invitrogen™

NativePure™ pcDNA™ Gateway® Vector Kit

For expression and purification of N- and C-terminal biotinylated fusion proteins and associated complexes in mammalian cells

Catalog nos. BN3002, BN3006

Version B
28 October 2010
25-0892

User Manual

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Experienced Users Procedure

Introduction

This quick reference procedure is provided for experienced users to create expression clones. If you are performing the LR recombination reactions for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Generate entry clones</td><td>Clone your gene of interest with and without a stop codon into Gateway® entry vectors to create two entry clones for recombination into pcDNA™3.2/capTEV™-NT/V5-DEST and pcDNA™3.2/capTEV™-CT/V5-DEST.</td></tr>
<tr><td>Perform LR recombination reactions</td><td>1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.
<table>
<tr><th>Component</th><th>NT-tag Entry Clone</th><th>CT-tag Entry Clone</th><th>Positive Control</th></tr>
<tr><td>Entry clone (without stop, 50-150 ng/rxn)</td><td>--</td><td>1-7 µl</td><td>--</td></tr>
<tr><td>Entry clone (with stop, 50-150 ng/rxn)</td><td>1-7 µl</td><td>--</td><td>--</td></tr>
<tr><td>pcDNA™3.2/capTEV™-NT/V5-DEST (150 ng/µl)</td><td>1 µl</td><td>--</td><td>1 µl</td></tr>
<tr><td>pcDNA™3.2/capTEV™-CT/V5-DEST (150 ng/µl)</td><td>--</td><td>1 µl</td><td>--</td></tr>
<tr><td>pENTR™-gus (50 ng/µl)</td><td>--</td><td>--</td><td>2 µl</td></tr>
<tr><td>TE Buffer, pH 8.0</td><td>to 8 µl</td><td>to 8 µl</td><td>5 µl</td></tr>
</table>
2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Briefly vortex the LR Clonase™ II enzyme mix twice (2 seconds each time).
4. To each sample, add 2 l of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 l of Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 l of the LR recombination reaction into a suitable E. coli host (follow the manufacturer's instructions) and select for expression clones.</td></tr>
<tr><td>Perform transient transfection</td><td>Transfect your NT- and CT-capTEV™ tagged expression clones into the mammalian cell line of choice (page 16).</td></tr>
<tr><td>Analyze proteins</td><td>Confirm biotinylation of your protein and complex formation by Western detection (page 18).</td></tr>
</table>

Kit Contents and Storage

Types of Kits

This manual is supplied with the kits listed below.

Product	Catalog no.
NativePure™ pcDNA™ Gateway® Vector Kit	BN3002
NativePure™ Mammalian Affinity Purification Kit	BN3006

Kit Components

The following table shows the components associated with the NativePure™ pcDNA™ Gateway® Vector and Affinity Purification Kits listed above. The NativePure™ Kits are shipped as described below. Upon receipt, store each item as detailed below.

Box	Component	Catalog no.		Shipping	Storage
		BN3002	BN3006		
1	NativePure™ pcDNA™ Gateway® Vector Kit	✓	✓	Room temperature	-20°C
2	NativePure™ Binding and Purification Module		✓	Blue ice	+4°C
3	NativePure™ AcTEV™ Protease Module		✓	Dry ice	-20°C

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Kit Contents and Storage, continued

NativePure™ pcDNA™ Gateway® Vectors

Each NativePure™ pcDNA™ Gateway® Vector Kit contains the following vectors. **Store the vectors at -20°C.**

Item	Amount
pcDNA™3.2/capTEV™-NT/V5-DEST	6 µg lyophilized in TE, pH 8.0
pcDNA™3.2/capTEV™-CT/V5-DEST	6 µg lyophilized in TE, pH 8.0
pcDNA™3.2/capTEV™-NT/V5-GW/ARPC2	10 µg lyophilized in TE, pH 8.0

NativePure™ Binding and Purification Module

The following reagents are included in the NativePure™ Binding and Purification Module (supplied with cat. no. BN3006 only). **Store at +4°C.**

Reagent	Composition	Amount
Streptavidin Agarose	10 ml of a 50% slurry containing 5 ml of packed Streptavidin Agarose beads in 0.1 M sodium phosphate, pH 7.5, 0.1 M NaCl, and 2 mM sodium azide.	5 ml packed resin
10% NP40	10% (v/v) NP40 in deionized water	8 ml
NativePure™ 5X Lysis/Binding Buffer	0.5 M Tris-HCl, pH 8.0 0.5 M KCl 1 mM EDTA 7.5 mM $MgCl_2$	100 ml
NativePure™ 10X TEV Buffer	0.1 M Tris-HCl, pH 8.0 1.5 M NaCl 5 mM EDTA	40 ml
NativePure™ Columns	Polypropylene columns	10
NativePure™ Concentrator	Includes a concentrator fitted with a membrane and a filtration chamber	10

NativePure™ AcTEV™ Protease Module

The following reagents are included in the NativePure™ AcTEV™ Protease Module (supplied with cat. no. BN3006 only). **Store at -20°C.**

Reagent	Composition	Amount
AcTEV™ Protease	10 U/µl AcTEV™ Protease in: 50 mM Tris-HCl, pH 7.5 1 mM EDTA 5 mM DTT 50% (v/v) glycerol 0.1% (w/v) Triton X-100	400 µl
100 mM DTT	100 mM DTT in deionized water	500 µl

Additional Products

Accessory Products

Some of the reagents supplied in the NativePure™ pcDNA™ Gateway® Vector Kit, as well as other products suitable for use are available separately from Invitrogen. For more information, go to www.invitrogen.com or contact Technical Service (see page 36).

Product	Amount	Catalog no.
pENTR™/D-TOPO® Cloning Kit	20 reactions	K2400-20
pCR8/GW/TOPO® TA Cloning Kit	20 reactions	K2500-20
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
One Shot® *ccd*B Survival T1^R Chemically Competent Cells	10 reactions	C7510-03
LB Media	500 ml	10855-021
Ampicillin	200 mg	11593-027
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
Lipofectamine™ 2000	0.75 ml	11668-027
Geneticin® Selective Antibiotic, liquid	20 ml	10131-035
Quant-iT™ Protein Assay Kit	1000 assays	Q33210
Streptavidin-AP Conjugate	125 µl	SA100-04
Streptavidin-HRP Conjugate	2.5 mg	43-4323
WesternBreeze® Chromogenic Kit, Anti-Rabbit	20 reactions	WB7105
WesternBreeze® Chemiluminescent Kit, Anti-Rabbit	20 reactions	WB7104
NuPAGE® MOPS SDS Running Buffer (20X)	500 ml	NP0001
NuPAGE® MES SDS Running Buffer (20X)	500 ml	NP0002
NuPAGE® LDS Sample Buffer (4X)	10 ml	NP0007
NuPAGE® Sample Reducing Agent (10X)	250 µl	NP0004
NuPAGE® Transfer Buffer (20X)	1 L	NP0006-1
HiMark™ Pre-Stained Protein Standard	250 µl	LC5699
Tris-Glycine SDS Running Buffer (10X)	500 ml	LC2675
Tris-Glycine SDS Sample Buffer (2X)	20 ml	LC2676
Nitrocellulose (0.45 µm) Membrane/Filter Paper Sandwiches	20 sandwiches	LC2001
Invitrolon™ PVDF (0.45 µm) Membrane/Filter Paper Sandwiches	20 sandwiches	LC2005
Phosphate-Buffered Saline (PBS), 1X	500 ml	10010-023
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-AP Antibody	125 µl	R961-25
Anti-V5-HRP Antibody	50 µl	R962-25

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Additional Products, continued

Products for Native Protein Analysis

A complete range of products for purification of native protein complexes and analysis using native gel electrophoresis is available from Invitrogen. For more information go to www.invitrogen.com or contact Technical Service (see page 36).

Item	Amount	Catalog no.
NativePure™ Affinity Purification Kit	1 kit	BN3003
NativeMark™ Unstained Protein Standard	5 × 50 µl	LC0725
NativePAGE™ Novex 3-12% Bis-Tris Gels, 10-well	10 gels	BN1001BOX
NativePAGE™ Novex 3-12% Bis-Tris Gels, 15-well	10 gels	BN1003BOX
NativePAGE™ Novex 4-16% Bis-Tris Gels, 10-well	10 gels	BN1002BOX
NativePAGE™ Novex 4-16% Bis-Tris Gels, 15-well	10 gels	BN1004BOX
NativePAGE™ Running Buffer (20X)	1 L	BN2001
NativePAGE™ Cathode Buffer Additive (20X)	250 ml	BN2002
NativePAGE™ Sample Buffer (4X)	10 ml	BN2003
NativePAGE™ 5% G-250 Sample Buffer Additive	0.5 ml	BN2004
NativePAGE™ Running Buffer Kit	1 kit	BN2007
NativePAGE™ Sample Prep Kit	1 kit	BN2008
10% DDM (n-dodecyl-β-D-maltoside)	1 ml	BN2005
5% Digitonin	1 ml	BN2006
Streptavidin Agarose (sedimented bead suspension)	5 ml	S-951
AcTEV™ Protease	1000 units	12575-015
	10,000 units	12575-023

Pre-Cast Gels

A large variety of pre-cast gels for SDS-PAGE, native PAGE, and pre-made buffers are available from Invitrogen.

