TA Cloning® Kit

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TA Cloning® Kit

Catalog nos. K2000-01, K2000-40, K2020-20, K2020-40, K2030-01 K2030-40, K2040-01, K2040-40

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Important Information

Type of Kits

This manual is supplied with the following kits.

Kit	Quantity	Catalog no.
TA Cloning® Kit	20 reactions 40 reactions	K2020-20 K2020-40
TA Cloning® Kit with One Shot® INVαF´ Chemically Competent *E. coli*	20 reactions 40 reactions	K2000-01 K2000-40
TA Cloning® Kit with One Shot® TOP10F´ Chemically Competent *E. coli*	20 reactions 40 reactions	K2030-01 K2030-40
TA Cloning® Kit with One Shot® TOP10 Chemically Competent *E. coli*	20 reactions 40 reactions	K2040-01 K2040-40

Storage Instructions

The TA Cloning® Kits 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). Catalog nos. K2020-20 and K2020-40 are **not** supplied with One Shot® Competent Cells.

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

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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 -20°C.

Item	Composition	Amount
pCR®2.1, linearized	25 ng/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8	5 x 10 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
10X Ligation Buffer	60 mM Tris-HCl, pH 7.5 60 mM $MgCl_2$ 50 mM NaCl 1 mg/ml bovine serum albumin 70 mM β-mercaptoethanol 1 mM ATP 20 mM dithiothreitol 10 mM spermidine	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
T4 DNA Ligase	4.0 Weiss units/µl	25 µl
Sterile Water	Deionized, autoclaved water	1 ml
Control DNA Template	0.1 µg/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 8	10 µl
Control PCR Primers	0.1 µg/µl each in 10 mM Tris-HCl, 1 mM EDTA, pH 8	10 µl

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One Shot® Reagents

The table below describes the items included in the One Shot® competent cell kit. Catalog nos. K2020-20 and K2020-40 are **not** supplied with competent cells. Forty reaction kits are supplied as two 20 reaction kits.

The transformation efficiency for TOP10F´ and TOP10 cells is 1 x 10^9 cfu/μg DNA. The transformation efficiency for INVαF´ is 1 x 10^8 cfu/μg DNA.

Store competent cells at -80°C.

Component	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	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´, TOP10F´, **or** TOP10 cells	--	21 x 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

Genotype of TOP10

F^- *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

Accessory Products

Additional Products

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

Item	Amount	Catalog no.
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

One Shot® Competent Cells

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

Item	Amount	Catalog 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

Introduction

Overview

Purpose

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

Advantages

Using the TA Cloning® Kit:

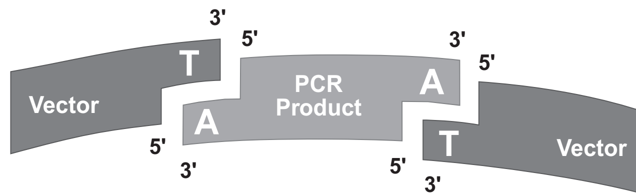
- Eliminates any enzymatic modifications of the PCR product
- Does not require the use of PCR primers that contain restriction sites

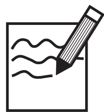
How TA Cloning® Works

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 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 as 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 incubation with *Taq* at the end of your cycling program. See page 14 for a protocol.

Alternatively, you may want to try the Zero Blunt® PCR Cloning Kit (Catalog 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 our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Experimental Outline

Introduction

To clone your gene of interest into pCR®2.1, you must first generate a PCR product. The PCR product is ligated into pCR®2.1 and transformed into competent cells. Since the PCR product can ligate into the vector in either orientation, individual recombinant plasmids need to be analyzed to confirm proper orientation. The correct recombinant plasmid is then purified for further subcloning or characterization.

Flow Chart

The table below describes the major steps necessary to clone your gene of interest into pCR®2.1.

