invitrogen™

NativePure™ Affinity Purification Kit

For affinity purification of biotinylated proteins and protein complexes

Catalog nos. BN3003, BN3006

Version A
23 January 2006
25-0895

User Manual

Table of Contents

Kit Contents and Storage

Types of Products

This manual is supplied with the following products:

Product	Catalog No.
NativePure™ Affinity Purification Kit	BN3003
NativePure™ Mammalian Affinity Purification Kit	BN3006

Shipping and Storage

The NativePure™ Affinity Purification Kit includes the NativePure™ Binding and Purification Module (Box 1) and NativePure™ AcTEV™ Protease Module (Box 2). The shipping and storage conditions are listed below: For contents, see next page.

Sufficient reagents are included in the kit for 10 purifications.

Module	Shipping	Storage
NativePure™ Binding and Purification Module	Blue ice	4ºC
NativePure™ AcTEV™ Protease Module	Dry ice	-20ºC

Product Qualification

The NativePure™ 5X Lysis/Binding Buffer and NativePure™ 10X TEV Cleavage Buffer are qualified by pH and conductivity measurements. The DTT and NP40 solutions are qualified by conductivity measurements. The reagents must meet the set specifications.

The Streptavidin Agarose is qualified by performing a biotin binding capacity assay and must meet the set specifications.

The AcTEV™ Protease must demonstrate functional absence of any non-specific protease activity in a quality control assay.

Continued on next page

Kit Contents and Storage, Continued

NativePure™ Binding and Purification Module

The contents of the NativePure™ Binding and Purification Module are listed in the table below.

Store the module at 4°C. Do not freeze the Streptavidin Agarose. You may store the NativePure™ Columns and Concentrators at room temperature.

Reagent	Composition	Quantity
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
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
10% NP40 (Nonidet P40)	10% NP40 in deionized water	8 ml
NativePure™ Columns	Polypropylene columns	10 columns
NativePure™ Concentrators	See page 6 for specifications	10 concentrators

NativePure™ AcTEV™ Protease Module

The contents of the NativePure™ AcTEV™ Protease Module are listed in the table below.

Store the module at -20°C. For long-term storage, store the AcTEV™ Protease at -80°C.

Reagent	Composition	Quantity
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	4 x 100 µl
DTT (Dithiothreitol)	100 mM DTT in deionized water	500 µl

Accessory Products

Additional Products

Additional products for use with the NativePure™ Affinity Purification Kit are available separately from Invitrogen. Ordering information is included in the table below. For details on the product, visit our web site at www.invitrogen.com or contact Technical Service (page 22).

Product	Quantity	Catalog No.
NativePure™ pcDNA™ Gateway® Vector Kit	1 kit	BN3002
AcTEV™ Protease	100 µl (1,000 units) 1 ml (10,000 units)	12575-015 12575-023
Streptavidin Agarose (sedimented bead suspension)	5 ml	S-951
NativePAGE™ Running Buffer (20X)	1 L	BN2001
NativePAGE™ Sample Buffer (4X)	10 ml	BN2003
NativePAGE™ 5% G-250 Sample Additive	0.5 ml	BN2004
NativePAGE™ Cathode Buffer Additive (20X)	250 ml	BN2002
NativeMark™ Unstained Protein Standard	5 x 50 µl	LC0725
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® Transfer Buffer (20X)	1 L	NP0006-1
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
WesternBreeze® Chromogenic Kit, Anti-Mouse	1 kit	WB7103
WesternBreeze® Chromogenic Kit, Anti-Rabbit	1 kit	WB7105
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	1 kit	WB7104
WesternBreeze® Chemiluminescent Kit, Anti-Rabbit	1 kit	WB7106
Phosphate-Buffered Saline (PBS), 1X	500 ml	10010-023
Quant-iT™ Protein Assay Kit	1000 assays	Q-33210

Continued on next page

Accessory Products, Continued

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 our web site at www.invitrogen.com or contact Technical Service (page 22).

Introduction

Overview

Introduction

The NativePure™ Affinity Purification Kit is suitable for purification of biotinylated proteins and protein complexes from mammalian cells. The kit includes Streptavidin Agarose which utilizes the high affinity and selectivity of streptavidin-biotin interaction to allow affinity chromatography of biotinylated proteins (Bayer & Wilchek, 1990).

The NativePure™ Affinity Purification Kit is a complete system that includes purification buffers and resin for purifying biotinylated proteins and protein complexes containing a TEV cleavage site under native conditions. The resulting purified proteins are ready for use in many downstream applications such as SDS-PAGE, Western analysis, native electrophoresis, and mass spectrometry.

