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Inclusion Body Isolation Maxi Kit — Product Insert

Product # 17700

The ProteoSpin™ Inclusion Body Isolation Maxi Kit facilitates the isolation of recombinant proteins in the form of inclusion bodies from *E. coli*. 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 Maxi Kit significantly reduces time and labour for isolating and purifying proteins of interest in a larger-scale format. The ProteoSpin™ Inclusion Body Isolation Maxi Kit employs spin-column chromatography using Norgen's proprietary resin as an ion-exchanger. Each spin column is able to purify up to 12 mg of recombinant proteins from 100 mL of culture. The kit is designed to purify both acidic and basic proteins.

Kit Components

Component	Product # 17700 (4 samples)
Column Activation and Wash Buffer (Acidic)	130 mL
Column Activation and Wash Buffer (Basic)	130 mL
pH Binding Buffer (Acidic)	3 mL
pH Binding Buffer (Basic)	20 mL
Elution Buffer	50 mL
Neutralizer	3 mL
Cell Lysis Reagent	110 mL
IB Solubilization Reagent	50 mL
Syringes (10 mL, Luer-Lok™ Tip)	4
Needles (18G x 1½ inch)	4
Maxi Spin Columns (filled with SiC) inserted into 50 mL collection tubes	4
Final Elution Tubes, 50 mL	4
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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 2 years 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 Maxi Kit:

- Benchtop centrifuge (capable of spinning 50 mL conical tubes)
- Centrifuge (capable of spinning 40 mL centrifuge tubes up to 27,000 x g and 250 mL centrifuge bottles up to 15,000 x g)
- pH Indicator Paper
- Micropipettors
- Sterile, deionized water or Milli-Q® water

Procedure

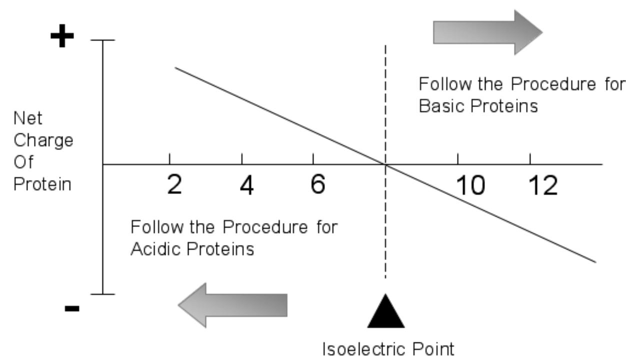
This section describes the procedure for the ProteoSpin™ Inclusion Body Isolation Maxi Kit and how to select the appropriate protocol for your sample.

Overview

The Proteospin™ Inclusion Body Isolation Maxi 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 12 mg 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 Maxi 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

The Proteospin™ Inclusion Body Isolation Maxi Kit is designed for testing larger volume cultures for the purposes of rapidly screening bacterial clones for expression of recombinant proteins in inclusion bodies and the subsequent purification of the proteins. Generally, starter cultures with 2 mL culture medium are initiated by inoculation with single colonies picked from culture plates. These starter cultures are then used to inoculate 100 mL of culture medium in a shake-flask.

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 and for repeating experiments, it is recommended that the user preserve stocks of uninduced bacteria for each clone tested.

Please note that centrifugation steps are carried out at various speeds throughout the procedure in two different centrifuges. Please check your centrifuge 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 100 mL of the bacterial culture into a 250 mL centrifuge bottle.
2. Centrifuge for five minutes at 15,000 x g and discard supernatant.

Note: Use of a 250 mL centrifuge bottle may be eliminated by initially spinning the bacterial culture in a 40 mL centrifuge tube. This would require three spins of five minutes each at 15,000 x g to accommodate the 100 mL of culture.

3. Freeze pellet at -20°C or lower using liquid N_2.

Note: This can be a convenient place to stop the protocol overnight. The procedure can then be carried on from this point the next day.

4. Thaw pellet at room temperature or 37°C to improve lysis efficiency.
5. Add 15 mL of Cell Lysis Reagent to the bacterial pellet.
6. Assemble a needle with a 10 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 centrifuge bottle. Pass through the needle 10 to 15 times.
7. Transfer resuspension into a 40 mL centrifuge tube and centrifuge for 5 minutes at 27,000 x g. 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 15 mL conical tube for comparative analysis of the soluble proteins present in this fraction.)

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 Milli-Q® water).
9. Using the needle-and-syringe technique described in step 6, add 5 mL of Cell Lysis Reagent and 10 mL of the diluted Cell Lysis Reagent to the tube and carefully resuspend the pellet. Approximately 10 passes through the needle should be sufficient to prepare a homogeneous suspension. You can use the same needle and syringe from step 6.
10. Centrifuge for 5 minutes at 27,000 x g and discard supernatant.

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

11. Add 15 mL of the diluted Cell Lysis Reagent to the pellet and resuspend using the needle and syringe until homogeneous.
12. Centrifuge for 5 minutes at 27,000 x g and discard supernatant.
13. 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 use in the ProteoSpin™ chromatography technology.
Cell lysis and inclusion body isolation must be completed before starting the solubilization process.

1. Add 10 mL 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, incubate on a rocker at room temperature to aid solubilization.

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

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 Maxi 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 12 mg of protein.

A. Sample Preparation

1. Transfer all 10 mL of the dissolved protein sample to a fresh 50 mL conical tube (not supplied).
2. Add 9.55 mL deionized or Milli-Q® water.
3. Prepare the protein sample by adding 450 µL of pH Binding Buffer (Acidic) to the sample and mix by inversion.

