Verve™ Mammalian Two-Hybrid Kit with TOPO® Tools Technology

Rapid, directional TOPO®-mediated generation of functional constructs for detecting protein-protein interactions

Catalog no. T501-100

Version D

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Kit Contents and Storage

Shipping and Storage

The Verve™ Mammalian Two-Hybrid Kit is shipped on dry ice. Upon receipt, remove the TOPO®-adapted elements and store the elements at -80°C. Store the remaining kit components at -20°C.

Kit Components

The reagents included in the Verve™ Mammalian Two-Hybrid Kit are described in the table below. Enough reagents are provided in the kit to perform 100 reactions. See next page for primer sequences.

Item	Concentration	Amount
PSV40-GAL4 5′ Element, TOPO®-adapted	75 ng/µl double-strand DNA in: 15.63 mM Tris-HCl, pH 8.0 3.85% glycerol (w/v) 0.78 mM EDTA 0.63 mM dithiothreitol (DTT) 0.04% Triton X-100 (v/v)	100 µl
PSV40-VP16 5′ Element, TOPO®-adapted	75 ng/µl double-strand DNA in: 15.63 mM Tris-HCl, pH 8.0 3.85% glycerol (w/v) 0.78 mM EDTA 0.63 mM dithiothreitol (DTT) 0.04% Triton X-100 (v/v)	100 µl
SV40 pA 3′ Element, TOPO®-adapted	75 ng/µl double-strand DNA in: 15.63 mM Tris-HCl, pH 8.0 3.85% glycerol (w/v) 0.78 mM EDTA 0.63 mM dithiothreitol (DTT) 0.04% Triton X-100 (v/v)	200 µl
PSV40-GAL4 5′ Element Forward Primer	Lyophilized in TE Buffer, pH 8.0	20 µg
SV40 pA 3′ Element Reverse Primer	Lyophilized in TE Buffer, pH 8.0	20 µg
pGAL/*lacZ* Reporter Plasmid	0.8 µg/µl in TE Buffer, pH 8.0	80 µg
CAT Control PCR Template	10 ng/µl in TE Buffer, pH 8.0	10 µl
CAT Control PCR Primers 5′ CAT Control and 3′ CAT Control Primer	100 ng/µl each in TE Buffer, pH 8.0	10 µl
pCR®2.1/p53 Bait Control Plasmid	1 µg/µl in TE Buffer, pH 8.0	10 µg
pCR®2.1/LgT (Large T Antigen) Prey Control Plasmid	1 µg/µl in TE Buffer, pH 8.0	10 µg
pCR®2.1/GAL4-VP16 Positive Control Plasmid	1 µg/µl in TE Buffer, pH 8.0	10 µg

Continued on next page

Kit Components, continued

Item	Concentration	Amount
pCR®2.1/VP16-CP (CP; Polyoma Viral Coat Protein, VP3) Negative Control Plasmid	1 µg/µl in TE Buffer, pH 8.0	10 µg
250 mM Tris-HCl	250 mM Tris-HCl, pH 7.5	250 µl

Sequence of PCR Primers

The sequences of the PCR primers included in the kit are listed in the table below.

Primer	Sequence	pmoles Supplied
PSV40-GAL4 5′ Element Forward Primer	5′-TATGTATCATACACATACGATTTAGGT-3′	2425
SV40 pA 3′ Element Reverse Primer	5′--CTCTGACTTGAGCGTCGATTTT-3′	449
5′ CAT Control Primer	5′-CGGAACAAGGGACCATGGAGAAAAAAATCACTGGATA-3′	87
3′ CAT Control Primer	5′-TGAGTCAAGGGCGCCCCGCCCTGCCACTCATCG-3′	100

Accessory Products

Additional Products

The table below lists reagents available from Invitrogen that may be used with the Verve™ Mammalian Two-Hybrid kit or any of the TOPO® Tools products. Ordering information is provided below. A large variety of TOPO® Tools products are available for different applications. Contact Technical Service for more information (see page 36) or visit our Web site at www.invitrogen.com.

Name	Quantity	Catalog no.
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
	500 units	11304-029
S.N.A.P.™ MiniPrep Kit	25 purifications	K1900-25
β-Gal assay Kit	100 reactions	K1455-01
β-gal Antiserum	50 µl*	R901-25
Lipofectamine™ 2000 Reagent	1.5 ml	11668-019
Zero Blunt® TOPO® PCR Cloning Kit for Sequencing	20 reactions	K2875-20
pUB/Bsd TOPO® Cloning Kit	20 reactions	K512-20

*Sufficient antiserum is provided for 25 Western blots

Introduction

Overview of TOPO® Tools Technology

Introduction

TOPO® Tools technology utilizes the unique properties of vaccinia virus DNA topoisomerase I to allow rapid, directional topoisomerase I-mediated joining ("TOPO® Joining") of your PCR products to a choice of 5′ and 3′ elements. You will choose the 5′ and 3′ elements depending on the application you wish to perform. Once your PCR product and the 5′ and 3′ elements are TOPO® Joined, the resulting linear DNA template is amplified to generate a linear DNA construct. This linear DNA construct can be used directly in the appropriate downstream application without the need for additional cloning or transformation steps.

To use the TOPO® Tools technology, you will need:

- Your PCR product(s) of interest containing the appropriate ends to allow efficient topoisomerase I-mediated joining
- TOPO®-adapted 5′ and 3′ elements specific for the application of interest

For more details on the TOPO® Tools technology, refer to the TOPO® Tools Technology manual.

Verve™ Mammalian Two-Hybrid Kit

The Verve™ Mammalian Two-Hybrid Kit uses TOPO® Tools technology to TOPO® Join your PCR products to the appropriate TOPO® Tools elements containing a DNA binding domain and an activation domain. Amplification of these resultant linear DNA templates produces linear DNA constructs. The linear DNA constructs can be directly transfected into a mammalian cell line of choice and used to detect potential protein-protein interactions between your genes of interest.

For more information on the mammalian two-hybrid system and creating the linear DNA constructs containing your genes of interest, refer to pages 3-4.

Features of 5′ and 3′ TOPO®-adapted Elements

The Verve™ Mammalian Two-Hybrid Kit is supplied with two 5′ elements (containing a DNA binding domain and an activation domain) and one 3′ element (containing the SV40 early polyadenylation signal). The important features of the 5′ and 3′ TOPO®-adapted Elements are listed below:

- Supplied as a linear, double-strand element
- Adapted with topoisomerase I to facilitate TOPO® Joining of your gene of interest
- Contains a six base pair single-strand overhang at one end which is complementary to the first six bases on your PCR product for directional TOPO® Joining
- Contains priming sites for secondary amplification using primers included in the kit

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Overview of TOPO® Tools Technology, Continued

Applications

The Verve™ Mammalian Two-Hybrid Kit can be used to:

- Detect protein-protein interactions in mammalian cells
- Confirm yeast two-hybrid interactions in mammalian cells
- Perform functional analysis of protein-protein interactions using site-directed mutagenesis
- Eliminate false positive clones obtained from yeast two-hybrid screening

Using this Manual

To create a linear DNA construct and use the linear DNA construct for an appropriate downstream application, you will need instructions from this manual and the TOPO® Tools Technology manual.

This manual provides the following information:

- Sequences of the 5′ and 3′ TOPO®-adapted elements
- Guidelines for primer design
- Transfection guidelines
- Using the linear DNA construct to detect protein-protein interactions
- Maps and features of the reporter and control plasmids

For information on the TOPO® Tools technology, refer to the TOPO® Tools Technology manual. You may download the manual from our Web site at www.invitrogen.com or contact Technical Service (see page 36). The TOPO® Tools manual includes the information to:

- Design PCR primers
- Amplify your sequence of interest
- TOPO®-Join your PCR product to the appropriate TOPO®-adapted 5′ and 3′ elements to create a linear DNA template
- Amplify the linear DNA template to generate a linear DNA construct
- Perform the control reaction using the CAT control PCR template

Overview of the Mammalian Two-Hybrid System

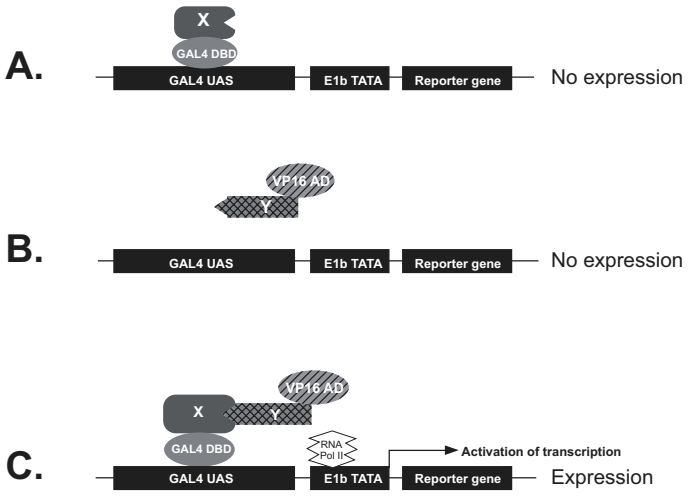
Introduction

The Verve™ Mammalian Two-Hybrid Kit provides a method for detecting interactions between two proteins in mammalian cells. The Verve™ Mammalian Two-Hybrid Kit utilizes the TOPO® Tools technology to generate linear DNA constructs containing your genes of interest (see next page). The mammalian two-hybrid system is similar to the yeast two-hybrid system (Fields and Song, 1989), except the interaction is assayed in mammalian cells. The mammalian two-hybrid system enables you to detect protein interactions in mammalian cells that:

- May not fold properly in yeast
- Involve post-translational modifications or subcellular localization for interaction
- Require certain mammalian cellular factors for interaction

Description

Two-hybrid systems exploit the fact that transcription factors are comprised of two functional domains, a DNA binding domain (DBD) and an activation domain (AD). The DBD recognizes a DNA sequence while the AD facilitates assembly of the transcription machinery and instructs RNA polymerase II to transcribe appropriate genes downstream of the DBD. In two-hybrid systems, the DBD and the AD are physically separated from each other until brought together by two interacting proteins, X and Y. These two interacting proteins, X and Y, are created as linear DNA constructs using the TOPO® Tools Technology (see next page). The first linear DNA construct consists of a *Saccharomyces cerevisiae* GAL4 DBD/protein X fusion known as the "bait" (Figure A) while the second linear DNA construct consists of a herpes simplex virus VP16 AD/protein Y fusion known as the "prey" (Figure B). These two linear DNA constructs and a reporter plasmid (see next page) containing the reporter gene, *lacZ*, are cotransfected into a mammalian host cell line of choice. If protein X interacts with protein Y, the activation domain is brought into close proximity of the DNA-binding domain to reconstitute transcriptional activation and result in expression of the reporter gene (Figure C). Positive interactions are detected by assaying for β-galactosidase activity.



