USER GUIDE

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TA Cloning® Kit
Dual Promoter (pCR®II)

Catalog Numbers K2050-01, K2050-40, K2060-01, K2060-40, K2070-20, and K2070-40

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Contents

Contents iii
Kit Contents and Storage iv
Introduction 1
Product Overview 1
Experimental Outline 2
Methods 3
Produce PCR Products 3
Clone into pCR®II 4
Transform Competent Cells 6
Analyze Transformants 9
Troubleshoot 10
Appendix 13
Perform the Self-Ligation Reaction 13
Perform the Control Reactions 14
Add 3´ A-Overhangs 16
Recipes 18
Map and Features of pCR®II 19
Accessory Products 21
Technical Support 22
Purchaser Notification 23
References 23

Kit Contents and Storage

Shipping and storage

The TA Cloning® Kits Dual Promoter are shipped on dry ice and contain a box of TA Cloning® Reagents (Box 1) and a box of One Shot® Competent Cells (Box 2). Cat. nos. K2070-20 and K2070-40 are **not** supplied with One Shot® Competent Cells.

Store Box 1 at −30°C to −10°C in a non-frost-free freezer and Box 2 at −85°C to −68°C.

Type of kits

This manual is supplied with the following kits.

Kit	Quantity	Cat. no.
TA Cloning® Kit Dual Promoter	20 reactions	K2070-20
	40 reactions	K2070-40
TA Cloning® Kit Dual Promoter with One Shot® INVαF´ Chemically Competent *E. coli*	20 reactions	K2050-01
	40 reactions	K2050-40
TA Cloning® Kit Dual Promoter with One Shot® TOP10F´ Chemically Competent *E. coli*	20 reactions	K2060-01
	40 reactions	K2060-40

Product use

For research use only. Not for use in diagnostic procedures.

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Kit Contents and Storage, Continued

TA Cloning® reagents

TA Cloning® reagents (Box 1) are listed below. Note that the user must supply *Taq* Polymerase. Forty reaction kits are supplied as two 20 reaction kits.

Store Box 1 at –30°C to –10°C.

Item	Composition	Amount
pCR®II , linearized	25 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5	5 × 10 µL
ExpressLink™ T4 DNA Ligase	5.0 Weiss units/µL	25 µL
5X Express Link™ T4 DNA Ligase Buffer	5X T4 DNA Ligase Buffer (50 mM Tris-HCl, pH 7.6 , 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25 % (w/v) polyethylene glycol-8000)	200 µL
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µL
50 mM dNTPs	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP (adjusted to pH 8.0)	10 µL
Control DNA Template	0.1 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5	10 µL
Water	Deionized, autoclaved water	1 mL
Control PCR Primers	0.1 µg/µL each in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5	10 µL

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Kit Contents and Storage, Continued

One Shot® reagents

The following table describes the items included in the One Shot® Competent Cell Kit. Forty reaction kits are supplied as two 20-reaction kits.

Note: Cat. nos. K2070-20 and K2070-40 are **not** supplied with competent cells.

The transformation efficiency for TOP10F´ cells is 1×10^9 cfu/µg DNA. The transformation efficiency for INVαF´ is 1×10^8 cfu/µg DNA.

Store competent cells at −85°C to −68°C.

Component	Composition	Amount
S.O.C. Medium (may be stored at room temperature or 4ºC)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose (dextrose)	6 mL
INVαF´ or TOP10F´ cells	—	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

Genotype of INVαF´

F´ *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 λ^-

Genotype of TOP10F´

F´ [*lac*I^q Tn*10* (Tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Introduction

Product Overview

Purpose

The TA Cloning® Kit Dual Promoter with pCR®II provides a quick, one-step cloning strategy for directly inserting a PCR product into a plasmid vector. The T7 and Sp6 promoters allow *in vivo* or *in vitro* transcription of the insert to produce sense or anti-sense products.

