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Inclusion Body Isolation Micro Kit — Product Insert

Product # 10300, 10600

The ProteoSpin™ Inclusion Body Isolation Micro Kit facilitates the screening of *E. coli* clones that express recombinant proteins in inclusion bodies. The kit includes reagents specially formulated to achieve rapid and high-quality purification of inclusion body proteins using three processes:

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

With optimized reagents and streamlined processes, the ProteoSpin™ Inclusion Body Isolation Micro Kit significantly reduces time and labour for screening and identifying clonal cell lines that can be used in scale-up production. The ProteoSpin™ Inclusion Body Isolation Micro Kit employs spin-column chromatography using Norgen's proprietary resin as an ion-exchanger. Each spin column is able to purify 2 – 50 µg of recombinant proteins from 1.5 mL of culture. The kit is designed to purify both acidic and basic proteins.

Kit Components

Component	Product # 10300 (20 Samples)	Product # 10600 (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, 1cc, slip tip	20	50
Needles (Bev, 20G x 1 inch)	20	50
Micro Spin Columns	20	50
Collection Tubes	20	50
Elution tubes (1.7 mL)	20	50
Product Insert	1	1

Storage Conditions and Product Stability

The Cell Lysis and IB Solubilization Reagents should be stored at 4°C upon receipt of this kit. For other unopened solution containers, the reagents should remain stable for 1 year when stored at room temperature. Once opened, the solutions should be stored at 4°C when not in use except for the pH Binding Buffers (Acidic and Basic). Some precipitation will occur with 4°C storage. This precipitation should be dissolved with slight heating to room temperature before using.

Precautions and Disclaimers

This kit is designed for research purposes only. It is not intended for human or diagnostic use.

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

Customer-Supplied Reagents and Equipment

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

- Benchtop microcentrifuge
- Micropipettors
- Sterile, deionized water or Milli-Q® water

Procedure

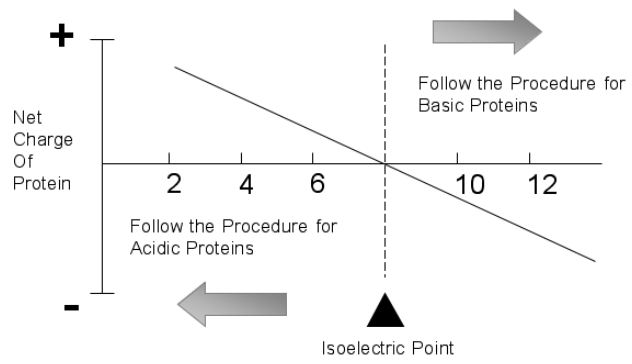
This section describes the procedure for the ProteoSpin™ Inclusion Body Isolation Micro Kit and how to select the appropriate protocol for your sample.

Overview

The Proteospin™ 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 recovered using the elution buffer. Each spin column is able to recover up to 50 µg of acidic or basic protein.

Choosing a ProteoSpin™ Procedure: Acidic or Basic 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 ≥ 8), a different condition for binding the protein to the resin has been developed. For the purposes of the Proteospin™ 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 greater than or equal to 8, use the basic protocol.



Protocols

To rapidly screen bacterial clones for expression of recombinant proteins in inclusion bodies, the ProteoSpin™ 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 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 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 homogeneous 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 water 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 µL of IB Solubilization Reagent to pelleted inclusion bodies.

2. Dissolve the pellet by pipetting and vortexing.

Note: If the pellet is not completely dissolved after ample pipetting and vortexing, add more IB Solubilization Reagent.

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

Now purify the recombinant protein of interest using either the acidic or basic purification procedure.

Protocol Three - Purification of Basic Inclusion Body Proteins

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 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 (Basic) to the sample and mix by vortexing.

Column Activation

1. Assemble a spin column with a provided collection tube. Open the cap on the column

   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 for 1 minute at maximum speed and discard the flowthrough.

4. Repeat steps 2 and 3 to complete the column activation step.

Protein Binding

1. Apply the 257.5 µL of prepared protein sample (from the Sample Preparation step) onto the activated column and centrifuge for 1 minute.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: The flowthrough can be saved in a fresh tube (not supplied) to asses the binding efficiency of the protein.