Use NuPAGE® and Novex® Tris-Glycine pre-cast gels for SDS-PAGE and Western analysis. Use NativePAGE™ pre-cast gels for native gel electrophoresis and Western analysis.

For details, visit www.invitrogen.com or contact Technical Service (page 36).

Introduction

Overview

Introduction

The NativePure™ Mammalian Affinity Purification Kit and NativePure™ pcDNA™ Gateway® Vector Kit contains Gateway®-adapted vectors for expression and purification of N- and C-terminal biotinylated fusion proteins in mammalian cells using Gateway® Technology (see page 6 for details on Gateway® Technology).

After transfection into your mammalian cell line of choice, the NativePure™ pcDNA™ Gateway® Vectors allow *in vivo* biotinylation and expression of the biotin-tagged protein of interest ("bait"). The biotin-tagged recombinant protein "bait" can be used to identify novel proteins that specifically interact with the protein of interest or to test complex formation between proteins or protein domains for which there is a prior reason to expect an interaction.

NativePure™ Mammalian Affinity Purification System

The NativePure™ Mammalian Affinity Purification System is based on the TAP (Tandem Affinity Purification) method used to purify native protein complexes (Puig *et al.*, 2001). The purification of native protein complexes requires the use of a high affinity tag that allows rapid affinity purification of the tagged protein and associated protein complexes when present in low concentrations from cells without any prior information on the protein complex. The purified protein complexes are released from the affinity resin using a highly-specific protease under native conditions.

The NativePure™ pcDNA™ Gateway® Vectors allow expression and *in vivo* biotinylation of your bait protein of interest. Rapid and efficient purification of the bait protein and associated complexes even when present at low concentrations is achieved using the streptavidin agarose included with the NativePure™ Affinity Purification Kit. The biotin-tagged protein and associated protein complexes may be analyzed by native gel electrophoresis or other techniques such as mass spectrometry.

The NativePure™ Mammalian Affinity Purification System when combined with mass spectrometry provides a novel experimental approach to identify interacting proteins for proteome analysis or examine protein complexes that are part of specific cellular pathways, differentiation stages, or cell types.

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Overview, continued

System Components

The NativePure™ Mammalian Affinity Purification System consists of two kits, the NativePure™ pcDNA™ Gateway® Vector Kit and NativePure™ Affinity Purification Kit.

The NativePure™ pcDNA™ Gateway® Vector Kit includes:

- Gateway®-adapted vectors that allow you to clone your gene of interest in frame with an N-terminal or C-terminal capTEV™ tag that allows *in vivo* biotinylation of your protein. See next page for a description of the capTEV™ tag
- A control vector expressing the *ARPC2* (actin related protein complex component p34, (Robinson *et al.*, 2001)) gene fused to the capTEV™ tag at the N-terminal end is included for use as a positive control for expression in the mammalian cell line of choice

The NativePure™ Affinity Purification Kit contains the following components to allow purification of biotinylated proteins and associated protein complexes expressed from vectors containing the capTEV™ tag:

- Streptavidin Agarose
- NativePure™ Columns
- Pre-made, ready-to-dilute lysis, binding and cleavage buffers
- AcTEV™ Protease
- NativePure™ Concentrators

For more information about each component and its use, see the NativePure™ Affinity Purification Kit manual. This manual is supplied with the NativePure™ Affinity Purification Kit but is also available for downloading at www.invitrogen.com.

Note

The NativePure™ pcDNA™ Gateway® Vector Kit is appropriate for use with established cell lines or cells that can be easily transfected. The user must optimize transfection conditions, and results may vary among cell types.

Continued on next page

Overview, continued

How the System Works

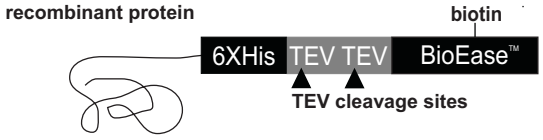
To express your biotinylated protein of interest in mammalian cells, construct N- and C-terminally tagged expression clones by performing LR recombination reactions between Gateway® entry vectors containing the gene of interest and both pcDNA™3.2/capTEV™/V5-DEST vectors. The resulting expression clones allow expression of N- and C-terminally biotinylated proteins ("bait" protein) in mammalian cells. Since individual protein expression and biotinylation may vary with an N- or C- terminal fusion tag in your cell line, it is necessary to construct both versions and determine which expression clone is best for your application.

Perform transfection of the pcDNA™3.2/capTEV™ N- and C-terminally tagged expression clones into mammalian cells to allow expression of biotinylated proteins and complex formation. Cells are lysed and the lysates are analyzed using Western analysis with a streptavidin conjugate to verify biotinylation of the protein of interest. The lysates are also analyzed using native gel electrophoresis to verify complex formation with the protein of interest.

After optimizing the expression and biotinylation of the bait protein of interest, the biotinylated protein and associated protein complexes are purified under native conditions using the NativePure™ Affinity Purification Kit (supplied with cat. no. BN3006 only, also available separately from Invitrogen). The associated complexes are analyzed by Western detection or mass spectrometry.

capTEV™ Tag

The NativePure™ pcDNA™ Gateway® Vectors allow N- and C- terminal fusion of your recombinant protein of interest to the capTEV™ Tag. The capTEV™ Tag consists of a BioEase™ *in vivo* biotinylation peptide, two Tobacco Etch Virus (TEV) protease recognition sites, and a 6XHis tag.



The capTEV™ tag facilitates *in vivo* biotinylation of the recombinant "bait" protein of interest. The biotin-tagged protein of interest forms complexes in your cell line of choice, which can be purified by binding to streptavidin agarose. The TEV sites allow removal of the bound biotinylated proteins/complexes of interest while endogenous biotinylated proteins remain bound to the streptavidin agarose column. After TEV cleavage, a 6xHis tag is present for potential removal of the "bait" protein under denaturing conditions after protein complex purification. These features are described in detail in the following sections.

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Overview, continued

BioEase™ Tag

The BioEase™ Tag is a 72 amino acid peptide derived from the C-terminus (amino acids 524-595) of *Klebsiella pneumoniae* oxalacetate decarboxylase α-subunit that contains a single covalent biotinylation site at lysine 561 of the protein (Schwarz *et al.*, 1988). When fused to a heterologous protein, the 72 amino acid BioEase™ domain is both necessary and sufficient to facilitate recognition and *in vivo* biotinylation of the recombinant protein of interest by cellular biotinylation enzymes. The high-affinity and selectivity of the streptavidin-biotin interaction is utilized to efficiently purify the biotinylated protein and associated complexes by streptavidin agarose affinity chromatography (supplied with cat. no. BN3006, also available separately from Invitrogen). For ore information about cellular biotinylation processes, refer to published reviews (Chapman-Smith and J.E. Cronan, 1999).

TEV Protease Recognition Site

Since the streptavidin/biotin interaction is extremely strong, removal of the bound protein complexes from the streptavidin agarose is achieved by cleavage with a protease. The TEV (Tobacco Etch Virus) Protease is a site-specific protease that allows efficient release of bound materials under native conditions (Rigaut *et al.*, 1999).

The NativePure™ pcDNA™ Gateway® Vectors are designed with two tandem TEV cleavage sites that promote >90% cleavage of the biotinylated recombinant protein and associated protein complexes from the streptavidin agarose during purification under native conditions.

AcTEV™ Protease, an enhanced form of TEV protease that is highly active and specific (Nayak *et al.*, 2003) is supplied with cat. no BN3006 and also available separately from Invitrogen (page vii).

Note: Upon TEV cleavage, the biotin tag is lost, and protein detection can be performed using the V5 tag, see below.

6XHis Tag

The capTEV™ Tag includes a polyhistidine region (6XHis) for potential removal of the "bait" protein under denaturing conditions using a nickel charged affinity resin after purifying the protein complex using streptavidin agarose.

V5 epitope

The NativePure™ pcDNA™ Gateway® Vectors contain a N- or C-terminal V5 epitope derived from the P and V proteins of the paramyxovirus SV5 (Southern *et al.*, 1991). Fusion proteins containing the 14 amino acid V5 epitope (GKPIPNPLLGLDST) can be identified using an anti-V5 antibody. See page vii for recommended antibodies and ordering information.

