Voyager™ NES Protein Production Kits

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Voyager™ NES Protein Production Kits

Rapid cloning and expression of VP22 fusion proteins in *E. coli* for translocation of purified recombinant protein into the cytoplasm of mammalian cells

Catalog nos. K4880-01, K4880-02

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Important Information

Kits

This manual is supplied with the following products.

Kit	Catalog no.
Voyager™ NES Protein Production Kit 1 with pCR®T7/VP22-NES-1-TOPO®	K4880-01
Voyager™ NES Protein Production Kit 2 with pCR®T7/VP22-NES-2-TOPO®	K4880-02

Shipping/Storage

Each Voyager™ NES Protein Production Kit is shipped on dry ice. Each kit contains three boxes as listed below. Upon receipt, store each box as described in the table below.

Box	Contents	Storage Temp
1	pCR®T7/VP22/NES-1 TOPO TA Cloning® Reagents **OR** pCR®T7/VP22/NES-2 TOPO TA Cloning® Reagents	-20°C
2	One Shot® TOP10 Competent Cells	-80°C
3	One Shot® BL21(DE3)pLysS Competent Cells	-80°C

pCR®T7/VP22/ NES TOPO TA Cloning® Reagents

The pCR®T7/VP22/NES TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.** Store Box 1 at -20°C.

Item	Concentration	Amount
pCR®T7/VP22/NES-1-TOPO® **OR** pCR®T7/VP22/NES-2-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.5 1 mM EDTA 1 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

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pCR®T7/VP22/NES TOPO TA Cloning® Reagents, continued

Item	Concentration	Amount
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml
VP22 Forward Sequencing Primer (supplied with the Voyager™ NES Protein Production Kit 1 only)	Lyophilized in TE, pH 8	2 µg
myc-His Reverse Sequencing Primer (supplied with the Voyager™ NES Protein Production Kit 1 only)	Lyophilized in TE, pH 8	2 µg
T7 Promoter Sequencing Primer (supplied with the Voyager™ NES Protein Production Kit 2 only)	Lyophilized in TE, pH 8	2 µg
VP22 Reverse 2 Sequencing Primer (supplied with the Voyager™ NES Protein Production Kit 2 only)	Lyophilized in TE, pH 8	2 µg
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Expression Control Plasmid (pCR®T7/VP22/NES-1 **OR** pCR®T7/VP22/NES-2)	0.5 µg/µl in TE Buffer, pH 8	10 µl
IPTG	1 M	1 ml

One Shot® TOP10 Reagents

The table below describes the items included in the One Shot® TOP10 chemically competent cell kit. Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

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One Shot® BL21(DE3)pLysS Reagents

The table below describes the items included in the One Shot® BL21(DE3)pLysS chemically competent cell kit. Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
BL21(DE3)pLysS 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 lists the sequence and pmoles for the primers included in the Voyager™ NES Protein Production Kits. If you wish to order additional primers, see **Additional Reagents** on the next page.

Primer	Sequence	Amount
VP22 Forward	5′-GGCCACGGCGACTCGA-3′	410 pmoles
myc-His Reverse	5′-ATGACCGGTATGCATATTCAG-3′	311 pmoles
T7 Promoter	5′-TAATACGACTCACTATAGGG-3′	327 pmoles
VP22 Reverse 2	5′-GGTGCTAAAGTGCAGC-3′	406 pmoles

Genotype of TOP10 Cells

Use this strain for general cloning. Note that this strain cannot be used for single-strand rescue of DNA.

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Genotype of BL21(DE3)pLysS Cells

Use these cells for expression purposes **only**. Do not use these cells for propagating or maintaining your construct. For more information about the BL21(DE3)pLysS strain, see page 5.

Genotype: F^- *omp*T *hsd*S_B ($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cam^R)

The DE3 designation means this strain contains the lambda DE3 lysogen that carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase.

The pLysS plasmid (Cam^R) carried by the BL21(DE3)pLysS strain produces T7 lysozyme to reduce basal level expression of the gene of interest. pLysS confers resistance to chloramphenicol and contains the replication origin from plasmid p15A. This origin allows pLysS to be compatible with plasmids containing origins derived from pUC or pBR322. For more information on pLysS, see page 5.

Accessory Products

Introduction

The products listed in this section are intended for use with the Voyager™ NES Protein Production Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Additional Reagents

Many of the reagents supplied in the Voyager™ NES Protein Production Kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Amount	Catalog no.
One Shot® Kit (TOP10 Chemically Competent Cells)	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® Kit (BL21(DE3)pLysS **Chemically Competent** Cells)	10 reactions	C6060-10
	20 reactions	C6060-03
T7 Promoter Sequencing Primer	2 µg (327 pmoles)	N560-02

Additional Products

The table below lists additional products available from Invitrogen which you may use in conjunction with the Voyager™ NES Protein Production Kits.

Item	Amount	Catalog no.
One Shot® Kit (BL21(DE3) **Chemically Competent** Cells)	20 reactions	C6000-03
One Shot® Kit (BL21(DE3)pLysE **Chemically Competent** Cells)	20 reactions	C6565-03
One Shot® Kit (TOP10 Electrocompetent Cells)	10 reactions	C4040-50
	20 reactions	C4040-52
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Kanamycin	5 g	11815-024
imMedia™ Kan Liquid	20 pouches	Q610-20
imMedia™ Kan Agar	20 pouches	Q612-20

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Detection of Fusion Protein

A number of antibodies are available from Invitrogen to detect expression of your VP22 fusion protein from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection in western blots using colorimetric or chemiluminescent detection methods. FITC-conjugated antibodies allow one-step immunofluorescence detection of VP22 fusion proteins. The amount of antibody supplied is sufficient for 25 westerns at a 10 ml working solution (primary and HRP-conjugated antibodies only) or for 25 immunostaining reactions at a 1 ml working solution (FITC-conjugated antibodies only).

Antibody	Epitope	Catalog no.
Anti-*myc*	Detects the 10 amino acid epitope derived from *c-myc* (Evans *et al.*, 1985): EQKLISEEDL	R950-25
Anti-*myc*-HRP		R951-25
Anti-*myc*-FITC		R953-25
Anti-His(C-term)	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25
Anti-His(C-term)-FITC		R933-25
Anti-VP22	Detects the epitope derived from amino acids 159-267 of the VP22 protein	R999-25

Purification of Fusion Protein

The polyhistidine (6xHis) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™. Ordering information for ProBond™ resin is provided below.

Item	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-*myc*-HRP Antibody	1 kit The amount of antibody supplied is sufficient for 25 westerns.	K852-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit The amount of antibody supplied is sufficient for 25 westerns	K853-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15
Purification Columns	50 polypropylene columns	R640-50

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Accessory Products, continued

Other Voyager™ Protein Production Kits

Additional Voyager™ Protein Production Kits are available from Invitrogen which allow production of N-terminal or C-terminal VP22 fusion proteins without the nuclear export signal (NES). Once purified from *E. coli*, the VP22 fusion protein can be applied directly to mammalian cells. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Item	Amount	Catalog no.
Voyager™ Protein Production Kit 1 with pCR®T7/VP22-1-TOPO®	20 reactions	K4860-01
Voyager™ Protein Production Kit 2 with pCR®T7/VP22-2-TOPO®	20 reactions	K4860-02

Other Voyager™ Kits for Expression in Mammalian Cells

Additional Voyager™ products are available from Invitrogen which allow cloning of N-terminal or C-terminal VP22 fusion proteins for transfection and direct expression of the gene of interest in mammalian cells. Once expressed, the VP22 fusion protein translocates into surrounding non-transfected cells. The Voyager™ kits listed below also facilitate creation of stable cell lines expressing VP22 fusion proteins. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Item	Amount	Catalog no.
pVP22/*myc*-His TOPO® TA Expression Kit	20 reactions	K4840-01
pVP22/*myc*-His Kit	20 µg	V484-01
pVP22/*myc*-His2 TOPO® TA Expression Kit	20 reactions	K4850-01
pVP22/*myc*-His2 Kit	20 µg	V485-01

Introduction

Overview

Introduction

The Voyager™ NES Protein Production Kits utilize the TOPO® Cloning technology to facilitate rapid cloning of *Taq* polymerase-amplified PCR products into the pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® vector. The pCR®T7/VP22/NES-1-TOPO® and pCR®T7/VP22/NES-2-TOPO® vectors allow cloning of PCR products as N-terminal or C-terminal fusions with the herpes virus VP22 protein. Once cloned into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®, the VP22 fusion protein can be expressed and purified from *E. coli*, then applied to the mammalian cells of interest where it is and imported and targeted to the cytoplasm. The translocation and import properties of the VP22 protein form the basis of the Voyager™ technology (see the next page).

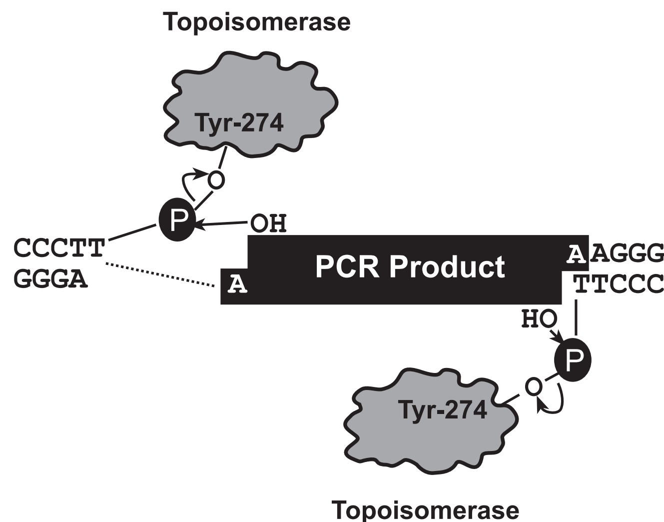
How TOPO® Cloning Works

The plasmid vector, pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-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 that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

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



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How TOPO® Cloning Works, continued

Once the PCR product is cloned into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® and transformants analyzed for the correct orientation, expression of the VP22 fusion protein can be induced in BL21(DE3)pLysS *E. coli* by addition of IPTG. Recombinant VP22 fusion protein is then purified using metal-chelating resin. Purified VP22 fusion protein can be applied to the mammalian cell line of choice where it is rapidly imported into the cytoplasm of virtually 100% of the cells.

