TOPO® Reporter Kits

Five-minute Cloning of PCR Products for Analysis of Promoter Function in Mammalian Cells

Catalog nos. K4830-01, K4831-01

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User Manual

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Kit Contents and Storage

Shipping and Storage

Each TOPO® Reporter Kit is shipped on dry ice. Each kit contains a box with TOPO® Reporter reagents (Box 1) and a box with One Shot® TOP10 chemically competent cells (Box 2). Store Box 1 at -20°C and **Box 2 at -80°C.**

TOPO® Reporter Kits

Ordering information for the TOPO® Reporter Kits is provided below.

Kit	Vector	Reactions	Catalog no.
pBlue TOPO® TA Expression Kit	pBlue-TOPO®	20	K4831-01
pGlow TOPO® TA Expression Kit	pGlow-TOPO®	20	K4830-01

TOPO® Reporter Reagents

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

Item	Concentration	Amount
pBlue-TOPO® **or** pGlow-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 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
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
Salt Solution	1.2 M NaCl; 0.06 M $MgCl_2$	50 µl
T7 Sequencing Primer	0.1 µg/µl in TE Buffer	20 µl
LacZ Reverse **or** GFP Reverse Sequencing Primer	0.1 µg/µl in TE Buffer	20 µl
Control PCR Template	0.05 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer	10 µl
Sterile Water	--	1 ml

Continued on next page

One Shot® Reagents

The table below describes the items included in the One Shot® TOP10 chemically 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
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

Sequencing Primers

The table below provides the sequence and pmoles of the T7, *LacZ* Reverse, and GFP Reverse sequencing primers.

Primer	Sequence	Amount
T7	5´-TAATACGACTCACTATAGGG-3´	328 pmoles
LacZ Reverse	5´-CAGTCATGCTAGCCATACC-3´	350 pmoles
GFP Reverse	5´-GGGTAAGCTTTCCGTATGTAGC-3´	296 pmoles

Genotype of TOP10 Cells

TOP10: Use this strain for general cloning. Please note that this strain cannot be used for single-strand rescue of DNA.

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

The table below lists additional products available from Invitrogen which you may use in conjunction with the TOPO® Reporter Kit.

Item	Amount	Catalog no.
One Shot® Kit (TOP10 **Electrocompetent** Cells)	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® Kit (TOP10 **Chemically Competent** Cells)	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
T7 Promoter Primer	2 µg (328 pmoles)	N560-02
S.N.A.P.™ MiniPrep Kit	20 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Anti-Xpress™ Antibody	25 Westerns	R910-25
β-galactosidase Antiserum	25 Westerns	R901-25
GFP Antiserum	25 Westerns	R970-01
β-Gal Assay Kit	100-400 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01

Methods

Overview

Introduction

TOPO® Reporter Kits provide a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of promoter sequences amplified by *Taq* polymerase into a reporter vector. Recombinant vectors can then be transfected into mammalian cells or transformed into *E. coli* (pGlow-TOPO only) and tested for promoter function *in vivo* or *in vitro*. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

Two types of TOPO® Reporter Kits are discussed in this manual:

- pBlue TOPO® TA Expression Kit containing pBlue-TOPO® for insertion of promoter sequences upstream of the *E. coli* β-galactosidase gene (*lacZ*) for *in vitro* or *in vivo* assay.
- pGlow TOPO® TA Expression Kit containing pGlow-TOPO® for insertion of promoter sequences upstream of GFP for *in vitro* or *in vivo* assay of promoter function.

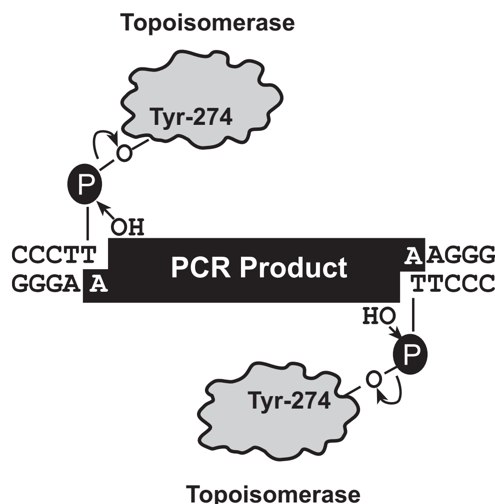
How It Works

Each reporter vector (pBlue-TOPO® or pGlow-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 products to ligate efficiently into the vector.

TOPO® Cloning exploits the ligation activity of topoisomerase I by providing an "activated", linearized TA vector using proprietary technology (Shuman, 1994). Ligation of the vector with a PCR product containing 3´ A-overhangs is very efficient and occurs spontaneously within 5 minutes at room temperature. The TOPO® Cloning Reaction can be transformed into chemically competent cells (provided) or electroporated directly into electrocompetent cells (available separately, see page vi).