The NativePure™ Affinity Purification Kit is designed for purifying biotinylated proteins and protein complexes expressed from mammalian cells using the NativePure™ Gateway® Vector Kits (see page 3 for details).

System Components

The NativePure™ Affinity Purification Kit contains the following components: For details on each component, see page 4.

- Streptavidin Agarose
- NativePure™ Columns
- Pre-made, Ready-to-dilute Buffers
- AcTEV™ Protease
- NativePure™ Concentrators

Continued on next page

Overview, Continued

Streptavidin-Biotin Interaction

Streptavidin is an avidin analog isolated from culture filtrates of *Streptomyces avidinii* (Chaiet & Wolf, 1964) that binds biotin with extremely high affinity, but exhibits little of avidin's non-specific binding at physiological pH. This is presumed to be due to streptavidin's:

- Low isoelectric point
- Lack of glycosylation

Streptavidin can be covalently bound to a solid phase support (*i.e.* agarose beads) with no effect on its biotin-binding activity or low non-specific binding characteristics (Haeuptle *et al.*, 1983; Updyke & Nicolson, 1984).

Important

We have found that the streptavidin-biotin complex is essentially covalent and **is not dissociable** even under the stringent elution conditions recommended in the literature (Heney & Orr, 1981). While this tight association may be useful for some applications (*e.g.* immunoprecipitation), it can restrict the use of the streptavidin-agarose for other affinity purification applications.

To allow elution of the biotinylated proteins from the Streptavidin Agarose under mild conditions, we recommend cleaving the biotin tag using proteases. The NativePure™ Affinity Purification Kit includes AcTEV™ Protease, a highly site-specific, active, protease that cleaves the biotin affinity tag from fusion proteins expressed using the NativePure™ Gateway® Vector Kits.

Continued on next page

Overview, Continued

System Overview

The NativePure™ Affinity Purification Kit 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 the freeze-thaw method. The lysate is centrifuged and NP40 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 containing a TEV cleavage site are released from the column by AcTEV™ Protease treatment. Endogenous biotinylated proteins remain bound to the column. The AcTEV™ Protease is removed from the eluted fractions and protein complexes are concentrated using NativePure™ Concentrators. The protein complexes are analyzed by native electrophoresis, Western detection, or mass spectrometry.

Biotinylating Proteins

To biotinylate your protein of interest, express your protein of interest using the NativePure™ Gateway® Vector Kits (page vii) for mammalian expression using the CMV promoter. These vectors allow fusion of your protein of interest to a BioEase™ tag, which facilitates *in vivo* biotinylation of your recombinant fusion protein. The vectors also include two tandem AcTEV™ Protease recognition sites to release the bound biotinylated proteins and protein complexes from Streptavidin Agarose during purification. For details on cloning and expression of your protein of interest using these Gateway® vectors, refer to the manuals supplied with the kit.

Note: Using other vector systems that allow *in vivo* biotinylation of your protein of interest such as the BioEase™ Gateway® vectors from Invitrogen will not allow release of the bound protein from Streptavidin Agarose due to the absence of a TEV cleavage site in these vectors.

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. Use of other commercially available streptavidin agarose may not provide optimal binding and recovery of protein complexes.

Description of Components

Introduction

The NativePure™ Affinity Purification Kit contains the following components:

- Streptavidin Agarose
- NativePure™ Columns
- Pre-made, Ready-to-dilute Buffers
- AcTEV™ Protease
- NativePure™ Concentrators

Details on each component are described in this section.

Streptavidin Agarose

The Streptavidin Agarose is a streptavidin-conjugate of 4% beaded crosslinked agarose. The Streptavidin Agarose is suitable for use in affinity chromatography to isolate and purify biotinylated molecules (Haeuptle *et al.*, 1983).

Binding Capacity

The binding capacity of Streptavidin Agarose is 15-20 µg (18-24 nmoles) of fluorescein biotin (MW 831) per ml of sedimented gel.

NativePure™ Columns

The NativePure™ Columns are used to prepare purification columns with Streptavidin Agarose for purifying biotinylated protein complexes. The NativePure™ Columns hold up to 2 ml of chromatography resin and contain a 10 ml reservoir for sample or buffer.