Advantages

Using the TA Cloning® Kit Dual Promoter:

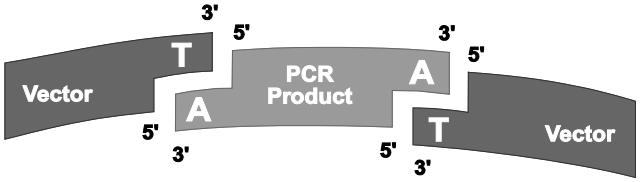
- Eliminates any enzymatic modifications of the PCR product
- Does not require the use of PCR primers that contain restriction sites
- Allows transcription from either direction into the insert

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 Platinum® *Pfx*, do not leave 3´ A-overhangs. PCR products generated with *Taq* polymerase have a high efficiency of cloning in the TA Cloning® system because the 3´ A-overhangs are not removed. However, if you use a proofreading polymerase or wish to clone blunt-ended fragments, you can add 3´ A-overhangs by incubating with *Taq* at the end of your cycling program. See page 16 for a protocol.

Alternatively, you may want to try the Zero Blunt® PCR Cloning Kit (Cat. nos. K2700-20 and K2750-20). This kit offers efficient cloning of blunt-end PCR products generated using thermostable, proofreading polymerases. For more information, visit **www.lifetechnologies.com/support** or contact Technical Support (page 22).

Experimental Outline

Introduction

To clone your gene of interest into pCR®II , you must first generate a PCR product. You will ligate the PCR product into pCR®II and transform into 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 may then be purified for further subcloning or characterization.

Flow chart

The table below describes the major steps necessary to clone your gene of interest into pCR®II .

Step	Action	Page
1	Amplify your PCR product using *Taq* polymerase and your own primers and parameters.	3
2	Ligate the PCR product into pCR®II .	4
3	Transform your ligation into competent *E. coli*.	5–8
4	Select colonies and isolate plasmid DNA. Analyze plasmid DNA for the presence and orientation of the PCR product by restriction enzyme digestion or sequencing.	9

When using the TA Cloning® Kit Dual Promoter Cloning® Kit for the first time, we recommend that you perform the control reactions to help you evaluate your results (pages 9–15).

Methods

Produce 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.

Materials supplied by the user

- DNA template and primers for PCR product
- *Taq* polymerase and appropriate 10X PCR buffer (see page 21 for ordering information)
- Thermocycler

Polymerase mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be in excess of a 10:1 ratio to ensure the presence of 3′ A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity (see page 21 for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhands using the method on page 16.

Produce PCR products

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
Water	to a total volume of 49 µL
Taq Polymerase	1 unit
Total Volume	50 µL

Gel purification

If you do not obtain a single, discrete band from the PCR, you may gel-purify your fragment before proceeding. Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Cat. no. K1220-01) can help you optimize your PCR. Contact Technical Support for more information (page 22).

Clone into pCR®II

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.

Take care when handling the pCR®II vector as loss of the 3´ T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency.

Calculating amount of PCR product to use

Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR®II vector:

$$x \text{ ng PCR product} = \frac{(y \text{ bp PCR product})(50 \text{ ng pCR®II vector})}{(\text{size in bp of the pCR®II vector: } \sim 3900)}$$

where *x* ng is the amount of PCR product of *y* base pairs to be ligated for a 1:1 (vector to insert) molar ratio.

Notes on efficiency

In general, 0.5–1.0 µL of a typical PCR sample with an average insert length (400–700 bp) will give the proper ratio of 1:1 (vector to insert) and an efficient ligation. If you are concerned about the accuracy of your DNA concentrations and want to increase efficiency, do a second ligation reaction at a ratio of 1:3 (vector to insert).

Do not use more than 2–3 µL of the PCR sample in the ligation reaction because salts in the PCR sample may inhibit T4 DNA Ligase.

Procedure

1. Determine the volume of PCR sample needed to reach the required amount of PCR product (see the preceding **Note**). Use sterile water to dilute the PCR sample if necessary.
2. Set up the 10 µL ligation reaction as follows:

Fresh PCR product	X µL
5X Express Link™ T4 DNA Ligase Buffer	2 µL
pCR®II vector (25 ng/µL)	2 µL
Water to a total volume of	9 µL
ExpressLink™ T4 DNA Ligase (5 units)	1 µL
Final volume	10 µL

3. Incubate the ligation reaction at room temperature for a minimum of 15 minutes. Longer incubation times increase the cloning efficiency, see page 17). Proceed to **Transform Competent Cells**, page 5.

   Note: You may store the ligation reaction at –20°C until you are ready for transformation.

Continued on next page

Clone into pCR®II, Continued

Notes about incubation times

Cloning efficiency can be optimized by changing incubation time and altering the vector to insert ratio. In the tables below, the ligation reactions were performed using a 1:1 vector to insert ratio **(Table 1)**, or using a 1:3 vector to insert ratio, which was achieved by reducing the pCR® II vector concentration to 25ng. **(Table 2)**.