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Overview, continued

Features of the Vectors

The pcDNA™3.2/capTEV™/V5-DEST vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer to control expression of your gene of interest in a wide range of mammalian cells
- N- or C-terminal capTEV™ Tag for *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes. The capTEV™ tag consists of:
  - BioEase™ Tag for *in vivo* protein biotinylation
  - 2 Tobacco Etch Virus (TEV) protease recognition sites to remove bound biotinylated protein complexes after affinity purification with streptavidin agarose
  - 6XHis tag for potential purification of protein complexes after TEV cleavage
- N or C-terminal V5 epitope tag for detection of recombinant protein using anti-V5 antibodies
- Two recombination sites, *att*R1 and *att*R2, for recombinational cloning of the gene of interest from an entry clone
- The *ccd*B gene located between the two *att*R sites for negative selection
- Chloramphenicol resistance gene located between the two *att*R sites for counterscreen
- The Herpes Simplex Virus (HSV) thymidine kinase polyadenylation signal (TKpA) for proper termination and processing of the recombinant transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- Neomycin resistance gene for selection of stable cell lines with Geneticin®
- pUC origin for high copy replication and maintenance of plasmid in *E. coli*
- Ampicillin (*bla*) resistance gene for selection in *E. coli*

For maps and features of the pcDNA™3.2/capTEV™-NT/V5-DEST and pcDNA™3.2/capTEV™-CT/V5-DEST vectors, see pages 29-35.

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Overview, continued

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989). Gateway® Technology enables rapid and highly efficient transfer of DNA sequences into multiple vector systems for protein expression and functional analysis while maintaining orientation and reading frame. To express your gene of interest using Gateway® Technology, simply:

1. Clone your gene of interest with and without a stop codon into Gateway® entry vectors to create two entry clones.
2. Generate two expression clones by performing LR recombination reactions between the appropriate entry clones and pcDNA™3.2/capTEV™/V5-DEST vectors.
3. Transfect each expression clone separately into the cell line of choice for transient or stable expression of your gene of interest.

For more information on the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. This manual is available for downloading (www.invitrogen.com) or by contacting Technical Service (page 36).

Experimental Outline

Experimental Outline

Outlined below are steps to clone your gene of interest into the pcDNA™3.2/capTEV™/V5-DEST vectors to express and analyze your recombinant biotinylated proteins of interest.

Step	Action
1	Clone your gene of interest with and without a stop codon into a Gateway® entry vector to create two entry clones.
2	Generate two expression clones (N and C-terminally tagged) by performing LR recombination reactions between the appropriate entry clones and the pcDNA™3.2/capTEV™-NT/V5-DEST and pcDNA™3.2/capTEV™-CT/V5-DEST vectors.
3	Transfect each of the expression clones separately into the cell line of choice for expression of your biotinylated protein of interest.
4	Confirm *in vivo* biotinylation of recombinant protein by SDS-PAGE and detection on a Western blot using a streptavidin conjugate.
5	Confirm complex formation with biotinylated recombinant protein by native gel electrophoresis and detection on a Western blot using a streptavidin conjugate.
6	Purify the biotinylated protein and associated protein complexes using NativePure™ Affinity Purification Kit.
7	Analyze protein complexes using native electrophoresis, SDS-PAGE, immunodetection, or mass spectrometry.

Methods

Generating Entry Clones

Introduction

To recombine your gene of interest into both pcDNA™3.2/capTEV™-NT/V5-DEST and pcDNA™3.2/capTEV™-CT/V5-DEST vectors, you will generate two entry clones containing your gene of interest with and without a stop codon. This section provides guidelines for generating entry clones.

Choosing an Entry Vector

Many entry vectors are available from Invitrogen to facilitate generation of entry clones. We recommend pENTR™/D-TOPO® or pCR8/GW/TOPO® for rapid cloning of your gene of interest using TOPO® technology (see page vii for ordering information).

You may also perform a BP recombination reaction using a PCR product containing *att*B sites and an *att*P-containing pDONR™ vector to create your entry clone. A large selection of pDONR™ vectors is available from Invitrogen.

For more information, go to www.invitrogen.com or contact Technical Service (page 36). Refer to the manual for the specific vector you are using for detailed instructions to construct entry clones.

Note

If you are using an Ultimate™ ORF (open reading frame) clone from Invitrogen as the source of your gene of interest, you may do the following:

For N-terminal tagged protein:

Use Ultimate™ ORF clones directly as an entry vector for LR recombination with pcDNA™3.2/capTEV™-NT/V5-DEST to generate your entry clone.

For C-terminal tagged protein:

- Do **NOT** use Ultimate™ ORF clones directly as an entry clone for LR recombination with pcDNA™3.2/capTEV™-CT/V5-DEST to generate your entry clone due to the presence of the TAG **stop codon**.
- Use Ultimate™ ORF clone as a template to amplify the gene of interest using primers that modify the stop codon, and clone the template **without** the stop codon into the entry vector of choice (page 8).

For more information about the Ultimate™ ORF collection, go to www.invitrogen.com or contact Technical Service (page 36).

Generating Entry Clones, continued

N- and C-Terminal Expression Clones

Individual expression and *in vivo* biotinylation of the protein of interest in your mammalian cell line may vary depending on whether your protein of interest is fused to an N-terminal or C-terminal tag. We strongly recommend that you recombine your gene of interest into both pcDNA™3.2/capTEV™-V5-DEST vectors to create both N- and C- terminally tagged expression clones, and determine which clone gives optimal protein biotinylation and complex formation after transfection into mammalian cells.

Therefore, you will need to create two entry vectors containing your gene of interest with either a stop codon (N-terminal tagged, recombine with pcDNA™3.2/capTEV™-NT/V5-DEST) or a Kozak translation initiation sequence and no stop codon (C-terminal tagged, recombine with pcDNA™3.2/capTEV™-CT/V5-DEST). These required elements are summarized below.

To make an entry clone to recombine with…	Then your gene of interest must contain…
pcDNA™3.2/capTEV™-NT/V5-DEST	• **Stop codon**
pcDNA™3.2/capTEV™-CT/V5-DEST	• **Kozak consensus sequence** (see below) • **No stop codon**

Make sure that your gene of interest is in frame with the N- or C- terminal capTEV™ tag and other vector elements after performing the LR recombination reaction with the pcDNA™3.2/capTEV™-V5-DEST vectors. Refer to pages 11-12 to see the recombination regions of the vectors.

Kozak Consensus Sequence

When recombining into the pcDNA™3.2/capTEV™-CT/V5-DEST vector, the design for your entry clone must contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

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

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

Creating N- and C-Terminal Tagged Expression Clones

Introduction

After you have generated both entry clones, perform LR recombination reactions to transfer the gene of interest into the pcDNA™3.2/capTEV™ V5/DEST vectors to create your expression clones. To ensure that you obtain the best results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reactions** (pages 13-15) before beginning.

Resuspending the Vectors

The pcDNA™3.2/capTEV™ V5/DEST vectors are supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, resuspend the plasmid in 40 µl sterile water to a final concentration of 150 ng/µl.

Propagating the Vectors

If you wish to propagate and maintain the pcDNA™3.2/capTEV™ V5-DEST vectors, we recommend using One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* from Invitrogen for transformation (see page vii for ordering information). The *ccd*B Survival T1^R *E. coli* strain is resistant to ccdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vectors, select for transformants in media containing 50-100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to ccdB effects.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using each *att*L-containing entry clone and the appropriate *att*R-containing pcDNA™3.2/capTEV™ V5/DEST vector.
2. Transform the reaction mixtures into a suitable *E. coli* host.
3. Select for expression clones (refer to the next pages for diagrams of the recombination regions of the resulting expression clones).

Continued on next page

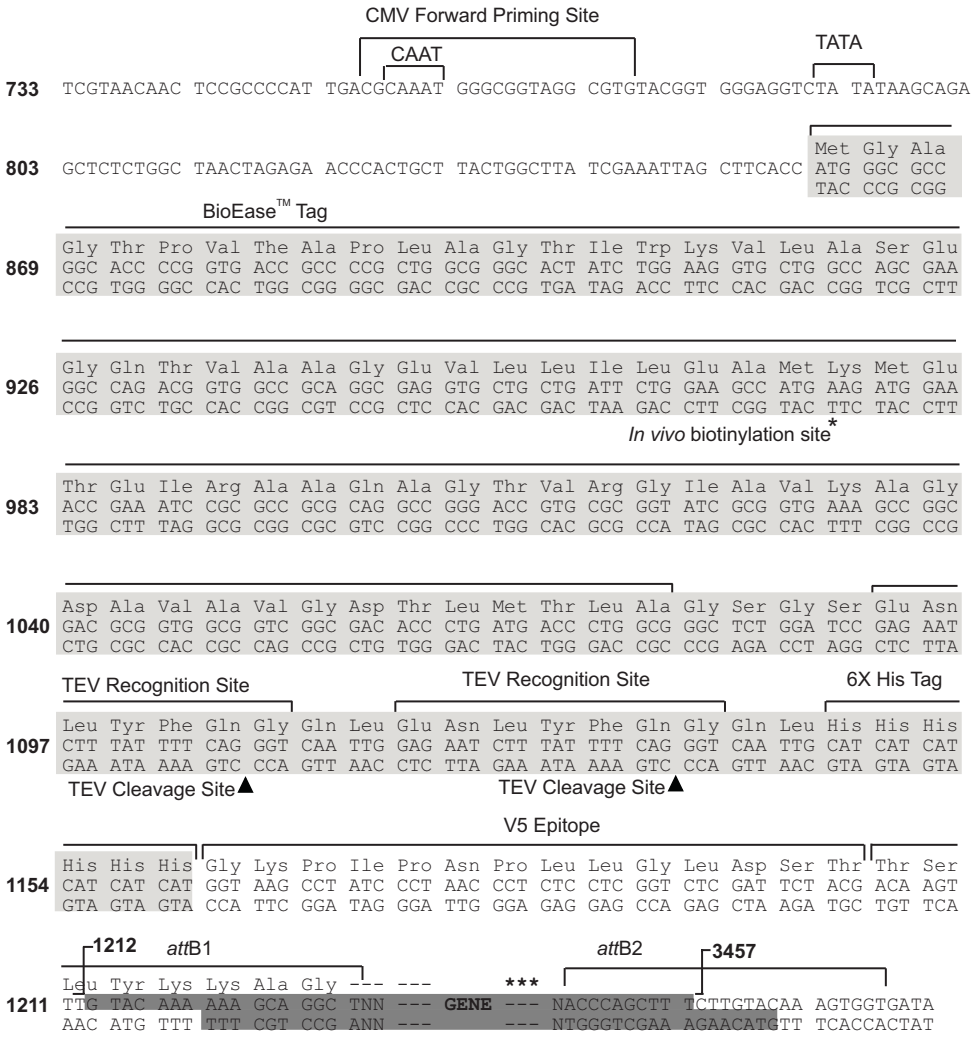
Creating N- and C-Terminal Tagged Expression Clones, continued

