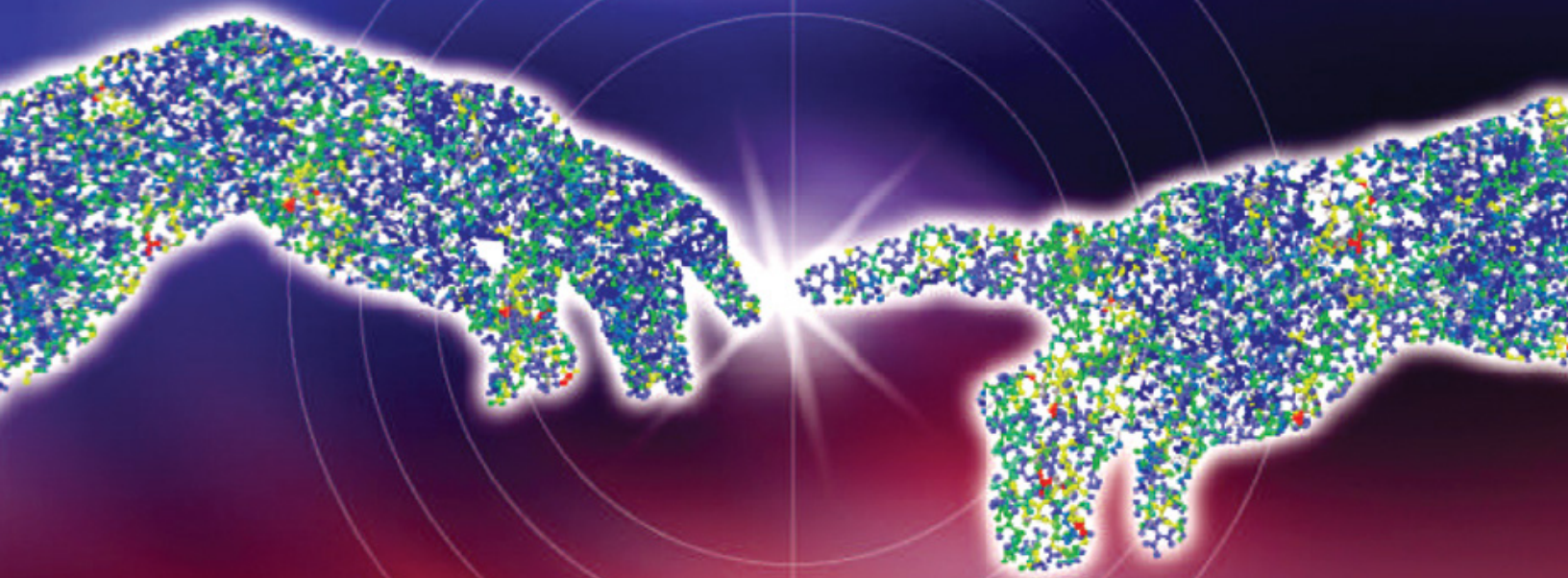


PIERCE

Protein Interactions

Protein Interactions

Extraordinary interactions are within reach.



Discover ... Confirm ... Characterize

Co-Immunoprecipitation (co-IP) ★ Cross-linking Reagents ★ Far-Western Analysis
Label Transfer ★ Protein Interaction Mapping ★ Pull-down Assays

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Protein Interactions

Introduction to Protein Interactions

Importance of Protein Interactions

The study of protein interactions has been vital to the understanding of how proteins function within the cell. Publication of the draft sequence of the human genome and proteomics-based protein profiling studies catalyzed a resurgence in protein interaction analysis. Characterizing the interactions of proteins in a given cellular proteome (now often referred to as the “interactome”) will be the next milestone along the road to understanding the biochemistry of the cell.

The ~30,000 genes of the human genome are speculated to give rise to 1×10^6 proteins through a series of post-translational modifications and gene-splicing mechanisms. Although a population of these proteins can be expected to work in relative isolation, the majority are expected to operate in concert with other proteins in complexes and networks to orchestrate the myriad of processes that impact cellular structure and function. These processes include cell-cycle control, differentiation, protein folding, signaling, transcription, translation, post-translational modification and transport.

Implications about function can be made via protein:protein interaction studies. These implications are based on the premise that the function of unknown proteins may be discovered if captured through their interaction with a protein target of known function.

Consequences of Protein Interactions

The result of two or more proteins interacting with a specific functional objective can be demonstrated in several different ways. The measurable effects of protein interactions have been outlined by Phizicky and Fields (see Recommended Reading List on page 11). Protein interactions can:

- Alter the kinetic properties of enzymes. This may be the result of subtle changes at the level of substrate binding or at the level of an allosteric effect.
- Allow for substrate channeling by moving a substrate between or among subunits, resulting ultimately in an intended end-product.
- Create a new binding site, typically for small effector molecules.
- Inactivate or destroy a protein.
- Change the specificity of a protein for its substrate through interaction with different binding partners; e.g., demonstrate a new function that neither protein can exhibit alone.
- Serve a regulatory role in either an upstream or a downstream action.

Types of Protein Interactions

Protein interactions fundamentally can be characterized as stable or transient. Both stable and transient interactions can be either strong or weak. Stable interactions are those associated with proteins that are purified as multi-subunit complexes. The subunits of the complex can be identical or different. Hemoglobin and core RNA polymerase are two examples of stable multi-subunit complex interactions. Stable interactions are best studied by co-immunoprecipitation, pull-down or far-Western methods.

Transient interactions are expected to control the majority of cellular processes. As the name implies, transient interactions are on/off or temporary in nature and typically require a specific set of conditions that promote the interaction. Transient interactions can be strong or weak, fast or slow. While in contact with their binding partners, transiently interacting proteins are expected to be involved in the whole range of cellular processes including protein modification, transport, folding, signaling, cell cycling, etc. Transient interactions can be captured by cross-linking or label transfer methods.

Protein Interactions

Introduction to Protein Interactions (continued)

The “Bait-Prey” Model — The Yeast Two-hybrid System

The yeast two-hybrid system uses the transcription process to make predictions about protein interaction. This method is based on the ability of an interacting protein pair to bring together the DNA-binding domain and activation domain of a transcription factor *in vivo* to produce a functional activator of transcription. The interaction can be detected by expression of the linked reporter genes.

The system requires that two yeast hybrids be prepared. One “bait” protein is fused to a transcription factor DNA-binding domain. The other “prey” protein is fused to a transcription factor activation domain. When expressed in a yeast cell containing the appropriate reporter gene, interaction of the “bait” with the “prey” brings the DNA-binding domain and the activation domain into close proximity, creating a functional transcription factor. This triggers transcription of the intended reporter gene (e.g., β -galactosidase). The “bait-prey” nomenclature has been adopted and applied to *in vitro* methods used to study protein interactions.

Many interactions currently known were first indicated by the yeast two-hybrid method. *In vitro* methods for protein interaction analysis are often employed to confirm interactions indicated by the yeast two-hybrid strategy described above.

In vitro Methods for Protein Interaction Analysis

The natural affinity of binding partners for each other is at the core of *in vitro* methods widely adopted for both interaction discovery and confirmation. *In vitro* methods span a broad range of techniques. At one end of the range are those methods that can be performed at the bench with basic laboratory skills and a small investment in reagents. The apparatus required for these methods can be found in most modern protein chemistry laboratories. At the other extreme are those methods that require special skills and knowledge and a substantial investment in specialized instrumentation. All methods within this spectrum have their place and can provide valuable insight into detailing protein interactions. Widely employed methods are listed and briefly described in Table 1.

Table 1. *In vitro* Methods for Protein Interaction Analysis

<i>In vitro</i> Methods	Description
Co-immunoprecipitation (Co-IP)	An immunoprecipitation (IP) experiment designed to affinity-purify a bait protein antigen together with its binding partner using a specific antibody against the bait.
Cross-linking Reagents	Strategies involve homo- or heterobifunctional reagents whose chemical cross-links may or may not be reversed. Nearest neighbors (suspected to interact) <i>in vivo</i> or <i>in vitro</i> can be trapped in their complexes for further study.
Far-Western Analysis	Similar strategy to Western blotting with one key difference. The antibody probe in a typical Western blot detection is substituted with an appropriately labeled bait protein as the probe. Detection can be radioisotopic, chemiluminescent or colorimetric, depending on the probe label.
Label Transfer	Involves a specialized cross-linking agent with several important features. These include hetero-bifunctionality for stepwise cross-linking, a detectable label and reversibility of the cross-link between binding partners. Upon reduction of the cross-linked complex, a binding partner (prey protein) acquires the label from a bait protein that was first modified with the reagent. The label is typically used in the detection process to isolate or identify the unknown prey protein.
Protein Arrays / Protein Chips	Antibody- or bait-based arrays that allow for screening and detection of specific interactions of proteins from complex mixtures. Primary applications include high-throughput assays of protein expression profiling, protein:protein interaction and enzyme activity. Most of the current protein chips are based on the binding between the capture proteins immobilized on a surface and the target proteins in the sample solution.
Protein Interaction Mapping	Uses an “artificial protease” on a bait protein to initiate contact-dependent cleavages in the prey protein in the presence of specific reactants. The nonspecific cleavage fragments produced by the artificial protease can be analyzed to map the contact sites or interface of a known protein:protein interaction.
Pull-down Assays	An affinity chromatography method that involves using a tagged or labeled bait to create a specific affinity matrix that will enable binding and purification of a prey protein from a lysate sample or other protein-containing mixture.
Surface Plasmon Resonance	Relates binding information to small changes in refractive indices of laser light reflected from gold surfaces to which a bait protein has been attached. Changes are proportional to the extent of binding. Special labels and sample purification are not necessary, and analysis occurs in real time.
NMR (Nuclear Magnetic Resonance)	Method that can provide insights into the dynamic interaction of proteins in solution.
Mass Spectroscopy	Used in concert with affinity-based methods (such as co-IPs) to isolate binding partners and complexes and to identify the component proteins using standard mass spectral methods; e.g., MALDI-TOF and mass searching of bioinformatics databases.
X-ray Crystallography	Crystallization of the interacting complex allows definition of the interaction structure.

Factors Affecting Success of *In vitro* Affinity-based Methods

In vitro affinity-based strategies can be direct, such as those used for pull-down assays or far-Western analysis, or indirect, such as the typical co-immunoprecipitation experiment that is mediated by an antibody against a target antigen that in turn precipitates an interacting protein. Affinity-based methods are sensitive with some methods capable of detecting weak interactions with dissociation constants in the range of 10^{-5} M. In addition, methods such as co-immunoprecipitations, pull-down assays, far-Western analyses and label transfer methods allow all proteins in the sample to compete equally for the bait protein. For these methods, success can be related to the following factors:

- **Purity of the tagged or labeled bait protein:** For example, using pure bait protein in a pull-down assay eliminates the possibility of selecting an interacting protein as a result of binding to a contaminant in the bait preparation.
- **Modification requirement of the bait or prey protein:** Interaction between binding partners may depend on one or the other or both partners presenting the appropriate post-translational modification.
- **Native state of the interacting pair:** Failure to detect an interaction may be the result of denaturation of one or the other binding partner. Binding conditions should be kept as close to physiological as possible.
- **Bait protein concentration:** To efficiently detect interactions in these systems, the bait protein concentration is required to be well above the dissociation constant (K_d) of the interaction.
- **Interaction conditions:** This refers to the buffer composition, pH and co-factor requirements necessary to promote the interaction under study.

Usually a combination of techniques is necessary to validate, characterize and confirm protein interactions. Previously unknown proteins may be discovered by their association with one or more proteins that are known. Protein interaction analysis may also uncover unique, unforeseen functional roles for well-known proteins.

The methods discussed in more detail in this brochure are supported by products developed to study biologically relevant protein interactions. Discovery or verification of an interaction is the first step on the road to understanding where, how and under what conditions these proteins interact *in vivo* and the functional implications of these interactions.

Protein Interactions Co-Immunoprecipitation

Immunoprecipitation

The topic of co-immunoprecipitation (co-IP) is best preceded by a discussion of immunoprecipitation (IP) to help frame an understanding of the principles involved.

IP is one of the most widely used methods for antigen detection and purification. An important characteristic of IP reactions is their potential to deliver not only the target protein, but also other macromolecules that interact with the target.

The IP Principle

The principle of an IP is very simple (Figure 1). An antibody (monoclonal or polyclonal) against a specific target antigen is allowed to form an immune complex with that target in a sample, such as a cell lysate. The immune complex is then captured on a solid support to which either Protein A or Protein G has been immobilized (Protein A or Protein G binds to the antibody, which is bound to its antigen). The process of capturing this complex from the solution is referred to as precipitation. Any proteins not “precipitated” by the immobilized Protein A or Protein G support are washed away. Finally, components of the bound immune complex (both antigen and antibody) are eluted from the support and analyzed by SDS-PAGE (gel electrophoresis), often followed by Western blot detection to verify the identity of the antigen.

Traditional immunoprecipitation involves the following steps:

1. Form the antigen-antibody complex (immune complex) by incubating specific antibody with the antigen-containing sample for 1 hour to several hours.
2. Capture the immune complex on an immobilized Protein A or Protein G agarose gel support by incubation for 0.5-2 hours.
3. Remove any non-bound protein (non-immune complex sample components) from the precipitated complex by washing gel support with additional sample buffer.
4. Boil gel support in reducing SDS-PAGE sample loading buffer.
5. Recover sample eluted in loading buffer from gel support and analyze by SDS-PAGE.
6. Perform Western blot analysis, probing with antigen-specific antibody.

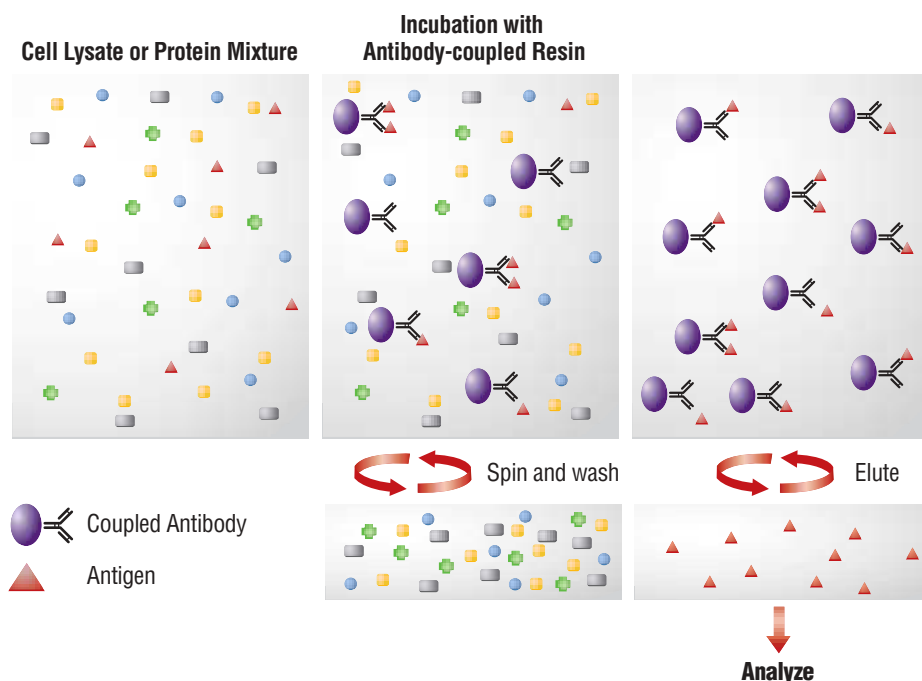


Figure 1. Summary of a traditional immunoprecipitation procedure.

