

ProteoSpin



Application Manual



Inclusion Body Isolation Micro Kit

For Use with P/Ns 10300 and 10600

ProteoSpin™ Inclusion Body Isolation Micro Kit Benefits

Basic Features

Benefits

Three kits in one for both acidic and basic proteins

The kit provides essential reagents for cell disruption, inclusion body solubilization and purification using spin column chromatography. The kit includes solutions and protocols for use with either acidic or basic proteins.

Gentle disruption procedure provides high purity final product

Cell lysis is accomplished through non-ionic detergent chemical disruption. Passing cells through a needle assists in mechanical disruption and reduces viscosity, producing protein often >95% pure. Proteins are then ready for SDS-PAGE, 2D gels, mass spectrometry analysis or other procedures.

Convenient process volume

Analyze 1.5 mL of microbial cell culture yielding 2-50 µg of protein.

Long shelf life

The kit uses an enzyme-free process with stable solutions for longer shelf life.

Fast process time

Process up to 12 samples in only 60 minutes.

NOTE: Not intended for testing protein activity.

Introduction and Description

1

The ProteoSpin™ Inclusion Body Isolation Micro Kit facilitates the screening of *E. coli* clones that express recombinant proteins in inclusion bodies. This kit procedure is designed to extract the expressed recombinant protein directly from whole bacterial cells, and purify using spin column chromatography, allowing SDS-PAGE analysis. With optimized reagents and streamlined processes, the ProteoSpin™ Inclusion Body Isolation Micro Kit significantly reduces time and labour for screening and identifying clonal lines that can be used in scale-up production.

Each ProteoSpin™ Inclusion Body Isolation Micro Kit contains sufficient materials to screen candidate clones from colonies on agar plates. The kit is designed to purify both acidic and basic proteins.

The three main processes for purification of recombinant proteins in inclusion bodies starting from whole cells are:

- Lysis of bacterial cells to release inclusion bodies in solid form.
- Solubilization of purified inclusion bodies.
- Purification of the recombinant protein using spin column chromatography.

Each process requires specialized reagents that are carefully formulated to achieve rapid and high quality purification. The ProteoSpin™ Inclusion Body Isolation Micro Kit combines these processes to allow for a rapid and convenient analysis of bacterial clones.

Inclusion Bodies

The production of recombinant proteins using gene expression systems constitutes a powerful technique of molecular biology. When host strains, vectors, and growth conditions are optimized, many recombinant proteins can be cloned and expressed in *E. coli* with high efficiency and impressive yields. Subsequent protein products may remain in the cytoplasm, be directed to the periplasm, or secreted from the cell and into the culture medium.

In many cases, overexpression of the recombinant protein leads to the formation of insoluble aggregates, termed inclusion bodies, that appear as distinct subcellular compartments in bacterial cells. Studies have shown that inclusion body recombinant proteins expressed by cells can be misfolded. This is due to the inappropriate formation of secondary structures and misplaced disulfide bonds that combine to render the protein highly insoluble. The insoluble proteins achieve a high degree of aggregation so that, as a whole, the *E. coli* expression system in inclusion bodies is more efficient and exhibits greater yields than other systems such as mammalian, yeast and insect cultures.

The insoluble nature of inclusion bodies in *E. coli* provides a means for their separation from other cellular components. With appropriate techniques, the bacterial cells can be disrupted chemically, enzymatically, or mechanically to release their inclusion bodies in solid form.

The purified inclusion bodies are often free of cellular components and contain mainly the incorrectly folded recombinant protein. In this state, the protein is inactive and insoluble. Because of this, the protein is protected from the harmful effects of proteolytic enzymes that are released during lysis of the cells.

Solubilization of inclusion bodies is a necessary step to release the recombinant protein, which is obtained traditionally through denaturation with chaotropic reagents. These reagents disrupt hydrogen bonds and hydrophobic interactions so that the protein can be further purified by chromatographic means. More importantly, denaturation of the incorrectly folded protein is often a step that is taken to initiate a refolding process so that the protein can achieve its native conformation for full biological activity.

