PCR-Script Amp Cloning Kit

INSTRUCTION MANUAL

Catalog #211188 (10 reactions), #211190 (25 reactions), and #211189 (50 reactions)
Revision A

For In Vitro Use Only
211190-12

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Agilent Technologies
Stratagene Products Division
11011 North Torrey Pines Road
La Jolla, CA 92037
Telephone (858) 373-6300
Order Toll Free (800) 424-5444
Technical Services (800) 894-1304
Internet techservices@agilent.com
World Wide Web www.stratagene.com

Europe

Location	Telephone	Fax	Technical Services
Austria	0800 292 499	0800 292 496	0800 292 498
Belgium	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 15775	0800 15740	0800 15720
France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

All Other Countries

Please contact your local distributor. A complete list of distributors is available at www.stratagene.com.

PCR-Script Amp Cloning Kit

CONTENTS

PCR-Script Amp Cloning Kit

MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]		
		#211188	#211190	#211189
Cloned *Pfu* DNA polymerase[b,c]	0.5 U/µl	10 µl	25 µl	2× 25 µl
dNTP mix[d]	10 mM (2.5 mM each)	10 µl	25 µl	2× 25 µl
Polishing buffer	10×	250 µl	250 µl	2× 250 µl
pPCR-Script Amp SK(+) cloning vector (predigested)	10 ng/µl	100 ng	250 ng	2× 250 ng
PCR-Script reaction buffer	10×	250 µl	250 µl	2× 250 µl
rATP	10 mM	250 µl	250 µl	2× 250 µl
Control PCR insert (1.1 kb) (chloramphenicol-resistance gene)	100 ng/µl	4 µl	4 µl	4 µl
Srf I restriction enzyme[b]	5 U/µl	50 U	125 U	2× 125 U
T4 DNA ligase[b]	4 U/µl	40 U	100 U	2× 100 U
XL10-Gold Kan ultracompetent cells[b,e,f]	—	2 × 0.2 ml	5 × 0.2 ml	10 × 0.2 ml
pUC18 control plasmid	0.1 ng/µl	10 µl	10 µl	2× 10 µl
XL10-Gold β-mercaptoethanol mix (β-ME)	—	50 µl	50 µl	2× 50 µl
StrataPrep PCR Purification Kit[b]				
DNA-binding solution[g]	—	2.5 ml	2.5 ml	5 ml
PCR wash buffer (5×)[g]	5×	5 ml	5 ml	10 ml
Microspin cups[h]	—	10	25	50
Receptacle tubes (2 ml)	—	10	25	50

[a] Catalog #211188 contains reagents sufficient for 10 reactions. Catalog #211190 contains reagents sufficient for 25 reactions. Catalog #211189 contains reagents sufficient for 50 reactions.

[b] Cloned Pfu DNA polymerase (2.5 U/µl) is also available separately (Stratagene catalog #600153).

[c] **The cloned *Pfu* DNA polymerase provided with these kits is formulated specifically for polishing; successful PCR results will not be achieved using this formulation**.

[d] Store long-term at –80°C or at –20°C for up to 3 months.

[e] 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.

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

[g] Contains enough reagents for 25 PCR purifications.

[h] 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

Revision A

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

INTRODUCTION

The Stratagene PCR-Script Amp cloning kit is a polymerase chain reaction (PCR) cloning method that can be performed in 1 hour without adding bases to the primers.[1] 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 Amp 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.[2] *Srf* I is a novel rare-cleavage restriction enzyme that recognizes the oligonucleotide sequence 5´-GCCC|GGGC-3´.[3]

