TA Cloning® Kit

Catalog nos. K2030-01 and K2030-40
Catalog nos. K2040-01 and K2040-40

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</table>
**Important Information**

**Storage Instructions**

The TA Cloning® Kit contains a box with TA Cloning® Reagents (Box 1) and a box with One Shot® Competent Cells (Box 2).

*Store Box 1 at -20°C in a non-frost-free freezer and Box 2 at -80°C.*

**TA Cloning® Reagents**

These reagents should be stored at -20°C in a non-frost-free freezer. Note that the user must supply *Taq* Polymerase. TA Cloning® reagents are supplied as follows:


*(Note that the 40-reaction kit is supplied as two 20-reaction kits)*

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
<th>Amount (10 rxn kit)</th>
<th>Amount (20 and 40 rxn kits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR®2.1, linearized</td>
<td>25 ng/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8</td>
<td>2 x 10 µl</td>
<td>5 x 10 µl</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>25 mM MgCl₂ 0.01% gelatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10X Ligation Buffer</td>
<td>60 mM Tris-HCl, pH 7.5 60 mM MgCl₂</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td>50 mM NaCl 1 mg/ml bovine serum albumin 70 mM β-mercaptoethanol 1 mM ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 mM dithiothreitol 10 mM spermidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM dNTPs</td>
<td>12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td></td>
<td>(adjusted to pH 8.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>4.0 Weiss units/µl</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>Deionized, autoclaved water</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Control DNA Template</td>
<td>0.1 µg/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>Control PCR Primers</td>
<td>0.1 µg/µl each in 10 mM Tris-HCl, 1 mM EDTA, pH 8</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

*continued on next page*
### Important Information, continued

**Sequence of Primers**

The amplification primers are used to make a control PCR product (page 15) and cannot be used to sequence inserts in pCR®2.1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification Primer #1,</td>
<td>5´-TTTACTGTTCGGTAACAGTTTTG-3´</td>
</tr>
<tr>
<td>Amplification Primer #2,</td>
<td>5´-CAACAACGCACAGAATCTAGC-3´</td>
</tr>
</tbody>
</table>

**One Shot® Reagents**

The table below describes the items included in the One Shot® competent cell kit.

Catalog nos. K2000-01 and K2000-40 contain INVαF’ cells
Catalog nos. K2030-01 and K2030-40 contain TOP10F’ cells
Catalog nos. K2040-01 and K2040-40 contain TOP10 cells

*Note that the 40-reaction kit is supplied as two 20-reaction kits.*

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition or Amount (10 rxn kit)</th>
<th>Composition or Amount (20 or 40 rxn kit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOC Medium, 6 ml</td>
<td>2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl₂ 10 mM MgSO₄ 20 mM glucose (dextrose)</td>
<td>2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl₂ 10 mM MgSO₄ 20 mM glucose (dextrose)</td>
</tr>
<tr>
<td>INVαF’, TOP10F’ or TOP10 cells</td>
<td>11 x 50 µl</td>
<td>21 x 50 µl</td>
</tr>
<tr>
<td>pUC19 Control DNA</td>
<td>10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

**Genotype of INVαF’**

F’ endA1 recA1 hsdR17 (rK₂, mK+) supE44 thi-1 gyrA96 relA1 φ80lacZΔM15 Δ(lacZYA-argF)U169 λ−

**Genotype of TOP10F’**

F’ [lacI4 Tn10 (TetR)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

**Genotype of TOP10**

F’ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

*continued on next page*
Important Information, continued

Be sure to have the following equipment and solutions on hand before starting.

**Equipment**
- microcentrifuge
- thermocycler
- 14°C water bath
- 42°C water bath
- 37°C water bath
- bucket with ice

**Reagents and Media**
- Taq DNA Polymerase
- TE Buffer
- mineral oil
- Luria-Bertani (LB) medium and agar
- dimethylformamide (DMF)
- 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in DMF
- 100 mg/ml ampicillin stock
- 50 mg/ml kanamycin
- 100 mM isopropyl-β-D-thiogalactoside (IPTG)

**One Shot® Cells**
One Shot® INVαF’, TOP10F’ and TOP10 Chemically Competent *E. coli* are available separately from Invitrogen. The table below provides the ordering information.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Shot® INVαF’ Chemically Competent <em>E. coli</em></td>
<td>21 x 50 µl</td>
<td>C2020-03</td>
</tr>
<tr>
<td>One Shot® TOP10F’ Chemically Competent <em>E. coli</em></td>
<td>21 x 50 µl</td>
<td>C3030-03</td>
</tr>
<tr>
<td>One Shot® TOP10 Chemically Competent <em>E. coli</em></td>
<td>21 x 50 µl</td>
<td>C4040-03</td>
</tr>
</tbody>
</table>
Introduction

Overview

Purpose
The TA Cloning® Kit with pCR®2.1 provides a quick, one-step cloning strategy for the direct insertion of a polymerase chain reaction (PCR*) product into a plasmid vector.

