INSTRUCTIONS

Pierce™ Pull-Down Biotinylated Protein:Protein Interaction Kit

21115

1378.4

Number	Description
21115	**Pierce Pull-Down Biotinylated Protein:Protein Interaction Kit,** contains sufficient material for conducting 25 pull-down assays using biotinylated protein as the bait
	Kit Contents:
	Immobilized Streptavidin, 1.5mL of settled gel, supplied as a 50% slurry
	BupH™ Tris Buffered Saline (TBS), 1 pack, 25mM Tris•HCl, 0.15M NaCl, pH 7 when reconstituted with 500mL of ultrapure water
	Biotin Blocking Solution, 15mL
	Wash Buffer, 100mL, pH 5.0
	Elution Buffer, 50mL, pH 2.8
	Pierce Spin Columns plus Accessories, 27 columns with pre-inserted frit and top and bottom caps
	Collection Tubes and Caps Accessory Pack, 2 packs, 100 graduated 2mL tubes and plug caps per pack
	Storage: Upon receipt store at 4°C. Product is shipped at ambient temperature.

Table of Contents

Introduction

The Thermo Scientific™ Pierce™ Pull-Down Biotinylated Protein:Protein Interaction Kit makes it possible to perform an efficient pull-down assay with high protein recovery and reproducibility. This kit uses immobilized streptavidin to capture a biotinylated protein and a spin column format that enables complete gel retention during each capture, wash and elution step. When the biotinylated bait protein is immobilized, it can withstand stringent washes and remains bound during prey protein elution, which results in an isolated prey protein without bait protein contamination. This technique is ideal when using native or recombinant proteins that are not expressed with a fusion tag. Additionally, prey protein is isolated from the immobilized bait protein using native conditions.

The pull-down assay uses a tagged or labeled bait protein coupled to resin to capture a prey protein contained in a cell lysate or other unpurified protein mixtures. This technique can identify novel interactions between a known protein (bait) and previously undiscovered target (prey) or it can confirm the interaction between a known (bait) and a known target (prey) protein.[1]

"Homemade" pull-down methodologies for confirming or identifying protein:protein interactions are common in contemporary scientific literature.[2,3,4] The homemade pull-down assay represents a collection of reagents from multiple commercial vendors that cannot be validated together as a functional assembly until they are incorporated into a protocol by the researcher. Troubleshooting this collection of reagents can be tedious and time-consuming. The Pierce Pull-Down Biotinylation Protein:Protein Interaction Kit contains a validated set of reagents specifically developed for performing pull-down assays. The kit format is highly flexible, allowing control in optimizing experimental conditions specific to the requirements of each unique protein:protein interaction.

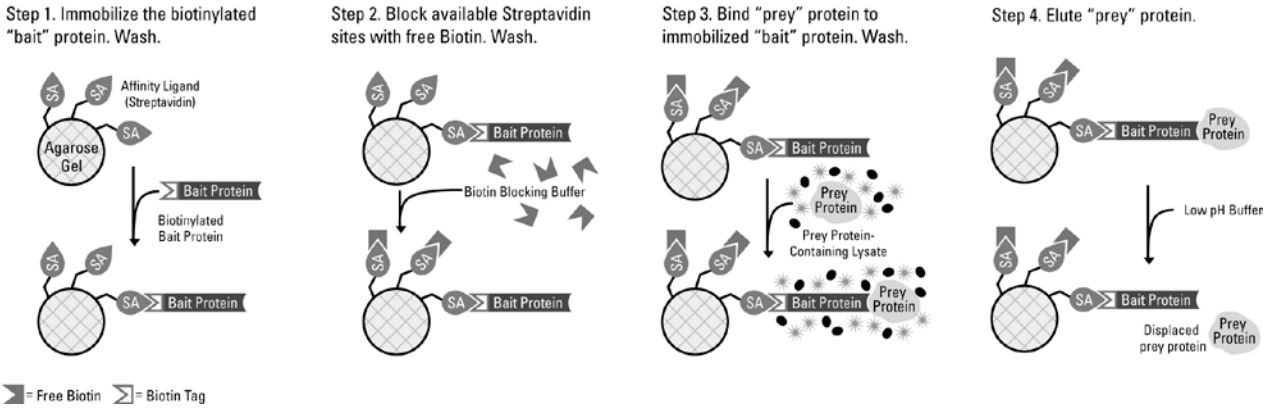


Figure 1. Schematic of the pull-down procedure.

