INSTRUCTIONS

Pierce™ GST Protein Interaction Pull-Down Kit

21516

1342.6

Number	Description
21516	**Pierce GST Protein Interaction Pull-Down Kit,** contains sufficient materials for conducting 25 pull-down assays using a GST-tagged fusion protein as the bait

Kit Contents:

Glutathione Agarose, 750µL settled resin (1.5mL of 50% slurry in preservative buffer); binding capacity ≥ 10mg GST-tagged protein per milliliter of settled resin

Pull-Down Lysis Buffer, 250mL

Glutathione, 1g

BupH™ Tris Buffered Saline, 1 pack, 25mM Tris•HCl, 0.15M NaCl, pH 7.2 when reconstituted with 500mL of ultrapure water

Pierce Spin Columns Accessory Pack, 27 columns with pre-inserted frit and top and bottom caps

Collection Tubes and Caps Accessory Pack, 100 graduated 2mL tubes and plug caps

Storage: Upon receipt store Pull-Down Lysis Buffer at room temperature. Store remaining kit components at 4°C. Kit is shipped at ambient temperature.

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Introduction

Elucidating gene function involves determining the function of each gene's encoded protein product. In the cell, proteins participate in extensive networks of protein:protein interactions. These interactions take the form of dynamic "protein machines", which assemble and disassemble in concert with an ever-changing influx of intra-, inter- and extracellular cues.[1] A preliminary step in understanding protein structure and function is to determine which proteins interact with each other, thereby identifying the relevant biological pathways. These pathways are further dissected for structure and function by experimental procedures such as epitope mapping, X-ray crystallography and enzymology of functional protein complexes.

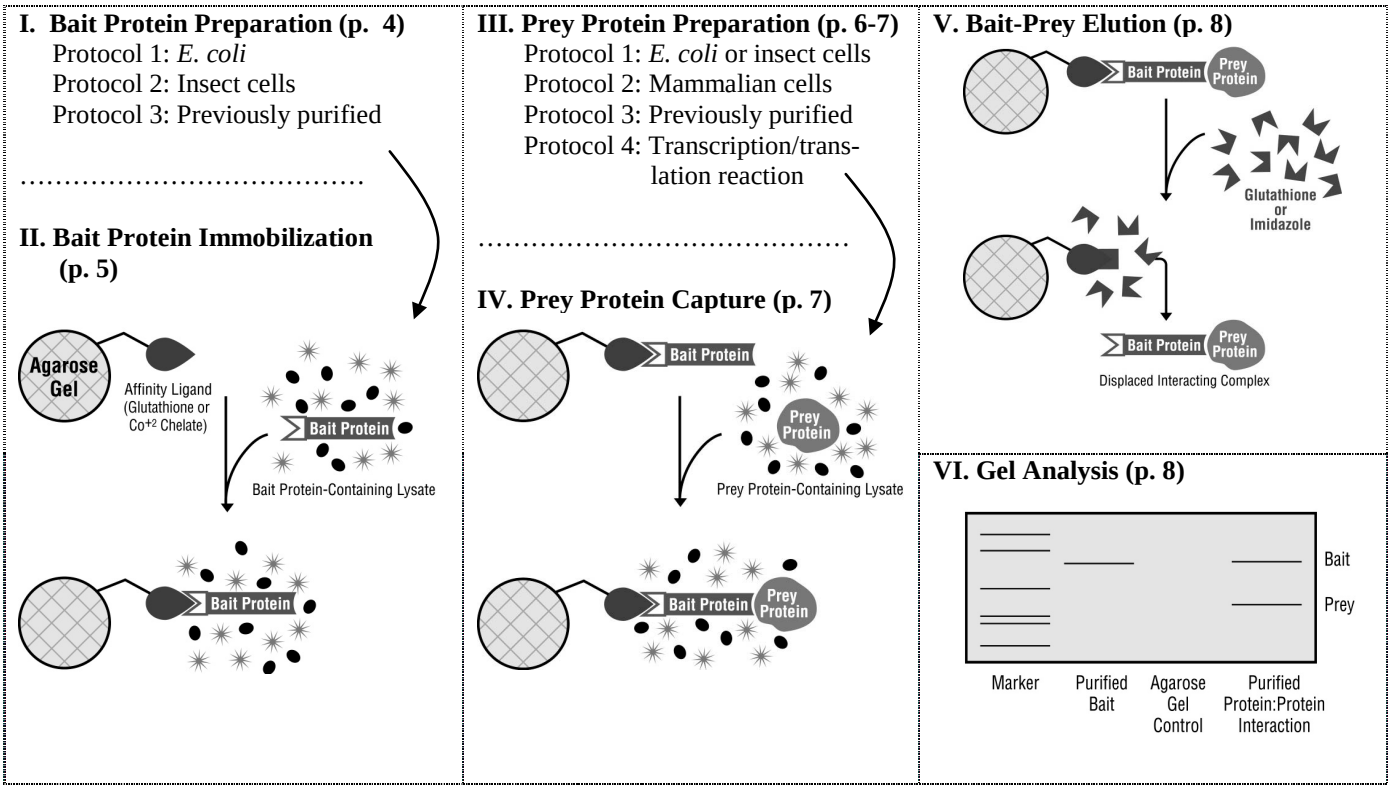
The glutathione S-transferase (GST) pull-down technique has become an invaluable tool for the life scientist interested in protein chemistry.[2] The basic pull-down assay is an *in vitro* technique that consists of a GST-tagged bait protein (the researcher's protein of interest) that can be used to identify putative binding partner(s) (the prey). The bait protein, purified from an appropriate expression system (e.g., *Escherichia coli* or baculovirus-infected insect cells), is immobilized to glutathione affinity resin. The bait serves as the secondary affinity support for confirming a previously suspected protein partner or for identifying new protein partners to the bait. Prey protein can be obtained from multiple sources, including previously purified proteins, cell lysates or *in vitro* transcription/translation reactions. Protein:protein interactions can be visualized by SDS-PAGE and associated detection methods depending on the sensitivity requirements of the interacting proteins. These methods include coomassie, silver and zinc staining; Western blotting; and [^{35}S] radioisotopic detection.[3] Experiments designed to identify new protein:protein interactions often entail protein band isolation from a polyacrylamide gel, tryptic digestion of the isolated protein and mass spectrometric identification of digested peptides.