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Overview of the Mammalian Two-Hybrid System, Continued

Creating Linear DNA Constructs

The Verve™ Mammalian Two-Hybrid Kit uses linear DNA constructs instead of plasmid DNA to express the bait and prey fusion proteins. To use the Verve™ Mammalian Two-Hybrid Kit, you will create two linear DNA constructs using the TOPO® Tools procedure.

- A bait linear DNA construct is generated by TOPO® Joining your gene of interest, X, with a 5′ DBD Element (PSV40-GAL4) and a 3′ pA Element (SV40 pA)
- A prey linear DNA construct is created by TOPO® Joining your gene of interest, Y, with a 5′ AD Element (PSV40-VP16) and a 3′ pA Element (SV40 pA).

The resulting linear DNA constructs and a reporter plasmid are directly cotransfected into a mammalian host cell line of choice without any additional cloning or transformation steps. The protein-protein interaction is detected by assaying for β-galactosidase activity. For sequence information and features of the 5′ and 3′ elements, refer to pages 9-12.

Features of the DBD and AD Elements

The features of the DNA binding domain and activation domain are described below:

- The GAL4 DNA binding domain consisting of GAL4 amino acid residues 1-147 (Sadowski *et al.*, 1992) allows binding to the *GAL4* UAS on the reporter plasmid and transcriptional activation of the PSV40-GAL4 5′ Element and your PCR product
- The VP16 transcriptional activation domain (Sadowski *et al.*, 1988) allows expression of the reporter gene which is activated when brought into proximity with the GAL4 DNA binding domain (DBD) by interacting bait and prey proteins

Reporter Plasmid

The reporter plasmid, pGAL/*lacZ,* contains the reporter gene, *lacZ*, which encodes the β-galactosidase protein. Expression of *lacZ* is controlled by a hybrid promoter consisting of *Saccharomyces cerevisiae* GAL4 upstream activating sequences (UAS) (Giniger *et al.*, 1985) linked to the TATA box sequence from the adenovirus E1b minimal promoter (Lillie and Green, 1989). Contained within the GAL4 UAS are 6 copies of the 17 nucleotide sequence, 5′-(T/C)GGAGTACTGTCCTCCG-3′ that constitute the binding site for the yeast GAL4 transcription factor. Each 17 nucleotide sequence serves as the binding site for two molecules of the GAL4 DBD (Marmorstein *et al.*, 1992). The background β-galactosidase activity is very low since expression of *lacZ* is minimal in the absence of activation from the GAL4 UAS. Interaction between the bait and prey protein brings the DBD and the AD into close proximity of each other to activate transcription of the reporter gene from the GAL4 UAS and E1b promoter. For more information on the specific features of the pGAL/*lacZ* plasmid, refer to pages 24-25.

Experimental Overview

Experimental Outline

The table below outlines the experimental steps necessary to detect protein-protein interactions using the Verve™ Mammalian Two-Hybrid Kit and your PCR product. Refer to the indicated manual for more details on each step. We recommend that you read the entire TOPO® Tools Technology manual to familiarize yourself with the technology and the various steps necessary to create the linear DNA construct containing your PCR product and a 5′ and 3′ element.

Refer to the figure on the next page for an illustrated depiction of the experimental outline.

Step	Action	Manual
1	Design PCR primers to link your gene of interest with the appropriate 5′ and 3′ elements. Consult the diagram in this manual for each element to help you design your PCR primers.	TOPO® Tools Technology Verve™ Mammalian Two-Hybrid Kit (pages 9-12)
2	Amplify your gene of interest with a thermostable polymerase.	TOPO® Tools Technology
3	TOPO® Join your PCR product (bait protein) to the PSV40-GAL4 5′ Element and SV40 pA 3′ Element to create a linear DNA template for the bait protein.	
4	TOPO® Join your PCR product (prey protein) to the PSV40-VP16 5′ Element and SV40 pA 3′ element to create a linear DNA template for the prey protein.	
5	Amplify both linear DNA templates with a thermostable proofreading polymerase to generate two linear DNA constructs.	
6	Verify the integrity and concentration of your PCR product.	
7	Cotransfect both linear DNA constructs and the reporter plasmid (pGAL/*lacZ*) into a cell line of choice.	Verve™ Mammalian Two-Hybrid Kit (pages 15-16)
8	Analyze a potential protein-protein interaction using an assay for β-galactosidase.	Verve™ Mammalian Two-Hybrid Kit (page 17)
9	TOPO® clone your linear DNA constructs into pCR®4Blunt-TOPO® or a vector of choice for sequencing, if desired.	Verve™ Mammalian Two-Hybrid Kit (page 19)

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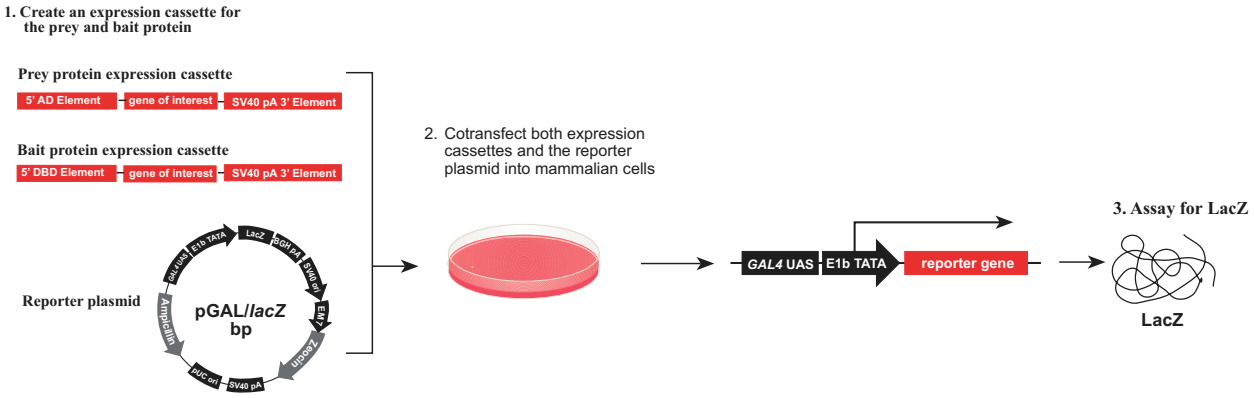
Experimental Overview, Continued

Experimental Outline, continued



High-Throughput Application

The Verve™ Mammalian Two-Hybrid Kit can be used to produce linear DNA constructs for mammalian transfections and detection of protein-protein interactions in a high-throughput format. You can perform the TOPO® Tools procedure, transfect mammalian cells, and assay for β-galactosidase activity in a high-throughput format. For more information and guidelines to use the TOPO® Tools Technology in a high-throughput format, refer to the TOPO® Tools Technology manual.

Materials Supplied by the User

You will need the following items on hand before using this kit:

- Mammalian cell line of choice
- Appropriate transfection reagent
- Assay kit for β-galactosidase
- Cell culture media
- Fetal Bovine Serum (FBS)
- Phosphate Buffered Saline (PBS)
- Cell culture equipment
- Centrifuge or microcentrifuge

Methods

Using the TOPO® Tools 5′ and 3′ Elements

Introduction

General guidelines are provided below to design primers to amplify your genes of interest. Sequence information for the 5′ and 3′ elements is provided on pages 9-12.

For more details and an example of designing PCR primers for proper TOPO® Joining and generation of the linear DNA construct, refer to the TOPO® Tools Technology manual.

Requirements for Directional TOPO® Joining

To perform directional TOPO® Joining, the 5′ ends of your forward and reverse PCR primers **MUST** contain an 11 base pair sequence consisting of the following:

- A six base pair sequence which base pairs with the overhang sequence on the TOPO®-adapted 5′ or 3′ element. The six base pair sequence facilitates directional TOPO® Joining and differs for the forward and reverse primer (see below)
- A five base pair sequence (AAGGG, shown in bold in the sequences below) which is complementary to the topoisomerase I recognition site (CCCTT) to allow double strand joining of the PCR product to the 5′ and 3′ elements

Forward Primer: 5′-CGGAAC**AAGGG**-3′

Reverse Primer: 5′-TGAGTC**AAGGG**-3′

Important

- **To obtain consistent and efficient results in the TOPO® Joining reaction, we highly recommend that you use HPLC-purified oligonucleotides to produce your PCR products**. Using a mixture of full-length and non full-length primers to produce your PCR products can reduce the efficiency of TOPO® Joining and result in poor yield of linear DNA construct after secondary amplification. Once you have obtained your HPLC-purified oligonucleotides, we also recommend that you use polyacrylamide gel electrophoresis to verify the length of your oligonucleotides.
- Do not add 5´ phosphates to your primers for PCR. This will prevent TOPO® Joining.

