pYES2.1 TOPO®
TA Expression Kit

Version J
7 April 2004
25-0261

pYES2.1 TOPO® TA Expression Kit

Five-minute cloning of *Taq* polymerase-amplified PCR products for regulated expression in *Saccharomyces cerevisiae*

Catalog no. K4150-01

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Kit Contents

Shipping/Storage

The pYES2.1 TOPO® TA Expression Kit is shipped on dry ice. Each kit contains a box with pYES2.1 TOPO TA Cloning® reagents (Box 1) and a box with TOP10F´ One Shot® chemically competent cells (Box 2).

Store Box 1 at -20°C and Box 2 at -80°C.

pYES2.1 TOPO TA Cloning® Reagents

pYES2.1 TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.**

Store at -20°C.

Item	Concentration	Amount
pYES2.1/V5-His-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	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
GAL1 Forward Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
V5 C-term Reverse Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8.0	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Sterile Water	--	1 ml
Expression Control Plasmid (pYES2.1/V5-His/*lacZ*)	0.5 µg/µl in TE Buffer, pH 8.0	10 µl

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Kit Contents, continued

One Shot® Reagents

The table below describes the items included in the TOP10F′ One Shot® Chemically Competent *E. coli* kit.

Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10F´ cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Sequencing Primers

The table below provides the sequence and total pmoles supplied of the *GAL1* Forward and the V5 C-term Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
GAL1 Forward	5´-AATATACCTCTATACTTTAACGTC-3´	332
V5 C-term Reverse	5´-ACCGAGGAGAGGGTTAGGGAT-3´	278

Genotype of TOP10F´ Cells

TOP10F´: Use this strain for general cloning of PCR products in pYES2.1/V5-His-TOPO®.

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

Introduction

Overview

Introduction

pYES2.1 TOPO TA Cloning® provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for regulated expression in *Saccharomyces cerevisiae*. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transformed into a *Saccharomyces cerevisiae* host strain, the PCR product can be expressed upon induction with galactose.

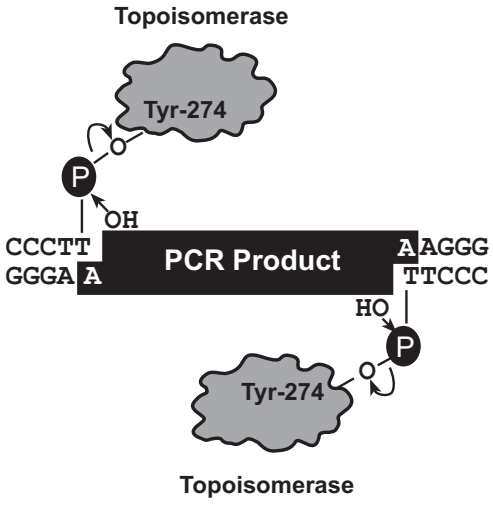
How It Works

The plasmid vector (pYES2.1/V5-His-TOPO®) is 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, which 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 (see below).



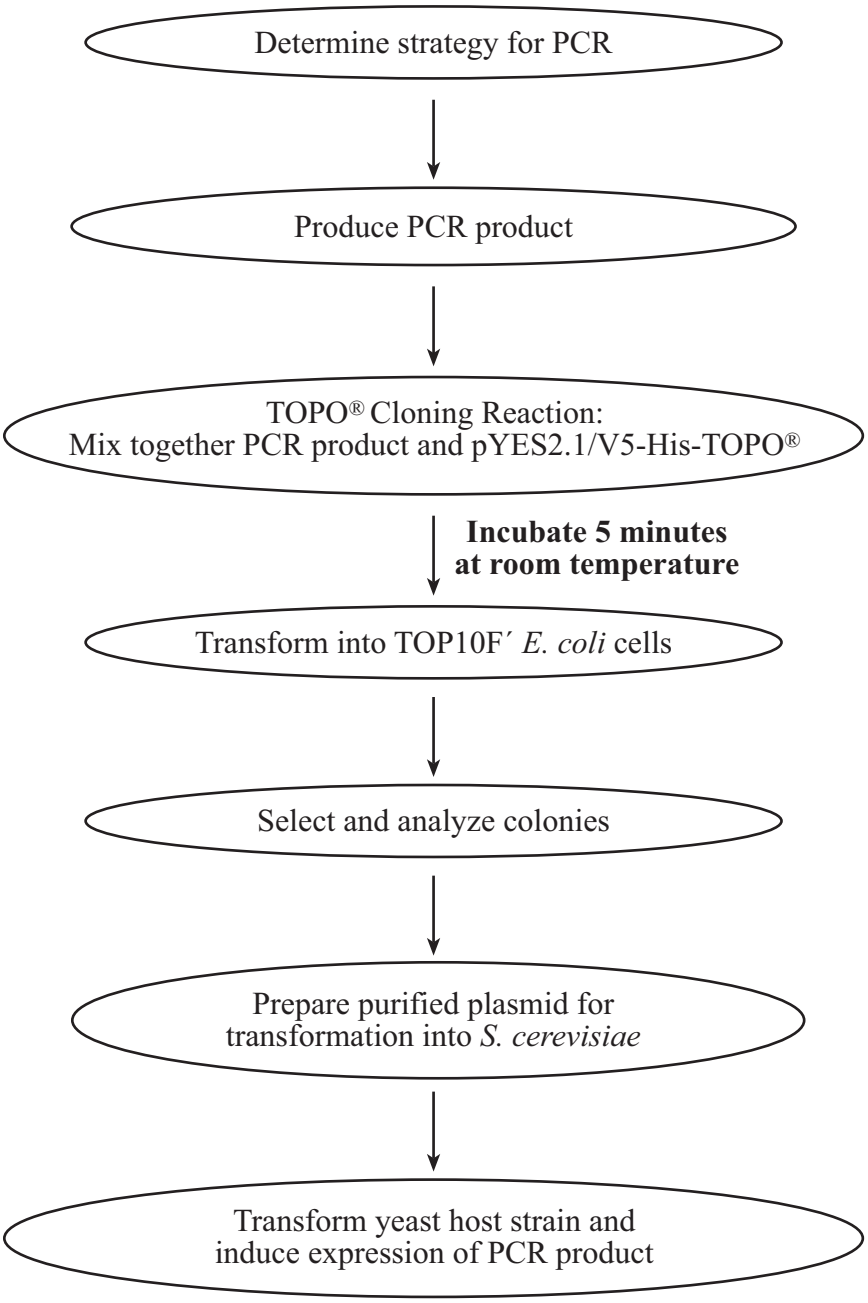
Once the PCR product is cloned into pYES2.1/V5-His-TOPO® and transformants analyzed for the correct orientation, the plasmid may be transformed into a yeast host strain for expression. Expression from the *GAL1* promoter in pYES2.1/V5-His-TOPO® is induced with galactose (Giniger *et al.*, 1985). The PCR product may be expressed as a fusion to the C-terminal V5 epitope and polyhistidine tag for detection and purification; or, by designing the 3´ PCR primer with a stop codon, the PCR product may be expressed as a native protein.

