pCMV-Script PCR Cloning Kit

INSTRUCTION MANUAL

Catalog #211199
Revision A

For In Vitro Use Only
211199-12

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.

pCMV-Script PCR Cloning Kit

CONTENTS

pCMV-Script PCR Cloning Kit

MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]
Cloned *Pfu* DNA polymerase[b,c]	0.5 U/µl	25 µl
dNTP mix[d]	10 mM (2.5 mM each)	25 µl
Polishing buffer	10×	250 µl
pCMV-Script mammalian expression vector (predigested)	10 ng/µl	250 ng
pCMV-Script reaction buffer	10×	250 µl
rATP	10 mM	250 µl
PCR test insert (1.1 kb ampicillin-resistance gene)	100 ng/µl	4 µl
Srf I restriction enzyme[b]	5 U/µl	125 U
T4 DNA ligase	4 U/µl	100 U
XL10-Gold Cam ultracompetent cells [b,e,f]	—	1 ml (10 × 0.1-ml)
pUC18 control plasmid	0.1 ng/µl	10 µl
XL10-Gold β-mercaptoethanol mix (β-ME)	—	50 µl
StrataPrep PCR Purification Kit[b]		
DNA-binding solution[g]	—	2.5 ml
PCR wash buffer (5×)[g]	5×	5 ml
Microspin cups[h]	—	25
Receptacle tubes (2 ml)	—	25

[a] The pCMV-Script PCR cloning kit contains sufficient reagents for 25 reactions.

[b] Also available separately from Agilent, Stratagene Products Division.

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

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

[e] The XL10-Gold ultracompetent cells must be stored at the bottom of a –80°C freezer immediately upon 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}*ZΔM15* Tn*10* (Tet^R) Amy Cam^R].

[g] Sufficient for 25 PCR purification reactions.

[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 for up to 3 months or –80°C for long-term storage
StrataPrep PCR Purification Kit: Room Temperature
All Other Reagents: –20°C

Revision A

ADDITIONAL MATERIALS REQUIRED

Sterile Media and Reagents

LB–ampicillin agar plates[§] (for control PCR insert)
LB–kanamycin agar plates[§]
NZY+ broth[§]
Elution buffer[§]

Equipment

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
Water baths (37°C, 42°C, and 72°C)

NOTICES TO PURCHASER

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

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

INTRODUCTION

The Stratagene pCMV-Script PCR 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. In the ligation reaction, PCR products are incubated with the predigested pCMV-Script mammalian expression vector, the restriction enzyme *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]

Description of the Vector

The pCMV-Script mammalian expression vector is derived from a high-copy-number pUC-based plasmid and is designed to allow protein expression in eukaryotic systems (see Figure 2). Eukaryotic expression is driven by the human cytomegalovirus (CMV) immediate early promoter to promote constitutive expression of cloned inserts in mammalian cells. Stable mammalian cell clones may be selected in the presence of the antibiotic G418, due to the presence of the neomycin-resistance gene, which is driven by the SV40 early promoter with thymidine kinase (TK) transcription termination and polyadenylation signals.[4]

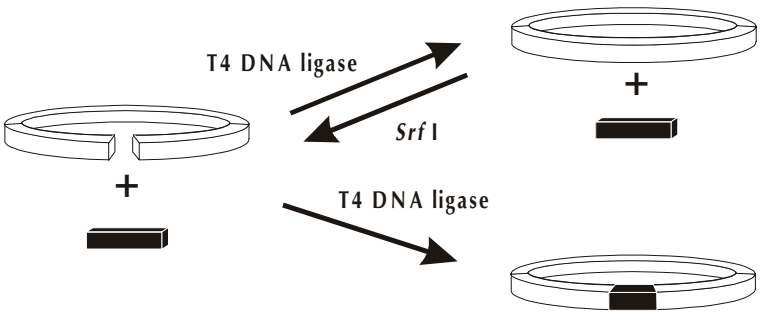


Figure 1 The pCMV-Script cloning method. An aliquot of a PCR product is added to 10 µl of a ligation reaction containing pCMV-Script 1× reaction buffer, 0.5 mM rATP, and 10 ng of *Srf* I-digested pCMV-Script mammalian expression vector. The enzymes *Srf* I and T4 DNA ligase are added. The reaction is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

The pCMV-Script mammalian expression vector does not contain an ATG initiation codon. If the PCR fragment to be cloned does not have an initiating ATG codon, a translation initiation sequence must be incorporated into the forward primer. For optimal translation, include a Kozak sequence.[5] A complete Kozak sequence includes CC$\frac{A}{G}$CC**ATG**G, although CC**ATG**G, or the core **ATG**, is sufficient.