Advantages
Using the TA Cloning® Kit:
• Eliminates any enzymatic modifications of the PCR product
• Does not require the use of PCR primers that contain restriction sites

How TA Cloning® Works
* Taq polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3’ ends of PCR products. The linearized vector supplied in this kit has single 3’ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Diagram
The diagram below shows the concept behind the TA Cloning® method.

Note
Thermostable polymerases containing extensive 3’ to 5’ exonuclease activity, such as Vent™ and Pfu, do not leave 3’ A-overhangs. PCR products generated with Taq polymerase have a high efficiency of cloning in the TA Cloning® system as the 3’ A-overhangs are not removed. However, if you use Vent™ or Pfu, or wish to clone blunt-ended fragments, you can add 3’ A-overhangs by incubation with Taq at the end of your cycling program. See page 17 for the protocol.

Alternatively, you may want to try the Zero Blunt® PCR Cloning Kit (Catalog no. K2700-20 and K2700-40). This kit offers efficient cloning of blunt-end PCR products generated using thermostable, proofreading polymerases such as Pfu and Vent™. For more information, contact Technical Service (see page 20).

Vent™ is a trademark of New England Biolabs.

* Performance of the polymerase chain reaction (“PCR”) is covered by one or more of the following U.S. Patents: Nos. 4,683,202, 4,683,195, and 4,899,818 issued to Cetus Corporation and owned and licensed by Hoffmann-LaRoche, Inc. and Roche Molecular Systems, Inc.
The pCR®2.1 Vector

Map of pCR®2.1

The map of the linearized vector, pCR®2.1, is shown below. The sequence of the multiple cloning site is shown with a PCR product inserted by TA Cloning®. EcoRI sites flank the inserted PCR product on each side. The arrow indicates the start of transcription for the T7 RNA polymerase. The complete sequence of pCR®2.1 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (page 20).

Comments for pCR®2.1

3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
Multiple Cloning Site: bases 234-355
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

continued on next page
The table below describes the features of pCR®2.1.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lac</em> promoter</td>
<td>For bacterial expression of the <em>lacZα</em> fragment for α-complementation (blue-white screening).</td>
</tr>
<tr>
<td><em>lacZα</em> fragment</td>
<td>Encodes the first 146 amino acids of β-galactosidase. Complementation in <em>trans</em> with the Ω fragment gives active β-galactosidase for blue-white screening.</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Selection and maintenance in <em>E. coli</em>, especially useful when cloning products amplified from ampicillin-resistant plasmids.</td>
</tr>
<tr>
<td>Ampicillin resistance gene</td>
<td>Selection and maintenance in <em>E. coli</em>.</td>
</tr>
<tr>
<td>pUC origin</td>
<td>Replication, maintenance, and high copy number in <em>E. coli</em>.</td>
</tr>
<tr>
<td>T7 promoter and priming site</td>
<td><em>In vivo</em> or <em>in vitro</em> transcription of anti-sense RNA. Sequencing of insert.</td>
</tr>
<tr>
<td>M13 Forward (-20) and M13 Reverse Priming Sites</td>
<td>Sequencing of insert.</td>
</tr>
<tr>
<td>f1 origin</td>
<td>Rescue of sense strand for mutagenesis and single-strand sequencing.</td>
</tr>
</tbody>
</table>
Methods

Experimental Outline

Description
To clone your gene of interest into pCR® 2.1, you must first generate a PCR product. Modification of the PCR primer by phosphorylating or addition of a restriction site is not necessary. The PCR product is ligated into pCR® 2.1 and transformed into One Shot® competent cells. Since the PCR product can ligate into the vector in either orientation, individual recombinant plasmids need to be analyzed by restriction mapping for orientation. The correct recombinant plasmid is then purified for further subcloning or characterization.

Summary
The table below summarizes the overall experimental process.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amplify your PCR product using Taq polymerase and your own primers and parameters.</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Ligate your PCR product into pCR® 2.1.</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Transform your ligation into the One Shot® competent cells supplied with the kit.</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Select colonies and isolate plasmid DNA for analysis.</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Analyze plasmid DNA for the presence and orientation of the PCR product by restriction enzyme digestion or sequencing.</td>
<td>9</td>
</tr>
</tbody>
</table>

When using the TA Cloning® Kit for the first time, we recommend that you perform the control reactions for PCR, ligation, and transformation along with your sample. This will help you analyze any possible problems. See pages 13-16.
Producing PCR Products

Guidelines for PCR

Generally 10-100 ng of DNA is sufficient to use as a template for PCR. If amplifying a pool of cDNA, the amount needed will depend on the relative abundance of the message of interest in your mRNA population. For optimal ligation efficiencies, we recommend using no more than 30 cycles of amplification.