Continued on next page

Overview, continued

Experimental Outline

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



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Overview, continued

Detection of Recombinant Proteins

Once cloned into pYES2.1/V5-His-TOPO®, expression of your PCR product can be detected using an antibody to the protein itself or to the appropriate epitope. The table below describes the antibodies available for use with pYES2.1/V5-His-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The amount of antibody supplied is sufficient for 25 Western blots.

Antibody	Epitope	Catalog no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991): GKPIPNPLLGLDST	R960-25
Anti-V5-HRP		R961-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed using pYES2.1/V5-His-TOPO®, the ProBond™ Purification System is available separately. Additional ProBond™ resin is available in bulk. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 Kit	K854-01
ProBond™ Metal-Binding Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Note

Note that the C-terminal tag cannot be cleaved off. If you wish to express your protein without the C-terminal tag, see **PCR Primer Design**, next page.

Methods

PCR Primer Design

ATG Initiation Codon

Design of the PCR primers to clone your PCR product of interest is critical for expression. This is a C-terminal fusion vector that does not contain an ATG initiation codon. If there is no initiating ATG codon, then this feature needs to be incorporated into your forward primer.

Some researchers prefer to add sequences for translation initiation (Kozak sequences) in the DNA to be amplified (Kozak, 1987; Kozak, 1990; Kozak, 1991). A simple Kozak consensus sequence is provided below. Note that other sequences are possible (see references above), but the G or A at position -3 and the G at position +4 are the most critical (shown in bold). The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Inclusion of C-Terminal Tag

Depending on the nature of your PCR product you have two options to consider:

- Clone in frame with the V5 epitope and polyhistidine tag (C-terminal peptide) in order to detect and/or purify your fusion PCR product.

OR

- Include the native stop codon to express the native protein.

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to **Producing PCR Products**, page 6.

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pYES2.1/V5-His-TOPO®.

Cloning efficiencies may vary depending on the primer nucleotide sequence (see page 20).

Continued on next page

PCR Primer Design, continued

TOPO® Cloning Site of pYES2.1/ V5-His-TOPO®

The diagram below is supplied to help you design appropriate PCR primers to correctly clone and express your PCR product. Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 512 and 513. This is the TOPO® Cloning site. For a map and a description of the features of pYES2.1/V5-His-TOPO®, refer to the **Appendix**, pages 26-27. **The complete sequence of pYES2.1/V5-His-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page** Error! Bookmark not defined.**).**

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 the PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 23.

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, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the pYES2.1 TOPO® TA Expression Kit (see page 21). 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. Call Technical Service for more information (page **Error! Bookmark not defined.**).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR product, ligate it into pYES2.1/V5-His-TOPO®, and transform the recombinant vector into *E. coli* all in one day. It is important to have everything you need set up and ready to use to ensure you obtain the best possible results. If this is the first time you have TOPO® Cloned, you may wish to perform the control reactions on pages 18-19 in parallel with your samples.

Note

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

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

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **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. Read the following information carefully.

Chemically Competent *E. coli*

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

Electrocompetent *E. coli*

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

Materials Supplied by the User

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

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

Continued on next page

TOPO® Cloning Reaction and Transformation, continued

Note

There is no blue-white screening for the presence of inserts. Individual recombinant colonies need to be analyzed by restriction analysis or sequencing for the presence and orientation of the insert in pYES2.1/V5-His-TOPO®. The *GAL1* Forward and V5 C-term Reverse sequencing primers supplied in the kit can be used to sequence across an insert in the TOPO® Cloning site to confirm orientation. Refer to page 5 for the location of the priming sites.

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 chemical transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent TOP10 One Shot® *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 11.

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

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

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

Performing the TOPO® Cloning Reaction

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

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

TOP10 One Shot® Chemical Transformation

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

   Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion (see page 11).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 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).

