invitrogen™

Vivid Colors™ pcDNA™ 6.2/ EmGFP and YFP-GW/TOPO Mammalian Expression Vectors

For TOPO Cloning and expression of PCR products fused to EmGFP or YFP in mammalian cells

Catalog nos. K359-20, K360-20, K361-20, K362-20

Version B
14 December 2010
25-0849

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits:

Product	Catalog no.
Vivid Colors™ pcDNA™6.2/C-EmGFP-GW/TOPO® Mammalian Expression Vector Kit	K359-20
Vivid Colors™ pcDNA™6.2/N-EmGFP-GW/TOPO® Mammalian Expression Vector Kit	K360-20
Vivid Colors™ pcDNA™6.2/C-YFP-GW/TOPO® Mammalian Expression Vector Kit	K361-20
Vivid Colors™ pcDNA™6.2/N-YFP-GW/TOPO® Mammalian Expression Vector Kit	K362-20

Shipping and Storage

The pcDNA™6.2/EmGFP and YFP-GW/TOPO® Mammalian Expression Vector Kits are shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store boxes as detailed below.

Box	Item	Storage
1	pcDNA™6.2/EmGFP or YFP-GW/TOPO® Cloning Reagents	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	-80°C

Continued on next page

Kit Contents and Storage, Continued

TOPO® Cloning Reagents

The pcDNA™6.2/EmGFP and YFP-GW/TOPO® Mammalian Expression Vector cloning reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase**. **Store the contents of Box 1 at -20°C.**

Item	Concentration	Amount
pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector, TOPO® adapted	5-10 ng linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 1 mM EDTA 1mM 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	50 µl
dNTP Mix	12.5 mM dATP 12.5 mM dATP 12.5 mM dATP 12.5 mM dATP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	---	1 ml
Control PCR template	50 ng/µl in TE buffer, pH 8.0	10 µl
Control PCR primers	100 ng/µl each in TE buffer, pH 8.0	10 µl
T7 promoter sequencing primer (pcDNA™6.2/C-EmGFP or YFP-GW/TOPO® only)	100 ng/µl in TE buffer, pH 8.0	20 µl
FP 2 Reverse sequencing primer (pcDNA™6.2/C-EmGFP or YFP-GW/TOPO® only)	100 ng/µl in TE buffer, pH 8.0	20 µl
FP 1 Forward sequencing primer (pcDNA™6.2/N-EmGFP or YFP-GW/TOPO® only)	100 ng/µl in TE buffer, pH 8.0	20 µl
TK PolyA Reverse sequencing primer (pcDNA™6.2/N-EmGFP or YFP-GW/TOPO® only)	100 ng/µl in TE buffer, pH 8.0	20 µl
pcDNA™6.2/EmGFP or YFP /GW/CAT Control Plasmid	0.5 µg/µl in TE buffer, pH 8.0	20 µl

Continued on next page

Kit Contents and Storage, continued

Primers

Each pcDNA™6.2/EmGFP or YFP-GW/TOPO® Mammalian Expression Vector Kit contains the following primers to sequence your insert:

Kit	Primer	Sequence	pMoles Supplied
pcDNA™6.2/C-EmGFP or YFP-GW/TOPO®	T7 Promoter	5′-TAATACGACTCACTATAGGG-3′	324
	FP 2 Reverse	5′-TCACCATGTTAACAGCATCAA-3′	315
pcDNA™6.2/N-EmGFP or YFP-GW/TOPO®	FP 1 Forward	5′-ACAAGGGCTCGAGCCCATCAA-3′	313
	TK PolyA Reverse	5′-CTTCCGTGTTTCAGTTAGC-3′	348

One Shot® TOP10 Reagents

The following reagents are included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store the contents of Box 2 at -80°C**.

Item	Concentration	Amount
TOP10 *E. coli*	---	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl
S.O.C. Medium	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

Genotype of TOP10 Strain

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 products listed in this section are available from Invitrogen and may be used with Vivid Colors™ pcDNA™6.2/EmGFP or YFP-GW/TOPO® Mammalian Expression Kit. Ordering information is provided below.

Product	Amount	Catalog no.
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
Taq DNA Polymerase, Recombinant	100 units	10342-053
	250 units	10342-012
	500 units	10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-020
	500 units	11304-011
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
	20 reactions	C4040-52
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
Lipofectamine™ 2000	0.75 ml	11668-027
	1.5 ml	11668-019
Blasticidin	50 mg	R210-01
CAT Antiserum	50 µl	R902-25
Anti-GFP Antibody; rabbit polyclonal sera	100 µl	A6455
Anti-GFP Antibody; rabbit IgG fraction	100 µl	A11122
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-HRP Antibody	50 µl	R961-25
Anti-V5-AP Antibody	50 µl	R962-25
WesternBreeze® Chemiluminescent Kit Anti-Rabbit	20 reactions	WB7106
WesternBreeze® Chromogenic Kit Anti-Rabbit	20 reactions	WB7105

Overview

Description

The Vivid Colors™ EmGFP or YFP-GW/TOPO® Mammalian Expression Vector Kits combine TOPO® Cloning with the brightness of Emerald Green Fluorescent Protein (EmGFP) or Yellow Fluorescent Protein (YFP) derived from *Aequorea victoria* GFP to express a fluorescent-tagged protein of interest.

Users can easily make an EmGFP or YFP N- or C-terminally tagged expression plasmid by amplifying the gene of interest with *Taq* polymerase and performing a 5-minute TOPO® Cloning reaction into pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector followed by transformation of competent *E. coli*. After screening clones for directionality of the insert, the expression plasmid can be transfected into the mammalian cell line of choice to express a fusion protein.

Your N- or C-terminal fluorescent-tagged protein of interest can be visualized using fluorescence detection methods or detected using commercially available antibodies in Western blot analysis.

A choice of kits allows you to fuse your gene of interest to an N-terminal or C-terminal tag for easy detection and purification of recombinant fusion proteins (see table below).

Vector	Fusion Peptide	Fusion Tag	Benefit
pcDNA™6.2/N-EmGFP-GW/TOPO®	N-terminal	EmGFP	Fluorescent Detection and Detection with Antibodies
	C-terminal	Optional V5	Detection with Antibodies
pcDNA™6.2/N-YFP-GW/TOPO®	N-terminal	YFP	Fluorescent Detection and Detection with Antibodies
	C-terminal	Optional V5	Detection with Antibodies
pcDNA™6.2/C-EmGFP-GW/TOPO®	C-terminal	EmGFP	Fluorescent Detection
pcDNA™6.2/C-YFP-GW/TOPO®	C-terminal	YFP	Fluorescent Detection

The Vivid Colors™ EmGFP or YFP-GW/TOPO® Mammalian Expression Vector Kits include a positive control expression plasmid for transfection and expression optimization (pcDNA™6.2/EmGFP or YFP /GW/CAT).