Amplifying Your Product

Perform the PCR in a 50 µl volume containing:

- DNA Template: 10-100 ng
- 10X PCR Buffer: 5 µl
- 50 mM dNTPs: 0.5 µl
- Primers: 1 µM each
- Sterile water: to a total volume of 49 µl
- Taq Polymerase: 1 unit

Total Volume: 50 µl

Gel Purification

Smearing or multiple banding of the PCR product may necessitate gel purification. If gel purification is necessary, be extremely careful to remove all sources of nuclease contamination. Do not use communal ethidium bromide baths. All solutions that come in contact with the gel and fragment should be free of nucleases, and high quality agarose should be used. We have found that either electroeluting or using a silica-based DNA purification system works well.

Optimization of PCR

Note that gel purification may decrease ligation efficiency. You may find that optimizing your PCR is preferential over gel purification. You may use a "hot start" step before the cycling program. Hot start PCR can be achieved using any of the following methods:

- Using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Catalog no. 11304-011) or Platinum® Pfx DNA polymerase (Invitrogen, Catalog no. 11708-013) that provide an automatic hot start. A thermolabile inhibitor containing monoclonal antibodies to Taq DNA polymerase is bound to these polymerases. Initial denaturing step of the PCR results in denaturation of the inhibitor, releasing active polymerase into the reaction.

- Withholding a key component of the reaction (e.g. thermostable polymerase or magnesium) until the denaturation temperature is reached.

Innis et al. (1990) provides suggestions to optimize your PCR reaction. Invitrogen also offers the PCR Optimizer™ Kit (Catalog no. K1220-01) which incorporates many of the recommendations found in this reference.
Cloning into pCR®2.1

**Before Starting**

For optimal ligation efficiencies, we recommend using fresh (less than 1 day old) PCR products. The single 3’ A-overhangs on the PCR products will be degraded over time, reducing ligation efficiency.

pCR®2.1 vector is stable for 6 months from date of purchase if it has not been subjected to freeze-thaw cycles. Take care when handling the linearized vector as loss of the 3’ T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency.

**Procedure to Clone into pCR®2.1**

1. Centrifuge one vial of pCR®2.1 to collect all the liquid in the bottom of the vial.
2. Mark the date of first use on the vial, and if there is any vector remaining after the experiment, store at -20°C or -80°C.
3. Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmols) of pCR®2.1 vector:

   \[
   X \text{ ng PCR product} = \frac{(Y \text{ bp PCR product})(50 \text{ ng pCR}®2.1 \text{ vector})}{(\text{size in bp of the pCR}®2.1 \text{ vector: } \sim 3900)}
   \]

   where \(X\) ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio. We recommend that a 1:1 (vector:insert) ratio be used; however, you may wish to include a 1:3 (vector:insert) ratio, so multiply \(X\) by 3 to get the amount needed for ligation.

4. Using the concentration previously determined for your PCR sample, calculate the volume needed to give the amount determined in step 3. Use sterile water to dilute your PCR sample if necessary.

5. Set up the 10 µl ligation reaction as follows:

   - Fresh PCR product \(X\) µl
   - 10X Ligation Buffer 1 µl
   - pCR®2.1 vector (25 ng/µl) 2 µl
   - Sterile water to a total volume of 9 µl
   - T4 DNA Ligase (4.0 Weiss units) 1 µl
   - Final volume 10 µl

6. Incubate the ligation reaction at 14°C for a minimum of 4 hours (preferably overnight). Proceed to Transformation, page 7.

7. If you are unable to transform immediately, you can store your ligation reaction at -20°C until ready for transformation.

In general, 0.5 to 1.0 µl of a typical PCR sample with an average insert length (400-700 bp) will give the proper ratio of 1:1 (vector:insert). The ratio of 1:1 (vector:insert) gives the best efficiency of ligation. You may wish to do a second ligation reaction at a ratio of 1:3 (vector:insert), if you are concerned about the accuracy of your DNA concentrations.

Do not use more than 2-3 µl of the PCR sample in the ligation reaction as salts in the PCR sample may inhibit the T4 DNA Ligase.

Ligation at higher or lower temperatures than 14°C may reduce the ligation efficiency.
Transformation

Before Starting

Once you have a ligation reaction that contains your insert ligated into pCR®2.1, you are ready to transform the reaction mixture into One Shot® cells (INVαF’, TOP10F’ or TOP10).

Note

INVαF’ and TOP10 do not express the lac repressor; therefore, your product may express from pCR®2.1 in the absence of IPTG because of the presence of the lac promoter. IPTG will not have any affect on INVαF’ or TOP10.

TOP10F’ does express the lac repressor (lacIq), which will repress transcription from the lac promoter. To perform blue-white screening for inserts, you must add IPTG to your plates to express LacZα.

Using Competent Cells

• Competent cells are sensitive to temperature and mechanical lysis caused by pipetting. Be extremely gentle when working with competent cells.
• Start transformation immediately after thawing the cells on ice. Mix any additions by stirring gently with a pipette tip. Keep the cells as cold as possible during all steps.
• Use sterile technique when handling and plating your transformations.

Use kanamycin to select transformants when PCR products amplified from ampicillin-resistant plasmids are cloned into pCR®2.1. For example, the Control PCR product amplified from the Control DNA Template included in the kit is a PCR product from an ampicillin-resistant plasmid. Selecting with kanamycin will prevent contamination of the transformation reaction by the original ampicillin-resistant plasmid.