Ion Exchange Chromatography

In ion exchange chromatography, ions that are electrostatically bound to an insoluble and chemically inert matrix are reversibly replaced by ions in solution. The matrix can be either an anion exchanger, consisting of positively charged groups that reversibly bind anions in solution, or it can be a cation exchanger bearing negatively charged groups that binds to mobile cations. Proteins are complex molecules that can have an overall positive or negative charge, depending on their amino acid composition and the pH of their solutions. In this case, any given protein will bind to both anion and cation exchangers depending on the net charge of the protein.

When purifying a protein, the mobile phase pH, salt concentration and buffer are chosen to bind the desired protein on the selected ion exchanger. The impure protein solution is applied to a column that contains the ion exchanger, and is washed with the same mobile phase solution. Proteins bind to the matrix and are eluted by changing either salt concentration or pH.

Silicon Carbide as an Ion Exchanger

The chromatographic material used in the ProteoSpin™ Inclusion Body Isolation Micro Kit consists of silicon carbide (SiC), a man-made material noted for its hardness (second only to diamond) and high resistance to chemical change. These properties make SiC highly suitable for spin column chromatography.

When processed appropriately, SiC becomes an effective cation exchanger for the purpose of purifying macromolecules. The surface of SiC is negatively charged and can bind positively charged macromolecules. SiC has poor affinity to monovalent and divalent cations, also making it an effective resin for the removal of salts.

Soluble proteins bind to SiC through interaction between the positively charged side-groups on their chain with the negative charges on the SiC surface. As a cation exchanger, SiC has an estimated effective pKa ~2 making the resin negatively charged over a wide range of pH. Therefore, with the exception of those peptides that are highly acidic, most soluble polypeptides can be bound to SiC.

Isoelectric Point

Proteins generally contain a number of ionizable groups with a variety of pKa values. Each protein has a particular pH where the negative charges on the molecule exactly balance the positive charges, therefore giving the protein no net charge. This is known as the isoelectric point, or pI of the protein.

The pI of a protein determines the ideal conditions for its purification by ion exchange chromatography. For example, based on pI, one can determine whether the pH of a binding buffer and an elution buffer are suitable for a particular protein. Isoelectric points for a number of proteins have already been determined. If the pI of a protein of interest is unknown, a theoretical pI can be determined using web-based tools found at the ExPASy site http://us.expasy.org/tools/pi_tool.html. Another useful pI-predictor method can be found at http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html.

Kit Description

The ProteoSpin™ Cell Lysis Reagent was carefully formulated to selectively dissolve the cells while keeping the released inclusion bodies intact. A subsequent centrifugation step separates the inclusion bodies from other cellular components, which are discarded with the liquid phase.

The ProteoSpin™ Inclusion Body Isolation Micro Kit provides a specially formulated reagent for the efficient solubilization of inclusion bodies with minimal use of chaotropes. The formulation is primarily intended for compatibility with ProteoSpin™ spin column technology. However, the reagent, when used intentionally to obtain a biologically active product, may also serve as the initiator for the refolding process, with or without an intermediate chromatographic purification step. For the purpose of screening bacterial clones, the solubilization reagent for inclusion bodies is intended to facilitate purification of the recombinant protein by spin column chromatography to prepare the protein for analysis by SDS-PAGE.

Kit Components

ProteoSpin™ inclusion Body Isolation Micro Kit

Component	ProteoSpin™ Inclusion Body Isolation Micro Kit	ProteoSpin™ Inclusion Body Isolation Micro Kit
	20 samples	50 samples
Column Activation and Wash Buffer (Acidic)	30 mL	60 mL
Column Activation and Wash Buffer (Basic)	30 mL	60 mL
pH Binding Buffer (Acidic)	1 mL	1 mL
pH Binding Buffer (Basic)	1 mL	1 mL
Elution Buffer	4 mL	8 mL
Neutralizer	1 mL	1 mL
Cell Lysis Reagent	15 mL	30 mL
IB Solubilization Reagent	2 mL	4 mL
Syringes 1 cc, slip tip	20	50
Needles (Bev, 20G x 1 inch)	20	50
Micro Spin Columns (filled with SiC) inserted into 2 mL collection tubes	20	50
Final Collection Tube, 1.7 mL	30	60
ProteoSpin™ Inclusion Body Isolation Micro Kit Application Manual	1	1
ProteoSpin™ Inclusion Body Isolation Micro Kit Protocol Card	1-Acidic Protocol 1-Basic Protocol	1-Acidic Protocol 1-Basic Protocol

Recommended Storage Conditions

The Cell Lysis and IB Solubilization reagent should be stored at 4°C upon receipt of this kit. For other unopened solution containers, the reagents should remain stable for at least six months when stored at room temperature. Once opened, the solutions should be stored at 4°C when not in use except for the pH binding buffer acidic and basic. Some precipitation will occur with 4°C storage. This precipitation should be dissolved with slight heating to room temperature before using.