Co-IP vs. IP

Co-IP is a popular technique for protein interaction discovery. Co-IP is conducted in essentially the same manner as IP (Figure 2). However, in a co-IP the target antigen precipitated by the antibody “co-precipitates” a binding partner/protein complex from a lysate; i.e., the interacting protein is bound to the target antigen, which becomes bound by the antibody that becomes captured on the Protein A or Protein G gel support. The assumption that is usually made when associated proteins are co-precipitated is that these proteins are related to the function of the target antigen at the cellular level. This is only an assumption, however, that is subject to further verification.

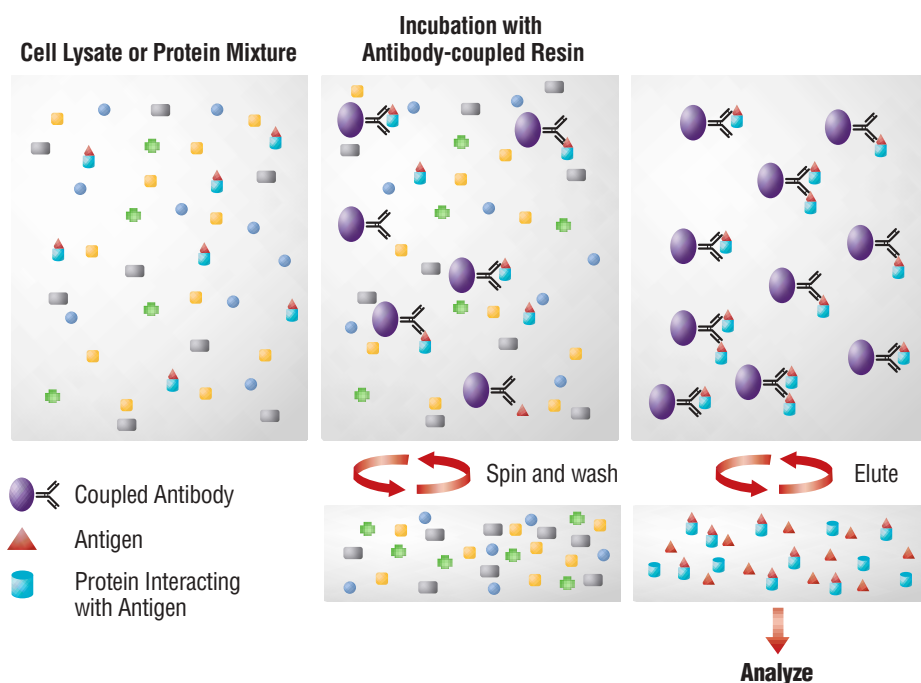


Figure 2. Summary of a traditional co-immunoprecipitation procedure.

Traditional Methods vs. Pierce Innovations for Co-IP

Problems with Traditional Co-IP Methods

The traditional co-IP protocol has certain deficiencies relating to the fundamental format of the assay, the antibody and associated chemistry. One of the most commonly encountered problems with the traditional IP and co-IP approach is interference from antibody bands in gel analysis. In those cases in which several proteins may be co-precipitated with the target, presence of the co-eluted antibody heavy and light chains (25 and 50 kDa bands in reducing SDS-polyacrylamide gel) in the preparation can obscure the results. The ideal situation would be to conduct the co-IP without contamination of the eluted antigen with antibody. With this potential interference eliminated, only the co-precipitated proteins will be present and detected on a gel. This and other shortcomings of the traditional protocol and their solutions are summarized in Table 2.

Protein Interactions Co-Immunoprecipitation (continued)

Table 2. Comparison of Traditional Co-IP and Pierce Products for Co-IP

Traditional Co-IP Problems	Pierce Product Solutions
Batch processing of the precipitated complex in a single tube: results in inefficient washing of non-bound proteins from the support and in resin loss due to decanting wash buffer from tube via a pipette	Spin cup or spin tube processing: dedicated IP and co-IP kits that contain spin-cup or spin tube devices that increase washing efficiency offer more effective elution of antigen and associated protein and eliminate resin loss, yielding more consistent results
Antibody fragment interference: co-elution of antibody fragments with antigen often results in bands interfering with detection of any co-precipitated proteins on SDS-PAGE	Antibody immobilization: chemistries designed to immobilize the antibody to the support, thereby allowing elution of only the target and any associated proteins in a co-IP complex
Antibody sacrificed: as a consequence of harsh elution conditions, the target antibody is destroyed; antibody loss by way of the protocol can be costly	Antibody re-used: immobilization chemistry and mild elution conditions for the target and associated proteins allow the immobilized antibody to be re-equilibrated and re-cycled several times in the co-IP protocol

Approaches to Co-IP Free of Antibody Interference

Three approaches have been incorporated into several products targeted to IP and co-IP applications.

Activated Support for Antibody Immobilization — Direct Strategy

In this approach, an antibody is immobilized directly through its surface amine groups (contributed primarily by the side chain epsilon-amino group of lysine) to a high-capacity aldehyde-activated beaded agarose support (AminoLink® Plus Coupling Gel). The support forms a Schiff's base with these available amines that is reduced to form stable secondary amine bonds during the immobilization process. The wide range of coupling conditions that can be used with this support make it ideal for maintaining biological binding activity critical to the successful execution of a co-IP experiment. ProFound™ Co-Immunoprecipitation Kits (Product # 23600, 23605) and Seize® Primary Immunoprecipitation Kits (Product # 45335, 45332) use this direct immobilization approach. The word "Primary" in the Seize® Primary IP Kit name connotes direct coupling of a primary antibody to the activated matrix. For co-IP applications, the flexibility, simplicity and durability of the direct method as an antibody-coupling strategy makes it the method of choice for delivering results free from antibody interference.

Antibody Orientation and Immobilization — Indirect Strategy

This strategy takes advantage of the binding characteristics of the traditional Immobilized Protein A- or Protein G-based support combined with chemical cross-linking to covalently link the antibody to the support. Protein A and Protein G bind IgG class antibodies through the Fc region that is characterized primarily by dimerized heavy chain modified by carbohydrate. Fc region binding naturally orients the antigen-binding domains of the antibody (Fab) away from the support, making them available for binding to their respective target antigen. To ensure that the antibody remains on the support during the requisite antigen binding, wash and elution steps of the protocol, this bound and oriented antibody is chemically cross-linked to the Protein A or Protein G with the bifunctional reagent disuccinimidyl suberate (DSS). Seize® X Immunoprecipitation Kits (e.g., Product # 45210, 45215) incorporate this strategy. The X in the name connotes use of a cross-linking agent.

Use of the Streptavidin:Biotin Interaction

This direct coupling approach incorporates the binding association between streptavidin and biotin. Streptavidin immobilized to beaded agarose gel or coated in microplate wells provides an alternative IP or co-IP strategy for obtaining results free from antibody interference. Biotinylated antibody is bound very strongly to each matrix and is not eluted when mild conditions are used to release the target antigen. The IP/co-IP is conducted by incubating the sample with the biotinylated antibody-loaded matrix. Elution of the target antigen and any interacting proteins is performed free of antibody contamination. ImmunoPure® Immobilized Streptavidin Gel (Product # 20347) and kit products that use this support, as well as the Seize® Streptavidin Coated Plate Immunoprecipitation Kit (Product # 45360), provide high-capacity biotin-binding matrices suitable for IP and co-IP applications. For a detailed description of the Seize® Immunoprecipitation Products, order the Pull-down Assay/Immunoprecipitation Handbook (Product # 1600852).

Optimization Parameters in IP and Co-IP

Classical Immune Complex Formation vs. Pre-binding of Antibody

A change in protocol from the classical immune complex precipitation is necessary when using immobilized antibody in the co-IP method. In the traditional co-IP protocol, the immune complex (antigen:antibody) is formed in solution before “precipitating” it with the immobilized Protein A or Protein G matrix. When using immobilized antibody, the immune complex is formed directly on the antibody-coupled matrix by incubation of the antigen-containing sample with the matrix. Formation of the immune complex (the target antigen and any target-associated protein) and its precipitation occurs in one step.

In the immobilized format, the antibody is allowed to incubate with the lysate. The matrix is washed using a spin cup format and the bound protein eluted for analysis. The target antigen and co-IP complex is recovered free of antibody or antibody fragment contamination, and the antibody is retained in an active form on the support to be used in another co-IP cycle.

Research conducted at Pierce indicates that pre-binding the antibody consistently results in the capture of more target antigen, even in coated plate IP procedures that do not require it. This approach is recommended for the Seize® Coated Plate IP Kits that use 96-well microplates coated with streptavidin, Protein G or Protein A/G (Products # 45360, 45355, 45350, respectively). Antibody is bound to the plate wells prior to the prescribed incubation with a lysate sample. Unbound protein is easily washed from the wells prior to the elution of the target and any co-precipitated proteins.

Evaluating a Co-IP-captured Interaction

In their review of protein interactions, Phizicky and Fields (see Recommended Reading below) present a discussion of the issues to consider in validating a suspected interaction obtained by a co-IP experiment. Ultimately, the following question must be answered: Does the interaction detected by co-IP occur *in vivo*, and what significance does it have at the cellular level? A summary of the Phizicky and Fields approach to verification of co-IP data follows.

Confirm that the co-precipitated protein is obtained only by antibody against the target

Use monoclonal antibodies in the co-IP protocol. When only a polyclonal antibody is available, pre-treatment of the antibody with sample devoid of the primary target (bait protein) may be required to assure that the polyclonal antibody does not contain clones or contaminants that bind prey protein(s) directly. Pre-adsorption to extracts devoid of target or pre-purification of polyclonal IP antibodies against an affinity column containing pure target antigen safeguards against a false-positive co-IP.

Conclude that antibody against the target antigen does not itself recognize the co-precipitated protein(s)

Use independently derived antibodies that have demonstrated specificities against different epitopes on the target protein. Their use serves as verification that the target (bait)-directed antibodies have no affinity for the target-associated prey proteins recovered during the co-IP. Alternatively, an antibody against the co-precipitated protein can be used to co-IP the same complex.

Determine if the interaction is direct or indirect

Is the interaction mediated through a third-party protein that contacts both target and co-precipitated protein? Immunological and other more sophisticated methods such as mass spectrometry may be necessary to answer this question.

Determine that the interaction takes place in the cell and not as a consequence of cell lysis

Suggested approaches here involve co-localization studies and site-specific mutagenesis giving rise to mutants that perturb the binding process.

Recommended Reading

Adams, P.D., *et al.* (2002). Identification of Associated Proteins by Coimmunoprecipitation. *In* Protein-Protein Interactions – A Molecular Cloning Manual. Golemis, E., Ed., Cold Spring Harbor Laboratory Press, pp 59-74. (Product # 20068)

Liebler, D.C. (2002). Identifying protein-protein interactions and protein complexes. *In* Introduction to Proteomics, Tools for the New Biology, Humana Press, pp.151-165. (Product # 20061)

Phizicky, E.M. and Fields, S. (1995). Protein-Protein Interactions: Methods for Detection and Analysis. *Microbiological Reviews* (Mar.), pp. 94-123.

Co-Immunoprecipitation

ProFound™ Co-Immunoprecipitation Kits

All the components you need to perform a properly controlled co-IP experiment directly out of the box with the antibody you provide.

Co-immunoprecipitation (co-IP) is the gateway method to all other *in vitro* methods for protein interaction analysis. As a result, co-IP is among the most common and fastest growing *in vitro* approaches to protein:protein interaction discovery and verification in use today. For example, protein interactions discovered through the use of yeast two-hybrid experiments are most often confirmed by co-IP experiments. The new Pierce ProFound™ Co-Immuno-precipitation Kit was configured to provide the essential tools to effectively perform a classical co-IP using the primary antibody of your choice. These kits provide those attempting a co-IP for the first time, as well as those experienced in the method, an ideal system for designing, constructing and performing a controlled co-IP experiment.

Benefits of target antibody immobilization

The heart of the new ProFound™ Co-Immunoprecipitation Kit is our AminoLink® Plus Coupling Gel that brings several benefits to the co-IP application.

- The AminoLink® Coupling Gel allows the user to confidently and successfully immobilize a purified antibody against the target protein directly onto the matrix and retain antibody activity.
- The antibody-coupled core support retains the antibody during elution of the co-IP complex, a feature considered ideal especially for the co-IP application.
- Detection of the co-precipitated products on the gel is accomplished without interference from antibody fragments commonly encountered with other methods based on immobilized Protein A or Protein G supports.
- Covalent attachment of antibody conserves costly antibody and allows the matrix to be used repeatedly in the co-IP application.

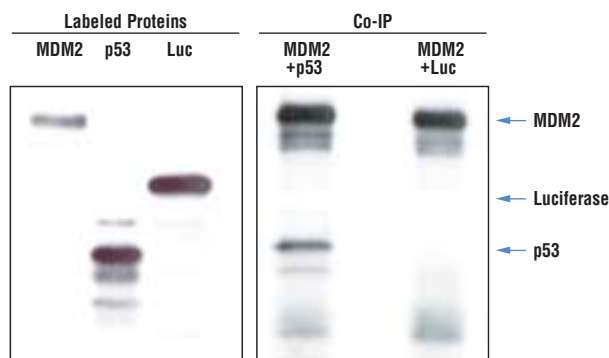


Figure 3. Co-IP of p53 and MDM2 with mouse MAAb to MDM2 using components of the ProFound™ Co-Immunoprecipitation Kit. Purified mouse anti-MDM2 (100 µg) was coupled to 200 µl settled AminoLink® Plus Coupling Gel. Luciferase, MDM2 and p53 were translated and ³⁵S-labeled using TNT Coupled Reticulocyte Lysate System (Promega). *In vitro* translated p53 (5 µl) and MDM2 (5 µl) were combined and incubated at 30°C for 30 minutes. Co-IP was performed at 4°C for 2 hours with 60 µl anti-MDM2 antibody-coupled resin. Luciferase was used as a negative control protein to incubate with MDM2. Eluted proteins were resolved over a 4-20% SDS-PAGE gel and visualized by autoradiography.



