Invitrogen™
life technologies

Instruction Manual

ProBond™ Purification System

For purification of polyhistidine-containing recombinant proteins

Catalog nos. K850-01, K851-01, K852-01, K853-01, K854-01,
R801-01, R801-15

Version K
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Table of Contents

Kit Contents and Storage

Types of Products

This manual is supplied with the following products:

Product	Catalog No.
ProBond™ Purification System	K850-01
ProBond™ Purification System with Antibody	
with Anti-Xpress™ Antibody	K851-01
with Anti-myc-HRP Antibody	K852-01
with Anti-His(C-term)-HRP Antibody	K853-01
with Anti-V5-HRP Antibody	K854-01
ProBond™ Nickel-Chelating Resin (50 ml)	R801-01
ProBond™ Nickel Chelating Resin (150 ml)	R801-15

ProBond™ Purification System Components

The ProBond™ Purification System includes enough resin, reagents, and columns for six purifications. The components are listed below. See next page for resin specifications.

Component	Composition	Quantity
ProBond™ Resin	50% slurry in 20% ethanol	12 ml
5X Native Purification Buffer	250 mM NaH_2PO_4, pH 8.0 2.5 M NaCl	1 × 125 ml bottle
Guanidinium Lysis Buffer	6 M Guanidine HCl 20 mM sodium phosphate, pH 7.8 500 mM NaCl	1 × 60 ml bottle
Denaturing Binding Buffer	8 M Urea 20 mM sodium phosphate, pH 7.8 500 mM NaCl	2 × 125 ml bottles
Denaturing Wash Buffer	8 M Urea 20 mM sodium phosphate, pH 6.0 500 mM NaCl	2 × 125 ml bottles
Denaturing Elution Buffer	8 M Urea 20 mM NaH_2PO_4, pH 4.0 500 mM NaCl	1 × 60 ml bottle
Imidazole	3 M Imidazole, 20 mM sodium phosphate, pH 6.0 500 mM NaCl	1 × 8 ml bottle
Purification Columns	10 ml columns	6

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Kit Contents and Storage, Continued

ProBond™ Purification System with Antibody

The ProBond™ Purification System with Antibody includes resin, reagents, and columns as described for the ProBond™ Purification System (previous page) and 50 µl of the appropriate purified mouse monoclonal antibody. Sufficient reagents are included to perform six purifications and 25 Western blots with the antibody.

For more details on the antibody specificity, subclass, and protocols for using the antibody, refer to the antibody manual supplied with the system.

Storage

Store ProBond™ resin at +4°C. Store buffer and columns at room temperature.

Store the antibody at 4°C. Avoid repeated freezing and thawing of the antibody as it may result in loss of activity.

The product is guaranteed for 6 months when stored properly.

Note

All native purification buffers are prepared from the 5X Native Purification Buffer and the 3 M Imidazole, as described on page 7.

The Denaturing Wash Buffer pH 5.3 is prepared from the Denaturing Wash Buffer (pH 6.0), as described on page 11.

Resin and Column Specifications

ProBond™ resin is precharged with Ni^{2+} ions and appears blue in color. It is provided as a 50% slurry in 20% ethanol.

ProBond™ resin and purification columns have the following specifications:

- Binding capacity of ProBond™ resin: 1–5 mg of protein per ml of resin
- Average bead size: 45–165 microns
- 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

Product Qualification

The ProBond™ Purification System is qualified by purifying 2 mg of myoglobin protein on a column and performing a Bradford assay. Protein recovery must be 75% or higher.

Accessory Products

Additional Products

The following products are also available for order from Invitrogen:

Product	Quantity	Catalog No.
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
Polypropylene columns (empty)	50	R640-50
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Purification System with Antibody		
with Anti-Xpress™ Antibody	1 kit	K951-01
with Anti-myc-HRP Antibody	1 kit	K952-01
with Anti-His(C-term)-HRP Antibody	1 kit	K953-01
with Anti-V5-HRP Antibody	1 kit	K954-01
Anti-*myc* Antibody	50 µl	R950-25
Anti-V5 Antibody	50 µl	R960-25
Anti-Xpress™ Antibody	50 µl	R910-25
Anti-His(C-term) Antibody	50 µl	R930-25
InVision™ His-tag In-gel Stain	500 ml	LC6030
InVision™ His-tag In-gel Staining Kit	1 kit	LC6033

Pre-Cast Gels and Pre-made Buffers

A large variety of pre-cast gels for SDS-PAGE and pre-made buffers for your convenience are available from Invitrogen. For details, visit our web site at www.invitrogen.com or contact Technical Service (page 23).

