TOPO TA Cloning®

Five-minute cloning of *Taq* polymerase-amplified PCR products

Catalog nos. K4500-01, K4500-40, K4520-01, K4520-40, K4550-01, K4550-40, K4560-01 (pCR®2.1-TOPO®)

Catalog nos. K4600-01, K4600-40, K4620-01, K4620-40, K4650-01, K4650-40, K4660-01 (pCR®II-TOPO®)

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Kit Contents and Storage

Shipping and Storage

TOPO TA Cloning® Kits are shipped on dry ice. Each kit contains a box with TOPO TA Cloning® reagents (Box 1) and a box with One Shot® Chemically Competent or Electrocomp™ cells (Box 2). Store Box 1 at -20°C and **Box 2 at -80°C.**

Types of TOPO TA Cloning® Kits

TOPO TA Cloning® Kits are available with either pCR®2.1-TOPO® or pCR®II-TOPO® and either DH5α™-T1^R, TOP10 or TOP10F′ One Shot® Chemically Competent cells or TOP10 One Shot® Electrocomp™ cells (see page vi for the genotypes of the strains).

Product	Reactions	One Shot® Cells	Type of Cells	Catalog no.
TOPO TA Cloning® Kit (containing pCR®2.1-TOPO®)	20	TOP10	chem. competent	K4500-01
	40	TOP10	chem. competent	K4500-40
	20	DH5α-T1^R	chem. competent	K4520-01
	40	DH5α-T1^R	chem. competent	K4520-40
	20	TOP10F′	chem. competent	K4550-01
	40	TOP10F′	chem. competent	K4550-40
	20	TOP10	electrocompetent	K4560-01
TOPO TA Cloning® Kit Dual Promoter (containing pCR®II-TOPO®)	20	TOP10	chem. competent	K4600-01
	40	TOP10	chem. competent	K4600-40
	20	DH5α-T1^R	chem. competent	K4620-01
	40	DH5α-T1^R	chem. competent	K4620-40
	20	TOP10F′	chem. competent	K4650-01
	40	TOP10F′	chem. competent	K4650-40
	20	TOP10	electrocompetent	K4660-01

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Kit Contents and Storage, continued

TOPO TA Cloning® Reagents

TOPO TA Cloning® reagents (Box 1) are listed below. **Please note that the user must supply *Taq* polymerase.** Store Box 1 at -20°C.

Item	Concentration	Amount
pCR®2.1-TOPO® or pCR®II-TOPO®	10 ng/µl plasmid DNA in:; 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA phenol red	20 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
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
M13 Forward (-20) Primer	0.1 µg/µl in TE Buffer	20 µl
M13 Reverse Primer	0.1 µg/µl in TE Buffer	20 µl
Control Template	0.1 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer	10 µl
Sterile Water	--	1 ml

Sequence of Primers

The table below describes the sequence and pmoles supplied of the sequencing primers included in this kit.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

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Kit Contents and Storage, continued

One Shot® Reagents

The table below describes the items included in each One Shot® competent cell kit. Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
DH5α-T1^R **OR** TOP10 cells **OR** TOP10F´ cells	Chemically Competent Chemically Competent or Electrocomp™ Chemically Competent only	21 x 50 µl
pUC18 **OR** pUC19 Control DNA	10 ng/µl in TE, pH 8 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	10 µl 50 µl

Genotypes of *E. coli* Strains

DH5α-T1^R: Use this strain for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage.

F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A (confers resistance to phage T1)

TOP10: Use this strain for general cloning and blue/white screening without IPTG.

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

TOP10F´: This strain overexpresses the Lac repressor (*lac*I^q gene). For blue/white screening, you will need to add IPTG to the plates to obtain expression from the *lac* promoter. This strain contains the F episome and can be used for single-strand rescue of plasmid DNA containing an f1 origin.