Highlights:

- AminoLink® Plus Coupling Gel for covalent attachment of primary antibody prevents co-elution of antibody or antibody fragments with the target protein interactors
- Couple any purified antibody regardless of species or Ig subclass (chicken IgY, human IgE, mouse IgG1, IgM, etc.)
- Physiologic co-IP buffer for optimal binding
- Control gel included, allowing for a properly controlled co-IP experiment
- Spin cup columns for efficient washing and elution of small gel volumes
- Coupled antibody support reusable up to 10 times, saving precious antibody

Ordering Information

Product #	Description	Pkg. Size
23600	ProFound™ Co-Immunoprecipitation Kit Sufficient material to immobilize up to 10 antibodies and perform a minimum of 40 co-IP reactions if 25 µl of antibody immobilized support is used. More co-IPs can be performed if smaller volumes are used. Includes: AminoLink® Plus Coupling Gel, (4 ml of a 50% slurry) Control Gel (4 ml of a 50% slurry) BupH™ Modified Dulbecco's PBS (Coupling Buffer and Co-IP Buffer) (1 liter total volume) Quenching Buffer Wash Solution Sodium Cyanoborohydride Solution (5 M) Handee™ Spin-Cup Columns Handee™ Microcentrifuge Tubes, 1.5 ml IgG Elution Buffer ImmunoPure® Lane Marker Sample Buffer, Non-Reducing (5X)	Kit 2 ml settled gel 2 ml settled gel 2 packs 60 ml 60 ml 0.5 ml 50 ea. 144 ea. 50 ml 5 ml
23605	ProFound™ Mammalian Co-Immunoprecipitation Kit Sufficient material to immobilize up to 10 antibodies and perform a minimum of 40 co-IP reactions if 25 µl of antibody immobilized support is used. More co-IPs can be performed if smaller volumes are used. Includes same components as Product # 23600 and also includes M-PER® Lysis Buffer, 25 ml	Kit

Related Pierce Products:

Product #	Description	Pkg. Size
28314	Surfact-Amps® X-100	6 x10 ml ampules
21027	ImmunoPure® Gentle Ag/Ab Elution Buffer	500 ml

ProFound™ HA- or c-Myc Tag IP/Co-IP Kit

**Need to perform IP or co-IP reactions with your HA- or c-Myc-tagged protein?
Open box ... Read instructions ... Start performing an IP or co-IP.
High-specificity immobilized antibodies make it easy.**

No tags are more popular for mammalian system protein expression than HA or c-Myc. Although these tags are extremely popular, a kit that allows you to conveniently perform immunoprecipitation (IP) or co-immunoprecipitation (co-IP) reactions using these tags has not been available. The new ProFound™ IP/Co-IP Kits from Pierce include all necessary reagents, buffers and hardware that allow efficient purification of a tagged target protein (i.e., IP) or confirmation of potential interactions indicated by yeast two-hybrid results (i.e., co-IP).

These four new kits, which are specifically for HA- and c-Myc-tagged proteins, allow you to easily perform an IP or co-IP experiment with minimal optimization. The kit includes everything needed for both the novice and the expert to get a quick start on whichever application chosen.

High-affinity, high-specificity antibodies immobilized onto an agarose matrix are at the heart of these new kits. In addition, the kits contain a full complement of buffers, eluents, a positive control and necessary hardware to efficiently perform the intended application.

Highlights:

IP and co-IP for HA- or c-Myc-tagged proteins directly out of the box

- Demonstrated utility in the IP and co-IP application benefits the novice and expert. The kits include all essential components to perform the assays. There's no need to formulate or validate raw materials.

Immobilized high-affinity antibodies with excellent specificity for the HA or c-Myc tag

- The immobilized anti-HA and anti-c-Myc monoclonals precipitate the appropriately tagged protein specifically and in high yield, resulting in clean Western blot detection.
- Excellent results with as little as 2.5 µg of anti-HA antibody and 1 µg of anti-c-Myc antibody in the IP mode with the respective positive control lysate.
- Limits possibility of nonspecific binding to other proteins in the lysate.
- Eliminates contamination from antibody or antibody fragments after elution of the precipitated protein or the co-IP complex. This benefit is especially important when interpreting protein interaction results.

Simple, flexible and easy-to-follow protocols

- Complete kit format offers optimum convenience in both the IP and co-IP modes.
- Eliminate Protein A or Protein G, reducing nonspecific binding and shortening the IP procedure.
- System demonstrates excellent flexibility with respect to the amount of antibody or amount of lysate used, enabling isolation of low-expression HA-/c-Myc-tagged targets.
- The use of Handee™ Mini-Spin Columns accelerate the IP/co-IP process.

Spin Columns

- Handee™ Mini-Spin Columns are very convenient for small sample handling.
- Allow more efficient washing.
- Eliminate resin losses.

ProFound™ HA- or c-Myc Tag IP/Co-IP Kit Descriptions

Each kit listed at right consists of two components: an Application Set and a Positive Control Lysate. The Application Set contains the immobilized support appropriate for the kit and all of the required buffers, eluents and hardware. The second component is a bacterial lysate containing an overexpressed GST with either HA or c-Myc as the c-terminal tag. The mammalian version of each kit contains M-PER® Protein Extraction Reagent for use with mammalian cell-based IP or co-IP applications. The Application Sets and Positive Control Lysates can also be ordered separately. See ordering information.

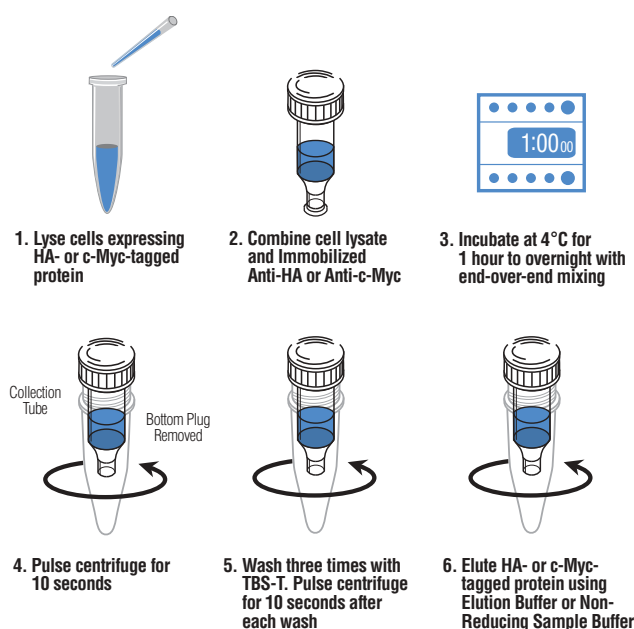


Figure 4. Protocol summary.

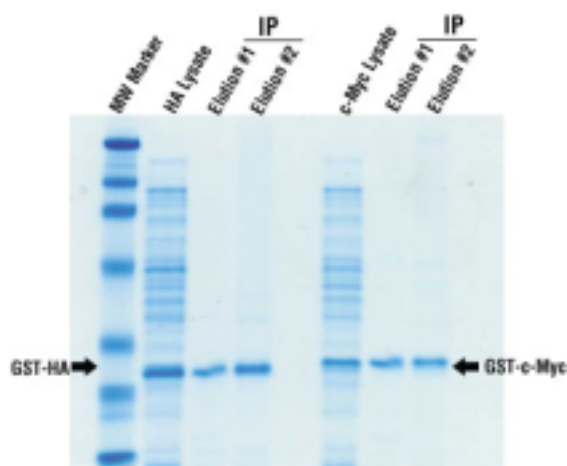


Figure 5. Effectiveness of elution options in the IP of GST-HA and GST-c-Myc from bacterial lysates. IP results achieved with the ProFound™ HA and c-Myc IP/Co-IP kits using the appropriate positive control lysate provided and suggested elution options for GST-HA and GST-c-Myc, respectively. The elution components are supplied with each kit. Elution Performed with #1: Elution Buffer or #2: 2X nonreducing sample buffer.

Ordering Information

Product #	Description	Pkg. Size
23610	ProFound™ HA-Tag IP/Co-IP Kit Sufficient material to conduct 25 IP/co-IP assays using proteins expressed with an HA tag. Kit is supplied complete with an HA-tagged positive control lysate. Kit contains: Immobilized Anti-HA 150 µl BupH™ Tris Buffered Saline Pack 1 pack Elution Buffer, pH 2.8 50 ml ImmunoPure® Lane Marker Non-Reducing Sample Buffer (5X) 5 ml Handee™ Mini-Spin Columns Accessory Pack, 27 columns with pre-inserted frit and top and bottom caps Collection Tubes and Caps Accessory Pack, 100 graduated 2 ml tubes and plug caps HA-Tagged Positive Control 500 µl Bacterial lysate containing over-expressed GST-HA	Kit
23615	ProFound™ Mammalian HA-Tag IP/Co-IP Kit Sufficient material to conduct 25 IP/co-IP assays using proteins expressed with an HA tag. Kit is supplied complete with a mammalian cell lysis buffer and an HA-tagged positive control lysate. Kit contains: Immobilized Anti-HA 150 µl BupH™ Tris Buffered Saline Pack 1 pack Elution Buffer, pH 2.8 50 ml ImmunoPure® Lane Marker Non-Reducing Sample Buffer (5X), Handee™ Mini-Spin Columns Accessory Pack, 27 columns with pre-inserted frit and top and bottom caps Collection Tubes and Caps Accessory Pack, 100 graduated 2 ml tubes and plug caps M-PER® Mammalian Protein Extraction Reagent 25 ml HA-Tagged Positive Control 500 µl Bacterial lysate containing over-expressed GST-HA	Kit
23620	ProFound™ c-Myc-Tag IP/Co-IP Kit Sufficient material to conduct 25 IP/co-IP assays using proteins expressed with a c-Myc tag. Kit is supplied complete with a c-Myc-tagged positive control lysate. Kit contains: Immobilized Anti-c-Myc 250 µl BupH™ Tris Buffered Saline Pack 1 pack Elution Buffer, pH 2.8 50 ml ImmunoPure® Lane Marker Non-Reducing Sample Buffer (5X), Handee™ Mini-Spin Columns Accessory Pack, 27 columns with pre-inserted frit and top and bottom caps Collection Tubes and Caps Accessory Pack, 100 graduated 2 ml tubes and plug caps c-Myc-Tagged Positive Control 500 µl Bacterial lysate containing over-expressed GST-c-Myc	Kit
23625	ProFound™ Mammalian c-Myc Tag IP/Co-IP Kit Sufficient material to conduct 25 IP/co-IP assays using proteins expressed with a c-Myc tag. Kit is supplied complete with a mammalian cell lysis buffer and a c-Myc-tagged positive control lysate. Kit contains: Immobilized Anti-c-Myc 250 µl BupH™ Tris Buffered Saline Pack 1 pack Elution Buffer, pH 2.8 50 ml ImmunoPure® Lane Marker Non-Reducing Sample Buffer (5X) Handee™ Mini-Spin Columns Accessory Pack, 27 columns with pre-inserted frit and top and bottom caps Collection Tubes and Caps Accessory Pack, 100 graduated 2 ml tubes and plug caps M-PER® Mammalian Protein Extraction Reagent 25 ml c-Myc-Tagged Positive Control 500 µl Bacterial lysate containing over-expressed GST-c-Myc	Kit

Protein Interactions Pull-Down Assays

Introduction to Pull-down Assays

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. The pull-down technique has become an invaluable tool for the life scientist interested in studying cellular pathways via protein:protein interactions.

The pull-down assay is an *in vitro* method used to determine physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein:protein interaction predicted by other research techniques (e.g., co-immunoprecipitation, yeast two-hybrid and density gradient centrifugation) and as an initial screening assay for identifying previously unknown protein:protein interactions. The minimal requirement for a pull-down assay is the availability of a purified and tagged protein (the bait) that will be used to capture and "pull-down" a protein-binding partner (the prey).

Pull-down assays are a form of affinity purification and are very similar to immunoprecipitation (see previous topic in this brochure) except that a bait protein is used instead of an antibody. Affinity chromatography (i.e., affinity purification) methodologies greatly enhance the speed and efficiency of protein purification and simultaneously provide the technology platform for performing a pull-down, or co-purification, of potential binding partners. In a pull-down assay, a tagged bait protein is captured on an immobilized affinity ligand specific for the tag, thereby generating a "secondary affinity support" for purifying other proteins that interact with the bait protein. The secondary affinity support of immobilized bait can be incubated with a variety of other protein sources that contain putative prey proteins. The source of prey protein at this step depends on whether the researcher is confirming previously suspected protein:protein interactions or identifying unknown protein:protein interactions.

The Pull-down Assay as a Confirmatory Tool

Confirmation of previously suspected interactions typically uses a prey protein source that has been expressed in an artificial protein expression system. This allows the researcher to work with a larger quantity of the protein than is typically available under endogenous expression conditions and eliminates confusing results, which could arise from interaction of the bait with other interacting proteins present in the endogenous system that are not under study. Protein expression system lysates (i.e., *E. coli* or baculovirus-infected insect cells), *in vitro* transcription/translation reactions, and previously purified proteins are appropriate prey protein sources for confirmatory studies.

The Pull-down Assay as a Discovery Tool

Discovery of unknown interactions contrasts with confirmatory studies because the research interest lies in discovering new proteins in the endogenous environment that interact with a given bait protein. The endogenous environment can entail a plethora of possible protein sources but is generally characterized as a complex protein mixture considered to be the native environment of the bait protein. Any cellular lysate in which the bait is normally expressed, or complex biological fluid (i.e., blood, intestinal secretions, etc.) where the bait would be functional, are appropriate prey protein sources for discovery studies.

Protein Interactions Pull-down Assays

Critical Components of Pull-down Assays

Bait Protein Criteria

Bait proteins for pull-down assays can be generated either by linking an affinity tag to proteins purified by traditional purification methods or by expressing recombinant fusion-tagged proteins. Researchers who have access to commercially available purified proteins or frozen aliquots of purified protein from an earlier study can design a pull-down assay without the need for cloning the gene encoding the protein of interest. The purified protein can be tagged with a protein-reactive tag (e.g., Sulfo-NHS-LC-Biotin, Product # 21335) commonly used for such labeling applications. Alternatively, if a cloned gene is available, molecular biology methods can be employed to subclone the gene to an appropriate vector with a fusion tag (e.g., 6xHis or glutathione S-transferase, GST). Recombinant clones can be overexpressed and easily purified, resulting in an abundance of bait protein for use in pull-down assays.