Introduction

Overview

Introduction

The ProBond™ Purification System is designed for purification of 6xHis-tagged recombinant proteins expressed in bacteria, insect, and mammalian cells. The system is designed around the high affinity and selectivity of ProBond™ Nickel-Chelating Resin for recombinant fusion proteins containing six tandem histidine residues.

The ProBond™ Purification System is a complete system that includes purification buffers and resin for purifying proteins under native, denaturing, or hybrid conditions. The resulting proteins are ready for use in many target applications.

This manual is designed to provide generic protocols that can be adapted for your particular proteins. The optimal purification parameters will vary with each protein being purified.

ProBond™ Nickel-Chelating Resin

ProBond™ Nickel-Chelating Resin is used for purification of recombinant proteins expressed in bacteria, insect, and mammalian cells from any 6xHis-tagged vector. ProBond™ Nickel-Chelating Resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins.

Proteins can be purified under native, denaturing, or hybrid conditions using the ProBond™ Nickel-Chelating Resin. Proteins bound to the resin are eluted with low pH buffer or by competition with imidazole or histidine. The resulting proteins are ready for use in target applications.

Binding Characteristics

ProBond™ Nickel-Chelating Resin uses the chelating ligand iminodiacetic acid (IDA) in a highly cross-linked agarose matrix. IDA binds Ni^{2+} ions by three coordination sites.

Note

The protocols provided in this manual are generic, and may not result in 100% pure protein. These protocols should be optimized based on the binding characteristics of your particular proteins.

Native Versus Denaturing Conditions

The decision to purify your 6xHis-tagged fusion proteins under native or denaturing conditions depends on the solubility of the protein and the need to retain biological activity for downstream applications.

- Use **native conditions** if your protein is soluble (in the supernatant after lysis) and you want to preserve protein activity.
- Use **denaturing conditions** if the protein is insoluble (in the pellet after lysis) or if your downstream application does not depend on protein activity.
- Use **hybrid protocol** if your protein is insoluble but you want to preserve protein activity. Using this protocol, you prepare the lysate and columns under denaturing conditions and then use native buffers during the wash and elution steps to refold the protein. Note that this protocol may not restore activity for all proteins. See page 14.

Methods

Preparing Cell Lysates

Introduction

Instructions for preparing lysates from bacteria, insect, and mammalian cells using native or denaturing conditions are described below.

Materials Needed

You will need the following items:

- Native Binding Buffer (recipe is on page 8) for preparing lysates under native conditions
- Sonicator
- 10 µg/ml RNase and 5 µg/ml DNase I (optional)
- Guanidinium Lysis Buffer (supplied with the system) for preparing lysates under denaturing conditions
- 18-gauge needle
- Centrifuge
- Sterile, distilled water
- SDS-PAGE sample buffer
- Lysozyme for preparing bacterial cell lysates
- Bestatin or Leupeptin, for preparing mammalian cell lysates

Processing Higher Amount of Starting Material

Instructions for preparing lysates from specific amount of starting material (bacteria, insect, and mammalian cells) and purification with 2 ml resin under native or denaturing conditions are described in this manual.

If you wish to purify your protein of interest from higher amounts of starting material, you may need to optimize the lysis protocol and purification conditions (amount of resin used for binding). The optimization depends on the expected yield of your protein and amount of resin to use for purification.

Perform a pilot experiment to optimize the purification conditions and then based on the pilot experiment results, scale-up accordingly.

Continued on next page

Preparing Bacterial Cell Lysate—Native Conditions

Follow the procedure below to prepare bacterial cell lysate under native conditions. Scale up or down as necessary.

1. Harvest cells from a 50 ml culture by centrifugation (*e.g.*, 5000 rpm for 5 minutes in a Sorvall SS-34 rotor). Resuspend the cells in 8 ml Native Binding Buffer (recipe on page 8).
2. Add 8 mg lysozyme and incubate on ice for 30 minutes.
3. Using a sonicator equipped with a microtip, sonicate the solution on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst.

   Alternatively, sonicate the solution on ice using two or three 10-second bursts at medium intensity, then flash freeze the lysate in liquid nitrogen or a methanol dry ice slurry. Quickly thaw the lysate at 37°C and perform two more rapid sonicate-freeze-thaw cycles.
4. **Optional**: If the lysate is very viscous, add RNase A (10 µg/ml) and DNase I (5 µg/ml) and incubate on ice for 10–15 minutes. Alternatively, draw the lysate through a 18-gauge syringe needle several times.
5. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.

   Note: Some 6xHis-tagged protein may remain insoluble in the pellet, and can be recovered by preparing a denatured lysate (page 4) followed by the denaturing purification protocol (page 12). To recover this insoluble protein while preserving its biological activity, you can prepare the denatured lysate and then follow the hybrid protocol on page 14. Note that the hybrid protocol may not restore activity in all cases, and should be tested with your particular protein.
6. Remove 5 µl of the lysate for SDS-PAGE analysis. Store the remaining lysate on ice or freeze at -20°C. When ready to use, proceed to the protocol on page 7.