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Note

- Make sure that the PCR product does not contain an ATG initiation codon.
- Design primers to link your PCR product in frame with the DNA binding domain or the activation domain to generate bait or prey fusion protein.
- If you design your primers such that the first three base pairs following the six base pair overhang and the topoisomerase recognition site encode a complete codon, your PCR product will automatically be linked in frame with the DNA binding domain or the activation domain.
- Be sure to include a stop codon in the reverse primer or design the reverse primer to hybridize downstream of the native stop codon. If your PCR product does not contain a stop codon, you may use a potential stop codon present in the SV40 pA 3′ Element that is in frame with your PCR product (see page 12). Note that using this stop codon will add 12 amino acids to the C-terminal of your PCR product.

Using the 5′ DNA Binding Domain Element

Introduction

This section provides sequence information and important features of the 5′ DNA Binding Domain Element, PSV40-GAL4 5′ Element supplied with the Verve™ Mammalian Two-Hybrid Kit. You will TOPO® Join the gene that encodes your bait protein to the PSV40-GAL4 5′ Element and SV40 pA 3′ Element to create the bait linear DNA template. Use the sequence of the PSV40-GAL4 5′ Element provided on the next page to properly design your PCR primers.

PSV40-GAL4 5′ Element

The PSV40-GAL4 5′ Element contains the following features:

- SV40 promoter for expression of your PCR product
- GAL4 DNA binding domain to allow binding to the *GAL4* UAS on the reporter plasmid and transcriptional activation of the PSV40-GAL4 5′ Element and your PCR product
- Topoisomerase binding site (CCCTT) for directional topoisomerase-I mediated joining of your PCR product to the 5′ element
- Priming site for secondary amplification of the linear DNA template using the PSV40-GAL4 5′ Element Forward Primer

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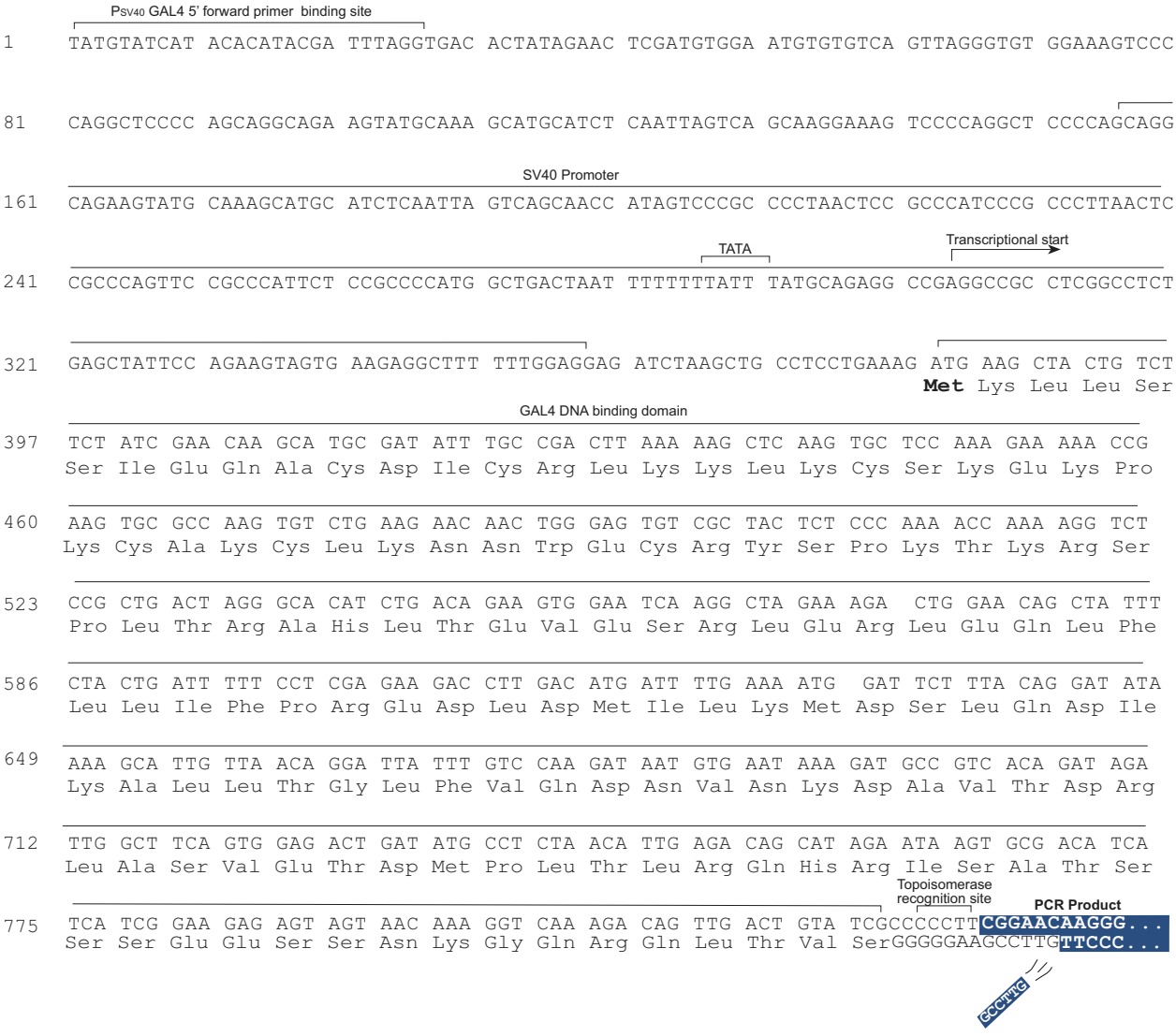
Using 5′ DNA Binding Domain Element, Continued

Sequence of PSV40-GAL4 5′ Element

The sequence of the PSV40-GAL4 5′ Element is shown below. The PSV40-GAL4 5′ Element is supplied as a linear fragment adapted with topoisomerase I on the 3′ end to facilitate TOPO® Joining to your PCR product (bait gene). **The complete sequence of the PSV40-GAL4 5′ Element is available for downloading from our Web site at www.invitrogen.com or by contacting Technical Service (see page 36)**.



Using the 5′ Activation Domain Element

Introduction

This section provides sequence information and important features of the 5′ Activation Domain Element, PSV40-VP16 5′ Element, supplied with the Verve™ Mammalian Two-Hybrid Kit. You will TOPO® Join the gene encoding your prey protein to the PSV40-VP16 5′ Element and SV40 pA 3′ Element to create the prey linear DNA template. Use the sequence of the PSV40-VP16 5′ Element provided below to properly design your PCR primers.

PSV40-VP16 5′ Element

The PSV40-VP16 5′ Element contains the following features:

- SV40 promoter for expression of your PCR product (prey protein)
- VP16 transcriptional activation domain to allow expression of the reporter gene which is activated when brought into proximity with the GAL4 DNA binding domain (DBD) by interacting bait and prey proteins
- Topoisomerase binding site (CCCTT) for directional topoisomerase-I mediated joining of your PCR product to the 5′ element

Sequence of PSV40-VP16 5′ Element

The sequence of the PSV40-VP16 5′ Element is shown below. The PSV40-VP16 5′ Element is supplied as a linear fragment adapted with topoisomerase I on the 3′ end to facilitate TOPO® Joining of your PCR product (prey gene). **The complete sequence of the PSV40-VP16 5′ Element is available for downloading from our Web site at www.invitrogen.com or by contacting Technical Service (see page 36).**

PSV40 GAL4 5′ forward primer binding site

```
1    TATGTATCAT ACACATACGA TTTAGGTGAC ACTATAGAAC TCGATGTGGA ATGTGTGTCA GTTAGGGTGT GGAAAGTCCC

81   CAGGCTCCCC AGCAGGCAGA AGTATGCAAA GCATGCATCT CAATTAGTCA GCAAGGAAAG TCCCCAGGCT CCCCAGCAGG
```

SV40 Promoter

```
161  CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC
```

TATA Transcriptional start

```
241  CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT

321  GAGCTATTCC AGAAGTAGTG AAGAGGCTTT TTTGGAGGAG ATCTAAGCTA GCGCCGCCACC ATG GGC CCT AAA AAG
                                                                        Met Gly Pro Lys Lys
```

VP16 activation domain

```
397  AAG CGT AAA GTC GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC
     Lys Arg Lys Val Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp

463  GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG GAT TCC
     Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp Ser

529  CCG GGT CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG
     Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu
```

Topoisomerase recognition site PCR Product

```
595  TTT GAG CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGCCCTTCGGAACAAGGG..
     Phe Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly CCGGGAAGCCTTGTTCCC..
```

CCCTTG

Using the 3′ Element

Introduction

This section provides sequence information and important features of the SV40 pA 3′ Element supplied with the Verve™ Mammalian Two-Hybrid Kit. You will TOPO® Join your bait and prey proteins to the SV40 pA 3′ Element and the appropriate 5′ element to create the prey and bait linear DNA template. Use the sequence of the SV40 pA 3′ Element provided below to properly design your PCR primers.