Binding Parameters: Stable vs. Transient Interactions

Discovery and confirmation of protein:protein interactions using the pull-down technique depend heavily on the nature of the interaction under study. Interactions can be stable or transient and this characteristic determines the conditions for optimizing binding between bait and prey proteins. Stable interactions make up most cellular structural features but can also occur in enzymatic complexes that form identifiable structures. Transient interactions are usually associated with transport or enzymatic mechanisms. The ribosome illustrates both examples because the structure consists of many stable protein:protein interactions, but the enzymatic mechanism that translates mRNA to nascent protein requires transient interactions.

Stable protein:protein interactions are easiest to isolate by physical methods like pull-down assays because the protein complex does not disassemble over time. Because these interactions often contribute to cellular structure, the dissociation constant between proteins is usually low, correlating to a strong interaction. Strong, stable protein complexes can be washed extensively with high-ionic strength buffers to eliminate any false-positive results due to nonspecific interactions. If the complex interaction has a higher dissociation constant and is a weaker interaction, the interaction strength and thus protein complex recovery can be improved by optimizing the assay conditions related to pH, salt species and salt concentration. Problems of nonspecific interactions can be minimized with careful design of appropriate control experiments.

Transient interactions are defined by their temporal interaction with other proteins and are the most challenging protein:protein interactions to isolate. These interactions are more difficult to identify using physical methods like pull-down assays because the complex may dissociate during the assay. Because transient interactions occur during transport or as part of enzymatic processes, they often require cofactors and energy via NTP hydrolysis. Incorporating cofactors and non-hydrolyzable NTP analogs during assay optimization can serve to “trap” interacting proteins in different stages of a functional complex that is dependent on the cofactor or NTP.

Elution of the Bait-prey Complex

Identification of bait-prey interactions requires that the complex is removed from the affinity support and analyzed by standard protein detection methods. The entire complex can be eluted from the affinity support by using SDS-PAGE loading buffer or a competitive analyte specific for the tag on the bait protein. SDS-PAGE loading buffer is a harsh treatment that will denature all protein in the sample, and it restricts analysis to SDS-PAGE. This method may also strip excess protein off the affinity support that is nonspecifically bound to the matrix, and this material will interfere with analysis. Competitive analyte elution is much more specific for the bait-prey interaction because it does not strip proteins that are nonspecifically bound to the affinity support. This method is non-denaturing; thus, it can elute a biologically functional protein complex, which could be useful for subsequent research.

An alternative elution protocol allows selective elution of prey proteins while the bait remains immobilized. This is accomplished using a step-wise gradient of increasing salt concentration or a step-wise gradient of decreasing pH. A gradient elution is not necessary once the critical salt concentration or pH has been optimized for efficient elution. These elution methods are also non-denaturing and can be informative in determining relative interaction strength.

Gel Detection of Bait-prey Complex

Protein complexes contained in eluted samples can be visualized by SDS-PAGE and associated detection methods including gel staining with Pierce GelCode™ Stains (e.g., Product # 24590, 24602), Western blotting detection with SuperSignal® Chemiluminescent Substrates (e.g., Product # 34080) and ³⁵S radioisotopic detection. Final determination of interacting proteins often entails protein band isolation from a polyacrylamide gel, tryptic digestion of the isolated protein and mass spectrometric identification of digested peptides.

Importance of Control Experiments for the Pull-down Assay

In all pull-down assays, carefully designed control experiments are absolutely necessary for generating biologically significant results. A negative control consisting of a non-treated affinity support (minus bait protein sample, plus prey protein sample) helps to identify and eliminate false-positives caused by nonspecific binding of proteins to the affinity support. The immobilized bait control (plus bait protein sample, minus prey protein sample) helps identify and eliminate false-positives caused by nonspecific binding of proteins to the tag of the bait protein. The immobilized bait control also serves as a positive control to verify that the affinity support is functional for capturing the tagged bait protein.

ProFound™ Pull-Down Protein:Protein Interaction Kits

“Homemade” pull-down methodologies for confirming or identifying protein:protein interactions are ubiquitous in contemporary scientific literature. The homemade pull-down assay represents a collection of reagents from multiple commercial vendors that cannot be validated together as a functional assembly except by extensive assay development by the researcher. Troubleshooting this mix of reagents can be tedious and time-consuming. ProFound™ Pull-Down Protein:Protein Interaction Kits (Product # 21115, 21277, 21516) contain complete, validated sets of reagents specifically developed for performing pull-down assays. The buffers provided in each kit allow complete flexibility to determine optimal conditions for isolating interacting proteins. The working solutions for washing and binding are physiologic in pH and ionic strength, providing a starting point from which specific buffer conditions for each unique interacting pair can be optimized. These kits incorporate the Handee™ Mini-Spin Column format for efficient handling of small volumes of affinity support. The format allows complete retention of the affinity support during the pull-down assay, eliminating one important source of variability common in traditional pull-down assay formats.

Reference

1. Einarson, M.B. and Orlinick, J.R. (2002). Identification of Protein-Protein Interactions with Glutathione S-Transferase Fusion Proteins. *In* Protein-Protein Interactions: A Molecular Cloning Manual, Cold Spring Harbor Laboratory Press, pp. 37-57.

Recommended Reading

- Einarson, M.B. (2001). Detection of Protein-Protein Interactions Using the GST Fusion Protein Pulldown Technique. *In* Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press, pp.18.55-18.59.

Protein Interactions Pull-Down Assays

ProFound™ GST- and PolyHis-Tagged Pull-Down Assay Kits

Prepare to discover a new protein:protein interaction with your GST- or polyHis-tagged bait protein.

Identifying and characterizing the interactions of a given protein has emerged as the most valuable information that can be developed in the post-genomic era. ProFound™ Pull-Down Kits contain the necessary components to capture interacting proteins using the popular pull-down method. The only item you provide is an appropriately tagged fusion protein as the “bait.” ProFound™ Pull-Down Kits are designed to teach the method to the first-time user and to shorten the time to a result for those experienced in this method.

Highlights:

- Provides a complete, affordable and easy-to-use strategy for discovery of protein:protein interactions
- Uses common laboratory equipment (e.g., microcentrifuges and mini-gels)
- Adapts to single- or multiple-sample demands
- Supplied complete with cell lysis buffer
- Flexible protocol aids in the capture of weak or transient interactions
- Efficient recovery of interacting complexes



Applications

- Discover a new protein:protein interaction from a cell lysate
- Confirm a putative interaction from a cell lysate or with a previously purified protein
- Extract protein:protein interaction information from *in vitro* transcription/translation lysates

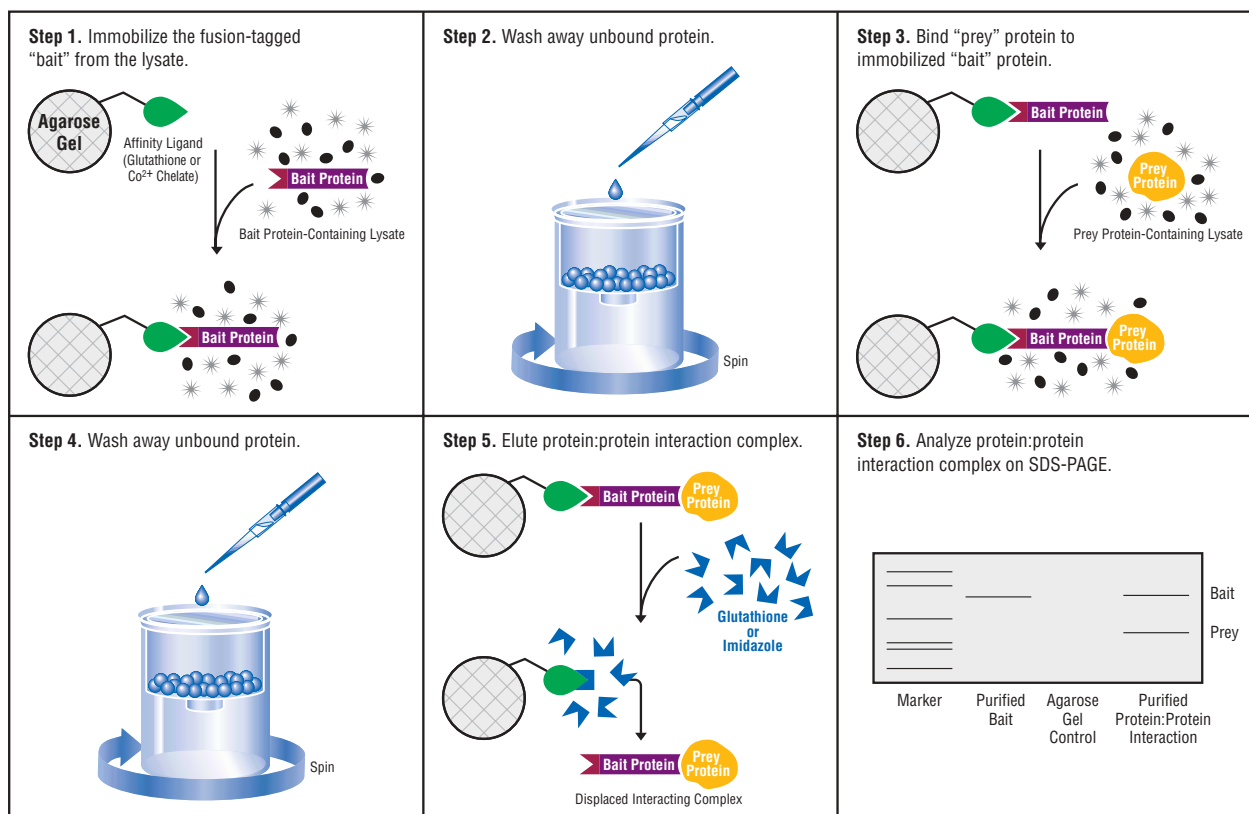
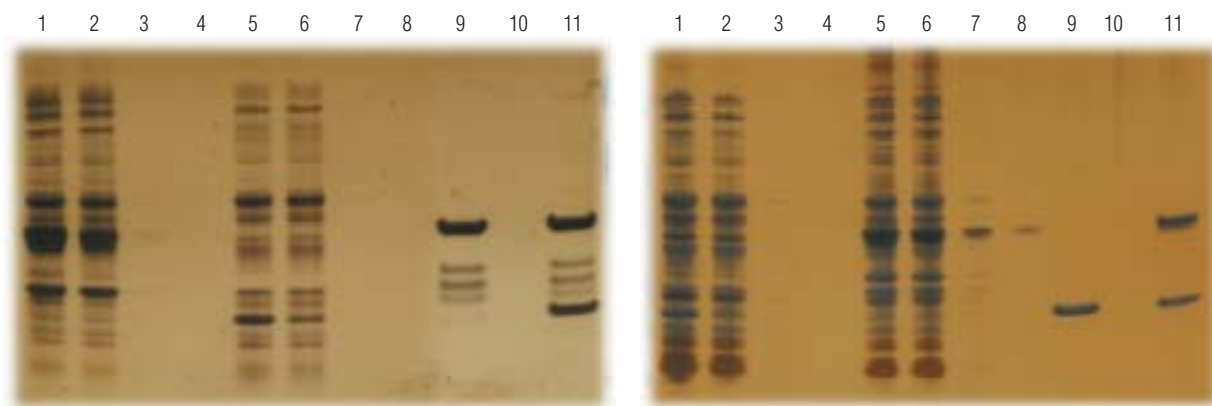


Figure 6. Generalized scheme for use of a ProFound™ Pull-Down Protein:Protein Interaction Kit using a GST-tagged or PolyHis-tagged protein as the “bait.”



Lane # A. GST-Tag Pull-Down

1	Lysate from <i>E. coli</i> expressing GST-tagged BIR2 (bait protein).
2	Flow-through from the lysate in Lane 1 bound to an immobilized reduced glutathione support for 1 hour at 4°C.
3	Wash #1 of the support.
4	Wash #2 of the support. (Washes 3-5 not shown.)
5	Lysate from <i>E. coli</i> expressing 9xHis-tagged wild-type Smac (prey protein).
6	Flow-through from the lysate in Lane 5 is allowed to interact with the immobilized bait for 1 hour at 4°C.
7	Wash #1 of the support.
8	Wash #2 of the support. (Washes 3-5 not shown.)
9	Bait control. Bait treated as described in Lanes 1-8 and subsequently eluted. No prey added – just binding buffer.
10	Prey control. Prey treated as described in Lanes 1-8 and subsequently eluted. No bait added – just binding buffer.
11	Elution of bait:prey complex (prepared in Lanes 1-8) from the support with 100 mM reduced glutathione. Western blotting confirms that the minor bands observed in Lanes 9 and 11 are degradation products of GST-tagged BIR2.

B. PolyHis-Tag Pull-Down

Lysate from <i>E. coli</i> expressing 9xHis-tagged wild-type Smac (bait protein).
Flow-through from the lysate in Lane 1 bound to an immobilized cobalt chelate support for 1 hour at 4°C.
Wash #1 of the support.
Wash #2 of the support. (Washes 3-5 not shown.)
Lysate from <i>E. coli</i> expressing GST-tagged BIR2 (prey protein).
Flow-through from the lysate in Lane 5 is allowed to interact with the immobilized bait for 1 hour at 4°C.
Wash #1 of the support.
Wash #2 of the support. (Washes 3-5 not shown.)
Bait control. Bait treated as described in Lanes 1-8 and subsequently eluted. No prey added – just binding buffer.
Prey control. Prey treated as described in Lanes 1-8 and subsequently eluted. No bait added – just binding buffer.
Elution of bait:prey complex (prepared in Lanes 1-8) from the support with 250 mM imidazole.

Figure 7. Validation of the ProFound™ Pull-Down Protein:Protein Interaction Kits using a known interacting pair.