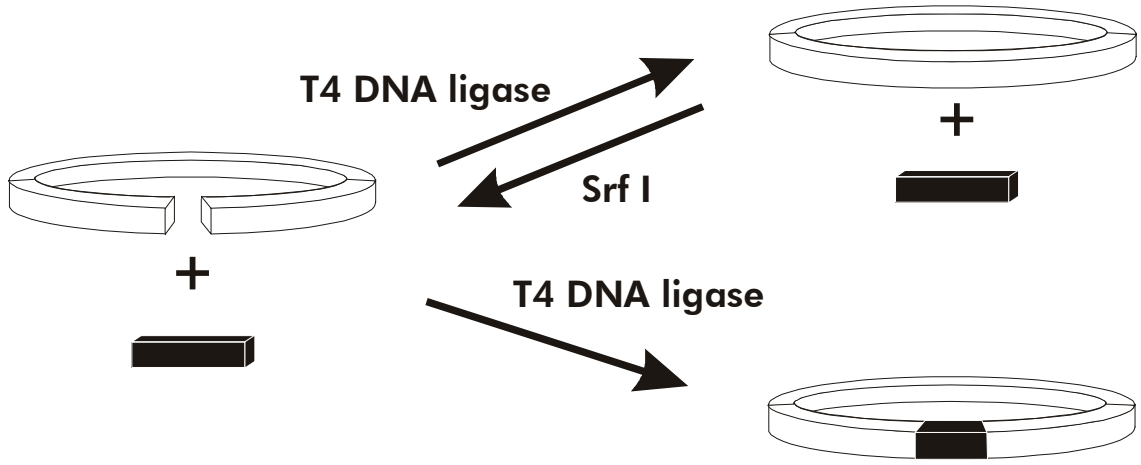


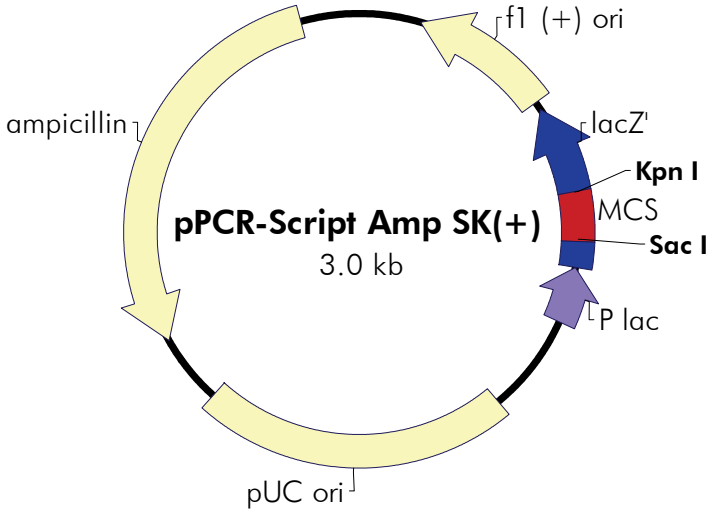
FIGURE 1 The PCR-Script Amp cloning method. An aliquot of a PCR product is added to 10 µl of a ligation reaction containing PCR-Script 1× reaction buffer, 0.5 mM rATP, and 10 ng of *Srf* I-digested pPCR-Script Amp SK(+) cloning vector. The enzymes *Srf* I and T4 DNA ligase are added. The reaction is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

Description of the Vector

The pPCR-Script Amp SK(+) cloning vector is derived from the pBluescript II SK(+) phagemid. This cloning vector includes an ampicillin-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 Amp SK(+) cloning vector available at *www.stratagene.com*.