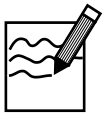
Ligation reactions were incubated at room temperature for 15 minutes, 30 minutes and 1 hour, transformed into One Shot® Top10 cells and 50µl plated for blue/white screening. The data at each time point shows the total colony number and percentage of white colonies for 3 replicates.

Table 1 Vector to Insert Ratio 1:1			**Table 1 Vector to Insert Ratio 1:3**		
Time	**Total Colonies**	**% White**	**Time**	**Total Colonies**	**% White**
15 min	272 ± 27	78± 2	15 min	104 ± 25	83± 5
30 min	168 ± 63	69± 7	30 min	122 ± 79	83± 8
1 hour	226 ±101	77± 9	1 hour	247 ±51	88± 1

Transform Competent Cells

Introduction

After ligating your insert into pCR®II , you are ready to transform the construct into competent *E. coli*. One Shot® cells are provided with Cat. nos. K2050-01, K2050-40, K2060-01, and K2060-40 to facilitate transformation. A protocol to transform One Shot® cells is provided in this section. To transform another competent strain, refer to the manufacturer's instructions.



Note

INVαF´ **does not** express the *lac* repressor. You may express your product from pCR®II in the absence of IPTG due to the presence of the *lac* promoter. IPTG will not have any effect on INVαF´ cells.

TOP10F´ **does** express the *lac* repressor (*lac*I^{q}), 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.

E. coli host strain

You may use any *rec*A, *end*A *E. coli* strain including TOP10, TOP10F´, INVαF´, DH5α™, or equivalent for transformation. Other strains are suitable. Refer to page 21 for a list of other available competent *E. coli*.

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.

If you amplified the PCR product from an ampicillin-resistant plasmid, use Kanamycin to select for transformants containing your pCR®II construct. Selecting with kanamycin will prevent contamination of the transformation reaction by the original ampicillin-resistant plasmid.

Transform Competent Cells, Continued

Materials supplied by the user

In addition to general microbiological supplies (e.g. plates, spreaders), you will need the following reagents and equipment. **Note:** The competent cells, SOC medium, and positive control are provided in kits with the One Shot chemically competent cells.

- Chemically competent *E. coli* suitable for transformation
- S.O.C. medium (warmed to room temperature)
- Positive control, optional (e.g. pUC19)
- LB plates containing 50 µg/mL kanamycin or 100 µg/mL ampicillin (two for each transformation)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Prepare for transformation

- Equilibrate a water bath to 42°C.
- Bring the S.O.C. medium to room temperature.
- If you are using INVαF´ cells, equilibrate LB plates containing antibiotic 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.
- If you are using TOP10F´ cells, equilibrate LB plates containing antibiotic 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.

One Shot® transformation protocol

Follow the protocol below to transform One Shot® Competent Cells. To transform another strain, refer to the manufacturer's instructions.

1. Centrifuge 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 transformation.
3. Pipet 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 the cells for 30 seconds at 42°C without shaking. Immediately transfer the vials to ice.
6. Add 250 µL of room temperature S.O.C. medium to each vial.
7. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 10–200 µL from each transformation vial on 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´ 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 S.O.C. to allow even spreading.
9. Incubate plates overnight at 37ºC. Transfer plates to 4°C for 2–3 hours to allow for proper color development.

Continued on next page

Transform Competent Cells, Continued

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.

Expected results

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

Analyze Transformants

Analyze positive clones

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 and analyze the plasmid by restriction mapping or sequencing for orientation of the insert. We recommend using the PureLink® HQ Mini Plasmid Purification Kit for purifying your plasmid DNA (see page 21 for ordering information).

Sequence the insert

If you wish to sequence your insert in pCR®II , you may use the Sp6 Promoter Primer or the M13 Reverse Primer to sequence into your insert from the *lac* promoter. To sequence into the insert from the *lac*Zα fragment, you can use either the T7 Promoter Primer or the M13 (–20) Forward Primer. Refer to the diagram on page 19 for the primer sequences and location of the primer binding sites. For information about our custom primer synthesis service, go to **www.lifetechnologies.com/support** or contact Technical Support (page 22).

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 14–15. These reactions will help you troubleshoot your experiment. Refer to the **Troubleshooting** section, page 10 for additional tips.