Ordering Information

Product #	Description	Pkg. Size
21516	ProFound™ Pull-Down GST Protein:Protein Interaction Kit Sufficient materials for performing 25 pull-down assays using a GST-tagged protein as the bait. Includes: Immobilized Glutathione ProFound™ Lysis Buffer Glutathione BupH™ Tris Buffered Saline Handee™ Spin Cup Columns Accessory Pack Collection Tubes and Caps Accessory Pack	Kit 750 µl settled gel 250 ml 1 g 1 pack (500 µl) 27 columns 100 tubes

Acknowledgment

Pierce gratefully acknowledges Dr. Yigong Shi of Princeton University for supplying the GST-BIR2- and 9xHis Smac/DIABLO-expressing clones. Dr. Shi's laboratory is engaged in research aimed at understanding the structural and molecular mechanisms involved in tumorigenesis and apoptosis.

Ordering Information

Product #	Description	Pkg. Size
21277	ProFound™ Pull-Down PolyHis Protein:Protein Interaction Kit Sufficient materials for performing 25 pull-down assays using a polyhistidine-tagged protein as the bait. Includes: Immobilized Glutathione ProFound™ Lysis Buffer 4 M Imidazole Stock Solution BupH™ Tris Buffered Saline Handee™ Spin Cup Columns Accessory Pack Collection Tubes and Caps Accessory Pack	Kit 750 µl settled gel 250 ml 5 ml 1 pack (500 µl) 27 columns 100 tubes

References

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Sambrook, J. and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual* 3rd Edition. Chapter 18: Protein Interaction Technologies, Protocol #3: Detection of Protein-Protein Interactions using the GST Fusion Protein Pull-Down Technique. Cold Spring Harbor Laboratory Press.
Soutoglou, *et al.* (2000). *Mol. Cell* **5**, 745-751.

Protein Interactions Pull-Down Assays

ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit

Pull-down a binding partner with the new Pierce Biotinylated-Protein:Protein Interaction Kit.

Identifying and characterizing the interactions of a given protein has emerged as the most valuable information that can be developed in the post-genomic era. This Pierce Protein:Protein Interaction Kit contains the essential components necessary to enable the capture of an interacting prey protein using the popular pull-down method. All you need to provide is an appropriately purified and biotinylated target protein as the "bait." The Pierce ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit is designed to teach the method to the first-time user and to shorten the time to a result for those more experienced in the method.

Highlights:

- Provides a complete, affordable and easy-to-use strategy for discovery of protein:protein interactions
- Uses common laboratory equipment and reagents (e.g., microcentrifuges, mini-gels, protein stains)
- Adaptable to single- or multiple-sample demands
- Flexible pull-down format
- Detects a potential binding partner of a biotinylated bait protein in one day

Applications

- Discover a new protein:protein interaction using an immobilized biotinylated bait and prey captured from a cell lysate
- Confirm a putative interaction with a prey protein captured from a cell lysate or with a previously purified prey protein
- Extract protein:protein interaction information from *in vitro* transcription/translation lysates

Ordering Information

Product #	Description	Pkg. Size
21115	ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit Sufficient materials for conducting 25 pull-down assays using a purified and biotinylated protein as the bait. Includes: Immobilized Streptavidin	Kit
	BupH™ Tris Buffered Saline	1.5 ml settled gel
	Biotin Blocking Buffer	1 pack (500 ml)
	Wash Buffer (Acetate, pH 5.0)	15 ml
	Elution Buffer (pH 2.8)	100 ml
	Handee™ Spin Cup Columns	50 ml
	Accessory Pack	27 columns
	Collection Tubes and Caps	200 x 2 ml tubes,
	Accessory Pack	graduated

See also: *GST- and PolyHis-Tagged Pull-Down Assay Kits, pages 18-19.*

References

Ducoux, M., et al. (2001). *J. Biol. Chem.* **276** (52), 49258-29266.
Jannatipour, M., et al. (2001). *J. Biol. Chem.* **276** (35), 33093-33100.

For a complete list of Pierce's biotinylation reagents, order a free *Avidin-Biotin Handbook* (Product # 1600941).

For more product information, or to download a product instruction booklet, visit www.piercenet.com.

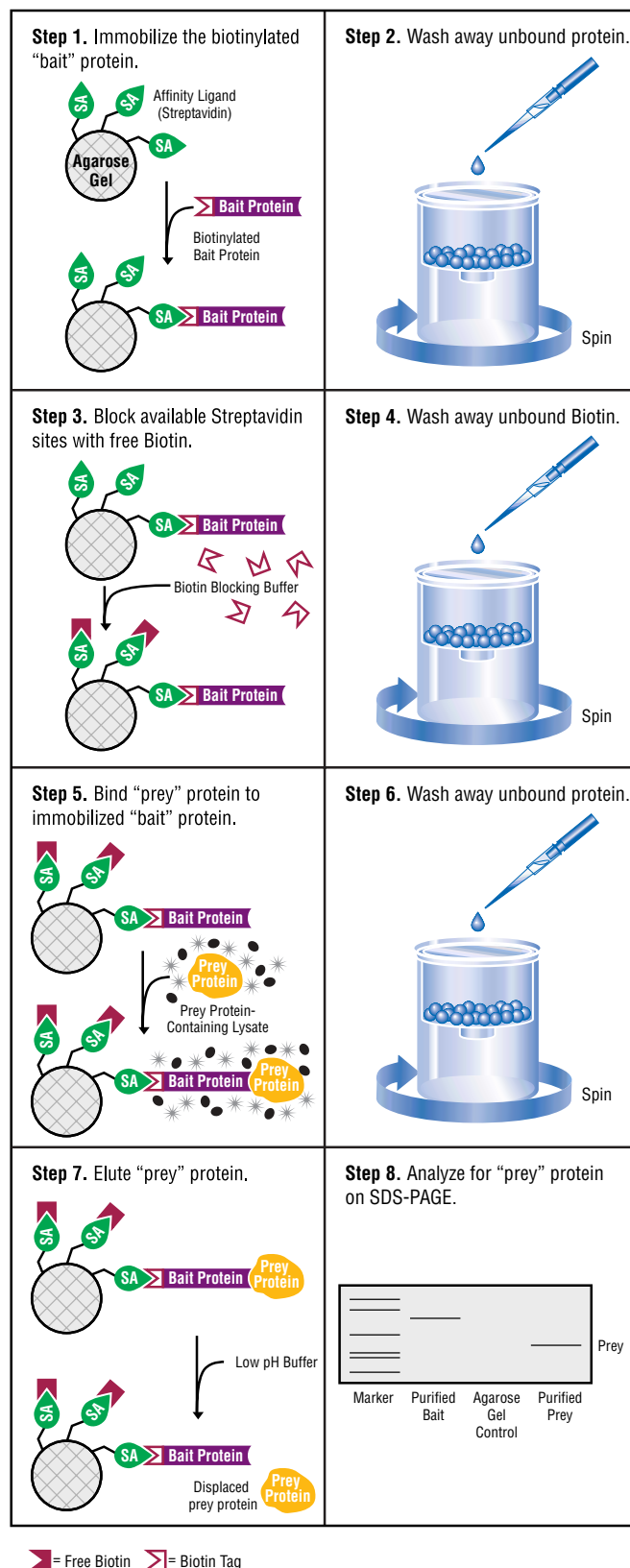


Figure 8. ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit Protocol.

Cross-linking Reagents

The Use of Cross-linking Agents to Study Protein Interactions

Covalently Bonding Interacting Proteins

When two or more proteins have specific affinity for one another that causes them to come together in biological systems, bioconjugation technology can provide the means for investigating those interactions. Most *in vivo* protein:protein binding is transient and occurs only briefly to facilitate signaling or metabolic function. Capturing or freezing these momentary contacts to study which proteins are involved and how they interact is a significant goal of proteomics research today.

Cross-linking reagents can provide the means for capturing protein:protein complexes by covalently bonding them together as they interact. The rapid reactivity of the common functional groups on cross-linkers allows even transient interactions to be frozen in place or weakly interacting molecules to be seized in a complex stable enough for isolation and characterization.

Targeting and Controlling Specificity of Cross-linking

The simple addition of homobifunctional or heterobifunctional cross-linkers to cell suspensions or cell lysates will cause many protein conjugates to be formed, not just those directly involved in the target protein:protein interaction. Many cell-surface protein interactions have been studied using this “shotgun” approach, but the challenge in this technique is the analysis of data after complexes have been isolated.

To help solve these problems, more sophisticated cross-linker designs were created that incorporate photoreactive groups, which can be made to react at selected times and only in response to irradiation by UV light. Heterobifunctional cross-linkers with a thermoreactive group (spontaneously reactive) at one end and a photoreactive group on the other end can be reacted first through the thermoreactive end with a protein that can be used as bait for other interacting proteins. The modified protein is introduced into a sample and allowed to interact with other proteins. Then the sample is exposed to UV light, which causes the photoreactive end of the modified protein to covalently link to nearby molecules, thus “freezing” in place any interacting protein as a complex.

Photoreactive Cross-linkers

The use of photoreactive cross-linkers for studying protein interactions is preferred over methods that use standard bifunctional thermoreactive cross-linkers because photoreactive cross-linkers limit the formation of conjugate polymer artifacts. However, the downside of some photoreactive coupling methods is that the yield of conjugate formation is typically low. Particularly, many aryl azide groups undergo an inefficient ring-expansion reaction, which directs their reactivity exclusively toward amine groups, therefore limiting their utility for nonselective insertion into any neighboring protein structures. In addition, solvent reactions that quench the photoreactive intermediate often exceed reactions with a desired target.

Some photoreactive groups, such as halogen-substituted aryl azides and benzophenones, have much better conjugation yields and can efficiently capture interacting molecules. For instance, cross-linkers that incorporate a perfluoroazidobenzamido photoreactive group do not undergo ring expansion after photolyzing, thus they create a highly reactive nitrene upon UV exposure that effectively couples to any protein structures nearby. An example of this type of photoreactive reagent is SFAD (Product # 27719), which has an amine-reactive sulfo-NHS-ester at one end and the halogen-substituted phenylazide group at the other end.

Pierce Bifunctional Cross-linking Reagents

The ability to selectively conjugate two or more proteins together using cross-linking reagents permits the study of interacting proteins in complex mixtures. As the proteome is better defined, the interactions those protein molecules undergo will become increasingly important to understand. Pierce bioconjugation reagents are a critical factor in facilitating this knowledge. Additional information on label transfer and cross-linking as it pertains to the study of protein interactions follows this section. Additional information on these reagents and cross-linking chemistry in general is provided in the Pierce Applications Handbook and Catalog.

Cross-linking Reagents

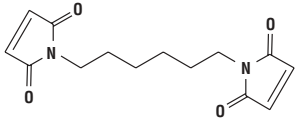
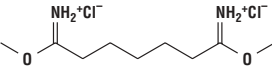
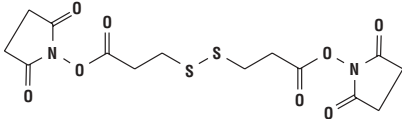
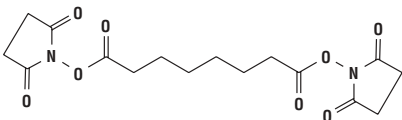
Cross-linking Reagents for Protein Interaction Capture

Homobifunctional cross-linking reagents and heterobifunctional photoreactive cross-linking agents are routinely applied to protein:protein interaction studies. The following table of homobifunctional cross-linkers represents a small sampling of the Pierce Products offering in this area. Specific application references to the use of these reagents in the elucidation of protein:protein interactions are provided below.

Protein interactions can also be trapped effectively with the use of heterobifunctional reagents. These reagents are used in a stepwise manner. Initially, a purified bait protein is modified by reaction with one of the reactive groups of the cross-linker. Most reagents target amine functions but other functional groups can be targeted as well. The initial reaction is usually carried out in the dark. The other reactive moiety common to reagents with application to protein interactions is photoreactive. The photoreactive group of the heterobifunctional reagent will ultimately cross-link a prey protein to the bait when exposed to light. Typically, this photoreactive group is an aryl azide-based moiety that reacts nonspecifically with proteins and other biomolecules upon photolysis.

The modified bait is incubated with a lysate or a suspected prey protein and allowed to interact in the dark. The complex is captured when the reaction is exposed to the proper light conditions to activate the cross-linker. Photoreactive, heterobifunctional reagents with a cleavable disulfide linkage will allow reversal of the bait:prey cross-link and recovery of the components of the interacting pair for further analysis.

Homobifunctional Cross-linking Reagents

Product #	Product Name	Structure M.W. Spacer Arm Length	Key Features	Ref.	Pkg. Size
22330	BMH Bis-Maleimido-hexane	 BMH M.W. 276.29 Spacer Arm 16.1 Å	<ul style="list-style-type: none"> Mild, irreversible -SH to -SH cross-linking agent 	1-3	50 mg
21666	DMP Dimethyl pimelimidate • 2 HCl	 DMP M.W. 259.17 Spacer Arm 9.2 Å	<ul style="list-style-type: none"> Reversible amidine bond retains net charge character of proteins Amine-reactive 	4-6	50 mg
22585	DSP Dithiobis-(Succinimidyl propionate)	 DSP M.W. 404.42 Spacer Arm 12.0 Å	<ul style="list-style-type: none"> Thiol-cleavable and amine-reactive 	7-9	1 g
21655 21555	DSS Disuccinimidyl suberate	 DSS M.W. 368.35 Spacer Arm 11.4 Å	<ul style="list-style-type: none"> Non-cleavable, amine-reactive homobifunctional 	10-12	50 mg 1 g

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Heterobifunctional Photoreactive Cross-linking Reagents

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
21510	ABH <i>p</i> -Azidobenzoyl hydrazide M.W. 177.17 Spacer Arm 11.96 Å		<ul style="list-style-type: none"> Glycoprotein-reactive Non-cleavable 	1	50 mg
20108	APG <i>p</i> -Azidophenyl glyoxal monohydrate M.W. 193.16 Spacer Arm 9.3 Å		<ul style="list-style-type: none"> Non-cleavable Arginine-reactive 	2	50 mg
21553	Sulfo-SADP Sulfo-succinimidyl-(4'-azidophenyl)1,3'-dithiopropionate M.W. 454.45 Spacer Arm 13.9 Å		<ul style="list-style-type: none"> Cleavable by 50 mM DTT, 100 mM BME or 1% NaBH4 Photolysis at 265-275 nm Amine-reactive 	3,4	50 mg
27735	Sulfo-NHS-LC-ASA Sulfosuccinimidyl (4-azido-salicylamido) hexanoate M.W. 491.41 Spacer Arm 18.0 Å		<ul style="list-style-type: none"> Can incorporate ¹²⁵I label before acylation step Photolysis initiated by long-wave UV Water-soluble Non-cleavable Amine-reactive 	5,6	50 mg
22589	Sulfo-SANPAH Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate M.W. 492.39 Spacer Arm 18.2 Å		<ul style="list-style-type: none"> Optimal photolysis occurs at 320-350 nm limiting damage to biomolecules by irradiation Water-soluble Non-cleavable Amine-reactive 	7,8	50 mg
27719	SFAD Sulfosuccinimidyl(perfluoro-azidobenzamido) ethyl-1,3'-dithiopropionate M.W. 597.48 Spacer Arm 14.6 Å		<ul style="list-style-type: none"> Improved photoconjugation Photolyzes at 320 nm Coupling ~70% efficient Water-soluble Cleavable Amine-reactive 	9,10	50 mg

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Protein Interactions Label Transfer

Label Transfer

Label transfer involves cross-linking interacting molecules (i.e., bait and prey proteins) with a labeled cross-linking agent and then cleaving the linkage between bait and prey such that the label remains attached to the prey (Figure 9). This method allows a label to be transferred from a known protein to an unknown, interacting protein. The label can then be used to purify and/or detect the interacting protein. Label transfer is particularly valuable because of its ability to identify proteins that interact weakly or transiently with the protein of interest. New non-isotopic reagents and methods continue to make this technique more accessible and simple to perform by any researcher.