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

Qualifying the Product

Restriction Digest

Supercoiled pCR®2.1-TOPO® and pCR®II-TOPO® are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pCR®2.1-TOPO®	pCR®II-TOPO®
*Hin*d III (linearizes)	3929 bp	3971 bp
Xba I (linearizes)	3929 bp	3971 bp
Nsi I	3929 bp	112, 3859 bp
Pst I	1190, 2739 bp	1190, 2781 bp
*Eco*R I and *Afl* III	16, 408, 716, 2789 bp	16, 450, 716, 2789 bp

TOPO® Cloning Efficiency

Once the vectors have been adapted with topoisomerase I, they are lot-qualified using the control reagents included in the kit. Under conditions described on pages 12-14, a 750 bp control PCR product was TOPO®-Cloned into each vector and subsequently transformed into the One Shot® competent *E. coli* included with the kit.

Each lot of vector should yield greater than 95% cloning efficiency.

Primers

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

One Shot® Competent *E. coli*

All competent cells are tested for transformation efficiency using the 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 10^9 cfu/µg DNA.

In addition, untransformed cells are tested for appropriate antibiotic sensitivity and lack of phage contamination. DH5α-$T1^R$ is also tested for resistance to phage T5, a standard test for resistance to phage T1.

Purchaser Notification

Cloning Technology Label License

The consideration paid for Cloning Technology products (e.g., TOPO® Cloning, TOPO TA Cloning®, TA Cloning®, TOPO® Tools, Directional TOPO® Cloning, Zero Background™, GATEWAY™ Cloning Systems and Echo™ Cloning Systems) grants a Limited License with a paid up royalty to use the product pursuant to the terms set forth in the accompanying Limited Label License (see below).

The Cloning Technology products and their use are the subject of U.S. Patent Nos. 5,888,732, 6,143,557, 6,171,861, 5,766,891, 5,487,993, 5,827,657, 5,910,438, 6,180,407, 5,851,808, and/or other pending U.S. and foreign patent applications owned by or licensed to Invitrogen Corporation. Use of these products requires a license from Invitrogen. Certain limited nontransferable rights are acquired with the purchase of these products (see below).

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Methods

Overview

Introduction

TOPO TA Cloning®* provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

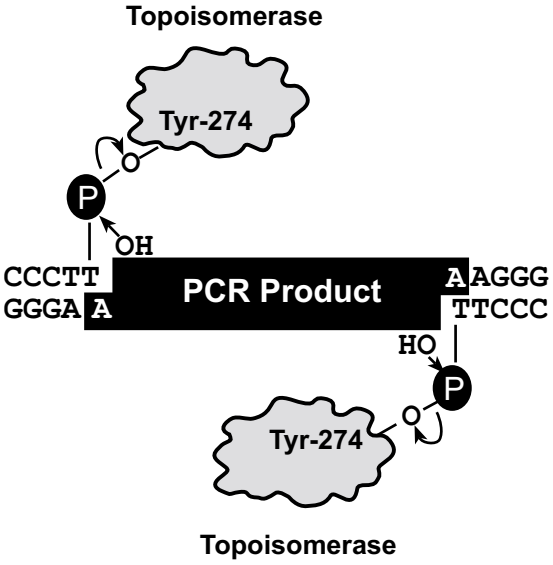
How It Works

The plasmid vector (pCR®II-TOPO® or pCR®2.1-TOPO®) is supplied linearized with:

- Single 3′-thymidine (T) overhangs for TA Cloning®
- Topoisomerase I covalently bound to the vector (referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994).



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Overview, continued

Experimental Outline

- Produce Your PCR Product
- Set Up TOPO® Cloning Reaction (Mix Together PCR Product and TOPO® Vector)
- Incubate 5 Minutes at Room Temperature
- Transform TOPO® Cloning Reaction into One Shot® Competent Cells
- Select and Analyze 10 White or Light Blue Colonies for Insert

Producing PCR Products

Introduction

It is important to properly design your PCR primers to ensure that you obtain the product you need for your studies. Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product. **Remember that your PCR product will have single 3′ adenine overhangs.**

Note

Do not add 5′ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®2.1-TOPO® or pCR®II-TOPO®.

Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3′ A-overhangs on the PCR product (i.e. Expand™ or eLONGase™).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 17.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3′ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers (100-200 ng each)	1 µM each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, please refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the TOPO TA Cloning® Kit (see page 15). 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. Please call Technical Service for more information (page 19).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into pCR®2.1-TOPO® or pCR®II-TOPO®, and transform the recombinant vector competent *E. coli* in one day. To ensure that you obtain the best possible results, it is important to have everything you need set up and ready to use. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 12-14 in parallel with your samples.

Note

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl; 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Inclusion of salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Please note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells or electrocompetent cells (see below).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Please read the following information carefully.

Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

Helpful Hint

If you use a plasmid template for your PCR that carries either the ampicillin or kanamycin resistance marker, we recommend that you use the other selection agent to select for transformants. For example, if the plasmid template contains the ampicillin resistance marker, then use kanamycin to select for transformants. The template is carried over into the TOPO® Cloning and transformation reactions, resulting in transformants that are ampicillin resistant and white, but are not the desired construct.

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TOPO® Cloning Reaction and Transformation, continued

Materials Supplied by the User

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

- Sterile microcentrifuge tubes
- 15 ml snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath or an electroporator and 0.1 or 0.2 cm cuvettes
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 40 mg/ml X-gal in dimethylformamide (DMF)
- 100 mM IPTG in water (for use with TOP10F´)
- 37°C shaking and non-shaking incubator

Preparation for Transformation

For each transformation, you will need one vial of competent cells and **two** selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g. add 5 µl of the Salt Solution to 15 µl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Spread 40 µl of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- For TOP10F´ cells, spread 40 µl of 100 mM IPTG in addition to X-gal on each LB plate and incubate at 37°C until ready for use. IPTG is required for blue/white screening.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent or electrocompetent TOP10 or chemically competent DH5α-T1^R or TOP10F′ One Shot® *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 9. **Note**: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution	--	1 µl
Sterile Water	add to a total volume of 5 µl	add to a total volume of 5 µl
TOPO® vector	1 µl	1 µl
Final Volume	**6 µl**	**6 µl**

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

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TOPO® Cloning Reaction and Transformation, continued

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for **5 minutes** at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to either the **One Shot® Chemical Transformation (below) or One Shot® Electroporation (next page)**. **Note**: You may store the TOPO® Cloning reaction at -20°C overnight.

One Shot® Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2 above into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion (see page 9).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 white or light blue colonies for analysis (see **Analysis of Positive Clones**, next page). Do not pick dark blue colonies.

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TOPO® Cloning Reaction and Transformation, continued

One Shot® Electroporation

1. Add 2 µl of the TOPO® Cloning reaction into a vial (50 µl) of One Shot® Electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Carefully transfer solution to a 0.1 cm cuvette to avoid formation of bubbles.
3. Electroporate your samples using your own protocol and your electroporator. **Note**: If you have problems with arcing, see below.
4. Immediately add 250 µl of room temperature SOC medium.
5. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
6. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
7. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 white or light blue colonies for analysis (see **Analysis of Positive Clones**, below). Do not pick dark blue colonies.

Note

Addition of the Dilute Salt Solution in the **TOPO® Cloning Reaction** brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try **one** of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analysis of Positive Clones

1. Take the 10 white or light blue colonies and culture them overnight in LB medium containing 50 µg/ml ampicillin or kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01)or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis (digest with *Eco*R I or refer to the vector map accompanying the manual for alternate sites) or by sequencing. M13 Forward (-20) and M13 Reverse primers are included to help you sequence your insert. Please refer to the maps on page 10 (pCR®2.1-TOPO®) or page 11 (pCR®II-TOPO®) for sequence surrounding the TOPO TA Cloning® site. For the full sequence of either vector, please see our Web site or contact Technical Service (page 19).