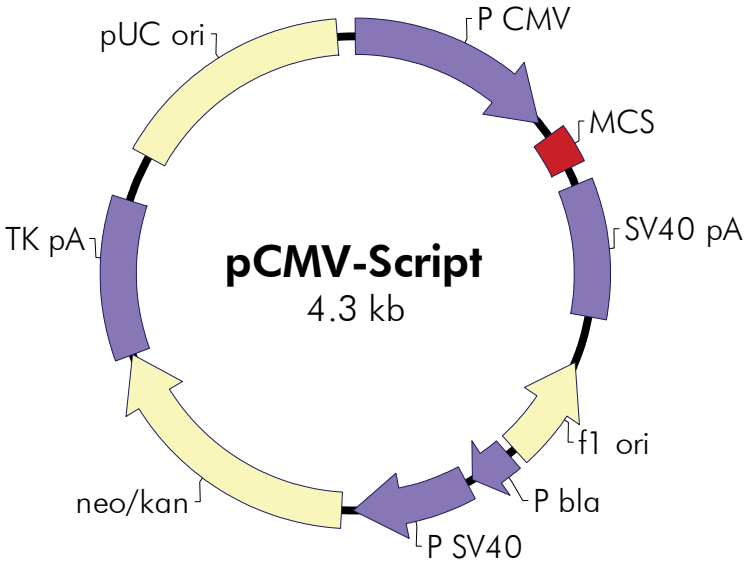
The multiple cloning site (MCS) contains a *Srf* I restriction enzyme recognition site for high-efficiency cloning of PCR fragments. In addition to the *Srf* I site, the polylinker of the pCMV-Script vector contains 14 other unique restriction enzyme recognition sites, organized with alternating 5´ and 3´ overhangs to allow serial exonuclease III/mung bean nuclease deletions. Flanking the polylinker are T3 and T7 RNA polymerase promoters that can be used to synthesize RNA in vitro. The choice of promoter used to initiate transcription determines which strand of the DNA insert will be transcribed.

Genotype of XL10-Gold Ultracompetent Cells

Host strain	References	Genotype
XL10-Gold Ultracompetent Cells	6–8	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^R) Amy Cam^R]

XL10-Gold ultracompetent cells feature the Hte phenotype, and exhibit increased transformation efficiencies with ligated DNA and large supercoiled DNA molecules.[6] XL10-Gold cells are tetracycline- and chloramphenicol-resistant and are both 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.[9–11] 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.[12] 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.

pCMV-Script Vector Map



pCMV-Script Multiple Cloning Site Region
(sequence shown 620–799)

```
                                                Sac I   BstX I  Sac II          Not I
          T3 promoter                             |       |       |              |
AATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTA...

      Srf I          BamH I              Pst I      EcoR I    EcoR V   Hind III                Acc I/Sal I
        |              |                   |          |         |        |                       |
...GCCCGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGAC...

      Xho I          Apa I     Kpn I
        |              |         |                                                  T7 promoter
...CTCGAGGGGGGGCCCGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
```

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site	620–639
multiple cloning site	651–758
T7 promoter and T7 primer binding site	778–799
SV40 polyA signal	811–1194
f1 origin of ss-DNA replication	1332–1638
bla promoter	1663–1787
SV40 promoter	1807–2145
neomycin/kanamycin resistance ORF	2180–2971
HSV-thymidine kinase (TK) polyA signal	2972–3421
pUC origin	3559–4226

Figure 2 Circular map and polylinker sequence of the pCMV-Script vector. The complete vector sequence is available at *www.stratagene.com*. The vector supplied in this kit has been digested with *Srf* I restriction enzyme.

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.[13] 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

- The pCMV-Script mammalian expression vector does not contain an ATG initiation codon. If the PCR fragment to be cloned does not have an initiating ATG codon or an optimal sequence for initiating translation, such as the Kozak sequence [GCC(A/G)CCATGG], a translation initiation sequence must be incorporated into the forward primer.

- 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,[14–16] 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 deoxynucleotidyltransferase (TdT) activity[17] 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,[18] which facilitates cloning into the pCMV-Script mammalian expression vector.

- 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.*

- 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.