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Preparing Bacterial Cell Lysate—Denaturing Conditions

Follow the procedure below to prepare bacterial cell lysate under denaturing conditions:

1. Equilibrate the Guanidinium Lysis Buffer, pH 7.8 (supplied with the system or see page 19 for recipe) to 37°C.
2. Harvest cells from a 50 ml culture by centrifugation (*e.g.*, 5000 rpm for 5 minutes in a Sorvall SS-34 rotor).
3. Resuspend the cell pellet in 8 ml Guanidinium Lysis Buffer from Step 1.
4. Slowly rock the cells for 5–10 minutes at room temperature to ensure thorough cell lysis.
5. Sonicate the cell lysate on ice with three 5-second pulses at high intensity.
6. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
7. Remove 5 µl of the lysate for SDS-PAGE analysis. Store the remaining lysate on ice or at -20°C. When ready to use, proceed to the denaturing protocol on page 11 or hybrid protocol on page 13.

   Note: To perform SDS-PAGE with samples in Guanidinium Lysis Buffer, you need to dilute the samples, dialyze the samples, or perform TCA precipitation prior to SDS-PAGE to prevent the precipitation of SDS.

Harvesting Insect Cells

For detailed protocols dealing with insect cell expression, consult the manual for your particular system. The following lysate protocols are for baculovirus-infected cells and are intended to be highly generic. They should be optimized for your cell lines.

For baculovirus-infected insect cells, when the time point of maximal expression has been determined, large scale protein expression can be carried out. Generally, the large-scale expression is performed in 1 liter flasks seeded with cells at a density of 2×10^6 cells/ml in a total volume of 500 ml and infected with high titer viral stock at an MOI of 10 pfu/cell. At the point of maximal expression, harvest cells in 50 ml aliquots. Pellet the cells by centrifugation and store at -70°C until needed. Proceed to preparing cell lysates using native or denaturing conditions as described on the next page.

Continued on next page

Preparing Insect Cell Lysate—Native Condition

1. Prepare 8 ml Native Binding Buffer (recipe on page 8) containing Leupeptin (a protease inhibitor) at a concentration of 0.5 µg/ml.
2. After harvesting the cells (previous page), resuspend the cell pellet in 8 ml Native Binding Buffer containing 0.5 µg/ml Leupeptin.
3. Lyse the cells by two freeze-thaw cycles using a liquid nitrogen or dry ice/ethanol bath and a 42°C water bath.
4. Shear DNA by passing the preparation through an 18-gauge needle four times.
5. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
6. Remove 5 µl of the lysate for SDS-PAGE analysis. Store remaining lysate on ice or freeze at -20°C. When ready to use, proceed to the protocol on page 7.

Preparing Insect Cell Lysate—Denaturing Condition

1. After harvesting insect cells (previous page), resuspend the cell pellet in 8 ml Guanidinium Lysis Buffer (supplied with the system or see page 19 for recipe).
2. Pass the preparation through an 18-gauge needle four times.
3. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
4. Remove 5 µl of the lysate for SDS-PAGE analysis. Store remaining lysate on ice or freeze at -20° C. When ready to use, proceed to the denaturing protocol on page 11 or hybrid protocol on page 13.

   Note: To perform SDS-PAGE with samples in Guanidinium Lysis Buffer, you need to dilute the samples, dialyze the samples, or perform TCA precipitation prior to SDS-PAGE to prevent the precipitation of SDS.

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Preparing Mammalian Cell Lysate—Native Conditions

For detailed protocols dealing with mammalian expression, consult the manual for your particular system. The following protocols are intended to be highly generic, and should be optimized for your cell lines.

To produce recombinant protein, you need between 5 x 10^6 and 1 x 10^7 cells. Seed cells and grow in the appropriate medium until they are 80–90% confluent. Harvest cells by trypsinization. You can freeze the cell pellet in liquid nitrogen and store at -70°C until use.

1. Resuspend the cell pellet in 8 ml of Native Binding Buffer (page 8). The addition of protease inhibitors such as bestatin and leupeptin may be necessary depending on the cell line and expressed protein.
2. Lyse the cells by two freeze-thaw cycles using a liquid nitrogen or dry ice/ethanol bath and a 42°C water bath.
3. Shear the DNA by passing the preparation through an 18-gauge needle four times.
4. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
5. Remove 5 µl of the lysate for SDS-PAGE analysis. Store the remaining lysate on ice or freeze at -20° C. When ready to use, proceed to the protocol on page 7.

Preparing Mammalian Cell Lysates—Denaturing Conditions

For detailed protocols dealing with mammalian expression, consult the manual for your particular system. The following protocols are intended to be highly generic, and should be optimized for your cell lines.