The column specifications are listed below:

Pore size of purification columns:	30–35 microns
Recommended flow rate:	0.5 ml/min
Maximum flow rate:	2 ml/min
Maximum linear flow rate:	700 cm/h
Column material:	Polypropylene
pH stability (long term):	pH 3–13
pH stability (short term):	pH 2–14

Continued on next page

Description of Components, Continued

Pre-made Buffers

The NativePure™ Affinity Purification Kit includes qualified reagents used in the binding, washing, AcTEV™ cleavage, and elution steps during the protein purification procedure. The pre-made, ready-to-dilute buffers provide consistent results and eliminate the time required to prepare reagents.

AcTEV™ Protease

AcTEV™ Protease is an enhanced form of Tobacco Etch Virus (TEV) protease that is highly site-specific, active, and more stable than native TEV protease (Nayak *et al.*, 2003). AcTEV™ Protease recognizes the seven-amino-acid sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly (Carrington & Dougherty, 1988; Dougherty *et al.*, 1988; Dougherty & Parks, 1989; Dougherty *et al.*, 1989) and cleaves between Gln and Gly with high specificity. The protease is used to cleave affinity tags from fusion proteins. The optimal temperature for cleavage is 30°C; however, the enzyme is active over wide ranges of temperature (4ºC to 30ºC) and pH (pH 6.0-8.5). Following digestion, AcTEV™ Protease is easily removed from the cleavage reaction by affinity chromatography using the polyhistidine tag at the N-terminus of the protease or size exclusion. AcTEV™ Protease is purified from *E. coli* by affinity chromatography using the polyhistidine tag. Using the AcTEV™ cleavage protocol in this manual, results in >95% cleavage efficiency.

Continued on next page

Description of Components, Continued

NativePure™ Concentrators

The NativePure™ Concentrators are disposable, ultrafiltration units used for concentrating protein samples. Each unit consists of a concentrator fitted with a vertical membrane that allows ultrafiltration of molecules based on size. The concentrator is assembled in a filtration chamber and the assembly is then placed in a centrifuge that provides high speed sample concentration.

The concentrator specifications are listed below:

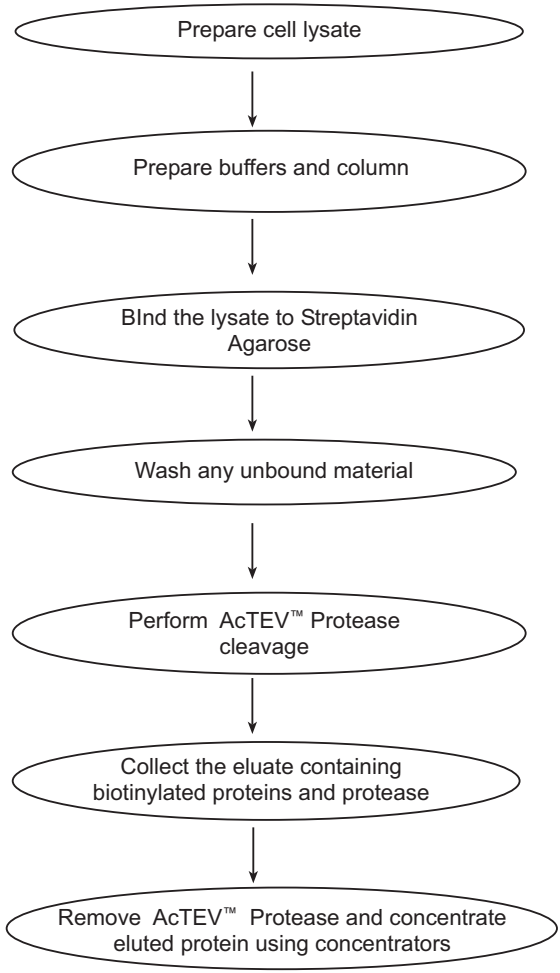
Molecular weight cutoff:	50 kDa
Maximum initial sample volume:	4 ml
Membrane material:	Polyethersulfone
Active membrane area:	2 cm^2
Hold-up volume:	<10 µl
Dead stop volume:	20 µl
Concentrate recovery:	>95%
Concentrator dimensions:	122 mm (l) x 12 mm (w)
Concentrator material:	Polycarbonate, polypropylene

The NativePure™ Concentrators are compatible with swing bucket or fixed angle centrifuge rotors capable of holding 15 ml conical centrifuge tubes.

Experimental Overview

Experimental Outline

The experimental outline for purifying biotinylated proteins and protein complexes using the NativePure™ Affinity Purification Kit is shown below.



Methods

Preparing Samples and Buffers

Introduction

Instructions are included in this section to prepare buffers using the pre-made buffers supplied with the kit.

