GFP Fusion TOPO®
TA Expression Kits

Version J

22 April 2004

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GFP Fusion TOPO® TA Expression Kits

For 5 minute cloning and expression of PCR products fused to GFP in mammalian cells

CT-GFP Fusion TOPO® TA Expression Kit: Catalog no. K4820-01
(for fusing GFP to the C-terminus of PCR products)

NT-GFP Fusion TOPO® TA Expression Kit: Catalog no. K4810-01
for fusing GFP to the N-terminus of PCR products)

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life technologies

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Important Information

Shipping and Storage

The GFP Fusion TOPO® TA Expression Kits are shipped on dry ice. Each kit contains a box with GFP Fusion TOPO® Cloning reagents (Box 1) and a box with One Shot® TOP10 Chemically Competent *E. coli* (Box 2). Store Box 1 at -20°C and **Box 2 at -80°C.**

Ordering Information

Ordering information for the GFP Fusion TOPO® TA Expression Kits is provided below.

Kit	Reactions	Catalog no.
CT-GFP Fusion TOPO® TA Expression Kit (contains pcDNA3.1/CT-GFP-TOPO®)	20	K4820-01
NT-GFP Fusion TOPO® TA Expression Kit (contains pcDNA3.1/NT-GFP-TOPO®)	20	K4810-01

GFP Fusion TOPO® Cloning Reagents

GFP Fusion TOPO® 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
pcDNA3.1/CT-GFP-TOPO® **OR** pcDNA3.1/NT-GFP-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 30 µM 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 (50 mM dNTPs)	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

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GFP Fusion TOPO® Cloning Reagents, Continued

Item	Concentration	Amount
Sterile Water	--	1 ml
Control PCR Template	0.05 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.2 µg/µl in TE Buffer (0.1 µg/µl each)	10 µl
Expression Control Plasmid (pcDNA3.1/CT-GFP, supercoiled **OR** pcDNA3.1/NT-GFP, supercoiled)	0.5 µg/µl in TE Buffer	10 µl

Primers

Each kit contains different primers to sequence your insert.

CT-GFP Fusion TOPO® TA Expression Kit

Item	Concentration	Amount
Forward Sequencing Primer (T7 Sequencing Primer)	0.1 µg/µl in TE Buffer	20 µl
Reverse Sequencing Primer (GFP Reverse Primer)	0.1 µg/µl in TE Buffer	20 µl

NT-GFP Fusion TOPO® TA Expression Kit

Item	Concentration	Amount
Forward Sequencing Primer (GFP Forward Primer)	0.1 µg/µl in TE Buffer	20 µl
Reverse Sequencing Primer (BGH Reverse Sequencing Primer)	0.1 µg/µl in TE Buffer	20 µl

Sequence of Primers

The table below provides the sequence and total pmoles of the sequencing primers.

Primer	Sequence	Amount
T7	5´-TAATACGACTCACTATAGGG-3´	328 pmoles
GFP Reverse	5´-GGGTAAGCTTTCCGTATGTAGC-3´	296 pmoles
GFP Forward	5´-CGACACAATCTGCCCTTTCG-3´	334 pmoles
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358 pmoles

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Important Information, continued

One Shot® Reagents

The table below describes the items included in the One Shot® Kit. Store at -80°C. The genotype of TOP10 is provided on the next page.

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

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

Additional Products

The table below lists additional products available from Invitrogen which you may use in conjunction with the GFP Fusion TOPO® Cloning Kits.

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
BGH Reverse primer	2 µg (358 pmoles)	N575-02
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
GFP Antiserum	25 westerns	R970-01

Methods

Overview

Introduction

GFP Fusion TOPO® Cloning provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct fusion of *Taq* polymerase-amplified PCR products to the green fluorescent protein (GFP). No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transfected, the GFP fusion protein will express directly in mammalian cell lines. Two kits are available that allow you to create N-terminal (NT-GFP Fusion TOPO® TA Expression Kit) or C-terminal (CT-GFP Fusion TOPO® TA Expression Kit) GFP fusions.

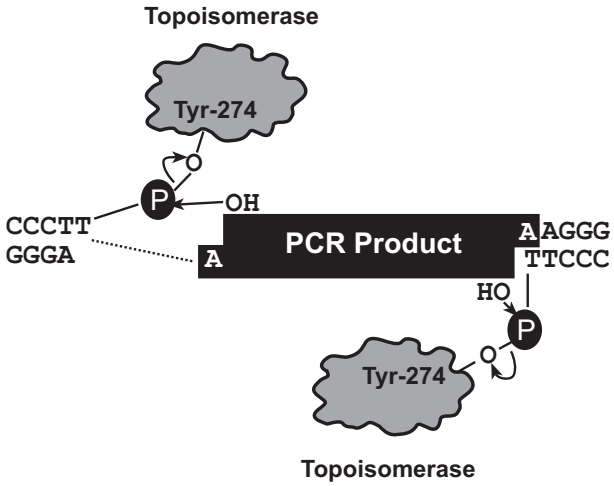
How It Works

The plasmid vectors (pcDNA3.1/CT-GFP-TOPO® or pcDNA3.1/NT-GFP-TOPO®) are supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase 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 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). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see below).



Once the PCR product is cloned into one of the GFP Fusion TOPO® vectors and transformants analyzed for correct orientation and reading frame, the plasmid may be transfected into mammalian cells for expression of the GFP fusion protein.