   If you need help with setting up restriction enzyme digests or DNA sequencing, please refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. Use either the M13 Forward (-20) or the M13 Reverse primer and a primer that hybridizes to your insert as PCR primers. If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that PCR gives you the correct result. False positive and false negative results can be obtained because of mispriming or contaminating template.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume. Multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. Don't forget to patch the colony onto a separate plate to preserve it for future use.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at +4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 12-14. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to 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 out on LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin or 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.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

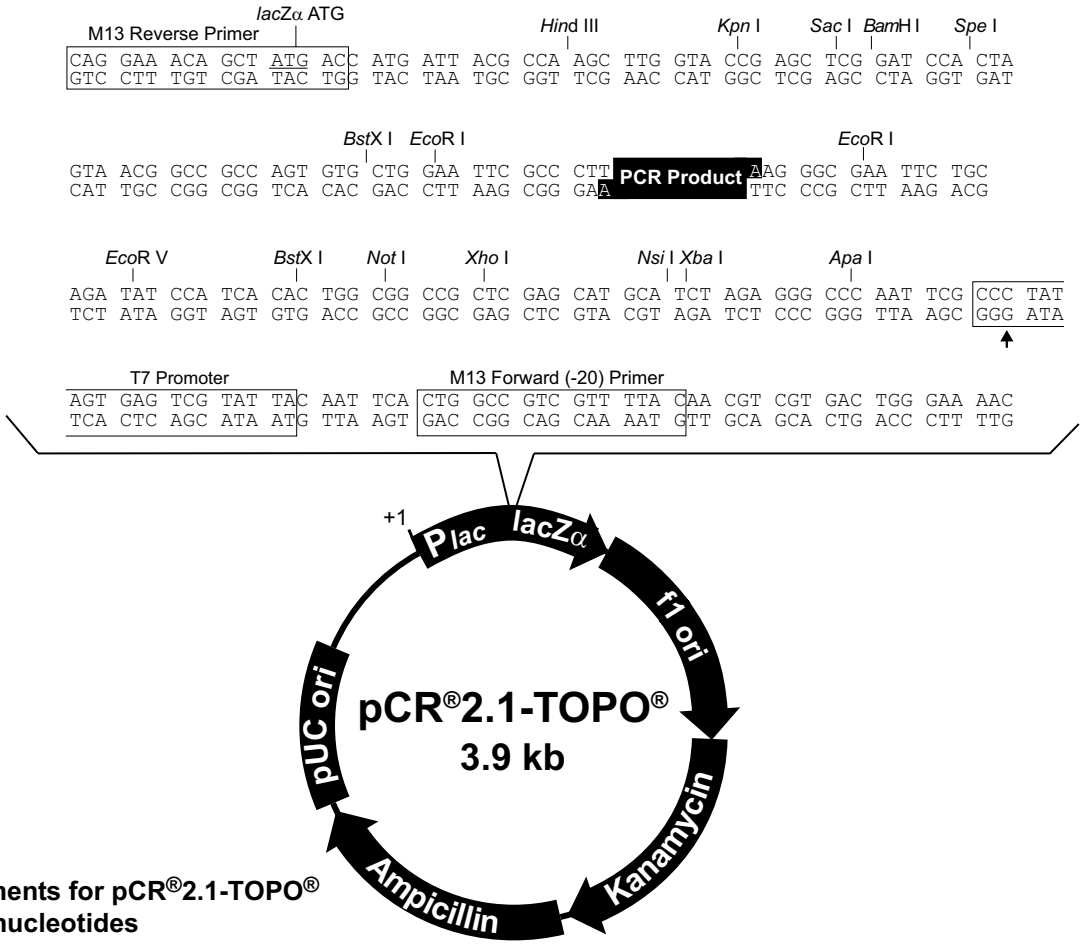
To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Map of pCR®2.1-TOPO®

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. For the full sequence of the vector, you may download it from our Web site or call Technical Service (page 19).