A protocol to prepare mammalian cell extract is on page 10.

Materials Needed

You will need the following items:

- Mammalian cells expressing your biotinylated protein of interest using a NativePure™ Gateway® vector
- Complete protease inhibitor (Roche cat. no 1697498 or equivalent)
- Pepstatin (Roche cat. no 1359053 or equivalent)
- Phosphate buffered saline (page vii)
- Deionized water

Components supplied with the kit

- NativePure™ 5X Lysis/Binding Buffer
- NativePure™ 10X TEV Cleavage Buffer
- 100 mM DTT
- 10% NP40

1X Lysis Buffer

100 mM Tris-HCl, pH 8.0
100 mM KCl
200 µM EDTA
1.5 mM $MgCl_2$
1X (700 ng/ml) Pepstatin
Complete protease inhibitor

1. For each purification, prepare 50 ml 1X Lysis Buffer using the NativePure™ 5X Lysis/Binding Buffer supplied in the kit as follows:

NativePure™ 5X Lysis/Binding Buffer	10 ml
Deionized water	40 ml

2. Mix well and adjust the pH to 8.0 with HCl if needed.
3. Filter-sterilize using a 0.22 micron filter unit.
4. Add 1 tablet of complete protease inhibitor (Roche) and 50 µl 1000X Pepstatin (Roche) to 50 ml 1X Lysis Buffer from Step 3.
5. Mix well. Store at 4ºC until use. You may aliquot the buffer into 5 ml and store the aliquots at -20ºC, if needed.

Continued on next page

Preparing Samples and Buffers, Continued

1X Binding Buffer

1X Lysis Buffer (see previous page)
0.1% NP40

1. For each purification, prepare 35 ml 1X Binding Buffer using the 1X Lysis Buffer prepared as described on the previous page and NP40 supplied in the kit as follows:

1X Lysis/Binding Buffer	34.6 ml
10% NP40	0.35 ml

2. Mix well. Store the buffer at 4ºC until use.

1X TEV Elution Buffer

10 mM Tris-HCl, pH 8.0
150 mM NaCl
500 µM EDTA

1. For each purification, prepare **fresh** 8 ml 1X TEV Elution Buffer using the NativePure™ 10X TEV Cleavage Buffer supplied in the kit as follows:

NativePure™ 10X TEV Cleavage Buffer	0.8 ml
Deionized Water	7.2 ml

2. Mix well and adjust the pH to 8.0 with HCl if needed.
3. Filter-sterilize using a 0.22 micron filter unit. Store the buffer at 4ºC until use.

1X TEV Cleavage Buffer

10 mM Tris-HCl, pH 8.0
150 mM NaCl
500 µM EDTA
0.1% NP40

1. For each purification, prepare **fresh** 32 ml 1X TEV Cleavage Buffer using the NativePure™ 10X TEV Cleavage Buffer and 10% NP40 supplied in the kit as follows:

NativePure™ 10X TEV Cleavage Buffer	3.2 ml
10% NP40	320 µl
Deionized Water	28.5 ml

2. Mix well and adjust the pH to 8.0 with HCl if needed.
3. Filter-sterilize using a 0.22 micron filter unit. Store the buffer at 4ºC until use.

Continued on next page

Preparing Samples and Buffers, Continued

Important

- 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.

Preparing Mammalian Cell Lysate

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 (page 8 for a recipe). Proceed to Step 5.
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.

   Tip: To ensure that most of the protein complexes are recovered in the post-nuclear supernatant, perform Western analysis using streptavidin detection after SDS-PAGE of the supernatant and pellet samples. If >50% protein complexes are remaining in the pellet, solubilize the protein complexes using mild, non-ionic detergents such as NP40, Triton X-100, or equivalent.
7. 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.
8. Adjust protein concentration of the sample to **40-50 mg total protein/8 ml**. Store aliquots at -80ºC until use.

Affinity Chromatography

Introduction

Instructions for affinity purification of biotinylated protein complexes using Streptavidin Agarose are described in this section.

Experimental Outline

1. Prepare the Streptavidin Agarose column in 1X Binding Buffer.
2. Bind the lysate to Streptavidin Agarose at 4ºC for 3 hours.
3. Wash any unbound material using two washes with 1X Binding Buffer and 3 washes with 1X TEV Cleavage Buffer.
4. Perform AcTEV™ Protease cleavage overnight at 4ºC on the column to release the bound, biotinylated proteins containing the AcTEV™ cleavage site.
5. Collect the eluate and perform 3 washes with 1X TEV Elution Buffer.
6. Remove AcTEV™ Protease from the eluted fractions and concentrate the eluted protein complexes using NativePure™ Concentrators.