Note

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

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

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

Continued on next page

TOPO® Cloning Reaction and Transformation, continued

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50-100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis or by sequencing. The *GAL1* Forward and V5 C-term Reverse sequencing primers are included to help you sequence your insert. Refer to the diagram on page 5 for the sequence surrounding the TOPO TA Cloning® site. For the complete sequence of the vector, see our Web site (www.invitrogen.com) or contact Technical Service (see page **Error! Bookmark not defined.**).

   If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology manuals (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 *GAL1* Forward or the V5 C-term Reverse sequencing primer 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 of your choice.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, see pages 18-20. Control reactions are described using reagents supplied in the kit. This will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long term storage. It is also a good idea to keep a DNA stock of your plasmid at -20°C.

1. Streak the original colony out on an LB plate 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 with 50-100 µg/ml ampicillin.
3. Grow the culture to saturation (OD_{600} = 1-2).
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 cloning 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

Yeast Transformation

Introduction

Once you have identified the correct clone, you will purify your pYES2.1/V5-His-TOPO® construct, transform it into the yeast host strain of your choice, and use galactose to induce expression of your PCR product.

Plasmid Preparation

You may use any method of your choice to prepare purified plasmid DNA for small-scale yeast transformation. Standard protocols may be found in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) or *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989). For your convenience, the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) is available from Invitrogen for isolation of plasmid DNA from 10-100 ml of bacterial culture.

Basic Yeast Molecular Biology

The user should be familiar with basic yeast molecular biology and microbiological techniques. Refer to *Current Protocols in Molecular Biology*, Unit 13 (Ausubel *et al.*, 1994) and the *Guide to Yeast Genetics and Molecular Biology* (Guthrie and Fink, 1991) for information on preparing yeast media and handling yeast.

Yeast Host Strain

You may use any *Gal*$^+$ or galactose-utilizing yeast host strain of your choice to transform your pYES2.1/V5-His-TOPO® construct. To allow selection of your pYES2.1/V5-His-TOPO® construct, remember that the strain must be auxotrophic for uracil.

Positive Control

pYES2.1/V5-His/*lacZ* is provided as a positive control vector for yeast transformation and expression. The β-galactosidase protein is fused to a C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag and is expressed from pYES2/V5-His/*lacZ* in yeast cells under the control of the inducible *GAL1* promoter. Successful transformation and induction will result in β-galactosidase expression that can be easily assayed (see pages 15-16).

Yeast Transformation

Use any method of your choice to transform your pYES2.1/V5-His-TOPO® construct into the appropriate yeast host strain. A small-scale yeast transformation protocol is included in the **Appendix** (see page 24) for your convenience. Refer to general reference sources (Ausubel *et al.*, 1994) or published references (Gietz *et al.*, 1992; Gietz *et al.*, 1995; Hill *et al.*, 1991; Schiestl and Gietz, 1989) for other protocols.

The *S. c.* EasyComp™ Kit from Invitrogen (Catalog no. K5050-01) provides a quick and easy method for the preparation of competent yeast cells that can be used immediately or stored frozen for future use. Transformation efficiency is guaranteed at >10^3 transformants per μg DNA.

Select for transformants on SC-U (SC minimal media lacking uracil) selective plates (see the **Appendix**, page 30 for a recipe). Transformants should exhibit uracil prototrophy. Once you have identified a transformant, be sure to prepare a glycerol stock for long-term storage.

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Yeast Transformation, continued

Maintenance of Transformants

Maintain yeast cells containing your pYES2.1/V5-His-TOPO® construct in SC-U medium containing 2% glucose or 2% raffinose (see the next page). See the **Appendix**, page 30 for a recipe for SC-U medium.

Expression of the PCR Product

Introduction

Once you have obtained a transformant containing your pYES2.1/V5-His-TOPO® construct, you are ready to induce expression of your recombinant protein of interest. This section provides information on how to induce and assay for expression of your protein of interest.

GAL1 Promoter

In typical *S. cerevisiae* laboratory strains (i.e. INVSc1), transcription from the *GAL1* promoter is repressed in the presence of glucose (West *et al.*, 1984). Removing glucose and adding galactose as a carbon source induces transcription (Giniger *et al.*, 1985). Maintaining cells in glucose gives the most complete repression and the lowest basal transcription of the *GAL1* promoter. Transferring cells from glucose- to galactose-containing medium causes the *GAL1* promoter to become derepressed and transcription to be induced.

Alternatively, cells may be maintained in medium containing raffinose as a carbon source. The presence of raffinose does not repress or induce transcription from the *GAL1* promoter. Addition of galactose to the medium induces transcription from the *GAL1* promoter even in the presence of raffinose. Induction of the *GAL1* promoter by galactose is more rapid in cells maintained in raffinose when compared to those maintained in glucose.

You may choose to grow cells containing your pYES2.1/V5-His-TOPO® construct in glucose or raffinose depending on how quickly you want to obtain your expressed protein after induction with galactose and on the toxicity of the expressed protein. For more information about expression in yeast, refer to the *Guide to Yeast Genetics and Molecular Biology* (Guthrie and Fink, 1991).

For a protocol to induce expression of your protein with galactose, proceed to **Time Course of Protein Induction by Galactose** on the next page.

Before Starting

Be sure you have the following reagents and equipment on hand before starting.