Voyager™ Technology

The Voyager™ technology utilizes the protein, VP22, one of the structural proteins that form the tegument of herpes simplex virus type 1 (HSV-1). The tegument is a region located between the capsid and the envelope. This novel protein possesses unusually potent translocation properties following transfection or infection (Elliott and O'Hare, 1997) or when simply applied to mammalian cells. The VP22 protein expressed in the Voyager™ NES Protein Production Kits exhibits the following properties:

- When applied to mammalian cells, the VP22 protein is generally rapidly imported and localized to the nucleus of the cell. With the inclusion of the nuclear export signal (NES), the VP22 fusion protein is predominantly targeted to the cytoplasm of the cell. Uptake occurs within 20 minutes after addition of VP22 protein to cells.
- Heterologous proteins fused to the VP22 protein can be similarly imported and localized to the cytoplasm of the cell, although some nuclear localization does occur.
- VP22 fusion proteins can be imported into a wide variety of mammalian cell lines including those that are typically refractory to standard transfection methods.
- Heterologous proteins fused to VP22 protein appear to retain their biological activity.

For more information about the translocation properties of the VP22 protein, refer to published references (Brewis *et al.*, 2000; Elliott and O'Hare, 1997; Phelan *et al.*, 1998).

Note that the VP22 protein expressed from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® is a truncated version of the native VP22 protein and does not contain the entire open reading frame. The VP22 protein expressed from pCR®T7/VP22/NES-1-TOPO® and pCR®T7/VP22/NES-2-TOPO® is derived from amino acids 159-301 of the native VP22 protein. This truncated VP22 protein appears to be sufficient to impart translocation properties to heterologous fusion partners (P. O'Hare, personal communication).

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Overview, continued

Cell Lines Tested

The table below lists the mammalian cell lines that have been successfully tested at Invitrogen for import of VP22 fusion proteins produced using the Voyager™ Protein Production Kits or the Voyager™ NES Protein Production Kits. All of these are established cell lines. To date, no pleiotrophic effects have been observed in any of the uptake experiments performed using VP22 fusion proteins. Primary cell lines have not been extensively tested.

Cell Line	Description	Adherent/Suspension
COS-1	African Green Monkey kidney fibroblast (SV40 Large T antigen transformed)	Adherent
Swiss 3T3	Murine embryo fibroblast	Adherent
CHO	Chinese hamster ovary	Adherent
PC12	Rat pheochromocytoma	Suspension
Jurkat	Human T-cell leukemia	Suspension

Proteins Expressed

The following proteins have been expressed as VP22 fusions in *E. coli* using the Voyager™ Protein Production Kits or the Voyager™ NES Protein Production Kits. Purified VP22 fusion proteins were imported into mammalian cells and shown to be biologically active.

Protein	Size (kDa)	Cell Line Tested
Human rhoA	22	Swiss 3T3
HIV Rev	14	CHO
Green Fluorescent Protein (GFP)	27	PC12, Jurkat, CHO
Bacteriophage T7 RNA polymerase	99	COS-1, 293
β-galactosidase alpha fragment	10	CHO

The pCR®T7/VP22/NES TOPO® Vectors

pCR®T7/VP22/NES-1-TOPO® and pCR®T7/VP22/NES-2-TOPO® are 4.9 kb expression vectors designed to facilitate rapid cloning and expression of PCR products as fusions to the VP22 protein. The vectors allow high-level, inducible expression of the gene of interest in *E. coli*. The vectors contain the following elements:

- T7 promoter for high-level, inducible expression of the gene of interest in *E. coli* (see below)
- TOPO® Cloning site for rapid cloning of *Taq*-amplified PCR products
- Truncated VP22 ORF (amino acids 159-301 only) to allow import of the gene of interest into a wide variety of mammalian cells
- HIV Rev nuclear export signal (NES) for targeted localization of the VP22 fusion protein to the cytoplasm of mammalian cells (see below)
- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant fusion protein
- Kanamycin resistance gene for selection in *E. coli*

A control plasmid (pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2) is included for use as a positive control for expression and purification (see page 17 for more information).

NES

The NES used in pCR®T7/VP22/NES-1-TOPO® and pCR®T7/VP22/NES-2-TOPO® is derived from the activation domain of the HIV Rev protein (Fischer *et al.*, 1995; Wen *et al.*, 1995) and functions to mediate RNA export. When coupled to a heterologous protein, the NES directs export of recombinant fusion proteins from the nucleus to the cytoplasm. Note that the NES is not cleaved after fusion protein export. For more information about the NES, refer to published reviews (Dingwall and Laskey, 1991; Gorlich and Mattaj, 1996).

Regulation of Expression of the Gene of Interest

Expression of the gene of interest from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® is controlled by the very strong bacteriophage T7 promoter that drives expression of gene 10 (ϕ10). T7 RNA polymerase specifically recognizes this promoter. For expression of the gene of interest, it is necessary to deliver T7 RNA polymerase to the cells by either inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the Voyager™ NES Protein Production Kits, addition of IPTG to transformed BL21(DE3)pLysS cells induces expression of T7 RNA polymerase. Once sufficient T7 RNA polymerase is produced, it binds to the T7 promoter in pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® and transcribes the gene of interest.

Use of TOP10 Cells

One Shot® TOP10 competent cells, which do not contain T7 polymerase, are included in the Voyager™ NES Protein Production Kits to provide a host for stable propagation and maintenance of recombinant plasmids. The presence of T7 polymerase, even at low basal levels, can lead to expression of the desired gene even in the absence of inducer (see below). In general, this is not a problem, but if the gene of interest is toxic to the *E. coli* host, plasmid instability and/or cell death results. **We recommend that you transform your TOPO® Cloning reaction into TOP10 cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into one of the expression strains described below.

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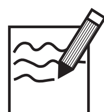
Regulation of Expression of T7 RNA Polymerase

The BL21(DE3)pLysS *E. coli* strain is specifically included in this kit for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This lambda lysogen contains the *lacI* gene, the T7 RNA polymerase gene under the control of the *lacUV5* promoter, and a small portion of the *lacZ* gene. This *lac* construct is inserted into the *int* gene, thus inactivating the gene. Disruption of the *int* gene prevents lysis in the absence of helper phage. The lac repressor represses expression of T7 RNA polymerase. Addition of the gratuitous inducer, isopropyl β-D-thiogalactoside (IPTG) allows expression of T7 RNA polymerase.

The BL21(DE3)pLysE strain is also available from Invitrogen. For more information on BL21(DE3)pLysS and BL21(DE3)pLysE , see below.

Regulation of T7 RNA Polymerase by T7 Lysozyme

There is always some basal level expression of T7 RNA polymerase. If a toxic gene is cloned downstream of the T7 promoter, basal expression of this gene may lead to reduced growth rates, increased cell death, or plasmid instability. T7 lysozyme has been shown to bind to T7 polymerase and inhibit transcription. This activity is exploited to reduce basal levels of T7 RNA polymerase.



Note

T7 lysozyme is a bifunctional enzyme. In addition to its T7 RNA polymerase binding activity, it also cleaves a specific bond in the peptidoglycan layer of the *E. coli* cell wall. This activity increases the ease of cell lysis by freeze-thaw cycles prior to purification, thus making cells more fragile.

pLysE and pLysS

The gene for T7 lysozyme has been cloned into the *Bam*H I site of pACYC184 (Chang and Cohen, 1978; Studier *et al.*, 1990). If the gene is oriented so that it is expressed from the constitutive *tet* promoter, the plasmid is called pLysE; if it is cloned in the opposite orientation so that it is expressed from the ϕ3.8 promoter, it is called pLysS. The plasmids confer resistance to chloramphenicol (34 μg/ml) and contain the origin of replication from plasmid p15A. This origin allows pLysS and pLysE to be stably maintained with pUC- and pBR322-derived plasmids in the same host. The differences between these two plasmids are summarized in the table below.

Feature	pLysS	pLysE
Relative amount of T7 lysozyme	Moderate (may not sufficiently suppress T7 RNA polymerase for expression of more toxic genes)	High
Growth rate of host	No or little effect	May cause a significant decrease and/or cell lysis
Stability of expression plasmid	Increases	Increases
Lag between addition of inducer and expression of desired gene	Short	Long
Maximum expression level of desired protein	No effect	May reduce

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Note that One Shot® BL21(DE3)pLysS competent cells are supplied in the Voyager™ NES Protein Production Kits. In most cases, pLysS supplies sufficient T7 lysozyme to reduce the activity of T7 RNA polymerase while maintaining good growth rates and maximum yield of recombinant protein.

If you discover that your gene is still toxic to *E. coli*, try BL21(DE3)pLysE cells (see page ix for ordering information). Call Technical Service for more information (see page 48).

Experimental Outline

Use the following outline to clone and express your gene of interest in pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the truncated VP22 ORF and the C-terminal peptide containing the NES, *c-myc* epitope, and polyhistidine (6xHis) tag. Consult the diagram of the TOPO® Cloning site on page 8 or page 9 to help you design your PCR primers.	7-9
2	Produce your PCR product.	10
3	TOPO® Clone your insert into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® and transform into One Shot® TOP10 *E. coli*. Select transformants on 50 μg/ml kanamycin.	11-14
4	Analyze your transformants for the presence and orientation of insert by restriction enzyme digestion.	14
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the truncated VP22 ORF and the C-terminal peptide.	14
6	Prepare purified plasmid and transform into One Shot® BL21(DE3)pLysS *E. coli*. Select transformants on 50 μg/ml kanamycin and 34 μg/ml chloramphenicol.	17-18
7	Induce expression of the VP22 fusion protein with IPTG and optimize expression conditions.	19-20
8	Scale-up expression and purify VP22 fusion protein from BL21(DE3)pLysS cells using metal-chelating resin such as ProBond™.	23-28
9	Apply purified VP22 fusion protein to mammalian cells.	29-30
10	Test for uptake of your recombinant VP22 fusion protein by immunofluorescence, western blot analysis, or functional assay.	30-31

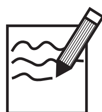
It is very unlikely that VP22 fusions can penetrate the skin; however, this has not been tested. Avoid contact with the skin or mucous membranes. We recommend that you wear a lab coat, gloves, and safety glasses when working with solutions containing VP22 fusions.

Methods

PCR Primer Design

Introduction

The design of the PCR primers to clone your DNA sequence of interest is critical for fusion to VP22 and expression. **Remember that your PCR product will have single 3´ adenine overhangs if *Taq* is used as your polymerase (see page 37).**



Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®.

Cloning in pCR®T7/VP22/NES-1-TOPO®

To fuse your gene to the C-terminus of VP22, design your 5´ PCR primer such that the PCR product will clone in frame with the VP22 ORF. pCR®T7/VP22/NES-1-TOPO® also contains a C-terminal peptide encoding the NES, *c-myc* epitope and a polyhistidine (6xHis) tag. Therefore, remember to design your PCR primers such that the PCR product will also clone in frame with the C-terminal peptide.

Note: Cloning efficiencies may vary depending on the 5´ nucleotide sequence of your primer (see page 34).