Preliminary Steps

1. Equilibrate a water bath to 42°C.
2. Bring the SOC medium to room temperature.

Preparation of plates for transformation (use two plates for each ligation/transformation):

3. If you are using INVαF’ or TOP10 cells, take LB plates containing 50 µg/ml of kanamycin or 100 µg/ml ampicillin and equilibrate at 37°C for 30 minutes. Spread each plate with 40 µl of 40 mg/ml X-Gal. Let the liquid soak into the plates. Plates may also be made ahead of time. See Recipes, page 18.
4. If you are using TOP10F’ cells, take LB plates containing antibiotic and equilibrate at 37°C for 30 minutes. Spread 40 µl each of 100 mM IPTG and 40 mg/ml X-Gal onto the plates. Let the liquid soak into the plates. Plates may also be made ahead of time. See Recipes, page 18.

continued on next page
Transformation, continued

**Procedure for Transformation**

Use the instructions below for the best results with TA Cloning®. Note that TOP10F’ and TOP10 cells have an efficiency of $1 \times 10^9$ cfu/µg DNA while INVαF’ have an efficiency of $1 \times 10^8$ cfu/µg DNA.

1. Centrifuge the vials containing the ligation reactions briefly and place them on ice.
2. Thaw on ice one 50 µl vial of frozen One Shot® competent cells for each ligation/transformation.
3. Pipette 2 µl of each ligation reaction directly into the vial of competent cells and mix by stirring gently with the pipette tip.
4. Incubate the vials on ice for 30 minutes. Store the remaining ligation mixtures at -20°C.
5. Heat shock for exactly 30 seconds in the 42°C water bath. Do not mix or shake. Remove the vials from the 42°C water bath and place on ice.
6. Add 250 µl of SOC medium (at room temperature) to each tube.
7. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 10 µl to 200 µl from each transformation vial on separate, labeled LB agar plates containing X-Gal and 50 µg/ml of kanamycin or 100 µg/ml ampicillin. Be sure to also include IPTG if you are using TOP10F’ cells. We recommend plating 10-50 µl for TOP10F’ or TOP10 cells and 50-200 µl for INVαF’ cells.
   **Note:** Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
9. Make sure the liquid is absorbed, then invert the plates and place them in a 37°C incubator for at least 18 hours. Shift plates to +4°C for 2-3 hours to allow for proper color development.

**Important**

Transformed INVαF’ cells may appear very small after overnight growth when compared to other *E. coli* strains. The transformants may need to grow an additional 2-3 hours before selecting colonies for analysis.
Analysis

Results of Transformation
For an insert size of 400-700 bp, you should obtain 50-200 colonies per plate depending on the volume plated and of these, approximately 80% should be white on X-Gal plates (INVαF’ and TOP10) or X-Gal/IPTG plates (TOP10F’). Note that ligation efficiency depends on insert size. As insert size increases, the efficiency will decrease.

Restriction Analysis
To determine the presence and orientation of insert:
1. Pick at least 10 white colonies for plasmid isolation and restriction analysis.
2. Grow colonies overnight in 2-5 ml LB broth containing either 100 µg/ml of ampicillin or 50 µg/ml kanamycin.
3. Isolate plasmid and analyze by restriction mapping or sequencing for orientation of the insert. For plasmid isolation and restriction enzyme digests see Ausubel, et al. (1990) or Sambrook, et al. (1989).

Important
To increase plasmid yield, use Terrific Broth or SOC medium containing antibiotic (see page 19).

Sequencing Your Insert
If you wish to sequence your insert in pCR® 2.1, you may use the M13 Reverse Primer to sequence into your insert from the lac promoter (see vector map, page 2). To sequence into the insert from the lacZα fragment, you can use either the T7 Promoter Primer or the M13 Forward (-20) Primer. The following primers are available from Invitrogen:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amount</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Promoter</td>
<td>2 µg, lyophilized</td>
<td>N560-02</td>
</tr>
<tr>
<td>Universal M13 Reverse</td>
<td>2 µg, lyophilized</td>
<td>N530-02</td>
</tr>
<tr>
<td>Universal M13 Forward (-20)</td>
<td>2 µg, lyophilized</td>
<td>N520-02</td>
</tr>
</tbody>
</table>

Note that not all T7 primers commercially available have the same sequence. If you substitute primers, check the sequence carefully.