Step	Action	Page
1	Amplify your PCR product using *Taq* polymerase and your own primers and parameters.	3
2	Ligate your PCR product into pCR®2.1.	4
3	Transform your ligation into competent *E. coli*.	5-6
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.	7

When using the TA Cloning® Kit for the first time, we recommend that you perform the control reactions to help you evaluate your results (pages 11-13).

Methods

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.

Materials Supplied by the User

You will need the following reagents and equipment.

- DNA template and primers for PCR product
- *Taq* polymerase and appropriate 10X PCR buffer (see page viii 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 available from Invitrogen (see page viii 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 14).

Producing 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
Sterile 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 your 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 (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Contact Technical Service for more information (page 18).

Cloning into pCR®2.1

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.

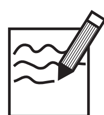
Take care when handling the pCR®2.1 vector as loss of the 3´ T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency.

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®2.1 vector:

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

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



Note

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.

Procedure

1. Centrifuge one vial of pCR®2.1 to collect all the liquid in the bottom of the vial.
2. Determine the volume of PCR sample needed to reach the required amount of PCR product (see above). Use sterile water to dilute your PCR sample if necessary.
3. 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

4. Incubate the ligation reaction at 14°C for a minimum of 4 hours (preferably overnight). Proceed to **Transforming Competent Cells**, next page.

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

Transforming Competent Cells

Introduction

Once you have a ligated your insert into pCR®2.1, you are ready to transform the construct into competent *E. coli*. One Shot® cells are provided with Catalog nos. K2000-01, K2000-40, K2030-01, K2030-40, K2040-01, K2040-40, K2040-01, and K2040-40 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´ and TOP10 *E. coli* **do not** express the *lac* repressor. You may express your product from pCR®2.1 in the absence of IPTG due to the presence of the *lac* promoter. IPTG will not have any affect on INVαF´ or TOP10 cells.

TOP10F´ **does** express the *lac* repressor (*lac*Iq), 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 viii for a list of competent *E. coli* available from Invitrogen.

If you amplified the PCR product from an ampicillin-resistant plasmid, use kanamycin to select for transformants containing your pCR®2.1 construct. Selecting with kanamycin will prevent contamination of the transformation reaction by the original ampicillin-resistant plasmid.

Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- 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

continued on next page

Transforming Competent Cells, continued

Preparing for Transformation

- Equilibrate a water bath to 42°C.
- Bring the S.O.C. medium to room temperature.
- If you are using INVαF´ or TOP10 cells, take LB plates containing antibiotic 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.
- 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.

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. 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 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 µl to 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´ 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 S.O.C. to allow even spreading.
9. Incubate plates overnight at 37ºC. 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.

Expected Results

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.

Analyzing Transformants

Analyzing 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 plasmid and analyze 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 viii for ordering information).

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. To sequence into the insert from the *lac*Zα fragment, you can use either the T7 Promoter Primer or the M13 Forward Primer. Refer to the diagram on page 16 for the primer sequences and location of the primer binding sites. For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 12-13. These reaction swill help you troubleshoot your experiment. Refer to the **Troubleshooting** section, page 8 for additional tips.

Long-Term Storage

Once you have identified the correct clone, be sure purify the colony and make 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 culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Troubleshooting

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 11-13) to help you evaluate your results.

Problem	Reason	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 (< 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 vector by performing the Self-Ligation Reaction, page 11.
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 (< 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* as 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:insert molar ratio.

continued on next page

Troubleshooting, continued

Problem	Reason	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 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®2.1.
	An Sp6 primer was used to sequence inserts in pCR®2.1.	Do not use an Sp6 primer to sequence pCR®2.1. There is no binding site for this primer.
No PCR product	Either the *Taq* polymerase is inactive or the conditions for your PCR are not optimal.	Perform the control reactions on pages 11-13 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®2.1 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. Transformation efficiency should be 1 x 10^8 cfu/μg DNA for INVαF′ and 1 x 10^9 cfu/μg DNA for TOP10 and TOP10F′.
Control PCR Product	Tests the PCR reagents including *Taq* polymerase.
Control Ligation Reaction	Tests the ligation reagents and pCR®2.1. Greater than 80% white colonies are produced and these colonies should contain vector with insert.