Long-term storage

After identifying the correct clone, purify the colony and prepare a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at –20°C.

1. Streak the original colony on LB plates containing 100 µg/mL ampicillin or 50 µg/mL kanamycin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 100 µg/mL ampicillin or 50 µg/mL kanamycin.
3. Grow until the culture reaches stationary phase.
4. Mix 0.85 mL of the culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Troubleshoot

Introduction

If you do not obtain the results you expect, use the following table to troubleshoot your experiment. We recommend performing the control reactions (pages 9–15) to help you evaluate your results.

Observation	Cause	Solution
No colonies obtained from transformation	Bacteria were not competent.	Use the pUC19 control vector included with the One Shot® Kit to test transformation efficiency.
	Incorrect concentration of antibiotic on plates or the plates are too old.	Use 100 µg/mL of ampicillin or 50 µg/mL kanamycin. Use fresh ampicillin plates (less than 1 month old).
White colonies do not have insert	Single 3´ T-overhangs on the vector degraded.	Use another tube of vector. Avoid storing the vector for longer than 6 months or subjecting it to repeated freeze/thaw cycles. Check the vector by performing the Self-Ligation Reaction, page 9.
Only white colonies obtained	No IPTG or X-Gal in plates.	Be sure to include X-Gal for blue/white screening and both IPTG and X-Gal if using TOP10F´.
Majority of colonies are blue or light blue with very few white colonies	The insert does not interrupt the reading frame of the *lac*Z gene.	If you have a small insert (less than 500 bp), you may have light blue colonies. Analyze blue colonies as they may contain insert.
	Used a polymerase that does not add 3´ A-overhangs.	Do not use proofreading polymerases such as Platinum® *Pfx* because they do not add 3´ A-overhangs. Use *Taq* polymerase.
	PCR products were gel-purified before ligation.	Gel purification can remove the single 3´ A-overhangs. If gel purification is needed, use nuclease-free solutions to purify fragment or optimize your PCR.
	The PCR products were stored for a long period of time before performing the ligation reaction.	Use fresh PCR products. Efficiencies are reduced after as little as 1 day of storage.
	Too much of the amplification reaction was added to the ligation.	The high salt content of PCR reactions can inhibit ligation. Do not use more than 2–3 µL of the PCR reaction in the ligation reaction.
	Incorrect molar ratio of vector: insert used in the ligation reaction.	Estimate the concentration of the PCR product. Set up the ligation reaction with a 1:1 or 1:3 vector to insert molar ratio.

Continued on next page

Troubleshoot, Continued

Observation	Cause	Solution
Some colonies have a light blue color or appear white with blue centers	Leaky expression of the *lac*Z fragment or only a partial disruption of *lac*Z by the insert.	If you are looking for a smaller size insert, 500 bp or less, analyze these colonies as they may contain insert.
White colonies or blue colonies of normal size are surrounded by smaller, white colonies	The smaller colonies are ampicillin-sensitive satellite colonies. Do not pick the small colonies as they do not contain any plasmid.	Use kanamycin selection. Be sure the stock solution of ampicillin and your plates are both fresh.
White colonies do not grow in liquid culture	Ampicillin-sensitive satellite colonies.	Be sure to pick large white colonies. Be sure the ampicillin is fresh. Use kanamycin to eliminate this problem.
No results from sequencing	Accidental use of the amplification primers in the kit for sequencing. These are for generating the control PCR product only.	Use the M13 Forward (−20) and Reverse Primers for sequencing. You may also use the T7 or SP6 promoter primer to sequence into the insert.
	The T7 primer used was not the right sequence.	Check the sequence of your T7 promoter primer and make sure it matches with the priming site on pCR®II .
No PCR product	Either the *Taq* polymerase is inactive or the conditions for your PCR are not optimal.	Perform the control reactions on pages 9–15 to test the activity of the *Taq* polymerase. If *Taq* polymerase is active, you may need to optimize the conditions for your PCR reaction.
Low plasmid yield	Cells do not grow well in LB.	Try using S.O.C. medium with the appropriate antibiotic.

Continued on next page

Explanation of control reactions

The following table describes the control reactions that can be performed to troubleshoot your TA Cloning® experiment and how to interpret the results from these control reactions.