- SC-U medium containing 2% raffinose or 2% glucose (see **Recipes**, page 30)
- 50 ml conical tubes
- SC-U medium containing 2% galactose (see **Recipes**, page 30)
- 250 ml culture flasks
- Table-top centrifuge
- 15 ml snap-cap, sterile plastic tubes
- Sterile water
- Sterile microcentrifuge tubes
- 30°C water bath

Continued on next page

Expression of the PCR Product, continued

Time Course of Protein Induction by Galactose

To induce expression of your protein of interest from the *GAL1* promoter, galactose is added to the medium. For cells that have been maintained in glucose, recombinant protein can be detected in as little as 4 hours after galactose induction. Recombinant protein can be detected in cells that have been cultured in raffinose by 2 hours after galactose induction.

If you are assaying for expression of your recombinant protein for the first time, we recommend that you perform a time course to optimize expression of your recombinant protein (e.g. 0, 4, 8, 12, 16, 24 hours after galactose induction). A standard protocol is provided below to perform a time course experiment. Other protocols are suitable.

1. Inoculate a single colony containing your pYES2.1/V5-His-TOPO® construct or pYES2.1/V5-His/*lacZ* into 15 ml of SC-U medium containing 2% raffinose or 2% glucose. Grow overnight at 30°C with shaking.
2. Determine the OD_{600} of your overnight culture. Calculate the amount of overnight culture necessary to obtain an OD_{600} of 0.4 in 50 ml of *induction medium* (SC-U medium containing 2% galactose).

   Example: Assume that the OD_{600} of your overnight culture is 3 OD_{600} per ml. Then, the amount of overnight culture needed to inoculate a 50 ml culture to OD_{600} = 0.4 is

   $$\frac{(0.4\ \text{OD/ml})\ (50\ \text{ml})}{3\ \text{OD/ml}} = 6.67\ \text{ml}$$
3. Remove the amount of overnight culture as determined in Step 2 and pellet the cells at 1500 x g for 5 minutes at +4°C. Discard the supernatant.
4. Resuspend the cells in 1-2 ml of *induction medium* and inoculate into 50 ml of *induction medium*. See the **Appendix**, page 30 for a recipe for *induction medium*. Grow at 30°C with shaking.
5. Harvest an aliquot of cells at 0, 4, 8, 12, 16, and 24 hours after addition of cells to the *induction medium*. For each time point, remove 5 ml of culture from the flask and determine the OD_{600} of each sample. You will use this information when assaying for your recombinant protein (see Step 3 on the next page).
6. Centrifuge the cells at 1500 x g for 5 minutes at +4°C.
7. Decant the supernatant. Resuspend cells in 500 µl of sterile water.
8. Transfer cells to a sterile microcentrifuge tube. Centrifuge samples for 30 seconds at top speed in the microcentrifuge.
9. Remove the supernatant.
10. Store the cell pellets at -80°C until ready to use. Proceed to page 16 to prepare cell lysates to detect your recombinant protein.

β-Galactosidase Assay

We recommend using the β-Gal Assay Kit (Catalog no. K1455-01) to assay for the positive control. Lyse your cell pellets using the protocol below. For more information on detecting β-galactosidase activity in yeast (Ausubel, *et al.*, 1994).

Continued on next page

Expression of the PCR Product, continued

Detection of Recombinant Protein

You may use an enzymatic assay to detect your protein or Western blot. To detect the recombinant protein by Western blot (see below), you may use the Anti-V5 Antibody available from Invitrogen (see page 3 for ordering information) or an antibody to your protein. You will also need to prepare a cell lysate from your yeast transformant. A general protocol for small-scale preparation of cell lysates using acid-washed glass beads is provided below for your convenience. Other protocols are suitable. Refer to (Ausubel *et al.*, 1994) for more information. For large-scale preparations (culture volumes over 1 liter), see **Scale-up** on the next page.

Materials Needed:

- Breaking buffer:
  50 mM sodium phosphate, pH 7.4, (see page 31 for recipe of the stock buffer)
  1 mM EDTA (omit EDTA if using this buffer for purification on metal-chelating resins)
  5% glycerol
  1 mM PMSF
- Acid-washed glass beads (0.4-0.6 mm size; Sigma-Aldrich, Catalog no. G8772)

Protocol:

1. You may prepare cell lysates from either frozen cells or fresh cells.

   Reminder: You will need to know the OD_{600} of your cell sample(s) before beginning (see Step 5, previous page).
2. Resuspend fresh or frozen cell pellets in 500 µl of breaking buffer. Centrifuge at 1500 x g for 5 minutes at +4°C to pellet cells.
3. Remove supernatant and resuspend the cells in a volume of breaking buffer to obtain an OD_{600} of 50-100. Use the OD_{600} determined in Step 5, previous page, to calculate the appropriate volume of breaking buffer to use.
4. Add an equal volume of acid-washed glass beads.
5. Vortex mixture for 30 seconds, followed by 30 seconds on ice. Repeat four times for a total of four minutes to lyse the cells. Cells will be lysed by shear force. You can check for the extent of lysis by checking a small aliquot under the microscope.
6. Centrifuge your sample(s) containing glass beads in a microcentrifuge for 10 minutes at maximum speed.
7. Remove supernatant and transfer to a fresh microcentrifuge tube. Assay the lysate for protein concentration using BSA as a standard.
8. Add SDS-PAGE sample buffer to a final concentration of 1X and heat the sample for 5 minutes at 70°C.
9. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your recombinant protein.

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine tag will add approximately 5 kDa to the size of your protein

Purification

Scale-up of Expression for Purification

Once you have determined the optimal induction time necessary to obtain maximal protein expression, you may increase the protein yield by scaling up the procedure described on page 15. To prepare cell lysates from culture volumes over 1 liter, we recommend that you use a bead beater (Biospec Products, Bartlesville, OK) to lyse the cells. Refer to *Current Protocols in Molecular Biology*, Unit 13.13 (Ausubel *et al.*, 1994) for a suitable protocol to lyse cells with a bead beater.