To produce recombinant protein, you need between 5 x 10^6 and 1 x 10^7 cells. Seed cells and grow in the appropriate medium until they are 80–90% confluent. Harvest cells by trypsinization. You can freeze the cell pellet in liquid nitrogen and store at -70°C until use.

1. Resuspend the cell pellet in 8 ml Guanidinium Lysis Buffer (supplied with the system or see page 19 for recipe).
2. Shear the DNA by passing the preparation through an 18-gauge needle four times.
3. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
4. Remove 5 µl of the lysate for SDS-PAGE analysis. Store the remaining lysate on ice or freeze at -20° C until use. When ready to use, proceed to the denaturing protocol on page 11 or hybrid protocol on page 13.

   Note: To perform SDS-PAGE with samples in Guanidinium Lysis Buffer, you need to dilute the samples, dialyze the samples, or perform TCA precipitation prior to SDS-PAGE to prevent the precipitation of SDS.

Purification Procedure—Native Conditions

Introduction

In the following procedure, use the prepared Native Binding Buffer, Native Wash Buffer, and Native Elution Buffer, columns, and cell lysate prepared under native conditions. Be sure to check the pH of your buffers before starting.

Buffers for Native Purification

All buffers for purification under native conditions are prepared from the 5X Native Purification Buffer supplied with the system. Dilute and adjust the pH of the 5X Native Purification Buffer to create 1X Native Purification Buffer (page 8). From this, you can create the following buffers:

- Native Binding Buffer
- Native Wash Buffer
- Native Elution Buffer

The recipes described in this section will create sufficient buffers to perform one native purification using one kit-supplied purification column. Scale up accordingly.

If you are preparing your own buffers, see page 18 for recipe.

Materials Needed

You will need the following items:

- 5X Native Purification Buffer (supplied with the system or see page 18 for recipe)
- 3 M Imidazole (supplied with the system or see page 18 for recipe)
- NaOH
- HCl
- Sterile distilled water
- Prepared ProBond™ columns with native buffers (next page)
- Lysate prepared under native conditions (page 2)

Imidazole Concentration in Native Buffers

Imidazole is included in the Native Wash and Elution Buffers to minimize the binding of untagged, contaminating proteins and increase the purity of the target protein with fewer wash steps. Note that, if your level of contaminating proteins is high, you may add imidazole to the Native Binding Buffer.

If your protein does not bind well under these conditions, you can experiment with lowering or eliminating the imidazole in the buffers and increasing the number of wash and elution steps.

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Purification Procedure—Native Conditions, Continued

1X Native Purification Buffer

To prepare 100 ml 1X Native Purification Buffer, combine:

- 80 ml of sterile distilled water
- 20 ml of 5X Native Purification Buffer (supplied with the system or see page 18 for recipe)

Mix well and adjust pH to 8.0 with NaOH or HCl.

Native Binding Buffer

Without Imidazole

Use 30 ml of the 1X Native Purification Buffer (see above for recipe) for use as the Native Binding Buffer (used for column preparation, cell lysis, and binding).

With Imidazole (Optional):

You can prepare the Native Binding Buffer with imidazole to reduce the binding of contaminating proteins. (Note that some His-tagged proteins may not bind under these conditions.).

To prepare 30 ml Native Binding Buffer with 10 mM imidazole, combine:

- 30 ml of 1X Native Purification Buffer
- 100 µl of 3 M Imidazole, pH 6.0

Mix well and adjust pH to 8.0 with NaOH or HCl.

Native Wash Buffer

To prepare 50 ml Native Wash Buffer with 20 mM imidazole, combine:

- 50 ml of 1X Native Purification Buffer
- 335 µl of 3 M Imidazole, pH 6.0

Mix well and adjust pH to 8.0 with NaOH or HCl.

Native Elution Buffer

To prepare 15 ml Native Elution Buffer with 250 mM imidazole, combine:

- 13.75 ml of 1X Native Purification Buffer
- 1.25 ml of 3 M Imidazole, pH 6.0

Mix well and adjust pH to 8.0 with NaOH or HCl.

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Note

Do not use strong reducing agents such as DTT with ProBond™ columns. DTT reduces the nickel ions in the resin. In addition, do not use strong chelating agents such as EDTA or EGTA in the loading buffers or wash buffers, as these will strip the nickel from the columns.

Be sure to check the pH of your buffers before starting.