Important Product Information

- It is often useful to establish experimental evidence that a protein of interest interacts with other proteins before attempting a pull-down assay for binding partners. Several methods, including yeast two-hybrid, density gradient centrifugation and immunofluorescence microscopy are valuable in predicting whether protein-protein interactions.
- Negative controls are absolutely necessary for generating biologically significant results. A non-treated gel control (minus bait or probe, plus prey) helps identify and eliminate false positives caused by nonspecific binding of proteins to the Immobilized Streptavidin. The immobilized bait control (plus bait, minus prey) serves as a positive control that verifies the Immobilized Streptavidin affinity gel can successfully capture adequately biotinylated bait protein.
- This kit incorporates the spin column format for handling small volumes of Immobilized Streptavidin. This format allows complete retention of resin during the pull-down assay and minimizes variability between experiments (See **Appendix C**).[5]
- 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 altering 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 requiring the removal of components. The working solution for binding is a Tris-buffered solution containing 150 mM NaCl. The working solution for the wash buffer is a pH 5.0acetate buffer that reduces the nonspecific interaction. Increasing the NaCl concentration (See **Appendix D**) can enhance the efficiency of the wash buffer to reduce hydrophobic nonspecific interactions and increase binding efficiency of target proteins. These buffers provide a starting point from which specific buffer conditions for each unique interacting pair can be optimized.
- This kit and instructions are designed with flexibility to adapt to multiple 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. Additonal analysis of experimental conditions may require additional tubes and caps. Many tube styles will accommodate the spin cup 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 spin columns and the solution retained in the Collection Tube.

Additional Materials Required

- Pipettes and disposable tips that can accurately deliver 2µL-1mL
- 0.2µm, 500mL filter sterilization unit
- Microcentrifuge capable of 5000 × *g*
- NaCl, ~8g
- Tris

Material Preparation

Biotinylated bait protein	Biotinylate the bait protein using standard procedures. Suggested biotinylation reagents are listed in **Appendix E**.
Binding Buffer (TBS)	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 buffer pack is sufficient for 25 pull-down assays.
Neutralization Buffer	Prepare 1M Tris [Tris(hydroxymethl)aminomethane] solution by dissolving 1.21g Tris in 10mL of ultrapure water.
NaCl, 5 M Stock Solution	Prepare a 5M NaCl stock solution by dissolving 7.30gm of NaCl in 20mL of ultrapure water. Once dissolved, adjust to 25mL of final volume.

Procedure for Pull-Down Biotinylation Protein:Protein Interaction Assay

A. Immobilized Streptavidin Preparation

The bait protein must have been already purified, biotinylated and the complex removed of nonreacted biotinylation reagent before beginning this procedure. See **Appendix E** for a list of suggested biotinylation reagents.

1. Label enough spin columns to include sample(s), a non-treated gel control and an immobilized bait control.
2. Thoroughly resuspend the Immobilized Streptavidin by inverting the tube several times. Pipette 50µL of the gel slurry into each labeled spin column and place them in the Collection Tubes.

   Note: To better facilitate slurry transfer, cut off 2-3mm from the narrow tip of a standard 200µL disposable pipette tip.
3. Add 250µL of the TBS to each of the spin columns. Place the top screw caps on the columns and mix by gently inverting the columns 3-5 times.
4. Remove the columns' screw caps. Place columns in the Collection Tubes, and centrifuge at 1250 × *g* for 30-60 seconds.
5. Repeat Steps 3 and 4 two additional times. Apply bottom plugs to the columns.