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Overview, continued

Green Fluorescent Protein

The GFP gene used in these vectors is described in Crameri *et al.*, 1996. In this paper, the codon usage was optimized for expression in *E. coli* and three cycles of DNA shuffling were used to generate a mutant form of GFP that expressed well in mammalian cells and has the following characteristics:

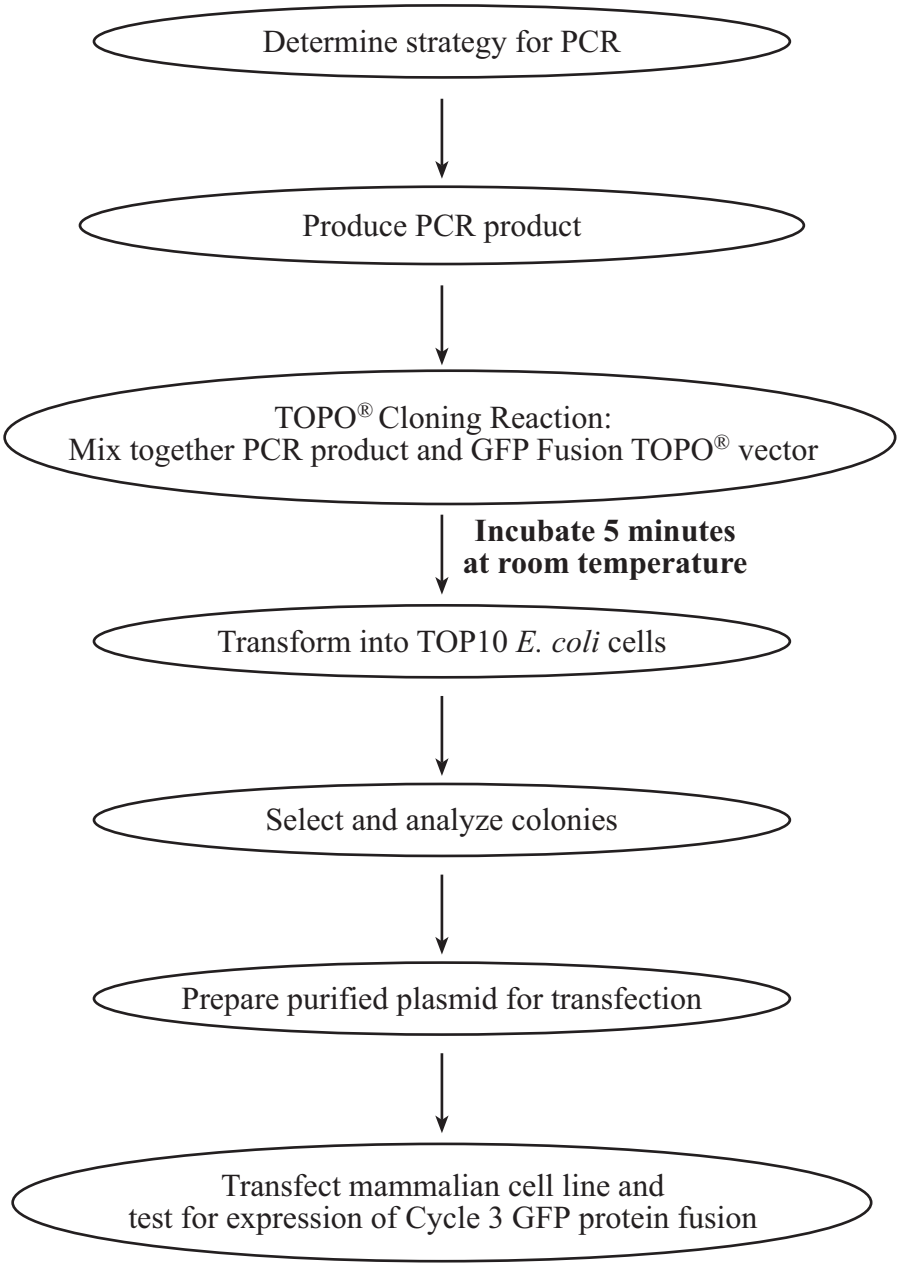
- Excitation and emission maxima that are the same as wild-type GFP (395 nm and 478 nm for primary and secondary excitation, respectively, and 507 nm for emission)
- High solubility in *E. coli* for visual detection of transformed cells (if expressed from a promoter recognized by *E. coli*. Please note that there is no bacterial promoter upstream of the multiple cloning site in the GFP Fusion TOPO® vectors.)
- >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.

Experimental Outline

Experimental Outline

The flow chart below outlines the experimental steps necessary to clone and express your PCR product.



Cloning into pcDNA3.1/CT-GFP-TOPO®

Introduction

The pcDNA3.1/CT-GFP-TOPO® vector is used to express your PCR product with Cycle 3 GFP fused to the C-terminus of your protein. Design of the PCR primers to clone your DNA sequence of interest upstream and in frame with Cycle 3 GFP is critical for expression.

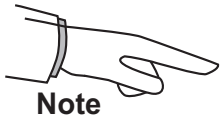
Designing Your PCR Primers

Important: You must consider the nontemplate 3´ A-residues on your PCR product to ensure that your PCR product is cloned in frame with the Cycle 3 GFP protein. The 3´ A-residues are shown with the corresponding 3´ T overhangs from the vector in the diagram on page 5.

To clone into pcDNA3.1/CT-GFP-TOPO®, please note that this is a C-terminal fusion vector that does not contain an ATG initiation codon. If there is no initiating ATG codon or optimal sequences for translation initiation (Kozak sequences) in the DNA to be amplified, then these features need to be incorporated into your forward primer (Kozak, 1987; Kozak, 1990).

Example: Kozak consensus sequence is (G/A)NNATGG

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to **TOPO® Cloning Reaction and Transformation**, page 9.



Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into either vector.