- A sample purification protocol is provided in this manual and may not result in 100% pure protein. Optimize the protocol based on the binding characteristics of your particular proteins.
- Always wear gloves during the purification procedure and while handling the columns to eliminate keratin contamination (for mass spectrometry analysis).
- Perform binding in the presence of 0.1% NP40 to prevent any non-specific binding.
- **Perform all purification steps at 4ºC and use chilled buffers.**
- **Do not** use protease inhibitors during TEV cleavage and **do not** use NP40 during the wash steps after TEV cleavage. This allows to minimize the amount of NP40 in the final eluate because in the subsequent step for protease removal and protein concentration, the NP40 is concentrated and forms micelles which can interfere with gel electrophoresis.

Continued on next page

Affinity Chromatography, Continued

Important

Do not re-use the Streptavidin Agarose after purification. Discard the column containing Streptavidin Agarose after each purification as endogenous biotinylated proteins are still bound to the column.

Materials Needed

You will need the following items:

- Lysate (40-50 mg total protein/8 ml) prepared in 1X Lysis Buffer (page 8), store on ice until use
- 1X Binding Buffer (see page 9 for recipe), store on ice until use
- 1X TEV Elution buffer and 1X TEV Cleavage Buffer (see page 9 for a recipe), store on ice until use
- Rotary shaker
- Sterile microcentrifuge tubes

Components supplied with the kit

- Streptavidin Agarose and NativePure™ Columns
- AcTEV™ Protease
- 100 mM DTT and 10% NP40
- NativePure™ Concentrators

Preparing the Column

1. Resuspend the Streptavidin Agarose by gently rocking the bottle until the resin is evenly suspended in the buffer. Avoid mixing by shaking or vortexing.
2. Transfer 750 µl of the Streptavidin Agarose into a 10-ml NativePure™ Purification Column supplied with the kit using a 1-ml pipette tip (cut off the end of the tip using clean scissors to prevent any keratin contamination).
3. Add 6 ml 1X Binding Buffer to the column. Cap the column and wash by gently inverting the column.
4. Uncap the column and allow the buffer to drip from the bottom outlet by gravity flow.
5. Repeat wash steps 3-4, twice using 6 ml 1X Binding Buffer each time.
6. Proceed immediately to **Binding Step**, next page.

Continued on next page

Affinity Chromatography, Continued

Note

Do not store the column containing Streptavidin Agarose. Use the column immediately for purification after preparing the column.

Binding Step

Perform all purification steps at 4ºC using chilled buffers.

1. Add 80 µl 10% NP40 to 8 ml of the lysate containing 40-50 mg total protein to obtain a final NP40 concentration of 0.1% in the lysate.
2. Save an aliquot (20-50 µl) of the lysate at -20ºC for analysis.
3. Add 8 ml of the lysate in 1X Lysis Buffer containing 0.1% NP40 to Streptavidin Agarose prepared as described on the previous page.
4. Cap the column and incubate the column at 4ºC for 3 hours with gentle agitation on a rotary shaker to allow binding of the biotinylated proteins to Streptavidin Agarose. Make sure the agarose is resuspended in the lysate without allowing the agarose to dry during the binding step.
5. Proceed to **Washing Step**, below.

Washing Step

1. Uncap the column and allow the unbound material to drip from the bottom outlet by gravity flow.
2. Save an aliquot (20-50 µl) of the unbound material at -20ºC for analysis.
3. Add 8 ml 1X Binding Buffer to the column. Cap the column and wash by gently inverting the column.
4. Uncap the column and allow the buffer to drip from the bottom outlet by gravity flow.
5. Repeat wash with 8 ml 1X Binding Buffer once.
6. Add 8 ml 1X TEV Cleavage Buffer to the column. Cap the column and wash by gently inverting the column.
7. Uncap the column and allow the buffer to drip from the bottom outlet by gravity flow.
8. Repeat wash with 1X TEV Cleavage Buffer twice using 8 ml buffer each time.
9. Proceed to **AcTEV™ Cleavage**, next page.

Continued on next page

Affinity Chromatography, Continued

AcTEV™ Cleavage

1. Add 1 ml 1X TEV Cleavage Buffer to the column, cap and gently invert the column to resuspend the beads.
2. Add 100 mM DTT to the column to obtain a final DTT concentration of 1 mM in the buffer.