B. Bait Protein Immobilization

1. Add at least 100µL of biotinylated protein to the appropriate spin columns. To a separate spin column designated as a gel control (no biotinylated protein added to the gel) add an equal volume of buffer that was added to the sample columns. Secure top screw caps onto the spin columns.

   Note: At least 100µL of liquid volume is required for adequate mixing, but do not exceed 500µL total volume. The concentration of bait protein needed is specific to each protein interaction model. A suggested starting concentration is 50µg/100µL of biotinylated bait protein. The Thermo Scientific™ BCA Protein Assay Kit (Product No. 23227) may be used to determine the protein concentration.
2. Incubate at 4°C for at least 30 minutes with gentle rocking motion on rotating platform. DO NOT VORTEX.

   Note: Maximal binding may require a longer incubation, which should be determined for each protein. Incubation may proceed at room temperature if protein is deemed stable.
3. During the incubation, label a series of Collection Tubes "bait flow-through (A)" for each spin column incubating.

4. After incubation, remove top caps then remove the bottom plug from the columns. Place columns in the pre-labeled Collection Tubes.

   Note: If subsequent analysis indicates excessive flow through of the biotinylated bait protein, the protein can be diluted in the TBS or a pre-determined binding buffer for the next time the assay is performed.

5. Centrifuge tubes at 1250 × *g* for 60 seconds.
6. Remove spin columns from the tubes and secure caps onto the Collection Tubes (A) and set them aside for later evaluation. Place spin columns in separate Collection Tubes for the Biotin Blocking.

C. Biotin Blocking

1. Add 250µL of Biotin Blocking Solution to each spin column. Secure the top screw caps onto the columns and mix by gently inverting the columns 3-5 times.
2. Incubate the spin columns at room temperature for 5 minutes. Remove the top screw caps from the columns, place columns in Collection Tubes and centrifuge at 1250 × *g* for 30-60 seconds. Repeat Steps 1 and 2 once.
3. Add 250µL of the TBS to each spin cup. Place the top screw caps on the columns and mix by gently inverting the column 3-5 times.

   Note: The TBS may be replaced in Steps 3 and 4 for column rinsing and pre-equilibration if an alternate Bait/Prey Binding Buffer is formulated for a particular interaction.

4. Remove top screw caps from columns. Place columns in Collection Tubes and centrifuge at 1250 × *g* for 30-60 seconds.
5. Repeat Steps 3 and 4 two additional times. Insert bottom plugs to the columns.

D. Prey Protein Capture

1. Add at least 100µL of lysate or other sample containing suspected prey protein to the appropriate spin columns. To a separate spin column designated as an immobilized bait control (no lysate added to the gel containing the bound biotinylated protein) add an equal volume of buffer that was added to the sample columns. Secure top screw caps onto the spin columns.
2. Incubate at 4°C for at least 60 minutes with gentle rocking motion on rotating platform. DO NOT VORTEX.

   Note: Maximal binding may require a longer incubation, which should be determined for each protein. Incubation may proceed at room temperature if lysate is deemed stable.

3. During the incubation, label a series of Collection Tubes “lysate flow-through (B)” for each spin column that is incubating.
4. After incubation, remove top cap from the spin columns, then remove the bottom plug from the columns and place in the pre-labeled tubes.

   Note: If subsequent analysis indicates excessive flow through of lysate protein, the protein can be diluted in the TBS or a pre-determined binding buffer for the next time the assay is performed.

5. Centrifuge tube at 1250 × *g* for 60 seconds.
6. Remove spin columns from the Collection Tubes (B), secure the caps and set the tubes aside for later evaluation. Place the spin columns in separate Collection Tubes for washing.