Traditional Label Transfer Reagents

The earliest examples of label transfer reagents incorporated a photoreactive phenyl azide group that contained a hydroxy-phenyl modification on the ring. The phenolic hydroxyl activates the ring for substitution reactions to occur ortho or para to its position. These compounds can be radioiodinated using typical oxidation reagents such as chloramine T or IODO-BEADS® Iodination Reagent (see Protein Structure section of the Pierce catalog for more information on iodination). Iodination of the cross-linker with ^{125}I prior to its use will result in a radioactive label transfer reagent that can tag an unknown interacting protein with a radiolabel after cleavage of the cross-linker's spacer arm.

In practice, the cross-linker is first radioiodinated and then reacted with a bait protein, typically through available amine groups. This modified protein is then introduced into a sample and allowed to interact with other proteins. The sample is exposed to UV light to photo-cross-link the interacting complex. At this point, the label can facilitate detection of the interacting proteins or the complex can be cleaved and the radiolabel transferred to the protein interacting with the bait. The now radiolabelled, unknown protein(s) can be detected by autoradiography after separation by electrophoresis and Western transfer.

The first reagents employed using this method were bifunctional. They were designed such that the photoreactive moiety bears the transferable label. These molecules are either amine-reactive or sulfhydryl-reactive and are labeled radioisotopically with ^{125}I . More recent offerings have been prepared as trifunctional reagents that more adequately segregate the reactive sites from the label. These trifunctional reagents can be designed to include non-radioisotopic labels such as biotin.

SASD and APDP: Radiolabel Transfer Reagents

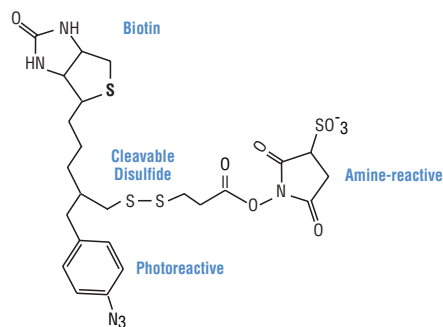
SASD and APDP (Product # 27716 and 27720, respectively) are heterobifunctional cross-linkers containing a photoreactive group that can be labeled with ^{125}I . They differ only in the functional group against which they are directed on the bait protein. SASD contains the amine-reactive sulfo-NHS group. APDP contains the sulfhydryl-reactive pyridyl-dithio group. The sulfhydryl-reactive group of APDP offers the advantage of allowing the course of the bait protein coupling to be monitored by following the loss of the 2-pyridyl-thione moiety (leaving group). The 2-pyridyl-thione can be detected at 343 nm (extinction coefficient: $8.08 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$).

Disadvantages of Traditional Bifunctional Label Transfer Reagents

Although these reagents have been used successfully to obtain data on protein interactions, they possess some inherent deficiencies compared to trifunctional reagents designed for label transfer applications. The user should be aware of the following characteristics of these reagents.

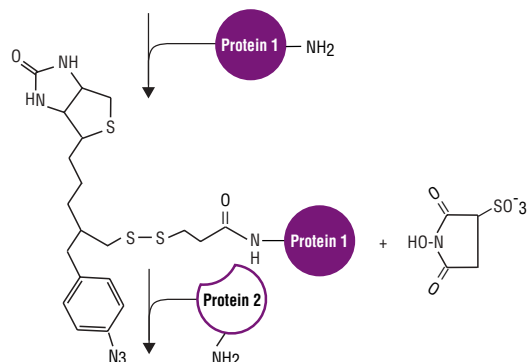
1. Photoreactive and labeled chemical groups are the same.
2. They require labeling with ^{125}I before use, and the efficiency of label incorporation is low.
3. The photoactivation step can result in several unproductive pathways that lower cross-linking yield between bait and prey.
4. The ^{125}I label can be released during the light reaction, causing nonspecific labeling of the protein(s) in the mix.

1. React available amine groups on a purified Bait Protein (**Protein 1**) with Sulfo-NHS containing Biotin Label Transfer Reagent [pH 7-9, in the dark, 30 minutes at RT].

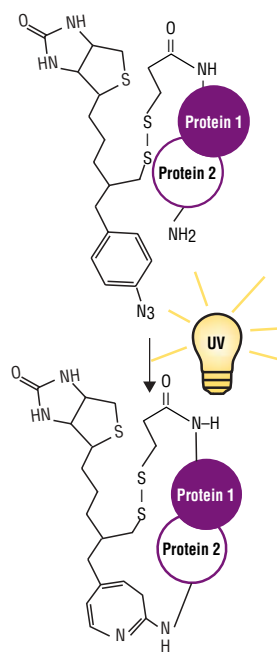


Biotin Label Transfer Reagent

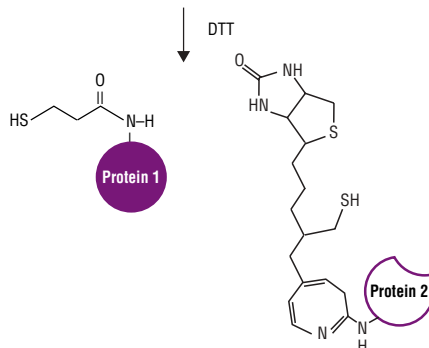
2. Introduce Biotinylated Bait Protein to Prey Protein (**Protein 2**) containing sample under conditions which promote favorable binding. Incubate in the dark 30-60 minutes.



3. Capture the Bait:Prey complex by irradiating the photoreactive aryl azide group with UV light.



4. Reduce the disulfide bond using DTT. Transfer the Biotin label from the **Bait Protein 1** to the **Bait Protein 2**.



5. Reduced sample is applied to a gel and separated by electrophoresis. Transfer proteins to a membrane. Detect Bait or Prey Proteins with the appropriate antibodies or Streptavidin-HRP.

Figure 9. General scheme for label transfer reactions.

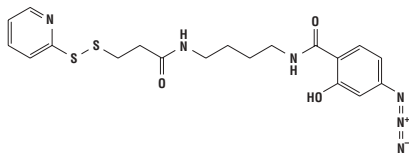
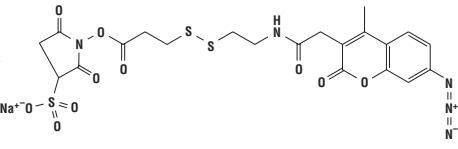
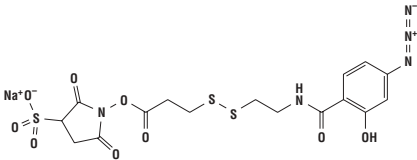
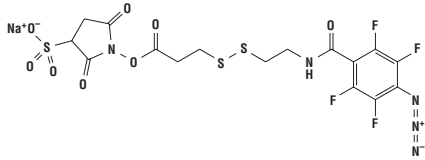
SAED: Fluorescent Label Transfer Reagent

Subsequent designs of bifunctional label transfer reagents used nonradioactive labels to avoid the safety issues posed by ¹²⁵I. Fluorescent constituents designed into cleavable photoreactive cross-linkers make possible transfer of a fluorescent label to an unknown interacting protein. An example of this type of reagent that incorporates a coumarin group is SAED (Product # 33030), which has been substituted with an azido group on the aromatic, photoreactive ring. The reagent is non-fluorescent prior to exposure to UV light, but upon photolyzing and coupling to interacting proteins, it becomes highly fluorescent. The reagent also has a disulfide bond that can be reduced, resulting in cleavage of the cross-linked proteins and transfer of the label to the unknown interacting species. In this case, the fluorescently labeled interacting proteins can be followed in cells to determine the site of interactions or the fate of the proteins after interacting.

Label Transfer Reagents

Bifunctional Label Transfer Reagents

Heterobifunctional, photoreactive, thiol-cleavable label transfer reagents enable the tagging of a prey protein. The photolysis wavelengths for these reagents are in the range between 320-400 nm, limiting damage to biomolecules by irradiation.

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
27720	APDP N-[4-(p-Azido-salicylamido) butyl]-3'-(2'-pyridyldithio) propionamide M.W. 446.55 Spacer Arm 21.0 Å		<ul style="list-style-type: none"> Radioiodinatable between -N₃ and -OH group of phenyl ring -SH-reactive Reaction monitored at 343 nm Membrane permeable 	1-5	50 mg
33030	SAED Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3'-dithiopropionate M.W. 621.6 Spacer Arm 23.6 Å		<ul style="list-style-type: none"> Water-soluble Amine-reactive Photoreactive Prey protein tracked by fluorescence Ex: 345-350 nm, Em: 440-460 nm No radiolabeling required AMCA moiety exhibits large Stokes shift 	16,17	5 mg
27716	SASD Sulfosuccinimidyl 2-[p-azidosalicylamido] ethyl-1,3'-dithiopropionate M.W. 541.51 Spacer Arm 18.9 Å		<ul style="list-style-type: none"> Radioiodinatable between -N₃ and -OH group of phenyl ring -NH₂-reactive Water-soluble 	3,6-13	50 mg
27719	SFAD Sulfosuccinimidyl(perfluoroazido)benzamidoethyl-1,3'-dithiopropionate M.W. 597.48 Spacer Arm 14.6 Å		<ul style="list-style-type: none"> Improved photoconjugation efficiency Photolyzes at 320 nm Label transfer monitored by ¹⁹F NMR Water-soluble Cleavable Amine-reactive 	9,10,14,15	50 mg

Compatible products for addition of ¹²⁵I to APDP or SASD.

Related Pierce Products:

Product #	Description	Pkg. Size
28601	ODO-GEN® Pre-Coated Iodination Tubes (12 mm x 75 mm glass test tubes coated with 50 µg ODO-GEN® Iodination Reagent in 100 µl volume)	10 tubes/pkg.
28665	ODO-BEADS® Iodination Reagent (N-Chloro-benzenesulfonamide modified non-porous polystyrene beads)	50/pkg.
28666	ODO-BEADS® Iodination Reagent	250/pkg.
28600	ODO-GEN® Iodination Reagent (1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril)	1 g

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Sulfo-SBED: ProFound™ Label Transfer Protein:Protein Interaction Reagent

Label transfer reagents can also have biotin built into their structure. This type of design allows the transfer of a biotin tag to an interacting protein after cleavage of a cross-bridge. Sulfo-SBED (Product # 33033) is an example of such a trifunctional reagent (Figure 10). It contains an amine-reactive sulfo-NHS-ester on one arm (built off the α -carboxylate of the lysine core), a photoreactive phenyl azide group on the other side (synthesized from the α -amine) and a biotin handle (connected to the ϵ -amino group of lysine). The arm containing the sulfo-NHS-ester has a cleavable disulfide bond, which permits transfer of the biotin component to any captured proteins.

In use, a bait protein first is derivatized with Sulfo-SBED through its amine groups, and the modified protein is allowed to interact with a sample. Exposure to UV light (300–366 nm) couples the photoreactive end to the nearest available C-H or N-H bond in the bait:prey complex, resulting in covalent cross-links between bait and prey. Upon reduction and cleavage of the disulfide spacer arm, the biotin handle remains attached to the protein(s) that interacted with the bait protein, facilitating isolation or identification of the unknown species using streptavidin, NeutrAvidin™ Protein or monomeric avidin.

The architecture of this trifunctional label transfer reagent differs substantially from the bifunctional counterparts discussed above. The advantages become almost immediately apparent just by examining the structure.

The reactive moieties are well-segregated within Sulfo-SBED. Most importantly, with a biotin label designed into Sulfo-SBED, radiolabeling with ^{125}I is no longer necessary. The biotin label can be used to significant advantage in a label transfer application. For example, biotin can operate as a handle for purification of the prey protein or prey protein fragments or as a detection target using streptavidin-HRP and colorimetric or chemiluminescent substrates.

Applications for Sulfo-SBED

Since the first availability of this patented reagent in 1994, the number of literature references for use of Sulfo-SBED in protein interaction-related applications has grown rapidly. Published applications show how Sulfo-SBED can be used to:

- Define interactions of complexes with activator domains¹
- Clarify the mechanism of protein complex assembly²
- Convert to a sulfhydryl-reactive trifunctional reagent to map interactions³
- Study docking site and factor requirements for binding⁴
- Describe binding contacts of interactors⁵
- Confirm recognition of a specific phosphopeptide⁶
- Search for putative binding partners⁷
- Gain insight into chaperone-mediated refolding interactions⁸
- Investigate mechanism of protein interaction⁹
- Facilitate receptor activity-directed affinity tagging (re-tagging)¹⁰
- Detect low-abundance protein receptors¹¹
- Find protein:carbohydrate interactions¹¹
- Understand drug-receptor interactions¹²
- Quantitate triple helix-forming oligonucleotides¹³

Routes to determining the prey protein identification using Sulfo-SBED are outlined schematically in Figure 11. Note that the biotin label is a purification handle for captured prey protein. In the trypsin digestion strategy, the peptide(s) trapped can offer information relating to the binding interaction interface. The biotin-labeled prey protein or prey protein peptides recovered as result of the strategies outlined below can be subjected to several detection and identification options designed to discover the identity of the prey protein.

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Tri-functional Label Transfer (continued)

ProFound™ Label Transfer Sulfo-SBED Protein:Protein Interaction Reagent

Biotin label transfer reagent proven to be a powerful tool in the pursuit of protein interactions.