Continued on next page

Features of the Vectors

The Vivid Colors™ pcDNA™6.2/EmGFP or YFP-GW/TOPO® vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level gene expression in a wide range of mammalian cells
- Emerald Green Fluorescent Protein (EmGFP) or Yellow Fluorescent Protein (YFP) derived from *Aequorea victoria* GFP for N- or C-terminal fusion to the protein of interest for fluorescent detection
- TOPO® Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products
- *att*B1 and *att*B2 sites to allow recombination-based transfer of the gene of interest into any Gateway® expression vector via an LR and BP reaction
- The V5 epitope tag for detection of recombinant protein using Anti-V5 antibodies (**N-terminal fusion vectors only**)
- The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the Blasticidin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Blasticidin resistance gene for selection of stable cell lines
- pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- Ampicillin (*bla*) resistance gene for selection in *E. coli*

For maps and features of the pcDNA™6.2/EmGFP or YFP-GW/TOPO® vectors, see pages 32-35.

Continued on next page

Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a naturally occurring bioluminescent protein derived from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). GFP emits fluorescence upon excitation, and the gene encoding GFP contains all of the necessary information for posttranslational synthesis of the luminescent protein. GFP is often used as a molecular beacon because it requires no species-specific cofactors for function, and the fluorescence is easily detected using fluorescence microscopy and standard filter sets. Commonly, GFP is fused to a protein of interest, and upon expression, the localization of the fusion protein can be detected in cells. GFP can also function as a reporter gene downstream of a promoter of interest.

GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include nucleic acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescence signal, resulting in "enhanced" GFP (Zhang *et al.*, 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998). The Emerald GFP (EmGFP) and Yellow Fluorescent Protein (YFP) are such variants of enhanced GFP.

EmGFP and YFP

The EmGFP and YFP variants have been described in a published review (Tsien, 1998) and the amino acid changes are summarized in the table below. The mutations are represented by the single letter abbreviation for the amino acid in the consensus GFP sequence, followed by the codon number and the single letter amino acid abbreviation for the substituted amino acid.

Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T
YFP	S65G, S72A, K79R, T203Y

*Mutations listed are as described in the literature. When examining the actual sequence, the vector codon numbering starts at the first amino acid **after** the initiation methionine of the fluorescent protein, so that mutations appear to be increased by one position. For example, the S65T mutation actually occurs in codon 66 of EmGFP.

Continued on next page

EmGFP and YFP Fluorescence

The fluorescent proteins from the Vivid Colors™ pcDNA™6.2/EmGFP or YFP-GW/TOPO® vectors have the following excitation and emission wavelengths, as published in the literature (Tsien, 1998):

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509
YFP	514	527

Filter Sets for Detecting EmGFP or YFP Fluorescence

The fluorescence signal from EmGFP and YFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for each of the fluorescent proteins. These filter sets and their manufacturer are listed below:

Fluorescent Protein	Filter Set for Fluorescence Microscopy	Manufacturer
EmGFP	Omega XF100	Omega (www.omegafilters.com)
YFP	Omega XF1042	Omega (www.omegafilters.com)
	Chroma 41028	Chroma (www.chroma.com)

Continued on next page

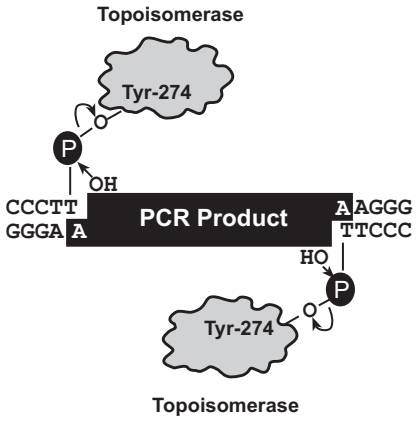
How TOPO® Works

The plasmid vectors (pcDNA™6.2/EmGFP or YFP-GW/TOPO®) are supplied linearized with:

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

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

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



Once the PCR product is cloned into a pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector and the transformants are analyzed for correct orientation and reading frame, the expression plasmid may be transfected into mammalian cells for expression of the GFP fusion protein.

Continued on next page

The Gateway® Technology

The Gateway® Technology is a universal cloning system that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems.

Once an expression vector is created by TOPO® Cloning into a pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector, the gene of interest is flanked by *att*B sites (see cloning region maps, pages 8-9). The expression vector can be used to generate an entry clone by BP recombination with a donor vector. The gene of interest can then be shuttled to numerous expression vectors by LR recombination.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual, available at www.invitrogen.com or by contacting Technical Service (page 41).

Experimental Outline

To TOPO® Clone your gene of interest into the pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector, you will perform the following steps:

1. Generate a PCR product with *Taq* polymerase.
2. TOPO® Clone your PCR product into pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector and use the reaction to transform *E. coli.*
3. Pick colonies, isolate plasmid DNA, and screen for insert directionality by sequencing expression clones.
4. Transfect your expression clone into the mammalian cell line of choice for transient or stable expression of your gene of interest.
5. Visualize fusion proteins by fluorescence microscopy or by Western blot.

The following sections of this manual provide instructions and guidelines for these steps.

Methods

Designing PCR Primers

Introduction

TOPO® Cloning provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. The section below will help you design primers to produce your PCR product for cloning into pcDNA™6.2/EmGFP or YFP-GW/TOPO®vectors.

Note

Do not add 5′ phosphate to your primers for PCR. The PCR product synthesized will not ligate into the vectors.

Cloning efficiencies may vary depending on the primer nucleotide sequences.

Points to Consider When Designing PCR Primers

To obtain the fusion protein that you want from the expression plasmid, consider the options below before designing your entry clone. For more information on the TOPO® cloning sites of the vectors, see the next pages.

To create an expression clone using…	And you wish to…	Then your insert…
pcDNA™6.2/N-EmGFP-GW/TOPO® or pcDNA™6.2/N-YFP-GW/TOPO®	Include the N-terminal fluorescent fusion and NOT the V5 epitope	• **Should** contain a stop codon
pcDNA™6.2/N-EmGFP-GW/TOPO® or pcDNA™6.2/N-YFP-GW/TOPO®	Include the N-terminal fluorescent fusion and the V5 epitope	• **Should not** contain a stop codon • **Should** be designed to be in frame with the V5 epitope
pcDNA™6.2/C-EmGFP-GW/TOPO®or pcDNA™6.2/C-YFP-GW/TOPO®	Express your protein of interest fused to EmGFP or YFP at the C-terminus	• **Should** contain a Kozak consensus sequence • **Should not** contain a stop codon