Homemade pull-down methodologies for confirming or identifying protein:protein interactions are present in contemporary scientific literature.[4,5,6] The homemade pull-down assay represents a collection of reagents from multiple commercial vendors that cannot be validated together as a functional assembly until the researcher incorporates them into a complete protocol. Troubleshooting this mix of reagents can be tedious and time-consuming. The Thermo Scientific™ Pierce™ GST Protein Interaction Pull-Down Kit contains a complete, validated set of reagents specifically developed for performing pull-down assays. The kit format is highly flexible, allowing complete control in optimizing experimental conditions specific to the requirements of each unique protein:protein interaction.

Important Product Information

- It is often useful to establish experimental evidence that a protein of interest interacts with other proteins before attempting to perform a pull-down assay for binding partners. Several methods, including yeast two-hybrid, density gradient centrifugation and indirect immunofluorescence microscopy are valuable in predicting whether the protein of interest is involved in a complex with other proteins.
- Detailed protocols necessary to clone a gene with a GST tag or to express the GST-tagged gene product are available in numerous molecular biology and recombinant protein handbooks.[3]
- For best results, determine optimal conditions for overexpression of soluble GST-tagged fusion protein before attempting pull-down.
- Negative controls are absolutely necessary for generating biologically significant results. A non-treated gel control (minus bait, plus prey) helps identify and eliminate false positives caused by nonspecific binding of proteins to the immobilized glutathione. The immobilized bait control (plus bait, minus prey) helps identify and eliminate false positives caused by nonspecific binding of proteins to the GST tag of the bait protein. The immobilized bait control also serves as a positive control that verifies the Glutathione Agarose affinity resin can successfully capture the GST-tagged bait protein.
- This kit incorporates the Pierce Spin Column format for handling small volumes of Glutathione Agarose affinity resin. This format allows complete retention of resin during the pull-down assay and eliminates variability between experiments that results from resin loss (See Appendix C).[7]
- The buffer provided in the kit allows complete flexibility to determine optimal conditions for isolating interacting proteins. Characteristics of interacting proteins (See Appendix A) may require alteration of the binding and wash buffers by adding components such as protein cofactors or additional salts and detergents. However, different interacting pairs may require less stringent conditions and fewer binding reaction components. The working solution for washing and binding, a 1:1 mix of TBS to Pull-Down Lysis Buffer, is a physiologically neutral, Tris buffered solution of 75mM NaCl with 1% of a proprietary nonionic detergent. This buffer provides a starting point from which specific buffer conditions for each unique interacting pair can be optimized.