If There is No Insert
The next chapter provides a trouble-shooting guide and some control reactions you can do to resolve any problems you might have. Call Technical Service for help with TA Cloning" (page 20).
# Troubleshooting Guide and Control Reactions

## Troubleshooting Guide

**Troubleshooting Your Experiment** If you do not obtain the results you expect, use the following table to troubleshoot your experiment. To confirm that your kit is working properly, perform the Control PCR and Ligation/Transformation Reaction on pages 15 and 16.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No colonies obtained from transformation</td>
<td>Bacteria were not competent.</td>
<td>Use the pUC control vector included with the One Shot® module to check</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the transformation efficiency of the cells. See page 14 for details.</td>
</tr>
<tr>
<td>Incorrect concentration of antibiotic on plates, or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>the plates are too old.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Use 100 µg/ml of ampicillin or 50 µg/ml kanamycin. Use fresh ampicillin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>plates (&lt; 1 month old).</td>
</tr>
<tr>
<td>White colonies do not have insert</td>
<td>Single 3´ T-overhangs on the vector</td>
<td>Use another tube of vector. Avoid storing the vector for longer than 6</td>
</tr>
<tr>
<td></td>
<td>degraded.</td>
<td>months or subjecting it to repeated freeze/thaw cycles. Check vector by</td>
</tr>
<tr>
<td>Only white colonies obtained</td>
<td>No IPTG or X-Gal in plates.</td>
<td>Be sure to include X-Gal for blue/white screening and both IPTG and X-Gal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>if using TOP10F®.</td>
</tr>
<tr>
<td>Majority of colonies are blue or light blue with very</td>
<td>The insert does not interrupt the reading</td>
<td>If you have a small insert (&lt; 500 bp), you may have light blue colonies.</td>
</tr>
<tr>
<td>few white colonies</td>
<td>frame of the lacZ gene.</td>
<td>Analyze some of these blue colonies as they may contain insert.</td>
</tr>
<tr>
<td></td>
<td>Used a polymerase that does not add 3´ A-</td>
<td>Do not use Vent™ or Pfu as they do not add 3´ A-overhangs. Use Taq</td>
</tr>
<tr>
<td></td>
<td>overhangs.</td>
<td>polymerase. See page 17 if you use Vent™ or Pfu.</td>
</tr>
<tr>
<td>PCR products were gel-purified before ligation.</td>
<td></td>
<td>If gel purification is needed, use nuclease-free solutions and either</td>
</tr>
<tr>
<td></td>
<td></td>
<td>electroelution or GeneClean to purify fragment. Otherwise, optimize your</td>
</tr>
<tr>
<td>The PCR products were stored for a long period of</td>
<td></td>
<td>PCR.</td>
</tr>
<tr>
<td>time before ligation reaction.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*continued on next page*
## Troubleshooting Your Experiment, continued

<table>
<thead>
<tr>
<th>Observation</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Majority of colonies are blue or light blue with very few white colonies, continued</td>
<td>Too much of the amplification reaction was added to the ligation. The high salt content of PCR can inhibit ligation.</td>
<td>Use no more than 2-3 µl of the PCR mixture in the ligation reaction.</td>
</tr>
<tr>
<td>Incorrect molar ratio of vector:insert used in the ligation reaction.</td>
<td>Estimate the concentration of the PCR product using the DNA DipStick™ Kit or agarose gel electrophoresis. Set up the ligation reaction with a 1:1 or 1:3 vector:insert molar ratio. See page 6.</td>
<td></td>
</tr>
<tr>
<td>Some colonies have a light blue color or appear white with blue centers</td>
<td>Leaky expression of the lacZ fragment or only a partial disruption of lacZ by the insert.</td>
<td>If you are looking for a smaller size insert, 500 bp or less, analyze these colonies as they may contain insert.</td>
</tr>
<tr>
<td>White colonies or blue colonies of normal size are surrounded by smaller, white colonies</td>
<td>The smaller colonies are ampicillin-sensitive satellite colonies. Do not pick the small colonies as they do not contain any plasmid.</td>
<td>Use kanamycin selection. Be sure the stock solution of ampicillin and your plates are both fresh.</td>
</tr>
<tr>
<td>White colonies do not grow in liquid culture</td>
<td>Ampicillin-sensitive satellite colonies.</td>
<td>Be sure to pick large white colonies. Be sure the ampicillin is fresh. Use kanamycin to eliminate this problem.</td>
</tr>
<tr>
<td>No results from sequencing</td>
<td>Accidental use of the amplification primers in the kit for sequencing. These are for generating the control PCR product only.</td>
<td>Use the M13 Forward (-20) and Reverse Primers for sequencing. You may also use the T7 promoter primer to sequence into the insert.</td>
</tr>
<tr>
<td>The T7 primer used was not the right sequence.</td>
<td>Check the sequence of your T7 promoter primer and make sure it matches with the priming site on pCR® 2.1.</td>
<td></td>
</tr>
<tr>
<td>An Sp6 primer was used to sequence inserts in pCR® 2.1.</td>
<td>Do not use an Sp6 primer to sequence pCR® 2.1. There is no binding site for this primer.</td>
<td></td>
</tr>
<tr>
<td>No PCR product</td>
<td>Either the Taq polymerase is inactive or the conditions for your PCR are not optimal.</td>
<td>Perform the PCR Control Reaction on page 15. If it works, then Taq polymerase is probably active and you need to optimize your PCR. If it does not work, try new enzyme.</td>
</tr>
<tr>
<td>Low plasmid yield</td>
<td>Cells do not grow well in LB.</td>
<td>Try using SOC medium. Remember to include antibiotic. See page 19 for recipe.</td>
</tr>
</tbody>
</table>

*continued on next page*
The following table describes the control reactions that can be performed to troubleshoot the TA Cloning® technology and how to interpret the results from these control reactions.