   Note: The volume of resuspended agarose is ~1.5 ml and includes 1 ml 1X TEV Cleavage Buffer and 0.5 ml packed resin. Add 15 µl 100 mM DTT to 1.5 ml column contents.
3. Save an aliquot (10-20 µl) of the material at -20ºC for analysis.
4. Add 400 units (40 µl) of AcTEV™ Protease included with the kit to the column. Cap the column.

   Note: Depending on your protein, you may need to optimize the conditions for AcTEV™ cleavage (page 17).
5. Incubate the column at 4ºC overnight with gentle agitation on a rotary shaker to elute biotinylated proteins. Make sure the beads are resuspended in the buffer without allowing the beads to dry during this step.
6. Proceed to **Elution Step**, next page.

Continued on next page

Affinity Chromatography, Continued

Elution Step

1. Save an aliquot (10-20 µl) of the column material at -20ºC for analysis.
2. Place a sterile microcentrifuge tube under the column to collect the eluate containing your protein complexes and AcTEV™ Protease. Uncap the column and allow the buffer to drip from the bottom outlet by gravity flow into the microcentrifuge tube. Store the eluate on ice.
3. Place another sterile microcentrifuge tube under the column. Add 1 ml 1X TEV Elution Buffer to the column and allow the buffer to drip from the bottom outlet by gravity flow into the microcentrifuge tube.

   Note: Do not invert the column or resuspend the beads at this point or between elutions to avoid any loss of protein.
4. Perform two additional washes with 1X TEV Elution Buffer into a new collection tube each time and allow each elution to drain completely into the tube prior to adding the next buffer aliquot.

   At the end of the elution step, you will have 4 collection tubes containing ~1 ml eluate each.
5. Save the eluted fractions on ice and discard the Streptavidin Agarose column. Do not re-use the column.
6. Proceed to **Removing AcTEV™ Protease** from the eluted samples as described in the next page.

Continued on next page

Removing AcTEV™ Protease

1. Remove one NativePure™ Concentrator (50 kDa cutoff) from the package.

   Note: The concentrator membrane contains trace amounts of glycerin and sodium azide. If these reagents interfere with your protein complex, pre-rinse concentrator with deionized water.

2. Combine the 4 elution fractions from Step 5, previous page, to obtain ~4 ml eluate and transfer the combined eluate to the NativePure™ Concentrator (50 kDa cutoff) supplied with the kit. Cap the concentrator.

3. Insert the assembled concentrator in a swinging bucket or fixed rotor that can accommodate 15 ml conical centrifuge tubes and centrifuge at 1600 x g for 25 minutes at 4ºC. Discard the contents of the filtration chamber.

   The AcTEV™ Protease passes through the concentrator membrane into the filtration chamber, while the protein complexes remain in the concentrator.

4. Add 4 ml 1X TEV Elution Buffer to the concentrator, cap, and mix by inverting the tube several times. Centrifuge the concentrator at 1600 x g for 25 minutes at 4ºC to remove any residual AcTEV™ Protease and wash the concentrated protein complexes.

5. Remove the concentrator assembly and transfer the concentrated protein complexes (~40-80 µl) from the concentrator into a sterile microcentrifuge tube using a thin pipette tip capable of reaching the bottom of the concentrator.

   Note: The NP40 detergent micelles are not removed using the NativePure™ Concentrator (50 kDa cutoff) membrane, and are recovered with the concentrated protein complexes during this step. Depending on the final volume obtained, the NP40 concentration in the concentrated complexes is ~2-4%.

6. **Optional:** Wash the concentrator membrane with 20 µl 1X TEV Elution Buffer to collect any protein complexes remaining on the membrane. Combine this wash with the protein complex from Step 5.

7. Store the purified, concentrated protein complexes in 1X TEV Elution Buffer at -20ºC or -80ºC depending on your sample. Discard the NativePure™ Concentrator after use.

Continued on next page

Affinity Chromatography, Continued

Analyzing Complexes

You can analyze the purified protein complexes using:

- SDS-PAGE and Western detection (page vii)
- NativePAGE™ Gel Electrophoresis (page vii)
- Mass Spectrometry

To determine the efficiency of purification and percent protein cleavage, we recommend analyzing the aliquots saved during the purification procedure using SDS-PAGE and Western detection. The percent protein cleavage is determined by analyzing the amount of cleaved products formed and amount of uncleaved protein remaining after digestion.

An example of results is shown on the next page.

Optimizing AcTEV™ Cleavage

After evaluating the initial results, you may optimize the cleavage reaction for your specific protein by optimizing the amount of AcTEV™ Protease, incubation temperature, or reaction time as follows to obtain >95% cleavage efficiency.