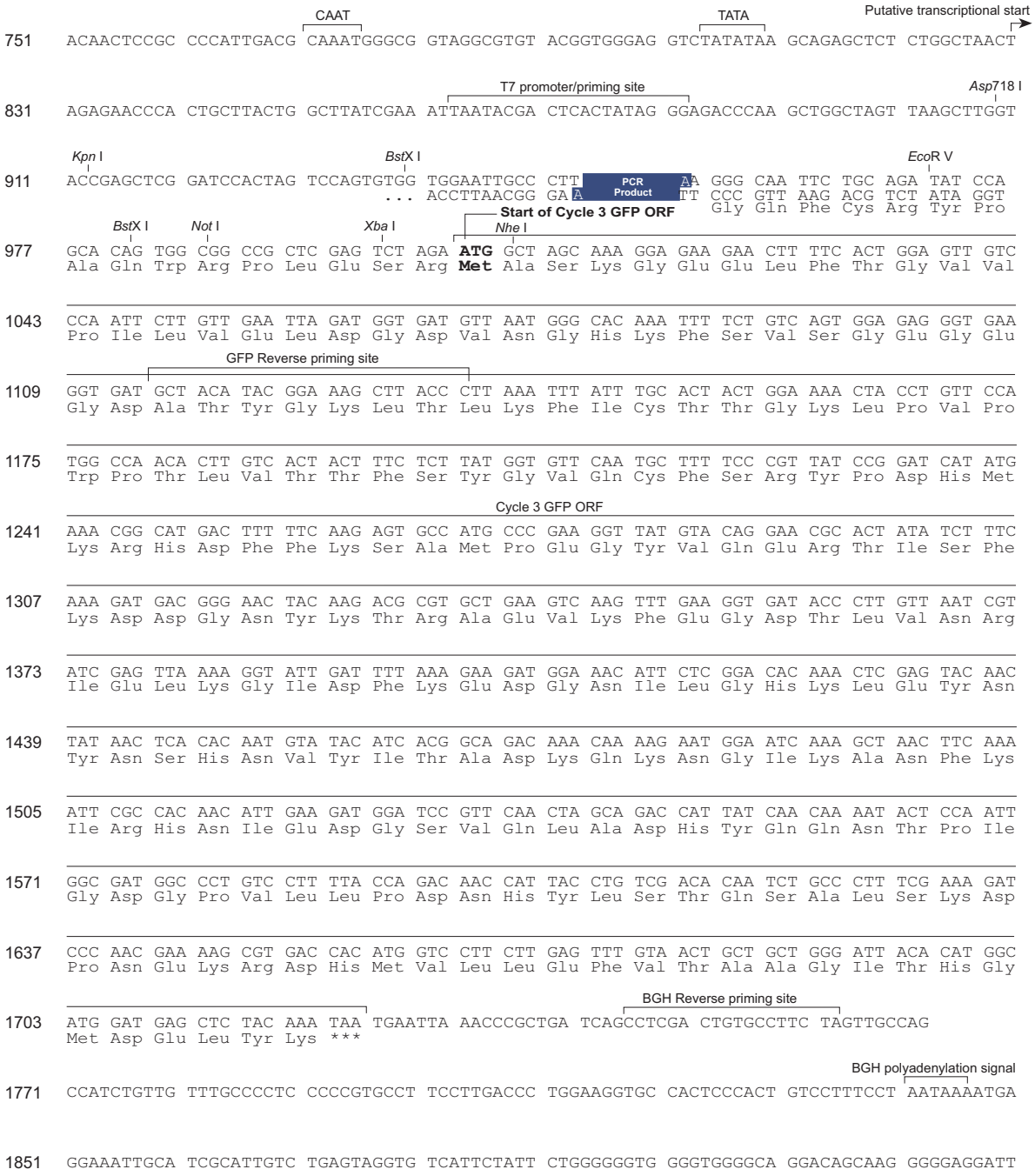
Cloning efficiencies may vary depending on the primer nucleotide sequences (see **Factors Affecting Cloning Efficiency**, page 22).

continued on next page

Cloning into pcDNA3.1/CT-GFP-TOPO®, continued

TOPO® Cloning Site of pcDNA3.1/ CT-GFP-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized at the TOPO® Cloning site. **Please note that the complete sequence of the vector may be downloaded from our Web site (www.invitrogen.com)** or requested from Technical Service (see page 32). A map of the vector is located on page 26.



Cloning into pcDNA3.1/NT-GFP-TOPO®

Introduction

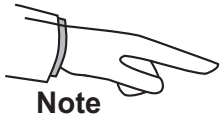
The pcDNA3.1/NT-GFP-TOPO® vector is used to express your PCR product with Cycle 3 GFP fused to the N-terminus of your protein. Design of the PCR primers to clone your DNA sequence of interest downstream and in frame with Cycle 3 GFP is critical for expression.

Designing Your PCR Primers

Important: You must consider the nontemplate 3´ A-residues on your PCR product to ensure that your PCR product is cloned in frame with the Cycle 3 GFP protein. The 3´ A-residues are shown with the corresponding 3´ T overhangs from the vector in the diagram on the next page.

To clone into pcDNA3.1/NT-GFP-TOPO®, your PCR product must be in frame with Cycle 3 GFP and contain a stop codon.

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to **TOPO® Cloning Reaction and Transformation**, page 9.



Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into either vector.

Cloning efficiencies may vary depending on the primer nucleotide sequences (see **Factors Affecting Cloning Efficiency**, page 22).