- Before use, spin down and gently mix all cloning reagents except the XL10-Gold ultracompetent cells and the XL10-Gold β•-mercaptoethanol mix.

- 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.[19]

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% ethanol to the container of 5× PCR wash buffer (part #400771-17). After adding the ethanol, mark 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. On removal 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 pCMV-Script Mammalian Expression 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 (5–500 ng)
   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 then 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 pCMV-Script Mammalian Expression Vector*) or store the polished PCR products at 4°C until ready for further use.

Inserting the PCR Products into the pCMV-Script Expression 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:

$$\text{X ng of PCR product} = \frac{(\text{number of base pairs in PCR product})\,(10\text{ ng of pCMV Script vector})}{4278\text{ bp of pCMV Script vector}}$$

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*X*–100*X*
250	23–58
500	47–117
750	70–175
1000	94–234
1500	140–351
2000	187–468
3000	281–701

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 pCMV-Script expression vector (10 ng/µl)
   1 µl of pCMV-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 (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 at 65°C for 10 minutes.

4. Store the ligation reaction on ice until ready to use for transformation into the XL10-Gold Cam 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 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 14-ml BD Falcon Polypropylene Round-Bottom Tubes

It is important that 14-ml BD Falcon polypropylene 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 BD Falcon polypropylene 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. (we 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.

TRANSFORMATION PROTOCOL

1. Thaw the XL10-Gold ultracompetent cells on ice.

2. Gently mix the cells. Aliquot 40 µl of cells into a chilled, 14-ml, BD Falcon polypropylene 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 bacteria. (we 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 µl of the cloning reaction from step 4 of the *Ligating the Insert* to the cells 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. 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-kanamycin 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 plate.*

b. Pipet 10 µl of the transformation reaction containing the PCR test insert into a 100-µl pool of media on an LB-kanamycin agar plate. Plate evenly using a sterile spreader. Plate 10 µl 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-kanamycin agar plates and on LB–ampicillin agar plates to verify that the transformed colonies are also ampicillin 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. Plate evenly using a sterile spreader.

12. Incubate the plates overnight at 37°C.

Transformation Summary and Expected Results

Control transformation	Transformation quantity	Plating quantity	Expected results	
			LB-ampicillin agar plates	LB-kanamycin agar plates
pUC18 control plasmid	1 µl of a 1:10 dilution	5 µl	100 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-ampicillin plates indicates successful insertion of the PCR test insert, which contains the ampicillin-resistance gene.

VERIFICATION OF INSERT PERCENTAGE, SIZE, AND ORIENTATION

Individual colonies can be examined by PCR directly from the colony or by restriction analysis to identify the vectors with inserts and determine the insert size and orientation. T3 and T7 primers are recommended for use in PCR amplification and sequencing from the pCMV-Script vector.

Polymerase Chain Reaction Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in a pCMV-Script vector may be determined by PCR amplification of DNA from individual colonies.

1. For each colony to be examined, prepare a PCR amplification reaction containing the following components:

   4.0 µl of 10× *Taq* DNA polymerase buffer
   0.4 µl of dNTP mix (25 mM each dNTP)
   40.0 ng of T3 primer
   40.0 ng of T7 primer
   0.4 µl of 10% (v/v) Tween® 20
   1.0 U of *Taq* DNA polymerase
   dH_2O to a final volume of 40 µl

Vector	Primer	Nucleotide sequence (5´ to 3´)
pCMV-Script vector	T3	AATTAACCCTCACTAAAGGG
	T7	GTAATACGACTCACTATAGGGC

2. Stab a transformed colony with a sterile toothpick and swirl cells from the colony into the amplification reaction mixture. Immediately following inoculation into the reaction mixture, remove the toothpick and streak onto antibiotic-containing patch plates for future reference.

3. Gently mix each reaction, then overlay the reactions with 30 µl of mineral oil and perform PCR using the following cycling parameters:

Number of cycles	Temperature	Length of time
1 cycle	94°C	4 minutes
	50°C	2 minutes
	72°C	2 minutes
30 cycles	94°C	1 minute
	56°C	2 minutes
	72°C	1 minute
1 cycle	72°C	5 minutes

4. Analyze the PCR products to determine insert sizes using standard 1% (w/v) agarose gel electrophoresis. Because the forward and reverse PCR/sequencing primers are located on both sides of the MCS, **the expected size of the PCR product should be 167 bp plus the size of the insert**. Additional information can be obtained by restriction analysis of the PCR products.