Note

If you are using the breaking buffer (see previous page) for purification of your recombinant protein using ProBond™, do not include EDTA in this buffer as it will interfere with binding of the proteins on ProBond™.

Purification

For help with purification of your recombinant protein, refer to the ProBond™ Purification System manual (ProBond™).

If you are using another type of resin, refer to the manufacturer's recommendations.

Appendix

pYES2.1 TOPO TA Cloning® Control Reactions

Introduction

If you have trouble obtaining transformants or vector containing insert, we recommend performing the following control TOPO® Cloning reactions to help you evaluate your experiment. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the LacZα protein using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing ampicillin, X-gal, and isopropyl β-D-thiogalactoside (IPTG).

Before Starting

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

- 40 mg/ml X-gal in dimethylformamide (see page 29 for a recipe)
- 100 mM IPTG in water (see page 29 for a recipe)
- LB plates containing 50-100 µg/ml ampicillin, X-gal, and IPTG

To add X-gal and IPTG to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of 40 mg/ml X-Gal and 40 µl of 100 mM IPTG onto the plate. Spread evenly and let dry 15 minutes. Protect plates from light.

Producing Control PCR Product

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

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg 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	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the Control TOPO® Cloning Reactions, next page.

Continued on next page

pYES2.1 TOPO TA Cloning® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and pYES2.1/V5-His-TOPO® 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
pYES2.1/V5-His-TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of TOP10 One Shot® chemically competent *E. coli* or electrocompetent *E. coli* (page 9).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 29). Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the "Vector + PCR Insert" reaction should be produced. Greater than 85% of these will be blue and contain the 500 bp insert.

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

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform with 10 pg per 50 µl of cells using the protocol on page 9. Plate 10 µl of the transformation mixture on LB plates containing 50-100 µg/ml ampicillin. Use 20 µl SOC to facilitate spreading. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Continued on next page

pYES2.1 TOPO TA Cloning® Control Reactions, continued

Factors Affecting Cloning Efficiency

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

Variable	Solution
pH>9 in PCR amplification reaction	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)	Increase amount of insert. Or gel-purify as described on page 21.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. **Note:** You may use up to 4 µl of your PCR reaction in a TOPO® Cloning reaction.
Cloning blunt-ended fragments	Add 3′ A-overhangs by incubating with *Taq* polymerase (page 23).
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 21) or optimize your PCR.
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).
	Do not use a 2-step cycling program (denaturation and annealing only) to produce PCR products. Use only a 3-step cycling program (denaturation, annealing, and extension). *Taq* polymerase is more likely to add nontemplate 3′ A-residues in a 3-step cycling program than in a 2-step cycling program.

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. 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.

Note

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

Using the S.N.A.P.™ MiniPrep Kit

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

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

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 8). Be sure to make the gel slice as small as possible for best results.

Continued on next page

Purifying PCR Products, continued

Low-Melt Agarose Method

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

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 8.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into chemically competent TOP10 One Shot® *E. coli* using the method on page 9.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO® TA expression vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the lack of the terminal transferase activity associated with proofreading polymerases which adds the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

You will need the following items:

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

Procedure

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

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
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 pYES2.1/V5-His-TOPO®

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

Note

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

Vent® is a registered trademark of New England Biolabs.

Small-Scale Yeast Transformation

Introduction

A small-scale yeast transformation protocol for routine transformations is provided below. Other protocols are suitable. The *S.c.* EasyComp™ Transformation Kit (Catalog no. K5050-01) is available from Invitrogen for rapid preparation of transformation-competent yeast cells. Visit our Web site (www.invitrogen.com) or call Technical Service for more information (see page **Error! Bookmark not defined.**).

Materials Needed

Be sure to have the following reagents on hand before starting.

- YPD liquid medium (see **Recipe**, page 31)
- 1X TE (see **Recipe**, page 31)
- 1X LiAc/0.5X TE (see **Recipe**, page 32)
- Denatured salmon sperm DNA (see recipe on the next page)
- pYES2.1/V5-His-TOPO® vector construct (or other plasmid DNA to be transformed)
- 1X LiAc/40% PEG-3350/1X TE (See **Recipe**, page 32)
- DMSO
- Selective plates

Protocol

1. Inoculate 10 ml of YPD medium with a colony of your yeast strain and shake overnight at 30°C.
2. Determine the OD_{600} of your overnight culture. Dilute culture to an OD_{600} of 0.4 in 50 ml of YPD medium and grow an additional 2-4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml 1X TE.
4. Pellet the cells at 2500 rpm and resuspend the pellet in 2 ml of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA with 100 µl of the yeast suspension from Step 5.
7. Add 700 µl of 1X LiAc/40% PEG-3350/1X TE and mix well.
8. Incubate solution at 30°C for 30 minutes.
9. Add 88 µl DMSO, mix well, and heat shock at 42°C for 7 minutes.
10. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 ml 1X TE and re-pellet.
12. Resuspend the cell pellet in 50-100 µl 1X TE and plate on a selective plate.

Note

To calculate the number of yeast cells, assume that 1 OD_{600} unit = ~2.0 x 10^7 yeast cells/ml.