Customer-Supplied Reagents and Equipment

You must have the following in order to use the ProteoSpin™ Inclusion Body Isolation Micro Kit:

- Benchtop microcentrifuge (e.g. Eppendorf)
- Micropipettors
- Sterile, deionized water or Milli-Q water

Procedure

2

This section describes the procedures for the ProteoSpin™ Inclusion Body Isolation Micro Kit and how to select the appropriate one for your sample.

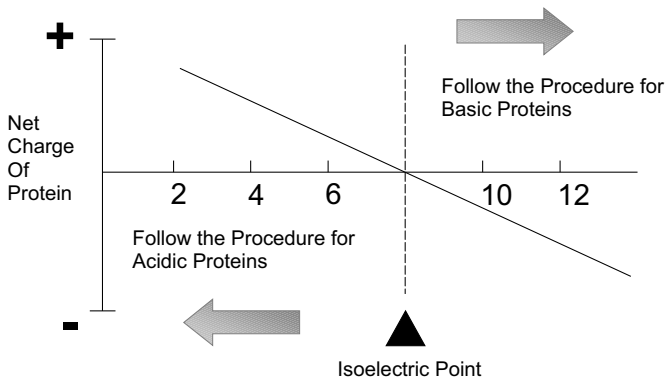
Overview

Procedure Overview

The Inclusion Body Isolation Micro Kit uses a proprietary cell lysis reagent to selectively lyse the cells and release inclusion bodies in their solid form. Using the IB Solubilization Reagent, inclusion bodies are dissolved and their contents released. Inclusion body proteins are then further purified by loading onto spin columns containing SiC. Non-specifically bound components in the solution can be washed from the column, leaving the inclusion body protein bound to the SiC. These specific proteins can then be removed using the elution buffer. Each spin column is able to recover up to 50 µg of acidic or basic protein.

Choosing a Basic or Acidic Protocol

The kit includes solutions for isolating inclusion bodies containing either acidic or basic proteins. In theory, the protocol for acidic proteins should apply to the majority of proteins since the resin is a cation exchanger. All proteins with a pI greater than the binding pH at 4.5 should bind. Basic proteins, however, bind strongly when they are used under these conditions, making their elution quite inefficient. Therefore, for soluble basic proteins ($pI \geq 8$), a different condition for binding the protein to the resin has been developed. For the purposes of the Inclusion Body Isolation Micro Kit, a protein with a pI less than 8 will be treated as acidic and will use the acidic protocol. For a protein with a $pI \geq 8$, use the basic protocol.



Protocols

To rapidly screen bacterial clones for expression of recombinant proteins in inclusion bodies, the Inclusion Body Isolation Micro Kit is designed for testing small bacterial cultures growing in test tubes. Test tube cultures with a 2 mL culture medium are normally initiated by inoculation with single colonies picked from culture plates.

Protocol One – Isolating Inclusion Bodies (for both Acidic and Basic Proteins)

The procedure for lysing bacterial cells to release their inclusion bodies is identical for all recombinant proteins to be screened. The procedure for purifying proteins from solubilized inclusion bodies using ProteoSpin™ columns, however, depends on the isoelectric point of the recombinant protein that is expressed in the inclusion bodies. The user must decide whether to use the acidic or basic procedures depending on the pI of the recombinant protein in question.

The efficiency of inclusion body extraction may vary from strain to strain. Growth and induction conditions are dependent upon host strain and gene expression vector utilized. The user must consult expression system instructions/literature for proper use. To ensure the option of scaling-up clones found to contain the protein of interest, it is recommended that the user preserve stocks of uninduced bacteria for each clone tested.