Preparing ProBond™ Column

When preparing a column as described below, make sure that the snap-off cap at the bottom of the column remains intact. To prepare a column:

1. Resuspend the ProBond™ resin in its bottle by inverting and gently tapping the bottle repeatedly.
2. Pipet or pour 2 ml of the resin into a 10-ml Purification Column supplied with the kit. Allow the resin to settle completely by gravity (5-10 minutes) or gently pellet it by low-speed centrifugation (1 minute at 800 × *g*). Gently aspirate the supernatant.
3. Add 6 ml of sterile, distilled water and resuspend the resin by alternately inverting and gently tapping the column.
4. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
5. For purification under **Native Conditions**, add 6 ml Native Binding Buffer (recipe on page 8).
6. Resuspend the resin by alternately inverting and gently tapping the column.
7. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
8. Repeat Steps 5 through 7.

Storing Prepared Columns

To store a column containing resin, add 0.02% azide or 20% ethanol as a preservative and cap or parafilm the column. Store at room temperature.

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Purification Under Native Conditions

Using the native buffers, columns and cell lysate, follow the procedure below to purify proteins under native conditions:

1. Add 8 ml of lysate prepared under native conditions to a prepared Purification Column (page 9).
2. Bind for 30–60 minutes using gentle agitation to keep the resin suspended in the lysate solution.
3. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis.
4. Wash with 8 ml Native Wash Buffer (page 8). Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis.
5. Repeat Step 4 three more times.
6. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein with 8–12 ml Native Elution Buffer (see page 2). Collect 1 ml fractions and analyze with SDS-PAGE.

   Note: Store the eluted fractions at 4°C. If -20°C storage is required, add glycerol to the fractions. For long term storage, add protease inhibitors to the fractions.

If you wish to reuse the resin to purify the same recombinant protein, wash the resin with 0.5 M NaOH for 30 minutes and equilibrate the resin in a suitable binding buffer. If you need to recharge the resin, see page 17.

Purification Procedure—Denaturing Conditions

Introduction

Instructions to perform purification using denaturing conditions with prepared denaturing buffers, columns, and cell lysate are described below.

Materials Needed

You will need the following items:

- Denaturing Binding Buffer (supplied with the system or see page 19 for recipe)
- Denaturing Wash Buffer, pH 6.0 (supplied with the system or see page 19 for recipe) and Denaturing Wash Buffer, pH 5.3 (see recipe below)
- Denaturing Elution Buffer (supplied with the system or see page 20 for recipe)
- Prepared ProBond™ columns with Denaturing buffers (see below)
- Lysate prepared under denaturing conditions (page 11)

Preparing the Denaturing Wash Buffer pH 5.3

Using a 10 ml aliquot of the kit-supplied Denaturing Wash Buffer (pH 6.0), mix well, and adjust the pH to 5.3 using HCl. Use this for the Denaturing Wash Buffer pH 5.3 in Step 5 next page.

Note

Be sure to check the pH of your buffers before starting. Note that the denaturing buffers containing urea will become more basic over time.

Preparing ProBond™ Column

When preparing a column as described below, make sure that the snap-off cap at the bottom of the column remains intact.

If you are reusing the ProBond™ resin, see page 17 for recharging protocol.

To prepare a column:

1. Resuspend the ProBond™ resin in its bottle by inverting and gently tapping the bottle repeatedly.
2. Pipet or pour 2 ml of the resin into a 10-ml Purification Column supplied with the kit. Allow the resin to settle completely by gravity (5-10 minutes) or gently pellet it by low-speed centrifugation (1 minute at 800 × *g*). Gently aspirate the supernatant.
3. Add 6 ml of sterile, distilled water and resuspend the resin by alternately inverting and gently tapping the column.
4. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant.
5. For purification under **Denaturing Conditions**, add 6 ml of Denaturing Binding Buffer.
6. Resuspend the resin by alternately inverting and gently tapping the column.
7. Allow the resin to settle using gravity or centrifugation as described in Step 2, and gently aspirate the supernatant. Repeat Steps 5 through 7.

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Purification Procedure—Denaturing Conditions, Continued

Purification Under Denaturing Conditions

Using the denaturing buffers, columns, and cell lysate, follow the procedure below to purify proteins under denaturing conditions:

1. Add 8 ml lysate prepared under denaturing conditions to a prepared Purification Column (page 11).
2. Bind for 15–30 minutes at room temperature using gentle agitation (*e.g.*, using a rotating wheel) to keep the resin suspended in the lysate solution. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant.
3. Wash the column with 4 ml Denaturing Binding Buffer supplied with the kit by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step one more time.
4. Wash the column with 4 ml Denaturing Wash Buffer, pH 6.0 supplied in the kit by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step one more time.
5. Wash the column with 4 ml Denaturing Wash Buffer pH 5.3 (see recipe on previous page) by resuspending the resin and rocking for 2 minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*), and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step once more for a total of two washes with Denaturing Wash Buffer pH 5.3.
6. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein by adding 5 ml Denaturing Elution Buffer supplied with the kit. Collect 1 ml fractions and monitor the elution by taking OD_{280} readings of the fractions. Pool the fractions that contain the peak absorbance and dialyze against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. Concentrate the dialyzed material by any standard method (i.e., using 10,000 MW cut-off, low-protein binding centrifugal instruments or vacuum concentration instruments).