<table>
<thead>
<tr>
<th>Control Reaction</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-Ligation</td>
<td>This control reaction shows if pCR® 2.1 has lost the 3’ T-overhangs. Loss of the T-overhangs results in blunt-end ligation and disruption of the lacZα reading frame. False white colonies will result. Normally, less than 5% of the colonies should be white.</td>
</tr>
<tr>
<td>Transformation Efficiency Control</td>
<td>Tests the competency of the One Shot® competent cells. INVαF’ should yield approximately 1 x 10^8 cfu/µg DNA. TOP10F’ should yield approximately 1 x 10^9 cfu/µg DNA.</td>
</tr>
<tr>
<td>PCR Control Reaction</td>
<td>Tests the PCR reagents except for Taq polymerase.</td>
</tr>
<tr>
<td>Ligation/Transformation Control Reaction</td>
<td>Tests the ligation reagents and pCR® 2.1. Greater than 80% white colonies are produced and these colonies should contain vector with insert.</td>
</tr>
</tbody>
</table>
Self-Ligation Reaction

Introduction

The TA Cloning® vector, pCR® 2.1, is supplied in five aliquots (or 2 aliquots if using the 10 reaction kit). It is stable for six months if not subjected to repeated freeze-thaw cycles. Vector that has been stored for longer periods or repeatedly frozen and thawed will lose the 3’ T-overhangs resulting in "false" white positives. Use the self-ligation reaction conditions described below and then transform into the One Shot® competent cells supplied with the kit. You may also wish to test the competency of the One Shot® cells along side the Self-Ligation Reaction by performing the Transformation Efficiency Control Reaction, page 14.

Procedure

1. Set up the 10 µl self-ligation reaction as follows:
   - Sterile water: 6 µl
   - 10X Ligation Buffer: 1 µl
   - pCR® 2.1 vector (25 ng/µl): 2 µl
   - T4 DNA Ligase (4.0 Weiss units): 1 µl
   - Total Volume: 10 µl

2. Incubate overnight at 14-15°C. Centrifuge the vials containing the ligation reactions briefly and place them on ice.

3. Thaw on ice one 50 µl vial of frozen One Shot® competent cells for each ligation/transformation.

4. Pipette 1 µl of the Control Ligation Reaction directly into the vial of competent cells and mix by stirring gently with the pipette tip.

5. Incubate the vial on ice for 30 minutes. Store the remainder of the ligation mixture at -20°C.

6. Heat shock for exactly 30 seconds in the 42°C water bath. Do not mix or shake. Remove the vial from the 42°C water bath and place on ice.

7. Add 250 µl of SOC medium (at room temperature) to the tube.

8. Shake the vial horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.

9. Spread 50 µl from the vial on a labeled LB agar plate containing 50 µg/ml of kanamycin or 100µg/ml ampicillin and X-Gal. Be sure to include IPTG if you are using TOP10F’.

10. Make sure the liquid is absorbed, and then invert the plate and place in a 37°C incubator overnight.

Results

You should expect about 5-25 colonies from the 50 µl plated. Most of these colonies should be blue; there should be less than 5% white colonies. The blue colonies result from supercoiled pCR® 2.1 vector. Over time, the 3’ T-overhangs will degrade, causing a blunt-end self-ligation of the vector. This can cause a frameshift of the lacZ gene, resulting in a “false” white or light blue colony with no insert.
Transformation Efficiency Control

Before Starting
A vial of supercoiled pUC19 is included with the One Shot® competent cells as a control for transformation.

- Use as supplied. No dilution is required.
- Prepare LB agar plates containing 100 µg/ml ampicillin.

Test Transformation
1. Thaw on ice one 50 µl vial of frozen One Shot® competent cells for each ligation/transformation.
2. Pipette 1 µl of undiluted pUC19 (10 pg/ul) directly into the vial of cells and mix by stirring gently with the pipette tip.
3. Incubate the vial on ice for 30 minutes.
4. Heat shock for exactly 30 seconds in the 42°C water bath. Do not mix or shake. Remove the vial from the 42°C water bath and place on ice.
5. Add 250 µl of SOC medium (at room temperature) to the tube.
6. Shake the vial horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
7. Spread 50 µl from the INVαF’ transformation and 10 µl from the TOP10F’ or TOP10 transformation on separate, labeled LB agar plates containing 100 µg/ml ampicillin. Make sure the liquid is absorbed, then invert the plates and place them in a 37°C incubator overnight.