Control Reaction	Explanation
Self-Ligation	This control reaction shows if pCR®II has lost the 3´ T-overhangs. Loss of the T-overhangs results in blunt-end ligation and disruption of the *lac*Zα reading frame. False white colonies will result. Normally, less than 5% of the colonies should be white.
Transformation Control	Tests the transformation efficiency of the One Shot® Competent Cells. The transformation efficiency should be 1×10^8 cfu/μg DNA for INVαF′ and 1×10^9 cfu/μg DNA for TOP10F′.
Control PCR Product	Tests the PCR reagents including *Taq* polymerase.
Control Ligation Reaction	Tests the ligation reagents and pCR®II . Up to 80% or greater white colonies can be produced and these colonies should contain vector with insert..

Appendix

Perform the Self-Ligation Reaction

Introduction

The TA Cloning® vector is stable for 6 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. Follow the protocol below to perform the self-ligation reaction and transform One Shot® Competent Cells. If you are using another *E. coli* strain, follow the manufacturer's instructions.

Procedure

1. Set up the 10 µL self-ligation reaction as follows:

Water	5 µL
5X ExpressLink™ T4 DNA Ligase Buffer	2 µL
pCR®II vector (25 ng/µL)	2 µL
ExpressLink™ T4 DNA Ligase (5 units)	1 µL
Total Volume	10 µL

2. Incubate the reaction at room temperature for 1 hour. Place the reaction on ice before transformation.
3. Thaw, on ice, one 50 µL vial of frozen One Shot® Competent Cells for each transformation.
4. Pipet 1 µL of the Control Ligation Reaction from step 1 of this procedure 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 the cells for 30 seconds at 42°C without shaking. Immediately transfer the vials to ice.
7. Add 250 µL of room temperature S.O.C. medium to the vial.
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 100 µg/mL ampicillin and X-Gal. Be sure to include IPTG if you are using TOP10F´.

Incubate the plates overnight at 37ºC.

Expected results

You should expect about 5–25 blue colonies from the 50 µL plated. There should be less than 5% white colonies which result from supercoiled pCR®II 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 *lac*Z gene, resulting in a "false" white or light blue colony with no insert.

Continued on the next page

Perform the Control Reactions

Introduction

We recommend performing the control reactions the first time you use the kit to help you evaluate results. Performing the control reactions involve producing a control PCR product using the reagents included in the kit and using this product in a ligation reaction.

Produce the control PCR product

Use *Taq* Polymerase and the protocol below to amplify the control PCR product.

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
Water	41.5 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. Overlay the reaction with 70 µL of mineral oil.
3. Amplify using the cycling parameters below:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1
Denature	1 minute	94°C	25
Anneal	1 minute	55°C	
Extend	1 minute	72°C	
Final Extension	7 minutes	72°C	1

4. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 700 bp band should be visible. Proceed to the **Control Ligation Reaction** on the next page.

Continued on next page

Perform the Control Reactions, Continued

Control ligation reaction

Using the control PCR product produced on page 14, set up the following control ligation reaction. In general, 1 µL of the Control PCR product should be sufficient for ligation. Alternatively, you may use the formula given on page 4 to estimate the amount of PCR product to ligate with 50 ng of pCR®II.

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

Water	4 µL
5X ExpressLink™ T4 DNA Ligase Buffer	2 µL
pCR®II vector (25 ng/µL)	2 µL
Control PCR Product	1 µL
ExpressLink™ T4 DNA Ligase (5 units)	1 µL
Total Volume	10 µL

2. Incubate the Control Ligation Reaction at room temperature for 1 hour (see notes on the next page).
3. Transform 1 µL of the Control Ligation Reaction into one vial of One Shot® Competent Cells or into another suitable competent *E. coli* strain.
4. Plate 10–50 µL of each transformation mix on LB agar plates containing 50 µg/mL kanamycin with X-Gal (and IPTG for TOP10F´ cells).
1. Incubate plates overnight at 37°C.

Transformation control

TA Cloning® Kits Dual Promoter supplied with One Shot® Competent Cells will also be supplied with pUC19 plasmid for use as a transformation control. Transform one vial of One Shot® cells with 10 pg of pUC19 using the protocol on page 7. Plate 10–50 µL of the transformation mixture on LB plates containing 100 µg/mL ampicillin. The transformation efficiency should be 1×10^9 cfu/µg DNA for TOP10F´ cells and 1×10^8 cfu/µg DNA for INVαF´.