Procedure Summary



Procedure for Pierce GST Pull-Down Assay

Note: This kit and these instructions may be adapted to different formats depending on the source of bait and prey protein. The protocols are general guidelines and will require varying degrees of experimental analysis to determine optimal conditions for specific interaction pairs. Sufficient Collection Tubes are provided to perform the pull-down assay as described. More vigorous analysis of experimental conditions may require additional tubes and caps. Many tube styles will accommodate the spin columns. Rotor dimensions of each microcentrifuge will dictate the collection tube style that can be substituted. A 2mL collection tube (with a detached plug-style cap) allows sufficient clearance between the bottom opening of the Pierce Spin Column and the solution retained in the Collection Tube.

Additional Materials Required

- Pipettes and disposable tips that can accurately deliver 2µL-1mL
- Vortex mixer
- 0.2µm, 500mL filter sterilization unit
- 1.5mL microcentrifuge tubes
- 2mL collection tubes
- Microcentrifuge capable of 12,000 × *g*
- Analytical balance capable of milligram quantitation

Buffer Preparation

Reconstitute contents of the BupH Tris Buffered Saline (TBS) pack with 500mL of ultrapure water. Filter sterilize solution using a 0.2µm filter apparatus and store at 4°C. When stored properly, the reconstitution of one pack of BupH TBS is sufficient for 25 pull-down assays.

I. Bait Protein Preparation

Choose one of the following three protocols for GST-tagged bait protein preparation; then proceed to **Section II. Bait Protein Immobilization** on the next page.

Protocol 1: Bait from *E. coli* Expression Systems

1. Grow and transform *E. coli* according to standard protocols.
2. Transfer 5mL of IPTG-induced *E. coli* culture to a sterile centrifuge tube.

   Note: This amount can be increased if the GST-tagged fusion protein exhibits low expression levels. Five milliliters of culture grown to OD_{600} = 1.0 (or greater) will suffice if the overexpressed GST-tagged fusion protein forms a distinguishable band when lysate is analyzed by SDS-PAGE and coomassie staining.
3. Centrifuge at 5000 × *g* for 5 minutes and discard culture supernatant.
4. Resuspend pellet in 1mL of TBS per 5mL of original culture volume. Mix using a pipette or vortex mixer.
5. Transfer 1mL of cell suspension to a 1.5mL microcentrifuge tube.
6. Centrifuge at 5000 × *g* for 5 minutes and discard supernatant.
7. Resuspend pellet in 200µL of ice-cold TBS per 5mL of original culture volume. Mix using a pipette or vortex mixer. Add protease inhibitors if desired. For optimal results, use protease inhibitor cocktail when preparing cell lysate.
8. Add 200µL of Pull-Down Lysis Buffer per 5mL of original culture volume. Immediately invert until thoroughly mixed.

   Note: The Pull-Down Lysis Buffer will not solubilize inclusion bodies. For inclusion body solubilization, use Thermo Scientific Inclusion Body Solubilization Reagent (Product No. 78115).
9. Incubate on ice for approximately 30 minutes. Invert tubes periodically.

   Note: Continue with **Section II. A. Equilibrate Glutathione Agarose** while performing this incubation.
10. Centrifuge at 12,000 × *g* for 5 minutes to clarify crude *E. coli* lysate.
11. Decant supernatant to a separate microcentrifuge tube and store on ice. Label this tube “bait lysate.”

Protocol 2: Bait from Insect Cell Expression Systems

1. Grow and infect insect cells according to standard protocols.
2. Transfer insect cell culture to a sterile centrifuge tube.
3. Centrifuge at 500 × *g* for 5 minutes and discard culture supernatant.
4. Add 1mL of ice-cold TBS per 5mL of cultured insect cells. Invert tube several times to thoroughly suspend cells.
5. Transfer cell suspension to an appropriately sized centrifuge tube.
6. Centrifuge at 500 × *g* for 5 minutes and discard wash supernatant.
7. Resuspend insect cell pellet with 2.5 volumes of ice-cold TBS per gram wet weight of insect cell pellet. Invert tube several times to thoroughly mix cells. For optimal results, use protease inhibitor cocktail when preparing cell lysate.
8. Add 2.5 volumes of Pull-Down Lysis Buffer per gram wet weight of insect cell pellet and immediately invert until thoroughly mixed.
9. Incubate on ice for approximately 30 minutes. Invert tubes periodically.