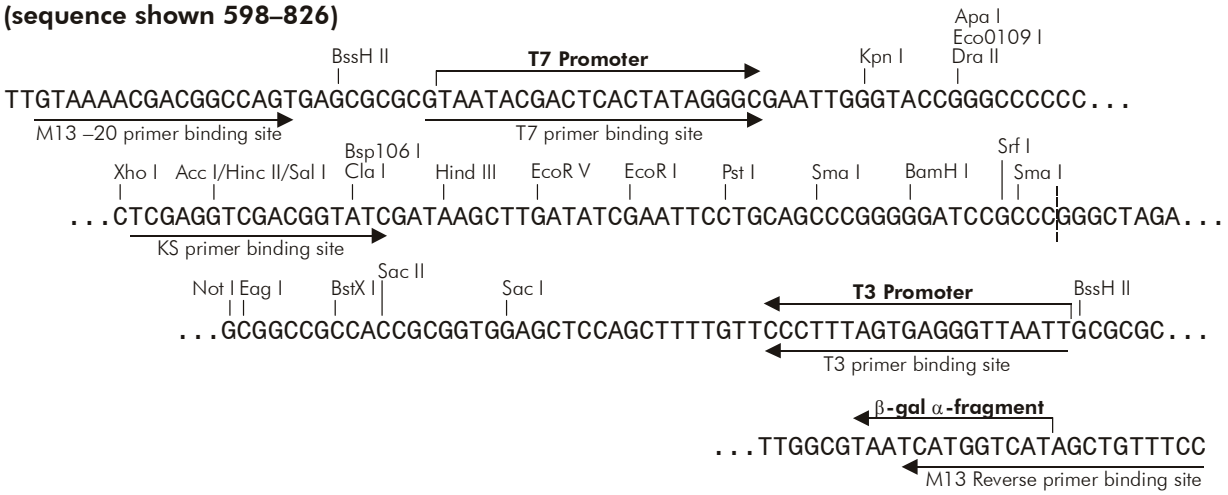
The pPCR-Script Amp SK(+) cloning vector works ideally with blunt-ended PCR products. Use of *Pfu* DNA polymerase, a proofreading enzyme with 3´ → 5´ exonuclease activity, to generate the inserts ensures the production of high-fidelity, blunt-ended PCR products.[4] When cloning *Pfu* DNA polymerase-generated PCR products purified with the StrataPrep PCR purification kit into the pPCR-Script Amp 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 Amp 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 Amp SK(+) cloning vector allows rapid and efficient blunt-ended cloning of PCR products.

pPCR-Script Amp SK(+) Vector Map



pPCR-Script Amp 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 (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 2 Circular map and polylinker sequence of the pPCR-Script Amp SK(+) cloning vector. The pPCR-Script Amp 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 both from the GenBank® database (Accession #U46017) and from *www.stratagene.com*.

Genotype of XL10-Gold Ultracompetent Cells

XL10-Gold Kan ultracompetent cells exhibit increased transformation efficiencies with ligated DNA and large supercoiled DNA molecules.[5] 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 *Eco*K (*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. [6, 7, 8, 9, 10] 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.[11] 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,[4,13,14] contributes to a lower error rate compared to *Taq* DNA polymerase. Studies also show that many species of DNA polymerases (e.g., *Taq* and Vent$_R$®) exhibit terminal deoxynucleotidyl-transferase (TdT) activity[15] and that 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 Amp 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 Amp 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 overall cloning efficiency.

  Note *The cloned* Pfu *DNA polymerase provided with these kits 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 the following to the container of the 5× PCR wash buffer: 20 ml of 100% (v/v) ethanol for the 10- and 25-reaction kits or 40 ml of 100% (v/v) ethanol for the 50-reaction kit. 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 tube[‖] 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.

[‖] 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 Amp 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 the polished PCR product directly to the ligation reaction (see *Inserting the PCR Products into the pPCR-Script Amp 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 Amp 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:

$$X \text{ ng of PCR product} = \frac{(\text{number of bp of PCR product})\,(10 \text{ ng of pPCR Script cloning vector})}{2961 \text{ bp of pPCR Script cloning vector}}$$

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	33–85
500	67–169
750	101–253
1000	135–338
1500	202–506
2000	270–675
3000	405–1013

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 Amp 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 control PCR insert
   1 µl of *Srf* I restriction enzyme (5 U/µl)
   1 µl of T4 DNA ligase (4U/µl)
   Distilled water (dH_2O) 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 perform the 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 provided β-ME mix. (Using an alternative source of β-ME may reduce transformation efficiency.)

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 µg/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-ampicillin 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 µl 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 µl of the XL10-Gold β-mercaptoethanol mix provided with the kit to the 40 µl of competent cells. (Using an alternative source of β-ME may reduce transformation efficiency.)

4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.

5. Add 2 µl 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 µl of pUC18 plasmid (diluted 1:10 in high-quality water) and 2 µl of the control ligation reaction containing the PCR test insert to separate 40-µl 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 µl of 2% X-gal and 100 µl of 10 mM IPTG on the LB-ampicillin 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-µl 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 µl (or less) of the experimental transformation reaction onto LB-ampicillin agar plates.