All centrifugation steps are carried out at 14,000 X g in a benchtop microcentrifuge. Please check your microcentrifuge specifications to ensure proper speed. All centrifugation steps are performed at room temperature. Centrifugation at 4°C will not adversely affect performance.

Cell Lysis and Isolation of Inclusion Bodies (Acidic and Basic Proteins)

1. At the end of the protein induction period, transfer 1.5 mL of the bacterial culture into a microcentrifuge tube.
2. Centrifuge for one minute and discard supernatant.
3. Freeze cell pellet at -20°C or lower using liquid N_2 . Then thaw at room temperature or at 37°C to improve lysis efficiency.
4. Add 200 μL of Cell Lysis Reagent to the bacterial pellet.
5. Assemble a needle with a 1 mL syringe (provided). Carefully disrupt the bacterial pellet by drawing it along with the Cell Lysis Reagent through the needle and ejecting the suspension back into the microcentrifuge tube. Pass through the needle 15 to 20 times.
6. Centrifuge the suspension for 10 minutes and carefully discard supernatant.

Important! This supernatant may be quite viscous. Do not disturb the pelleted material when discarding the supernatant. (The supernatant may be saved in a fresh microcentrifuge tube for comparative analysis of the soluble proteins present in this fraction.)

7. Using the needle-and-syringe technique described in step 5, again add 200 μL of Cell Lysis Reagent to the tube and carefully resuspend the pellet. A few passes through the needle is sufficient to prepare a homogenous suspension. You can use the same needle and syringe from step 5.
8. Prepare a 10-fold dilution of the Cell Lysis Reagent (mix one part of the stock Cell Lysis Reagent to nine parts of sterile deionized or MilliQ water). Add 600 μL of this solution to the suspension prepared in step 7 and pass through the needle a few times.
9. Centrifuge for 10 minutes and discard supernatant.

Important! Use caution to avoid accidental removal of pelleted material.

10. Add 800 μL of the diluted Cell Lysis Reagent to the pellet and resuspend using the needle and syringe until homogeneous.
11. Centrifuge for 10 minutes and discard supernatant.
12. Ensure that the pellet is relatively dry by tapping out residual liquid or by careful use of aspiration.

Protocol Two – Solubilization of Inclusion Bodies (Acidic and Basic Proteins)

The ProteoSpin™ IB solubilization reagent has demonstrated an exceptional ability to dissolve inclusion bodies. This step is necessary before proceeding with the purification of the recombinant protein using the ProteoSpin™ chromatography technology.

Cell lysis and inclusion body isolation must be completed before starting the solubilization process.

1. Add 50 μ L of IB Solubilization Reagent to pelleted inclusion bodies
2. Dissolve the pellet by pipetting and vigorous vortexing.

Note: If the pellet is not completely dissolved, add more IB Solubilization Reagent

3. Once the pellet is dissolved, add 50 μ L of sterile deionized water (or a volume equal to the amount of IB Solubilization Reagent used in step 1), and mix by vortexing.

Purify the recombinant protein of interest, using the basic or acidic purification procedure.

Protocol Three – Purification of Basic Protein

Proteins with isoelectric points (pI) greater than 7 are considered basic; however proteins with a pI greater than or equal to 8 should be treated as basic proteins when using the ProteoSpin™ Inclusion Body Isolation Micro Kit. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based applications at http://us.expasy.org/tools/pi_tool.html or http://www.up.univmrs.fr/~wabim/d_abim/compo-p.html.

Each column is capable of processing up to 50 µg of protein.

Sample Preparation

1. Transfer 50 µL of the dissolved protein sample to a fresh microcentrifuge tube.
2. Add 200 µL deionized or Milli-Q water.
3. Prepare the protein sample by adding 7.5 µL of pH Binding Buffer (basic) to the sample and mix by vortexing.

Column Activation

1. Open the cap on the pre-assembled spin column with its 2 mL collection tube.

Note: The collection tube will be used for the binding and wash steps.

2. Add 250 μL of Column Activation and Wash Buffer (basic) to the column and close the cap.
3. Centrifuge at room temperature or 4°C for one minute at maximum speed (14,000 x g) and discard the flowthrough.
4. Repeat steps 2 and 3 to complete the column activation step.