Preparation of Denatured Salmon Sperm DNA

Introduction

A convenient protocol to make denatured salmon sperm DNA (Schiestl and Gietz, 1989) is provided for your convenience. You may also purchase denatured salmon sperm DNA from Sigma-Aldrich (Catalog no. D9156). Alternatively, some researchers have found that using yeast transfer RNA (Sigma-Aldrich, Catalog no. R9001) as a carrier results in a cleaner transformation although there are fewer total colonies.

Materials Needed

Prepare or have the following reagents on hand before starting.

- Salmon Sperm DNA (Sigma-Aldrich, Catalog no. D1626)
- 1X TE
- Sonicator
- 50 ml conical centrifuge tubes; 250 ml centrifuge bottle
- TE-saturated phenol; TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- Chloroform
- Low-speed centrifuge
- 3 M sodium acetate, pH 6.0
- 95% ethanol (-20°C)
- Boiling water bath

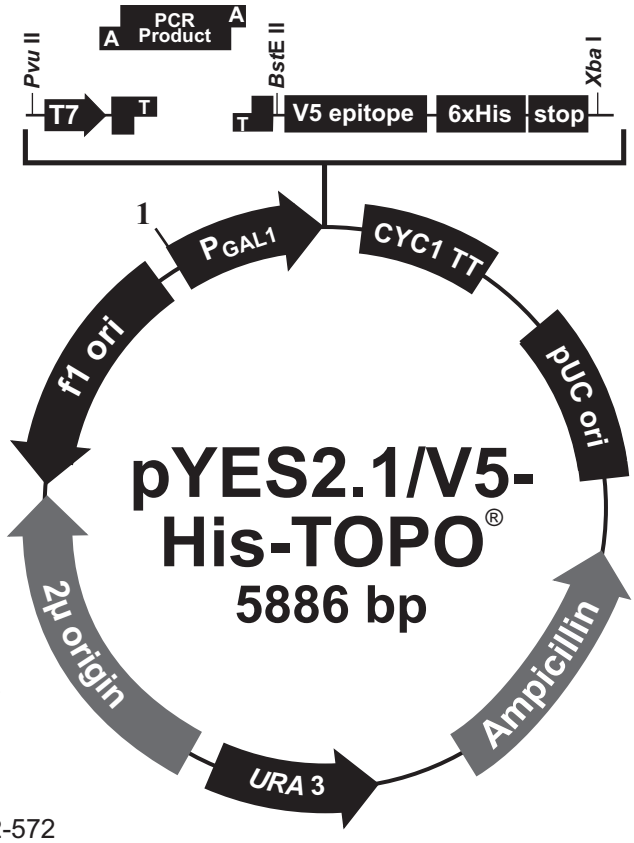
Protocol

1. In a 250 ml flask, dissolve 1 g salmon sperm DNA into 100 ml of TE (10 mg/ml). Pipet up and down with a 10 ml pipet to dissolve completely.
2. Incubate overnight at +4°C.
3. Using a sonicator with a large probe, sonicate the DNA twice for 30 seconds at 3/4 power. The resulting DNA will have an average size of 7 kb. You may verify the size of the DNA on a gel.
4. Aliquot the sonicated DNA into four 50 ml conical centrifuge tubes (25 ml per tube).
5. Extract with 25 ml of TE-saturated phenol. Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
6. Extract with 25 ml of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
7. Extract with 25 ml of chloroform. Centrifuge at 10,000 x g for 5 minutes at +4°C. Transfer the DNA (upper layer) to a 250 ml centrifuge bottle.
8. Add 5 ml of 3 M sodium acetate, pH 6.0 (1/10 volume) and 125 ml ice-cold (-20°C) 95% ethanol (2.5 volume) to precipitate DNA.
9. Pellet the DNA at 12,000 x g for 15 minutes at +4°C.
10. Wash the DNA once with 200 ml 70% ethanol and centrifuge as described in step 9.
11. Partially dry DNA by air or in a Speed-Vac (cover tubes with Parafilm® and poke holes in top) for 20 minutes.
12. Transfer DNA to a 250 ml sterile flask and dissolve DNA in 100 ml sterile TE (10 mg/ml).
13. Boil for 20 minutes to denature DNA. Immediately place on ice, aliquot in 1 ml samples, and freeze at -20°C.

pYES2.1/V5-His-TOPO® Vector

Map of pYES2.1/V5-His-TOPO®

The figure below summarizes the features of the pYES2.1/V5-His-TOPO® vector. The vector is supplied linearized between base pairs 512 and 513. This is the TOPO® Cloning site. **The complete sequence for pYES2.1/V5-His-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**



Comments for pYES2.1/V5-His-TOPO®: 5886 nucleotides

GAL1 promoter: bases 1-451
GAL1 Forward priming site: bases 414-437
T7 promoter: bases 475-494
TOPO® Cloning site: bases 512-513
V5 epitope: bases 543-584
V5 C-term Reverse priming site: bases 552-572
Polyhistidine (6xHis) region: bases 594-611
CYC1 transcription termination signal: bases 633-886
pUC origin: bases 1068-1741
Ampicillin resistance gene: bases 1886-2746 (complementary strand)
URA3 promoter: bases 3647-3872 (complementary strand)
URA3 gene: bases 2764-3645 (complementary strand)
2µ origin: bases 3875-5346
f1 origin: bases 5414-5869 (complementary strand)