E. Spin Column Wash

The wash buffer needs to be stringent enough to reduce nonspecific binding, while not disrupting the bait:prey protein interaction. If the ideal wash conditions are unknown, use the provided acetate Wash Buffer with varying concentrations of NaCl. The chart in **Appendix D** lists formulations for acetate-NaCl buffers. In some instances, the TBS provided may be the best option. In other instances, only minimal washing is necessary because of minimal non-target binding. Wash conditions must be determined empirically to ensure a successful result.

Note: It may be helpful to monitor protein loss during wash steps by collecting the wash fractions. Extra Collection Tubes and Caps are provided to reserve wash fractions for subsequent analysis.

1. Add 250µL of Wash Buffer to each spin column. Secure the top screw caps onto the columns and mix by gently inverting the columns 5-7 times.
2. Incubate spin columns for 1 minute at room temperature. Remove the top screw caps from the columns, place columns in "waste" Collection Tubes and centrifuge at 1250 × *g* for 30-60 seconds. Repeat Steps 1-2, three additional times.
3. During the washing steps, label a series of Collection Tubes "elution 1 (C)" and "elution 2 (D)."
4. After the wash steps, place spin columns in separate Collection Tubes. Two tubes are needed for each spin column.

F. Prey Protein Elution

Note: Before elution, add 10µL of the Neutralization Buffer to each elution Collection Tube, which will neutralize the pH of the contents upon elution.

1. Add 250µL of Elution Buffer to each spin column. Secure the top screw caps onto the columns and mix by gently inverting the columns 5-7 times.
2. Incubate the spin column for 3-5 minutes at room temperature. Remove the top screw caps from the columns, place columns in Collection Tubes (C) and centrifuge at 1250 × *g* for 30-60 seconds.
3. Remove spin columns from the first elution tubes (C), cap tubes (C) and place spin cups in elution tubes (D). Repeat Step 1.

G. Sample Preparation for Gel Electrophoresis

Sample Buffer Compatibility: The elution buffer provided is compatible with known popular native and denaturing sample buffer recipes.

1. Pipette 20µL of the sample buffer into a microcentrifuge tube.
2. Allow Sample Buffer to equilibrate to room temperature and gently mix by inverting the tube 5-10 times. Pipette into the microcentrifuge tube containing the sample, the appropriate amount of sample buffer to achieve a 1X final concentration.

Troubleshooting

Problem	Possible Cause	Solutions
Expression level of target protein in cell lysate is low	Expression conditions are not optimal	Optimize expression conditions
High background or many contaminating bands on the polyacrylamide gel	Inadequate spin column washing after adding the lysate	Increase number of washes or increase NaCl concentration of the Wash Buffer (see **Appendix D**) or use a different wash buffer
	Bait protein was not completely purified before biotinylation	Optimize bait protein purification
Interacting protein was not isolated	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
	Excessive biotinylation hinders target protein binding site	Reduce molar excess of biotinylation reagent or use a reagent that targets a different functional group
	Insufficient biotinylation of bait protein	Increase molar excess of biotinylation reagent or use a reagent that targets a different, more abundant functional group
	Cofactors may be essential for "trapping" interaction	Test the affects of adding possible cofactors
	The binding conditions are insufficient	Dialyze prey protein against a suitable buffer determined empirically to be conducive to binding and rewash spin column before adding prey protein with this same buffer
Resin foams during wash	Detergent sensitivity	Remove detergent or other denaturing agents
	Top cap to column is still in place during centrifugation	Remove top cap to spin column before centrifugations

Appendix

A. Considerations for Protein:Protein Interactions

- **Strong:** Interactions that exhibit strong binding affinity (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 to occur 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 unique molecular interaction site. The recruited protein is held at this site only until it has performed its cognate event. These transient interactions are frequently associated with NTP hydrolysis and, as such, the inclusion of NTP and non-hydrolysis NTP analogs can be crucial to "trapping" a complex in a particular conformation conducive for the docking event under study. Other cofactor such as hormones and divalent cations can have similar effects on binding conformations depending on the system.
- The abundance of the target protein, the strength of the interaction and the efficiency of biotinylation determine the amount of material required for the assay.