Use the diagram on the next page to design your PCR primers. Once you have designed your PCR primers, proceed to page 10.

Cloning in pCR®T7/VP22/NES-2-TOPO®

You will need to consider the following when cloning your PCR product into pCR®T7/VP22/NES-2-TOPO®:

- The T7 leader contains an ATG initiation codon and surrounding sequences that allow optimal expression of heterologous fusion proteins in *E. coli*. We recommend that you design your 5′ PCR primer such that your PCR product will clone in frame with the T7 leader.
- To fuse your gene to the N-terminus of VP22, design your 3′ PCR primer such that the PCR product will clone in frame with the VP22 ORF. Note that if you clone your PCR product in frame with the VP22 ORF, your PCR product will automatically be in frame with the C-terminal peptide encoding the NES, *c-myc* epitope, and a polyhistidine (6xHis) tag.

Note: Cloning efficiencies may vary depending on the 5´ nucleotide sequence of your primer (see page 34).

Use the diagram on page 9 to design your PCR primers. Once you have designed your PCR primers, proceed to page 10.

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PCR Primer Design, continued

TOPO® Cloning Site of pCR®T7/VP22/NES-1-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 597 and 598. This is the TOPO® Cloning site**. Note that the complete sequence of pCR®T7/VP22/NES-1-TOPO® may be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 48).** For a map and a description of the features of pCR®T7/VP22/NES-1-TOPO®, refer to pages 38-39.

```
                  VP22 Forward priming site
441  CAG GAC GTC GAC GCG GCC ACG GCG ACT CGA GGG CGT TCT GCG GCG TCG CGC CCC ACC GAG CGA CCT

                          VP22 159-301                           Asp718 I  Kpn I   Sac I       Spe I
507  CGA GCC CCA GCC CGC TCC GCT TCT CGC CCC AGA CGG CCC GTC GAG GGT ACC GAG CTC GGA TCC ACT
     Arg Ala Pro Ala Arg Ser Ala Ser Arg Pro Arg Arg Pro Val Glu Gly Thr Glu Leu Gly Ser Thr

                                                                     Pst I               Not I
573  AGT CCA GTG TGG TGG AAT TGC CCT T  PCR Product  AAG GGC AAT TCT GCA GAT ATC CAG CAC AGT GGC
                         TTA ACG GGA A               TTC CCG TTA
     Ser Pro Val Trp Trp Asn Cys Pro                 Lys Gly Asn Ser Ala Asp Ile Gln His Ser Gly

                     Nuclear export signal (NES)                                c-myc epitope
631  GGC CGC CTA CCA CCG CTT GAG AGA CTT ACT CTT GAT CTA GAG GGC CCG CGG TTC GAA CAA AAA CTC
     Gly Arg Leu Pro Pro Leu Glu Arg Leu Thr Leu Asp Leu Glu Gly Pro Arg Phe Glu Gln Lys Leu

                myc-His Reverse priming site   Age I      Polyhistidine (6xHis) tag
697  ATC TCA GAA GAG GAT CTG AAT ATG CAT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTTGAGCA
     Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly His His His His His His ***

761  ATAACTAGCA TAACCCCTTG GGGCCTCTAA ACGGGTCTTG AGGGGTTTTT TGCTGAAAGG AGGAACTATA TCCGGATATC
```

continued on next page

PCR Primer Design, continued

TOPO® Cloning Site of pCR®T7/VP22/NES-2-TOPO®

Restriction sites are labeled to indicate the actual cleavage site. The ATG initiation codon in the T7 leader is shown in bold. The vector is supplied linearized between base pair 180 and 181. This is the TOPO® Cloning site**. Note that the complete sequence of pCR®T7/VP22/NES-2-TOPO® may be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 48).** For a map and a description of the features of pCR®T7/VP22/NES-2-TOPO®, refer to pages 40-41.

```
     T7 promoter/primer binding site
1    TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC TAGAAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACAT

     T7 leader                                         Asp718 I  Kpn I           Sac I   Dra I
81   ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTT GGT ACC GCT GGA GCT CTC TTT AAA
     Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Gly Thr Ala Gly Ala Leu Phe Lys

     BamH I  Spe I                                                          Pst I
147  GGA TCC ACT AGT CCA GTG TGG TGG AAT TGC CCT T [PCR Product] AAG GGC AAT TCT GCA GAT ATC CAG CAC
                                 TTA ACG GGA A               TTC CCG TTA
     Gly Ser Thr Ser Pro Val Trp Trp Asn Cys Pro             Lys Gly Asn Ser Ala Asp Ile Gln His
          Not I
208  AGT GGC GGC CGC CCG TCG ACG GCG CCA ACC CGA TCC AAG ACA CCC GCG CAG GGG CTG GCC AGA AAG
     Ser Gly Gly Arg Pro Ser Thr Ala Pro Thr Arg Ser Lys Thr Pro Ala Gln Gly Leu Ala Arg Lys
     VP22 reverse 2 priming site                 VP22 159-301
274  CTG CAC TTT AGC ACC GCC CCC CCA AAC CCC GAC GCG CCA TGG ACC CCC CGG GTG GCC GGC TTT AAC
     Leu His Phe Ser Thr Ala Pro Pro Asn Pro Asp Ala Pro Trp Thr Pro Arg Val Ala Gly Phe Asn

340  AAG CGC GTC TTC TGC GCC GCG GTC GGG CGC CTG GCG GCC ATG CAT GCC CGG ATG GCG GCG GTC CAG
     Lys Arg Val Phe Cys Ala Ala Val Gly Arg Leu Ala Ala Met His Ala Arg Met Ala Ala Val Gln

406  CTC TGG GAC ATG TCG CGT CCG CGC ACA GAC GAA GAC CTC AAC GAA CTC CTT GGC ATC ACC ACC ATC
     Leu Trp Asp Met Ser Arg Pro Arg Thr Asp Glu Asp Leu Asn Glu Leu Leu Gly Ile Thr Thr Ile

472  CGC GTG ACG GTC TGC GAG GGC AAA AAC CTG CTT CAG CGC GCC AAC GAG TTG GTG AAT CCA GAC GTG
     Arg Val Thr Val Cys Glu Gly Lys Asn Leu Leu Gln Arg Ala Asn Glu Leu Val Asn Pro Asp Val

538  GTG CAG GAC GTC GAC GCG GCC ACG GCG ACT CGA GGG CGT TCT GCG GCG TCG CGC CCC ACC GAG CGA
     Val Gln Asp Val Asp Ala Ala Thr Ala Thr Arg Gly Arg Ser Ala Ala Ser Arg Pro Thr Glu Arg
                                                                  Nuclear export signal (NES)
604  CCT CGA GCC CCA GCC CGC TCC GCT TCT CGC CCC AGA CGG CCC GTC GAG TTC GGA CTA CCA CCG CTT
     Pro Arg Ala Pro Ala Arg Ser Ala Ser Arg Pro Arg Arg Pro Val Glu Phe Gly Leu Pro Pro Leu
                                          c-myc epitope                            Age I
670  GAG AGA CTT ACT CTT GAT ATC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT ATG CAT ACC GGT
     Glu Arg Leu Thr Leu Asp Ile Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly
          Polyhistidine (6xHis) tag
736  CAT CAT CAC CAT CAC CAT TGA GTTTTGAGCA ATAACTAGCA
     His His His His His His ***
```

Producing PCR Products

Introduction

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

Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

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

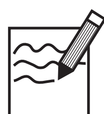
If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 37.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using a plasmid for 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 (0.1-0.2 µg each)	1 µM 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, discrete band, plase refer to the **Note** below.



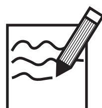
Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before TOPO® Cloning into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® (see pages 35-36). 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 48).

TOPO® Cloning and Transformation

Introduction

TOPO® Cloning technology allows you to ligate your PCR products into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-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, we recommend performing the control reactions on pages 32-33 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 results in the following:

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

Including salt in the TOPO® Cloning reaction prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

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

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **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 reaction protocols are provided to help you obtain the best possible results. Please read the following information carefully.

Chemically Competent *E. coli*

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

Electrocompetent *E. coli*

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

continued on next page

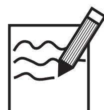
Materials Supplied by the User

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 μg/ml kanamycin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubators

imMedia™ Agar Plates

For fast and easy microwaveable preparation of Low Salt LB media and agar containing kanamycin, imMedia™ Kan Liquid (Catalog no. Q610-20) and imMedia™ Kan Agar (Catalog no. Q611-20) are available from Invitrogen. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 48).


Note

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

Preparation for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g. add 5 μl of the Salt Solution to 15 μl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® TOP10 cells for each transformation.

Important

Remember to use One Shot® **TOP10** *E. coli* to transform your TOPO® Cloning reaction. The BL21(DE3)pLysS strain supplied with the kit should **only** be used for expression purposes and **not** for general cloning purposes. For more information about BL21(DE3)pLysS cells, refer to pages 5 and 17.

continued on next page

TOPO® Cloning and Transformation, continued

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 16.

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 (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl

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

Performing the TOPO® Cloning Reaction

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

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.
2. Place the reaction on ice and proceed to **One Shot® Chemical Transformation** (below) or **Transformation by Electroporation** (next page). **Note:** You may store the TOPO® Cloning reaction at -20°C overnight.

One Shot® Chemical Transformation

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

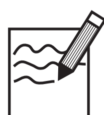
   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion (see above).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature 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. 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.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

continued on next page

Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction to 50 µl of electrocompetent *E. coli* in a microcentrifuge tube. Transfer the mixture to a 0.1 cm cuvette 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 genes.
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**, below).



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

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50 µg/ml kanamycin (3-5 ml).
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01).
3. Analyze the plasmids by restriction enzyme analysis or by sequencing.
   - If you are TOPO® Cloning into pCR®T7/VP22/NES-1-TOPO®, the VP22 Forward and *myc*-His Reverse sequencing primers are included in the kit to help you sequence your insert.
   - If you are TOPO® Cloning into pCR®T7/VP22/NES-2-TOPO®, the T7 Promoter and VP22 Reverse 2 sequencing primers are included in the kit to help you sequence your insert.

   Refer to the diagram on page 8 or page 9 for restriction sites and sequence surrounding the TOPO® Cloning site of pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®, respectively.

   Note: Resuspend each primer in 20 µl of sterile water to prepare a 0.1 µg/µl stock solution.

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TOPO® Cloning and Transformation, continued

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

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use a combination of one of the primers included with the kit with a primer that binds within your insert. You will have to determine the amplification conditions. If this is the first time you have used this technique, we recommend that you perform restriction enzyme 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 and multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. (Don't forget to make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using parameters previously determined (see text, above).
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 32-33. These reactions 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. We recommend that you also store the purified plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on LB plates containing 50 µg/ml kanamycin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.

- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Note: 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

Expression of the PCR Product

Introduction

To express your VP22 fusion product from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®, you will use BL21(DE3)pLysS cells included with the kit as the host strain. You will need purified plasmid DNA of your construct to transform into BL21(DE3)pLysS for expression studies. Since each recombinant protein has different characteristics that may affect optimal expression, we recommend that you perform a time course of expression to determine the optimal conditions for expression of your protein.

BL21(DE3)pLysS

This *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter. **Each time** you wish to perform an expression experiment, you will transform your plasmid into BL21(DE3)pLysS. **Do not use this strain for propagation and maintenance of your plasmid. Use TOP10 for propagation and maintenance of your plasmid.** Basal level expression of T7 RNA polymerase may lead to plasmid instability if your gene of interest is toxic to *E. coli*. For more information on this strain, refer to page 5.

Positive Control

The supercoiled pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2 vector provided with the kit produces a fusion protein consisting of the truncated VP22 ORF, NES, and the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag and may be used as a positive control for expression and purification. The table below shows the predicted size of the fusion protein that is produced from each vector. For a detailed map and a description of the features of the vectors, see pages 42 or 43.

Vector	Size of Fusion Protein
pCR®T7/VP22/NES-1	24 kDa
pCR®T7/VP22/NES-2	24 kDa

To propagate and maintain the pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2 vector, transform 10 ng of the plasmid into One Shot® TOP10 cells using the procedure on page 13.

Experimental Outline

The table below outlines the basic steps needed to induce expression of your gene of interest in *E. coli*.

Step	Action
1	Isolate plasmid DNA using standard procedures and transform your pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® construct and the appropriate positive control separately into One Shot® BL21(DE3)pLysS cells. Select transformants using 50 μg/ml kanamycin and 34 μg/ml chloramphenicol.
2	Grow the transformants and induce expression with IPTG over several hours. Take several time points to determine the optimal time of expression.
3	Optimize expression to maximize the yield of protein.

continued on next page

Materials to Have on Hand

Be sure to have the following solutions and equipment on hand before starting the experiment:

- 34 mg/ml chloramphenicol in ethanol (**Note:** Chloramphenicol is **required** to ensure the presence of pLysS)
- SOB or LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol
- 37°C incubator (shaking and nonshaking)
- 42°C water bath
- 1 M IPTG
- Bacterial Cell Lysis Buffer (see page 44 for recipe)
- Liquid nitrogen
- 1X and 2X SDS-PAGE sample buffer (see page 45 for a recipe for 2X SDS-PAGE sample buffer)
- Reagents and apparatus for SDS-PAGE (see page 19)
- Boiling water bath
- Sterile water

Plasmid Preparation

Purified plasmid DNA may be isolated using your method of choice. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 μg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 μg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

One Shot® BL21(DE3)pLysS Transformation Reaction

To transform your construct or the positive control (10 ng each) into One Shot® BL21(DE3)pLysS cells, follow the instructions below. You will need one vial of cells per transformation.

Note that you will not plate the transformation reaction, but inoculate it into medium for growth and subsequent expression.

1. Thaw on ice, one vial of One Shot® BL21(DE3)pLysS cells per transformation.
2. Add 5-10 ng DNA in a 1 to 5 μl volume into each vial of One Shot® BL21(DE3)pLysS cells and mix by stirring gently with the pipette tip. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 μl of room temperature SOC medium.
7. Cap the tube tightly, tape the tube on its side (for better aeration), and incubate at 37°C for 30 minutes with shaking (200 rpm).
8. Add the **entire** transformation reaction to 10 ml of LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol.
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, next page.

continued on next page

Pilot Expression

1. Inoculate 10 ml of LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with 500 μl of the overnight culture from Step 9, previous page.
2. Grow two hours at 37°C with shaking. OD_{600} should be about 0.5-0.7 (mid-log).
3. Split the culture into two 5 ml cultures. Add IPTG to a final concentration of 1 mM to one of the cultures. You will now have two cultures: one induced, one uninduced.
4. Remove a 500 μl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
5. Freeze the cell pellets at -20°C. These are the zero time point samples.
6. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
7. For each time point, remove 500 μl from the induced and uninduced cultures and process as described in Steps 4 and 5. Proceed to **Preparation of Samples**, below.

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The patented NuPAGE® Gel System prevents the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits for visualization of proteins. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Preparation of Samples

Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see above) to analyze all the samples you collected. If you are preparing your own SDS-PAGE gel, prepare 1X SDS-PAGE sample buffer. **Note**: If you wish to analyze your samples for soluble protein, see the next section.

1. When all the samples have been collected from Steps 5 and 7, above, resuspend each cell pellet in 80 μl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 μl of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing at -20°C.

continued on next page

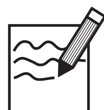
Expression of the PCR Product, continued

Preparation of Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µl of Bacterial Cell Lysis Buffer (see recipe on page 44).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times. Cells will easily lyse because some of the T7 lysozyme will leak out during the freeze-thaw cycle and digest the cell wall.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer (see recipe on page 45) and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE gel and electrophorese. The SeeBlue® Plus2 Pre-Stained Standard (Catalog no. LC5925) is available from Invitrogen for use as a molecular weight protein marker.

Analysis of Samples

1. Stain the gel with Coomassie™ blue and look for a band of increasing intensity in the expected size range for the recombinant fusion protein. Use the uninduced culture as a negative control.
2. In addition, you may perform a western blot (see below) to confirm that the overexpressed band is your desired protein.
3. Use the positive control to confirm that growth and induction were performed properly. The pCR®T7/VP22/NES-1 and pCR®T7/VP22/NES-2 vectors should both produce a 24 kDa protein when induced with IPTG.



Note

Fusion of your PCR product with the truncated VP22 ORF and the C-terminal peptide containing the NES, *c-myc* epitope, and polyhistidine tag will increase the size of your protein by approximately 24 kDa if you are using pCR®T7/VP22/NES-1-TOPO® and 25 kDa if you are using pCR®T7/VP22/NES-2-TOPO®.

Western Blot Analysis

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-VP22 Antibody, Anti-*myc* antibodies, or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a *c-myc* epitope or a polyhistidine tag. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

The Next Step

If you are satisfied with expression of your gene of interest, proceed to purification, page 23.

If you have trouble expressing your protein, or wish to optimize expression, see the next page.

Troubleshooting Expression

Introduction

Information is provided below to help you troubleshoot your expression experiment.

No Expression

- If you are cloning into pCR®T7/VP22/NES-2-TOPO®, sequence your construct to make sure that your PCR product is in frame with the T7 leader.
- If you are cloning into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®, sequence your construct to make sure it is in frame with the truncated VP22 ORF and the C-terminal peptide containing the NES, *c-myc* epitope, and polyhistidine tag.
- If the positive control is expressed, but you don't see any expression from your construct on a Coomassie™ blue-stained gel, re-run your samples on an SDS-PAGE gel and perform a western blot. Use the Anti-*myc* antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein.

Low Expression

If your protein is expressed, but the levels are low, it is possible that expression of your gene may be toxic to *E. coli*. This is the most common reason for poor expression. Evidence of toxicity may include the following:

- Slow growth relative to the control
- Loss of plasmid

To reduce the toxicity of your gene, basal levels of T7 RNA polymerase must be reduced. There are a number of methods to reduce basal level expression of T7 RNA polymerase. The choice of method depends on the relative toxicity of your gene product to *E. coli*. The table below outlines the method choices.

Relative Toxicity	Method	Comments
Moderate	Transformation into a pLysE-containing strain	Substantial levels of T7 lysozyme produced. Growth rate may be reduced.
High	Infect TOP10F′ (or any other suitable host strain) with M13 or lambda phage expressing T7 RNA polymerase	T7 RNA polymerase is not present in the cell until infection. Requires growth and maintenance of phage stocks.

Note

Many researchers use the leakiness of the T7 system to their advantage. In some cases, basal level, constitutive expression produces sufficient protein for analysis and purification, particularly if the host strain containing the construct of interest is grown at room temperature. We recommend growing the strain for 24-48 hours at room temperature to produce sufficient protein. Expression of your construct using this method can result in substantial production of soluble protein.

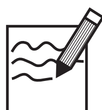
Tip: To optimize production of soluble protein using the above method, try BL21(DE3) cells, which do not express T7 lysozyme.

continued on next page

Obtaining Other BL21 Strains

BL21(DE3) and BL21(DE3)pLysE cells are available from Invitrogen in the One Shot® format (see below). Refer to our Web site (www.invitrogen.com) or contact Technical Service for more information (see page 48).

Cells	Catalog no.
BL21(DE3)	C6000-03
BL21(DE3)pLysE	C6565-03



Note

Do not use BL21(DE3), BL21(DE3)pLysS, or BL21(DE3)pLysE to propagate or maintain your pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® construct. Use TOP10 cells instead (see page 4).

Infection with Phage

In about 5% of all cases, there will be some genes that are so toxic that they require infection with phage expressing T7 RNA polymerase (Tabor, 1990). You will need to use an *E. coli* host strain that contains the F′ episome (e.g. TOP10F′ or DH5αF′). Remember that the BL21(DE3)pLysS and BL21(DE3)pLysE strains should **not** be used in this situation. A protocol for infecting *E. coli* with M13 phage expressing T7 polymerase can be found in *Current Protocols in Molecular Biology*, pp. 16.2.1-16.2.11 (Ausubel *et al.*, 1994). Information for infecting *E. coli* with lambda phage expressing T7 polymerase is also available (Studier *et al.*, 1990). TOP10F′ cells are available from Invitrogen (Catalog no. C615-00) for use in phage infections.

Purification of VP22 Fusion Proteins

Introduction

Once you have expressed your recombinant fusion protein from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®, you are ready to scale-up expression for purification of the fusion protein from the bacterial culture. The presence of the C-terminal polyhistidine (6xHis) tag in your VP22 fusion protein allows you to use a metal-chelating resin such as ProBond™ to purify your fusion protein. After purification, the VP22 fusion protein can be applied directly to the mammalian cells of choice.

Important

This section provides guidelines to purify your VP22 fusion protein using ProBond™. If you are using another metal-chelating resin, follow the manufacturer's instructions. Other purification methods are also suitable. Note that some empirical experimentation may be necessary to determine the optimum conditions required to purify your fusion protein.

ProBond™

ProBond™ is a nickel-charged Sepharose® resin that can be used for affinity purification of fusion proteins containing the 6xHis tag. Proteins bound to the resin can be eluted by competition with imidazole. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Capacity of ProBond™

The capacity of ProBond™ is approximately 1 mg of recombinant protein per milliliter of bed volume. Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein to the column.