Continued on next page

pYES2.1/V5-His-TOPO® Vector, continued

Features of pYES2.1/V5-His-TOPO®

pYES2.1/V5-His-TOPO® (5886 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
GAL1 promoter	Allows inducible expression of genes cloned into pYES2.1/V5-His-TOPO® (West *et al.*, 1984)
GAL1 Forward priming site	Allows sequencing through the insert
T7 promoter	Allows for *in vitro* transcription in the sense orientation
TOPO® Cloning site	Allows insertion of your PCR product in frame with the C-terminal V5 epitope and polyhistidine (6xHis) tag
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the fusion protein with the Anti-V5 Antibody (Catalog no. R960-25) or the Anti-V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991)
V5 C-term Reverse priming site	Allows sequencing of the non-coding strand
C-terminal polyhistidine (6xHis) tag	Allows purification of your fusion protein on metal-chelating resin (i.e. ProBond™). In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25) (Lindner *et al.*, 1997)
CYC1 transcription termination signal	Allows efficient termination and stabilization of mRNA
pUC origin	Maintenance and high copy replication in *E. coli*
Ampicillin resistance gene	Selection of transformants in *E. coli*
URA3 gene	Selection of yeast transformants in uracil-deficient medium
2µ origin	Maintenance and high copy replication in yeast
f1 origin	Rescue of single-stranded DNA

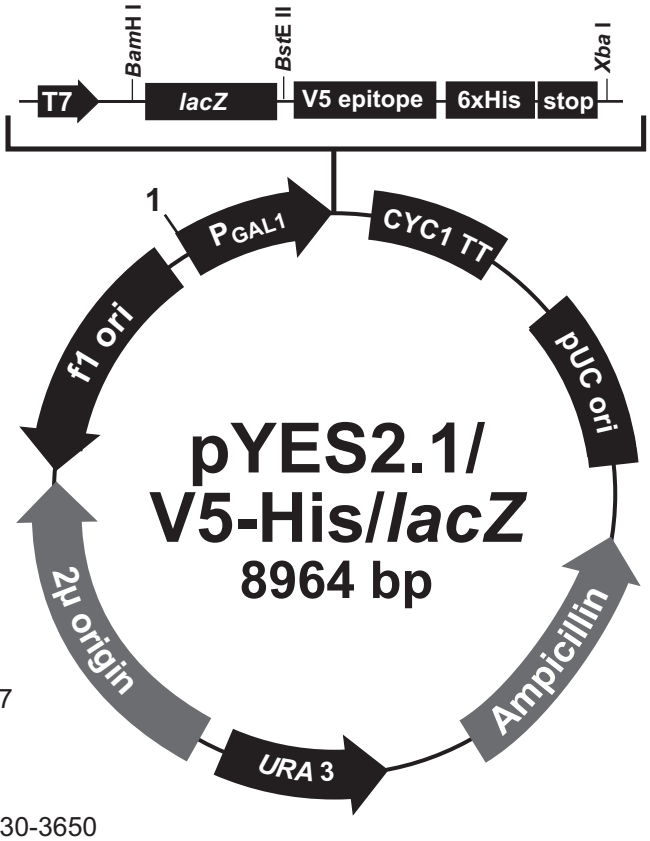
pYES2.1/V5-His/*lacZ* Vector

Description

pYES2.1/V5-His/*lacZ* is an 8964 bp control vector containing the gene for β-galactosidase. The *lacZ* gene was amplified and TOPO® Cloned into pYES2.1/V5-His-TOPO® such that it is in frame with the C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag.

Map of pYES2.1/ V5-His/*lacZ*

The figure below summarizes the features of the pYES2.1/V5-His/*lacZ* vector. **The complete sequence for pYES2.1/V5-His/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**



Comments for pYES2.1/V5-His/*lacZ*: 8964 nucleotides

GAL1 promoter: bases 1-451
GAL1 Forward priming site: bases 414-437
T7 promoter: bases 475-494
LacZ ORF: bases 522-3578
V5 epitope: bases 3621-3662
V5 C-term Reverse priming site: bases 3630-3650
Polyhistidine (6xHis) region: bases 3672-3689
CYC1 transcription termination signal: bases 3708-3956
pUC origin: bases 4146-4819
Ampicillin resistance gene: bases 4964-5824 (complementary strand)
URA3 promoter: bases 6724-6949 (complementary strand)
URA3 gene: bases 5842-6723 (complementary strand)
2µ origin: bases 6953-8424
f1 origin: bases 8492-8947 (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 (50-100 µg/ml ampicillin).
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50-100 µg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

X-Gal Stock Solution

1. To make a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethylformamide.
2. Protect from light by storing in a brown bottle at -20°C.
3. To add to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

IPTG Stock Solution

1. To make a 100 mM stock solution, dissolve 23.8 mg IPTG in 1 ml sterile water.
2. Filter-sterilize.
3. To add to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 100 mM stock solution onto the plate, spread evenly, and let dry 15 minutes.

Continued on next page

Recipes, continued

SC Minimal Medium and Plates

SC is synthetic minimal defined medium for yeast.

0.67% yeast nitrogen base (**without** amino acids **with** ammonium sulfate; Invitrogen, Catalog no. Q300-07)
2% carbon source (i.e. glucose or raffinose)
0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil)
0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine)
2% agar (for plates)

1. Dissolve the following reagents in 900 ml deionized water (800 ml if preparing medium containing raffinose). **Note:** We make medium and plates as we need them and weigh out each amino acid. Many researchers prepare 100X solutions of each amino acid that they need.

Reminder: Omit uracil to make selective plates for growing pYES2.1/V5-His-TOPO® transformants.