Continued on next page

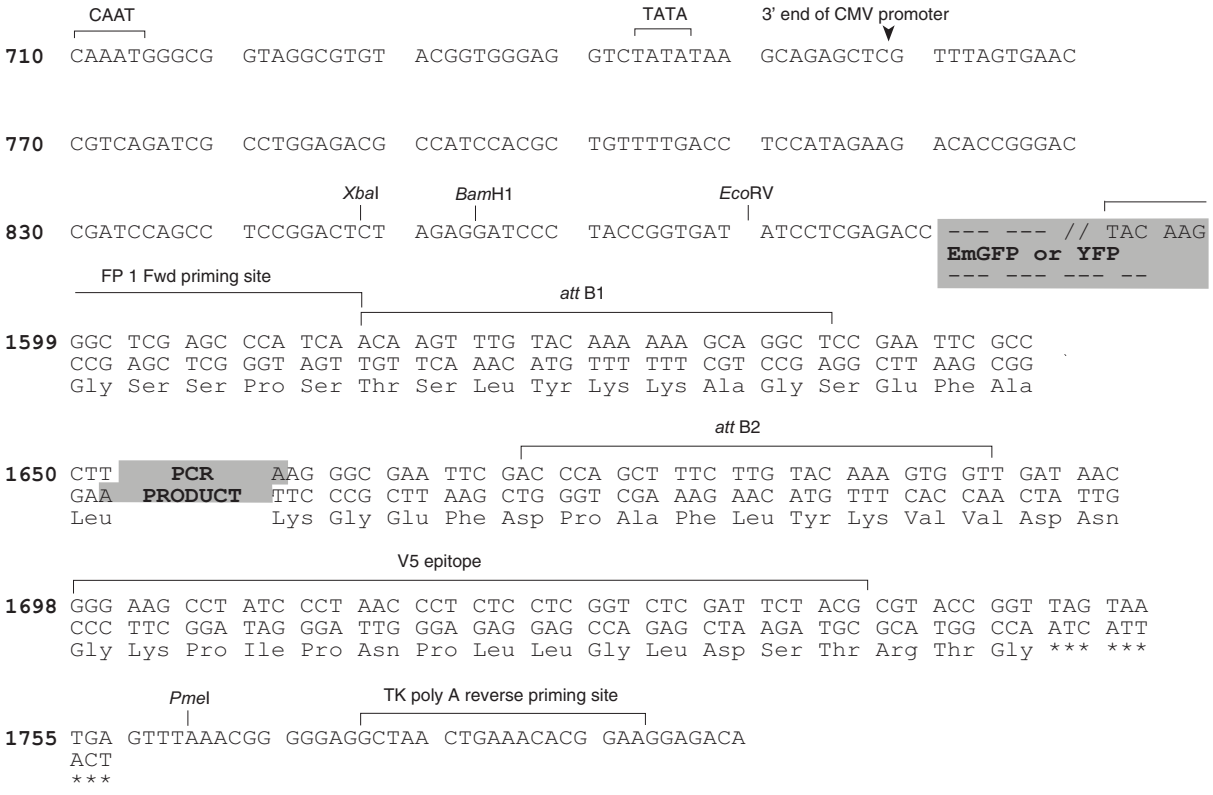
Designing PCR Primers, Continued

Points to Consider Before Cloning into N- Terminal Vectors

If you are cloning your PCR product into the pcDNA™6.2/N-EmGFP-GW/TOPO® or pcDNA™6.2/N-YFP-GW/TOPO® vector, you may express the V5 epitope located at the C-terminal end of the fusion by omitting a stop codon in your gene of interest. The V5 epitope is a 14 amino acid epitope (GKPIPNPLLGLDST) derived from the P and V proteins of the SV5 paramyxovirus (Southern *et al.*, 1991). Fusion proteins containing the V5 epitope can be detected using specific antibodies (see page viii for ordering information).

TOPO® Cloning Site for N-Terminal Fusion

Use the diagram below to help you design your PCR product for TOPO® Cloning into the pcDNA™6.2/N-EmGFP-GW/TOPO® or the pcDNA™6.2/N-YFP-GW/TOPO® vectors. The complete sequences of the vectors are available from www.invitrogen.com or by contacting Technical Service (page 41).



Continued on next page

Designing PCR Primers, Continued

Points to Consider Before Cloning into C- Terminal Vectors

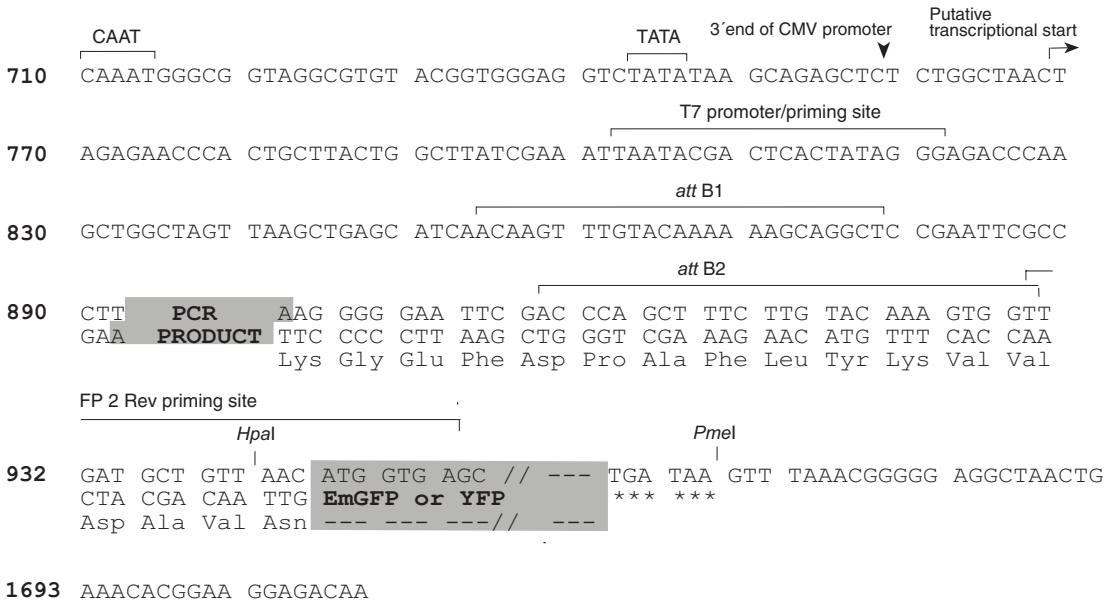
If you are cloning your PCR product into the pcDNA™6.2/C-EmGFP-GW/TOPO®or pcDNA™6.2/C-YFP-GW/TOPO® vector, the design for your PCR should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

(G/A)NNATG**G**

Other sequences are possible, but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold).

TOPO® Cloning Site for C-Terminal Fusion

Use the diagram below to help you design your PCR product for TOPO® Cloning into the pcDNA™6.2/C-EmGFP-GW/TOPO® or the pcDNA™6.2/C-YFP-GW/TOPO® vectors. The complete sequences of the vectors are available from www.invitrogen.com or by contacting Technical Service (page 41).



Producing PCR Products

Introduction

After you have designed primers to amplify your gene of interest, you are ready to produce your PCR product for TOPO® Cloning into a pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector.

Materials Supplied by User

You will need the following reagents and equipment:

- *Taq* polymerase, such as Platinum® *Taq* (Catalog no. 10966-018)
- Thermocycler
- DNA template
- Primers for PCR product

Polymerase Mixtures

You may use an enzyme mixture containing *Taq* polymerase and a proofreading polymerase, however *Taq* must be used in excess of 10:1 to ensure the presence of 3′ A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity available from Invitrogen (see page viii for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or use a proofreading polymerase only, you can add 3′ A-overhangs after amplification using the method on page 31.

Continued on next page

Producing PCR Products

1. Set up the following reaction in a 50 µl volume. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template.

Reagent	Amount
DNA template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
PCR Primers	100-200 ng each
Sterile water	to final volume of 49 µl
Taq polymerase (1 unit/µl)	1 µl
Total Volume	**50 µl**

2. Perform amplification using the cycling parameters suitable for your primers and template. Be sure to include a 7-30 minute extension at 72° after the last cycle to ensure that all PCR products are full length and 3′ adenylated.
3. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR reaction, try the following:

- The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR to eliminate multiple bands and smearing.
- Gel-purify your fragment before performing the TOPO® Cloning reaction. We recommend using the S.N.A.P.™ Gel Purification Kit from Invitrogen (Catalog no. K1999-25).