If you wish to reuse the resin to purify the same recombinant protein, wash the resin with 0.5 M NaOH for 30 minutes and equilibrate the resin in a suitable binding buffer. If you need to recharge the resin, see page 17.

Purification Procedure—Hybrid Conditions

Introduction

For certain insoluble proteins, use the Hybrid protocol to restore protein activity following cell lysis and binding under denaturing conditions. Note that this procedure will not work for all proteins and should be tested using your particular recombinant proteins.

Note

Be sure to check the pH of your buffers before starting. Note that the denaturing buffers containing urea will become more basic over time.

Materials Needed

You will need the following items:

- Denaturing Binding Buffer (supplied with the system or see page 19 for recipe)
- Denaturing Wash Buffer, pH 6.0 (supplied with the system or see page 19 for recipe)
- Native Wash Buffer (page 8 for recipe)
- Native Elution Buffer (page 8 for recipe)
- Prepared ProBond™ Columns under denaturing conditions (page 11)
- Lysate prepared under denaturing conditions (page 2)

ProBond™ Columns

Prepare the ProBond™ columns using Denaturing Binding Buffer as described on page 11.

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Purification Procedure—Hybrid Conditions, Continued

Purification Under Hybrid Conditions

Using the denaturing buffers, columns and cell lysate prepared under denaturing conditions, follow the purification procedure below:

1. Add 8 ml of lysate (page 2) to a prepared ProBond™ Column (page 11).
2. Bind for 15–30 minutes at room temperature using gentle agitation (*e.g.*, on a rotating wheel) to keep the resin suspended in the lysate solution. Settle the resin by gravity or low speed centrifugation (800 × *g*) and carefully aspirate the supernatant.
3. Wash the column with 4 ml Denaturing Binding Buffer supplied with the kit by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*) and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step one more time.
4. Wash the column with 4 ml Denaturing Wash Buffer, pH 6.0 supplied with the kit by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*) and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step one more time.
5. Wash the column with 8 ml Native Wash Buffer (page 8 for recipe) by resuspending the resin and rocking for two minutes. Settle the resin by gravity or low speed centrifugation (800 × *g*) and carefully aspirate the supernatant. Save supernatant at 4°C for SDS-PAGE analysis. Repeat this step three more times for a total of four native washes.
6. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein with 8–12 ml Native Elution Buffer (see page 8 for recipe). Collect 1 ml fractions and analyze with SDS-PAGE.

If you wish to reuse the resin to purify the same recombinant protein, wash the resin with 0.5 M NaOH for 30 minutes and equilibrate the resin in a suitable binding buffer. If you need to recharge the resin, see page 17.

Troubleshooting

Introduction

Review the information below to troubleshoot your experiments with the ProBond™ Purification System.

For troubleshooting problems with antibody detection, see the antibody manual supplied with the system.

Problem	Probable Cause	Possible Solution
• No recombinant protein recovered following elution.	• Nothing bound because of protein "folding."	• Try denaturing conditions.
	• Expression levels too low.	• Optimize expression levels using the guidelines in your expression manual.
	• Protein washed out by too stringent washing.	• Raise pH of wash buffer in high-stringency wash step. Wash less extensively in high-stringency wash step.
	• Not enough sample loaded.	• Increase amount of sample loaded or lysate used.
	• Recombinant protein has very high affinity for ProBond™ resin.	• Increase stringency of elution by decreasing the pH or increasing the imidazole concentration. • To preserve activity, use EDTA or EGTA (10-100 mM) to strip resin of nickel ions and elute the protein.
	• Protein degraded.	• Perform all purification steps at 4°C. • Check to make sure that the 6xHis-tag is not cleaved during processing or purification. • Include protease inhibitors during cell lysis.
• Good recombinant-protein recovery but contaminated with non-recombinant proteins.	• Wash conditions not stringent enough.	• Lower pH of wash buffer in high-stringency wash step. Wash more extensively.
	• Other His-rich proteins in sample.	• Consider an additional high stringency wash at a lower pH (i.e., between pH 6 and pH 4) before the elution step. • Further purify the eluate on a new ProBond™ column after performing dialysis of the eluate against the binding buffer and equilibrating the column with binding buffer. • Perform second purification over another type of column.
	• Recombinant protein has low affinity for resin; comes off in wash with many contaminating proteins.	• Try denaturing conditions. • Try an imidazole step gradient elution. • Try a pH gradient with decreasing pH.