TRANSFECTION INTO MAMMALIAN CELLS

For protocols for transfection into mammalian cell lines please see Sambrook, *et al*. (1989).[20]

TROUBLESHOOTING

Observation	Suggestion
Low colony yield	Plate a larger volume of the transformation reaction to compensate for low transformation efficiencies
	Visually check the PCR fragment on a 1% (w/v) agarose gel to ensure that the PCR product was added in sufficient quantity
Wrong insert size	Gel isolate the desired PCR fragment band or optimize PCR conditions to eliminate multiple bands
Bacterial lawn on selective agar plates	Plate a smaller volume of the transformation reaction
Low ultracompetent cell efficiency	Store the ultracompetent cells immediately at the bottom of a –80°C freezer
	Monitor the duration and temperature of the heat pulse carefully; heat-pulse in a 42°C water bath for 30 seconds
	Ensure that the transformation was performed in 14-ml BD Falcon polypropylene tubes, which are resistant to the strong reducing agent β-ME
Low ligation efficiency	Purify the PCR product with the provided StrataPrep PCR Purification Kit
	Ensure that PCR products are blunt ended. Generate the PCR products with *Pfu* DNA polymerase or polish the PCR products with the reagents provided in this kit to create blunt-ended DNA fragments
Colonies with no insert	Increase the insert-to-vector ratio during ligation
Low recovery of the PCR product	Verify that the PCR product was synthesized properly by running a portion of the unpurified PCR product on an agarose gel
	Failure to dilute the 5× PCR wash buffer properly may result in the PCR product being washed from the microspin cup. The 5× PCR wash buffer must be diluted with four volumes of 100% ethanol (to achieve a 1× solution) prior to use
	Use a low-ionic-strength (≤10 mM) buffer, pH 7–9. Using an elution buffer with higher ionic strength or lower pH will reduce PCR product recovery
	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 (do NOT dispense the buffer down the side of the microspin cup)
	Incubate the fiber-bound DNA with elution buffer for 5 minutes. Reducing the incubation time will reduce recovery of the PCR product
DNA floats out of the wells of agarose gel	The PCR product is contaminated with ethanol from the wash solution. 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–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml, filter-sterilized kanamycin
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

10× *Taq* DNA Polymerase Buffer

100 mM Tris-HCl (pH 8.8)
15 mM $MgCl_2$
500 mM KCl
0.01% (w/v) gelatin

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. Alting–Mees, M. A., Sorge, J. A. and Short, J. M. (1992) *Methods Enzymol* 216:483–95.
5. Kozak, M. (1991) *J Biol Chem* 266(30):19867–70.
6. Jerpseth, B., Callahan, M. and Greener, A. (1997) *Strategies* 10(2):37–38.
7. Greener, A. and Jerpseth, B. (1993) *Strategies* 6(2):57.
8. Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987) *Biotechniques* 5(4):376–378.
9. 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.
10. Kretz, P. L., Kohler, S. W. and Short, J. M. (1991) *J Bacteriol* 173(15):4707–16.
11. Raleigh, E. A. and Wilson, G. (1986) *Proc Natl Acad Sci U S A* 83(23):9070–4.
12. Gough, J. A. and Murray, N. E. (1983) *J Mol Biol* 166(1):1–19.
13. Vogelstein, B. and Gillespie, D. (1979) *Proc Natl Acad Sci U S A* 76(2):615–9.
14. Flaman, J. M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C. *et al.* (1994) *Nucleic Acids Res* 22(15):3259–60.
15. Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A. *et al.* (1991) *Gene* 108(1):1–6.
16. Scott, B., Nielson, K., Cline, J. and Kretz, K. (1994) *Strategies* 7(3):62–63.
17. Hu, G. (1993) *DNA Cell Biol* 12(8):763–70.
18. Costa, G. L. and Weiner, M. P. (1994) *Nucleic Acids Res* 22(12):2423.
19. Wong, K. K. and McClelland, M. (1991) *Nucleic Acids Res* 19(5):1081–5.
20. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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

pCMV-Script PCR Cloning Kit

Catalog #211199

QUICK-REFERENCE PROTOCOL

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 the pCMV-Script mammalian expression vector (10 ng/ µl), 1 µl of pCMV-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 dH2O 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 Cam 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 the pCMV-Script mammalian expression vector (10 ng/µl), 1 µl of pCMV-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 dH2O 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 Cam ultracompetent cells