   Note: Continue with **Section II. A. Equilibrate Glutathione Agarose** while performing this incubation.
10. Centrifuge at 12,000 × *g* for 5 minutes to clarify crude insect cell lysate.
11. Decant supernatant to a new microcentrifuge tube and store on ice. Label this tube “bait lysate.”

Protocol 3: Bait from Previously Purified Protein

1. Remove reduced glutathione from previously purified protein sample by dialysis against TBS.
2. Determine protein concentration of GST-tagged fusion protein sample using a protein assay method such as the Thermo Scientific™ BCA Protein Assay Kit (Product No. 23227) or Coomassie Plus™ (Bradford) Protein Assay Kit (Product No. 23236).

II. Bait Protein Immobilization

A. Equilibrate Glutathione Agarose

1. Label a sufficient number of Pierce Spin Columns to include a sample, non-treated resin control and immobilized bait control for each experiment.
2. Prepare a 1:1 wash solution of TBS:Pull-Down Lysis Buffer. For each spin column prepare approximately 8mL of wash solution.
3. Thoroughly resuspend the Glutathione Agarose resin using a vortex mixer. Pipette 50µL of the slurry into each labeled spin column. (For best results, use a cut or wide-bore pipette tip.)

   Note: Glutathione Agarose is supplied as a 50% slurry. Settled resin volume per assay is 25µL.
4. Add 400µL of the wash solution to each spin column. Cap both ends of the column and invert several times to equilibrate Glutathione Agarose resin. Remove both caps and place spin column in a Collection Tube.
5. Centrifuge at 1250 × *g* for 30 seconds to 1 minute. Replace bottom cap. Discard wash solution from Collection Tube and reinsert spin column.

   Note: Wash solution can be retained for analysis, but this will require additional collection tubes. (See note under Procedure for Pierce GST Pull-Down Assay, p. 3.)
6. Repeat wash Steps 4 and 5 for a total of 5 washes.

B. Immobilize Bait Protein

1. Apply bottom cap and remove top cap for each Pierce Spin Column.
2. Add prepared GST-tagged fusion protein (BAIT) to the Pierce Spin Column.
   - For cell lysates (Section I, Protocol 1or 2), add at least 300µL of GST-tagged fusion protein lysate.

     Note: Retain sufficient lysate to analyze the fusion protein expression by SDS-PAGE. If desired, determine lysate protein concentration using a BCA Protein Assay Kit (Product No. 23227) or Coomassie Plus (Bradford) Protein Assay Kit (Product No. 23236).
   - For previously purified GST-tagged fusion protein (Section I, Protocol 3), use a sufficient volume to ensure addition of approximately 100-150µg of bait protein. (The Pierce Spin Column has a maximum capacity of ~800µL.)

   Note: 25µL of settled Glutathione Agarose resin can bind up to 200µg of GST-tagged protein. However, GST-tagged fusion proteins may exhibit lower binding capacity because of conformational differences. Additionally, larger fusion proteins may cause steric hindrance, blocking some immobilized glutathione sites from binding to the GST tag.
3. Replace top cap of each column.
4. Incubate at 4°C for at least 30 minutes with gentle rocking motion on a rotating platform. Maximal binding may require a longer incubation time, which should be determined for each new GST-tagged fusion protein.

   Note: Continuing with **Section III. Prey Protein Preparation** while performing this incubation will shorten the total time for protocol completion.
5. Remove both caps from each column and place each in a Collection Tube.
6. Centrifuge at 1250 × g for 30 seconds to 1 minute. Label this tube “bait flow-through” and place on ice.
7. Replace bottom cap on spin column.

8. Add 400µL of wash solution and replace top cap. Invert several times to mix thoroughly.

   Note: Multiple washes with resin inversion are required to effectively eliminate nonspecific interactions. For additional information regarding interaction characteristics and wash requirements, see Appendix A, p. 9.

9. Remove both caps and place spin column in the Collection Tube.
10. Centrifuge at 1250 × g for 30 seconds to 1 minute. Label Collection Tube "wash," discard the wash volume and reuse tube for all wash collections.
11. Repeat wash steps 7-10 for a total of five washes.