Appendix

Performing the Self-Ligation Reaction

Introduction

The TA Cloning® vector 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. 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:

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 transformation.
4. Pipette 1 µl of the Control Ligation Reaction from Step 1, above, 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 cells for 30 seconds at 42°C without shaking. Immediately transfer 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 100µg/ml ampicillin and X-Gal. Be sure to include IPTG if you are using TOP10F´.
10. Incubate 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®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 *lac*Z gene, resulting in a "false" white or light blue colony with no insert.

Performing the Control Reactions

Introduction

We recommend performing the control reactions the first time you use the kit to help you evaluate your 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.

Producing 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
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:

Step	Time	Temperature	Cycles
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

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**, next page.

continued on next page

Performing the Control Reactions, continued

Control Ligation Reaction

Using the control PCR product produced on the previous page, set up the following 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®2.1.

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

Sterile water	5 µl
10X Ligation Buffer	1 µl
pCR®2.1 vector (25 ng/µl)	2 µl
Control PCR Product	1 µl
T4 DNA Ligase	1 µl
Total Volume	10 µl

2. Incubate the Control Ligation Reaction at 14°C for a minimum of 4 hours (preferably overnight).
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).
5. Incubate plates overnight at 37°C.

Transformation Control

TA Cloning® Kits 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 6. Plate 10 µl to 50 µl of the transformation mixture on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be 1×10^9 cfu/µg DNA for TOP10F´ and TOP10 cells and 1×10^8 cfu/µg DNA for INVαF´.

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 **Performing the Self-Ligation Reaction**, page 11). If this occurs, use another vial of pCR®2.1 and avoid repeated freeze-thaw cycles.

Adding 3´ A-Overhangs

Introduction

Direct cloning of DNA amplified by proofreading 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 that removes 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 nos. K2700-20 and K2750-20) for optimal cloning of blunt PCR products.

Materials Supplied by the User

You will need the reagents and equipment.

- *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 a proofreading 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.

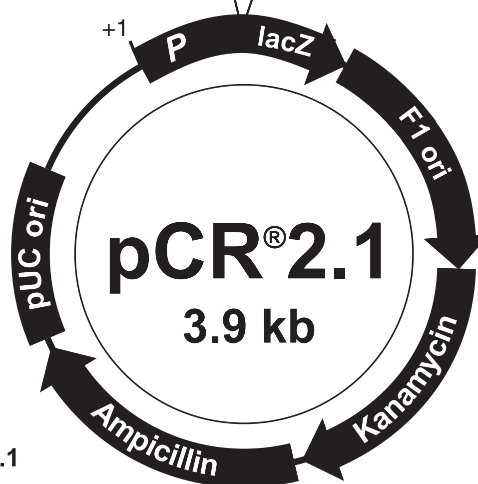
LB agar plates

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.

Map and Features of pCR®2.1

Map of pCR®2.1

The map of the linearized vector, pCR®2.1, is shown below. 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 18).**



Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
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

Map and Features of pCR®2.1, continued

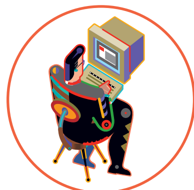
Features of pCR®2.1

The table below describes the features of pCR®2.1. 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.*
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.

Technical Service

World Wide Web



Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
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- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

continued on next page

Technical Service, continued

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Product Qualification

Restriction Digest

The parental supercoiled pCR®2.1 vector is qualified by restriction digest prior to TA adaptation. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

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 11-13, a 750 bp control PCR product is ligated into pCR®2.1 and 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*

All competent cells are qualified as follows:

- Competent cells are tested for transformation efficiency using the control plasmid included in the kit. 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:

  1×10^8 cfu/μg DNA for INVαF´

  1×10^9 cfu/μg DNA for TOP10F´

  1×10^9 cfu/μg DNA for TOP10
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates containing 100 μg/ml ampicillin, 25 μg/ml streptomycin, 50 μg/ml kanamycin, or 15 μg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

References

General Reference for PCR Technology

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.) (1990) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., San Diego, CA.