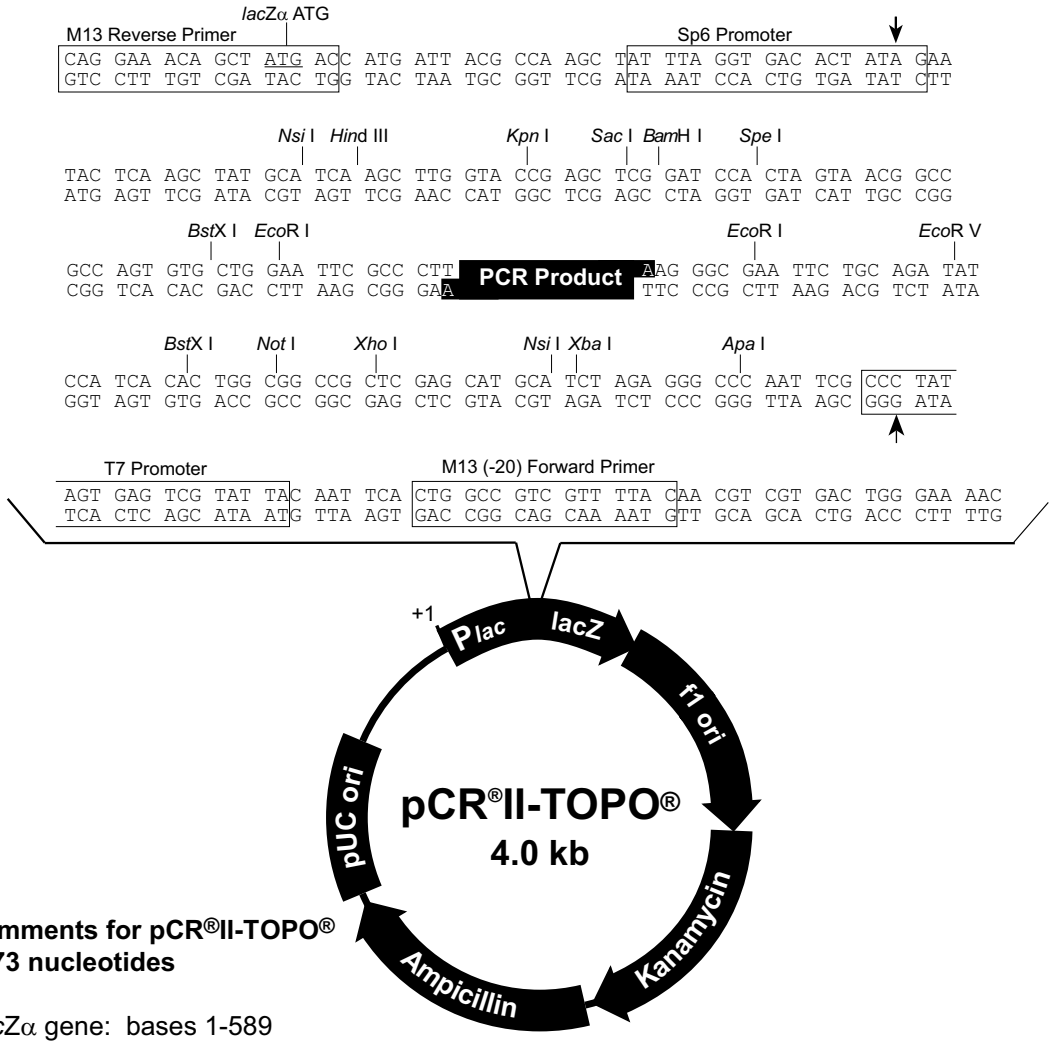
Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Map of pCR®II-TOPO®

pCR®II-TOPO® Map

The map below shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymerases. For the full sequence of the vector, you may download it from our Web site or call Technical Service (page 19).



Comments for pCR®II-TOPO®
3973 nucleotides

*Lac*Zα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

TOPO TA Cloning® Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using it directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Note: Do not use plates containing ampicillin. The control template is a plasmid that encodes ampicillin resistance. This template is carried over into the TOPO® Cloning and transformation reactions. Transformants carrying this plasmid will also be ampicillin resistant and white, resulting in an apparent increase in TOPO® Cloning efficiency, but upon analysis, colonies do not contain the desired construct.