6.7 g Yeast Nitrogen Base	**0.1 g each**	**0.05 g each**
	adenine	aspartic acid
	arginine	histidine
	cysteine	isoleucine
	leucine	methionine
	lysine	phenylalanine
	threonine	proline
	tryptophan	serine
	uracil (U)	tyrosine
		valine

2. If you are making plates, add the agar after dissolving the reagents above.
3. Autoclave at 15 psi, 121°C for 20 minutes.
4. Cool to 50°C and add 100 ml of filter-sterilized 20% glucose or 200 ml of filter-sterilized 10% raffinose.
5. Pour plates and allow to harden. Invert the plates and store at +4°C. Plates are stable for 6 months.

Induction Medium

If you are making induction medium, follow Steps 1-3 above except dissolve the reagents in 800 ml of deionized water. Cool the medium to 50°C and add 100 ml of filter-sterilized 20% galactose and 100 ml of filter-sterilized 10% raffinose to the medium.

When making stock solutions of raffinose, do not autoclave the stock solution. Autoclaving the solution will convert the raffinose to glucose. Filter-sterilize the stock solution.

Continued on next page

Recipes, continued

YPD

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (D-glucose)

1. Dissolve 10 g yeast extract, 20 g peptone, and 20 g dextrose (see note below if making plates) in 1000 ml of water.
2. Optional: Add 20 g agar, if making plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Store medium at room temperature or cool the medium and pour plates. The shelf life is approximately one to two months.

Note: If making plates, omit dextrose from Step 1. Autoclaving agar and dextrose together will cause the dextrose to caramelize. Prepare a separate stock solution of 20% dextrose and autoclave or filter-sterilize. After the YPD broth has been autoclaved, add 100 ml of 20% dextrose to the medium.

0.1 M Sodium Phosphate, pH 7.4

Before beginning, have the following reagents on hand.

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$; Sigma-Aldrich S9638)
Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$; Sigma-Aldrich S9390)

1. Prepare 100 ml of 1 M $NaH_2PO_4 \cdot H_2O$ by dissolving 13.8 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
2. Prepare 100 ml of 1 M $Na_2HPO_4 \cdot 7H_2O$ by dissolving 26.81 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
3. For 1L of 0.1 M sodium phosphate, pH 7.4, mix together 22.6 ml of 1 M NaH_2PO_4 and 77.4 ml of 1 M Na_2HPO_4. Bring up the volume to 1 L with deionized water.
4. Filter-sterilize and store at room temperature.

10X TE

100 mM Tris, pH 7.5
10 mM EDTA

1. For 100 ml, dissolve 1.21 g of Tris base and 0.37 g of EDTA in 90 ml of deionized water.
2. Adjust the pH to 7.5 with concentrated HCl and bring the volume up to 100 ml.
3. Filter sterilize and store at room temperature.

Alternatively, you can make the solution using 1 M Tris-HCl, pH 7.5 and 0.5 M EDTA, pH 8.0.

1X TE

10 mM Tris, pH 7.5
1 mM EDTA

Dilute 10X TE 10-fold with sterile water.

Continued on next page

Recipes, continued

10X LiAc

1 M Lithium Acetate, pH 7.5

1. For 100 ml, dissolve 10.2 g of lithium acetate in 90 ml of deionized water.
2. Adjust pH to 7.5 with dilute glacial acetic acid and bring up the volume to 100 ml.
3. Filter sterilize and store at room temperature.

1X LiAc

100 mM Lithium Acetate, pH 7.5

Dilute 10X LiAc solution 10-fold with sterile, deionized water.

1X LiAc/0.5X TE

100 mM Lithium Acetate, pH 7.5
5 mM Tris-HCl, pH 7.5
0.5 mM EDTA

1. For 100 ml, mix together 10 ml of 10X LiAc and 5 ml of 10X TE.
2. Add deionized water to 100 ml.
3. Filter-sterilize and store at room temperature.

50% PEG-3350

1. For 100 ml, dissolve 50 g of PEG-3350 in 90 ml of deionized water. You may have to heat the solution to fully dissolve the PEG.
2. Bring up the volume to 100 ml with deionized water.
3. Autoclave at 121°C, 15 psi for 20 minutes. Store at room temperature.

1X LiAc/40% PEG-3350/1X TE

100 mM Lithium Acetate, pH 7.5
40% PEG-3350
10 mM Tris-HCl, pH 7.5
1 mM EDTA

1. Prepare solution immediately prior to use. For 100 ml, mix together 10 ml of 10X LiAc, 10 ml of 10X TE, and 80 ml of 50% PEG-3350.
2. Filter-sterilize.

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Product Qualification

Introduction

Invitrogen qualifies the pYES2.1 TOPO® TA Expression Kit as described below.

Restriction Digest

The parental supercoiled pYES2.1/V5-His vector is qualified by restriction digest prior to adaptation with topoisomerase. **Note**: Some restriction sites may be removed during the adaptation process and will not be present in the sequence of the linearized, adapted vector.

The expression control vector, pYES2.1/V5-His/*lacZ,* is also qualified by restriction digestion. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pYES2.1/V5-His	pYES2.1/V5-His/*lacZ*
Nsi I	1936, 3925 bp	--
*Hin*d III	5861 bp (linearizes)	--
Xmn I	1174, 1348, 3339 bp	--
Ase I	--	1235, 1774, 2251, 3704 bp
Cla I	--	3353, 5611 bp
Xba I	--	8964 bp (linearizes)

TOPO® Cloning Efficiency

Once the vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 18-19, a 500 bp control PCR product was TOPO® Cloned into the 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® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

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