Recombination Region of pcDNA™3.2/ capTEV™-NT/V5-DEST

The recombination region of the expression clone resulting from pcDNA™3.2/capTEV™-NT/V5-DEST × entry clone is shown below.

Features of the Recombination Region:

- Light shaded regions correspond to the capTEV™ tag
- Dark shaded regions between the *att*B sites correspond to DNA sequences transferred from the entry clone into pcDNA™3.2/capTEV™-NT/V5-DEST by recombination. Non-shaded and light shaded regions are derived from the pcDNA™3.2/capTEV™-NT/V5-DEST vector.
- The overlined nucleotides flanking the shaded region correspond to bases 1212 and 3457 of the pcDNA™3.2/capTEV™-NT/V5-DEST vector sequence.



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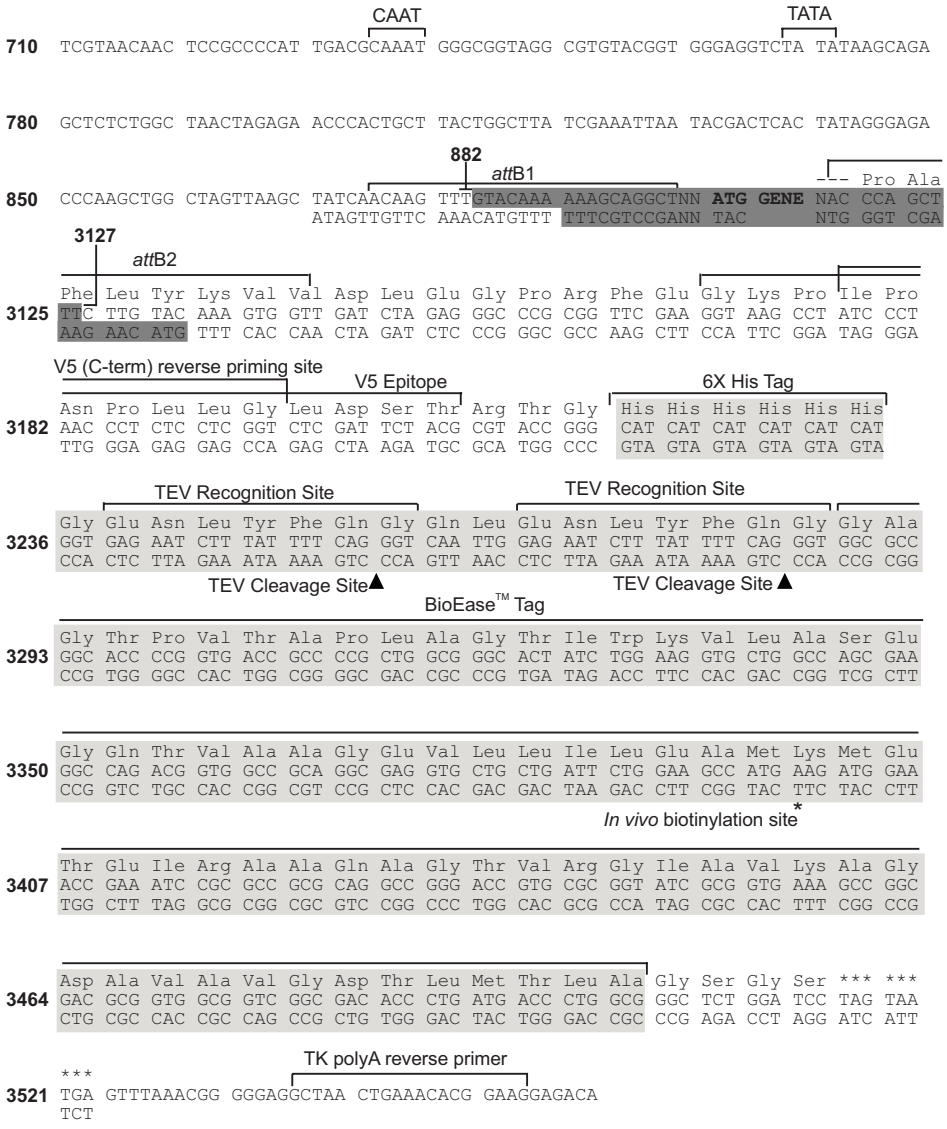
Creating N- and C-Terminal Tagged Expression Clones, continued

Recombination Region of pcDNA™3.2/ capTEV™-CT/V5-DEST

The recombination region of the expression clone resulting from pcDNA™3.2/capTEV™-CT/V5-DEST × entry clone is shown below.

Features of the Recombination Region:

- Light shaded regions correspond to the capTEV™ tag
- Dark shaded regions between the *att*B sites correspond to DNA sequences transferred from the entry clone into pcDNA™3.2/capTEV™-CT/V5-DEST by recombination. Non-shaded and light shaded regions are derived from the pcDNA™3.2/capTEV™-CT/V5-DEST vector.
- The overlined nucleotides flanking the shaded region correspond to bases 882 and 3127 of the pcDNA™3.2/capTEV™-CT/V5-DEST vector sequence.



Performing the LR Recombination Reactions

Introduction

Once you have obtained the entry clones containing your gene of interest, you may perform LR recombination reactions between the entry clones and pcDNA™3.2/capTEV™ V5-DEST vectors, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for expression clones. We recommend including a negative control (no LR Clonase™ II) in your experiment to help evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation (page vii). **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is available separately from Invitrogen (page vii) to catalyze the LR recombination reactions. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on the next page to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Materials Needed

You will need the following materials before beginning:

- Purified plasmid DNA of your entry clone with stop codon (50-150 ng/µl in TE, pH 8.0)
- Purified plasmid DNA of your entry clone without stop codon (50-150 ng/µl in TE, pH 8.0)
- pcDNA™3.2/capTEV™-NT/V5-DEST (150 ng/µl in TE, pH 8.0)
- pcDNA™3.2/capTEV™-CT/V5-DEST (150 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- pENTR™-gus (supplied with LR Clonase™ II enzyme mix; use as a control for the LR reaction; 50 ng/µl)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- Selective LB agar plates containing 100 µg/ml ampicillin

Continued on next page

Performing the LR Recombination Reactions continued

LR Reaction

Follow this procedure to perform both LR reactions between each of your entry clones and pcDNA™3.2/capTEV™-V5-DEST vectors. To include a negative control, set up a second sample reaction, but omit the LR Clonase™ II enzyme mix.

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

Component	NT-tag Entry Clone	CT-tag Entry Clone	Positive Control
Entry clone (without stop, 50-150 ng/rxn)	--	1-7 µl	--
Entry clone (with stop, 50-150 ng/rxn)	1-7 µl	--	--
pcDNA™3.2/capTEV™-NT/V5-DEST (150 ng/µl)	1 µl	--	1 µl
pcDNA™3.2/capTEV™-CT/V5-DEST (150 ng/µl)	--	1 µl	--
pENTR™-gus (50 ng/µl)	--	--	2 µl
TE Buffer, pH 8.0	to 8 µl	to 8 µl	5 µl

2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Briefly vortex the LR Clonase™ II enzyme mix twice (2 seconds each time).
4. To each sample above, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to -20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µl of the LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   Note: You may store the LR reaction at -20°C for up to 1 week before transformation, if desired.

Continued on next page

Performing the LR Recombination Reactions, continued

What You Should See

If you use *E. coli* cells with a transformation efficiency of ≥ 1 x 10^8 cfu/µg, the LR reaction should give > 5,000 colonies if the entire reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

Sequencing

To confirm that your gene of interest is in frame with the N- and C-terminal capTEV™ Tag, you may sequence your expression construct. We suggest using the following primer sequences. Refer to the diagrams on pages 11-12 for the location of the primer binding sites.