Alternatively, refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel, et. al., 1994) for other common protocols for isolating DNA fragments.

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector and use this plasmid for transformation of competent *E. coli*. It is important to have everything you need to set up the reaction so that you can obtain the best results. We suggest that you read this entire section and the next section about transformation before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions detailed on pages 29-30 in parallel with your samples.

Note

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt 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.

Including salt in the TOPO® Cloning reaction 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.

Using Salt in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). 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 page viii for ordering information).

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied, and set up the TOPO® Cloning reaction as directed on the next page.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction must be reduced to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed on the next page.

Continued on next page

Setting Up the TOPO® Cloning Reaction, Continued

Materials Needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- pcDNA™6.2/EmGFP or YFP-GW/TOPO® vector (supplied with the kit, Box 1; stored at -20°C)
- Salt Solution (supplied with the kit, Box 1) or Dilute Salt Solution (see previous page)
- Sterile water (supplied with the kit, Box 1)

Performing the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) to use for transformation of either chemically competent or electrocompetent *E. coli*.

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent	Chemically Competent *E. coli*	Electrocompetent *E. coli*
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 total volume of 5 µl	Add to total volume of 5 µl
TOPO® Vector	1 µl	1 µl
Final Volume	**6 µl**	**6 µl**

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

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

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. 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 larger PCR products (>1kb) or if TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to Transforming One Shot® Competent *E. coli*, next page.

   Note: You may store the TOPO® Cloning reaction overnight at -20°C.

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction you are ready to use your construct to transform competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit (Box 2) to facilitate transformation. You may also transform One Shot® Electrocompetent cells if desired (see page viii for ordering information). Protocols for transforming chemically competent and electrocompetent *E. coli* are provided in this section.

Selecting a One Shot® Chemical Transformation Protocol

Two protocols are provided to transform One Shot® TOP10 chemically competent *E. coli*. Consider the following factors and choose the protocol that best suits your needs.

If you wish to…	Then use the…
Maximize the number of transformants	Regular chemical transformation protocol, page 15.
Clone large PCR products (>1000 bp)	
Obtain transformants as quickly as possible	Rapid chemical transformation protocol, page 16. **Note:** This procedure is less efficient; the total number of transformants obtained may be lower than that obtained with the regular chemical transformation protocol.

Materials Needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following:

- TOPO® Cloning reaction (From Step 2, previous page)
- One Shot® TOP10 *E. coli*, either chemically competent (supplied with the kit, Box 2) or electrocompetent (purchased separately, see page viii)
- S.O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (supplied with the kit, Box 2)
- 42°C water bath (chemically competent cells only)
- Electroporator with cuvettes (electrocompetent cells only)
- 15 ml sterile, snap cap plastic culture tubes (electrocompetent cells only)
- For each transformation reaction, 2 selective LB or low-salt LB plates containing either 100 µg/ml ampicillin or 50 µg/ml Blasticidin (see page 38 for a recipe to prepare selective LB and low-salt LB plates)
- 37°C shaking and non-shaking incubators

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned into the vector. Sequencing primers are included in the kit to allow you to sequence across the insert in the TOPO® Cloning site to confirm orientation and reading frame.

Continued on next page

Transforming One Shot® Competent *E. coli*, Continued

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective LB plates.

- Equilibrate a water bath to 42°C if using chemically competent *E. coli*, or set up your electroporator if using electrocompetent *E. coli*
- Warm the vial of S.O.C. Medium to room temperature
- Warm selective LB plates at 37°C for 30 minutes
- Thaw one vial of One Shot® cells **on ice** for each transformation

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® Chemically Competent *E. coli* with a sterile pipette tip and mix gently. Do not mix by pipetting up and down.

   Note: If you are using the pUC19 control plasmid for transformation, use 1 µl (10 pg).
2. Incubate cells/plasmid mix on ice for 5-30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency.
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 S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally at 200 rpm in a 37°C shaking incubator for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective LB plate. To ensure even spreading of small volumes, you may add 20 µl of S.O.C. Medium to the transformation mixture. We recommend that you plate two different volumes to ensure that at least one plate contains well-spaced colonies. Incubate plates overnight at 37°C.

Continued on next page

Rapid One Shot® Chemical Transformation Protocol

Use the alternative protocol below to rapidly transform One Shot® TOP10 chemically competent *E. coli*. Before beginning, prewarm LB or low salt LB agar plates containing 100 µg/ml ampicillin or 50 µg/ml Blasticidin at 37°C for 30 minutes.

1. Add 4 µl of the TOPO® Cloning reaction 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 5 minutes.
3. Spread 50 µl of cells on a prewarmed selective plate and incubate overnight at 37°C.

One Shot® Electroporation Protocol

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation. We recommend using One Shot® TOP10 Electrocompetent cells for electroporation (Cat. no C4040-50).

1. Add 2 µl of the TOPO® Cloning reaction from the previous section into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* with a sterile pipette tip and mix gently. Do not mix by pipetting up and down and take care not to introduce bubbles.
2. Transfer the cells to a 0.1 cm electroporation cuvette.
3. Electroporate your sample using your own protocol and electroporator.
   Note: If you have problems with arcing, see below.
4. Immediately add 250 µl of room temperature S.O.C. Medium to the cuvette.
5. Transfer the solution to a 15 ml snap-cap tube and shake for at least 1 hour at 37°C.
6. Spread 10-50 µl from each transformation on a prewarmed selective LB plate. To ensure even spreading of small volumes, you may add 20 µl of S.O.C. Medium to the transformation mixture. We recommend that you plate two different volumes to ensure that at least one plate contains well-spaced colonies. Incubate plates overnight at 37°C.

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 µl and 80 µl (0.1 cm cuvettes) or 100 and 200 µl (0.2 cm cuvettes).

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

- Make sure that the salt levels in the TOPO® Cloning Reaction are reduced as suggested on page 13.
- 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 it in water prior to electroporation.

Selecting Clones

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 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 using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01). See page viii for ordering information.
3. Analyze the plasmids by sequencing to confirm the presence and correct orientation of the insert.

Sequencing

To confirm that your gene of interest is in frame with the N- or C-terminal fluorescent fusion, you may sequence your expression construct using the following primers included with the kit.

Expression Clone	Primer
pcDNA™6.2/C-EmGFP or YFP-GW/TOPO®	T7 Promoter
pcDNA™6.2/C-EmGFP or YFP-GW/TOPO®	FP 2 Reverse
pcDNA™6.2/N-EmGFP or YFP-GW/TOPO®	FP 1 Forward
pcDNA™6.2/N-EmGFP or YFP-GW/TOPO®	TK PolyA Reverse

Refer to page vii for the sequences of the primers and the diagrams on pages 8-9 for the location of the primer binding sites.

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. We also recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on an LB plate containing 100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
3. Grow at 37°C with shaking until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol.
5. Transfer to a cryovial and store at -80°C.

Transfecting Cells

Introduction

This section provides general information for transfecting your expression clone into the mammalian cell line of choice. We recommend that you include the positive control vector (pcDNA™6.2/EmGFP/GW/CAT or pcDNA™6.2/YFP/GW/CAT, below) and a mock transfection (negative control) in your experiments to evaluate your results.