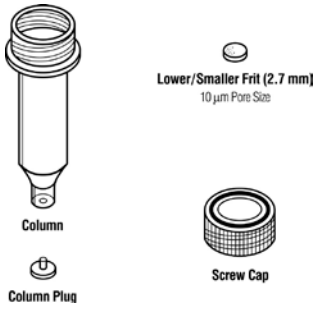
  When attempting to identify novel interaction between a known and unknown proteins, it is possible that the unknown protein will be in limited concentrations. To identify a novel interaction, the unknown protein must be present in sufficient quantities to allow the interaction to be visualized with the chosen method of detection. One important consideration is whether the bait and prey exist in the same lysate source. Another consideration is the state of the cells at the time of harvest and if an induction chemical or biological growth factor influences the expression levels.[2]

  The avidity of the potential interacting pairs can be greatly improved *in vitro* if information is known about the environment from which the bait protein and potential target proteins originate. Buffers, pH gradients, temperature, protein concentration, detergent sensitivity, needed cofactor and coenzymes, and sensitivity to chaotrophic salts are all determinants of whether the interaction will occur and thus subsequent detection possible.

B. Endogenous Protein Expression

Translational levels of different proteins, even in the same functional complex, can vary extensively when compared directly to each other, when 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 Columns



D. Acetate/NaCl dilution chart

Prepare a 5M NaCl stock solution by dissolving 7.30g of NaCl in 20mL of ultrapure water. Once dissolved, adjust to a final volume of 25mL.

Acetate Buffer (mL)	5.0M NaCl (mL)	Final Molarity of NaCl
32	8.0	1.0
34	6.0	0.75
36	4.0	0.50
38	2.0	0.25
39	1.0	0.125
39.2	0.8	0.100
39.4	0.6	0.075
39.6	0.4	0.050
39.8	0.2	0.025
39.9	0.01	0.012

E. Biotinylation

To Biotinylate proteins in the laboratory, use one of the following reagents:

- Thermo Scientific™ EZ-Link™ Sulfo-NHS-LC Biotin (Product No. 21335): This reagent biotinylates through primary amine groups. This reagent is also available in kit formats (Product No. 21435 and 21935).
- EZ-Link NHS-PEG_4-Biotin (Product No. 21330): This reagent biotinylates through primary amine groups with a hydrophilic spacer arm that reduces the chance of protein precipitation in cases of high biotinylation efficiency. This reagent is also available in kit formats (Product No. 21455 and 21955).
- EZ-Link PEG-Iodoacetyl Biotin (Product No. 21334): This reagent biotinylates through the sulfhydryl groups and contains a PEG spacer arm that increases the hydrophilic properties of the final conjugate.

Related Thermo Scientific Products

78425	**Halt™ Protease Inhibitor Single-Use Cocktail EDTA-Free**
78430	**Halt Protease Inhibitor Single-Use Cocktail**
78443	**Halt Protease and Phosphatase Inhibitor Single-Use Cocktail, EDTA-Free**
69570	**Slide-A-Lyzer™ MINI Dialysis Unit, 10K MWCO,** 10-100 µl capacity, 50 units/pkg
66382	**Slide-A-Lyzer Dialysis Cassette, 10K MWCO,** 0.5-3.0 ml, 1 Kit
23227	**BCA Protein Assay Kit**
24590	**GelCode™ Blue Stain Reagent,** 500mL
24612	**Pierce Silver Stain Kit**
24582	**Pierce Zinc Reversible Stain Kit**
39000	**Lane Marker Reducing Sample Buffer,** 5mL
39001	**Lane Marker Non-Reducing Sample Buffer,** 5mL

References

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