III. Prey Protein Preparation

Choose one of the following four protocols for prey protein sample preparation; then, when bait protein has been prepared and immobilized (Sections I and II), proceed to **Section IV. Prey Protein Capture**.

Protocol 1: Prey Protein from *E. coli* or Baculovirus-Infected Insect Cell Lysate

For best results, determine optimal conditions for overexpression of soluble prey protein before attempting pull-down procedure. The confirmation of suspected protein:protein interactions may be validated using expression lysate proteins as the prey protein; however, identification of novel protein:protein interactions is not recommended.

1. Grow and transform *E. coli* or grow and infect insect cells according to standard protocols.

   Transfer cell culture to a sterile centrifuge tube. (Amount can be increased depending on expression level of protein.)

2. Centrifuge for 5 minutes and discard supernatant. (Centrifuge *E. coli* at 5000 × *g*. Centrifuge insect cells at 500 × *g*.)
3. Add 1mL of TBS per 5mL of cell culture. Mix using a pipette or vortex mixer.
4. Transfer cell suspension to a sterile centrifuge tube.
5. Centrifuge for 5 minutes and discard supernatant. (Centrifuge *E. coli* at 5000 × *g*. Centrifuge insect cells at 500 × *g*.)
6. Resuspend *E. coli* with 200µL of ice-cold TBS per 5mL of starting culture. Resuspend insect cells with 2.5mL of ice-cold TBS per gram wet weight of cells. For best results, use a protease inhibitor when preparing cell lysate.
7. Add 200µL of Pull-Down Lysis Buffer per 5mL of *E. coli* starting culture. Add 2.5mL of Pull-Down Lysis Buffer per gram wet weight of insect cells. Immediately invert until thoroughly mixed.
8. Incubate on ice for approximately 30 minutes. Invert tubes periodically.
9. Centrifuge at 12,000 × *g* for 5 minutes to clarify crude cell lysate.
10. Decant supernatant to a separate microcentrifuge tube and store on ice. Label this tube "prey lysate."

Protocol 2: Prey Protein from Mammalian Cell Lysate

Confirmation of suspected protein:protein interactions and identification of novel protein:protein interactions is possible using mammalian cell lysate as the source of prey protein.

1. Grow mammalian cells according to standard protocols.
2. Transfer cell culture to a sterile centrifuge tube.

   Note: If cells are adherent, release them from the surface of the flask by trypsin digestion.

3. Centrifuge at 500 × *g* for 5 minutes and discard culture supernatant.
4. Add 1mL of TBS per 5mL of original cell culture volume. Mix using a pipette or a vortex mixer.
5. Transfer cell suspension to a sterile centrifuge tube.
6. Centrifuge at 500 × *g* for 5 minutes and discard wash supernatant.

   Resuspend mammalian cell pellet with 2.5mL of ice-cold TBS per gram wet weight of cells using a pipette or vortex mixer. For optimal results, use protease inhibitor cocktail when preparing cell lysate.

7. Add 2.5mL of Pull-Down Lysis Buffer per gram wet weight of cells and immediately invert until thoroughly mixed.
8. Incubate on ice for approximately 30 minutes. Invert tubes periodically.
9. Centrifuge at 12,000 × *g* for 5 minutes to clarify crude mammalian lysate.
10. Decant supernatant to a separate microcentrifuge tube and store on ice. Label this tube "mammalian prey lysate."

Protocol 3: Prey from Previously Purified Protein

Confirmation of suspected protein:protein interactions may be validated using purified proteins as the bait and prey proteins; however, identification of novel protein:protein interactions is not recommended.

1. Determine protein concentration of purified prey protein sample using the BCA Protein Assay Kit (Product No. 23227) or the Coomassie Plus (Bradford) Protein Assay Kit (Product No. 23236).
2. Calculate the volume of purified prey protein sample that contains approximately 100-150µg of protein.

   Note: This is a starting concentration and may have to be adjusted based on the molecular mass of the bait and prey protein as well as the nature of the interaction between the two proteins. The Pierce Spin Column has a maximum capacity of ~800µL. Previously purified protein may have to be concentrated or applied to the column in multiple batches for optimal binding. For concentrated protein samples, dilute sample up to 800µL with wash solution described in Section II A: Equilibrate Glutathione Agarose, Step 2.