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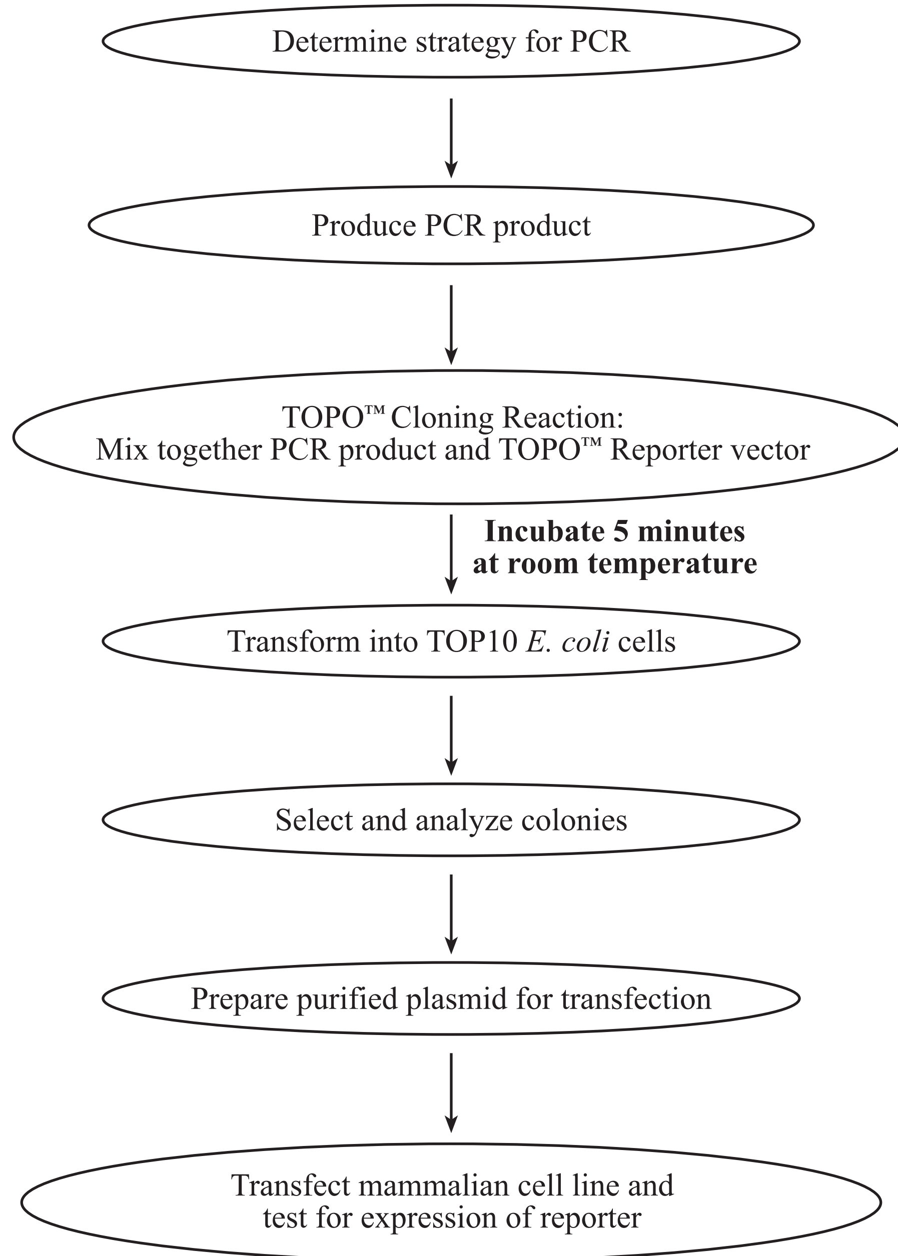
Experimental Outline

The flow chart below outlines the experimental steps necessary to clone and test your promoter sequences.



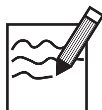
General Cloning Considerations

Introduction

In general, promoter reporter vectors can be used to analyze--

- Tissue and cell-specific promoter function
- Transcriptional enhancers in a known promoter
- Deletions within a promoter

Please note that each TOPO® Reporter vector contains not only a TOPO® Cloning site but also additional unique restriction sites upstream of the TOPO® Cloning site. These may be exploited to analyze promoter function.



Note

When analyzing promoters in a reporter vector, it is important to realize that sequences within the native gene can influence regulation of its own promoter. In addition, sequences within the reporter gene can also affect transcription from the promoter under study. We recommend that you verify any observations of transcriptional control of the fusion gene with expression of the native gene. S1 mapping (*Current Protocols in Molecular Biology*, pages 4.6.1 to 4.6.13) can be used to confirm that the subcloned promoter initiates transcription at the correct site.

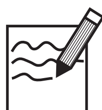
Important

Since initiation of translation in eukaryotes occurs at the first available AUG codon, it is important that there are no AUG codons between the start of transcription and the AUG of the reporter gene.

PCR Primer Design

Use the diagrams on pages 4 and 5 and the sequence of your promoter to design PCR primers. Unique restriction sites may be included in the 5´ and 3´ primers to excise the fragment or facilitate analysis once it is TOPO® Cloned.

For analysis of promoter function in *E. coli*, please use pGlow-TOPO and read page 5 before designing your primers.