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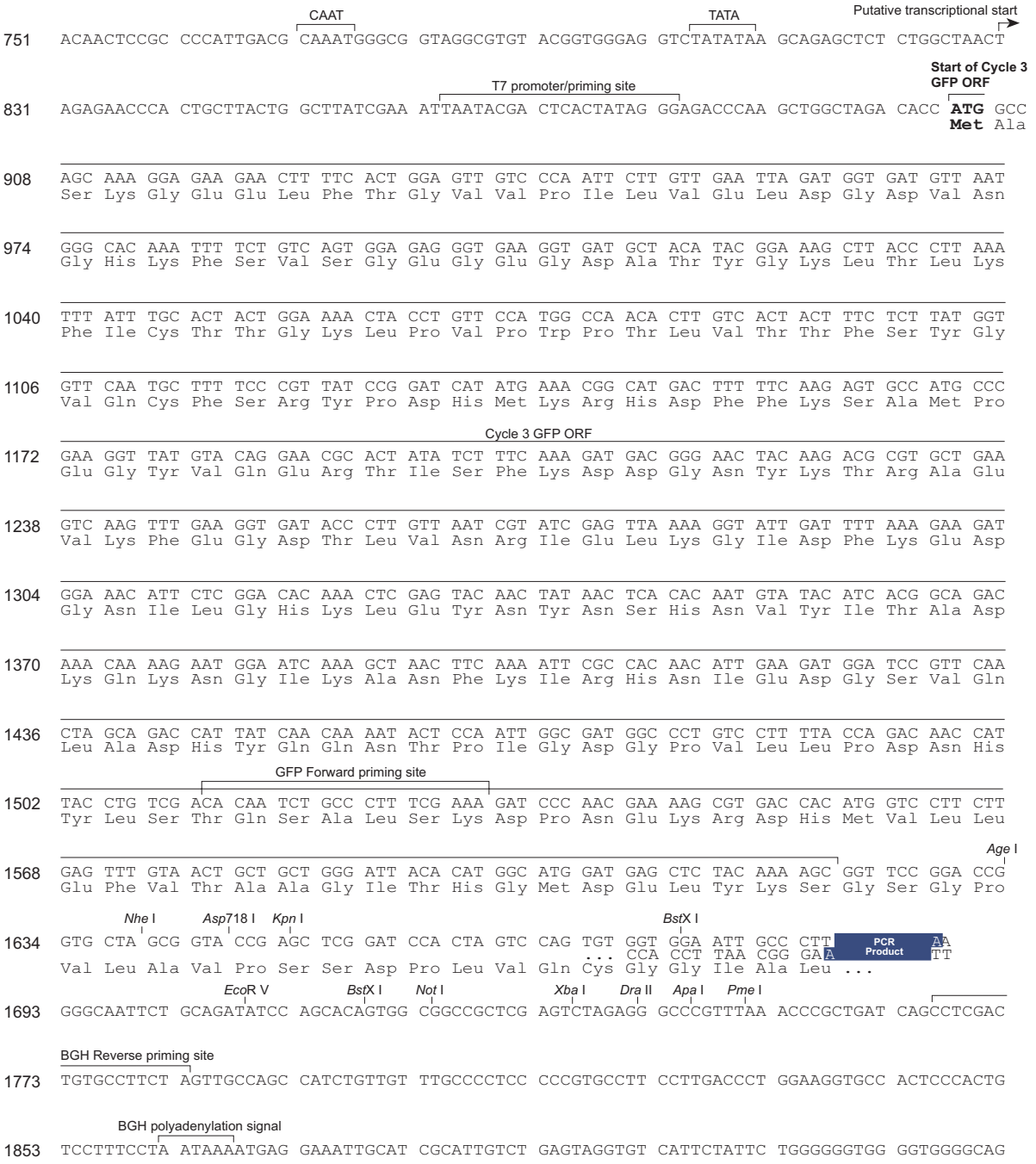
Cloning into pcDNA3.1/NT-GFP-TOPO®, continued

TOPO® Cloning Site of pcDNA3.1/ NT-GFP-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized at the TOPO® Cloning site. **Please note that the complete sequence of the vector may be downloaded from our Web site (www.invitrogen.com)** or requested from Technical Service (see page 32). A map of the vector is located on page 27.



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 (i.e. Platinum® *Taq* DNA Polymerase High Fidelity).

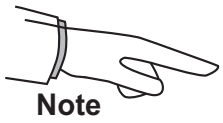
If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 25.

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.

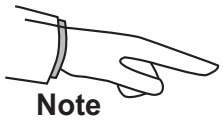


If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the GFP TOPO® TA Expression Kits (see page 23). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple 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 32).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into either pcDNA3.1/CT-GFP-TOPO® or pcDNA3.1/NT-GFP-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, include the control reactions on pages 20-21 with your samples.



Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction results in the following:

- a 2- to 3-fold increase in the number of transformants.
- allows for longer incubation times (up to 30 minutes). Longer incubation times can result in an increase in the number of transformants obtained.

Including salt in the TOPO® Cloning reaction 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.

If you do **not** include salt in the TOPO® Cloning reaction, the number of transformants obtained generally decreases as the incubation time increases beyond 5 minutes.

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

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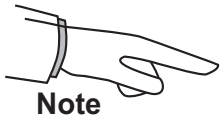
TOPO® Cloning Reaction and Transformation, continued



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. The T7 and GFP Reverse primers can be used to sequence inserts in pcDNA3.1/CT-GFP-TOPO®. The GFP Forward and BGH Reverse primers can be used to sequence inserts in pcDNA3.1/NT-GFP-TOPO®.

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 One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 14.

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.

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TOPO® Cloning Reaction and Transformation, continued

One Shot® Chemical Transformation

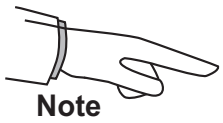
1. Add 2 µl of the TOPO® Cloning reaction from Step 2 previous page into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   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 14).
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 prewarmed 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 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.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).



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

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TOPO® Cloning Reaction and Transformation, continued

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in 3-5 ml of LB medium containing 50-100 µg/ml ampicillin.
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. Analyze the plasmids by restriction analysis or by sequencing to verify the orientation of your insert. Refer to the table below for the correct primers to use for sequencing.

If your insert is in....	Then use...
pcDNA3.1/CT-GFP-TOPO®	T7 and GFP Reverse primers
pcDNA3.1/NT-GFP-TOPO®	GFP Forward and BGH Reverse primers

Please refer to the diagram on either page 5 or page 7 for restriction sites and sequence surrounding the TOPO® Cloning site. For the complete sequence of either vector, please see our Web site or contact Technical Service (page 32).

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).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use a combination of either the Forward or the Reverse sequencing primer provided in the kit with a primer that binds within your insert. 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 determined in the text above.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

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TOPO® Cloning Reaction and Transformation, continued

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long term storage.

1. Streak the original colony on LB plates containing 50-100 µg/ml ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
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

Transfection

Introduction

Once you have the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. Please note the following guidelines for transfection. Included in each kit is an expression control vector (supercoiled pcDNA3.1/CT-GFP or pcDNA3.1/NT-GFP) that you can use to check both transfection efficiencies and expression of Cycle 3 GFP in your particular 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 lipids, 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 resin-based DNA purification systems.