Continued on next page

Troubleshooting, Continued

Problem	Probable Cause	Possible Solution
• Low recombinant protein recovery and contaminated with non-recombinant proteins.	• Recombinant protein not binding tightly to resin.	• Try denaturing conditions. • Try "reverse-chromatography": bind lysate, including recombinant protein; allow recombinant protein to come off in low stringency washes; collect these fractions; re-do chromatography on saved fractions on new or stripped and recharged column. Works for native purification only.
	• Expression levels too low.	• Consider an additional high stringency wash at a lower pH (i.e., between pH 6 and pH 4) before elution step.
• Some recombinant protein in the flow through and wash fractions	• Protein overload.	• Load less protein on the column or use more resin for purification.
• Column turns reddish brown.	• DTT is present in buffers.	• Use β-mercaptoethanol as a reducing agent.
• Column turns white.	• Chelating agents present in buffer that strip the nickel ions from the column.	• Recharge the column as described on page 17.
• Protein precipitates during binding.	• Temperature is too low.	• Perform purification at room temperature.
	• Protein forms aggregates.	• Add solubilization reagents such as 0.1% Triton X-100 or Tween-20 or stabilizers such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility. • Run column in drip mode to prevent protein from dropping out of solution.

Appendix

Additional Protocols

Cleavage of the Fusion Peptide

If your recombinant fusion protein contains the recognition sequence for enterokinase (EnterokinaseMax™) or AcTEV™ Protease between the 6xHis-tag and the protein, you may cleave the 6xHis-tag from the fusion protein using the specific protease. You can cleave the tag after obtaining the purified recombinant fusion protein or while the protein is bound to the nickel-chelating resin.

EnterokinaseMax™ is a recombinant preparation of the catalytic subunit of enterokinase. This enzyme recognizes -Asp-Asp-Asp-Asp-Lys- and cleaves after the lysine. It has high specific activity, leading to more efficient cleavage, and requires less enzyme.

Description	*Catalog no.*
EnterokinaseMax™, 250 units	E180-01
EnterokinaseMax™, 1000 units	E180-02

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. AcTEV™ Protease recognizes the seven-amino-acid sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly and cleaves between Gln and Gly with high specificity.

Description	*Catalog no.*
AcTEV Protease™, 1,000 units	12575-015
AcTEV Protease™, 10,000 units	12575-023

Recharging ProBond™ Resin

ProBond™ resin can be used for up to three or four purifications of the same protein without recharging. Wash the resin with 0.5 M NaOH for 30 minutes and equilibrate the resin with the appropriate binding buffer, if you are reusing the resin.

We recommend not recharging the resin more than three times and only reusing it for purification of the same recombinant protein. If the resin turns white due to the loss of nickel ions from the column, recharge the resin.

To recharge 2 ml of resin in a purification column:

1. Wash the column two times with 8 ml 50 mM EDTA to strip away the chelated nickel ions.
2. Wash the column two times with 8 ml 0.5 M NaOH.
3. Wash the column two times with 8 ml of sterile, distilled water.
4. Recharge the column with two washes of 8 ml $NiCl_2$ hexahydrate at a concentration of 5 mg/ml prepared in sterile, distilled water.
5. Wash the column two times with 8 ml distilled water.
6. Add 0.02% azide or 20% ethanol as a preservative and cap or apply a parafilm to the column. Store at room temperature.

Recipes

Buffer Stock Solutions (10X)

To prepare the buffer solutions described below, you need to prepare sodium phosphate stock solutions:

Stock Solution A (10X)

200 mM sodium phosphate, monobasic (NaH_2PO_4)
5 M NaCl

Dissolve 27.6 g of monobasic sodium phosphate (NaH_2PO_4) and 292.9 g of NaCl in 800 ml deionized water. Mix well and adjust the volume to 1 L with deionized water. Store solution at room temperature.

Stock Solution B (10X)

200 mM sodium phosphate, dibasic (Na_2HPO_4)
5 M NaCl

Dissolve 28.4 g dibasic sodium phosphate (Na_2HPO_4) and 292.9 g of NaCl in 800 ml of deionized water. Mix well and adjust the volume to 1 L with deionized water. Store solution at room temperature.

5X Native Purification Buffer

250 mM NaH_2PO_4, pH 8.0
2.5 M NaCl

Prepare 200 ml solution as follows:

1. To 180 ml deionized water, add

Sodium phosphate, monobasic	7 g
NaCl	29.2 g

2. Mix well and adjust the pH with NaOH to pH 8.0.
3. Bring the final volume to 200 ml with deionized water.
4. Store buffer at room temperature.