Protein Binding

During the binding step, the protein solution is passed through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most proteins tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the 257.5 μL of prepared protein sample onto the activated column and centrifuge for one minute.
2. Discard the flowthrough. Reassemble the spin column with its collection tube.

Note: You may want to save the flowthrough to check for binding.

Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 250 μ L of Column Activation and Wash Buffer (basic) to the column and centrifuge for one minute.
2. Discard the flowthrough and reassemble the spin column with its collection tube.
3. Repeat steps 1 and 2.
4. Inspect the column to ensure that all liquid has passed through the resin and into the collection tube. There should not be any liquid in the column. If necessary, spin for an additional minute to dry.

Protein Elution and pH Adjustment

1. Add 5 μ L of Neutralizer to a fresh 1.7 mL microcentrifuge tube.
2. Transfer the spin column from the Column Wash procedure into the microcentrifuge tube.
3. Apply 25 μ L of the Elution Buffer to the column and centrifuge for one minute to elute bound protein.
4. Add another 25 μ L of Elution Buffer and centrifuge for one minute into the same microcentrifuge tube.

Note: Approximately 95% of bound protein is recovered in the first two elutions. If desired, a third elution using 50 μ L of Elution Buffer may be carried out. This should be collected into a different tube (to which 5 μ L of Neutralizer is pre-added) to prevent dilution of the first two elutions.

Protein samples are now ready to be analyzed by SDS-PAGE and stained using Coomassie Blue for visualization.



Protocol Three – Purification of Acidic Proteins

Proteins with isoelectric points (pI) less than 7 are considered acidic; however, proteins with pI of less than 8 may be treated as acidic when using the Inclusion Body Isolation Micro Kit. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based application at http://us.expasy.org/tools/pi_tool.html or http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html.

Each column is capable of processing up to 50 μg of protein.

Sample Preparation

1. Transfer 50 μL of the dissolved protein sample to a fresh microcentrifuge tube.
2. Add 200 μL deionized or Milli-Q water.
3. Prepare the protein sample by adding 7.5 μL of pH Binding Buffer (acidic) to the sample and mix by vortexing.

This step should bring the pI of your sample to 4.5. If the protein of interest has a pI less than 4.5, refer to page 28 for further adjustment details.

Column Activation

1. Open the cap on the pre-assembled spin column with its 2 mL collection tube.
Note: The collection tube will be used for the binding and wash steps.
2. Add 250 μL of Column Activation and Wash Buffer (acidic) to the column and close the cap.
3. Centrifuge for one minute at room temperature or 4°C at maximum speed (14,000 x g) and discard the flowthrough.
4. Repeat steps 2 and 3 to complete the column activation step.

Protein Binding

During the binding step, the protein solution passes through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most proteins tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the 257.5 μL of prepared protein sample onto the activated column and centrifuge for one minute.
2. Discard the flowthrough.

Note: You may want to save the flowthrough to check for binding.



Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 250 μ L of the Column Activation and Wash Buffer (acidic) to the column and centrifuge for one minute.
2. Discard the flowthrough and reassemble the spin column with its collection tube.
3. Repeat steps 1 and 2 to complete the column wash.
4. Inspect the column and ensure that all liquid has passed through the resin and into the collection tube. There should not be any liquid in the column. If necessary, spin an additional minute to dry.

Protein Elution and pH Adjustment

1. Add 5 μL of Neutralizer to a fresh 1.7 mL microcentrifuge tube.
2. Transfer the spin column from the Column Wash procedure into the microcentrifuge tube.
3. Apply 25 μL of Elution Buffer to the column and centrifuge for one minute to elute the bound protein.
4. Add another 25 μL of Elution Buffer and centrifuge for one minute into the same microcentrifuge tube.

Note: Approximately 95% of bound protein is recovered in the first two elutions. If desired, a third elution using 50 μL of Elution Buffer may be carried out. This should be collected into a different tube (to which 5 μL of Neutralizer is pre-added) to prevent dilution of the first two elutions.