Methods of Transfection

For established mammalian cell lines (*e.g.* HeLa), please consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow the protocol for your cell line. Pay particular attention to medium requirements, cell density at transfection, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

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 a large selection of reagents for transfection including the Calcium Phosphate Transfection Kit (Catalog no. K2780-01). For more information about the different reagents available, please see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Positive Controls

pcDNA3.1/CT-GFP and pcDNA3.1/NT-GFP are provided as positive control vectors in the CT-GFP Fusion TOPO® TA Expression Kit and NT-GFP Fusion TOPO® TA Expression Kit, respectively. Both vectors allow you to evaluate the efficiency of your transfection by assaying for expression of Cycle 3 GFP (see pages 29-30 for maps of each vector). The control vectors may be used to optimize transfection conditions for your cell line. Please note that the gene encoding Cycle 3 GFP is expressed in mammalian cells under control of the CMV promoter. Transfected cells expressing Cycle 3 GFP can be easily assayed (see next page).

Expression of Cycle 3 GFP Fusion Proteins

Introduction

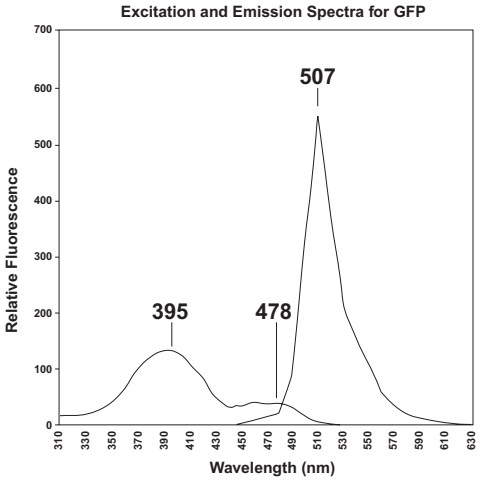
Expression of your Cycle 3 GFP fusion protein can be performed in either transiently transfected cells or stable cell lines (see page 19 for guidelines to create stable cell lines). To detect the protein encoded by your PCR product, you may use fluorescence, western blot analysis, or a functional assay specific for your protein of interest. Fusion of your PCR product to Cycle 3 GFP allows detection by fluorescence or by western blot (using the GFP Antiserum, next page). If you have antibody to the protein encoded by your PCR product, you may use immunofluorescence or western blot techniques.

Expression of Cycle 3 GFP Fusion Proteins

Since your Cycle 3 GFP fusion protein may express differently from the control, we recommend that you perform a time course to optimize expression of the Cycle 3 GFP fusion protein (*e.g.* 24, 48, 72 hours, etc. after transfection). Use one of the following techniques to evaluate expression.

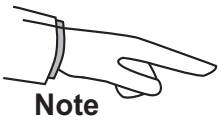
Detection of Cycle 3 GFP Fluorescence

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, please refer to Current Protocols in Molecular Biology, pages 9.7.22 to 9.7.28 (Ausubel et al., 1994).



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.

continued on next page

Expression of Cycle 3 GFP Fusion Proteins, continued

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.

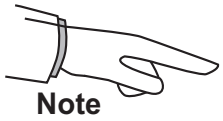
Estimate the total number of cells before assaying for fluorescence. Then check your plate for fluorescent cells. You can use fluorescence to estimate transfection efficiency and normalize any subsequent assay for your gene of interest.

Cells can be incubated further in order to optimize expression of the Cycle 3 GFP fusion protein.

Detection of Fusion Proteins by Western Blot

To detect the fusion protein by western blot, you will need an antibody to the protein encoded by your PCR product or antibody to Cycle 3 GFP (see below). You will also need to prepare cell lysates. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.* 24, 48, 72 hours, etc. after transfection). The cell lysis protocol below is provided for your convenience. Other protocols may be suitable.

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl NP-40 Cell Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8, see page 31).
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge resulting cell lysate at 10,000 x g for 10 minutes to pellet nuclei and transfer the post-nuclear lysate to a fresh tube. Assay the lysate for protein concentration. **Note**: Do not use assays containing Coomassie® Blue (*i.e.* Bradford assay) because NP-40 interferes with the binding of dye to the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and heat the sample at 70°C for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your Cycle 3 GFP fusion protein.



Cycle 3 GFP will add at least 27 kDa to the protein encoded by your PCR product. Remember to account for any additional amino acids located between your protein and Cycle 3 GFP. Please refer to the diagram on either page 5 (pcDNA3.1/CT-GFP-TOPO®) or page 7 (pcDNA3.1/NT-GFP-TOPO®). Please note that you can use Cycle 3 GFP expressed from either positive control vector as a marker.

Antiserum for Detection

The GFP Antiserum (Catalog no. R970-01) is a purified polyclonal rabbit antiserum raised against recombinant Xpress™-tagged Cycle 3 GFP. It has been tested in both immunoblotting and ELISA procedures.

Coomassie® is a registered trademark of Imperial Chemical Industries PLC.

Troubleshooting

Troubleshooting Table

If you have trouble expressing your fusion protein, try some of the suggestions listed below. Please be sure to include positive and negative controls when testing for expression of your protein to ensure that the cells can express Cycle 3 GFP and that the cells were grown, transfected, and assayed correctly.

Problem	Possible Cause	Solution
Recombinant protein is not detected on a western blot	PCR product is out of frame with Cycle 3 GFP (pcDNA3.1/NT-GFP-TOPO® only)	Sequence your construct to confirm the protein is in frame with Cycle 3 GFP. **Remember that *Taq* adds 3´ A-residues to your PCR product.**
Recombinant protein is not active	Cycle 3 GFP interferes with activity or tertiary structure	Try fusing Cycle 3 GFP to the other end of the protein. For example, if you fused Cycle 3 GFP to the C-terminus and the fusion protein is not active, try fusing Cycle 3 GFP to the N-terminus.
Cycle 3 GFP does not fluoresce	Fusion protein interferes with Cycle 3 GFP activity or structure	See above.