3 M Imidazole pH 6.0

3 M Imidazole
500 mM NaCl
20 mM Sodium Phosphate Buffer, pH 6.0

Prepare 100 ml solution as follows:

1. To 90 ml deionized water, add

Imidazole	20.6 g
Stock Solution A (10X)	8.77 ml
Stock Solution B (10X)	1.23 ml

2. Mix well and adjust the pH to 6.0 with HCl or NaOH as necessary.
3. Bring the final volume to 100 ml with deionized water. If the solution forms a precipitate, heat solution until the precipitate dissolves.
4. Store buffer at room temperature.

Continued on next page

Guanidinium Lysis Buffer

6 M Guanidine Hydrochloride
20 mM Sodium Phosphate, pH 7.8
500 mM NaCl

Prepare 100 ml solution as follows:

1. To 90 ml deionized water, add

Stock Solution A (10X)	0.58 ml
Stock Solution B (10X)	9.42 ml
Guanidine Hydrochloride	57.3 g

2. Stir the solution until completely dissolved. Adjust the pH to 7.8 using 1 N NaOH or 1 N HCl.
3. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45 µm filter (autoclaving the solution will alter the pH of the buffer).
4. Store buffer at room temperature.

Denaturing Binding Buffer

8 M Urea
20 mM Sodium Phosphate pH 7.8
500 mM NaCl

Prepare 100 ml solution as follows:

1. To 90 ml deionized water, add

Stock Solution A (10X)	0.58 ml
Stock Solution B (10X)	9.42 ml
Urea	48.1g

2. Stir the solution with gentle heating (50-60°C, do not overheat) until completely dissolved. When cooled to room temperature, adjust the pH to 7.8 using 1 N NaOH or 1 N HCl.
3. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45 µm filter (autoclaving the solution will alter the pH of the buffer).
4. Store buffer at room temperature.

Denaturing Wash Buffer

8 M Urea
20 mM Sodium Phosphate, pH 6.0
500 mM NaCl

Prepare 100 ml solution as follows:

1. To 90 ml deionized water, add

Stock Solution A (10X)	7.38 ml
Stock Solution B (10X)	2.62 ml
Urea	48.1g

2. Stir the solution with gentle heating (50-60°C, do not overheat) until completely dissolved. Adjust the pH to 6.0 using 1 N NaOH or 1 N HCl.
3. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45 µm filter (autoclaving the solution will alter the pH of the buffer).
4. Store buffer at room temperature.

Continued on next page

Denaturing Elution Buffer

8 M Urea
20 mM Sodium Phosphate, pH 4.0
500 mM NaCl

Prepare 100 ml as follows:

1. To 90 ml deionized water, add

Stock Solution A (10X)	10 ml
Urea	48.1g

2. Stir the solution with gentle heating (50-60°C, do not overheat) until completely dissolved. Adjust the pH to 4.0 using 1 N NaOH or 1 N HCl.
3. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45 µm filter (autoclaving the solution will alter the pH of the buffer).
4. Store buffer at room temperature.

Frequently Asked Questions

For denatured conditions, why is Guanidinium used for lysis of cells?	We have found that guanidinium works better for cell lysis than urea; however, urea works well for the remaining steps.
Can proteins bind to the resin at a pH lower than 7.8?	The optimal binding range is pH 7.2–7.8. However, we have performed purifications with columns equilibrated to pH 6.0. Some proteins bind well under these conditions and will remain bound to the column following a pH 6.0 wash.
Can glycine be used instead of sodium phosphate in the purification system binding buffers?	No, because glycine is a competitive ligand for nickel. People have successfully used: • Tris-HCl • Tris-Phosphate • Tris-Acetate • Sodium Acetate • Sodium Borate • MES-NaOH • Pipes-HCl • HEPES
Can I use the resin to purify a protein with fewer than six histidine residues?	We have not tried to purify proteins with less than six histidines. However, if several histidines are near each other, you may be able to attach the protein to the resin well enough for purification.
Is there a cell lysis procedure that will liberate microsome-bound proteins for subsequent purification using ProBond™?	If solubility is a problem, you can include up to 0.2% Sarkosyl in the 6M Guanidinium Lysis Buffer—this should solubilize everything and may still be compatible with purification on the ProBond™ columns. In general, anionic detergents are incompatible with nickel chelating columns, but up to 0.2% Sarkosyl has been used in some cases.
What are recommended elution conditions for His-tagged proteins that are unstable at a pH<7.0?	You can elute with a stepped imidazole gradient at a neutral pH of 7.0-7.5). Use 10 mM imidazole, then 50 mM, 75 mM, and so on until the protein elutes. Note that more contaminating proteins that would have been washed off at pH 6.0 will remain on the resin at pH 7.0.
What is the importance of NaCl in the binding buffer?	ProBond™ resin has a net positive charge, and 500 mM NaCl is used to prevent the nonspecific binding of negatively charged proteins.

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