5. Protein samples are now ready to be analyzed by SDS-PAGE and stained using Coomassie Blue for visualization.

Optional Elution Solutions

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Proteins bound to SiC via interactions with electrostatic charges are eluted through pH-dependent mechanisms. The efficiency of protein elution depends on high pH above the pI of the protein to be purified. The pH of the elution buffer chosen must be at least one unit higher than the pI (isoelectric point) of the protein of interest. Solutions not provided with the ProteoSpin™ Inclusion Body Isolation kit may be utilized if they are more appropriate for your needs. The table below describes optional elution buffers and their observed efficiency when BSA is used as a test protein.

Optional Elution Buffers

Volatile Elution Buffers	Approximate Protein Recovery (based on 50 µg input BSA)
50 mM ammonium hydroxide (approximate pH 11)	70%
250 mM ammonium hydroxide (approximate pH 11)	70%
1 M ammonium hydroxide (approximate pH 11)	90%
1 M ethanolamine (approximate pH 9)	70-80%

Proteins with Established Isoelectric Points

B

Protein	Molecular Weight (kDa)	Iso-Electric Point (pI)
Albumin, bovine serum	67	5.5
Albumin, human serum	66.5	4.8
Carbonic anhydrase	30	7.3
Carboxypeptidase	34	6.0
Catalase	250	5.6
Cytochrome C	13	10.6
Fibrinogen	330	5.5
Growth hormone, human	21.5	6.9
Hemoglobin, horse	65	6.9
Immunoglobulins G	150	6.4-7.2
Insulin	5.7	5.3
Lysozyme, hen egg white	14.3	11.0
Myoglobin, horse	17	7.0
Ovalbumin	40	4.6
Pepsin	35.5	< 1.0
Ribonuclease	14	7.8
Thyroglobulin	660	4.6
Trypsin inhibitor, soybean	22.5	4.55
Urease	480	5.1



Frequently Asked Questions and Troubleshooting



What is the black powder that is packed into the spin columns?

The black chromatography material is silicon carbide (SiC). It is processed using proprietary methods to function as an ion exchange chromatography resin.

What proteins can be processed using this kit?

All proteins isolated from inclusion bodies (acidic and basic) can be processed using this kit.

What can I do if I do not know the isoelectric point (pI) of my protein of interest?

The isoelectric point of a protein (pI) may be theoretically computed from its amino acid composition through the aid of web-based applications such as http://us.expasy.org/tools/pi_tool.html.

Another site at http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html determines isoelectric points (pI) with considerations for position of residues in the polypeptide chain. The calculated pI should help determine whether the acidic or basic protocol is to be followed.

Alternatively, if there is sufficient protein sample, the protein can be bound and eluted using first the acidic protocol, and then the basic protocol, and the recovery compared to determine which protocol gives better results.

Can the columns be re-used?

The columns are designed for single use only. This minimizes sample carryover.

How should I store the Inclusion Body Isolation Micro Kit solutions?

Upon arrival, the IB Solubilization Reagent and the Cell Lysis Reagent should be stored at 4°C. Once opened, the other solutions should be stored at 4°C when not in use except for the pH binding buffer acidic and basic. If any precipitation of the reagents occurs, warm to room temperature and dissolve crystals prior to use. If unopened, the solution containers can be stored at room temperature.

Do I need to pre-filter my sample before loading it onto the column?

Insoluble materials, if present in the protein solution, should be spun down and the clarified solution applied to the column.

How should I prepare my sample before loading it onto the column?

To 50 µL of the solubilized inclusion body sample, add 200 µL of deionized water and 7.5 µL of pH Binding Buffer. This will ensure that your sample is at the correct pH prior to loading onto the column.

What are the maximum and minimum sample volumes that I can load onto the column?

The sample prepared with the ProteoSpin™ Inclusion Body Screening Kit has a volume of 257.5 µL, which can be fully applied to the columns. If for any reason the actual amount substantially differs, we recommend that no more than 1 mL of sample be loaded onto the column for one spin cycle. This ensures that the flowthrough volume does not exceed the collection tube's capacity. We also recommend that at least 100 µL of the sample is applied to the column as this volume is required to completely cover the resin bed.

What are the highest and lowest recommended column microcentrifuge speeds?

We recommend a speed between 10,000 and 14,000 rpm in microcentrifuges (maximum 15,000 X g). Speeds below 10,000 rpm may be insufficient to completely move the liquid phase through the resin bed. Additional spinning times may be required to remove this liquid.

