to-vector molar ratio is used.
- ◆ The PCR fragment to be cloned must be free of *Srf*I sites. If necessary, use 5-methyl-dCTP in the PCR amplification reaction.¹⁷
- ◆ Polish the ends of *Taq* DNA polymerase-generated PCR products with the reagents provided in this kit to create the blunt ends needed to improve the overall cloning efficiency.

Note *The cloned Pfu DNA polymerase provided with this kit is formulated specifically for polishing; successful PCR results will not be achieved using this formulation.*

CLONING PROTOCOL

Purifying the PCR Products with the StrataPrep PCR Purification Kit

1. Add a volume of DNA-binding solution equal to the volume of the aqueous portion of the PCR product to the microcentrifuge tube containing the PCR product and mix the two components.

Note *Mineral oil from the PCR reaction does not affect the purification process (Avoiding the mineral oil overlay, however, is recommended.). Do not include the volume of the mineral oil overlay when calculating the quantity of DNA-binding solution to add to the PCR product.*

2. Using a pipet, transfer the PCR product–DNA-binding-solution mixture to a microspin cup that is seated in a 2-ml receptacle tube. (Exercise caution to avoid damaging the fiber matrix with the pipet tip.) Snap the cap of the 2-ml receptacle tube onto the top of the microspin cup.
3. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

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

4. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the DNA-binding solution.
5. Prepare the 1× PCR wash buffer by adding 20 ml of 100% (v/v) ethanol to the container of the 5× PCR wash buffer. After adding the ethanol, mark the box on the label on the container—
[] 1× (Ethanol Added). Store the 1× PCR wash buffer at room temperature.
6. Open the cap of the 2-ml receptacle tube and add 750 µl of 1× PCR wash buffer to the microspin cup. Snap the cap of the receptacle tube onto the top of the microspin cup.
7. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.
8. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the wash buffer.
9. Place the microspin cup back in the 2-ml receptacle tube and snap the cap of the receptacle tube onto the microspin cup.
10. Spin the tube in a microcentrifuge at maximum speed for 30 seconds. After removing the tube from the centrifuge, make sure that all of the wash buffer is removed from the microspin cup.

11. Transfer the microspin cup to a fresh 1.5-ml microcentrifuge tube¹¹ and discard the 2-ml receptacle tube.
12. Add 50 µl of elution buffer directly onto the top of the fiber matrix at the bottom of the microspin cup.

Note *For eluting DNA from the microspin cup, use a low-ionic-strength buffer (≤ 10 mM in concentration, pH 7–9) or sterile deionized water. For most applications 10 mM Tris base (pH adjusted to 8.5 with HCl) is recommended; however, TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) may be used for applications in which EDTA will not interfere with subsequent reactions (see Preparation of Media and Reagents).*

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

Note *Maximum recovery of the PCR product from the microspin cup depends on the pH, the ionic strength, and the volume of the elution buffer added to the microspin cup; the placement of the elution buffer into the microspin cup; and the incubation time. Maximum recovery is obtained if the elution buffer is ≤ 10 mM in concentration with pH 7–9, not less than 50 µl of elution buffer is added directly onto the fiber matrix at the bottom of the microspin cup, and the tube is incubated for 5 minutes.*

14. Snap the cap of the 1.5-ml microcentrifuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.
15. Open the lid of the microcentrifuge tube and discard the microspin cup.

Notes *The purified PCR product is in the bottom of the 1.5-ml microcentrifuge tube. Snap the lid of the microcentrifuge tube closed to store the purified PCR product.*

The binding capacity of the microspin cup is ~ 10 µg of DNA.

¹¹ 1.5-ml flat snap cap microcentrifuge tubes from Continental Laboratory Products, Inc. are recommended.

Polishing the Purified PCR Products

Polish the ends of purified PCR products generated with either *Taq* DNA polymerase or other low-fidelity DNA polymerases as indicated in the following protocol.

Note *Pfu* DNA polymerase-generated PCR products do not require polishing. Proceed directly to Inserting the PCR Products into the pPCR-Script Cam SK(+) Cloning Vector.