Note

Once you have decided on the sequence of your PCR primers, do not add 5´ phosphates to your primers. Phosphates will inhibit topoisomerase I and the synthesized PCR product will not ligate into the TOPO® Reporter vectors. Please note that cloning efficiencies may vary depending on the 5´ nucleotide in the primers (see page 19).

Cloning into pBlue-TOPO®

Important

There is a cryptic prokaryotic promoter upstream of the *lacZ* reporter gene. *E. coli* transformants may appear to be light blue when screened on plates containing X-Gal. We do not recommend using pBlue-TOPO® to evaluate promoter function in *E. coli*. Please use pGlow-TOPO® for these studies (next page). Please note that background expression of β-galactosidase does not occur in mammalian cells.

TOPO® Cloning Site of pBlue-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 116 and 117. This is the TOPO® Cloning site. **Please note that the full sequence of pBlue-TOPO® may be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 27).** A map of pBlue-TOPO® is provided on page 23.

```
                 Bgl II*   T7 promoter/priming site                           Afl II Hind III* BamH I
1     GACGGATCGG GAGATCTAAT ACGACTCACT ATAGGGAGAC CCAAGCTGGC TAGCGTTTAA ACTTAAGCTT GGTACCGAGC

               Spe I                                               Pst I* Hind III* Nco I*  Start of lacZ fusion
81    TCGGATCCAC TAGTCCAGTG TGGTGGAATT GCCCTT  PCR   AAGG GCAATTCTGC AGAAAGCTTA CC ATG GGG
                               ... CGGGAA Product TTCC ...                        Met Gly

                  Polyhistidine region            LacZ Reverse priming site
149   GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT
      Gly Ser His His His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp

             Xpress™ epitope                                              lacZ ORF
215   CTG TAC GAC GAT GAC GAT AAG GTA CCT AAG GAT CAG CTT GGA GTT GAT CCC GTC GTT TTA CAA CGT
      Leu Tyr Asp Asp Asp Asp Lys Val Pro Lys Asp Gln Leu Gly Val Asp Pro Val Val Leu Gln Arg
                 EK Recognition site

281   CGT GAC TGG GAA AAC CCT ...
      Arg Asp Trp Glu Asn Pro ...
```

*These sites are not unique, but they may be used to excise the promoter sequence after TOPO® Cloning.

Cloning into pGlow-TOPO®

Using pGlow-TOPO in *E. coli*

To use pGlow-TOPO as a reporter in *E. coli*, you must engineer your PCR product to ensure expression of GFP in the event that the sequences you are testing contain a promoter. Please note that there is no prokaryotic ribosomal binding site upstream of the GFP initiation codon. Your reverse PCR primer must include a ribosomal binding site (-AGGA-) and an initiation codon (ATG) in frame with the GFP initiation codon. Allow 8-12 nucleotides between the ribosomal binding site and the initiation codon to ensure proper spacing. There should not be any palindromic sequences within this region. Successful expression of GFP will result in additional amino acids at the N-terminus. This has been shown not to affect fluorescence. We recommend that you use a known promoter as a positive control and DNA sequences that do not contain a promoter as a negative control.

TOPO® Cloning Site of pGlow-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 116 and 117. This is the TOPO® Cloning site. **Please note that the full sequence of pGlow-TOPO® may be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page** Error! Bookmark not defined.**).** A map of pGlow-TOPO® is provided on page 24.

```
                 Bgl II*  T7 promoter/priming site                              Pme I*  Afl II    Asp718 I  Kpn I
1    GACGGATCGG GAGATCTAAT ACGACTCACT ATAGGGAGAC CCAAGCTGGC TAGCGTTTAA ACTTAAGCTT GGTACCGAGC

               Spe I            BstX I*                                    Bgl II*  Pst I* Xba I*     GFP ORF
81   TCGGATCCAC TAGTCCAGTG TGGTGGAATT GCCCTT  PCR   AAGG GCAATTCTGC AGATCTAGA ATG GCT AGC
                                 ... CGGGAA Product TTCC ...                   Met Ala Ser

149  AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAT GGG
     Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly
                                                         GFP Reverse priming site
215  CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT GAT GCT ACA TAC GGA AAG CTT ACC CTT AAA TTT
     His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe

281  ATT TGC ACT ACT GGA AAA ...
     Ile Cys Thr Thr Gly Lys ...
```

*These sites are not unique, but they may be used to excise the promoter sequence after TOPO® Cloning.

Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

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.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 22.

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
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.

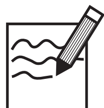
Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the TOPO® Reporter Kits (see page 20). 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 **Error! Bookmark not defined.**).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into either pBlue-TOPO® or pGlow-TOPO®, and transform the recombinant vector into *E. coli* all in one day. It is important to have everything you need set up and ready to use to ensure you obtain the best possible results. If this is the first time you have TOPO® Cloned, you may wish to perform the control reactions on pages 17-19 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 (provided) 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).

Materials Supplied by the User

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

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubator

Continued on next page

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in the kit can be used to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

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 if you are using electrocompetent *E. coli*.
- 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.
- 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 TOP10 One Shot® *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 11.