Calculation
Use the formula below to calculate transformation efficiency. The transformation efficiencies should be approximately:

- 1 x 10⁸ cfu/µg DNA for INVαF’
- 1 x 10⁹ cfu/µg DNA for TOP10F’
- 1 x 10⁹ cfu/µg DNA for TOP10

\[
\frac{\text{# of colonies}}{10 \text{ pg transformed}} \times \frac{10^6 \text{ pg}}{\mu \text{g}} \times \frac{300 \text{ µl transformed cells}}{X \mu \text{l plated}} = \frac{\text{# transformants}}{\mu \text{g plasmid DNA}}
\]
PCR Control Reaction

Control PCR Reaction

To test the components of the kit, a DNA template and primers are included to generate a control PCR product for ligating into pCR® 2.1. The control primers amplify a 700 bp fragment which when cloned into pCR® 2.1 will give about 80% white colonies when plated on selective medium with X-Gal (and IPTG, if using TOP10F`).

Procedure

1. Set up the 50 µl PCR as follows:
   - Control DNA Template (100 ng) 1 µl
   - 10X PCR Buffer 5 µl
   - 50 mM dNTPs 0.5 µl
   - Control PCR Primers 1 µl
   - Sterile water 41.5 µl
   - *Taq* Polymerase (1 unit/µl) 1 µl
   - Total Volume 50 µl

2. Overlay with 70 µl of mineral oil.

3. Amplify using the cycling parameters below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>1 minute</td>
<td>94°C</td>
<td>25X</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 minute</td>
<td>55°C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>1 minute</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>7 minutes</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

4. Remove 10 µl from the PCR sample and analyze by gel electrophoresis in a 0.8-1.5% agarose gel.

5. A 700 bp band should be visible. Quantify the amount of DNA by measuring against a known standard run on the same gel or using Invitrogen's DNA DipStick™ Kit (Catalog no. K5632-01). You should get a concentration of about 20 ng/µl for your Control PCR Reaction. Proceed to the Control Ligation Reaction.
Ligation/Transformation Control Reaction

Control Ligation Reaction

To test the efficiency of the vector, use fresh PCR product and ligate into pCR®2.1. In general, 1 µl of the Control PCR Reaction should be sufficient for ligation. Alternatively, you may use the formula given in Cloning into pCR®2.1, page 6 to estimate the amount of PCR product to ligate with 50 ng of pCR®2.1.

Procedure

1. Set up the 10 µl Control Ligation Reaction as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>10X Ligation Buffer</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pCR®2.1 vector (25 ng/µl)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Control PCR Product</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total Volume</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

2. Incubate the Control Ligation Reaction at 14°C for a minimum of 4 hours (preferably overnight). Proceed to Control Transformation.

Control Transformation for Ligation

To determine if your control ligation worked, it must be transformed into competent E. coli cells and plated on LB/kanamycin/X-Gal plates (INVαF´ or TOP10) or LB/kanamycin/X-Gal/IPTG plates (TOP10F´). Before starting:

1. Make LB plates with 50 µg/ml kanamycin, X-Gal, and IPTG (if necessary) for plating the transformation mix from the Control Ligation Reaction. See Recipes, page 18.

2. Centrifuge the ligation reactions briefly and place them on ice.

Procedure for Transformation

1. Thaw on ice one 50 µl vial of frozen One Shot® competent cells for each ligation/ transformation.

2. Pipette 1 µl of the Control Ligation Reaction into the vial of competent cells.

3. Incubate the vial on ice for 30 minutes. Store the remaining ligation mixture at -20°C.

4. Heat shock for exactly 30 seconds in the 42°C water bath. Do not mix or shake.

5. Remove the vial from the 42°C water bath and place on ice.

6. Add 250 µl of SOC medium (at room temperature) to each vial.

7. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a rotary shaking incubator.

8. Plate 10-50 µl from the Control Ligation/Transformation Reaction on LB agar plates containing 50 µg/ml kanamycin with X-Gal (and IPTG for TOP10F´ cells). Incubate at least 18 hours at 37°C.

Expected Results

The Control Ligation Reaction should produce >80% white colonies. Over time, the 3´ T-overhangs will degrade, causing an increase in the number of background white colonies (those without inserts). The number of background colonies should not exceed 10% (see the Self-Ligation Reaction, page 13). If this happens, use another vial of pCR®2.1 and try to avoid repeated freeze-thaw cycles.
Appendix

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by Vent™ or Pfu polymerases into pCR®2.1 is often difficult due to very low cloning efficiencies. These low efficiencies are caused by the 3´ to 5´ exonuclease proofreading activity of Vent™ and Pfu polymerases that remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

If you routinely clone blunt PCR products, we recommend the Zero Blunt® PCR Cloning Kit (Catalog no. K2700-20) for optimal cloning of blunt PCR products.