SV40 pA 3′ Element

The SV40 pA 3′ Element contains the following features:

- SV40 early polyadenylation sequence for efficient polyadenylation of mRNA
- Topoisomerase binding site (CCCTT) for directional topoisomerase-I mediated joining of your PCR product to the 3′ element
- Priming site for secondary amplification of the linear DNA construct using the SV40 pA 3′ Element Reverse Primer

Sequence of SV40 pA 3′ Element

The sequence of the SV40 pA 3′ Element is provided below. The SV40 pA 3′ Element is supplied as a linear fragment adapted with topoisomerase I on the 5′ end to facilitate TOPO® Joining of your PCR product. The potential stop codon is underlined. **The complete sequence of the SV40 pA 3′ Element is available for downloading from our Web site at www.invitrogen.com or by contacting Technical Service (see page 36)**.

Propagating and Maintaining Plasmids

Introduction

The amount of the reporter plasmid included in the Verve™ Mammalian Two-Hybrid Kit is sufficient to perform 100 transfections. Depending on your transfection method and the number of cells you are transfecting, you may need to propagate the reporter or control plasmids. Guidelines for propagating and maintaining the control and reporter plasmids are provided below. If you do not need to propagate the plasmids, proceed to **Transfecting the Linear DNA Construct**, next page.

E. coli Strain

Many *E. coli* strains are suitable for propagating the control and reporter plasmids including TOP10 (Catalog no. C610-00), TOP10F´ (Catalog no. C615-00), DH5α, JM109, or equivalent. We recommend propagating the control and reporter plasmids in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). TOP10 *E. coli* are available from Invitrogen (see the table below).

Item	Quantity	Catalog no.
One Shot™ TOP10 Chemically Competent *E. coli*	21 x 50 µl	C4040-03
One Shot™ TOP10 Electrocomp™ *E. coli*	10 x 50 µl	C4040-50

Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of Plasmids

Use the following steps to maintain the control and reporter plasmids:

1. Use the stock solution of the plasmids to transform a *rec*A, *end*A *E. coli* strain like TOP10, TOP10F´, or equivalent. Use 10 ng of each plasmid for transformation of *E. coli*.
2. Select transformants on LB plates containing 50 µg/ml ampicillin.
3. Store the stock solution at -20°C when finished.
4. Prepare a glycerol stock of each strain containing plasmid for long-term storage (see below for a protocol).

Preparing a Glycerol Stock

Once you have transformed the control and reporter plasmids into a suitable *E. coli* strain, purify a single transformant and 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 the appropriate antibiotic. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing the appropriate antibiotic.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial. Store at -80°C.

Transfecting the Linear DNA Construct

Introduction

At this point you should have generated two linear DNA constructs using the 5′ and 3′ TOPO®-adapted elements provided in the kit and your PCR products. The bait linear DNA construct contains your bait PCR product TOPO® Joined to the PSV40-GAL4 5′ Element and the SV40 pA 3′ Element, and the prey linear DNA construct contains your prey PCR product TOPO® Joined to the PSV40-VP16 5′ Element and SV40 pA 3′ Element. After generating the two linear DNA constructs, you must purify the linear DNA constructs before using them for transfection (see below).

This section provides transfection guidelines and recommendations to cotransfect the bait and prey linear DNA constructs into a mammalian cell line of choice. We recommend including positive controls for transfection in your experiments. An example of setting up the cotransfection with the appropriate controls in a mammalian cell line is provided on page 20.

Before Starting

- You need to purify your linear DNA constructs prior to cotransfection. A purification protocol using the S.N.A.P.™ Miniprep Kit is provided in the **Appendix**, page 23. Other purification protocols are suitable.
- You need to have the appropriate amount of DNA for transfection based on the number of cells you are transfecting and the transfection reagent (see next page for an example).
- You should have the appropriate cell line in a log phase of growth at 50-80% confluency at the time of transfection.

Generating a Reporter Stable Cell Line

The reporter plasmid, pGAL/*lacZ*, contains the Zeocin™ resistance gene to allow for selection of stable cell lines using Zeocin™. If you wish to create stable cell lines, transfect pGAL/*lacZ* into a mammalian cell line of choice and select for stable transfectants using Zeocin™. General information and guidelines for generating a reporter stable cell line are provided on page 30. For more information on Zeocin™ and its mechanism of action, refer to pages 31-32.

Follow the recommendations given below to obtain optimal results:

- Use a molar ratio of 1:1:1 to 2:2:1 (prey:bait:reporter) for cotransfecting the linear DNA constructs and the reporter plasmid.
- Use a cell line that does not contain any proteins that may interfere with the protein-protein interaction or does not exhibit any basal expression of the reporter gene, β-galactosidase. We recommend using CHO-K1 (ATCC number: CRL-1573) or HEK-293 (ATCC number: CCL-61) cell lines.
- Perform transfections in duplicates or triplicates.
- Perform transfections with the control plasmids included in the kit to verify that the system is working properly (see page 20).
- Optimize transfection of your cell line before performing the two-hybrid assay.

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Transfecting the Linear DNA Construct, Continued

Guidelines for Transfection

Refer to the guidelines below to cotransfect the linear DNA constructs and the reporter plasmid into a mammalian cell line of choice.

- Purify the linear DNA constructs prior to transfection (see previous page).
- The amount of the linear DNA construct you need to use for transfection will depend on the cell number (see below for an example). You need to optimize the amount of DNA used for transfection for a particular cell line.
- Use a molar ratio of 1:1:1 to 2:2:1 (prey:bait:reporter) to cotransfect the linear DNA constructs and the reporter plasmid.
- You may use any mammalian cell line of choice (see previous page for cell line recommendations). For high-throughput applications, we recommend using CHO-K1 suspension cells.
- To obtain high transfection efficiency across a wide variety of mammalian cell lines, we recommend using lipid-based reagents for transfection. We typically use Lipofectamine™ 2000 Reagent to transfect HEK-293 or CHO-K1 cells. Lipofectamine™ 2000 Reagent is available from Invitrogen (see page vi for ordering information). Other transfection methods are suitable.
- The sensitivity of the β-galactosidase assay is dependent on the transfection efficiency. A high transfection efficiency will increase the sensitivity of the assay and will require smaller sample volumes for assay.
- If you need to perform transfection in a high-throughput format, you may directly transfect the linear DNA constructs amplified in a 96-well plate. We recommend using Lipofectamine™ 2000 Reagent for transfection as Lipofectamine™ 2000 Reagent can be added directly to the cells in the presence of serum and does not require removal of the lipid after transfection or addition of medium following transfection.

Example of a Cotransfection

The following example demonstrates the amount of prey linear DNA construct, bait linear DNA construct, and reporter plasmid used for cotransfection using different numbers of CHO-K1 cells. The bait linear DNA construct is PSV40-GAL4 5′ Element-p53-SV40 pA 3′ Element (2400 bp). The prey linear DNA construct is PSV40-VP16 5′ Element-Large T antigen-SV40 pA 3′ Element (3300 bp). The reporter plasmid is pGAL/*lacZ* (7623 bp).

Cell Number	Type of Plate	Prey Linear DNA construct (µg)	Bait Linear DNA construct (µg)	Reporter Plasmid (µg)
7.5×10^5 cells/well	6-well	0.8	0.8	2.0
3×10^5 cells/well	12-well	0.4	0.4	1.0
1.5×10^5 cells/well	24-well	0.2	0.2	0.5
2×10^3 cells/well	96-well	0.03	0.03	0.06

Transfecting the Linear DNA Construct, Continued

Setting up the Cotransfection

1. Use the table below to set up cotransfection of the linear DNA constructs and the reporter plasmid. Be sure to set up the cotransfections in duplicates or triplicates and use a molar ratio of 1:1:1 - 2:2:1 (prey:bait:reporter). We recommend transfecting the cells with the control plasmids as described in the table below. For more information on the control plasmids, refer to page 20.

Sample	Prey	Bait	Reporter Plasmid	Purpose
Test	PSV40-VP16 5′ Element-**Y**-SV40 pA 3′ Element	PSV40-GAL4 5′ Element-**X**-SV40 pA 3′ Element	pGAL/*lacZ*	To detect a protein-protein interaction between your test proteins
Background Control 1	PSV40-GAL4 5′ Element-**Y**-SV40 pA 3′ Element	--	pGAL/*lacZ*	To check if your protein Y functions as a transcriptional activator
Background Control 2	--	PSV40-VP16 5′ Element-**X**-SV40 pA 3′ Element	pGAL/*lacZ*	To check if your protein X functions as a transcriptional activator
Background LacZ Control	--	--	--	To determine the background β-galactosidase activity in your cell line
Positive Control	pCR®2.1/GAL4-VP16 Control Plasmid	--	pGAL/*lacZ*	To verify induction of the reporter gene
Positive Interaction Control	pCR®2.1/LgT Prey Control Plasmid	pCR®2.1/p53 Bait Control Plasmid	pGAL/*lacZ*	To verify that the cell line and detection are working properly
Negative Control	pCR®2.1/VP16-CP Plasmid	pCR®2.1/p53 Bait Control Plasmid	pGAL/*lacZ*	To detect levels of false positive interactions

2. Once you transfect the cells as described above, incubate the cells for 24-72 hours at 37°C in a CO_2 incubator. You may have to optimize the incubation time for your cell type and for the proteins you are testing. If the interaction is strong, you should be able to detect a signal after 24 hours. For weak interactors, you may need to incubate the cells for 48-72 hours.
3. Harvest cells after the appropriate incubation time and perform the β-galactosidase assay to detect a protein-protein interaction (see next page).