We generally use 2 ml of ProBond™ resin to purify VP22 fusion proteins from 50 ml of bacterial culture. The amount of purified fusion protein obtained from 50 ml of bacterial culture is generally sufficient to treat up to 50 dishes of cells (plated in 35 mm dishes or tissue culture wells). Note that yields of purified recombinant protein will vary depending on the nature of the protein. If you need to purify larger amounts of recombinant protein, you may need more ProBond™ resin (see page ix for ordering information).

Positive Control

We recommend that you also purify the control VP22 fusion protein from the pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2 plasmid while purifying your recombinant fusion protein from pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO®. You may use the purified VP22 fusion protein (expressed from pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2) as a control to assay uptake in your mammalian cell line of interest.

continued on next page

Purification of VP22 Fusion Proteins, continued

Materials Supplied by the User

You will need to have the following reagents and equipment on hand before beginning.

- One vial of One Shot® BL21(DE3)pLysS cells and transformation reagents for each expression experiment (included with the kit)
- pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® construct (and positive control)
- LB medium containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol
- 1 M IPTG
- 37°C shaking incubator
- VP22 Lysis Buffer (see page 45 for a recipe)
- VP22 Wash Buffer (see page 46 for a recipe)
- Three different VP22 Elution Buffers (100 mM Elution Buffer, 200 mM Elution buffer, and 500 mM Elution Buffer; see page 46 for a recipe)
- 0.5 M β-mercaptoethanol
- 1 mg/ml leupeptin (prepare in sterile water; store at –20°C)
- 1 mg/ml pepstatin (prepare in methanol; store at –20°C)
- 0.5 M phenyl methyl sulfonyl fluoride (PMSF; prepare in methanol)
- 100 mg/ml lysozyme (prepare in VP22 Lysis Buffer immediately before use)
- 10 mg/ml DNase I
- 10 mg/ml RNase A
- 21-gauge needles
- 5 ml syringes
- Sonicator
- ProBond™ (resin or columns)
- 15 ml and 50 ml sterile conical tubes
- 1.5 ml microcentrifuge tubes
- Appropriate equipment to hold ProBond™ column and collect fractions
- 1X and 4X SDS-PAGE sample buffer
- Reagents and apparatus for SDS-PAGE gel
- Boiling water bath

continued on next page

Scale-up of Expression for Purification on ProBond™

Use the procedure below to grow and induce expression of your VP22 fusion protein from 50 ml of bacterial culture. If you need to purify larger amounts of recombinant protein, you may scale-up the volume of bacterial culture accordingly.

1. Inoculate 10 ml of SOB or LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with a BL21(DE3)pLysS transformation reaction (see protocol on page 18).
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD_{600} = 1-2.
3. The next day, inoculate 50 ml of SOB or LB containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol with 2 ml of the overnight culture. **Note:** You can scale up further and inoculate all of the 10 ml overnight culture into 500 ml of medium, but you will need a larger bed volume for your ProBond™ column.
4. Grow the culture at 37°C with shaking (225-250 rpm) to an OD_{600} = ~0.5 (2-3 hours). The cells should be in mid-log phase. Remove a 500 μl aliquot of the culture and centrifuge at 10,000 x g for 2 minutes. Discard the supernatant and resuspend the pellet in 50 μl of 1X SDS/PAGE sample buffer. This will be your gel sample of uninduced cells.
5. Add IPTG to the remaining culture to a final concentration of 1 mM to induce expression.
6. Grow at 37°C with shaking until the optimal time point determined by the pilot expression is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. Proceed directly to **Preparation of Cell Lysate** (see the next page) or store the cell pellets at -80°C for future use. If you are using another metal-chelating resin, refer to the manufacturer's instructions.

continued on next page

Purification of VP22 Fusion Proteins, continued

Preparation of Cell Lysate

Before beginning, we recommend that you read through the protocol. Be sure to have all of your solutions prepared.

1. Add 4 ml of ice cold VP22 Lysis Buffer to each cell pellet from Step 6, previous page. To each 4 ml sample, add the following:

0.5 M β-mercaptoethanol	40 µl
1 mg/ml Leupeptin	4 µl
1 mg/ml Pepstatin	4 µl
0.5 M PMSF	4 µl
100 mg/ml Lysozyme	40 µl

2. Resuspend the cell pellet making sure the cell pellet is fully dispersed. Keep samples on ice.
3. Incubate the cell lysate on ice for 20 to 30 minutes.
4. Sonicate the cell lysate for 3 x 10 seconds. Keep the samples on ice.
5. Add DNase I and RNase A to a final concentration of 10 µg/ml each. Incubate on ice for 20 minutes.
6. Pass the cell lysate through a 21-gauge needle on a 5ml syringe. Repeat twice.
7. Centrifuge the cell lysate at 20,000 x g for 15 minutes at +4°C.
8. Transfer the supernatant to a new tube. Remove and save an aliquot of the supernatant for later analysis. This sample contains your soluble fusion protein. Store samples at +4°C. For long-term storage, store the samples at -80°C. Proceed to **Purification on ProBond™**, next page.

   Note: If you wish to determine the amount of expressed fusion protein that is insoluble, resuspend the cell pellet in 4 ml of VP22 Lysis Buffer. Remove and save an aliquot of the lysate for later analysis.

continued on next page

Purification on ProBond™

1. To equilibrate the ProBond™ resin, add 10 ml of ice cold VP22 Lysis Buffer to the column. Cap the top of the column and place the column horizontally on ice. Rock gently on a shaking platform for 2-3 minutes. Attach the column vertically to a stand and allow the resin to settle. Carefully remove the Lysis Buffer by pipetting.
2. Apply the supernatant containing your soluble fusion protein from Step 8, previous page to the column. Cap the top of the column and place horizontally on ice. Place on a shaking platform and allow the resin and supernatant to mix for one to two hours at +4°C.
3. Clamp the column vertically and allow the resin to settle. Remove the caps from the top then the bottom of the column and allow the supernatant to pass through the column. Collect the flow-through and remove an aliquot for later analysis. This sample contains unbound protein.
4. Add 10 ml of ice cold VP22 Lysis Buffer to the column. Collect the flow-through and remove an aliquot for later analysis.
5. Add 20 ml of ice cold VP22 Wash Buffer to the column. Collect the flow-through and remove an aliquot for later analysis.
6. To elute your recombinant fusion protein, use the three VP22 Elution Buffers that you have prepared. Apply 3 ml of ice cold 100 mM VP22 Elution Buffer to the column. Collect the flow-through in one tube. Remove an aliquot of the flow-through and save for later analysis.

   Note: Generally, the majority of the fusion protein will not elute from the ProBond™ column until the 500 mM VP22 Elution Buffer is used. This may vary depending on the nature of your protein.
7. Apply 3 ml of ice cold 200 mM VP22 Elution Buffer to the column. Collect the flow-through in one tube. Remove an aliquot of the flow-through and save for later analysis.
8. Apply 3 ml of ice cold 500 mM VP22 Elution Buffer to the column. Collect the flow-through as 0.5 ml fractions. These fractions should contain your eluted fusion protein. Remove an aliquot from each fraction for SDS-PAGE analysis.
9. Remove 10 μl of the resin from the column. Add 20 μl sterile water and 10 μl of 4X SDS-PAGE sample buffer (see recipe on page 45). This sample will contain protein that has not eluted from the resin.
10. For SDS-PAGE, add SDS-PAGE sample buffer to the various eluted fractions to a final concentration of 1X and boil the samples for 5 minutes. Load samples onto an SDS-PAGE gel and electrophorese. Stain gel with Coomassie™ blue and identify the fraction containing the most concentrated eluted recombinant protein. If protein is not detectable by Coomassie™ blue staining, you may want to perform Western blot analysis (see page 20) to gauge the success of your purification. To determine the concentration of your fusion protein, see the next page.
11. Once you have identified the fractions containing your eluted fusion protein, pool the fractions and store them at +4°C for immediate use. For long-term storage, store the purified protein at -80°C (see the next page).

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Purification of VP22 Fusion Proteins, continued

Long-Term Storage of Purified Fusion Protein

We have stored purified VP22 fusion protein at -80°C for up to 4 weeks, therefore, it may be possible to store your purified fusion protein at -80°C. Storage conditions may vary depending on the nature of your protein. Note that freezing your purified fusion protein may affect its biological activity. If you plan to store your purified fusion protein at -80°C, avoid repeated freezing and thawing as it may result in loss of activity or protein integrity.

Note

We have found that the degree of purification achieved for VP22 fusion proteins using ProBond™ is sufficient for most applications in mammalian cells. In general, no further purification steps are required. This may vary depending on the nature of your protein and your specific application.

Determining Fusion Protein Concentration

You may use any method of your choice to determine the concentration of your purified fusion protein. We typically use the following techniques to determine the concentration of purified VP22 fusion proteins.

1. For a rough estimate of fusion protein concentration, run samples of the purified fusion protein and appropriate standards containing known amounts of protein (e.g. BSA) on an SDS/PAGE gel. Stain the gel with Coomassie™ blue or Simply Blue™ Safe Stain and use the standards to estimate the concentration of your fusion peptide.
2. To more accurately determine your fusion peptide concentration, we recommend using the BCA-200 Protein Assay Kit (Pierce, Catalog no. 23225ZZ). We do not recommend performing Bradford or Lowry assays because the presence of imidazole may interfere with accurate protein concentration determination.

Yield of Purified Fusion ProteiN

When purifying VP22 fusion proteins using the protocol on pages 25-27, we generally obtain 1-10 mg of purified protein at a concentration ranging from 1-10 μg/μl. Note that protein yields will vary depending on the nature of the protein and its solubility. We have found that the purified fusion protein is generally concentrated enough to add directly to mammalian cells and no further concentration is required. If you need a more concentrated protein solution, refer to general reference texts (Coligan *et al.*, 1998; Deutscher, 1990) for guidelines and protocols to help you concentrate your fusion protein.

Amount of Fusion Protein to Add to Mammalian Cells

We typically add 1-10 μg of purified fusion protein to 2 x 10^5 cells plated in a 35 mm dish or tissue culture well. Larger amounts of purified fusion protein may be added, if desired. The amount of purified fusion protein you need to add to your mammalian cells may vary depending on the nature of your protein of interest and your application.

Application and Detection of Purified Protein in Mammalian Cells

Introduction

Once you have obtained your purified VP22 fusion protein, you are ready to add the purified fusion protein to cultured mammalian cells. The section below provides information on application and detection of your fusion protein in mammalian cells.

Detection of Fusion Protein

Before plating cells, you will need to determine the technique that you will use to assay for uptake of your fusion protein (e.g. immunofluorescence, western analysis). Once you have designed your experiment, seed your cells accordingly.