Before Starting

You will need the following items:

- Taq polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- TE buffer

Procedure

1. After amplification with Vent™ or Pfu polymerase, place vials on ice and add 0.7-1 unit of Taq polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Extract immediately with an equal volume of phenol-chloroform.
4. Add 1/10 volume of 3 M sodium acetate and 2X volume of 100% ethanol.
5. Centrifuge at maximum speed for 5 minutes at room temperature to precipitate the DNA.
6. Remove the ethanol, rinse the pellet with 80% ethanol, and allow to air dry.
7. Resuspend the pellet in TE buffer to the starting volume of the DNA amplification reaction. The DNA amplification product is now ready for ligation into pCR®2.1.
Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 lbs./sq. inch. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 lbs./sq. in.
3. After autoclaving, cool to ~55°C, add antibiotic (100 µg/ml of ampicillin or 50 µg/ml kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

X-Gal Stock Solution

1. To make a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethylformamide.
2. Protect from light by storing in a brown bottle at -20°C.
3. To add to previously made agar plates, pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

IPTG Stock Solution

1. Prepare a 100 mM stock solution by dissolving 238 mg of IPTG in 10 ml deionized water.
2. Filter-sterilize and store in 1 ml aliquots at -20°C.

X-Gal/IPTG LB Plates

1. Warm an LB plate with the appropriate antibiotic at 37°C for 10 minutes.
2. Pipet 40 µl of the X-Gal stock solution and 40 µl of the IPTG stock solution onto the center of the plate and spread evenly with a sterile spreader.
3. Allow the solution to diffuse into the plate by incubating at 37°C for 20-30 minutes. Plates are now ready to use.

continued on next page
## Recipes, continued

### SOC Medium

**Composition:**

- 2.0% Tryptone
- 0.5% Yeast Extract
- 10.0 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl₂•6H₂O
- 20 mM glucose

1. For 1 liter, dissolve 20 g Tryptone, 5 g Yeast Extract, and 0.5 g NaCl in 950 ml deionized water.
2. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.0 with 5 M NaOH, then bring the volume to 980 ml with deionized water.
4. Prepare a 1 M solution of MgCl₂ by dissolving 20.33 g MgCl₂•6H₂O in 100 ml deionized water.
5. Autoclave both solutions on liquid cycle at 15 lbs./sq. in. for 20 minutes.
6. Meanwhile, make a 2 M solution of glucose by weighing out 36 g glucose and dissolving in a final volume of 100 ml deionized water. Filter-sterilize this solution.
7. Let the autoclaved solutions cool to about 55°C, then add 10 ml of the filter-sterilized 2 M glucose solution and 10 ml 1 M MgCl₂. Store at room temperature or +4°C.

### Terrific Broth

1.2% Tryptone
2.4% Yeast extract
0.4% glycerol
17 mM KH₂PO₄
72 mM K₂HPO₄

To prepare 1 liter of Terrific Broth:

1. Dissolve 2.31 g KH₂PO₄ and 12.54 g K₂HPO₄ in 90 ml deionized water.
2. Adjust the volume to 100 ml with deionized water.
3. Dissolve 12 g tryptone, 24 g yeast extract, and 4 ml glycerol in 900 ml of deionized water.
4. Autoclave both solutions for 20 minutes on liquid cycle.
5. Cool to ~60°C or less and add the sterile, 100 ml solution of KH₂PO₄ and K₂HPO₄ to the tryptone/yeast extract/glycerol solution.
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4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the ‘Submit’ button. Your MSDS will be sent within 24 hours.

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Technical Service, continued

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No. 8: Cells

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continued on next page
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Product Qualification

Restriction Digest

The parental supercoiled pCR®2.1 vector is qualified by restriction digest prior to TA adaptation. The table below lists the restriction enzymes and the expected fragments.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>pCR®2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hind</em> III (linearizes)</td>
<td>3929 bp</td>
</tr>
<tr>
<td><em>Xba</em> I (linearizes)</td>
<td>3929 bp</td>
</tr>
<tr>
<td><em>Nsi</em> I</td>
<td>3929 bp</td>
</tr>
<tr>
<td><em>Pst</em> I</td>
<td>1190, 2739 bp</td>
</tr>
<tr>
<td><em>EcoR</em> I and <em>Afl</em> III</td>
<td>16, 408, 716, 2789 bp</td>
</tr>
</tbody>
</table>

Cloning Efficiency

Once the vector has been adapted, they are lot-qualified using the control reagents included in the kit. Under conditions described on pages 13-16, a 750 bp control PCR product was ligated into each vector and subsequently transformed into the One Shot® competent *E. coli* included with the kit. To qualify the vector, the following criteria must be met:

- Self-ligation: < 5% white transformants
- PCR product ligation: > 80% white transformants
- Cloning efficiency: > 90% of the white transformants contain pCR®2.1 with the correct PCR product

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Chemically Competent *E. coli*

50 µl of competent cells are transformed with 10 pg of supercoiled control plasmid. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:

- 1 x 10^8 cfu/µg DNA for INVαF’
- 1 x 10^9 cfu/µg DNA for TOP10F’
- 1 x 10^9 cfu/µg DNA for TOP10

Untransformed cells are plated on:

- LB plates containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, 25 µg/ml streptomycin, and 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.
- LB plates as a lawn to verify the absence of phage contamination.
References

General Reference for PCR Technology


General Molecular Biology Techniques


TA Cloning


continued on next page
References, continued


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