Analyzing Protein-Protein Interactions

Introduction

Once you have cotransfected the linear DNA constructs and the reporter plasmid into a cell line of choice, you are ready to detect protein-protein interaction using an assay for the reporter gene, *lacZ*. The *lacZ* gene encodes β-galactosidase protein. β-galactosidase catalyzes the hydrolysis of β-galactosides such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG). Hydrolysis of ONPG to the ONP anion produces a bright yellow color with a peak absorbance at 420 nm that can be quantified using a spectrophotometer.

Detecting β-galactosidase

β-galactosidase can be detected by assaying the enzymatic activity of β-galactosidase or immunodetecting the expression of β-galactosidase using β-gal Antiserum.

A protocol is provided below for detecting β-galactosidase activity using the β-Gal Assay Kit from Invitrogen (see page vi for ordering information). For more details on the β-Gal Assay Kit and the procedure, download the β-Gal Assay manual from our Web site at www.invitrogen.com or contact Technical Service (see page 36). For **Immunodetection of β-galactosidase**, see page 19.

Materials Supplied by the User

Be sure to have the following items on hand before starting:

- 37°C water bath
- β-Gal Assay Kit (Catalog no. K1455-01)
- Spectrophotometer capable of measuring at 420 nm
- Cuvettes
- Tubes of appropriate size or microtiter plate

Note: Cleavage Buffer, Lysis Buffer, and Stop Buffer are supplied with the β-Gal Assay Kit from Invitrogen. If you are using any other method for assaying β-galactosidase activity, you may need to prepare appropriate solutions.

Before Starting

Prepare the following solutions before starting:

- Dilute the 10X PBS and 10X Cleavage Buffer from the β-Gal Assay Kit to make 1X solutions by adding 90 ml deionized water to 10 ml of the stock solution.
- Add 270 µl β-mercaptoethanol to 100 ml of 1X Cleavage Buffer before use.

Unused 1X solutions may be stored at +4°C for 6 months for use in future assays.

Continued on next page

Harvesting the Cells

1. Remove the growth medium from the transfected cells and wash the transfected cells once with 1X PBS.
2. Harvest cell monolayers with trypsin/EDTA or by scraping cells into 1 ml of 1X PBS.
3. Centrifuge cells at 250 x g for 5 minutes. Aspirate the supernatant.
4. Resuspend the pellet in 1X Lysis Buffer included in the β-Gal Assay Kit. The amount of Lysis Buffer used depends on the size of the cell pellet harvested 24-72 hours posttransfection (see the table below). Keep the sample at +4°C.

Size of Cell Pellet	Amount of 1X Lysis Buffer
6 well plate	250 µl
12 well plate	125 µl
24 well plate	62 µl
96 well plate	16 µl

5. Freeze the sample on dry ice and thaw at 37°C. Repeat the freeze-thaw procedure 2 more times.
6. Pellet the insoluble cell material by centrifugation at maximum speed at +4°C for 5 minutes. Transfer the supernatant to a new microcentrifuge tube.

β-galactosidase Assay

If you have a large number of samples or wish to perform high-throughput analysis, you may perform the β-galactosidase assay in a microtiter plate. If you decide to use a microtiter plate for the assay, you will need a spectrophotometer that is capable of reading microtiter plates at 420 nm.

1. For each sample (see Step 5, above), assay at least three different volumes of lysate (*e.g.* 1, 5, and 10 µl). Transfer cell lysate to a fresh microcentrifuge tube.
2. Add 17 µl of ONPG and 50 µl 1X Cleavage Buffer with β-mercaptoethanol. Mix by gently flicking the tube and centrifuging briefly.
3. Incubate the tube at 37°C for 5-20 minutes. You should see a faint yellow color develop if β-galactosidase is present.
4. To stop the reaction, add 125 µl of Stop Buffer. There may be some intensifying of the color. The final volume is 202 µl.
5. Read the absorbance at 420 nm against a blank containing ONPG and Cleavage Buffer without lysate. Be sure to assay a sample of the untransfected cell lysate as a control. Changes in absorbance should be linear with respect to the amount of lysate assayed to get an accurate determination of activity.
6. Once you have obtained an accurate reading of your lysate, determine the protein concentration of the lysate using your method of choice.
7. Calculate the specific activity of the lysate using the formula provided on the next page.

If you are having problems obtaining any β-galactosidase activity, refer to the **Troubleshooting** section on page 21 for more information.

Continued on next page

Calculating the Specific Activity

Use the formula given below to calculate the specific activity of your sample.

Specific activity = nmoles of ONPG hydrolyzed/t/mg protein

$$\text{nmoles of ONPG hydrolyzed} = \frac{(OD_{420})\ (2.02 \times 10^5\ \text{nl})}{(4500\ \text{nl/nmole-cm})\ (1\ \text{cm})}$$

where 4500 is the extinction coefficient, t = the time of incubation in minutes at 37°C, and mg protein is the amount of protein assayed. Be sure to subtract the background activity of the untransfected cell lysate.

Immunodetection of β-galactosidase

You can detect expression of β-galactosidase by immunodetection using β-gal Antiserum available from Invitrogen (see page vi for ordering information).

For more details on the western blot protocol using the β-gal Antiserum, refer to the β-gal Antiserum manual. You may download the manual from our Web site at www.invitrogen.com or contact Technical Service (see page 36). For information about SDS-polyacrylamide gel electrophoresis, western blotting, and immunodetection, see Ausubel, *et al.*, 1994.

The Next Step

Once you have identified the proteins that interact with each other using the Verve™ Mammalian Two-Hybrid Kit, you may clone the bait and prey linear DNA constructs into an appropriate vector to sequence the constructs or create an "archival copy" of your linear DNA constructs. We recommend using the pCR®4Blunt-TOPO® vector available with the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (see page vi for ordering information). The important features of pCR®4Blunt-TOPO® are listed below:

- Rapid ligation of blunt-end PCR products
- TOPO® activated vector to enable rapid ligation of PCR products with compatible ends
- T7, T3, M13 forward, and M13 reverse sequencing primer sites close to the PCR product insertion site for streamlined sequence analysis
- pUC origin of replication for high-copy replication and maintenance of the plasmid in *E. coli*

If you wish to generate stable cells using your linear DNA constructs, you may use the pUB/Bsd TOPO® Cloning Kit (see page vi for ordering information). The pUB/Bsd TOPO® vector contains the blasticidin resistance gene for selection of stable cell lines.

Using the Control Plasmids

Control Plasmids

The control reactions help you to verify if the mammalian two-hybrid system is functioning well in your cell type using your assay conditions and to identify any level of false positive interactions. We strongly recommend performing cotransfections with the control plasmids if you are a first time user of the mammalian two-hybrid system or are working with a unique cell type.

The following control plasmids are included in the Verve™ Mammalian Two-Hybrid Kit:

- The pCR®2.1/p53 Bait Control Plasmid contains a fusion of the GAL4 DBD and amino acids 72-390 of murine p53 (Iwabuchi *et al.*, 1993)
- The pCR®2.1/LgT Prey Control Plasmid contains a fusion of the VP16 AD and amino acids 84-708 of the SV40 large T antigen (Li and Fields, 1993)
- The pCR®2.1/GAL4-VP16 Positive Control Plasmid contains a fusion of the GAL4 DBD and VP16 AD
- The pCR®2.1/VP16-CP Negative Control Plasmid contains a fusion of the VP16 AD and the polyoma viral coat protein, VP3 (Arrand *et al.*, 1980)

For descriptions, maps, and features of the different control plasmids, refer to pages 26-29. Instructions to propagate and maintain the control plasmids in *E. coli* are provided on page 13.

Setting up the Control Cotransfection

Use the table described below to set up cotransfection for the control plasmids and the reporter plasmid. Be sure to perform the assay in duplicates.

Bait Plasmid	Prey Plasmid	Reporter Plasmid	Interaction	Expected Results
pCR®2.1/p53 Bait Control Plasmid	pCR®2.1/LgT Prey Control Plasmid	pGAL/*lacZ*	p53 and LgT interact *in vivo*	Positive
pCR®2.1/p53 Bait Control Plasmid	pCR®2.1/VP16-CP Plasmid	pGAL/*lacZ*	p53 and CP do not interact *in vivo*	Negative
pCR®2.1/GAL4-VP16 Plasmid	--	pGAL/*lacZ*	GAL4 and VP16 strongly interact *in vivo*	Strongly positive

Amount of Control Plasmids used for Cotransfection

The table below lists the amount of control plasmid DNA used to cotransfect different numbers of adherent CHO-K1 cells. You may have to optimize the amount of DNA used for transfection depending upon your cell type and transfection reagent.

Cell Number	Bait Plasmid (µg)	Prey Plasmid (µg)	Reporter Plasmid (µg)
7.5×10^5 cells/well (6-well plate)	1.3	1.3	1.0
3×10^5 cells/well (12-well plate)	0.65	0.65	0.5
1.5×10^5 cells/well (24-well plate)	0.3	0.3	0.25
2×10^4 cells/well (96-well plate)	0.045	0.045	0.06

Troubleshooting

Introduction

Use the information provided below to troubleshoot your mammalian two-hybrid experiments. If you are having problems generating the linear DNA constructs or performing secondary amplification of the linear DNA templates, refer to the **Troubleshooting** section of the TOPO® Tools Technology manual.

False Negative Results

False negative results can occur when there is a failure to detect interactions between two proteins that normally interact *in vivo*. Protein interactions may not be detected due to the following reasons:

- Hybrid proteins are not stably expressed
- The site of interaction is blocked due to fusion to the bait or prey protein
- High level expression of the bait or prey is toxic to the cells

To overcome false negative results, it may help to construct hybrids using truncated proteins or to use a different cell type.