- Increase the amount of protease (up to 500 units per purification). AcTEV™ Protease is available separately from Invitrogen (page vii).
- Increase the incubation time to 16-24 hours.
- Increase the incubation temperature to 12ºC to 16ºC.

Expected Results

Example of Results

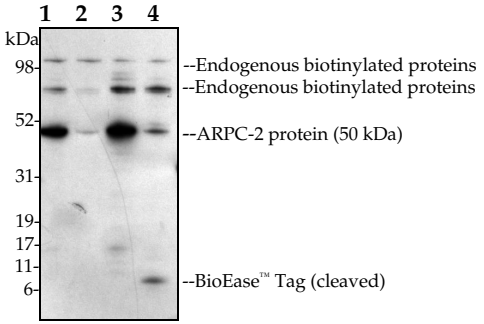
An example of Western blotting results obtained after analyzing samples purified by Streptavidin Agarose are shown below.

ARPC-2 (actin related protein complex p34) was expressed in GripTite™ 293 MSR Cell Line using the NativePure™ Gateway® Vector Kit. The cells were lysed using the lysis protocol described in this manual and biotinylated proteins and protein complexes were purified using the NativePure™ Affinity Purification Kit as described in this manual.

Samples were analyzed by Western blotting to assess the purification protocol. Samples (5 µl) were electrophoresed on a NuPAGE® Novex 4-12% Bis-Tris Gel with NuPAGE® SDS MES Running Buffer. The proteins were transferred to (0.45 µm) nitrocellulose membranes and subjected to Western detection with 1:4000 streptavidin-alkaline phosphatase conjugate using the WesternBreeze® Chemiluminescent Kit.

The results show proper binding of the protein to the Streptavidin Agarose and efficient cleavage of the biotinylated protein. Only biotinylated proteins containing a TEV site are released from the Streptavidin agarose column while endogenous biotinylated proteins remain bound to the column.

Lane 1: Cell lysate
Lane 2: Flow through fraction
Lane 3: Streptavidin Agarose beads before AcTEV™ cleavage
Lane 4: Streptavidin Agarose beads after AcTEV™ cleavage



Continued on next page

Expected Results, Continued

Example of Results, continued

An example of results obtained after analyzing protein complexes on NativePAGE™ and NuPAGE® Gel are shown.

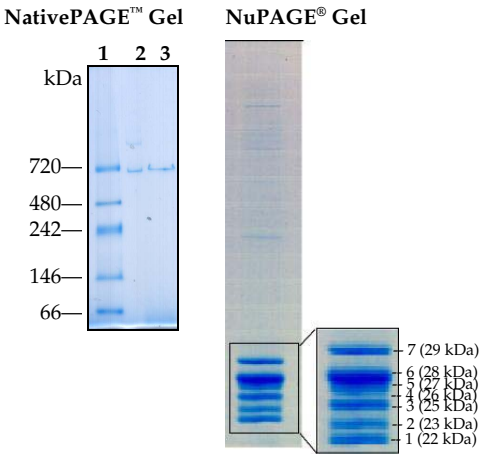
20S Proteasome was expressed in GripTite™ 293 MSR Cell Line using the NativePure™ Gateway® Vector Kit. The cells were lysed using the lysis protocol described in this manual and biotinylated proteins and protein complexes were purified using the NativePure™ Affinity Purification Kit as described in this manual.

Purified protein complex sample was analyzed by native gel electrophoresis on a NativePAGE™ Novex 3-12% Bis-Tris Gel. The gel is stained with Coomassie® stain. The results indicate that the intact 20S Proteasome complex (~700 kDa) was purified.

SDS-PAGE analysis of the purified 20 S Proteasome protein complex was also performed on a NuPAGE® Novex Bis-Tris Gel. The proteins were stained with Coomassie® stain and each individual band was excised from the gel. The excised protein bands were subjected to mass spectrometry analysis. The SDS-PAGE results indicate that the 20S Proteasome is composed of 7 individual polypeptides ranging from 20-30 kDa. Mass spectrometry results (not shown) confirmed that each polypeptide is a component of the 20S Proteasome.

NativePAGE™ Gel:
Lane 1: NativeMark™ Unstained Protein Standard (5 µl)
Lanes 2-3 : Purified 20S Proteasome (5 µg)

NuPAGE® Gel: Purified 20S Proteasome (5 µg). Inset shows the 7 polypeptides.