Positive Control Plasmid

Each kit contains a positive control plasmid expressing CAT for use in mammalian cell transfection and expression (see pages 36-37 for maps) and to optimize recombinant protein expression levels in your cell line. These vectors allow expression of an N- or C-terminally tagged chloramphenicol acetyl transferase (CAT) fusion protein that may be detectable by fluorescence microscopy, Western blot, or functional assay.

The control vector is supplied at 0.5 µg/µl in TE, pH 8.0. You can transfect mammalian cells with this stock or propagate and maintain the plasmid as described below:

1. Use 1 µl of the control vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, JM109, or equivalent.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.

Prepare a glycerol stock of a transformant containing plasmid for long-term storage (see previous page for a protocol for preparing glycerol stocks).

Plasmid Preparation

Once you have generated your expression vector, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free of contamination 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 using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01). Other methods of obtaining high quality plasmid DNA may be suitable.

Continued on next page

Transfecting Cells, Continued

Methods of Transfection

For established cell lines, consult the original references or the supplier of your cell line for the optimal method of transfection. We recommend 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* (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). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and other transfection reagents, go to www.invitrogen.com or contact Technical Service (page 41).

Recombinant Protein Expression

In general, recombinant protein can be visualized by UV fluorescence at 24 hours post transfection (see page 22). However for some cell lines, protein may be detected in as little as 6-8 hours after transfection.

Creating Stable Cell Lines

Introduction

The Vivid Colors™ pcDNA™6.2/EmGFP and YFP-GW/TOPO® vectors contain the Blasticidin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your expression construct into the mammalian cell line of choice and select for stable transfectants using Blasticidin. General information and guidelines are provided below.

Linearizing the Plasmid

To obtain stable transfectants, we recommend that you linearize your pcDNA™6.2/EmGFP or YFP-GW/TOPO® expression construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. Cut at a unique site that is not located within a critical element or within your gene of interest. Restriction site information for plasmid vectors is available at www.invitrogen.com.

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *bsd* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a nontoxic deaminohydroxy derivative (Izumi *et al.*, 1991). Blasticidin is available separately from Invitrogen (see page viii for ordering information). For information on preparing and handling Blasticidin see the Appendix, page 40.

Determining Blasticidin Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you first need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line. Most mammalian cells are killed by 2-10 µg/ml Blasticidin. Test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line (see protocol below). Refer to page 40 for instructions on how to prepare and store Blasticidin.

1. Prepare 6 plates of cells so that each plate will be approximately 25% confluent.
2. Replace the growth medium with fresh growth medium containing a range of Blasticidin concentrations: 0, 1, 3, 5, 7.5, and 10 µg/ml.
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.

Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that kills your cells within 1-3 weeks after addition of Blasticidin.

Continued on next page

Generating Stable Cell Lines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your Vivid Colors™ pcDNA™6.2/EmGFP or YFP expression construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/EmGFP or YFP expression construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium without Blasticidin.
3. 48 hours after transfection, split the cells into fresh growth medium without Blasticidin such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2-3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Blasticidin at the predetermined concentration required for your cell line (see previous page).
6. Feed the cells with selective media every 3-4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 10 Blasticidin-resistant colonies and expand them to assay for recombinant protein expression.

Refer to page 40 for instructions on how to prepare and store Blasticidin.

Detecting Vivid Colors™ Fusion Proteins

Introduction

You can perform detection and analysis of your recombinant protein of interest from the expression clone in either transiently transfected cells or stable cell lines. Once you have transfected your expression clone into mammalian cells, you may:

- Detect protein expression and localization directly in cells by fluorescence microscopy or other methods that use light excitation and detection of emission. See below for recommended fluorescence microscopy filter sets.
- Detect protein expression by Western blot of cell lysates using an antibody. See the table on the next page for suggested antibodies.

Filters for use with EmGFP YFP

Both the EmGFP and YFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for each of the fluorescent proteins. These filter sets are listed in the table below:

Fluorescent Protein	Filter Set for Fluorescence Microscopy
EmGFP	Omega XF100
YFP	Omega XF1042 Chroma 41028

For information on obtaining these filter sets, contact Omega Optical, Inc. (www.omegafilters.com) or Chroma Technology Corporation (www.chroma.com).

Continued on next page

Antibodies for Detection of Recombinant Protein

You may detect expression of your recombinant fusion protein using an antibody against your protein of interest, or by using the following antibodies available from Invitrogen:

Note: You can use an antibody against the V5 epitope **ONLY** for pcDNA™6.2/N-EmGFP-GW/TOPO®and YFP-GW/TOPO® if you have included the V5 epitope in your fusion construct. See page 9 for details on this option.

Antibody	Description	Epitope	Catalog Number
Anti-GFP	Rabbit polyclonal antiserum	Recognizes GFP and derivatives	A6455
Anti-GFP	Rabbit polyclonal IgG	Recognizes GFP and derivatives	A11122
Anti-V5	Mouse monoclonal IgG_{2a}	Detects 14 amino acid V5 epitope	R960-25
Anti-V5 HRP	Mouse monoclonal IgG_{2a}, conjugated to HRP	Detects 14 amino acid V5 epitope	R961-25
Anti-V5-AP	Mouse monoclonal IgG_{2a}, conjugated to AP	Detects 14 amino acid V5 epitope	R692-25

Continued on next page

Preparing Cell Lysates for Western Blot Detection

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols may be suitable. To lyse cells:

1. Wash cell monolayer (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS; Catalog no. 10010-023).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see page 39 for a recipe). Other cell lysis buffers may be suitable. Vortex.
4. To lyse cells, perform 3 freeze thaw cycles by incubating the samples in a dry ice/ethanol bath for 2 minutes, then incubating the sample in a 37°C water bath for one minute. Perform this step 3 times to ensure complete cell lysis without protein degradation.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C 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. Add SDS-PAGE sample buffer (see page 39 for a recipe) to a final concentration of 1X and boil the sample 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 fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. For more information, refer to www.invitrogen.com or contact Technical Service (page 41).

Western Blot Detection of Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use an antibody to your protein of interest, an Anti-GFP antibody available from Invitrogen (see previous page) or an Anti-V5 antibody if your fusion protein contains this epitope (see page 8 for this option). The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods (see page viii for ordering information).

Note

The N or C-terminal peptide fusion containing EmGFP or YFP will add approximately 27 kDa to your protein.

The fusion peptide including the V5 epitope will add approximately 4 kDa to your protein, if it is included in your fusion construct.

Continued on next page

Detecting CAT Protein

If you use the provided positive control vector in your experiment, you may assay for CAT expression using your method of choice. CAT Antiserum is also available separately from Invitrogen (see page viii for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the EmGFP or YFP/CAT fusion protein is approximately 57 kDa.

Troubleshooting

Introduction

The table below lists some potential problems solutions that may help you troubleshoot protein expression with a Vivid Colors™ pcDNA™6.2/EmGFP or YFP TOPO® vector construct.