1. To prepare the polishing reaction, add the following components *in order* to a 0.5-ml microcentrifuge tube:
 - 10 µl of the purified PCR product
 - 1 µl of 10 mM dNTP mix (2.5 mM each)
 - 1.3 µl of 10× polishing buffer
 - 1 µl of cloned *Pfu* DNA polymerase (0.5 U)
2. Mix the polishing reaction gently and add a 20-µl mineral oil overlay.
3. Incubate the polishing reaction for 30 minutes at 72°C in a water bath.
4. Add an aliquot of polished PCR product directly to the cloning reaction (see *Inserting the PCR Products into the pPCR-Script Cam SK(+) Cloning Vector*) or store the polished PCR products at 4°C until ready for further use.

Inserting the PCR Products into the pPCR-Script Cam SK(+) Cloning Vector

Calculating the Insert-to-Vector Molar Ratio

This kit requires an insert-to-vector molar ratio for ligation that is higher than the molar ratios used in many other cloning procedures. The ideal molar ratio of insert-to-vector DNA is variable. The control ligation in this kit is optimized to use an ideal test insert-to-vector ratio of 108:1. For the sample DNA, a range from 40:1 to 100:1 insert-to-vector ratio is recommended.

Use the following equation to optimize conditions for the insert:

$$X \text{ ng of PCR product} = \frac{(\text{number of bp of PCR product}) (10 \text{ ng of pPCR Script cloning vector})}{3399 \text{ bp of pPCR Script cloning vector}}$$

where *X* is the quantity of PCR product (in nanograms) required for a 1:1 insert-to-vector molar ratio. The following table provides examples of optimal insert-to-vector molar ratios calculated using the above equation:

Size of PCR product (bp)	Quantity of PCR product (ng), 40×–100×
250	29–74
500	59–147
750	88–221
1000	118–294
1500	177–441
2000	235–588
3000	353–883

Ligating the Insert

1. To prepare the ligation reaction, add the following components *in order* in a 0.5-ml microcentrifuge tube:
 - 1 µl of the pPCR-Script Cam SK(+) cloning vector (10 ng/µl)
 - 1 µl of PCR-Script 10× reaction buffer
 - 0.5 µl of 10 mM rATP
 - 2–4 µl of the blunt-ended PCR product or 4 µl of the PCR test insert
 - 1 µl of *Srf*I restriction enzyme (5 U/µl)
 - 1 µl of T4 DNA ligase (4U/µl)
 - Distilled water (dH₂O) to a final volume of 10 µl
2. Mix the ligation reaction gently and incubate this reaction for 1 hour at room temperature.
3. Heat the ligation reaction for 10 minutes at 65°C.
4. Store the ligation reaction on ice until ready to use for transformation into the XL10-Gold Kan ultracompetent cells.

TRANSFORMATION GUIDELINES

Storage Conditions

Ultracompetent cells are sensitive to even small 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. Ultracompetent cells should be placed at -80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the chilled tubes. It is also important to use at least 40 μl of ultracompetent cells/transformation.

Use of the 14-ml BD Falcon Polypropylene Tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by the β -mercaptoethanol used in step 3 of the *Transformation Protocol*. In addition, the duration of the heat-pulse step is critical and has been optimized specifically for the thickness and shape of the 14-ml BD Falcon tubes.

Use of β -Mercaptoethanol

β -Mercaptoethanol (β -ME) has been shown to increase transformation efficiency. The XL10-Gold β -mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 1.6 μl of the β -ME mix. (Stratagene cannot guarantee highest efficiencies with β -ME from other sources.)

Length and Temperature of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat pulsed for 30 seconds. Heat pulsing for at least 30 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease when the duration of the heat pulse is <30 seconds or >40 seconds. Do not exceed 42°C .