Column Wash

1. Apply 250 µL of Column Activation and Wash Buffer (Basic) to the column and centrifuge for 1 minute.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Repeat steps 1 and 2.

4. Inspect the column and ensure that all the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin for an additional minute to dry.

Protein Elution and pH Adjustment
The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. Please refer to Appendix 1 (Optional Elution Buffers) for a list of alternate elution solutions that have been tested with the kit.

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 1 minute to elute bound protein.

4. Add another 25 µL of Elution Buffer and centrifuge for 1 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.

Protein samples are now ready for downstream applications.

Protocol Three - Purification of Acidic Inclusion Body 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 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 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.

Column Activation

1. Assemble a spin column with a provided collection tube. Open the cap on the column

   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 1 minute at maximum speed and discard the flowthrough.

4. Repeat steps 2 and 3 to complete the column activation step.

Protein Binding

1. Apply the 257.5 µL of prepared protein sample (from the Sample Preparation step) onto the activated column and centrifuge for 1 minute.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: The flowthrough can be saved in a fresh tube (not supplied) to asses the binding efficiency of the protein.

Column Wash

1. Apply 250 µL of Column Activation and Wash Buffer (Acidic) to the column and centrifuge for 1 minute.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Repeat steps 1 and 2.

4. Inspect the column and ensure that all the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin for an additional minute to dry.

Protein Elution and pH Adjustment
The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. Please refer to Appendix 1 (Optional Elution Buffers) for a list of alternate elution solutions that have been tested with the kit.

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 1 minute to elute bound protein.

4. Add another 25 µL of Elution Buffer and centrifuge for 1 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.

Protein samples are now ready for downstream applications.

Troubleshooting Guide

Problem	Causes	Solution and Explanation
No inclusion body pellet is observed	Improper induction of gene expression.	Consult the manufacturer's expression system literature.
	Gene product does not produce inclusion bodies.	Reassess the expression cassette.
Inefficient Cell Lysis	Kit solutions were improperly stored.	Keep the lysis and solubilization reagent at 4°C 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 efficieny.	Add lysozyme to concentrations recommended by the supplier.
	Mechanical disruption of cells was inefficient.	Increase the number of passages through the needle and syringe.
Protein Solution Does Not Flow Through the Column	Centrifugation speed was too low.	Check the centrifuge to ensure that it is capable of generating 14,000 x 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 µM 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.
Supernatant Following First Spin of Cell Lysis is Too Viscous	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.
Eluted Protein Forms Precipitate	Protein too concentrated.	Vortex and repeatedly pipette to try and create a homogeneous solution. If needed, heat slightly to return the protein into solution.
	pH of eluted protein is close to the protein's pI.	Check the pH to verify it is the same as the pI of the protein. Add additional Neutralizer to bring the pH away from the pI of the protein (~1 pH lower or higher).

Problem	Causes	Solution and Explanation
Poor Protein Recovery	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 sample is the same as the pI of your protein, precipitation may occur. In this case, adjust the pH of your sample to at least one pH unit lower than the pI of your protein.
Eluted Protein is Degraded	Eluted protein solution was not neutralized.	Add 5 µL of Neutralizer to each 50 µL 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 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.
Too Many Gel Bands	Inefficient cell lysis.	See the "Problem: Inefficient Cell Lysis" table.

Appendix 1

Optional Elution Buffers

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 Micro 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.

Elution Buffers	Approximate Protein Recovery
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%
50 mM sodium phosphate (approximate pH 12.5)	95%
500 mM sodium phosphate (approximate pH 12.5)	<70%
100 mM sodium borate (approximate pH 12.5)	95-100%
1 M Tris (approximate pH 12.5)	95%

Appendix 2

Proteins with Established Isoelectric Points

Protein	Molecular Weight (kDa)	Isoelectric 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
Immunoglobulin 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

Related Products	Product #
ProteoSpin™ Inclusion Body Isolation Maxi Kit (4 samples)	17700
Inclusion Body Solubilization Reagent (25 mL, 100 mL)	18700, 18701
Cell Lysis Reagent (100 mL, 500 mL)	18800, 18801

Technical Support
Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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