Note: The red or yellow 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 final volume of 5 µl	Add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl

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

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 **One Shot® Chemical Transformation** (next page) or **Transformation by Electroporation** (next page). **Note**: You may store the TOPO® Cloning reaction at -20°C overnight.

Continued on next page

TOPO® Cloning Reaction and Transformation, continued

One Shot® Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2 previous page into a vial of One Shot® TOP10 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 seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion (see page 11).
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 25-200 µl from each transformation on a pre-warmed selective plate and incubate overnight at 37°C. 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 colonies for analysis (see **Analysis of Positive Clones**, next page).

Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction into a 0.1 cm cuvette containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature SOC medium.
4. 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 gene.
5. Spread 10-50 µl from each transformation on a pre-warmed 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.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

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
- Ethanol-precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Continued on next page

TOPO® Cloning Reaction and Transformation, continued

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50-100 µg/ml ampicillin (3-5 ml). **Note**: In cells transformed with pGlow-TOPO, a hand-held UV light can be used to detect fluorescence.
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).
3. Please note that PCR products will clone bidirectionally. Analyze the plasmids for insertion and orientation by restriction analysis or by sequencing. Use the sequencing primers included in the kit to help you sequence your insert. Please refer to the diagrams on page 4 and page 5 for restriction sites and sequence surrounding the TOPO Cloning® site. For the complete sequence of either vector, please see our Web site (www.invitrogen.com) or contact Technical Service (page 27).

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

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. Use a combination of either the T7 or *LacZ* Reverse (or GFP Reverse) sequencing primer and a primer that binds within your insert as PCR primers. You will have to determine the amplification conditions. 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. Artifacts may 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 make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using parameters previously determined (see text, above).
5. For the final extension, incubate at 72°C for 10 minutes. Store 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 17-19. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to isolate a single 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 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin. Grow until culture reaches stationary phase.
3. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
4. 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

Transfection

Introduction

Once you obtain the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. Please note the following guidelines for transfection.

We recommend that you include a positive and a negative control to evaluate expression of the reporter genes. A negative control can be either a mock transfection, or TOPO® Clone a PCR product that does not contain a promoter (stuffer DNA) into the desired TOPO® Reporter vector. For a positive control, we recommend cloning a known promoter that is active in your cell line.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipid complexing decreasing transfection efficiency. We recommend isolating plasmid DNA (up to 200 μg) using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), please consult original references or the supplier of your cell line for the optimal method of transfection. It is recommended that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section, page 31).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) and a large selection of reagents for transfection. For more information on the reagents available, please visit our Web site (www.invitrogen.com) or call Technical Service (see page 27).

Detection of Reporter

Once you have transfected your cell line with one of the TOPO® Reporter vectors and the appropriate controls, you are ready to assay for reporter function. See the next page for information on how to assay for β-galactosidase activity and page 14 for GFP assays.

Detection of β-Galactosidase Activity

Introduction

β-galactosidase is one of the most versatile reporters available. It can be assayed both *in vitro* and *in vivo* and a wide variety of substrates are available for detection. A few assays and substrates are described below. Other assays and substrates may be used. In addition to its use as a reporter for uncharacterized promoters, constitutive promoters may be cloned upstream of the *lacZ* gene for use as an internal control to normalize variability with other promoter reporter assays (Alam and Cook, 1990).

Choosing an *In Vitro* Assay

β-galactosidase activity can be detected using cell-free lysates and *o*-nitrophenyl-β-D-galactopyranoside (ONPG). This colorimetric assay is easy to perform and useful for determining whether or not a promoter is active, but it lacks the sensitivity needed for promoter analysis (detects about 100 pg of β-galactosidase). Invitrogen offers a β-Gal Assay Kit (Catalog no. K1455-01) which contains ONPG and all the buffers necessary to assay for β-galactosidase activity. Instructions are also included for a 96-well format (see page vi for ordering information).

If you need greater sensitivity for promoter analysis, we recommend using chemiluminescent 1,2-dioxetane substrates (i.e. Galacton, Tropix) (Beale *et al.*, 1992; Jain and Magrath, 1991). Use of these substrates increases the sensitivity of the assay and extends the range of detection (Bronstein *et al.*, 1994). If endogenous enzyme activity is minimized, sensitivity is enhanced (Young *et al.*, 1993). As little as 2 fg of β-galactosidase can be detected using chemiluminescent substrates. For more information on this assay, please see the references cited above and *Current Protocols in Molecular Biology*, pages 9.7.15 to 9.7.21.

Choosing an *In Vivo* Assay

In vivo detection systems are defined as those in which the reporter gene is detected in live cells or tissues or in cells or tissues fixed for histochemical staining. This is a less quantitative approach but provides important information about cell-type specificity, temporal and tissue expression patterns, and distribution of transcription factors.

The precipitating substrate X-Gal may be used to determine *in vivo* levels of β-galactosidase in eukaryotic cells, tissue sections, and intact embryos (Alam and Cook, 1990). Please note that staining with X-Gal requires that the cells or tissue be fixed. Invitrogen offers the β-Gal Staining Kit (Catalog no. K1465-01) to stain cells expressing β-galactosidase (see page vi for ordering information).