Preparing the Agar Plates for Color Screening

To prepare the LB agar plates for blue–white color screening, add 80 $\mu\text{g}/\text{ml}$ of X-gal, 20 mM IPTG, and the appropriate antibiotic to the LB agar. Alternatively, 100 μl of 10 mM IPTG and 100 μl of 2% X-gal can be spread on the LB-chloramphenicol agar plates 30 minutes prior to plating the transformations. Prepare the X-gal in dimethylformamide (DMF). Prepare the IPTG in sterile dH_2O .

Some β -galactosidase fusion proteins are toxic to the host bacteria. If an insert is suspected to be toxic to the bacteria, do not plate the transformations with X-gal and IPTG. Color screening will be eliminated, but the recombinants will express lower levels of potentially toxic proteins.

TRANSFORMATION PROTOCOL

1. Thaw the XL10-Gold Kan ultracompetent cells on ice.
2. Gently mix the cells by hand. Aliquot 40 μ l of the cells into a chilled 14-ml BD Falcon polypropylene round-bottom tube for each of the following reactions: the experimental ligation reaction, the ligation reaction containing the PCR test insert, and the pUC18 control plasmid.
3. Add 1.6 μ l of the XL10-Gold β -mercaptoethanol mix provided with the kit to the 40 μ l of competent cells. (Stratagene cannot guarantee highest efficiencies with β -ME from other sources.)
4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 2 μ l of the cloning reaction from step 4 of *Ligating the Insert* to the transformation reaction and swirl the reaction gently.

Note *As controls, add 1 μ l of pUC18 plasmid (diluted 1:10 in high-quality water) and 2 μ l of the control ligation reaction containing the PCR test insert to separate 40- μ l aliquots of the cells and swirl gently.*

6. Incubate the tubes on ice for 30 minutes.
7. Preheat NZY⁺ broth in a 42°C water bath for use in step 10.
8. Heat pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is *critical* for obtaining the highest efficiencies. Do not exceed 42°C.
9. Incubate the tubes on ice for 2 minutes.
10. Add 0.45 ml of preheated (42°C) NZY⁺ broth to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.
11. If color screening is desired and if the agar plates were not prepared with X-gal and IPTG, spread 100 μ l of 2% X-gal and 100 μ l of 10 mM IPTG on the LB-chloramphenicol agar plates 30 minutes prior to plating the transformations (see *Preparing the Agar Plates for Color Screening*).

Note *For consistent color development across the plate, pipet the X-gal and the IPTG into a 100- μ l pool of NZY⁺ broth and then spread the mixture across the plate. Do not mix the IPTG and the X-gal before pipetting them into the pool of NZY⁺ broth because these chemicals may precipitate.*

12. Plate the experimental transformation reaction, the transformation reaction containing the PCR test insert, and the transformation reaction containing the pUC18 control plasmid:

- a. Use a sterile spreader to plate 200 μ l (or less) of the experimental transformation reaction onto LB-chloramphenicol agar plates.

Note *The cells may be concentrated by centrifuging at 1000 rpm for 10 minutes if desired. Resuspend the pellet in 200 μ l of NZY⁺ broth prior to plating.*

- b. Pipet 10 μ l of the transformation reaction containing the PCR test insert into a 100- μ l pool of media on an LB-chloramphenicol agar plate. Spread the cells evenly across the plate using a sterile spreader. Plate 10 μ l of the transformation reaction containing the PCR test insert onto an LB-ampicillin agar plate.

Note *The control PCR test insert is a Pfu DNA polymerase-generated PCR product that contains an ampicillin-resistance gene. This control transformation reaction should be plated on LB-chloramphenicol agar plates and on LB-ampicillin agar plates to verify that the transformed colonies are also ampicillin-resistant.*

- c. Pipet 5 μ l of the transformation reaction containing the pUC18 control plasmid into a 100- μ l pool of NZY⁺ broth on an LB-ampicillin agar plate. Spread evenly using a sterile spreader.

13. Incubate the plates overnight at 37°C. For blue-white color screening, incubate the plates for 17 hours. Colonies containing plasmids without inserts will be blue after the 17-hour incubation. Colonies containing plasmids with inserts will remain white. The blue color can be enhanced by incubating the plates for two hours at 4°C following the overnight incubation at 37°C.