General Molecular Biology Techniques

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., eds. (1990) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Laboratory Press, Plainview, New York.

TA Cloning®

Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smeland, T. E., Cremers, F. P. M., and Goldstein, J. L. (1993) cDNA Cloning of Component A of Rab Geranylgeranyl Transferase and Demonstration of Its Role as a Rab Escort Protein. *Cell* **73**: 1091-1099.

Bates, M. D., Olsen, C. L., Becker, B. N., Albers, F. J., Middleton, J. P., Mulheron, J. G., Jin, S.-L. C., Conti, M., and Raymond, J. R. (1993) Elevation of cAMP Is Required for Down-regulation, but not Agonist-induced Desensitization, of Endogenous Dopamine D1 Receptors in Opossum Kidney Cells. *J. Biol. Chem.* **268**: 14757-14763.

Chen, Y., Weeks, J., Mortin, M. A., and Greenleaf, A. L. (1993) Mapping Mutations in Genes Encoding the Two Large Subunits of Drosophila RNA Polymerase II Defines Domains Essential for Basic Transcription Functions and for Proper Expression of Developmental Genes. *Molec. Cell. Biol.* **13**: 4214-4222.

Clark, J. M. (1988) Novel Non-Templated Nucleotide Addition Reactions Catalyzed by Procaryotic and Eucaryotic DNA Polymerases. *Nuc. Acids Res.* **16**: 9677-9686.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993) ARIA, a Protein that Stimulates Acetylcholine Receptor Synthesis, is a Member of the Neu Ligand Family. *Cell* **72**: 801-815.

Hake, L. E. and Hecht, N. B. (1993) Utilization of an Alternative Transcription Initiation Site of Somatic Cytochrome c in the Mouse Produces a Testis-Specific Cytochrome c mRNA. *J. Biol. Chem.* **268**: 4788-4797.

Liang, P. and Pardee, A. B. (1992) Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction. *Science* **257**: 967-973.

Lin, X.-L., Lin, Y.-Z., Koelsch, G., Gustchina, A., Wlodawer, A., and Tang, J. (1992) Enzymic Activities of Two-chain Pepsinogen, Two-chain Pepsin, and the Amino-terminal Lobe of Pepsinogen. *J. Biol. Chem.* **267**: 17257-17263.

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References, continued

Mahendroo, M. S., Mendelson, C. R., and Simpson, E. R. (1993) Tissue-specific and Hormonally Controlled Alternative Promoters Regulate Aromatase Cytochrome P450 Gene Expression in Human Adipose Tissue. *J. Biol. Chem.* **268**: 19463-19470.

Mallick, C. A., Dudley, E. C., Viney, J. L., Owen, M. J., and Hayday, A. C. (1993) Rearrangement and Diversity of T Cell Receptor β Chain Genes in Thymocytes: A Critical Role for the β Chain in Development. *Cell* **73**: 513-519.

Mead, D. A., Pey, N. K., Herrnstadt, C., Marcil, R. A., and Smith, L. M. (1991) A Universal Method for the Direct Cloning of PCR Amplified Nucleic Acid. *Bio/Technology* **9**: 657-663.

Miller, W. H., Kakizuka, A., Frankel, S. Warrell, R. P., DeBlasio, A., Levine, K., Evans, R. M., and Dmitrovsky, E. (1992) Reverse Transcription Polymerase Chain Reaction for the Rearranged Retinoic Acid Receptor α Clarifies Diagnosis and Detects Minimal Residual Disease in Acute Promyelocytic Leukemia. *Proc. Natl. Acad. Sci USA* **89**: 2694-2698.

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