Alternatively, detection in live cultured cells may be achieved with the substrate fluorescein di-β-D-galactopyranoside (FDG) (Jongkind *et al.*, 1986). Using hypotonic loading, FDG is introduced into the cell and cleaved by β-galactosidase. The resulting fluorescein compound is trapped in the cell because of its hydrophobic nature and easily assayed using fluorescence.

Detection of β-galactosidase

If you do not detect activity of β-galactosidase, check for expression by Western blot. You may use antibody to β-galactosidase (see page vi for ordering information) , or, since β-galactosidase is expressed as a fusion to an N-terminal peptide containing the Xpress™ epitope, use the Anti-Xpress™ Antibody (see page vi for ordering information).

Detection of GFP

Introduction

Green fluorescent protein (GFP) is very useful for *in vivo* or *in vitro* assay of promoter function. *In vivo* assays, while less quantitative than *in vitro* assays, provide information regarding cell-type specificity of promoters/enhancers and the tissue distribution of specific transcription factors. Use of pGlow-TOPO® allows you to monitor transcriptional changes in real time. Please note that detection of GFP *in vivo* will depend on the strength of the promoter. For low-level expression it may be necessary to prepare cell lysates and assay in a fluorimeter. For detection in *E. coli*, assay cell lysates.

GFP Gene Used in pGlow-TOPO®

The GFP gene used in pGlow-TOPO® is described in Crameri *et al.*, 1996. The codon usage was optimized for expression in *E. coli* and three cycles of DNA shuffling were used to generate a collection of mutants. The GFP mutant that exhibited the greatest fluorescence in mammalian cells is utilized in pGlow-TOPO®. This mutant form of GFP has the following characteristics:

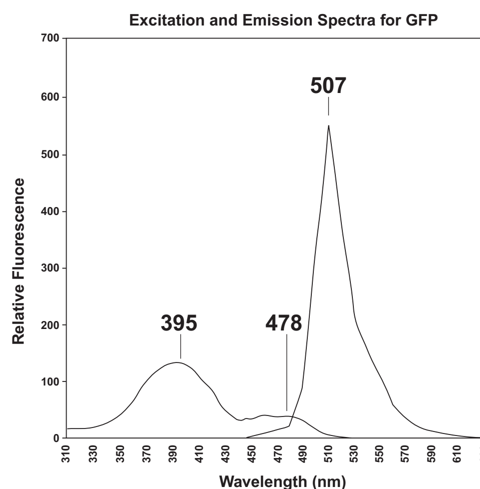
- Excitation and emission maxima that are the same as wild-type GFP (395 nm and 478 nm for primary and secondary excitation, respectively, and 507 nm for emission)
- High solubility in *E. coli* for visual detection of transformed cells (if expressed from a promoter recognized by *E. coli*)
- >40-fold increase in fluorescent yield over wild-type GFP

This GFP protein will be subsequently referred to as Cycle 3 GFP to differentiate it from wild-type GFP.

In Vivo Detection of Cycle 3 GFP Fluorescence

To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of Cycle 3 GFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at either of these wavelengths yields a fluorescent emission peak with a maximum at 507 nm, as shown in the figure below. **Please note that the quantum yield can vary as much as 5- to 10-fold depending on the wavelength of light that is used to excite the GFP fluorophore.**

Use of the best filter set will ensure that the optimal regions of the Cycle 3 GFP spectra are excited and passed (emitted). For best results, use a filter set designed to detect fluorescence from wild-type GFP (e.g. XF76 filter from Omega Optical, www. omegafilters.com). FITC filter sets can also be used to detect Cycle 3 GFP fluorescence. For example, the FITC filter set that we use excites Cycle 3 GFP with light from 460 to 490 nm, which covers the secondary excitation peak. The filter set passes light from 515 to 550, allowing detection of most of the Cycle 3 GFP fluorescence.



For general information about GFP fluorescence and detection, refer to *Current Protocols in Molecular Biology*, pages 9.7.22 to 9.7.28 (Ausubel *et al.*, 1994).

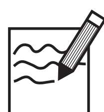
Continued on next page

Detection of Transformed *E. coli*

After transformation of *E. coli*, screen colonies using a hand-held UV light and select glowing cells. To quantitatively assay fluorescence, prepare cell lysates (10^8 to 10^9 cells/ml) from mid-log phase cells using your method of choice. Pellet cell debris and assay supernatant for fluorescence. Be sure to include positive and negative controls.

Detection of Transfected Cells

After transfection, allow the cells to recover and monitor the cells by fluorescence for expression of Cycle 3 GFP. Please note that the CMV promoter is a strong promoter and usually allows detection of Cycle 3 GFP by 24 hours posttransfection. If your promoter is not as strong as CMV, it will take longer to observe fluorescence.



Note

Most media fluoresce because of the presence of riboflavin (Zylka and Schnapp, 1996) and may interfere with detection of Cycle 3 GFP fluorescence. Medium can be removed and replaced with PBS during the assay to alleviate this problem. If cells will be cultured further after assaying, do not keep cells in PBS for a prolonged time. Remove PBS and replace with fresh medium prior to re-incubation.