Creation of Stable Cell Lines

Introduction

The pcDNA3.1/NT-GFP-TOPO® and pcDNA3.1/CT-GFP-TOPO® vectors contain a neomycin resistance gene to allow for selection of cells with Geneticin®. If you wish to create stable cell lines, select foci using Geneticin®. 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® (Southern and Berg, 1982).

Geneticin® Selection Guidelines

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

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

Cells will divide once or twice in the presence of lethal doses of Geneticin®, 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 unique sites that can be used to linearize your construct prior to transformation. Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Restriction Enzyme	Site in pcDNA3.1/ CT-GFP-TOPO®	Site in pcDNA3.1/ NT-GFP-TOPO®	Location	Supplier
Bgl II	13	13	backbone	Invitrogen, Catalog no. 15213-010
Dra III	2255	2264	f1 origin	Amersham, Boehringer
*Eam*1105 I	5235	5242	ampicillin gene	AGS*, Fermentas, Takara
Pvu I	5605	5612	ampicillin gene	Invitrogen, Catalog no. 25420-068
Sca I	5715	5722	ampicillin gene	Invitrogen, Catalog no. 15436-017
Ssp I	6039	6046	backbone	Invitrogen, Catalog no. 15458-011

*Angewandte Gentechnologie Systeme

Appendix

GFP Fusion TOPO® 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 using the reagents included in the kit involves producing a control PCR product that expresses the LacZα peptide and using it in a TOPO® Cloning reaction. Successful TOPO® Cloning of the control PCR product will yield > 90% blue recombinants on 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 31 for recipe)
- LB plates containing 50-100 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, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	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 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 5-10 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

GFP Fusion TOPO® Cloning Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and either the pcDNA3.1/CT-TOPO® or the pcDNA3.1/NT-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
GFP Fusion 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 One Shot® TOP10 chemically competent *E. coli* or electrocompetent *E. coli* (page 11).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 31). 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 90% of the colonies should be blue.

The "Vector Only" plate should contain very few colonies (<10% of the number of colonies on the "Vector + PCR Insert" plate).

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 11. Plate 10 µl of the transformation reaction plus 20 µl of SOC on LB plates containing 50-100 µg/ml ampicillin. The transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

continued on next page

GFP Fusion TOPO® Cloning Control Reactions, continued

Factors Affecting Cloning Efficiency

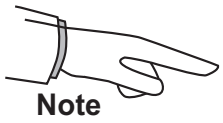
Please note that lower transformation and/or cloning efficiencies will result from the following variables. Most of these are easily corrected, but if you are cloning large inserts (>1 kb), you may not obtain the expected 90% (or more) cloning efficiency.

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 23.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. **Note**: You may use as much as 4 µl of your PCR in the TOPO® Cloning reaction.
Cloning blunt-end PCR products	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 25).
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 23).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*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).

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.



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 8).

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 10.

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 10). 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

If you prefer to use low-melt agarose, use the procedure below. Please note that the gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

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 10.
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® TOP10 *E. coli* using the method on page 11.

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. Proofreading polymerases lack the terminal transferase activity that adds the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-end fragments.

Before Starting

You will need the following items:

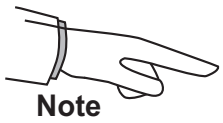
- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

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 pcDNA3.1CT-TOPO® or pcDNA3.1/NT-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.

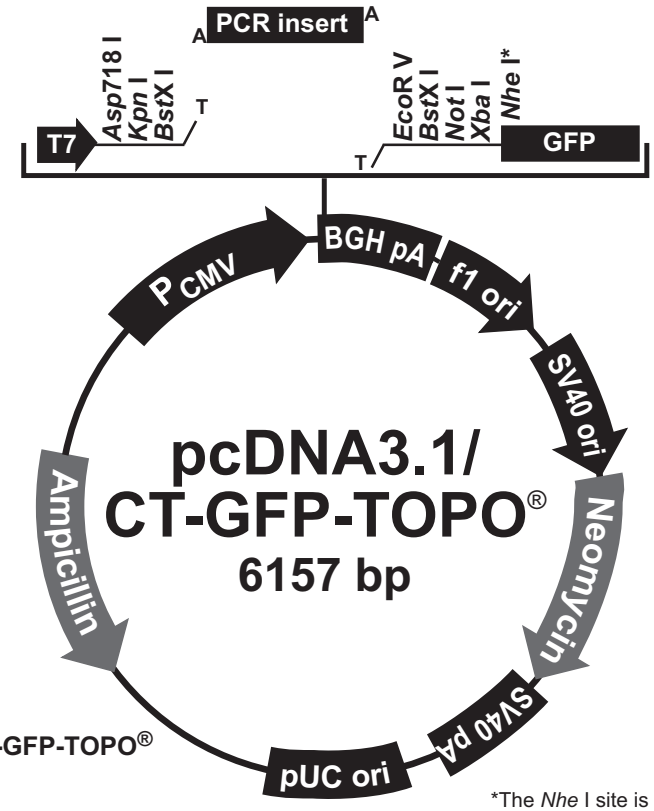


You may also gel-purify your PCR product after amplification with *Vent*™ or *Pfu* (see previous page). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

pcDNA3.1/CT-GFP-TOPO® Map

Map

The figure below summarizes the features of the pcDNA3.1/CT-GFP-TOPO® vector. The vector is supplied linearized between base pairs 953 and 954. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our World Wide Web site (www.invitrogen.com)** or from Technical Service (page 32).