TOPO® Cloning Reaction and Transformation

Problem	Possible Cause	Solution
Few or no colonies obtained from sample reaction, but transformation control yielded colonies	Incomplete extension during PCR	Include a final extension step of 7-30 minutes during PCR. Longer PCR products will need a longer extension time.
	Excess or dilute PCR product used in the TOPO® Cloning reaction	Reduce or concentrate the amount of PCR product.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Used a proofreading polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use *Taq* polymerase to add 3′A-overhangs to your PCR product by following the method on page 31.
	Large PCR product	• Increase the amount of PCR product used in the TOPO® Cloning reaction. • Increase the incubation time of TOPO® Cloning reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers or other artifacts.
	PCR reaction contains artifacts (i.e. not a single band on an agarose gel)	• Optimize your PCR conditions. • Gel-purify your PCR product.
	PCR product does not contain sufficient 3′A-overhangs even though you used *Taq* polymerase	• Increase the final extension time to ensure that all 3′ ends are adenylated. *Taq* polymerase is most efficient at adding a non-template 3′A next to a C, and less efficient at adding a nontemplate 3′ A next to another A (Brownstein *et al.*, 1996). • You may redesign your primers so that they contain a 5′ G instead of a 5′ T.

Continued on next page

TOPO® Cloning Reaction and Transformation, Continued

Problem	Possible Cause	Solution
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and other artifacts. • Optimize your PCR conditions. • Include a final extension step of 7-30 minutes during PCR.
Few or no colonies obtained from sample reaction and the transformation control gave no colonies	One Shot® competent *E. coli* stored incorrectly	• Store One Shot® competent *E. coli* at -80°C. • If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates with the wrong antibiotic	Use the appropriate antibiotic for selection.

Continued on next page

Detection of Vivid Colors™ Fusion Proteins

Problem	Possible Cause	Solution
Recombinant fusion protein not expressed	No Kozak consensus sequence added to C-terminal fusion	Make sure a Kozak consensus sequence is present (see page 9).
	Gene of interest not in frame with fluorescent protein	Make sure gene of interest is in frame with fluorescent protein.
No fluorescence signal detected after transfecting control or experimental expression clone	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter sets for detection of fluorescence (see page 4).
Transfected control gives fluorescent signal, but experimental expression clone does not	Fusion protein interferes with EmGFP or YFP	Try fusing EmGFP or YFP to the other end (N- or C- terminal) of your protein.
Recombinant protein is not detectable with specific antibodies	EmGFP or YFP interferes with fusion protein	Try fusing EmGFP or YFP to the other end (N- or C- terminal) of your protein.
Recombinant protein is not localizing correctly within cells	EmGFP or YFP interferes with fusion protein	Try fusing EmGFP or YFP to the other end (N- or C- terminal) of your protein.
	Mutation in localization signals or critical elements in your gene of interest	Confirm sequence and reclone if needed.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product expressing the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield > 85% blue colonies on LB plates containing ampicillin and X-gal.

Before Starting

The following reagents should be prepared before performing the control reaction:

- Prepare stock X-gal solution (40 mg/ml X-gal in dimethylformamide)
- For each transformation, you will need two LB plates containing 100 µg/ml ampicillin and X-gal

To add X-gal to previously made LB plates, warm the plate to 37°C, add 40 µl of the stock X-gal solution with a sterile pipette, spread evenly and let dry 15 minutes. Store plates at +4°C, protected from light.

Producing the Control PCR Product

1. In a 0.5 ml microcentrifuge tube, set up the following reaction in a 50 µl volume. Overlay reaction with 1 drop of mineral oil if required.

Reagent	Amount
Control DNA Template	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. Amplify the control PCR product using the following cycling parameters:

Step	Time	Temp.	Cycles
Initial Denaturation	2 min.	94°C	1X
Denaturation	1 min.	94°C	25X
Annealing	1 min.	60°C	
Extension	1 min.	72°C	
Final Extension	7 min.	72°C	1X

3. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible.

Continued on next page

Control TOPO® Cloning Reactions

Using the control PCR product generated in the steps above and the control vector, set up two 6 µl TOPO® Cloning reactions as described below:

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

1. Incubate at room temperature for 5 minutes and place on ice.
2. Use 2 µl of the reaction to transform two separate vials of One Shot® competent cells using the procedure on page 14.
3. Spread 10-50 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin and X-gal. When plating small volumes, add 20 µl of S.O.C. Medium to ensure even spreading. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
4. Incubate plates overnight at 37°C.

What You Should See

The "vector + PCR insert" reaction should produce hundreds of colonies. Greater than 85% of these will be blue.

The "vector only" reaction should yield very few colonies (<15% of the vector + PCR insert plate) and these should be white.

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 14. Plate 10 µl of the transformation reaction plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. The transformation efficiency should be 1 x 10^9 cfu/µg DNA.

Addition of 3' A-Overhangs Post-Amplification

Introduction

TOPO® Cloning DNA amplified by proofreading polymerases into TOPO® Cloning vectors often results in very low cloning efficiencies. Proofreading polymerases remove the 3´ A-overhangs necessary for TOPO® Cloning. A method for adding 3'As post-amplification is provided below.

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´ A-overhangs. Other protocols may be suitable.

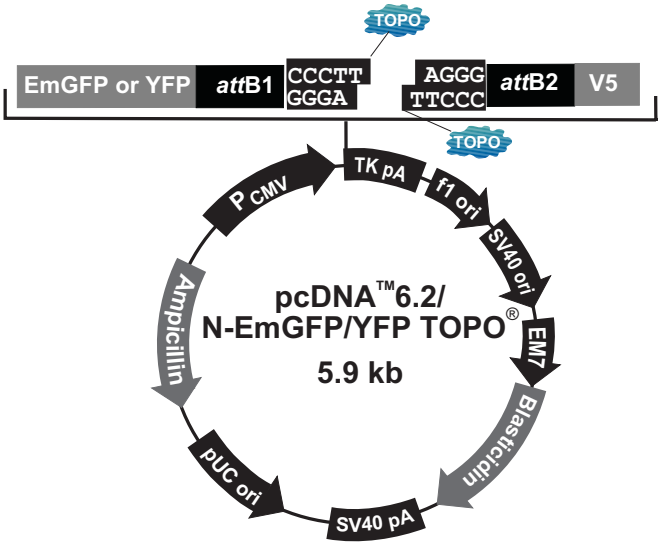
1. After amplification with a proofreading polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pcDNA™6.2/EmGFP or YFP-GW/TOPO®.

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

Map and Features of pcDNA™6.2/N-EmGFP/YFP-GW/TOPO®

Map

The map below shows the elements of the Vivid Colors™ pcDNA™6.2/N-EmGFP or YFP-GW/TOPO® vectors. **The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 41).**



Comments for:	pcDNA™6.2/ N-EmGFP TOPO® 5900 bp	pcDNA™6.2/ N-YFP TOPO® 5902 bp
CMV promoter:	174-761	174-761
EmGFP or YFP	882-1598	882-1598
FP 1 forward priming site:	1594-1614	1594-1614
*att*B1 site:	1614-1638	1614-1638
TOPO® cloning site:	1653	1653
*att*B2 site:	1666-1690	1666-1690
V5 epitope:	1698-1739	1698-1739
TK polyA reverse priming site:	1773-1791	1773-1791
TK polyadenylation signal:	1776-2037	1776-2037
f1 origin:	2073-2501	2073-2501
SV40 early promoter and origin:	2526-2834	2528-2836
EM7 promoter:	2889-2955	2891-2957
Blasticidin resistance gene:	2962-3354	2964-3356
SV40 early polyadenylation signal:	3512-3642	3514-3644
pUC origin (c):	4025-4698	4027-4700
Ampicillin (*bla*) resistance gene (c):	4843-5703	4845-5205
bla promoter (c):	5698-5802	5700-5804