   Note: This step should bring the pH of your sample to 4.5. If the protein of interest has a pI less than 4.5, refer to the Frequently Asked Questions for further adjustment details.

B. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Unscrew the cap.
2. Apply 5 mL of Column Activation and Wash Buffer (Acidic) to the column. Screw the cap back on **LOOSELY**.
3. Centrifuge at 1,000 x g for two minutes.

Note: Start timing spins only after centrifuge has reached desired speed.

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

C. Protein Binding

1. Apply the protein sample (from the Sample Preparation step) onto the column and centrifuge for five minutes at 1,000 x g.
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.

D. Column Wash

1. Apply 10 mL of Column Activation and Wash Buffer (Acidic) to the column and centrifuge for two minutes at 1,000 x g.
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 the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

E. Protein Elution

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. The proteins in the buffer should be neutralized immediately following elution. It is therefore recommended that the Neutralizer that is provided be added to the tube directly after elution.

1. Transfer the spin column, with bound protein, into the 50 mL elution tube (supplied).
2. Apply 8 mL of Elution Buffer to column and centrifuge for 2 minutes at 1,000 x g to elute bound protein.
3. Add 200-300 µL Neutralizer to the eluted protein and mix.

Note: Verify that the pH of the adjusted elution sample is approximately pH 7-9. If necessary, add additional Neutralizer to achieve a neutral pH. If slight precipitation occurs during elution or after neutralization, simply resuspend by vortexing. If precipitation persists, it may be due to the pH of the eluted protein solution being close to the pI of the protein of interest. In this case, adjust the pH of the eluted protein to ~ 1 pH unit higher or lower than the protein's pI. It may be desirable to determine the pH of the eluted protein solution before neutralizing and add Neutralizer until the appropriate pH (neutral or ~1 pH unit higher or lower than the protein's pI) is achieved.

4. A second elution is optional. Repeat steps 1 to 3 if desired, using only 2 mL of Elution Buffer and 170 µL of Neutralizer. Keep separate from first elution.

Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution using Elution Buffer may be carried out. This should be collected into a different tube to prevent dilution of the first two elutions.

Protein samples are now ready for downstream applications.

Protocol Three - Purification of Basic Inclusion Body Proteins
Proteins with isoelectric points (pI) greater than 7 are considered basic; however, proteins with pI greater than or equal to 8 should be treated as basic when using the Proteospin™ Inclusion Body Isolation Maxi 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 12 mg of protein.

A. Sample Preparation

1. Transfer all 10 mL of the dissolved protein sample to a fresh 50 mL conical tube (not supplied).
2. Add 36.5 mL deionized or Milli-Q® water.
3. Prepare the protein sample by adding 3.5 mL of pH Binding Buffer (Basic) to the sample and mix by inversion.

B. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Unscrew the cap.
2. Apply 5 mL of Column Activation and Wash Buffer (Basic) to the column. Screw the cap back on **LOOSELY**.
3. Centrifuge at 1,000 x g for two minutes.

Note: Start timing spins only after centrifuge has reached desired speed.

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

C. Protein Binding

1. Apply up to 20 mL of the protein sample (from the Sample Preparation step) onto the column and centrifuge for five minutes at 1,000 x g.
2. Discard the flowthrough. Reassemble the spin column with its collection tube.
3. Repeat steps 1 and 2 until the entire protein sample has been applied to the column.

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

D. Column Wash

1. Apply 10 mL of Column Activation and Wash Buffer (Basic) to the column and centrifuge for two minutes at 1,000 x g.
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 the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

E. Protein Elution

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. The proteins in the buffer should be neutralized immediately following elution. It is therefore recommended that the Neutralizer that is provided be added to the tube directly after elution.

1. Transfer the spin column, with bound protein, into the 50 mL collection tube (supplied).
2. Apply 6 mL of Elution Buffer to column and centrifuge for 2 minutes at 1,000 x g to elute bound protein.
3. Add 300 µL Neutralizer to the eluted protein and mix.

   Note: Verify that the pH of the adjusted elution sample is approximately pH 7-9. If necessary, add additional Neutralizer to achieve a neutral pH. If slight precipitation occurs during elution or after neutralization, simply resuspend by vortexing. If precipitation persists, it may be due to the pH of the eluted protein solution being close to the pI of the protein of interest. In this case, adjust the pH of the eluted protein to ~ 1 pH unit higher or lower than the protein's pI. It may be desirable to determine the pH of the eluted protein solution before neutralizing and add Neutralizer until the appropriate pH (neutral or ~1 pH unit higher or lower than the protein's pI) is achieved.

4. A second elution is optional. Repeat steps 1 to 3 if desired, using only 2 mL of Elution Buffer and 200 µL of Neutralizer. Keep separate from first elution.

   Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution using Elution Buffer may be carried out. This should be collected into a different tube 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 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 the required speed for a particular step. Sufficient centrifugal force is required to move the liquid phase through the resin.
	Inadequate spin time.	Spin an additional 2 minutes 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.	Repeatedly vortex and pipette to try and dissolve inclusion bodies. If needed, incubate on rocker at room temperature to aid in solubilization. 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 an appropriate amount of Neutralizer to the eluted protein. 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 Maxi 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 Micro Kit (20 samples)	10300
ProteoSpin™ Inclusion Body Isolation Micro Kit (50 samples)	10600

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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