Vector	Primer	Sequence
pcDNA™3.2/capTEV™-NT/V5-DEST	CMV forward	5′-CGCAAATGGGCGGTAGGCGTG-3′
pcDNA™3.2/capTEV™-CT/V5-DEST	TK polyA	5′-CTTCCGTGTTTCAGTTAGC-3′
	V5 (C-term) reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, go to www.invitrogen.com or contact Technical Service (page 36).

Long-Term Storage

Once you have identified the correct clone, be sure to purify the DNA and make a glycerol stock for long-term storage. We also recommend that you store a stock of plasmid DNA at -20°C.

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

Transient Transfection of Cells

Introduction

After generating your expression clones, we recommend performing transient transfection of your cell line of choice followed by Western detection of biotinylated protein to determine which expression clone is optimal. This section provides general information for transiently transfecting your expression clones into the mammalian cell line of choice. We recommend that you include the positive control vector (pcDNA™3.2/capTEV™-NT-GW/ARPC2, see below) and a mock transfection (negative control) in your experiments to evaluate your results.

Plasmid Preparation

Once you have generated your expression vectors, isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free of contamination from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (page vii). Other methods of obtaining high quality plasmid DNA may be suitable.

Positive Control Plasmid

The NativePure™ pcDNA™ Gateway® Vector Kits contain a positive control plasmid (pcDNA™3.2/capTEV™-NT-GW/ARPC2) expressing the ARPC2 (actin related protein complex component p34, Robinson *et. al*. 2001) with an N-terminal capTEV™ tag.

The control vector is supplied lyophilized. Resuspend the vector in 10 µl TE or sterile water to a final concentration of 1 µg/µl. You can transfect mammalian cells with this stock or propagate and maintain the plasmid as described below:

1. Use 1 µl of the control vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, or equivalent.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage (see previous page for a protocol for preparing glycerol stocks).

Continued on next page

Transient Transfection, continued

Methods of Transfection

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

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1987; Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988).

For high-efficiency transfection in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine™ 2000 Reagent (Ciccarone *et al.*, 1999) available from Invitrogen. Using Lipofectamine™ 2000 (see page vii) to transfect plasmid DNA into eukaryotic cells offers the following advantages:

- Provides the highest transfection efficiency in many mammalian cell types.
- DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes, medium change, or medium addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity.

For more information on Lipofectamine™ 2000 Reagent, go to www.invitrogen.com or contact Technical Service (see page 36).

After transfecting your cells with the pcDNA™ 3.2/capTEV™ N- and C-terminal expression constructs, we strongly recommend that you confirm the biotinylation of your protein of interest and the ability of the tagged protein to form complexes prior to proceeding with purification or analysis experiments.

Transient vs. Stable Transfection

It is not necessary to create stable cell lines for purification or analysis experiments. However you may transiently transfect your cells, confirm biotinylation of your protein of interest and the ability of the tagged protein to form complexes (see next section), then transfect your mammalian cells with the optimal expression construct and select for stable transfectants using Geneticin® selective antibiotic. See the protocol in the Appendix (page 29).

Detecting Protein Biotinylation and Complex Formation

Introduction

After transfecting your cells with the pcDNA™ 3.2/capTEV™ N- and C-terminal expression constructs, we strongly recommend that you confirm the biotinylation of your protein of interest and the ability of the tagged protein to form complexes prior to proceeding with purification or analysis experiments.

This section includes instructions to verify biotinylation using SDS-PAGE and to verify complex formation using native electrophoresis followed by Western detection with streptavidin conjugate.

Experimental Outline

To detect protein biotinylation and complex formation, you will:

1. Prepare cell lysate using freeze-thaw cycles (no SDS buffers).
2. Analyze lysate by:
   - SDS-PAGE
   - Native gel electrophoresis using NativePAGE™ gels
3. Perform two Western blots, one using the SDS-PAGE gel and other using the NativePAGE™ gel.
4. Develop the blots with streptavidin conjugate using the WesternBreeze® Kits.

Important

- The cell lysate is prepared using mild conditions for lysis to enable analysis of protein complexes. The cell lysis protocol included in this section allows you to use the same lysate for analysis using native (non-denaturing) electrophoresis and denaturing SDS-PAGE.
- We recommend using freeze-thaw cycles for cell lysis to obtain intact protein complexes. Trypsin treatment or scraping the cells is not recommended as these methods cause cell damage and dissociation of protein complexes.
- If you have already performed trypsin treatment, inactivate trypsin using medium with 10% FBS. Wash cells three times with 1X PBS before lysing the cells.
- Perform cell lysis in the absence of NP40 as some protein complexes maybe unstable in the presence of NP40.
- During lysate preparation, avoid vortexing the lysate as it can dissociate the protein complexes.
- If your sample is in a SDS-PAGE sample buffer, prepare a fresh lysate **without** SDS using the protocol on page 21 for native electrophoresis. **Do not** use SDS-PAGE samples for native gel electrophoresis.

Continued on next page

Detecting Native Protein Expression and Biotinylation, continued

Streptavidin Conjugates

Use the strong interaction between biotin and streptavidin to easily detect your recombinant biotinylated protein with one of the following streptavidin conjugates:

Conjugate	Catalog Number
Streptavidin-AP	SA100-01
Streptavidin-HRP	SA100-03

Ready-to-use WesternBreeze® Chromogenic and Chemiluminescent Kits are available from Invitrogen to facilitate detection of streptavidin conjugates (see page vii for ordering information).

NativePAGE™ Gel Electrophoresis

The NativePAGE™ Novex® Bis-Tris Gel system is a near neutral pH, pre-cast polyacrylamide mini gel system to perform **native** (non-denaturing) electrophoresis. The near neutral pH 7.5 environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems including the traditional Tris-glycine native electrophoresis (Laemmli) system. The NativePAGE™ Novex® Bis-Tris Gel system provides a sensitive and high-resolution method for analysis of native membrane protein complexes, native soluble proteins, molecular mass estimations, and assessing the purity of native proteins.

A variety of NativePAGE™ gels and pre-made buffers for native electrophoresis are available from Invitrogen (page viii).

SDS-PAGE

You may use NuPAGE® Novex Bis-Tris Gel (page vii), Novex® Tris-Glycine Gel (page vii), or any other SDS/PAGE gel of choice for performing SDS/PAGE. Use an appropriate percentage of acrylamide gel that will best resolve your proteins of interest.

Note

The N-terminal fusion tag (including capTEV™ and V5 epitope tag) adds approximately 12.5 kDa to the size of your protein. The C-terminal fusion tag (including capTEV™ and V5 epitope tag) adds approximately 15.1 kDa to the size of your protein.

Continued on next page

Detecting Native Protein Expression and Biotinylation, continued

Materials Needed

You will need the following materials and equipment:

- Transiently transfected cells (*i.e.* 24 hours after transfection)
- 1X phosphate-buffered saline (PBS, see page vii)
- Complete protease inhibitor (Roche cat. no 1697498 or equivalent)
- Pepstatin (Roche cat. no 1359053 or equivalent)
- Deionized water
- NativePure™ 5X Lysis/Binding Buffer (supplied with BN3006 or see below for buffer composition)
- Protein quantification kit (such as Quant-iT™ Protein Assay Kit, page vii)
- Protein standards (page viii)
- *Optional:* Benzonase nuclease
- NativePAGE™ gels (page viii) or equivalent for native electrophoresis
- NuPAGE® Novex Bis-Tris Gels or Tris-Glycine gels (page vii) for SDS-PAGE
- Appropriate units for electrophoresis and blotting
- Streptavidin conjugate (page vii)
- WesternBreeze® Detection Kits (page vii) or equivalent

1X Lysis Buffer

For each experiment, you will have 4 transiently transfected cell samples: (N- and C-terminal, positive and negative controls). You will need to make ~2-4 ml of 1X Lysis Buffer per sample, depending on the volume of your samples (*i.e.* 30 ml flask, 10 cm dish, T-175 flask, see next section).

Prepare 1X Lysis Buffer to obtain the following 1X final concentration. For cat. no. BN3006, refer to the manual supplied with the NativePure™ Affinity Purification Kit for details.

100 mM Tris-HCl, pH 8.0

100 mM KCl

200 µM EDTA

1.5 mM $MgCl_2$

1X (700 ng/ml) Pepstatin (Roche cat. no 1359053 or equivalent)

Complete protease inhibitor (Roche cat. no 1697498 or equivalent)

Store the buffer on ice until use. You may aliquot the buffer and store the aliquots at -20ºC, if needed.