Continued on next page

Map and Features of pcDNA™6.2/N-EmGFP/YFP-GW/TOPO®

Features

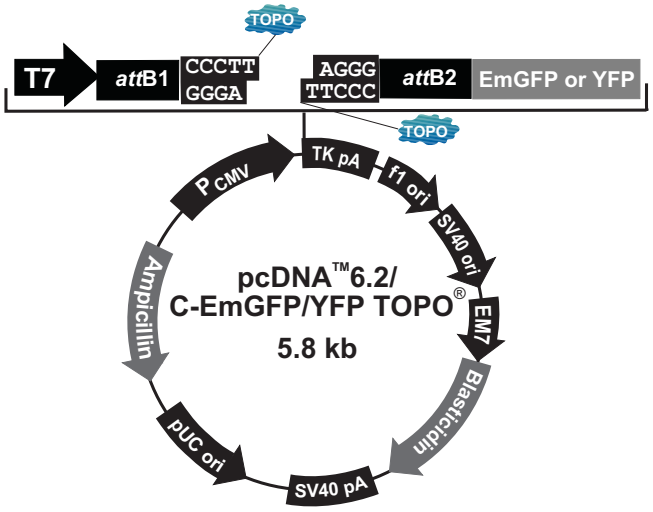
Vivid Colors™ pcDNA™6.2/N-EmGFP-GW/TOPO® (5900 bp) and YFP-GW/TOPO® (5902 bp) vectors contain the following elements. All features have been functionally tested and the vectors have been fully sequenced.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
EmGFP or YFP	N- terminal fusion to the gene of interest for fluorescent detection
FP 1 Forward primer binding site	Allows sequencing of the insert
*att*B1 and *att*B2 sites	Allows recombination with a pDONR™ plasmid to create a Gateway®entry clone
TOPO® Cloning site	Allows insertion of your PCR product in frame with EmGFP or YFP
V5 epitope	Allows detection of recombinant fusion protein with Anti-V5 antibodies (Southern *et al.*, 1991)
TK Poly A Reverse primer binding site	Allows sequencing of the insert
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map and Features of pcDNA™6.2/C-EmGFP/YFP-GW/TOPO®

Map

The map below shows the elements of the Vivid Colors™ pcDNA™/6.2 N-EmGFP or YFP-GW/TOPO® vectors. **The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 41).**



Comments for:	pcDNA™6.2/ C-EmGFP TOPO® 5814 bp	pcDNA™6.2/ C-YFP TOPO® 5814 bp
CMV promoter:	174-761	174-761
T7 promoter/priming site:	802-821	802-821
*att*B1 site:	854-878	854-878
TOPO® cloning site:	893	893
*att*B2 site:	906-930	906-930
FP 2 reverse priming site:	930-950	930-950
EmGFP/YFP:	944-1663	944-1663
TK polyadenylation signal:	1678-1949	1678-1949
f1 origin:	1985-2413	1985-2413
SV40 early promoter and origin:	2440-2748	2440-2748
EM7 promoter:	2803-2869	2803-2869
Blasticidin resistance gene:	2876-3268	2876-3268
SV40 early polyadenylation signal:	3426-3556	3426-3556
pUC origin (c):	3939-4612	3939-4612
Ampicillin (*bla*) resistance gene (c):	4757-5617	4757-5617
bla promoter (c):	5612-5716	5612-5716

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™6.2/C-EmGFP/YFP-GW/TOPO®

Features

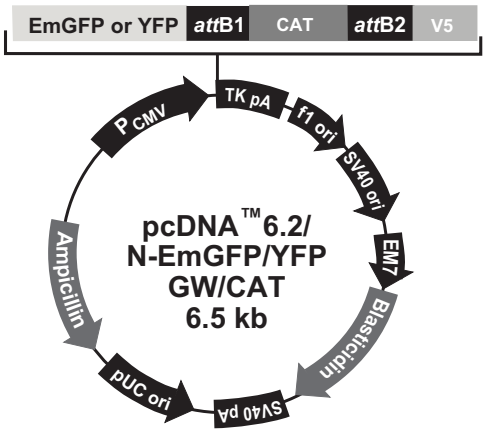
Vivid Colors™ pcDNA™6.2/C-EmGFP-GW/TOPO® (5814 bp) and YFP-GW/TOPO® (5814 bp) vectors contain the following elements. All features have been functionally tested, and the vectors have been fully sequenced.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows 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 *in vitro* transcription in the sense orientation and sequencing through the insert
*att*B1 and *att*B2 sites	Allows recombination with a pDONR™ plasmid to create a Gateway®entry clone
TOPO® Cloning site	Allows insertion of your PCR product in frame with EmGFP or YFP
EmGFP or YFP	C-terminal fusion to the gene of interest for fluorescent detection
T7 Promoter Forward primer binding site	Allows sequencing of the insert
FP 2 Reverse primer binding site	Allows sequencing of the insert
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map of pcDNA™6.2/N-EmGFP or YFP/GW/CAT

Map

The map below shows the elements of Vivid Colors™ pcDNA™6.2/N-EmGFP and YFP/GW/CAT vectors. The plasmids were generated by PCR amplification of the CAT gene followed by TOPO® Cloning into the pcDNA™6.2/N-EmGFP or YFP-GW/TOPO® vector. **The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 41).**



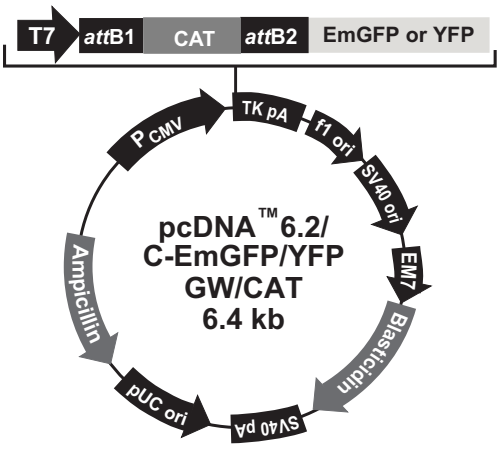
Comments for:	pcDNA™6.2/ N-EmGFP/GW/CAT 6557 bp	pcDNA™6.2/ N-YFP/GW/CAT 6559 bp
CMV promoter:	171-758	174-846
EmGFP/YFP:	882-1598	882-1598
*att*B1 site:	1614-1638	1614-1638
CAT gene:	1653-2312	1653-2312
*att*B2 site:	2323-2347	2323-2347
V5 epitope:	2355-2396	2355-2396
TK polyadenylation signal:	2423-2694	2423-2694
f1 origin:	2730-3158	2730-3158
SV40 early promoter and origin:	3183-3491	3185-3493
EM7 promoter:	3546-3612	3548-3614
Blasticidin resistance gene:	3619-4011	3621-4013
SV40 early polyadenylation signal:	4169-4299	4171-4301
pUC origin (c):	4682-5355	4684-5357
Ampicillin (*bla*) resistance gene (c):	5500-6360	5502-6362
bla promoter (c):	6355-6459	6357-6461