What is the typical amount of protein recovered that I can expect with the ProteoSpin™ columns?

Between 40 and 50 µg of recombinant protein can be recovered per column for a typical bacterial producer. However, poor producers can have a much lower yield.

Can I autoclave the columns?

No. The columns are not designed to be autoclaved.

How can I tell if my protein bound correctly to the column?

Save your flowthrough from the binding step and perform a protein assay to determine the amount of protein. To eliminate inaccuracies, make sure that the protein standard used is in the same buffer condition as the flowthrough.

I used the recommended binding pH, but my protein still did not bind correctly.

Can I use a different binding pH?

Yes. The binding pH may be adjusted depending on the pI of your protein. It is suggested to start with a pH that is one unit lower than the pI, and if necessary, lowering the pH from there. If the provided pH binding buffer is used, it can be adjusted to a pH as low as 3.7 by titrating it with HCl or glacial acetic acid. For proteins with an even lower pI, the pH binding buffer may be substituted with a 500 mM phosphate buffer (prepared by mixing 9 parts of 500 mM phosphoric acid with 1 part 500 mM trisodium phosphate) and used as a 10-fold concentrate.

What if no pellet is observed after the second spin of Protocol One?

If no pellet is apparent by this stage of the protocol, it is likely that the protein of interest does not form inclusion bodies, or that induction of the expression system was unsuccessful. Review the procedures of the expression system being used. If the soluble fraction of the cell lysate was saved during Protocol One, this can be run on SDS-PAGE to assess presence of the desired protein. If clearly present on the gel, the protein is soluble.

Can the Inclusion Body Isolation Micro Kit be used to screen for tagged proteins?

Yes. The user must take into account the amino acid sequence of the tag when calculating isoelectric points and selecting the proper protocol (acidic or basic).

Why is the supernatant so viscous following the first round of centrifugation?

Nucleic acids are liberated upon cell lysis and contribute to the observed viscosity. This is usually not a problem with subsequent steps of the protocol. However, if viscosity is a persistent problem, an appropriate amount of DNase I can be added to the supernatant.

Am I able to check the activity of my protein using the eluted protein directly?

The protocol has been designed for directly loading the protein samples onto SDS-PAGE gels for analytical purposes only. Other uses of the recovered protein are entirely up to the user. Protein samples recovered from the column may not have refolded properly, and therefore, may not retain their biological activity.

I use a colorimetric protein assay to determine concentration of eluted proteins. Is there an accurate way of doing it?

The colorimetric protein assays available in the market are quite accurate, however, there are a number of ways to improve them. First, using a linear regression to fit the points for the standard curve is often less accurate than using a second-degree polynomial. Microsoft Excel™ has full features for curve fitting. The goodness-of-fit for the regression should determine whether first-degree (linear) or second-degree polynomial would be used. Second, the protein used as the standard should closely match the dye-binding characteristics of the protein of interest. For example, using BSA as a standard to determine concentrations of immunoglobulins may provide erroneous results. This is because immunoglobulins do not bind some dyes efficiently. Third, ensure that the protein standard (and standard curve) is in a solution that resembles the solution of the test protein. Sometimes buffer constituents can have drastic effects on dye binding properties.

The acidic and basic methods are almost identical, except for the solutions used. Why is it necessary to separate the two?

In ion exchange chromatography, it is customary to use either a cation or anion exchanger resin, depending on the type of target protein. For SiC resin, which carries negative charges on its surface, proteins bind through positive charges on their side chains that are exposed on the surface. In theory, the acidic method should suffice to capture all basic and acidic proteins. In practice, however, we find that basic proteins that are bound under acidic conditions are difficult to elute, and therefore decrease the overall performance of the columns. Binding basic proteins at pH 7 reduces column affinity for such proteins, making elution quite efficient.

The binding pH is 4.5, yet my protein has pI of 4. Do I need to adjust the pH of my binding solution to lower than 4.5? If so, how would I do that?