***In Vitro* Detection of Cycle 3 GFP**

If promoter activity is too low to be detected *in vivo*, you may prepare mammalian cell lysates and assay fluorescence in a fluorimeter if available. A sample protocol is provided below to prepare lysates.

1. Wash cell monolayers (~10^6 cells) two times with PBS.
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 100 µl Cell Lysis Buffer (see recipe on page 26).
4. Incubate cell suspension on ice or at room temperature for 5 to 10 minutes to lyse the cells.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Assay 20 to 100 µg protein in 0.5-1 ml of PBS. Excite at 395 nm and detect at 510 nm.

Detection of Cycle 3 GFP by Western

If you do not detect fluorescence activity, check for expression of Cycle 3 GFP by Western blot. Antiserum to Cycle 3 GFP is available from Invitrogen as a rabbit polyclonal antibody (see page vi for ordering information).

Creation of Stable Cell Lines

Introduction

If you wish to create stable cell lines, select for foci using Geneticin® Selective Antibiotic. General information and guidelines are provided below for your convenience.

Geneticin® Selective Antibiotic

Geneticin® Selective Antibiotic blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® Selective Antibiotic (Southern and Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® Selective Antibiotic is available from Invitrogen (Catalog no. 11811-031). Use as follows:

1. Prepare Geneticin® Selective Antibiotic in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
2. Use 100 to 1000 µg/ml of Geneticin® Selective Antibiotic in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Test varying concentrations of Geneticin® Selective Antibiotic on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin® Selective Antibiotic.

Note: Cells will divide once or twice in the presence of lethal doses of Geneticin® Selective Antibiotic, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 4 weeks of growth in selective medium.

Possible Linearization Sites

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve your chances of obtaining stable transfectants, it ensures that the vector does not integrate in a way that disrupts the gene of interest. The table below lists some unique sites that can be used to linearize your construct prior to transformation. Other sites are possible. Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Site in pBlue-TOPO®	Site in pGlow-TOPO®	Location	Supplier
Aat II	--	5333	Backbone	Many
Afl II	63	63	Upstream of TOPO® Cloning site	Many
*Alw*N I	--	3934	pUC origin	Many
*Bam*H I	84	--	Upstream of TOPO® Cloning site	Invitrogen, Cat. no. 15201-023
Bgl II	13	--	Upstream of TOPO® Cloning site	Invitrogen, Cat. no. 15213-010
*Eam*1105 I	6871	4411	Ampicillin gene	AGS*, Fermentas, Takara
*Hin*d III	66	--	Upstream of TOPO® Cloning site	Invitrogen, Cat. no. 15207-012
Kpn I	--	76	Upstream of TOPO® Cloning site	Invitrogen, Cat. no. 15232-010
Pvu I	--	4781	Ampicillin gene	Invitrogen, Cat. no. 25420-019
Sap I	5862	3402	Backbone	New England Biolabs
Sca I	7351	4891	Ampicillin gene	Invitrogen, Cat. no. 15436-017
Spe I	90	90	Upstream of TOPO® Cloning site	Invitrogen, Cat. no. 15443-013

Appendix

TOPO® Reporter 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 using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing ampicillin and X-gal.

Before Starting

Be sure to prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page 26 for recipe)
- LB plates containing 100 µg/ml ampicillin and X-gal (two per transformation)

  To add X-gal 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.

Producing the Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZα, set up the following 50 µl PCR:

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl each)	2 µl
Sterile Water	40.5 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 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°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 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

Continued on next page

TOPO® Reporter Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and either the pBlue-TOPO® or the pGlow-TOPO® vectors set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
Control PCR Product	--	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 TOP10 One Shot® cells (page 9).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 26). 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 85% of these will be blue and contain the 500 bp insert. Very few colonies (<10% of the vector + PCR insert) will be present on the vector only plate. These colonies should be white.

Note: pBlue-TOPO® will yield dark blue colonies on the "vector + PCR insert" plate and light blue colonies on the "vector only" plate. This is apparently because of a cryptic prokaryotic promoter upstream of the reporter. Please note that no expression of β-galactosidase has been detected in mammalian cells without a promoter.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent *E. coli*. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 9 Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Continued on next page

TOPO® Reporter Control Reactions, continued

Factors Affecting Cloning Efficiency

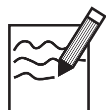
Please note that lower transformation and/or cloning efficiencies will result from the following variables. Most of these are easily corrected, but if you are cloning large inserts, you may not obtain the expected 85% (or more) cloning efficiency.

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 (>3 kb)	Gel-purify as described on page 20.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. Please note that you can add up to 4 µl of the PCR to the TOPO® Cloning reaction.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 22).
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 20).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	Increase the time of the final extension to ensure that the 3´ ends are adenylated. Please note that *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).
Size of promoter sequences cloned	For large plasmids, you may have to use electroporation to transform into *E. coli.* **Do not use the chemically competent TOP10 cells included in the kit for electroporation.** Use electrocompetent TOP10 cells (see page vi for ordering information).