To detect the VP22 fusion protein in mammalian cells, you may use the Anti-VP22 Antibody, Anti-*myc* antibodies, or the Anti-His(C-term) antibodies available from Invitrogen (see page ix for ordering information) or an antibody to your protein. The Anti-VP22 Antibody, Anti-*myc* antibodies, and the Anti-His(C-term) antibodies can be used to detect your protein using immunofluorescence or western blotting.

Don't forget to include a sample of purified control VP22 fusion protein (expressed from the parental pCR®T7/VP22/NES-1 or pCR®T7/VP22/NES-2 vector) to help you evaluate your results. The control VP22 fusion protein serves as a positive control for translocation and as a negative control for potential biological effects observed with your fusion protein. Also include a sample of 500 mM VP22 elution buffer containing no protein as a negative control for uptake and biological effects observed with your fusion protein.

The presence of serum in the medium can inhibit uptake of VP22 fusion proteins into mammalian cells. You will need to remove the serum-containing medium from your cells and replace with medium containing no serum immediately prior to addition of purified fusion protein. Since uptake occurs within 20 minutes after addition of VP22 fusion protein to the cells, we have not observed any deleterious phenotypic effects caused by the short-term removal of serum in the cell lines tested. You may want to keep this in mind if your cells are particularly sensitive to removal of serum.

Experimental Outline

The table below outlines the basic steps needed to add and detect VP22 fusion proteins in your mammalian cells of interest.

Step	Action
1	Decide on the technique that you will use to assay for uptake of your VP22 fusion protein and seed your mammalian cells accordingly. Incubate cells overnight at 37°C.
2	The next day, remove serum-containing medium from the cells and replace with medium containing no serum.
3	Add purified VP22 fusion protein to the cells.
4	Incubate cells at 37°C for 20 minutes.
5	Assay for uptake of your fusion protein by immunofluorescence, western blot, or other method of your choice.

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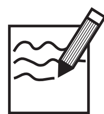


You will be adding purified fusion protein directly to the culture medium of your mammalian cells. Remember that your protein solution contains imidazole. Although we have not observed any pleiotrophic effects of imidazole on cells, this may vary depending on the nature of your cell line. If the presence of imidazole is a concern, you may dialyze your fusion protein solution to remove the imidazole. **When applying the VP22 fusion protein to mammalian cells, we recommend that the amount of VP22 fusion protein solution added to the culture medium not exceed 5% of the culture volume.**

Immunofluorescence

A sample protocol is provided below using the Anti-VP22 Antibody. Other protocols and antibodies (see below) may be suitable.

1. Plate cells (~10^5) in a 35 mm dish or a single well in a six-well tissue culture plate. Incubate cells overnight at 37°C in serum-containing medium.
2. The next day, remove the serum-containing medium and replace with medium containing no serum (1 ml for a 35 mm dish or a single well in a six-well tissue culture plate) immediately before addition of the purified VP22 fusion protein.
3. Add the appropriate amount of VP22 fusion protein directly to the medium of the cells. Swirl gently to mix. Incubate at 37°C for 20 minutes.
4. Remove the medium and wash cells twice with PBS (see page 47 for a recipe). Fix the cells by adding 2 ml of room temperature, 100% methanol. **Note**: The best results have been obtained with fixation using methanol rather than formaldehyde.
5. Incubate for 5 minutes at room temperature. Do not exceed 5 minutes.
6. After incubation, wash cells 5 times with PBS (2 ml/wash).
7. Add 2 ml PBS containing 10% fetal bovine serum (FBS) (blocking solution) and incubate for 15 minutes at room temperature to reduce non-specific binding of antibody.
8. Remove the blocking solution and add 1 ml of PBS/10% FBS containing the Anti-VP22 Antibody (1:500 dilution of antibody). Incubate for 20 minutes at room temperature.
9. Wash cells 2 x 5 minutes with PBS.
10. Dilute goat anti-mouse Oregon Green conjugate (Molecular Probes; Catalog no. O-6383) 1:500 in PBS/10% FBS. **Note**: Other fluorescent conjugates can be used.
11. Add to cells and incubate for 20 minutes at room temperature in the dark.
12. Wash cells 2 x 5 minutes with PBS and observe cells with fluorescence microscope equipped with a FITC filter (or appropriate filter).

For your convenience, FITC-conjugated antibodies are available from Invitrogen (see page ix for ordering information) to facilitate direct immunofluorescence detection of VP22 fusion proteins without the need for secondary fluororescent conjugates. If you are using the Anti-*myc*-FITC Antibody or Anti-His(C-term)-FITC Antibody for immunofluorescence, refer to the FITC-conjugated Antibodies manual for instructions for use.

continued on next page

Application and Detection of Purified Protein in Mammalian Cells, continued

What You Should See

Cells which have taken up the native VP22 protein typically show nuclear staining (Brewis *et al.*, 2000; Elliott and O'Hare, 1997; Phelan *et al.*, 1998). However, the presence of the NES directs export of your VP22 fusion protein to the cytoplasm. The VP22 fusion protein should be predominantly localized in the cytoplasm, although some of the fusion protein may also be found in the nucleus. If you have included the control VP22/NES fusion protein (expressed from pCR®T7/VP22/NES-1) in your experiment, the cells should also show predominantly cytoplasmic staining.

No Fluorescence

Assuming the VP22 fusion protein has been imported into the cells, review the immuno-fluorescence procedure.

- Try a different fixation method
- Try a different antibody

Preparation of Cytoplasmic and Nuclear Extracts

To detect your VP22 fusion protein by western blot, you will need to prepare a cytoplasmic extract from treated cells. We recommend that you also prepare a nuclear extract from treated cells to allow you to compare the distribution of VP22 fusion protein in the cells. A sample protocol is provided below. Other protocols and fractionation buffers may be suitable.

1. Seed cells in 35 mm culture dishes at 50% confluency (approximately 1 x 10^5 cells) in 2 ml of culture medium. Allow cells to adhere overnight.
2. The next day, remove serum-containing medium from the cells and replace with 1.5 ml of serum-free medium.
3. Add the appropriate amount of VP22 fusion protein directly to the culture medium. Swirl gently to mix. Incubate cells at 37°C for 20 minutes.
4. Wash cell monolayer twice with phosphate-buffered saline (PBS; see page 47 for a recipe).
5. Scrape cells into 1 ml PBS and pellet the cells at 500 x g for 5 minutes. Gently resuspend the cells in 10 ml PBS and centrifuge at 500 x g for 5 minutes.
6. Resuspend in 100 µl ice cold Mammalian Cell Fractionation Buffer (see page 47 for a recipe). Other cell fractionation buffers may be suitable.
7. Incubate cell suspension on ice for 10 minutes.
8. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet the nuclei. Remove the supernatant containing soluble cytoplasmic proteins to a new tube and add 4X SDS-PAGE sample buffer to a final concentration of 1X. Resuspend the pellet containing cell nuclei in 100 µl of 1X SDS-PAGE sample buffer. **Note:** Cell nuclei must be resuspended in a large enough volume of 1X SDS-PAGE sample buffer to facilitate gel loading.
9. Boil the cytoplasmic and nuclear samples for 5 minutes. Load 20 µl onto an SDS-PAGE gel and electrophorese. Perform western blot analysis using your desired protocol.

Appendix

pCR®T7/VP22/NES TOPO TA Cloning® Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing kanamycin and X-gal.

Before Starting

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

- 40 mg/ml X-gal in dimethylformamide (see page 44 for recipe)
- LB plates containing 50 µg/ml kanamycin and X-gal (two per transformation)
- To add X-gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Producing Control PCR Product

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

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl)	1 µl
Sterile Water	41.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 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

pCR®T7/VP22/NES TOPO TA Cloning® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

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

2. Incubate at 25°C (room temperature) for 5 minutes and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (page 13).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin and X-gal. 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' plate should contain only a few colonies (<15% of the vector + PCR insert plate).

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® cells with 10 pg of pUC19 per 50 µl of cells using the protocol on page 13. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 50 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

continued on next page

pCR®T7/VP22/NES 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% cloning efficiency.

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>3 kb)	Try one or all of the following: Increase amount of insert. Incubate the TOPO® Cloning reaction longer. Gel-purify the insert as described on pages 35-36.
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 in the TOPO® Cloning reaction.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 37).
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 (pages 35-36) 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).

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 for your convenience.

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 10).

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

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

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

   Note: Do not use TBE. Borate will interfere with the NaI step (Step 2.)
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M NaI.
3. Add 1.5 volumes of Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 μl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 μl of TE or sterile water. Use 4 μl for the TOPO® Cloning reaction and proceed as described on page 13.

Quick S.N.A.P.™ Method

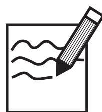
An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 μl of the flow-through in the TOPO® Cloning reaction (page 13). Be sure to make the gel slice as small as possible for best results.

continued on next page

Low-Melt Agarose Method

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

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 μl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 13.
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 One Shot® TOP10 cells using the method on page 13.



Note

Note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

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

Before Starting

You will need the following items:

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

Procedure

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

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

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

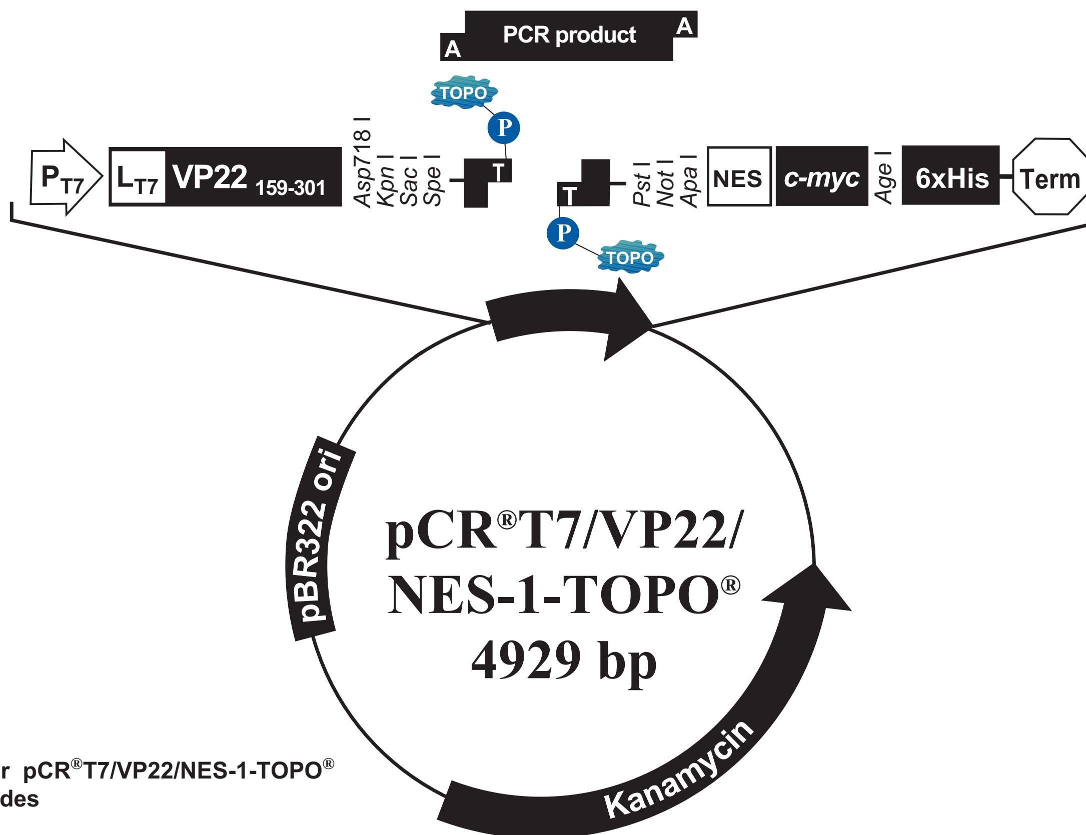
You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see previous page). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 μl in the TOPO® Cloning reaction.