Continued on next page

Detecting Native Protein Expression and Biotinylation, continued

Preparing Cell Lysate Under Native Conditions

1. Harvest **suspension cells** by centrifugation. We generally use cells from a 30 ml flask. Wash the cells twice in phosphate buffered saline (PBS). Resuspend the cell pellet in 4 ml 1X Lysis Buffer (see previous page for a recipe). Proceed to Step 4.
2. Wash **adherent cells** with PBS. Remove the PBS and add 0.5-1 ml 1X Lysis Buffer/10 cm culture dish containing adherent cells. For a T-175 flask, use 2 ml 1X Lysis Buffer.
3. Harvest cells by pipetting up and down. Transfer the cells to a sterile tube.
4. Perform 3 freeze-thaw cycles to lyse the cells.
5. Centrifuge the lysate at 10,000 x g for 10 minutes at 4ºC.
6. Transfer the post-nuclear supernatant to a sterile tube. Aliquot the supernatant and perform protein estimation on an aliquot of the lysate using the Quant-iT™ Protein Kit (page vii) or Bradford protein assay. Store aliquots at -80ºC until use.

Electrophoresis and Blotting

Guidelines are provided to prepare samples for native electrophoresis and SDS-PAGE. For details, refer to the manuals supplied with the gels.

NativePAGE™ Electrophoresis

For samples with high DNA content such as tissue or cell samples, we recommend a benzonase (endonuclease) treatment to reduce protein streaking as follows:

To the sample from Step 6, add $MgCl_2$ to a final concentration of 2 mM and 1-2 units benzonase per µl of sample. Mix well and incubate at room temperature for 30-60 minutes. Centrifuge the lysate at 20,000 x g for 30 minutes at 4°C.

For NativePAGE™ electrophoresis, add NativePAGE™ Sample Buffer (4X) to obtain a final concentration of 1X in the sample. **Do not heat the samples.** Load the samples onto the NativePAGE™ Gel and load NativeMark™ Unstained Protein Standard (page vii). Perform electrophoresis using the conditions listed in the NativePAGE™ manual.

SDS-PAGE

To the sample from Step 6, above, add NuPAGE® LDS Sample Buffer (4X) or Tris-Glycine SDS Sample Buffer (2X) to obtain a final concentration of 1X in the sample.

Add reducing agent (DTT) to a final concentration of 50 mM. Heat the samples at 85°C for 2-5 minutes. Load the samples onto the SDS gel and load appropriate molecular weight standard (page vii). Perform electrophoresis using the conditions listed in the manual supplied with the gel.

Western Analysis

Perform Western blotting with nitrocellulose or PVDF membranes (page vii). After blocking, probe the blot with a suitable dilution of streptavidin-AP or -HRP conjugate and develop the blot using the WesternBreeze® Chromogenic or Chemiluminescent Kits.

Continued on next page

Detecting Native Protein Expression and Biotinylation, continued

What You Should See

After SDS-PAGE and Western blotting with streptavidin conjugate, the protein of interest should exhibit biotinylation of the protein. Note that you will see background bands due to endogenous biotinylated proteins. The level of biotinylation may vary between the N- and C-terminally tagged protein or may be similar. If you do not observe any biotinylation on your protein of interest, see T**roubleshooting**, page 27.

After native electrophoresis and Western blotting with streptavidin conjugate, the protein of interest should migrate as a complex on the native gel, indicating the ability to interact with endogenous binding partners. The ability for complex formation may vary between the N- and C-terminally tagged proteins or may be similar.

The next page shows results of a SDS-PAGE and native electrophoresis experiment and provides guidelines on interpreting your results.

The Next Step

Based on the level of biotinylation for your protein and complex formation, choose the appropriate N-or C-terminal construct for further purification or analysis experiments.

You should select the construct that provides better biotinylation signal and demonstrates complex formation.

Expected Results

Introduction

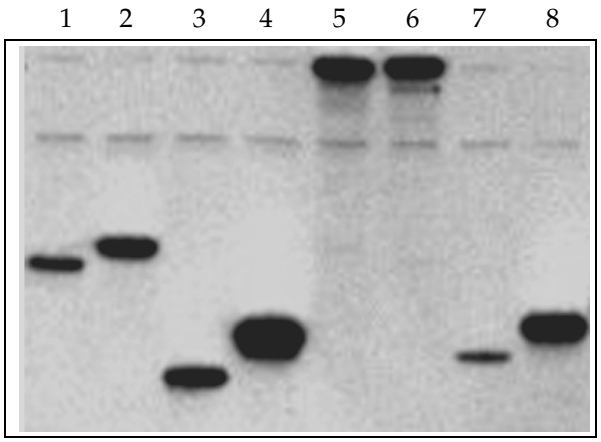
Examples of results obtained by SDS-PAGE and native gel electrophoresis followed by Western blot detection to confirm biotinylation and native complex formation of a number of N- and C-terminally tagged proteins of interest are shown in this section.

Protein Biotinylation

N- and C- terminal (NT and CT) tagged expression clones for the following genes were constructed as described in this manual: actin related protein complex component p34 (ARPC2), Golgi associated protein (Bet-3), β-galactosidase (LacZ), and human proteosome subunit beta-2 (PSMB2). Freestyle™293 cells were transiently transfected using Lipofectamine™ 2000.

At 24 hours post transfection, cells were harvested and lysed using the protocol on page 21. Ten micrograms of post-nuclear supernatant was loaded per well on a 4-12% NuPAGE® Novex® Bis-Tris gel and electrophoresed.

Proteins were transferred to a nitrocellulose membrane (0.45 µm) and subjected to Western detection using streptavidin-alkaline phosphatse conjugate (1:4000) and the WesternBreeze® Chemiluminescent Kit.



Lane 1: ARPC2 NT, 47.4 kDa
Lane 2: ARPC2 CT, 49.4 kDa
Lane 3: Bet-3 NT, 32.8 kDa
Lane 4: Bet-3 CT, 35.5 kDa
Lane 5: LacZ NT, 129 kDa
Lane 6: LacZ CT, 131.6 kDa
Lane 7: PSMB2 NT, 35.3 kDa
Lane 8: PSMB2 CT, 37.9 kDa

MultiMark™ multicolored protein standard (not shown, see page vii) was used to determine the molecular weights of the N- and C-terminally tagged proteins. The faint band detected in all lanes is endogenous biotinylation from the lysate. The presence of multiple bands in lane 5 represents slight protein degradation.

Results

These results show that the proteins of interest are biotinylated. For some proteins, there is a difference in the level of biotinylation in the N- and C-terminally tagged versions (*e.g.* lane 1 vs. lane 2, lane 3 vs. lane 4, and lane 7 vs. lane 8). However, for LacZ (lane 5 vs. lane 6), both N- and C- terminally tagged constructs have similar levels of biotinylation.

Continued on next page

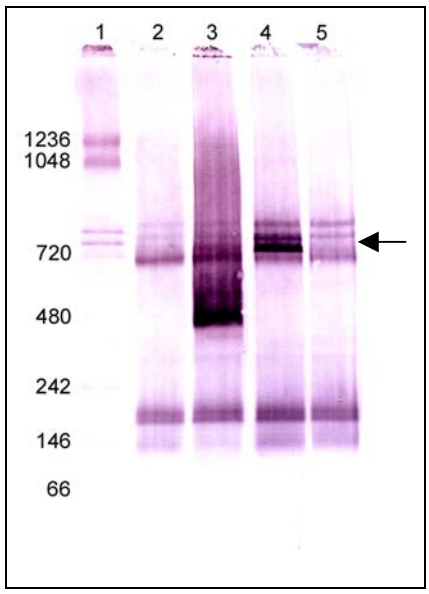
Expected Results, continued

Native Complex Formation

N- and C- terminal (NT and CT) tagged expression clones for the following genes were constructed as described in this manual: actin related protein complex component p34 (ARPC2), human proteosome subunit beta-2 (PSMB2). GripTite™293 cells were transiently transfected using Lipofectamine™ 2000.

At 24 hours post transfection, cells were harvested and lysed using the native protocol on page 21. Ten micrograms of post- nuclear supernatant was loaded per well on a 3-12% NativePAGE® Novex Bis-Tris gel and electrophoresed.

Proteins were transferred to Invitrolon™ PVDF membrane and subjected to Western detection using streptavidin-alkaline phosphatse conjugate (1:4000) and the WesternBreeze® Chromogenic Kit. Apparent molecular weights (kDa) are listed on the left, which correspond to NativeMark™ Unstained Protein Standard.



Lane 1: 5 µl of 1:20 diluted NativeMark™ Unstained Protein Standard

Lane 2: Untransfected cell lysate (negative control)

Lane 3: APRC2 CT

Lane 4: PSMB CT tagged

Lane 5: PSMB NT tagged

Results

These results show that in the case of the human proteasome subunit beta-2 protein (PSMB), the C-terminally tagged protein forms a complex, while the N-terminally tagged protein does not. The 20S proteosome complex (shown by arrow, above) is detected only in lane 4 and not in lane 5 of the streptavidin Western blot. These data also show the size of the protein complex formed. Background bands detected in all lanes may be protein present in the cell lysate with endogenous phosphatase activity, endogenous biotinylation, or nonspecific binding.