*The *Nhe* I site is at the N-terminus of GFP

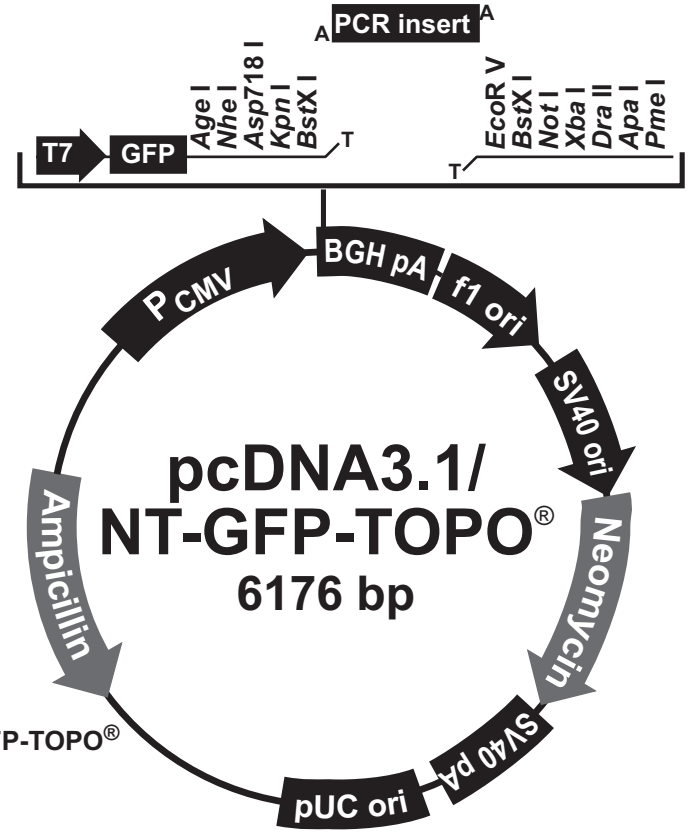
Comments for pcDNA3.1/CT-GFP-TOPO®
6157 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 908-1012
TOPO® Cloning site: bases 953-954
GFP Reverse priming site: bases 1115-1136
GFP ORF: bases 1004-1723
BGH reverse priming site: bases 1745-1762
BGH polyadenylation sequence: bases 1748-1975
f1 origin of replication: bases 2021-2449
SV40 promoter and origin: bases 2477-2785
Neomycin resistance gene: bases 2860-3654
SV40 polyadenylation sequence: bases 3830-3960
pUC origin: bases 4343-5016 (opposite strand)
Ampicillin resistance gene: bases 5161-6021 (opposite strand)

pcDNA3.1/NT-GFP-TOPO® Map

Map

The figure below summarizes the features of the pcDNA3.1/NT-GFP-TOPO® vector. The vector is supplied linearized between base pairs 1678 and 1679. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our World Wide Web site (www.invitrogen.com)** or from Technical Service (page 32).



Comments for pcDNA3.1/NT-GFP-TOPO®
6176 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
GFP ORF: bases 905-1621
GFP Forward priming site: bases 1512-1529
Multiple cloning site: bases 1645-1754
TOPO® Cloning site: 1690-1691
BGH reverse priming site: bases 1766-1783
BGH polyadenylation sequence: bases 1769-1996
f1 origin of replication: bases 2042-2470
SV40 promoter and origin: bases 2498-2806
Neomycin resistance gene: bases 2881-3675
SV40 polyadenylation sequence: bases 3849-3979
pUC origin: bases 4362-5035 (opposite strand)
Ampicillin resistance gene: bases 5180-6040 (opposite strand)

Features of GFP Fusion TOPO® Vectors

Features of GFP Fusion TOPO® Vectors

GFP Fusion TOPO® vectors contain the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
Cycle 3 GFP ORF (pcDNA3.1/NT-GFP-TOPO® only)	Allows fusion of Cycle 3 GFP to the N-terminus of your PCR product
GFP Forward priming site (pcDNA3.1/NT-GFP-TOPO® only)	Permits sequencing of your insert from Cycle 3 GFP into your insert from the 5´ end
TOPO® Cloning site	Allows insertion of your PCR product in frame with Cycle 3 GFP
GFP Reverse priming site (pcDNA3.1/CT-GFP-TOPO® only)	Permits sequencing of your insert from Cycle 3 GFP into your insert from the 3´ end
Cycle 3 GFP ORF (pcDNA3.1/CT-GFP-TOPO® only)	Allows fusion of Cycle 3 GFP to the C-terminus of your PCR product
BGH reverse priming site	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*

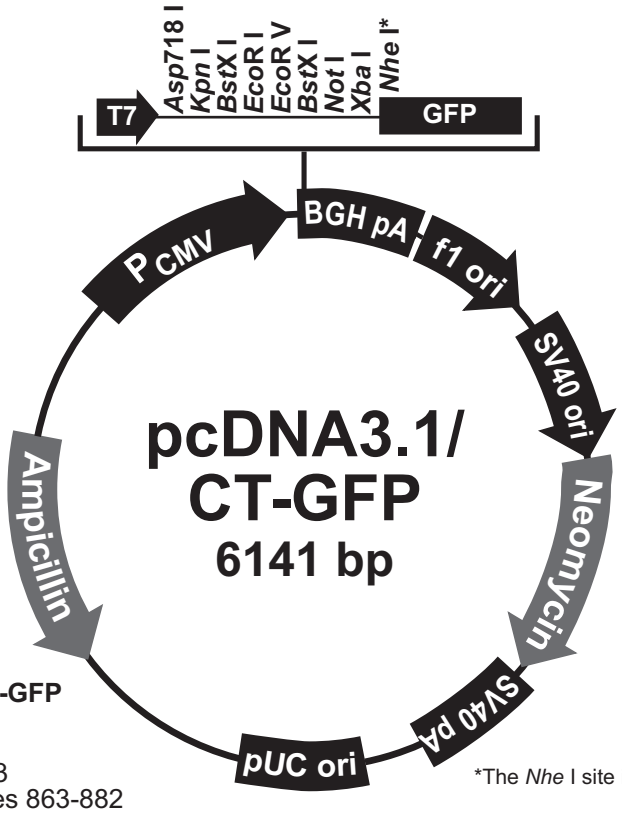
pcDNA3.1/CT-GFP Map

Description

pcDNA3.1/CT-GFP is a 6141 bp control vector containing the gene for Cycle 3 GFP. The vector was constructed by cloning an *Xba* I-*Eco*R I/Klenow fragment containing Cycle 3 GFP (with initiation codon) into pcDNA3.1/V5-His B digested with *Xba* I and *Pme* I. Expression and fluorescence of Cycle 3 GFP has been confirmed.