Vent® is a registered trademark of New England Biolabs.

PCR®T7/VP22/NES-1-TOPO®

Map

The figure below summarizes the features of the pCR®T7/VP22/NES-1-TOPO® vector. The vector is supplied linearized between base pairs 597 and 598. This is the TOPO® Cloning site. **The complete nucleotide sequence for pCR®T7/VP22/NES-1-TOPO® is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 48).**



Comments for pCR®T7/VP22/NES-1-TOPO®
4929 nucleotides

T7 promoter: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Truncated VP22 ORF (amino acids 159-301 only): bases 126-551
VP22 forward priming site: bases 455-470
TOPO® Cloning site: bases 597-598
HIV Rev nuclear export signal (NES): bases 637-666
c-myc epitope: bases 685-714
Polyhistidine (6xHis) tag: bases 730-747
myc-His reverse priming site: bases 712-732
Kanamycin resistance gene: bases 1223-2038 (complementary strand)
pBR322 origin: bases 2134-2807

continued on next page

PCR®T7/VP22/NES-1-TOPO ®, continued

Features

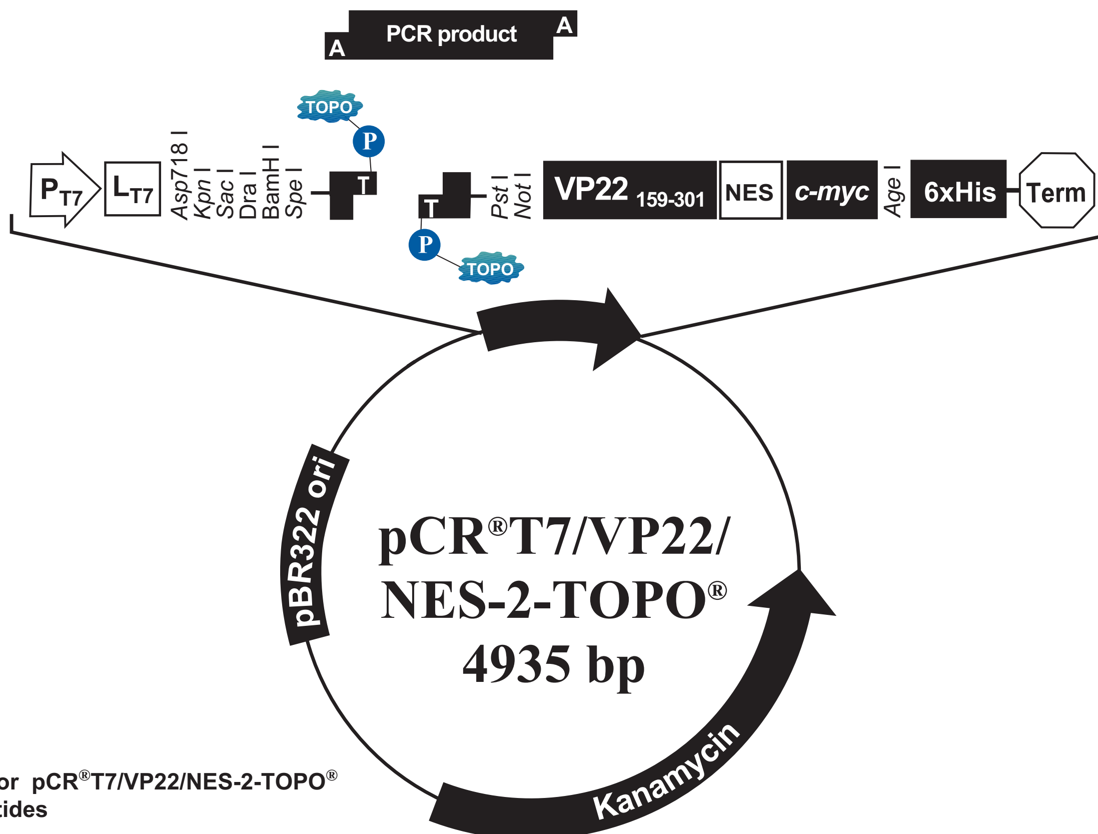
The pCR®T7/VP22/NES-1-TOPO® vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase
T7 leader	Improves translation efficiency of the gene of interest
Truncated VP22 ORF (amino acids 159-301 only)	Fusing your gene to the truncated VP22 ORF permits translocation of the protein into mammalian cells (Elliott and O'Hare, 1997)
VP22 Forward priming site	Permits sequencing of your insert to confirm that it is in frame with the truncated VP22 ORF
TOPO® Cloning site	Allows insertion of your PCR product in frame with the truncated VP22 ORF, NES, and the C-terminal peptide containing the *c-myc* epitope and polyhistidine tag
HIV Rev nuclear export signal (NES)	Directs VP22 fusion protein to the cytoplasm of mammalian cells (Fischer *et al.*, 1995; Wen *et al.*, 1995)
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant protein with the Anti-*myc* Antibody (Catalog no. R950-25) or Anti-*myc*-HRP Antibody (Catalog no. R951-25) (Evans *et al.*, 1985)
C-terminal polyhistidine (6xHis) tag	Permits purification of your recombinant VP22 fusion protein on metal-chelating resin such as 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)
myc-His Reverse priming site	Permits sequencing of your insert
Kanamycin resistance gene	Allows selection of the vector in *E. coli*
pBR322 origin	Permits replication and low-copy maintenance of the plasmid in *E. coli*

pCR®T7/VP22/NES-2-TOPO®

Map

The figure below summarizes the features of the pCR®T7/VP22/NES-2-TOPO® vector. The vector is supplied linearized between base pairs 180 and 181. This is the TOPO® Cloning site. **The complete nucleotide sequence for pCR®T7/VP22/NES-2-TOPO® is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 48).**



Comments for pCR®T7/VP22/NES-2-TOPO®
4935 nucleotides

T7 promoter: bases 1-20
T7 promoter/priming site: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
TOPO® Cloning site: bases 180-181
Truncated VP22 ORF (amino acids 159-301 only): bases 226-651
VP22 reverse 2 priming site: bases 273-288
HIV Rev nuclear export signal (NES): bases 658-687
c-myc epitope: bases 691-720
Polyhistidine (6xHis) tag: bases 736-753
Kanamycin resistance gene: bases 1229-2044 (complementary strand)
pBR322 origin: bases 2140-2813

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pCR®T7/VP22/NES-2-TOPO®, continued

Features

The pCR®T7/VP22/NES-2-TOPO® vector contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
T7 promoter/priming site	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase and allows sequencing of your insert
T7 leader	Improves translation efficiency of the gene of interest
TOPO® Cloning site	Allows insertion of your PCR product in frame with the truncated VP22 ORF and the C-terminal peptide containing the *c-myc* epitope and polyhistidine tag
Truncated VP22 ORF (amino acids 159-301 only)	Fusing your gene to the truncated VP22 ORF permits translocation of the protein into mammalian cells (Elliott and O'Hare, 1997)
VP22 Reverse 2 priming site	Permits sequencing of your insert
HIV Rev nuclear export signal (NES)	Directs VP22 fusion protein to the cytoplasm of mammalian cells (Fischer *et al.*, 1995; Wen *et al.*, 1995)
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of your recombinant protein with the Anti-*myc* Antibody (Catalog no. R950-25) or Anti-*myc*-HRP Antibody (Catalog no. R951-25) (Evans *et al.*, 1985)
C-terminal polyhistidine (6xHis) tag	Permits purification of your recombinant VP22 fusion protein on metal-chelating resin such as 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)
Kanamycin resistance gene	Allows selection of the vector in *E. coli*
pBR322 origin	Permits replication and low-copy maintenance of the plasmid in *E. coli*

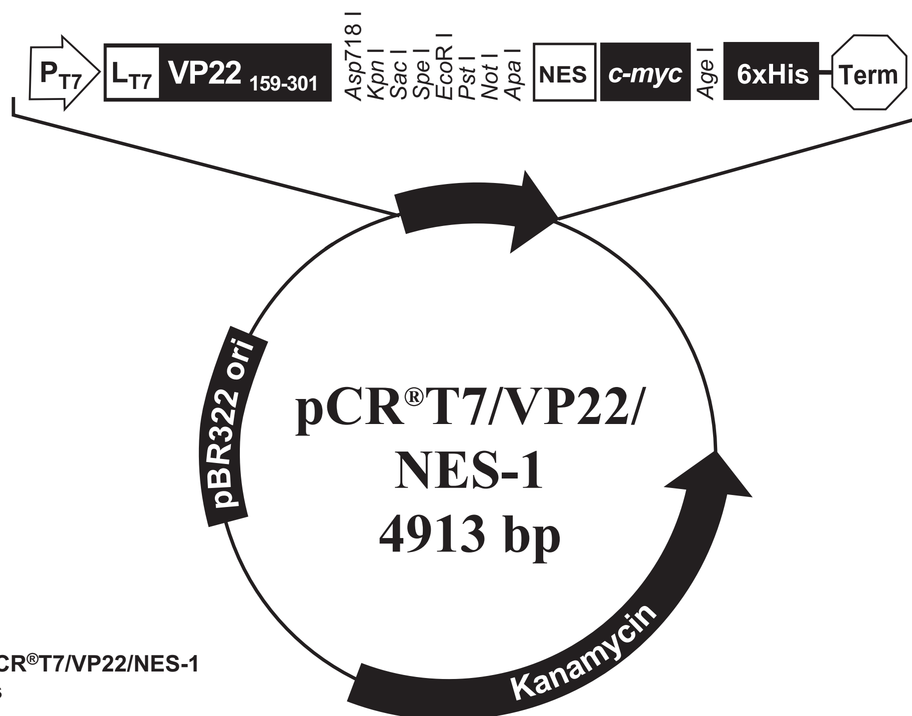
Map of pCR®T7/VP22/NES-1

Description

pCR®T7/VP22/NES-1 is a 4913 bp control vector containing the truncated VP22 ORF (amino acids 159-301) fused to the C-terminal peptide containing the NES, *c-myc* epitope, and the polyhistidine tag. pCR®T7/VP22/NES-1 is the parent vector of pCR®T7/VP22/NES-1-TOPO® and may be used as a positive expression control. The recombinant VP22 fusion protein produced from pCR®T7/VP22/NES-1 is 24 kDa in size. **Note**: The multiple cloning site maintains the frame through the C-terminal tag.