Native Protein Analysis

Introduction

You may analyze protein complexes as follows:

- Analyze the protein complexes directly from cell lysates using SDS-PAGE or native gel electrophoresis and Western immunodetection
- Purify protein complexes from cell lysates under native conditions using the NativePure™ Affinity Purification kit (supplied with cat. no. BN3006) and analyze purified protein complexes by SDS-PAGE, native gel electrophoresis or mass spectrometry.

NativePure™ Affinity Purification Kit

The NativePure™ Affinity Purification Kit is included with catalog no. BN3006 and is also available separately from Invitrogen (see page vii for ordering information). The NativePure™ Affinity Purification system is based on the selective binding of Streptavidin Agarose to biotinylated proteins and protein complexes. The lysate is prepared from mammalian cells in Lysis Buffer using a freeze-thaw method. The lysate is centrifuged and NP-40 is added to the post-nuclear supernatant for subsequent protein binding. The biotinylated proteins and protein complexes bind to the Streptavidin Agarose column and impurities are removed by thorough washing with buffers. The bound biotinylated proteins and protein complexes are released from the column by AcTEV™ Protease treatment. The AcTEV™ Protease is removed from the eluted fractions and protein complexes are concentrated using NativePure™ Concentrators. The resulting purified protein complexes are analyzed by native electrophoresis, Western analysis, or mass spectrometry.

For more information about the NativePure™ Affinity Purification Kit, refer to the manual, also supplied with cat. no. BN3006. Manuals are also available from www.invitrogen.com or by contacting Technical Service (page 36).

Important

For affinity purification of native protein complexes expressed using the NativePure™ Gateway® Vector Kits, use the Streptavidin Agarose included in the NativePure™ Affinity Purification Kit, or Streptavidin Agarose (cat. no S-951, page vii). Use of other commercially available streptavidin agarose may not provide optimal binding and recovery of protein complexes.

NativePAGE™ Gels

The NativePAGE™ Novex Bis-Tris Gel system is a near neutral pH, pre-cast polyacrylamide mini gel system to perform native (non-denaturing) electrophoresis. The NativePAGE™ Novex Bis-Tris Gel system provides a sensitive and high-resolution method for analysis of native membrane protein complexes, native soluble proteins, molecular mass estimations, and determining the purityof native proteins.

For more information about the NativePAGE™ Gels see page vii or go to www.invitrogen.com.

Troubleshooting

Introduction

The following sections list potential problems and solutions for problems you may encounter in your experiments. For more information to troubleshoot affinity purification, refer to the NativePure™ Affinity Purification manual (supplied with cat. no BN3006 or available at www.invitrogen.com or by contacting Technical Service).

LR Recombination Reaction

The table below lists some potential problems and solutions for troubleshooting your LR recombination reactions. For more information on troubleshooting within the Gateway® system, refer to the Gateway® Technology with Clonase™ II manual, which is available from www.invitrogen.com or by contacting Technical Service.

Problem	Possible Cause	Solution
Few or no colonies obtained from sample reaction	Incorrect antibiotic used to select for transformants	Use ampicillin (100 µg/ml) to select for clones.
	Recombinant reactions were not treated with Proteinase K	Treat reactions with Proteinase K before transformation.
High background in the absence of entry clone	LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use an *E. coli* strain that does not contain the F′ episome for transformation.
	Deletion of the *ccd*B gene from the destination vector	• To maintain the integrity of the vector, propagate in media containing ampicillin and 15-30 µg chloramphenicol. • Prepare plasmid DNA from one or more colonies and verify the integrity of the vector before use.
	Contamination of solution(s) with another plasmid with amp resistance, or by bacteria carrying a resistance plasmid	• Test for plasmid contamination by transforming *E. coli* with aliquots of each of the separate solutions used in the LR reaction. • Test for bacterial contamination by plating an aliquot of each solution directly onto LB amp plates.

Continued on next page

Troubleshooting, continued

Protein Expression

The table below lists some potential problems and solutions for troubleshooting protein expression from your pcDNA™3.2/capTEV™ expression construct.

Problem	Possible Cause	Solution
Recombinant protein not expressed from either pcDNA™3.2/capTEV™ expression clone	Poor transfection efficiency	• Make sure your cells are healthy prior to transfection. • Optimize transfection conditions for the method you are using. • Use Lipofectamine™ 2000 for transfecting your cells (see page 17).
	Incorrect detection method	Use streptavidin conjugated to alkaline phosphatase or horseradish peroxidase (page vii) followed by Western detection method of choice.
	Gene of interest not in frame with capTEV™ Tag	Make sure that the gene of interest is in frame with the capTEV™ Tag as shown on pages 11-12.
Recombinant protein not expressed from pcDNA™3.2/capTEV™-CT expression clone	No Kozak consensus sequence added to C-terminal fusion	Make sure a Kozak consensus sequence is present in your entry clone design (see page 9).
	Stop codon inserted	Make sure no stop codon is at the end of your gene (see page 9).
	Gene of interest not in frame with capTEV™ Tag	Make sure that the gene of interest is in frame with the capTEV™ Tag as shown on pages 11-12.

Continued on next page

Troubleshooting, continued

Protein Biotinylation and Complex Formation

The table below lists some potential problems and solutions for troubleshooting biotinylation and complex formation from your pcDNA™3.2/capTEV™ expression construct.

Problem	Possible Cause	Solution
No biotinylation of recombinant protein observed	Incorrect detection method	Use streptavidin conjugated to alkaline phosphatase or horseradish peroxidase followed by Western detection as described (page 19).
	Protein not expressed	Use antibodies to the V5 epitope (page vii) followed by western detection to ensure that protein is expressed.
	Gene of interest not in frame with capTEV™ Tag	Make sure that the gene of interest is in frame with the capTEV™ Tag as shown on pages 11-12.
Recombinant protein recovered but not as a complex	N- or C-terminal tag interfering with complex formation	Test both N- and C-terminal tagged constructs to determine optimal complex formation (page 18).
	Complexes dissociated during lysate preparation	To avoid dissociation of protein complexes: • Perform cell lysis using freeze-thaw cycles. Avoid trypsinizing the cells or scraping the cells. • Perform cell lysis in the absence of NP40 as some protein complexes maybe unstable in the presence of NP40. • Avoid vortexing the lysate during lysate preparation. **Perform all purification steps at 4°C and use chilled buffers.**
	Complexes unable to form in mammalian cell line of choice	Optimize using another mammalian cell line
Protein complexes not observed	Protein degraded	• Perform all purification steps at 4°C. • Check to make sure that the BioEase™-tag is not cleaved during processing or purification. • Include protease inhibitors during cell lysis.

Appendix

Creating Stable Cell Lines

Introduction

The pcDNA™3.2/capTEV™/V5-DEST vectors contain the neomycin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your expression construct into the mammalian cell line of choice and select for stable transfectants using Geneticin®. General information and guidelines are provided below.

Important

It is not necessary to create stable cell lines for purification or analysis of complexes. If you wish to create stable cell lines, make sure to assay clones for expression of biotinylated protein and complex formation (page 18), and expand those clones.

Linearizing the Plasmid

To obtain stable transfectants, we recommend that you linearize your pcDNA™ 3.2/capTEV™-N or CT expression construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. Cut at a unique site that is not located within a critical element or within your gene of interest. Restriction site information for plasmid vectors is available at www.invitrogen.com.

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern and Berg, 1982).

Determining Geneticin® Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Geneticin® required to kill your untransfected host cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 6-7 plates. Add the following concentrations of antibiotic to each plate: 0, 50, 125, 250, 500, 750, and 1000 µg/ml Geneticin®.
2. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents growth within 1-3 weeks after addition of the antibiotic.

Continued on next page

Creating Stable Cell Lines, continued

Geneticin® Selection Guidelines

Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line expressing your construct. Geneticin® is available separately from Invitrogen (see page vii). Use as follows:

1. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
2. Use the predetermined concentration of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 3 weeks of growth in selective medium.

Generating Stable Cell Lines

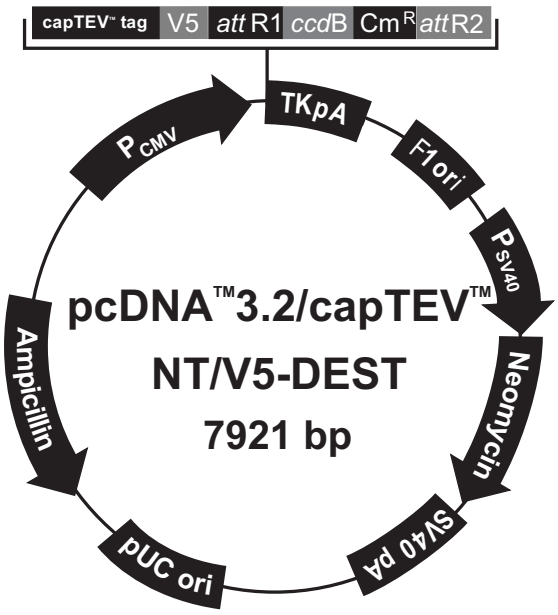
Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line expressing your expression construct.