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. Three simple protocols are provided below for your convenience.



Note

Please note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 6).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

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

   Note: Do not use TBE. Borate will interfere 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 of Binding Buffer.
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 3000 x g 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.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. 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 page 8.

Quick S.N.A.P.™ Method

An even 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 8). 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 chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
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. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 8.
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 TOP10 One Shot® *E. coli* using the method on page 9.

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. These low efficiencies are caused by the 3´ to 5´ exonuclease activity associated with proofreading polymerases which removes the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

Before Starting

You will need the following items:

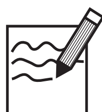
- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

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.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pBlue-TOPO® or pGlow-TOPO®

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.



Note

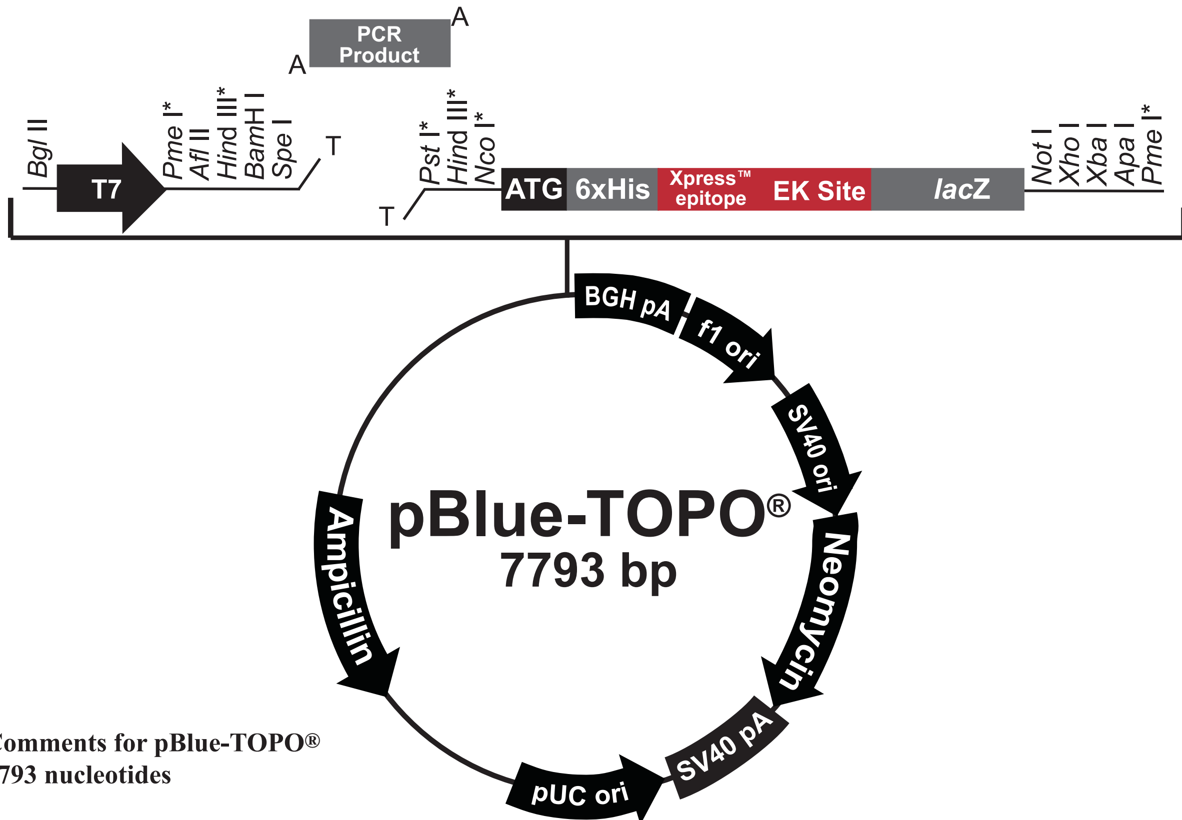
You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see previous page). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

Vent® is a registered trademark of New England Biolabs.

pBlue-TOPO® Map

Map

The figure below summarizes the features of the pBlue-TOPO® vector. The vector is supplied linearized between base pairs 116 and 117. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our Web site (www.invitrogen.com)** or from Technical Service (see page 27.).