If your protein has a pI lower than 4.5, it is almost always necessary to lower the pH of the protein solution so that it is one pH unit lower than the protein's pI. If the ProteoSpin™ pH Binding Buffer for Acidic Proteins is used, the final pH may be adjusted to as low as 3.75 by titrating with 100 mM HCl. If a lower binding pH is desired, you may use different buffers, such as phosphate ($pK_{a1} = 2.15$). Many acidic proteins tend to precipitate as the pH of the solution approaches their pI. If protein precipitation is apparent (solution becomes cloudy), diluting the solution to a lower protein concentration may solve the problem. Using a binding pH of one-half unit below the protein's pI is also feasible, although the expected recovery for the bound protein will be lower.

Troubleshooting Guide

Each of the following tables discusses a problem which can occur, its possible causes, and presents solutions and explanations.

Problem: No Inclusion Body Pellet is Observed

Possible Causes	Solution & Explanation
Improper induction of gene expression.	Consult the manufacturer's expression system literature.
Gene product does not produce inclusion bodies.	Reassess the expression cassette.

Problem: Inefficient Cell Lysis

Possible Causes	Solution & Explanation
Kit solutions were improperly stored.	Keep the lysis and solubilization reagent at 4 degrees at all times, when not in use. The two binding buffers are kept at room temperature.
Freeze / thaw step was not performed.	The freeze / thaw step is known to increase lysis efficiency. Repeat the protocol using the recommended freeze / thaw conditions.
Lysozyme may be required to increase lysis efficiency.	Add lysozyme to concentrations recommended by the supplier.
Mechanical disruption of cells was inefficient.	Increase the number of passes through the needle and syringe.

Problem: Protein Solution Does Not Flow Through the Column

Possible Causes	Solution & Explanation
Centrifugation speed was too low.	Check the centrifuge to ensure that it is capable of generating $14,000 \times g$. Sufficient centrifugal force is required to move the liquid phase through the resin.
Inadequate spin time.	Spin an additional minute to ensure that the liquid is able to flow completely through the column.
Protein solution is too viscous.	Dilute the protein solution and adjust the pH accordingly. Highly viscous materials due to high protein concentration can retard the flow rate.
Cellular debris is present in protein solution.	Filter the sample in a $0.45 \mu\text{m}$ filter or spin down insoluble materials and transfer the liquid portion to the column. Solid, insoluble materials can cause severe clogging problems.
Protein solution is not completely dissolved.	Dissolve the sample in a larger volume of buffer. Solid, insoluble materials can cause severe clogging problems.



Problem: Supernatant Following First Spin of Cell Lysis is Too Viscous

Possible Causes	Solution & Explanation
Liberation of nucleic acids following lysis.	Increase the degree of mechanical disruption by passing bacteria / lysis reagent through the needle at least five more times. Alternatively, add an appropriate amount of DNaseI.

Problem: Poor Protein Recovery

Possible Causes	Solution & Explanation
Incorrect procedure was used.	Ensure that the acidic protocol was used for an acidic protein, and the basic protocol for a basic protein. It is known that when basic proteins are bound using the acidic protocol, elution is inefficient because the basic proteins are bound tightly.
Incorrect pH adjustment of sample.	Ensure that the pH of the sample is 4.5 for acidic proteins and 7.0 for basic proteins.
Protein may have precipitated prior to loading onto the column	If the pH of the protein solution is the same as the <i>pI</i> of your protein, precipitation may occur. In this case, adjust the pH of your sample to at least one pH unit lower than the <i>pI</i> of your protein.

Problem: Eluted Protein is Degraded

Possible Causes	Solution & Explanation
Eluted protein solution was not neutralized.	Add 5 μ L of Neutralizer to each 50 μ L of eluted protein in order to adjust the pH to neutral. Some proteins are sensitive to high pH, such as the elution buffer at pH 12.5.
Eluted protein solution was not neutralized quickly enough.	If eluted protein is not used immediately, degradation will occur. We strongly suggest adding Neutralizer in order to lower the pH.
Proteases may be present	Use protease inhibitors during all steps of sample preparation.
Bacterial contamination of the protein solution.	Prepare the protein samples with 0.015 % sodium azide. The Elution Buffer already contains sodium azide.

Problem: Too Many Gel Bands

Possible Causes	Solution & Explanation
Inefficient cell lysis.	See the "Problem: Inefficient Cell Lysis" table of page 33



Other Kits:

- Concentration, Desalting and Buffer Exchange Micro Kit
- Detergent Clean-up Micro Kit

Notes:

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