Map of Control Vector

The figure below summarizes the features of the pcDNA3.1/CT-GFP vector. **The complete nucleotide sequence for pcDNA3.1/CT-GFP is available for downloading from our World Wide Web site (www.invitrogen.com)** or by contacting Technical Service (page 32).



*The *Nhe* I site is at the N-terminus of GFP

Comments for pcDNA3.1/CT-GFP
6141 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 908-996
GFP ORF: bases 988-1707
BGH reverse priming site: bases 1729-1746
BGH polyadenylation signal: bases 1732-1959
f1 origin of replication: bases 2005-2433
SV40 promoter and origin: bases 2461-2769
Neomycin resistance gene: bases 2844-3638
SV40 polyadenylation signal: bases 3814-3944
pUC origin: bases 4327-5000
Ampicillin resistance gene: bases 5145-6005

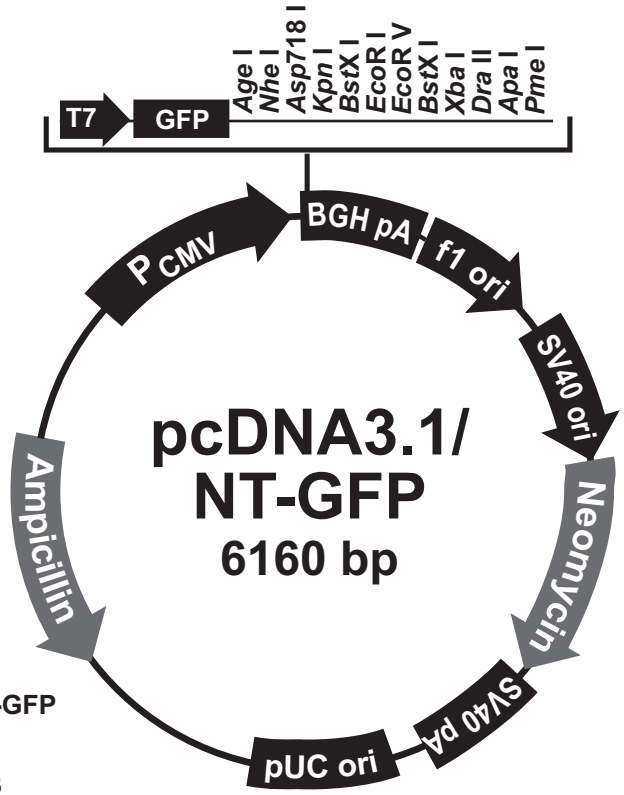
pcDNA3.1/NT-GFP Map

Description

pcDNA3.1/NT-GFP is a 6148 bp control vector containing the gene for Cycle 3 GFP. The vector was constructed by cloning an *Xba* I-*Hin*d III fragment containing Cycle 3 GFP (no stop codon included) into pcDNA3.1 (+) digested with *Nhe* I and *Hin*d III. Subcloning destroyed the *Xba* I site and the *Nhe* I site. Translation of Cycle 3 GFP results in the addition of 29 amino acids to the C-terminus of Cycle 3 GFP (Ser-Gly-Ser-Gly-Pro-Val-Leu-Ala-Val-Pro-Leu-Val-Gln-Cys-Gly-Gly-Ile-Leu-Gln-Ile-Ser-Ser-Thr-Val-Ala-Ala-Ala-Arg-Val). Expression and fluorescence of the modified Cycle 3 GFP has been confirmed.

Map of Control Vector

The figure below summarizes the features of the pcDNA3.1/NT-GFP vector. **The complete nucleotide sequence for pcDNA3.1/NT-GFP is available for downloading from our World Wide Web site (www.invitrogen.com)** or by contacting Technical Service (page 32).



Comments for pcDNA3.1/NT-GFP
6160 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
GFP ORF: bases 905-1621
Multiple cloning site: bases 1645-1738
BGH reverse priming site: bases 1750-1767
BGH poly A: bases 1753-1980
f1 origin of replication: bases 2026-2454
SV40 promoter and origin: bases 2482-2790
Neomycin resistance gene: bases 2865-3659
SV40 polyadenylation signal: bases 3833-3963
pUC origin: bases 4346-5019 (opposite strand)
Ampicillin resistance gene: bases 5164-6024 (opposite strand)

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 (50-100 µg/ml ampicillin) if needed.
4. Store at room temperature or at +4°C. Medium is stable for 1-2 weeks.

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 (50-100 µg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark. Medium is stable for 1-2 weeks.

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
150 mM NaCl
1% Nonidet P-40
pH 7.8

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

World Wide Web

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

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

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

http://www.invitrogen.com

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

Contact Us

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

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MSDS Requests

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

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Technical Service, continued

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Product Qualification

Introduction

Invitrogen qualifies the GFP TOPO® TA Expression Kits as described below.

Restriction Digest

The parental supercoiled pcDNA3.1/CT-GFP and pcDNA3.1/NT-GFP vectors are qualified by restriction digest prior to adaptation with topoisomerase. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pcDNA3.1/CT-GFP	pcDNA3.1/NT-GFP
*Eco*R I	6141 bp (linearized)	6148 bp (linearized)
*Hin*d III	5932, 209 bp	6148 bp (linearized)
Apa I	3 kb (supercoiled)	6148 bp (linearized)

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 20-21, 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

All 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 1×10^9 cfu/µg plasmid DNA.

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

Purchaser Notification

Limited Use Label License No: 55 Cycle 3 GFP

The 'cycle 3' mutant GFP was produced by Maxygen, Inc. using DNA shuffling technology. Commercial licensing inquiries should be directed to: Affymax Research Institute, 4001 Miranda Avenue, Palo Alto, CA 94304, U.S.A.

Limited Use Label License No: 127 GFP

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