Comments for pBlue-TOPO®
7793 nucleotides

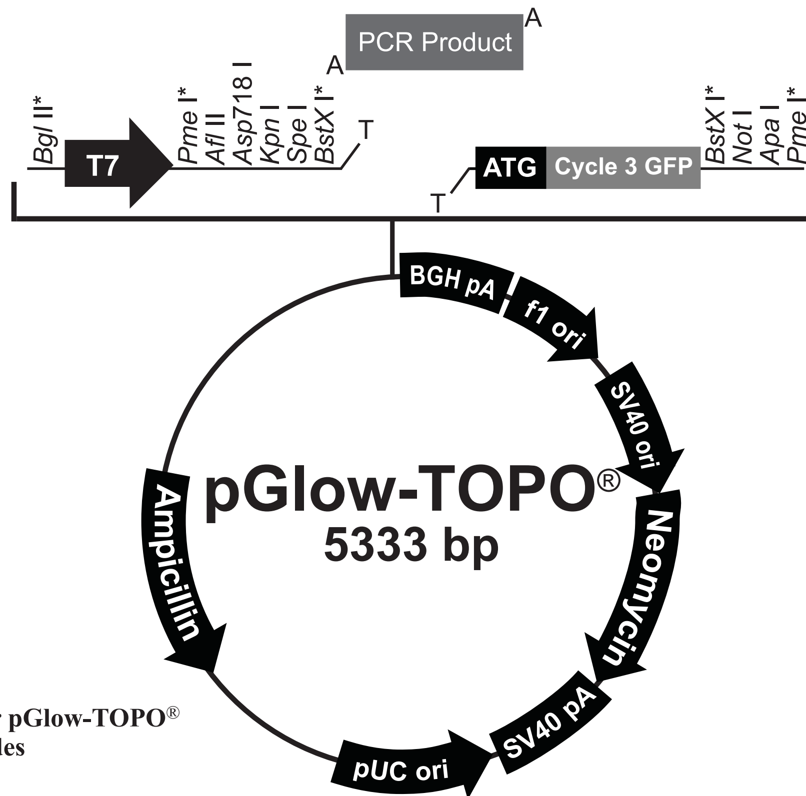
T7 promoter/priming site: bases 17-36
TOPO® Cloning site: bases 116-117
ATG initiation codon: bases 143-145
Polyhistidine region: bases 155-172
LacZ Reverse priming site: bases 173-191
Xpress™ epitope: bases 212-235
Enterokinase recognition site: bases 221-235
LacZ ORF: bases 264-3313
BGH polyadenylation sequence: bases 3386-3613
f1 origin: bases 3659-4087
SV40 promoter and origin: bases 4141-4423
Neomycin resistance gene: bases 4498-5292
SV40 polyadenylation sequence: bases 5466-5596
pUC origin: bases 5979-6652 (complementary strand)
Ampicillin resistance gene: bases 6797-7657 (complementary strand)

* These sites are not unique but may be used to excise the PCR product. The *Pme* I sites may be used to excise the reporter cassette, providing there are no *Pme* I sites in the PCR product.

pGlow-TOPO® Map

Map

The figure below summarizes the features of the pGlow-TOPO® vector. The vector is supplied linearized between base pairs 116 and 117. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our Web site (www.invitrogen.com)** or from Technical Service (see page 27.).



Comments for pGlow-TOPO®
5333 nucleotides

T7 promoter/priming site: bases 17-36
TOPO® Cloning site: bases 116-117
Initiation ATG: bases 140-142
Cycle 3 GFP ORF: bases 140-859
GFP Reverse priming site: bases 251-272
BGH polyadenylation sequence: bases 926-1153
f1 origin: bases 1199-1627
SV40 promoter and origin: bases 1681-1963
Neomycin resistance gene: bases 2038-2832
SV40 polyadenylation sequence: bases 3006-3136
pUC origin: bases 3519-4192 (complementary strand)
Ampicillin resistance gene: bases 4337-5197 (complementary strand)

*pGlow-TOPO™ has only two *Pme* I sites as shown.

Features of the TOPO® Reporter Vectors

Features

The TOPO® Reporter vectors pBlue-TOPO® (7793 bp) and pGlow-TOPO® (5333 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
TOPO® Cloning site	Allows insertion of your PCR product containing the promoter of interest upstream of the reporter gene.
β-galactosidase (pBlue-TOPO®) **or** Cycle 3 GFP (pGlow-TOPO®)	Allows assay of promoter function either *in vitro* or *in vivo*.
LacZ Reverse priming site (pBlue-TOPO®) **or** GFP Reverse priming site (pGlow-TOPO®)	Permits sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

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 (100 µg/ml ampicillin) 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 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (100 µg/ml of ampicillin), 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.

Cell Lysis Buffer

50 mM Tris-HCl, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine:

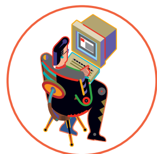
1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

Note: Protease inhibitors may be added at the following concentrations: 1 mM PMSF; 1 µg/ml pepstatin; 1 µg/ml leupeptin.

Technical Service

Web Resources



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Continued on next page

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Product Qualification

Introduction

Invitrogen qualifies the TOPO® Reporter Kits as described below.

Restriction Digest

The parental supercoiled pBlue and pGlow vectors are qualified by restriction digest prior to adaptation with topoisomerase. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pBlue	pGlow
*Eco*R I	7777 bp (linearizes)	5317 bp (linearizes)
*Bam*H I	7777 bp (linearizes)	--
Xho I	7777 bp (linearizes)	323, 4994 bp
Kpn I	--	5317 bp (linearizes)

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 17-19, a 500 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 85% cloning efficiency.

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 tested for transformation efficiency using the control plasmid included in the One Shot® 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 greater than 1 x 10^9 cfu/µg plasmid DNA.

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

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