Troubleshooting

Introduction

Review the information below to troubleshoot your experiments with the NativePure™ Affinity Purification Kit.

Problem	Cause	Solution
No recombinant protein or protein complexes recovered following elution	Expression levels too low	Optimize expression levels using the guidelines in your expression manual. Ensure the protein of interest is *in vivo* biotinylated.
	AcTEV™ cleavage not efficient or enzyme is inactive	Be sure the AcTEV™ cleavage was performed as described on page 14. Increase the incubation time or amount of enzyme to optimize cleavage. Store the AcTEV™ Protease at -20ºC or -80ºC to prevent any loss in activity.
	Protein complex not recovered in the soluble fraction	Perform western analysis to check that protein complexes are recovered in the post-nuclear supernatant. If >50% protein complexes are remaining in the pellet, solubilize the protein complexes using mild, non-ionic detergents such as NP40, Triton X-100, or equivalent.
	Not enough sample loaded	Increase the amount of sample loaded or lysate used.
	Adherent cells difficult to harvest using mild conditions	Some adherent cells maybe difficult to harvest without trypsin. If you need to use trypsin treatment to harvest cells, perform trypsin treatment for a short time.
	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.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
Recombinant protein recovered but not as a complex	Complexes dissociated during lysate preparation or purification	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.**
Some recombinant protein in the flow through and wash fractions	Protein overload	Load less protein on the column or use more resin for purification.
Additional or non-specific proteins observed in the eluate	Non-specific binding of proteins or protein degradation	Include 0.1% NP40 during the binding step to eliminate non-specific binding. Include protease inhibitors during cell lysis to prevent protein degradation.
Protein loss after column elution	Concentrator membrane damaged resulting in protein loss	Load the sample into the concentrator without damaging the membrane.

Appendix

Technical Service

Web Resources

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

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

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

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

MSDS Requests

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

Continued on next page

Technical Service, Continued

Limited Warranty

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

This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

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

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

Purchaser Notification

Limited Use Label License No: 5 Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. Email: outlicensing@invitrogen.com

Limited Use Label License No: 135 Recombinant TEV Protease

This product is licensed under U.S. Patent No. 5,532,142.

Continued on next page

Purchaser Notification, Continued

Limited Use Label License No: 163 Enhanced TEV Protease

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned by Invitrogen Corporation and claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

References

Bayer, E. A., and Wilchek, M. (1990) Application of Avidin-biotin Technology to Affinity-based Separations. J Chromatogr. *510*, 3-11

Carrington, J. C., and Dougherty, W. G. (1988) A Viral Cleavage Site Cassette: Identification of Amino Acid Sequences Required for Tobacco Etch Virus Polyprotein Processing. Proc. Natl. Acad. Sci. USA *85*, 3391-3395

Chaiet, L., and Wolf, F. J. (1964) Arch. Biochem. Biophys. *106*, 1-5

Dougherty, W. G., Carrington, J. C., Cary, S. M., and Parks, T. D. (1988) Biochemical and Mutational Analysis of a Plant Virus Polyprotein Cleavage Site. EMBO J. *7*, 1281-1287

Dougherty, W. G., and Parks, T. D. (1989) Molecular Genetic and Biochemical Evidence for the Involvement of the Heptapeptide Cleavage Sequence in Determining the Reaction Profile at Two Tobacco Etch Virus Cleavage Sites in Cell-free Assays. Virology *172*, 145-155

Dougherty, W. G., Parks, T. D., Cary, S. M., Bazan, J. F., and Fletterick, R. J. (1989) Characterization of the Catalytic Residues of the Tobacco Etch Virus 49-kDa Proteinase. Virology *172*, 302-310

Haeuptle, M. T., Aubert, M. L., Djiane, J., and Kraehenbuhl, J. P. (1983) Binding Sites for Lactogenic and Somatogenic Hormones from Rabbit Mammary Gland and Liver. J. Biol. Chem. *258*, 305-314

Heney, G., and Orr, G. A. (1981) The Purification of Avidin and its Derivatives on 2-iminobiotin-6-aminohexyl-Sepharose 4B. Anal. Biochem. *114*, 92-96

Nayak, S., Li, L., and Lee, J. (2003) Enhanced TEV Protease Extends Enzyme Stability for Long-Term Activity. Focus *25.3*, 12-14

Updyke, T. V., and Nicolson, G. L. (1984) Immunoaffinity Isolation of Membrane Antigens with Biotinylated Monoclonal Antibodies and Immobilized Streptavidin Matrices. J. Immunol. Methods *73*, 83-95

invitrogen™

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

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