Expected results

The Control Ligation Reaction should produce approximately 80% white colonies depending on the incubation time and vector to insert ratio (see next page). 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 **Perform the Self-Ligation Reaction**, on the previous page). If this occurs, use another vial of pCR®II and avoid repeated freeze-thaw cycles.

Add 3´ A-Overhangs

Introduction

Direct cloning of DNA amplified by proofreading polymerases into pCR®II is often difficult due to very low cloning efficiencies. These low efficiencies are caused by the 3´ to 5´ exonuclease proofreading activity that removes the 3´ A-overhangs necessary for TA Cloning®. We have 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 (see page 21) for optimal cloning of blunt PCR products.

Materials supplied by the user

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

Procedure

1. After amplifying with a proofreading polymerase, place the 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 the vials 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®II .

Notes about incubation times

Cloning efficiency can be optimized by changing incubation time and altering the vector to insert ratio. In the tables below, the ligation reactions were performed using a 1:1 vector to insert ratio **(Table 1)**, or using a 1:3 vector to insert ratio, which was achieved by reducing the pCR® II vector concentration to 25ng. **(Table 2)**.

Ligation reactions were incubated at room temperature for 15 minutes, 30 minutes and 1 hour, transformed into One Shot® Top10 cells and 50µl plated for blue/white screening. The data at each time point shows the total colony number and percentage of white colonies for 3 replicates.

Table 1 Vector to Insert Ratio 1:1			**Table 1 Vector to Insert Ratio 1:3**		
Time	**Total Colonies**	**% White**	**Time**	**Total Colonies**	**% White**
15 min	272 ± 27	78± 2	15 min	104 ± 25	83± 5
30 min	168 ± 63	69± 7	30 min	122 ± 79	83± 8
1 hour	226 ±101	77± 9	1 hour	247 ±51	88± 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 the solution to cool to 55°C and add antibiotic if needed.
4. Store LB medium at room temperature or at 4°C.