False Positive Results

False positive results can occur when the proteins do not specifically interact with each other but activate transcription in some other non-specific manner (see below, **Non-Specific Activation of Transcription**). You may have to perform additional analyses to eliminate false positives.

Non-Specific Activation of Transcription

The bait or prey protein may have some non-specific transcriptional activation activity, especially if one of the proteins is a transcription factor. To prevent non-specific transcriptional activity, you may have to truncate the protein or switch the proteins between the bait and prey linear DNA constructs.

Transfection Efficiency

It is important to obtain high a transfection efficiency to increase the sensitivity of the assay. You can improve the transfection efficiency by:

- Using clean, good quality DNA. We recommend purifying the linear DNA constructs before transfection.
- Optimizing the transfection protocol for your cell type before performing the transfection used for the two-hybrid assay.
- Using lipid-based transfection reagents. We recommend using Lipofectamine™ 2000 Reagent for transfection.

Continued on next page

β-galactosidase Activity

If you see no color development or very intense color development, see the table below for some possible solutions:

Problem	Cause	Solution
No color development	No ONPG added to the reaction tubes or wells	Be sure to add the correct amount of ONPG.
	Low expression levels of the prey or the bait proteins	Repeat the assay using more lysate or incubate the cells for a longer period of time (48-72 hours) after transfection.
	Low transfection efficiency	See previous page for some recommendations.
	Incomplete cell lysis	Repeat freeze-thaw procedure or use sonication for cell lysis.
	Cell line may contain proteins or molecules that interfere with the system	Switch to another cell line.
Color development is too intense	High expression levels	Repeat the assay with a diluted sample or decrease the incubation time of the assay. Remember to factor in the dilution factor or the decreased time in your specific activity calculations.
	High background levels of β-galactosidase in your cell type	Switch to another cell line.

Appendix

Using the S.N.A.P.™ MiniPrep Kit to Purify PCR Products

Introduction

We recommend purifying the linear DNA construct prior to transfection to obtain optimal transfection efficiencies. You may use the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-25) and the protocol below to purify your linear DNA construct. If the yield of your PCR product is low, you may also use the S.N.A.P.™ MiniPrep Kit to concentrate the linear DNA construct before transfection. Other resin-based purification kits are suitable.

Materials Needed

You should have the following reagents on hand before beginning:

- Isopropanol
- Binding Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Wash Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Final Wash Buffer (supplied with the S.N.A.P.™ MiniPrep Kit)
- Sterile water
- S.N.A.P.™ MiniPrep columns (supplied with the S.N.A.P.™ MiniPrep Kit)

Purification Protocol

Follow the protocol below to purify your linear DNA construct using the S.N.A.P.™ MiniPrep Kit. The protocol provides instructions to purify PCR products from a 50 µl reaction volume. To purify PCR products from larger reaction volumes (*e.g.* several PCR reactions pooled together), scale up the volumes of each buffer accordingly. **Note:** Details about the components of the S.N.A.P.™ MiniPrep Kit may be found in the S.N.A.P.™ MiniPrep Kit manual. The manual is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 36).

1. Mix the 50 µl amplification reaction with 150 µl of Binding Buffer. Mix well.
2. Add 350 µl of isopropanol. Mix well.
3. Load solution from Step 2 onto a S.N.A.P.™ MiniPrep column. Centrifuge for 30 seconds at 1000 x g in a microcentrifuge and discard the flow-through.
4. Add 250 µl of the Wash Buffer and centrifuge for 30 seconds at 1000 x g in a microcentrifuge. Discard the flow-through.
5. Add 450 µl of the Final Wash Buffer and centrifuge for 30 seconds at 1000 x g in a microcentrifuge. Discard the flow-through.
6. Centrifuge for an additional 10 seconds at full-speed in a microcentrifuge to dry the column.
7. Transfer the column to a new collection tube. Add 30 µl of sterile water to the column. Incubate at room temperature for 1 minute. **Note:** If you are purifying PCR products from pooled PCR reactions, do not increase the volume of sterile water that you use to elute the DNA.
8. Centrifuge for 30 seconds at full-speed in a microcentrifuge to elute the DNA. Collect the flow-through. Use appropriate amount of DNA for transfection.

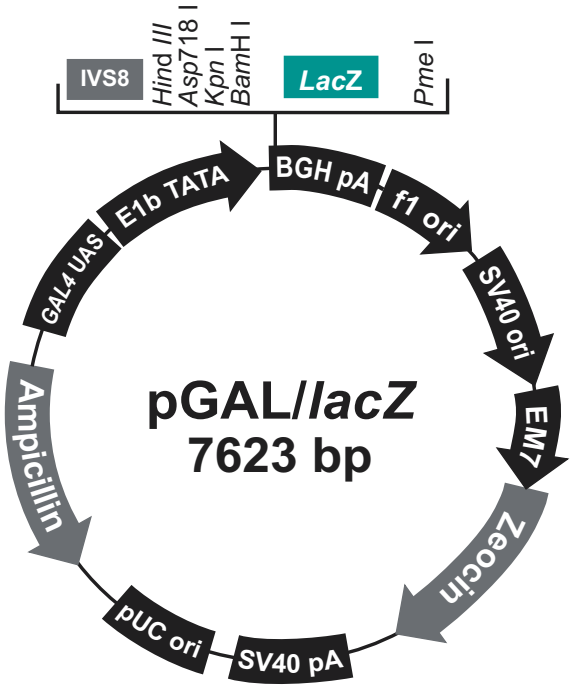
Map of pGAL/*lacZ*

Description

pGAL/*lacZ* is 7623 bp LacZ reporter plasmid containing 6 GAL4 DNA binding sites upstream of the *lacZ* gene. Expression of the *lacZ* gene is controlled by GAL4 UAS containing 6 binding sites for GAL4 and E1b TATA box The *lacZ* gene was cloned into pGene/V5-His/LacZ using *Bam* HI and *Pme* I sites to create pGAL/*lacZ*. The size of the β-galactosidase protein is approximately 120 kDa. For more details on the individual features of pGAL/*lacZ*, refer to the next page.

Map of Reporter Plasmid

The figure below summarizes the features of pGAL/*lacZ*. The complete nucleotide sequence for pGAL/*lacZ* is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 36).



Comments for pGAL/*lacZ*:
7623 nucleotides

GAL4 Upstream Activating Sequences (UAS)
GAL4 binding site: bases 24-40
GAL4 binding site: bases 43-59
GAL4 binding site: bases 62-78
GAL4 binding site: bases 94-110
GAL4 binding site: bases 113-129
GAL4 binding site: bases 132-148
Adenovirus E1b TATA sequence: bases 161-174
Synthetic intron IVS8: bases 329-446
LacZ ORF: bases 524-3588
BGH polyadenylation sequence: bases 3621-3845
f1 origin: bases 3891-4319
SV40 early promoter and origin: bases 4346-4654
EM7 promoter: bases 4709-4775
Zeocin™ resistance gene: bases 4776-5150
SV40 early polyadenylation sequence: bases 5280-5410
pUC origin: bases 5793-6466 (complementary strand)
bla promoter: bases 7463-7567 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6608-7468 (complementary strand)

Features of pGAL/*lacZ*

Introduction

The table below describes the features of the reporter plasmid, pGAL/*lacZ*. All features have been functionally tested.

Feature	Benefit
GAL4 Upstream Activating Sequences (UAS)	Contains six copies of a 17 bp sequence that allows binding and transcriptional activation of the gene of interest by the VP16 AD fusion protein (Giniger *et al*., 1985)
Adenovirus E1b TATA sequence	Allows transcriptional initiation of the gene of interest (Lillie and Green, 1989)
Synthetic intron IVS8	Enhances expression of the gene of interest
lacZ reporter gene	Allows detection of protein-protein interactions
Bovine growth hormone (BGH) polyadenylation sequence	Allows efficient polyadenylation of mRNA (Goodwin and Rottman, 1992)
SV40 early promoter and origin	Allows efficient, high-level expression of the Zeocin™ resistance gene in mammalian cells and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Synthetic prokaryotic promoter for expression of the Zeocin™ resistance gene in *E. coli*
Zeocin™ resistance (*Sh ble*) gene	Allows selection of stable transfectants in mammalian cells (Mulsant *et al.*, 1988) and transformants in *E. coli* (Drocourt *et al*., 1990)
SV40 early polyadenylation signal	Allows polyadenylation of mRNA
pUC origin	Allows high-copy number replication and maintenance in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