Protocol 4: Prey Protein from *In vitro* Transcription/Translation Reaction

Confirmation of suspected protein:protein interactions may be validated using *in vitro* transcribed/translated proteins as the prey protein; however, identification of novel protein:protein interactions is not recommended.

1. Perform *in vitro* transcription/translation reaction according to standard protocols.

   Note: *In vitro* translated proteins are either [^{35}S]-labeled or biotinylated, therefore, autoradiography or Western blotting is required for detection. These reactions are typically 50µL.
2. Dilute 10µL of an *in vitro* transcription/translation reaction in up to 400µL of TBS/Pull-Down Lysis Buffer. This will serve as the prey protein sample.

   Note: Efficiency of the *in vitro* transcription/translation reaction and strength of the protein:protein interaction being confirmed may require using more than 10µL of the reaction.

IV. Prey Protein Capture

1. Apply bottom cap and remove top cap on the Pierce Spin Column containing the immobilized GST-tagged bait protein.
2. Add up to 800µL of prepared prey protein sample.

   Note: Retain enough sample to analyze prey protein via SDS-PAGE.
3. Replace top cap to the column.
4. Incubate at 4°C for at least 1 hour with gentle rocking motion on a rotating platform. Maximal binding may require a longer incubation time and should be determined for each new prey protein sample.
5. Remove both caps from each column and place column in a Collection Tube.
6. Centrifuge at 1250 × *g* for 30 seconds to 1 minute. Label this tube "prey flow-through" and place on ice.
7. Replace bottom cap to spin column.

8. Add 400µL of wash solution and replace top cap. Invert several times to mix thoroughly.

   Note: Multiple washes with resin inversion are required to effectively eliminate nonspecific interactions. For additional information regarding interaction characteristics and wash requirements, See Appendix A, p. 9.

9. Remove both caps and place spin column in the Collection Tube labeled "wash."
10. Centrifuge at 1250 × *g* for 30 seconds to 1 minute. Discard the wash volume.
11. Repeat wash steps 7-10 for a total of five washes.
12. Proceed to **Section V. Bait-Prey Elution**.

V. Bait-Prey Elution

The following procedure is applicable to all methods of bait and prey preparations.

1. To prepare 1mL of 10mM Glutathione Elution Buffer, use an analytical balance to weigh 3.1mg of Glutathione (molecular mass of Glutathione is 307.3Da) and add to 1mL of TBS included in the kit.

   Note: DO NOT reconstitute entire bottle of Glutathione. Prepare fresh elution buffer for each experiment.

   Note: Adding glutathione alters the buffer's pH. Adjust the Elution Buffer's final pH to 8.0 with NaOH before use.

2. Apply bottom cap and remove top cap of spin column.
3. Add 250µL of the Elution Buffer to the spin column. Replace top cap to the column.
4. Incubate spin column for 5 minutes with gentle rocking on a rotating platform.
5. Remove both caps and place spin column in a Collection Tube.
6. Centrifuge at 1250 × *g* for 30 seconds to 1 minute. Label this tube "Elution 1" and place on ice.

   Note: Although multiple elutions may be performed for each assay this is usually unnecessary because most interacting proteins will elute in the first elution. This kit contains sufficient Glutathione for 3-4 elutions per assay so that a complete elution profile can be established for each protein assayed.

7. Prepare samples for SDS-PAGE.

   Note: The high glutathione concentration in the Elution Buffer may cause lane widening during electrophoresis in Tris-Glycine pre-cast gels. Also, some protein stains will detect Glutathione as a major constituent of the gel migration front and this staining may mask low molecular mass proteins. Sample dialysis with a Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Unit (Product No. 69560) before SDS-PAGE analysis will eliminate these anomalies.

VI. Gel Analysis

1. Electrophorese samples including non-treated Glutathione Agarose and immobilized bait controls.
2. Choose a detection method based on sensitivity requirements.
3. Protein bands, which are not present in the control lanes, are candidate interacting proteins.