Map of Control Vector

The figure below summarizes the features of the pCR®T7/VP22/NES-1 vector. **The complete nucleotide sequence for pCR®T7/VP22/NES-1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pCR®T7/VP22/NES-1
4913 nucleotides

T7 promoter: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Truncated VP22 ORF (amino acids 159-301 only): bases 126-551
VP22 forward priming site: bases 455-470
Multiple cloning site: bases 552-620
HIV Rev nuclear export signal (NES): bases 621-650
c-myc epitope: bases 669-698
Polyhistidine (6xHis) tag: bases 714-731
myc-His reverse priming site: bases 696-716
Kanamycin resistance gene: bases 1207-2022 (complementary strand)
pBR322 origin: bases 2118-2791

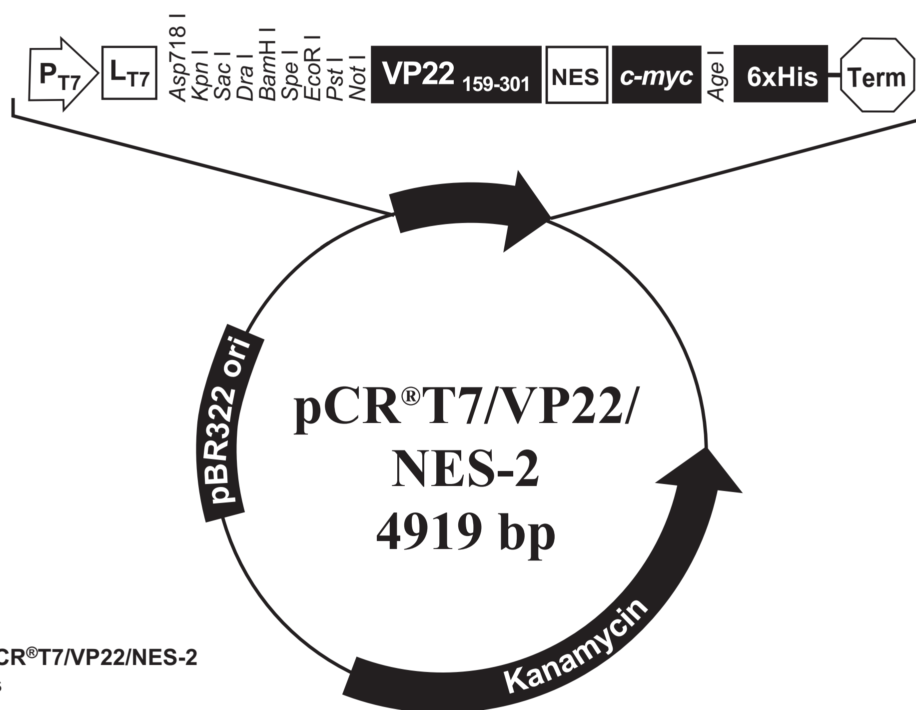
Map of pCR®T7/VP22/NES-2

Description

pCR®T7/VP22/NES-2 is a 4919 bp control vector containing the truncated VP22 ORF (amino acids 159-301) fused to the C-terminal peptide containing the NES, *c-myc* epitope, and the polyhistidine tag. pCR®T7/VP22/NES-2 is the parent vector of pCR®T7/VP22/NES-2-TOPO® and may be used as a positive expression control. The recombinant VP22 fusion protein produced from pCR®T7/VP22/NES-2 is 24 kDa in size. **Note**: The multiple cloning site maintains the frame through the C-terminal tag.

Map of Control Vector

The figure below summarizes the features of the pCR®T7/VP22/NES-2 vector. **The complete nucleotide sequence for pCR®T7/VP22/NES-2 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pCR®T7/VP22/NES-2
4919 nucleotides

T7 promoter: bases 1-20
T7 promoter/priming site: bases 1-20
Ribosome binding site: bases 66-71
T7 leader: bases 81-113
Multiple cloning site: bases 123-203
Truncated VP22 ORF (amino acids 159-301 only): bases 210-635
VP22 reverse 2 priming site: bases 257-272
HIV Rev nuclear export signal (NES): bases 642-671
c-myc epitope: bases 675-704
Polyhistidine (6xHis) tag: bases 720-737
Kanamycin resistance gene: bases 1213-2028 (complementary strand)
pBR322 origin: bases 2124-2797

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (50 μg/ml kanamycin) if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 μg/ml of kanamycin), 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.

Bacterial Cell Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   0.3 ml of 1 M KH_2PO_4
   4.7 ml of 1 M K_2HPO_4
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

continued on next page

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5.0 ml
Glycerol (100%)	4.0 ml
β-mercaptoethanol	0.8 ml
Bromophenol blue	0.04 g
SDS	0.8 g

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

2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2.0 ml
β-mercaptoethanol	0.4 ml
Bromophenol blue	0.02 g
SDS	0.4 g

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

0.1 M Sodium Phosphate, pH 8.0

Before beginning, have the following reagents on hand.

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$, Sigma, Catalog no. S9638)
Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$, Sigma, Catalog no. 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 1 liter of 0.1 M sodium phosphate, pH 8.0, mix together 6.8 ml of 1 M $NaH_2PO_4 \cdot H_2O$ and 93.2 ml of 1 M $Na_2HPO_4 \cdot 7H_2O$. Bring volume up to 1 liter with sterile water. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at room temperature.

VP22 Lysis Buffer

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
5 mM Imidazole (Sigma, Catalog no. I0125)

1. To 150 ml of deionized water, add the following and dissolve:

   250 ml of 0.1 M Sodium phosphate, pH 8.0 (see recipe above)
   8.77 g NaCl
   0.17 g Imidazole

2. Bring the volume up to 500 ml with deionized water.
3. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at +4°C.

continued on next page

VP22 Wash Buffer

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
40 mM Imidazole (Sigma, Catalog no. I0125)
10% glycerol

1. To 150 ml of deionized water, add the following and dissolve:
   250 ml of 0.1 M Sodium phosphate, pH 8.0 (see recipe on the previous page)
   8.77 g NaCl
   0.17 g Imidazole
   50 ml glycerol
2. Bring the volume up to 500 ml with deionized water.
3. Check to make sure the pH is 8.0.
4. Filter-sterilize and store at +4°C.

VP22 Elution Buffers

Note that three different Elution Buffers should be prepared (100 mM Elution Buffer, 200 mM Elution Buffer, and 500 mM Elution Buffer). The three Elution Buffers contain varying amounts of imidazole (see below).

50 mM Sodium phosphate, pH 8.0
300 mM NaCl (Sigma, Catalog no. S9888)
10% glycerol
Imidazole (100 mM, 200 mM or 500 mM; Sigma, Catalog no. I0125)

1. To prepare the VP22 Elution Buffers, add the following to three separate sterile beakers and mix until dissolved:

Reagent	100 mM Elution Buffer	200 mM Elution Buffer	500 mM Elution Buffer
0.1 M Sodium phosphate, pH 8.0	100 ml	100 ml	100 ml
NaCl	3.51 g	3.51 g	3.51 g
Glycerol	20 ml	20 ml	20 ml
Imidazole	1.36 g	2.72 g	6.8 g
Deionized water	Volume up to 200 ml	Volume up to 200 ml	Volume up to 200 ml

2. Check to make sure the pH is 8.0.
3. Filter-sterilize and store at +4°C.

continued on next page

Mammalian Cell Fractionation Buffer

10 mM HEPES-KOH, pH 7.9
1.5 mM $MgCl_2$
10 mM KCl
0.5 mM DTT
1% Triton X-100

1. Prepare 1 M stock solutions of HEPES and KOH. Use the 1 M stock solution of KOH to adjust the pH of the 1 M HEPES to pH 7.9.
2. For 100 ml, combine the following reagents in 90 ml of deionized water:

1M HEPES-KOH, pH 7.9	1 ml
1 M $MgCl_2$	150 µl
1 M KCl	1 ml
1 M DTT	50 µl
100% Triton X-100	1 ml

3. Mix thoroughly and bring the volume to 100 ml with deionized water.
4. Filter-sterilize and store at +4°C.

Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve the following in 800 ml of deionized water:
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at room temperature or at +4°C.

Technical Service

World Wide Web

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

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

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

http://www.invitrogen.com

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

Contact Us

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

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MSDS Requests

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

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Limited Warranty

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Purchaser Notification

Information for European Customers

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Product Specifications

Introduction

This section describes the criteria used to qualify the components in the Voyager™ NES Protein Production Kits.

Vectors

The pCR®T7/VP22/NES-1 and pCR®T7/VP22/NES-2 plasmids (parental vectors of pCR®T7/VP22/NES-1-TOPO® and pCR®T7/VP22/NES-2-TOPO®, respectively) are qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. The table below lists the restriction enzymes used to digest each vector and the expected fragments. Please note that restriction sites used to qualify the parental vectors may no longer be present in the topoisomerase I-adapted vector.

Vector	Restriction Enzyme	Expected fragments (bp)
pCR®T7/VP22/NES-1	*Eco*R I	4913
	*Eco*R V	191, 222, 4500
	Nsi I	266, 423, 772, 3452
	Sac II	404, 4509
pCR®T7/VP22/NES-2	*Eco*R I	4919
	*Eco*R V	156, 191, 489, 4083
	Nsi I	266, 345, 772, 3536
	Sac II	4919

TOPO® Cloning Efficiency

Once the supercoiled vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 32-33, a 500 bp control PCR product is TOPO®-Cloned into pCR®T7/VP22/NES-1-TOPO® or pCR®T7/VP22/NES-2-TOPO® and subsequently transformed into the One Shot® TOP10 competent *E. coli* included with the kit.

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

Sequencing Primers

The sequencing primers are 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 ~1×10^9 cfu/μg DNA for chemically competent cells and >1×10^9 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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