14. Choose white colonies for examination (**avoid colonies with a light blue appearance or colonies with a blue center**). If necessary, patch white colonies onto a new LB-ampicillin agar plate containing X-gal and IPTG to verify the Lac⁻ phenotype.

Note *Colonies containing inserts which were initially pure white may turn a light blue after ~2–5 days on the plate.*

Transformation Summary and Expected Results

Control transformation	Quantity of DNA added to the transformation	Plating quantity	Expected results	
			LB-ampicillin agar plates	LB-chloramphenicol agar plates
pUC18 control plasmid	1 μ l of a 1:10 dilution	5 μ l	100 colonies ($\geq 1 \times 10^9$ cfu/ μ g DNA)	—
Control PCR test insert	2 μ l	10 μ l	>100 colonies ^a	>100 colonies

^a Growth of colonies on LB-ampicillin plates indicates successful insertion of the PCR test insert, which contains the ampicillin-resistance gene.

TROUBLESHOOTING

Observation	Suggestion
Low colony yield	Low transformation efficiency; plate a larger volume of the transformation reaction
	Low PCR yield; visually check the PCR fragment on a 1% (w/v) agarose gel
Wrong insert size	Multiple PCR products; gel isolate the correct band or optimize the PCR conditions
Bacterial lawn on selected agar plates	Plate a lower volume of the transformation reaction
	Prepare new LB-chloramphenicol agar plates
Low ligation efficiency	Purify the PCR product with the provided StrataPrep PCR purification kit
	To ensure the PCR products are blunt-ended, generate the PCR product with <i>Pfu</i> DNA polymerase or polish the PCR product with the reagents provided in this kit
White colonies with no insert	Increase the insert-to-vector ratio
Low recovery of the PCR product	Verify that the PCR product is synthesized by running a portion of the unpurified PCR product on an agarose gel
	Ensure that the 1 × PCR wash buffer was prepared correctly: four volumes of 100% (v/v) ethanol must be added to the 5 × PCR wash buffer provided to prepare the 1 × PCR wash buffer
	Use a low-ionic-strength (≤ 10 mM) buffer, pH 7–9
	Add ≥ 50 μ l of elution buffer to the microspin cup
	Add the elution buffer directly onto the fiber matrix of the microspin cup to ensure complete coverage of the membrane
	Incubate the tube for 5 minutes after adding the elution buffer
DNA floats out of the wells of agarose gel	To ensure complete ethanol removal, remove all wash buffer from the microspin cup before adding elution buffer

PREPARATION OF MEDIA AND REAGENTS

<p>LB Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Add deionized H₂O 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)</p>	<p>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)</p>
<p>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)</p>	<p>NZY⁺ Broth (per Liter) 10 g of NZ amine (casein hydrolysate) 5 g of yeast extract 5 g of NaCl Add deionized H₂O to a final volume of 1 liter Adjust to pH 7.5 using NaOH Autoclave Add the following filter-sterilized supplements prior to use: 12.5 ml of 1 M MgCl₂ 12.5 ml of 1 M MgSO₄ 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)</p>
<p>TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA</p>	<p>Elution Buffer 10 mM Tris base Adjust pH to 8.5 with HCl <i>or</i> 10 mM Tris base 1 mM EDTA Adjust pH to 8.0 with HCl <i>or</i> Sterile ddH₂O</p>
<p>2× Wash Buffer 10 mM Tris HCl (pH7.5) 100 mM NaCl 2.5 mM EDTA</p>	