    Note *The cells may be concentrated by centrifuging at 1000 rpm for 10 minutes if desired. Resuspend the pellet in 200 µl of NZY⁺ broth and then plate the cells.*

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

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

    c. Pipet 5 µl of the transformation reaction containing the pUC18 control plasmid into a 100-µl 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 µl of a 1:10 dilution	5 µl	50 colonies (≥1 × 10^9 cfu/µg DNA)	—
Control PCR test insert	2 µl	10 µl	>100 colonies[a]	>100 colonies

[a] Growth of colonies on LB-chloramphenicol plates indicates successful insertion of the PCR test insert, which contains the chloramphenicol-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-ampicillin 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 *Pfu* 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 µl 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

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)

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)

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:

12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

2× Wash Buffer

10 mM Tris HCl (pH7.5)
100 mM NaCl
2.5 mM EDTA

Elution Buffer

10 mM Tris base
Adjust pH to 8.5 with HCl
or
10 mM Tris base
1 mM EDTA
Adjust pH to 8.0 with HCl
or
Sterile ddH_2O

REFERENCES

1. Bauer, J. C., Deely, D., Braman, J., Viola, J. and Weiner, M. P. (1992) *Strategies* 5(3):62-64.
2. Liu, Z. G. and Schwartz, L. M. (1992) *Biotechniques* 12(1):28, 30.
3. Simcox, T. G., Marsh, S. J., Gross, E. A., Lernhardt, W., Davis, S. *et al.* (1991) *Gene* 109(1):121-3.
4. Scott, B., Nielson, K., Cline, J. and Kretz, K. (1994) *Strategies* 7(3):62-63.
5. Jerpseth, B., Callahan, M. and Greener, A. (1997) *Strategies* 10(2):37–38.
6. Kelleher, J. E. and Raleigh, E. A. (1991) *J Bacteriol* 173(16):5220-3.
7. Kohler, S. W., Provost, G. S., Kretz, P. L., Dycaico, M. J., Sorge, J. A. *et al.* (1990) *Nucleic Acids Res* 18(10):3007-13.
8. Kretz, P. L., Kohler, S. W. and Short, J. M. (1991) *J Bacteriol* 173(15):4707-16.
9. Raleigh, E. A. and Wilson, G. (1986) *Proc Natl Acad Sci U S A* 83(23):9070-4.
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. Gough, J. A. and Murray, N. E. (1983) *J Mol Biol* 166(1):1-19.
12. Vogelstein, B. and Gillespie, D. (1979) *Proc Natl Acad Sci U S A* 76(2):615-9.
13. Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A. *et al.* (1991) *Gene* 108(1):1-6.
14. Flaman, J. M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C. *et al.* (1994) *Nucleic Acids Res* 22(15):3259-60.
15. Hu, G. (1993) *DNA Cell Biol* 12(8):763-70.
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

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

PCR-Script Amp Cloning Kit

Catalog #211188 (10 reactions), #211190 (25 reactions), and #211189 (50 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 µl of pPCR-Script Amp 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 (40:1 to 100:1 insert-to-vector ratio), 1 µl of *Srf* I restriction enzyme (5 U/µl), 1 µl of T4 DNA ligase, and dH_2O to a final volume of 10 µl
- 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 µl of purified PCR product, 1 µl of 10 mM dNTP mix (2.5 mM each), 1.3 µl of 10× polishing buffer, and 1 µl of cloned Pfu DNA polymerase (0.5 U)
  - Gently mix the reaction and add a 20-µl mineral oil overlay
  - Incubate the polishing reaction for 30 minutes at 72°C in a water bath
- Perform the ligation reaction
  - Mix 1 µl of pPCR-Script Amp 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 (40:1 to 100:1 insert-to-vector ratio), 1 µl of *Srf* I restriction enzyme (5 U/µl), 1 µl of T4 DNA ligase, and dH_2O to a final volume of 10 µl
  - Mix gently, incubate 1 hour at room temperature, and heat 10 minutes at 65°C
- Transform into XL10-Gold Kan ultracompetent cells