A proven reagent with exceptional versatility, Sulfo-SBED combines four properties into a powerful protein interaction tool:

- Amine group-specific reactivity
- Nonspecific photoreactivity
- Transferable biotin handle
- Thiol-cleavable disulfide linkage

Sulfo-SBED completely eliminates the need to radiolabel. Inclusion of a segregated biotin nucleus (vs. an integrated radioiodinatable one) offers the user a ready handle for purification of the protein to which the biotin is transferred.

Ordering Information

Product #	Description	Pkg. Size
33033	ProFound™ Label Transfer Sulfo-SBED Protein:Protein Interaction Reagent* Sulfo-NHS-2-(6-[Biotinamido]-2-(<i>p</i> -azido benzamido)-hexanoamido)ethyl-1,3'-dithiopropionate.	10 mg
33034	No-Weigh™ Sulfo-SBED Label Transfer Reagent	8 x 1 mg
33073	ProFound™ Sulfo-SBED Biotin Label Transfer Kit—Western Blot Application Sufficient reagents to perform eight label transfer reactions for subsequent Western blot analysis. Contains: No-Weigh™ Sulfo-SBED 8 x 1 mg BupH™ Phosphate Buffered Saline 1 pack (makes 500 ml) Label Transfer Buffer (20X) 200 ml (makes 4 liters) ImmunoPure® Streptavidin-HRP 0.1 mg No-Weigh™ Dithiothreitol (DTT) 8 x 7.7 mg Slide-A-Lyzer® MINI Dialysis Units** 10 units/pack Plus Float, 10K MWCO, 10-100 µl	Kit

*ProFound™ Label Transfer Technology is protected by U.S. Patent # 5,532,379.

**Slide-A-Lyzer® MINI Dialysis Unit Technology is protected by U.S. Patent # 6,039,871.

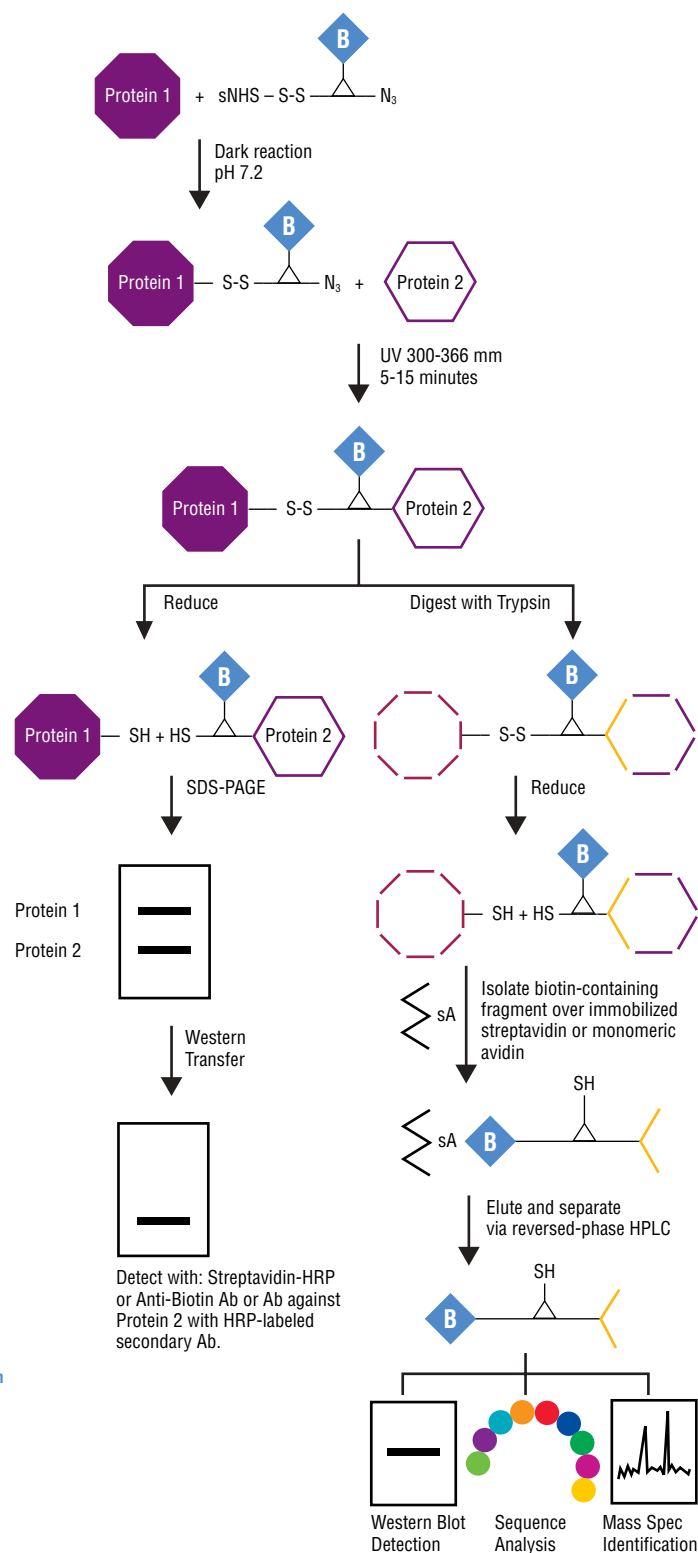
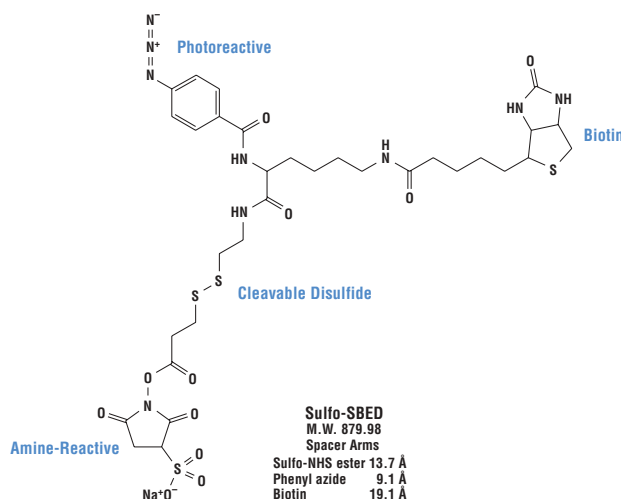


Figure 11. Applications of Sulfo-SBED in protein interaction studies.

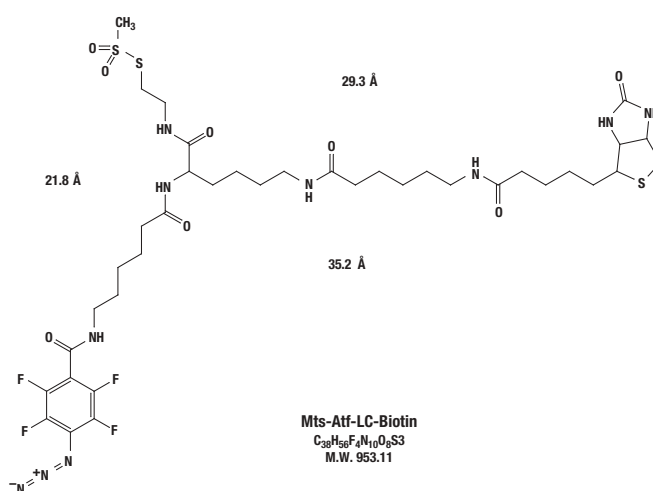
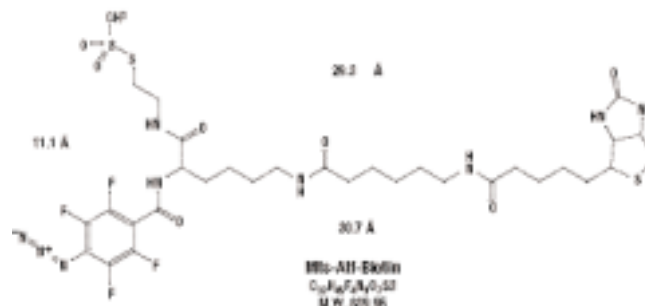
ProFound™ Mts-Atf-Biotin Label Transfer Reagents

Sulfhydryl-directed, photoreactive biotin label transfer reagents.

These two new biotin-containing reagents for use in label transfer applications incorporate the known features and benefits of the sulfhydryl-specific methanethiosulfonate (Mts) group and the high-yielding photoreactive tetrafluorophenylazide moiety. By combining these reactive groups with a biotin tag, powerful new reagents for protein interaction analysis were created. Purified bait protein is first labeled at reduced cysteine residues, then allowed to form an interaction complex with the target prey protein. When exposed to UV-light, the photoreactive group activates to form covalent bonds to adjacent sites on the prey protein. Reducing the disulfide-bond releases the bait protein and leaves the biotin label on the prey.

Highlights:

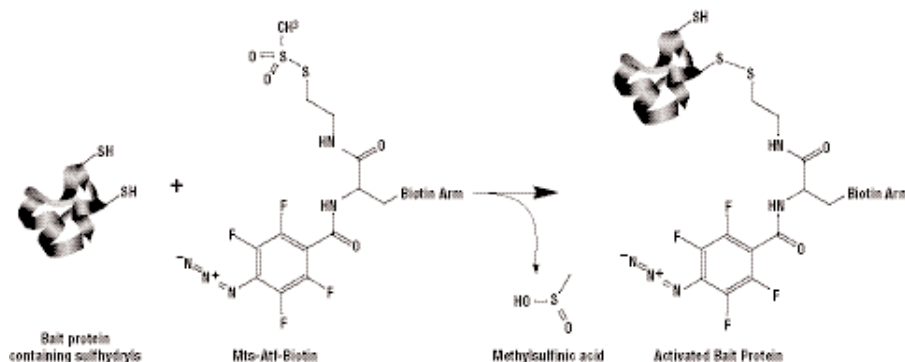
- Methanethiosulfonate (Mts) moiety is highly specific for the sulfhydryl (–SH) group that occurs in the side chain of reduced cysteine residues, enabling precise, rapid and quantitative labeling of the bait protein
- Tetrafluorophenyl azide moiety reacts 3-4 times more efficiently than regular phenyl azide moieties, increasing the likelihood of capturing sufficient bait:prey complex to detect
- Sulfinic acid by-products of the Mts reaction with bait protein do not interfere with disulfide bond formation or the activity of the bait protein and decompose quickly to a volatile low molecular weight product
- Resulting disulfide bond spacer arm connecting bait and prey proteins is easily reversed with commonly used reducing agents (DTT, 2-mercaptoethanol, TCEP)
- Mts reaction and photoreaction are compatible with physiological aqueous buffer conditions required to establish most protein interactions
- Long chain (LC) and short chain photoreactive versions are offered, allowing one to more precisely explore interaction distances



Ordering Information

Product #	Description	Pkg. Size
33093	Mts-Atf-Biotin Label Transfer Reagent* 2-[N2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-N6-(6-biotinamidocaproyl)-L-lysiny]ethyl methanethio-sulfonate	5 mg
33083	Mts-Atf-LC-Biotin Label Transfer Reagent* 2-[N2-[N6-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-6-aminocaproyl]-N6-(6-biotinamidocaproyl)-L-lysinylamido] ethyl methanethiosulfonate	5 mg

* Scheduled for availability on or before before March 2005.
Call our toll-free number or check our web site for status.



Reaction of Mts-Atf-Biotin with bait protein containing sulfhydryls (reduced disulfide bonds). Once desalted to remove excess nonreacted Mts-Atf-Biotin and byproducts (methylsulfinic acid), the activated bait protein may be allowed to interact with other proteins (the prey) and then cross-linked together by UV-activation of the tetrafluorophenyl azide group. If desired, the disulfide bond in the Mts-Atf-Biotin may be cleaved with a reducing agent, transferring the biotin label to the prey protein. In this figure, the protein molecule is not drawn to scale; in reality, it is many times larger than the labeling reagent.

Protein Interactions Far-Western Blotting

Studying Protein Interactions by Far-Western Blotting

Far-Western blotting was originally developed to screen protein expression libraries with ^{32}P -labeled-glutathione S-transferase (GST)-fusion protein.^{1,2} Far-Western blotting is now used to identify protein:protein interactions. In recent years, far-Western blotting has been used to determine receptor:ligand interactions and to screen libraries for interacting proteins.³ With this method of analysis, it is possible to study the effect of post-translational modifications on protein:protein interactions, examine interaction sequences using synthetic peptides as probes³ and identify protein:protein interactions without using antigen-specific antibodies.

Far-Western Blotting vs. Western Blotting

The far-Western blotting technique is quite similar to Western blotting. In a Western blot, an antibody is used to detect the corresponding antigen on a membrane. In a classical far-Western analysis, a labeled or antibody-detectable “bait” protein is used to probe and detect the target “prey” protein on the membrane. The sample (usually a lysate) containing the unknown prey protein is separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or native PAGE and then transferred to a membrane. When attached to the surface of the membrane, the prey protein becomes accessible to probing. After transfer, the membrane is blocked and then probed with a known bait protein, which usually is applied in pure form. Following reaction of the bait protein with the prey protein, a detection system specific for the bait protein is used to identify the corresponding band (Table 3).

Table 3. Comparison of Western Blotting and Far-Western Blotting Methods

Step	Western Blotting	Far-Western Analysis
Gel Electrophoresis	Native or Denaturing (usually)	Native (usually) or Denaturing
Transfer System	Optimal membrane and transfer system determined empirically	Optimal membrane and transfer system determined empirically
Blocking Buffer	Optimal blocking system determined empirically	Optimal blocking system determined empirically
Detection (several possible strategies)*	Unlabeled primary antibody → Enzyme-labeled secondary antibody → Substrate Reagent	Unlabeled bait protein → Enzyme-labeled bait-specific antibody → Substrate Reagent
[Arrows designate sequence of steps of detection strategy]	Enzyme-labeled primary antibody → Substrate Reagent	Radiolabeled bait protein → Exposure to film
	Biotinylated antibody → Enzyme-labeled streptavidin → Substrate Reagent	Biotinylated bait protein → Enzyme-labeled streptavidin → Substrate Reagent
		Fusion-tagged bait protein → Tag-specific antibody → Enzyme-labeled secondary antibody → Substrate Reagent

* Labeled antibodies generally are enzyme-labeled (either horseradish peroxidase or alkaline phosphatase). By contrast, bait proteins generally are not enzyme-labeled because a large enzyme label is likely to sterically hinder unknown binding sites between bait and prey proteins. Other labeling and detection schemes are possible.