REFERENCES

1. Costa, G. L., Sanchez, T. and Weiner, M. P. (1994) *Strategies* 7(2):52.
2. Bauer, J. C., Deely, D., Braman, J., Viola, J. and Weiner, M. P. (1992) *Strategies* 5(3):62-64.
3. Liu, Z. G. and Schwartz, L. M. (1992) *Biotechniques* 12(1):28, 30.
4. Simcox, T. G., Marsh, S. J., Gross, E. A., Lernhardt, W., Davis, S. *et al.* (1991) *Gene* 109(1):121-3.
5. Scott, B., Nielson, K., Cline, J. and Kretz, K. (1994) *Strategies* 7(3):62-63.
6. Jerpseth, B., Callahan, M. and Greener, A. (1997) *Strategies* 10(2):37-38.
7. Kelleher, J. E. and Raleigh, E. A. (1991) *J Bacteriol* 173(16):5220-3.
8. Kohler, S. W., Provost, G. S., Kretz, P. L., Dyaico, M. J., Sorge, J. A. *et al.* (1990) *Nucleic Acids Res* 18(10):3007-13.
9. Kretz, P. L., Kohler, S. W. and Short, J. M. (1991) *J Bacteriol* 173(15):4707-16.
10. Raleigh, E. A., Murray, N. E., Revel, H., Blumenthal, R. M., Westaway, D. *et al.* (1988) *Nucleic Acids Res* 16(4):1563-75.
11. Raleigh, E. A. and Wilson, G. (1986) *Proc Natl Acad Sci U S A* 83(23):9070-4.
12. Vogelstein, B. and Gillespie, D. (1979) *Proc Natl Acad Sci U S A* 76(2):615-9.
13. Iaman, J. M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C. *et al.* (1994) *Nucleic Acids Res* 22(15):3259-60.
14. Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A. *et al.* (1991) *Gene* 108(1):1-6.
15. Hu, G. (1993) *DNA Cell Biol* 12(8):763-770.
16. Costa, G. L. and Weiner, M. P. (1994) *Nucleic Acids Res* 22(12):2423.
17. Wong, K. K. and McClelland, M. (1991) *Nucleic Acids Res* 19(5):1081-5.

ENDNOTES

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MSDS INFORMATION

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STRATAGENE

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PCR-Script Cam Cloning Kit

Catalog #211192 (25 reactions)

QUICK-REFERENCE PROTOCOLS

PCR Products Generated with *Pfu* DNA Polymerase

- ♦ Purify the PCR product with the StrataPrep PCR Purification Kit
- ♦ Perform the ligation reaction
 - ♦ Mix 1 μ l of pPCR-Script Cam SK(+) cloning vector (10 ng/ μ l), 1 μ l of PCR-Script 10 \times reaction buffer, 0.5 μ l of 10 mM rATP, 2–4 μ l of the blunt-ended PCR product (40:1 to 100:1 insert-to-vector ratio), 1 μ l of *Srf* I restriction enzyme (5 U/ μ l), 1 μ l of T4 DNA ligase, and dH₂O to a final volume of 10 μ l
- ♦ Mix gently, incubate 1 hour at room temperature, and heat 10 minutes at 65°C
- ♦ Transform into XL10-Gold Kan ultracompetent cells

PCR Products Generated with *Taq* DNA Polymerase or Other Low-Fidelity Polymerases

- ♦ Purify the PCR product with the StrataPrep PCR Purification Kit
- ♦ Polish the ends of the purified PCR product
 - ♦ Add 10 μ l of purified PCR product, 1 μ l of 10 mM dNTP mix (2.5 mM each), 1.3 μ l of 10 \times polishing buffer, and 1 μ l of cloned *Pfu* DNA polymerase (0.5 U)
 - ♦ Gently mix the reaction and add a 20- μ l mineral oil overlay
 - ♦ Incubate the polishing reaction for 30 minutes at 72°C in a water bath
- ♦ Perform the ligation reaction
 - ♦ Mix 1 μ l of pPCR-Script Cam SK(+) cloning vector (10 ng/ μ l), 1 μ l of PCR-Script 10 \times reaction buffer, 0.5 μ l of 10 mM rATP, 2–4 μ l of the blunt-ended PCR product (40:1 to 100:1 insert-to-vector ratio), 1 μ l of *Srf* I restriction enzyme (5 U/ μ l), 1 μ l of T4 DNA ligase, and dH₂O to a final volume of 10 μ l
 - ♦ Mix gently, incubate 1 hour at room temperature, and heat 10 minutes at 65°C
- ♦ Transform into XL10-Gold Kan ultracompetent cells