1. Transfect the mammalian cell line of interest with the pcDNA™ 3.2/capTEV™expression construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium without Geneticin®.
3. 48 hours after transfection, split the cells into fresh growth medium without Geneticin® such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2-3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Geneticin® at the predetermined concentration required for your cell line.
6. Feed the cells with selective media every 3-4 days until Geneticin®-resistant colonies can be identified.
7. Pick at least 3-4 Geneticin®-resistant colonies and expand them in culture
8. Analyze clones as described on page 18 for biotinylation of recombinant protein and complex formation. Select clones that exhibit biotinylation and complex formation for further experiments.

Map and Features of pcDNA™3.2/capTEV™-NT/V5-DEST

Map

The map below shows the elements of the pcDNA™3.2/capTEV™-NT/V5-DEST vector (7921 bp). DNA from the entry clone replaces the region between bases 1212 and 3457 after performing the LR reaction. **The complete sequence of this vector is available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).**



Comments for pcDNA™3.2/capTEV™-NT/V5-DEST
7921 nucleotides

CMV promoter: bases 219-806
capTEV™ tag: bases 863-1162
BioEase™ tag: bases 863-1078
TEV cleavage: bases 1091-1111
TEV cleavage: bases 1118-1138
6XHis tag: bases 1145-1162
V5 epitope: bases 1163-1204
*att*R1: bases 1205-1329
*ccd*B gene: bases 1758-2063
Chloramphenicol resistance gene (CmR): bases 2408-3066
*att*R2: bases 3347-3471
TK polyA: bases 3479-3750
f1 origin: bases 3786-4214
SV40 early promoter: bases 4241-4549
Neomycin resistance gene: bases 4624-5418
SV40 polyA: bases 5594-5724
pUC origin: bases 6107-6780 (c)
Ampicillin (*bla*) resistance gene: bases 6925-7785 (c)
bla promoter: bases 7780-7884 (c)

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™3.2/capTEV™-NT/V5-DEST

Features

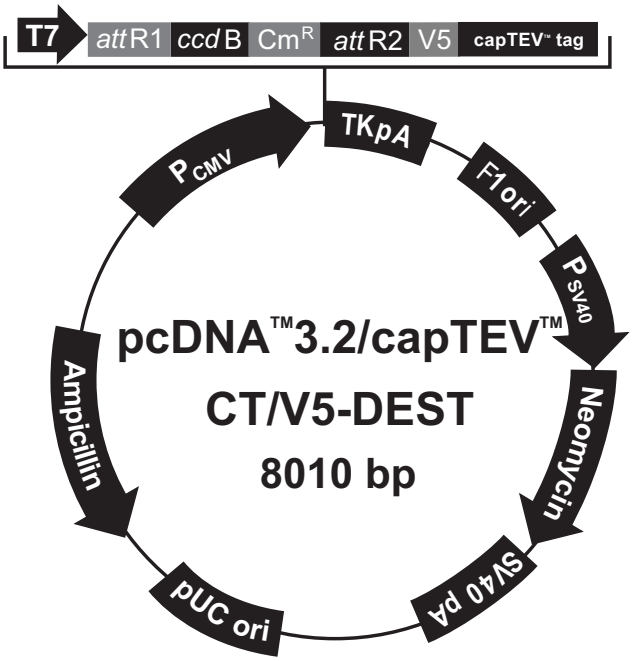
The pcDNA™3.2/capTEV™-NT/V5-DEST vector contains the following elements. Features have been functionally tested and the vector has been fully sequenced.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
CMV forward primer binding site	Allows sequencing of the insert
capTEV™ Tag: BioEase™ tag TEV cleavage sites 6XHis tag	Allows *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes
*att*R1 and *att*R2 sites	Allows recombinatorial cloning of the gene of interest from an entry clone
*ccd*B gene	Allows negative selection of plasmid
Chloramphenicol resistance gene	Allows counterscreening of plasmid
V5 epitope	Allows detection of recombinant fusion protein with Anti-V5 antibodies (Southern *et al.*, 1991)
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map and Features of pcDNA™3.2/capTEV™-CT/V5-DEST

Map

The map below shows the elements of the pcDNA™3.2/capTEV™-CT/V5-DEST vector (8010 bp). DNA from the entry clone replaces the region between bases 882 and 3127 after performing the LR reaction **The complete sequence of this vector is available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).**



Comments for pcDNA™3.2/capTEV™-CT/V5-DEST
8010 nucleotides

CMV promoter: bases 199-786
T7 promoter/priming site: bases 827-846
*att*R1: bases 875-999
*ccd*B gene: bases 1428-1733 (c)
Chloramphenicol resistance gene (Cm^R): bases 2078-2736 (c)
*att*R2: bases 3017-3141
V5 epitope: bases 3167-3208
capTEV™ tag: bases 3218-3502
- 6XHis tag: bases 3218-3235
- TEV cleavage: bases 3239-3259
- TEV cleavage: bases 3266-3286
- BioEase™ tag: bases 3287-3502

TK polyA: bases 3542-3813
f1 origin: bases 3839-4267
SV40 early promoter: bases 4294-4602
Neomycin resistance gene: bases 4677-5471
SV40 polyA: bases 5647-5777
pUC origin: bases 6160-6833 (c)
Ampicillin (*bla*) resistance gene: bases 6978-7838 (c)
bla promoter: bases 7833-7937 (c)

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™3.2/capTEV™-CT/V5-DEST

Features

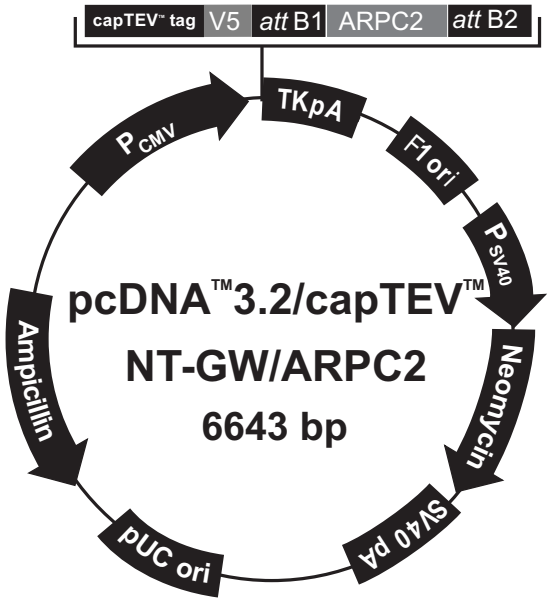
The pcDNA™3.2/capTEV™-CT/V5-DEST vector contains the following elements. Features have been functionally tested and the vector has been fully sequenced.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
*att*R1 and *att*R2 sites	Allows recombinatorial cloning of the gene of interest from an entry clone
*ccd*B gene	Allows negative selection of plasmid
Chloramphenicol resistance gene	Allows counterscreening of plasmid
V5 epitope	Allows detection of recombinant fusion protein with Anti-V5 antibodies (Southern *et al.*, 1991)
capTEV™ Tag: BioEase™ tag TEV cleavage sites 6XHis tag	Allows *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes
V5 (C-term) reverse primer binding site	Allows sequencing of the insert
TK polyA reverse primer binding site	Allows sequencing of the insert
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map of pcDNA™3.2/capTEV™-NT-GW/ARPC2

Map

The map below shows the elements of the pcDNA™3.2/capTEV™-NT-GW/ARPC2 vector (6643 bp). The plasmid was generated by performing an LR recombination reaction between an entry vector containing the *ARPC2* gene and the pcDNA™3.2/capTEV™-NT/V5-DEST vector. **The complete sequence of this vector is available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).**



Comments for pcDNA™3.2/capTEV™-NT-V5GW/ARPC2
6643 nucleotides

CMV promoter: bases 219-806
capTEV™ tag: bases 863-1162
- BioEase™ tag: bases 863-1078
- TEV cleavage: bases 1091-1111
- TEV cleavage: bases 1118-1138
- 6XHis tag: bases 1145-1162

V5 epitope: bases 1163-1204
*att*B1: bases 1205-1229
ARPC2 gene: bases 1250-2152
*att*B2: bases 2169-2193
TK polyA: bases 2201-2472
f1 origin: bases 2508-2936
SV40 early promoter: bases 2963-3271
Neomycin resistance gene: bases 3346-4140
SV40 polyA: bases 4316-4446
pUC origin: bases 4829-5502 (c)
Ampicillin (*bla*) resistance gene: bases 5647-6507 (c)
bla promoter: bases 6502-6606(c)

(c) = complementary strand

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

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Introduction

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy** on page 40.

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

Product Qualification

Vectors

The pcDNA™3.2/capTEV™ Gateway® destination vectors as well as the corresponding control plasmid are qualified by restriction endonuclease digestion. pcDNA™3.2/capTEV™-NT/V5-DEST and pcDNA™3.2/capTEV™-CT/V5-DEST are further qualified in a recombination assay using Gateway® LR Clonase™ II enzyme mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

NativePure™ Affinity Purification Reagents

For details on the qualification of NativePure™ Affinity Purification reagents, refer to the NativePure™ Affinity Purification Kit manual.

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