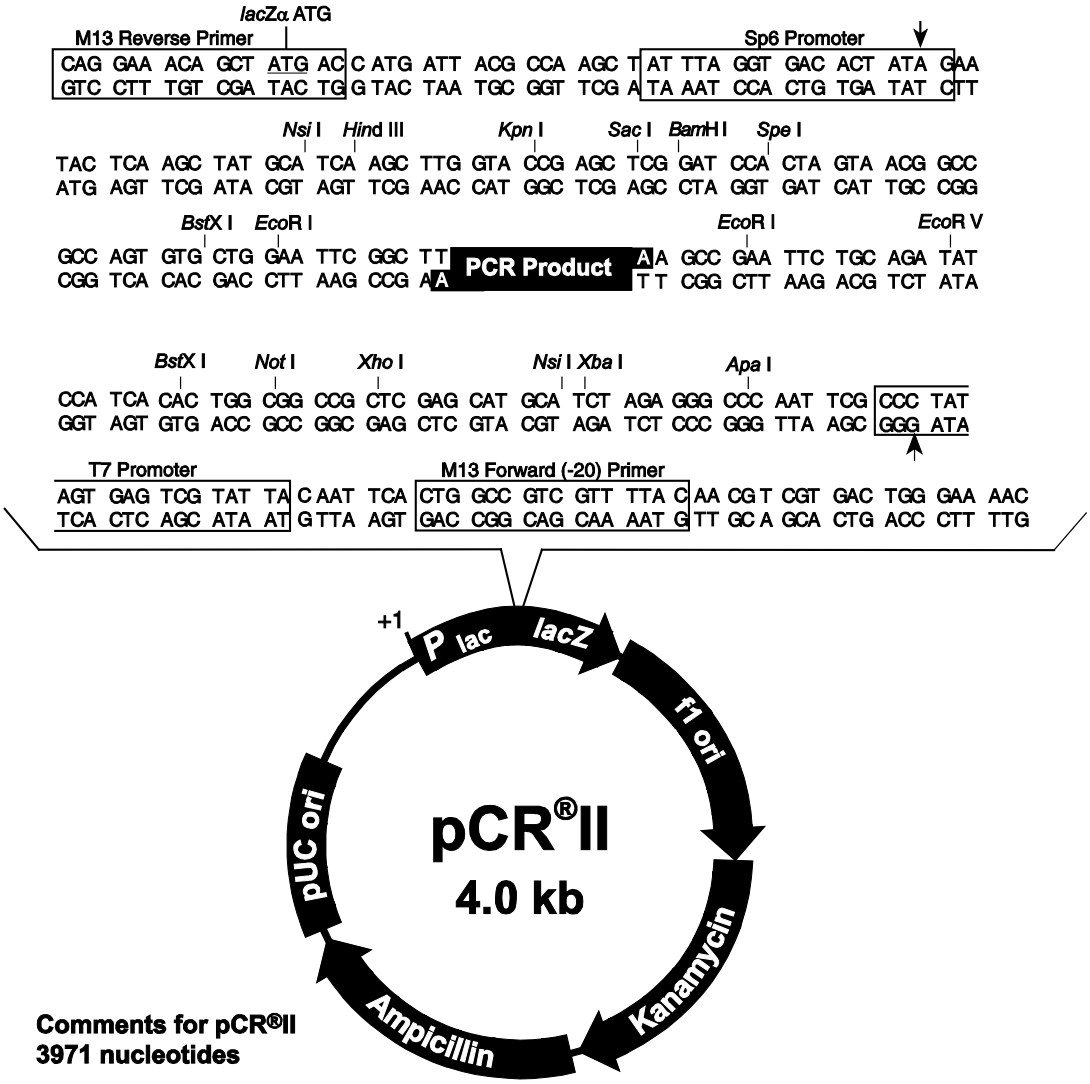
LB agar plates

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 the plates at 4°C.

Map and Features of pCR®II

Map of pCR®II

The map of the linearized vector, pCR®II , is shown below. The arrow indicates the start of transcription for the T7 RNA polymerase. The sequence of pCR®II is available at www.lifetechnologies.com or by contacting Technical Support (page 22).



Comments for pCR®II
3971 nucleotides

LacZα gene: bases 1-587
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
T7 promoter: bases 404-423
M13 (-20) Forward priming site: bases 431-446
f1 origin: bases 588-1025
Kanamycin resistance ORF: bases 1359-2153
Ampicillin resistance ORF: bases 2171-3031
pUC origin: bases 3176-3849

Continued on next page

Map and Features of pCR®II , Continued

Features of pCR®II

The following table describes the features of pCR®II . All features have been functionally tested.

Feature	Benefit
lac promoter	Allows bacterial expression of the *lac*Zα fragment for α-complementation (blue-white screening).
*lac*Zα fragment	Encodes the first 146 amino acids of β-galactosidase. Complementation in *trans* with the Ω fragment gives active β-galactosidase for blue-white screening.
Kanamycin resistance gene	Allows selection and maintenance in *E. coli;* useful when cloning products amplified from ampicillin-resistant plasmids.
Ampicillin resistance gene	Allows selection and maintenance in *E. coli.*
pUC origin	Allows replication, maintenance, and high copy number in *E. coli.*
Sp6 promoter and priming site	Allows *in vivo* or *in vitro* transcription of sense RNA. Allows sequencing of the insert.
T7 promoter and priming site	Allows *in vivo* or *in vitro* transcription of anti-sense RNA. Allows sequencing of the insert.
M13 Forward (–20) and M13 Reverse Priming Sites	Allows sequencing of the insert.
f1 origin	Allows rescue of sense strand for mutagenesis and single-strand sequencing.

Accessory Products

Additional products

Reagents supplied with the TA Cloning® Kit Dual Promoter and other reagents suitable for use with the kit are available separately. Ordering information is provided below.

Item	Quantity	Cat. no.
TA Cloning Dual Promoter Kit	40 reactions	K460040
ExpressLink™ T4 DNA Ligase	30 reactions	A13726
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
	5000 reactions	10966-083
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
IPTG	1 g	11529-019
X-gal	100 mg	15520-034
	1 g	15520-018
Bluo-gal	1 g	15519-028
Kanamycin	5 g	11815-024
	25 g	11815-032
Ampicillin	200 mg	11593-019
Zero Blunt® PCR Cloning Kit	20 reactions	K2700-20
	20 reactions	K2750-20

One Shot® competent cells

Chemically Competent *E. coli* are available separately in convenient One Shot® formats.

Item	Quantity	Cat. no.
One Shot® INVαF´ Chemically Competent *E. coli*	20 reactions	C2020-03
	40 reactions	C2020-06
One Shot® TOP10F´ Chemically Competent *E. coli*	20 reactions	C3030-03
	40 reactions	C3030-06
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03

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