Troubleshooting

Problem	Possible Cause	Solutions
Expression levels of target protein in *E. coli* or baculovirus-infected insect cells are low	Expression conditions are not optimized	Optimize expression conditions
High background or many contaminating bands	Inadequate resin washing	Increase wash volume Increase ionic strength of wash buffer
No isolation of interacting protein	Weak or transient interaction	Wash conditions too stringent - lower number of washes and ionic strength of wash buffer
	Poor expression level of prey protein	Apply more protein sample
	Cofactor may be essential for "trapping" interaction	Experiment with addition of possible cofactors
Resin foams during wash	Top cap to column is still in place during centrifugation	Remove top cap to column

Appendix

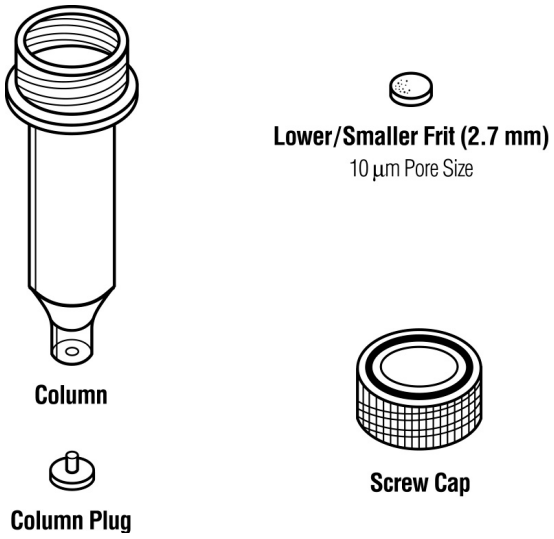
A. Considerations for Strong, Weak or Transient Protein:Protein Interactions

- **Strong:** Interactions that exhibit strong binding affinities (low dissociation constants) are the easiest to identify. These interactions can be washed extensively with high-ionic strength buffers to clear nonspecific proteins, eliminating false positive results.
- **Weak:** Interactions with weak binding affinities (high dissociation constants) require careful evaluation of binding and wash buffer conditions to allow the interaction to remain intact while removing nonspecific proteins. False positive results are more likely when identifying weak interactions. Eliminating false positive results requires careful design of appropriate control experiments.
- **Transient:** These interactions represent the most challenging to isolate and can be strong or weak in binding affinity, but are defined by their brief temporal interaction with other proteins. Transient interactions are common to enzymatic protein complexes that undergo dynamic rearrangements as the complex cycles through a particular biological process. A protein that transiently interacts with another protein or protein complex will often "dock" at a unique molecular interaction site. The recruited protein is held at this site only until it has performed its cognate enzymatic function after which it is allowed to recycle (deassemble from the complex) and await the next docking event. These transient interactions are frequently associated with NTP hydrolysis and, as such, the inclusion of NTP and non-hydrolyzable NTP analogs can be crucial to "trapping" a complex in a particular conformation conducive for the docking event under study. Other co-factors such as hormones and divalent cations can have similar effects on binding conformations depending on the system.

B. Endogenous Protein Expression

- Translation levels of different proteins, even ones in the same functional complex, can vary extensively when compared directly to each other, assayed during the cell cycle, and when calculated between different cell types. This variance can encompass a continuum from highly abundant to single molecule expression or no expression. Excessive translation of some proteins can potentially mask the interaction of other biologically significant interactions, while ultra-low expression can push the identification of biologically significant interactions below the threshold of current detection capabilities. These factors must be determined empirically for each protein.

C. Schematic of the Pierce Spin Column



Related Thermo Scientific Products

16100	**Pierce Glutathione Agarose,** 10mL
87785	**Halt™ Protease Inhibitor Cocktail, EDTA-Free**, 100X
69570	**Slide-A-Lyzer MINI Dialysis Unit,** 10K MWCO, 10-100µL capacity
66382	**Slide-A-Lyzer Dialysis Cassette,** 10K MWCO, 0.5-3mL capacity
23227	**Pierce BCA Protein Assay Kit**
23236	**Coomassie Plus (Bradford) Protein Assay Kit**
24590	**GelCode™ Blue Stain Reagent,** 500mL, sufficient for 20 mini gels
24612	**Pierce Silver Stain Kit**
24582	**Pierce Zinc Reversible Stain Kit**
34080	**SuperSignal™ West Pico Chemiluminescent Substrate,** Luminol/Enhancer 250mL, Stable Peroxide Buffer, 250mL
69705	**Pierce Spin Columns Plus Accessories,** 25 units
28376	**BupH Tris Buffered Saline Packs,** 40 packs

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