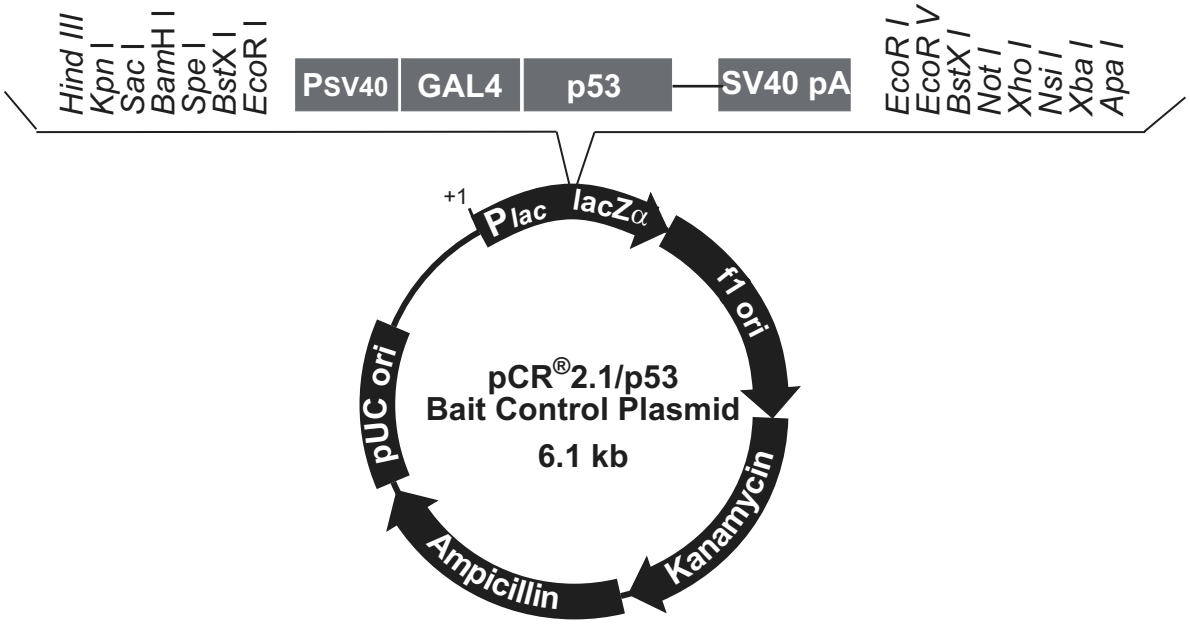
Map of Bait Control Plasmid

Description

pCR®2.1/p53 is 6100 bp Bait Control Plasmid that expresses the bait fusion protein, GAL4-p53. A PCR product containing the SV40 promoter, GAL4-p53 fusion protein, and SV40 pA was cloned into pCR®2.1-TOPO® to create pCR®2.1/p53.

Map of pCR®2.1/p53

The figure below summarizes the features of pCR®2.1/p53. The complete nucleotide sequence for pCR®2.1/p53 is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 36).



Comments for pCR®2.1/p53 Bait Control Plasmid
6100 nucleotides

lac promoter: bases 143-172
SV40 early promoter: bases 360-660
GAL4 DNA binding domain: bases 662-1118
murine p53 (amino acids 72-390): bases 1127-2087
SV40 early polyadenylation sequence: bases 2140-2382
LacZα fragment: bases 2564-2724
f1 origin: bases 2725-3139
Kanamycin promoter: bases 3335-3472
Kanamycin resistance ORF: bases 3473-3472
Ampicillin resistance ORF: bases 4285-5145
pUC origin: bases 5290-5963

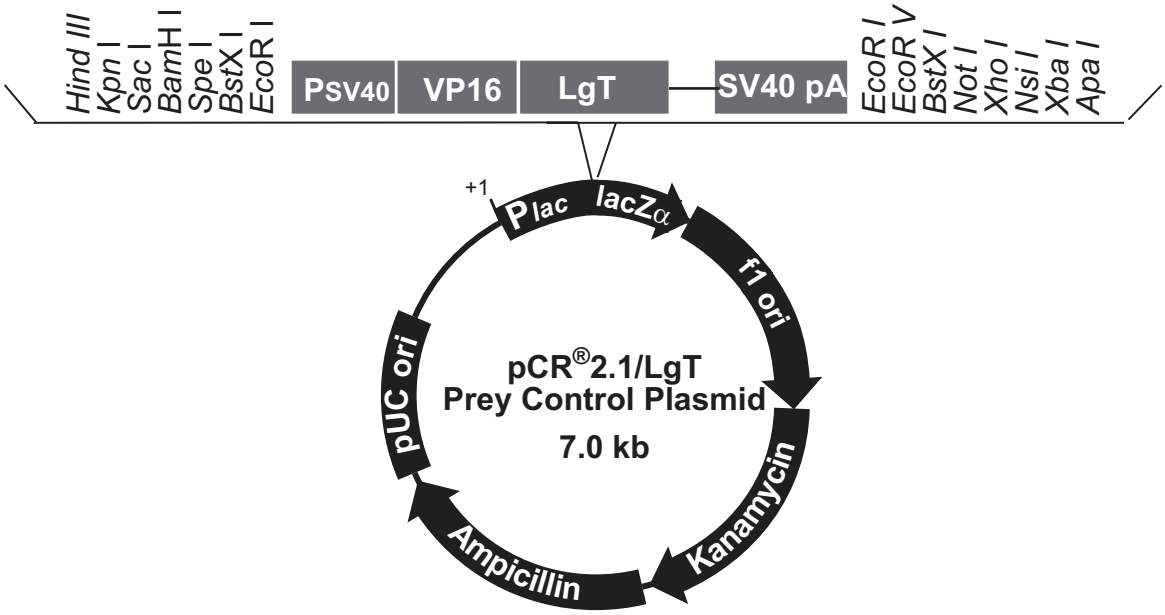
Map of Prey Control Plasmid

Description

pCR®2.1/LgT (SV40 large T antigen) is 6967 bp Prey Control Plasmid that expresses the prey fusion protein, VP16-LgT. A PCR product containing the SV40 promoter, VP16-LgT fusion protein and SV40 pA was cloned into pCR®2.1-TOPO® to create pCR®2.1/LgT.

Map of pCR®2.1/LgT

The figure below summarizes the features of pCR®2.1/LgT. The complete nucleotide sequence for pCR®2.1/LgT is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 36).



Comments for pCR®2.1/LgT Prey Control Plasmid
6967 nucleotides

lac promoter: bases 143-172
SV40 early polyadenylation sequence: bases 389-519 (C)
SV40 large T antigen (amino acids 84-708): bases 813-2681 (C)
VP16 activation domain: bases 2698-2972(C)
SV40 early promoter: bases 3000-3321 (C)
LacZα fragment: bases 3446-3606
f1 origin: bases 3607-4021
Kanamycin promoter: bases 4217-4354
Kanamycin resistance ORF: bases 4355-5149
bla promoter: bases 5150-5172
Ampicillin resistance ORF: bases 5167-6027
pUC origin: bases 6172-6842
(C)= complementary strand

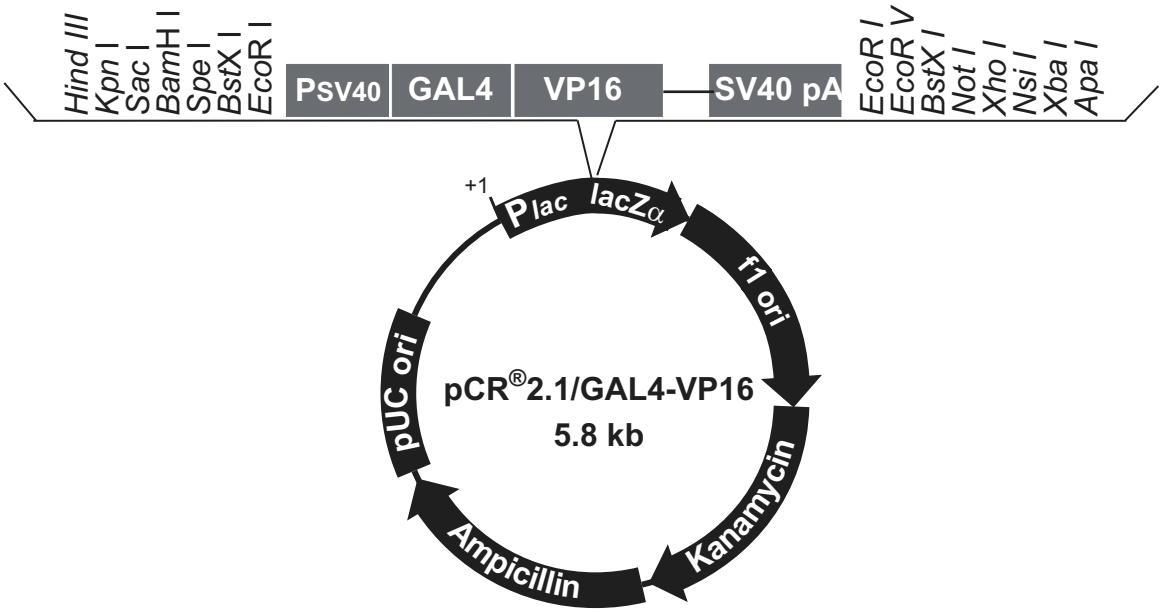
Map of Positive Control Plasmid

Description

The pCR®2.1/GAL4-VP16 is 5842 bp Positive Control Plasmid that expresses the GAL4 DNA binding domain and the VP16 transcription activation domain. A PCR product containing the SV40 promoter, GAL4-VP16 fusion protein, and SV40 pA was cloned into pCR®2.1-TOPO® to create pCR®2.1/GAL4-VP16.

Map of pCR®2.1/GAL-4-VP16

The figure below summarizes the features of pCR®2.1/GAL-4-VP16. The complete nucleotide sequence for pCR®2.1/GAL-4-VP16 is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 36).