(c) = complementary strand

Map of pcDNA™6.2/C-EmGFP or YFP/GW/CAT

Map

The map below shows the elements of Vivid Colors™ pcDNA™6.2/C-EmGFP and YFP/GW/CAT vectors. The plasmids were generated by PCR amplification of the CAT gene followed by TOPO® Cloning into the pcDNA™6.2/C-EmGFP or YFP-GW/TOPO® vectors. **The complete sequences of these vectors are available for downloading from www.invitrogen.com or by contacting Technical Service (page 41).**



Comments for:	pcDNA™6.2/ C-EmGFP/GW/CAT 6471 bp	pcDNA™6.2/ C-YFP/GW/CAT 6471 bp
CMV promoter:	174-761	174-761
T7 promoter/priming site:	802-821	802-821
*att*B1 site:	854-878	854-878
CAT gene:	893-1552	893-1552
*att*B2 site:	1563-1587	1563-1587
EmGFP/YFP:	1601-2320	1601-2320
TK polyadenylation signal:	2335-2606	2335-2606
f1 origin:	2642-3070	2642-3070
SV40 early promoter and origin:	3097-3405	3097-3405
EM7 promoter:	3460-3526	3460-3526
Blasticidin resistance gene:	3533-3925	3522-3925
SV40 early polyadenylation signal:	4083-4213	4083-4213
pUC origin (c):	4596-5269	4596-5269
Ampicillin (*bla*) resistance gene (c):	5414-6274	5414-6274
bla promoter (c):	6269-6373	6269-6373

(c) = complementary 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 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 if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

Low Salt LB Plates with Blasticidin

Composition:

10 g Tryptone

5 g NaCl

5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH and bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 50-100 µg/ml final concentration.

Store plates and unused medium at +4°C in the dark. Plates and medium containing Blasticidin S HCl are stable for up to 2 weeks.

Continued on next page

Cell Lysis Buffer

Composition:

50 mM Tris, 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.

To prevent proteolysis, you may add 1 mM PMSF, 1 μM leupeptin, or 0.1 μM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

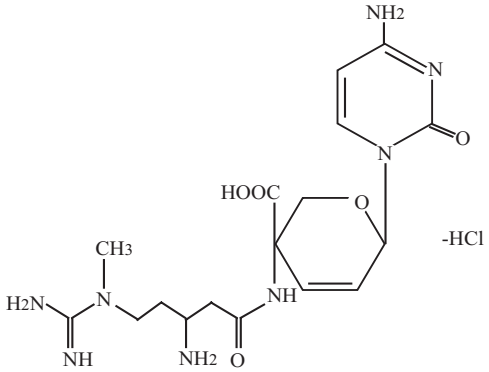
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Blasticidin

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot solution in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.

Medium containing Blasticidin may be stored at +4°C for up to 2 weeks.

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).

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS Requests

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

Continued on next page

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

Use of the Vivid Colors™ pcDNA™6.2/ N- or C-EmGFP or YFP-GW/TOPO®Vectors is covered under the licenses detailed below

Limited Use Label License No. 19: Gateway® Cloning Products

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of ClonaseTM purchased from Life Technologies Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Life Technologies under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200.

Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy** on page 46.

Continued on next page

Purchaser Notification, Continued

Limited Use Label License No. 51: Blasticidin & the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (bsd) are the subject of U.S. Patent No. 5,527,701 sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Limited Use Label License No 127: GFP with Heterologous Promoter

This product and its use is the subject of one or more of U.S. Patent Nos. 5,491,084 and 6,146,826, and foreign equivalents. This product is sold under license from Columbia University. Rights to use this product are limited to research use only, and expressly exclude the right to manufacture, use, sell or lease this product for use for measuring the level of toxicity for chemical agents and environmental samples in cells and transgenic animals. No other rights are conveyed. Not for human use or use in diagnostic or therapeutic procedures. Inquiry into the availability of a license to broader rights or the use of this product for commercial purposes should be directed to Columbia Innovation Enterprise, Columbia University, Engineering Terrace-Suite 363, New York, New York 10027.

Limited Use Label License No. 198: Fluorescent Proteins and Stable Cell Lines Expressing Such Proteins (but not for vectors that contain the genes for such fluorescent proteins)

This product and its use is the subject of one or more of U.S. Patent Nos. 5,777,079, 6,066,476, and 6,319,669 and foreign equivalents. The purchase of this product conveys to the buyer the nontransferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). No rights are conveyed to modify or clone the gene encoding GFP contained in this product. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of this product, or any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies Corporation is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200 or outlicensing@lifetech.com.

Continued on next page

Purchaser Notification, Continued

Limited Use Label LicenseNo 267: Mutant Green Fluorescent Products

This product and its use is the subject of one or more of U.S. Patent Nos. 6,090,919, 5,804,387, 5,994,077, and foreign equivalents.

Limited Use Label License No 272: Humanized GFP

This product is the subject of one or more of U.S. Patent Numbers 5,786,464, 5,795,737, 5,874,304, and 6,114,148 and foreign equivalents licensed by Life Technologies Corporation. This product is sold for research use only. Not for therapeutic or diagnostic use in humans.

Limited Use Label License No 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the Vivid Colors™ pcDNA™6.2/EmGFP or YFP-GW/TOPO® Mammalian Expression Vector Kit.

Vector

- Prior to adaptation with topoisomerase I, the parental supercoiled vectors are qualified by restriction enzyme digestion to verify identity and structure.
- Each control vector is qualified by restriction enzyme digestion to verify identity and structure.

TOPO® Cloning Efficiency

After adaptation with topoisomerase, each lot of the pcDNA™6.2/EmGFP or YFP-GW/TOPO® vectors are functionally qualified using the control reagents included in the kit. Under conditions described on pages 29-30, a 500-bp control PCR product is amplified, TOPO® Cloned into the vector, and transformed into One Shot® TOP 10 Chemically Competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency.

Primers

Primers are lot-qualified by DNA sequencing using the dideoxy chain termination technique.

One Shot® TOP10 Chemically Competent *E. coli*

One Shot® TOP10 Chemically Competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be 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.

Notes

Notes

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996). Modulation of Non-Templated Nucleotide Addition by *Taq* DNA Polymerase: Primer Modifications that Facilitate Genotyping. BioTechniques *20*, 1004-1010.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Cole, C. N., and Stacy, T. P. (1985). Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233.

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659.

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

References

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Shimomura, O., Johnson, F. H., and Saiga, Y. (1962). Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous hHydromedusan, Aequorea. Journal of Cellular and Comparative Physiology *59*, 223-239.

Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. J. Biol. Chem. *269*, 32678-32684.

Shuman, S. (1991). Recombination Mediated by Vaccinia Virus DNA Topoisomerase I in *Escherichia coli* is Sequence Specific. Proc. Natl. Acad. Sci. USA *88*, 10104-10108.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5.

Tsien, R. Y. (1998). The Green Fluorescent Protein. Annu. Rev. Biochem. *67*, 509-544.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677.

Zhang, G., Gurtu, V., and Kain, S. (1996). An Enhanced Green Fluorescent Protein Allows Sensitive Detection of Gene Transfer in Mammalian Cells. Biochem. Biophys. Res. Comm. *227*, 707-711.

invitrogen™

Corporate Headquarters
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

For country-specific contact information visit our web site at **www.invitrogen.com**