Producing Control PCR Product

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minute	94°C	1X
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 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

TOPO TA Cloning® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Control PCR Product	--	1 µl
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of DH5α-T1^R, TOP10, or TOP10F´ One Shot® cells (pages 6-7).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin and X-Gal (and IPTG, if using TOP10F´ cells) (see page 18). 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.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than ninety-five percent of these colonies will be white and 90% (or more) of these will contain the 750 bp insert when analyzed by *Eco*R I digestion and agarose gel electrophoresis.

Relatively few colonies will be produced in the vector-only reaction and most of these will be dark blue. You may observe a few white colonies. This results from removal of the 3´ deoxythymidine overhangs creating a blunt-end vector. Ligation (re-joining) of the blunt ends will result in disruption of the LacZα reading frame leading to the production of white colonies.

Transformation Control

Either pUC18 or pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform with 10 pg per 50 µl of cells using the protocols on page 6 or 7. **pUC18 will need to be diluted with sterile water or TE before use**.

Use LB plates containing 100 µg/ml ampicillin. Just before plating the transformation mix for electrocompetent cells, dilute 10 µl of the mix with 90 µl of SOC.

Type of Cells	Volume to Plate	Transformation Efficiency
Chemically Competent	10 µl + 20 µl SOC	~1 x 10^9 cfu/µg DNA
Electrocompetent	20 µl (1:10 dilution)	> 1 x 10^9 cfu/µg DNA

continued on next page

TOPO TA Cloning® Control Reactions, continued

Factors Affecting Cloning Efficiency

Please note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 95% (or more) cloning efficiency.

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>1 kb)	Try one or all of the following: Increase amount of insert. Incubate the TOPO® Cloning reaction longer. Gel-purify the insert as described on page 15.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning blunt-ended fragments	Add 3′ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (page 17).
	Use the Zero Blunt® PCR Cloning Kit to clone blunt PCR products (Catalog no. K2700-20).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 15).
PCR product does not contain sufficient 3′ A-overhangs even though you used *Taq* polymerase	Increase the final extension time to ensure all 3′ ends are adenylated. *Taq* polymerase is less efficient at adding a nontemplate 3′ A next to another A. *Taq* is most efficient at adding a nontemplate 3′ A next to a C. You may have to redesign your primers so that they contain a 5′ G instead of a 5′ T (Brownstein *et al.*, 1996).

Appendix

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Please refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are provided below for your convenience.

Using the S.N.A.P.™ MiniPrep Kit

The S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) allows you to rapidly purify PCR products from regular agarose gels. You will need to prepare 6 M sodium iodide, 10 mM sodium sulfite solution in sterile water before starting. Sodium sulfite prevents oxidation of NaI.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE. Borate interferes with the NaI step (Step 2).
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M NaI.
3. Add 1.5 volumes Binding Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at full speed in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the supernatant. Repeat.
8. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on pages 5-6.

Quick S.N.A.P.™ Method

An easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 5). Be sure to make the gel slice as small as possible for best results.

continued on next page

Purifying PCR Products, continued

Low-Melt Agarose Method

Please note that gel purification will result in a dilution of your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese all of your PCR reaction on a low-melt TAE agarose gel (0.8 to 1.2%).
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µl of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 5).
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes.** This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into chemically competent One Shot® cells using the method on page 6.

Note

Please note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. This is because proof-reading polymerases remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

You will need the following items:

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

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

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. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu*. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C and use as is in the TOPO® Cloning reaction.

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 psi. Allow solution to cool to 55°C and add antibiotic if needed (50 µg/ml of either ampicillin or kanamycin).
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 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml of either ampicillin or kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

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, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light. **Note**: If you need to add IPTG, see below.

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.
3. Add 40 µl of the IPTG stock solution onto the center of the plate and spread evenly with a sterile spreader.
4. Allow the solution to diffuse into the plate by incubating at 37°C for 20-30 minutes. Plates are now ready for use.

Technical Service

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Technical Service, continued

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