Comments for pCR®2.1/GAL4-VP16
5842 nucleotides

lac promoter: bases 143-172
SV40 early promoter: bases 360-660
GAL4 DNA binding domain: bases 676-1116
VP16 activation domain: bases 1135-1368
SV40 early polyadenylation sequence: bases 1898-2141
LacZα fragment: bases 2321-2481
f1 origin: bases 2482-2896
Kanamycin promoter: bases 3092-3229
Kanamycin resistance ORF: bases 3230-4024
bla promoter: bases 4025-4047
Ampicillin resistance ORF: bases 4042-4092
pUC origin: bases 5047-5720

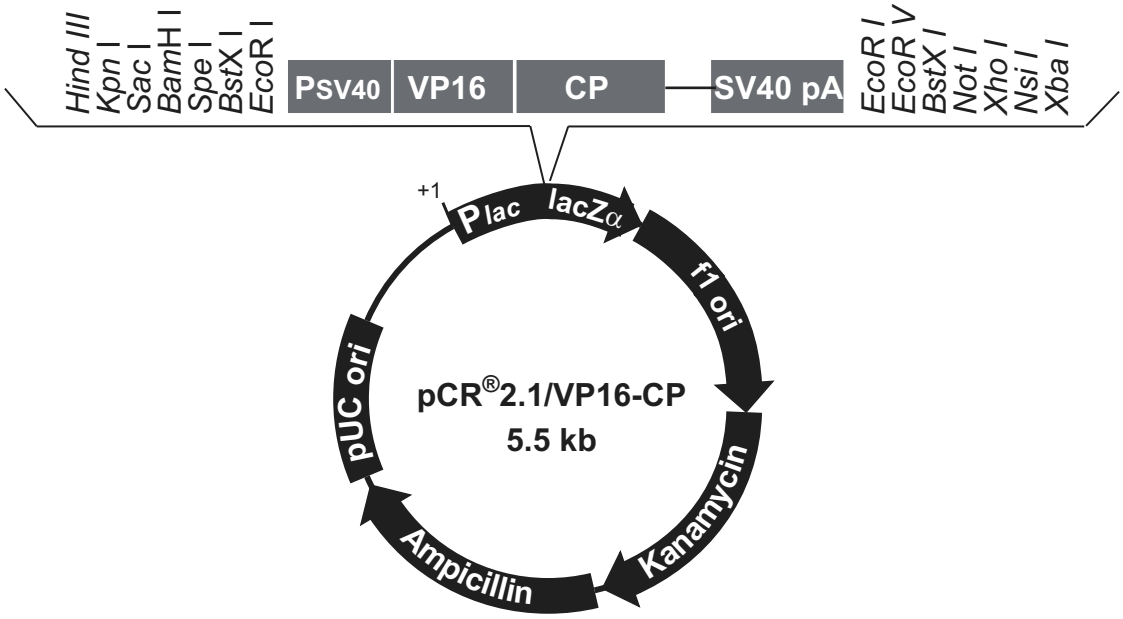
Map of Negative Control Plasmid

Description

The pCR®2.1/VP16-CP (CP; Polyoma viral coat protein, VP3) is 5504 bp Negative Control Plasmid that expresses the fusion protein, VP16-CP. A PCR product containing the SV40 promoter, VP16-CP fusion protein, and SV40 pA was cloned into pCR®2.1-TOPO® to create pCR®2.1/VP16-CP.

Map of pCR®2.1/VP16-CP

The figure below summarizes the features of pCR®2.1/VP16-CP. The complete nucleotide sequence for pCR®2.1/VP16-CP is available for downloading from our Web site at www.invitrogen.com or from Technical Service (see page 36).



Comments for pCR®2.1/VP16-CP
5504 nucleotides

lac promoter: bases 143-172
SV40 early polyadenylation sequence: bases 389-519 (C)
Polyoma viral coat protein, VP3 (amino acids 1-204): bases 631-1242 (C)
VP16 activation domain: bases 1243-1509 (C)
SV40 early promoter: bases 1537-1858 (C)
LacZα fragment: bases 1983-2143
f1 origin: bases 2144-2558
Kanamycin promoter: bases 2754-2891
Kanamycin resistance ORF: bases 2892-3686
bla promoter: bases 3687-3710
Ampicillin resistance ORF: bases 3704-4564
pUC origin: bases 4709-5384
(C)= complementary strand

Creating a Stable Cell Line Containing the Reporter Plasmid

Introduction

The pGAL/*lacZ* plasmid contains the Zeocin™ resistance gene for selection of stable cell lines using Zeocin™. Before transfection, we recommend that you test the sensitivity of your mammalian host cell to Zeocin™ as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Zeocin™ Selection Guidelines

Zeocin™ is available from Invitrogen (Catalog no. R250-01). For more information on Zeocin™ and its mechanism of action, see pages 31-32. Use as follows:

- Zeocin™ is supplied as sterile solution at a concentration of 100 mg/ml in water. Prepare a working solution of Zeocin™ at the required concentration.
- Test varying concentrations of Zeocin™ on your cell line to determine the concentration that kills your cells (see below). Cells differ in their susceptibility to Zeocin™.
- Use 50-1000 µg/ml of Zeocin™ in complete medium depending on your cell line.

Cells will divide once or twice in the presence of lethal doses of Zeocin™, so the effects of the drug take several days to become apparent. Complete selection can take up to 3 weeks of growth in selective medium.

Important

It is necessary to have cells at 25% confluency for Zeocin™ selection to work effectively. The method of killing by Zeocin™ is quite different from Geneticin® or hygromycin. Cells do not round up and detach from the plate.

Determination of Zeocin® Sensitivity

To successfully generate a stable cell line, you need to determine the minimum concentration of Zeocin™ required to kill your untransfected host cell line. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ (0, 50, 100, 200, 400, 600, 800 µg/ml Zeocin™).
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the concentration of Zeocin™ that prevents growth within 7-14 days after addition of Zeocin™.

Continued on next page

Creating a Stable Cell Line Containing the Reporter Plasmid,
Continued

Possible Sites for Linearization

We have found that it is not necessary to linearize the plasmid prior to transfection. However, if you would like to linearize, you can do so at any unique restriction site within the donor vector backbone or using one of the sites listed in the table below. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your fusion plasmid.

Enzyme	Location	Supplier
Ahd I	Ampicillin gene	NEB
Bsa I	Ampicillin gene	NEB
Sap I	Backbone	NEB

Selecting Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use for selection in your host cell line, you are ready to generate a reporter stable cell line.

1. Transfect your mammalian host cell line with your pGAL/*lacZ* plasmid using the desired protocol. Remember to include a plate of untransfected cells as a negative control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Feed the cells with selective medium every 3-4 days until Zeocin™-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

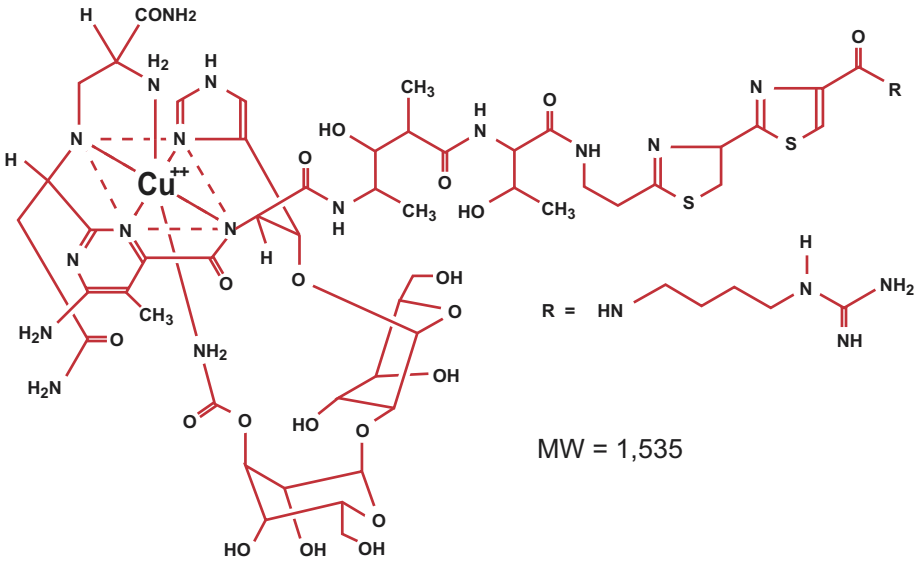
Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).
The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Continued on next page

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Handling Zeocin™

- High salt and acidity or basicity inactivate Zeocin™; therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active. Note that the salt concentration and pH do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates, and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.
- Store tissue culture medium containing Zeocin™ at +4°C in the dark. Medium containing Zeocin™ is stable for 1-2 weeks.

Product Qualification

Introduction

Invitrogen qualifies the Verve™ Mammalian Two-Hybrid Kit as described below.

Plasmids

The plasmids are qualified by restriction digestion. The table below lists the restriction enzymes and the expected fragments.

Plasmid	Restriction Enzyme	Expected Fragments (bp)
pCR®2.1/p53 bait Control Plasmid	*Eco*R I	4000, 1500, 85
	Not I	6400
pCR®2.1/LgT Prey Control Plasmid	*Eco*R I	4000, 2500, 650
	Not I	7200
pCR®2.1/GAL4-VP16 Positive Control Plasmid	*Eco*R I	4000, 1200
	Not I	5200
pCR®2.1/VP16-CP Negative Control Plasmid	*Eco*R I	4000, 1000, 650
	Not I	5650
pGAL/*lac*Z Reporter Plasmid	*Not* I	9000
	Xba I	4000, 5000

TOPO®-adapted Elements

The 5′ and 3′ elements included in the Verve™ Mammalian Two-Hybrid Kit are qualified before adaptation with topoisomerase I using agarose gel electrophoresis to verify the size of the element, and sequencing to confirm the integrity of the elements.

After adaptation with topoisomerase I, the 5′ elements are used in a TOPO® Joining reaction with a control PCR product and a TOPO® Tools 3′ element. The resulting linear DNA template is PCR amplified using element specific primers to produce a linear DNA construct. The linear DNA construct is analyzed using agarose gel electrophoresis to verify its size and sequencing to confirm end-specific linkage of the elements to the control PCR product.

Primers

The primers included in this kit have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

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