Specialized Far-Western Analysis

By creative design of bait protein variants and other controls, the far-Western blotting method can be adapted to yield specific information about protein:protein interactions. For example, Burgess, *et al.*⁴ used a modified far-Western blotting approach to determine sites of contact among subunits of a multi-subunit complex. By an “ordered fragment ladder” far-Western analysis, they were able to identify the interaction domains of *E. coli* RNA polymerase β' subunit. The protein was expressed as a polyhistidine-tagged fusion, then partially cleaved and purified using a Ni^{2+} -chelate affinity column. The polyhistidine-tagged fragments were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The fragment-localized interaction domain was identified using a ^{32}P -labeled protein probe.

Importance of Native Prey Protein Structure in Far-Western Analysis

Far-Western blotting procedures must be performed with care and attention to preserving as much as possible the native conformation and interaction conditions for the proteins under study. Denatured proteins may not be able to interact, resulting in a failure to identify an interaction. Alternatively, proteins presented in non-native conformations may interact in novel, artificial ways, resulting in “false-positive” interactions. The prey protein in particular is subjected to preparative processing steps for far-Western blotting that can have significant effects on detection of protein:protein interactions. This is not to imply that identification of valid interactions is not possible but only to stress the importance of appropriate validation and use of controls.

Critical Steps in Far-Western Analysis

Gel Electrophoresis

Separation of proteins by SDS-PAGE (i.e., denaturing conditions with or without a reducing agent) offers more information about MW, presence of disulfides and subunit composition of a prey protein, but may render the prey protein unrecognizable by the bait protein. In these cases, the proteins may need to be subjected to electrophoresis under native conditions; i.e., nondenaturing and without reducing agent.

Transfer to Membrane

After separation on the gel, proteins are electrophoretically transferred from the gel to a membrane in two to 16 hours. The type of membrane (e.g., nitrocellulose or PVDF) used for the transfer of proteins is critical, as some proteins bind selectively or preferably to a particular membrane.⁵ The efficiency and protein transfer rate is inversely proportional to the molecular weight of the protein. In some cases, transfer conditions alter the conformation of the protein and destroy or sterically hinder the interaction site on the protein. For far-Western analysis, it is essential that at least the interaction domain of the prey protein is not disrupted by the transfer or is able to re-fold on the membrane to form a three-dimensional (3-D) structure comprising an intact interaction site.⁴ Generally, a significant percentage of the protein population renatures upon removal of SDS. When SDS is eliminated during the transfer process, transferred proteins generally renature with greater efficiency and are, therefore, more easily detected by far-Western blotting. In the event that the protein is unable to re-fold to create an intact binding site, it may be necessary to add a denaturation/renaturation step to the procedure or to perform the protein:protein interaction in-gel without transfer (See In-Gel far-Western Detection on page 33). Denaturation/renaturation is typically accomplished using guanidinium hydrochloride.⁶

Blocking Buffer

After transferring proteins to the membrane, Western blotting procedures require that unreacted binding sites on the membrane be blocked with a non-relevant protein solution. In addition to blocking all remaining binding sites on the membrane, a blocking buffer reduces nonspecific binding and aids in protein renaturation during the probing procedure. A variety of different protein blockers may be used, and no one blocking protein solution will work for all blotting experiments. Any given protein blocker may cross-react or otherwise disrupt the specific probing interaction being studied. Determination of an effective blocking buffer must be made empirically. Often, bovine serum albumin (BSA) is used as a starting point for many membrane-probing reactions. Insufficient blocking may result in high background, whereas prolonged blocking could result in a weak or masked signal. Renaturation of the protein also appears to occur during the blocking step so it is important to optimize the blocking conditions to obtain the best signal-to-noise ratio for each application and then not deviate from the method.³

Binding and Wash Conditions

Protein:protein interactions vary depending on the nature of the interacting proteins. The strength of the interactions may depend on the pH, salt concentrations and the presence of certain co-factors during incubation with the bait protein. Some protein:protein interactions may also require the presence of additional proteins. Whatever the necessary conditions, they must be maintained throughout the procedure to maintain the interaction until it can be detected. This may influence the formulation of washing buffer used between probing steps.

Protein Interactions Far-Western Blotting [continued]

Detection Methods

Depending on the presence of a label or tag on the bait protein, one of four detection methods is used to detect far-Western blot protein:protein interactions:

- Direct detection of prey protein with a radioactive bait protein
- Indirect detection with antibody to the bait protein
- Indirect detection with antibody to the tag of a fusion-tagged bait protein
- Indirect detection with biotinylated bait protein and enzyme (HRP/AP) labeled with avidin or streptavidin

Each method has its own advantages and disadvantages.

Several methods are used to generate radioactive isotope labels on bait proteins. The isotope ^{32}P is commonly used to label fusion-tagged protein probes at phosphorylation sites on the tag. This method of phosphorylation has little effect on the protein:protein interaction because the phosphorylation site is located in the fusion tag portion of the protein. Another radioactive method involves direct labeling of bait protein using endogenous phosphorylation sites. However, this technique can be used only if ^{32}P labeling of these sites does not interfere with protein:protein interactions.⁶ Radioactive detection has also been used when probes are made by incorporation of ^{35}S -methionine during *in vitro* translation. One disadvantage of this method is that it can be used only for protein probes that have multiple methionine or cysteine residues.³ Although radioactive isotopes generally do not interfere with interactions because they alter MW by a only few atomic mass units, isotopic detection methods have several disadvantages including health hazards and disposal issues.

GST-tagged or polyhistidine-tagged recombinantly expressed bait proteins are often detected with a primary or enzyme-labeled antibody specific to the tag. Antibodies to both these popular fusion tags are commercially available. When recombinant techniques cannot be used to create fusion-tagged bait proteins and bait-specific antibodies are not available, bait proteins can be biotinylated and detected with labeled avidin or streptavidin. Pierce offers a full line of biotinylation reagents and enzyme-labeled avidin and streptavidin. Although lysate containing the bait protein can be used for probing membranes, this can result in high background (low signal-to-noise); therefore, it is preferable to purify the bait protein before probing.

Whatever the method of non-isotopic labeling used, the last probing step usually involves use of an antibody or streptavidin probe that is conjugated (labeled) with an enzyme whose localized activity on the membrane can be detected by incubation with a suitable colorimetric, chemiluminescent or fluorogenic substrate. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the most popular enzyme labels used for this purpose, with HRP being the most versatile. As with traditional Western blotting, sensitivity in far-Western blotting depends largely on the enzyme:substrate system used for detection. Patented SuperSignal[®] Chemiluminescent Substrate Technology enables unmatched sensitivity and lower limits of detection for HRP-based conjugates.

Controls

When identifying protein:protein interactions by the far-Western technique, it is important to always include appropriate controls to distinguish true protein:protein interaction bands from nonspecific artifactual ones. For example, experiments involving detection with recombinant GST fusion proteins should be replicated with GST alone. A bait protein with a mutation in the predicted interaction domain can be processed as a control to determine specificity of the protein:protein interaction. A non-relevant protein can be processed alongside the prey protein sample to act as a negative control. Ideally, the control protein would be of similar size and charge to the protein under investigation and would not interact nonspecifically with the bait protein.⁶

In approaches that use a secondary system for detection of the prey protein, such as enzyme-labeled streptavidin with a biotinylated bait protein, it is important to include a duplicate control membrane that is probed only with the labeled streptavidin. This would reveal any bands resulting from endogenous biotin in the sample or nonspecific binding of the labeled streptavidin. When a fusion tag is used with a corresponding antibody, it is critical to probe one of the control membranes with the labeled antibody alone. This control helps to confirm that the relevant band is not due to nonspecific binding of the labeled secondary antibody. To obtain meaningful results, appropriate test and control experiments should be subjected to gel electrophoresis, transfer and probing in parallel.³

In-Gel Far-Western Detection*

Advantages of In-Gel Detection

Because of restrictions associated with the transfer process, blocking and the possibility of nonspecific binding of bait proteins to unrelated bands on the membranes, it is sometimes advantageous to perform far-Western detection within the gel. In this procedure prey protein samples are separated in precast gels using either native or denaturing conditions. Following electrophoresis, the gels are pre-treated with 50% isopropyl alcohol and water to remove SDS from the gel and allow the prey protein to renature. The gel is then incubated with the bait protein (usually in the pure form). If the bait protein is biotinylated, it is subsequently detected with streptavidin-HRP and a highly sensitive formulation of Pierce's patented SuperSignal® Chemiluminescent Substrate. If the bait protein is fusion-tagged, detection is with an anti-tag HRP-conjugated antibody and the chemiluminescent substrate.

The same controls and experimental conditions necessary for optimization of membrane-based far-Westerns apply to in-gel detection. With in-gel detection the blocking step can be eliminated, but the "bait" protein and the labeled detection protein must be diluted in the blocking buffer to reduce nonspecific binding. Also, higher amounts of prey and bait proteins are often required for detection compared to membrane detection with the equivalent chemiluminescent substrate.

* U.S. patent pending

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Far-Western Analysis

ProFound™ Far-Western Protein:Protein Interaction Kits

Discover your next important protein interaction with the new Pierce far-Western kits.

The first non-isotopic far-Western protein:protein interaction kits that detect interactions on-membrane or in-gel

The ProFound™ Far-Western Biotinylated-Protein:Protein Interaction Kit and the ProFound™ Far-Western GST-Protein:Protein Interaction Kit represent a novel non-isotopic approach to far-Western analysis that can be performed either directly in the gel or, like the classical method, on a membrane. In both cases, interactions are discovered using sensitive chemiluminescent detection.

ProFound™ Far-Western Biotinylated-Protein:Protein Interaction Kit

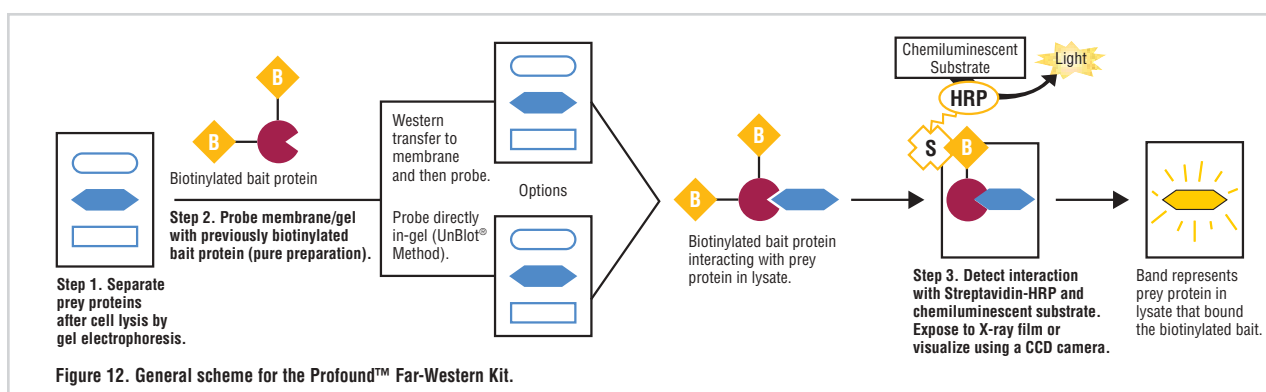
This kit uses biotin modification of a purified "bait" protein probe. Prey proteins in-gel or on a membrane can be probed with a biotinylated bait. Detection of a bait interaction with "prey" protein(s) is achieved with a Streptavidin-HRP conjugate and a chemiluminescent substrate.



▲ ProFound™ Far-Western Biotinylated-Protein:Protein Interaction Kit



◀ ProFound™ Far-Western GST-Protein:Protein Interaction Kit



Highlights:

- On-membrane or in-gel detection options – on-membrane detection offers greater sensitivity; in-gel detection method offers speed and prevents problems associated with incomplete or inefficient transfer
- Nonradioactive alternative for far-Western analysis – reliable and sensitive biotin/Streptavidin-HRP or anti-GST-HRP chemistry combined with chemiluminescent detection offers a practical and safe alternative to radiolabeling the bait protein
- Useful interaction range – kit targets moderate to strong associations between a prey and the biotinylated bait protein or GST-tagged probe protein
- Primary antibody-free detection – kit uses a biotinylated or GST-tagged protein as the probe, eliminating the need for antibody production
- Compatible with both SDS-PAGE and native gels – provides option to probe for prey proteins in a more native environment because reduced or denaturing systems may not always present an interface that promotes the intended interaction
- Reduced nonspecific binding – biotin/Streptavidin-HRP systems demonstrate less nonspecific binding compared to antibodies directed against the bait protein; the anti-GST antibody conjugate is highly specific for the GST tag
- Compatible with protein staining – can be used for total protein staining after the chemiluminescent detection step, eliminating the need to run two gels

Ordering Information

Product #	Description	Pkg. Size
23500	ProFound™ Far-Western Biotinylated Protein:Protein Interaction Kit <i>Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a biotinylated bait protein as the probe.</i> Includes: Streptavidin-HRP 0.1 mg 10X Dilution Buffer 50 ml BupH™ Phosphate Buffered Saline 17 packs 10% Tween®-20 6 x 10 ampules UnBlot® Stable Peroxide 55 ml UnBlot® Luminol Enhancer 55 ml Cellophane Enhancer Sheets 10 pack	10 mini gels
23505	ProFound™ Far-Western GST-Protein:Protein Interaction Kit <i>Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a GST-tagged bait protein as the probe.</i> Includes: Anti-Glutathione S-Transferase 0.25 mg 10X Dilution Buffer 50 ml BupH™ Phosphate Buffered Saline 17 packs 10% Tween®-20 6 x 10 ampules UnBlot® Stable Peroxide 55 ml UnBlot® Luminol Enhancer 55 ml Cellophane Enhancer Sheets 10 pack	10 mini gels

Protein Interactions Protein Arrays

Introduction to Protein Arrays

Microarrays were first developed as a tool for high-throughput genotyping and gene expression analysis. By combining small sample volumes and the ability to generate massive amounts of information in a single experiment, microarrays vastly accelerated the search for functional effects of SNPs (single nucleotide polymorphisms) and modified gene expression (increases and decreases in mRNA production) in normal and diseased physiological states.

Gene expression arrays operate under the pretense that changes in mRNA levels ultimately correlate to changes in encoded protein levels; often this assumption does not hold true. Additionally, gene expression arrays provide no information on protein post-translational modifications (phosphorylation, glycosylation, etc.) that affect cell function. The protein microarray was developed to examine expression at the protein level and otherwise acquire quantitative and qualitative information on proteins of interest.

A protein microarray consists of antibodies, proteins, protein fragments, peptides, aptamers or carbohydrate elements that are coated or immobilized in a grid-like pattern on small surfaces. The arrayed molecules are then used to screen and assess patterns of interaction with samples containing distinct proteins or classes of proteins. A brief overview of each type of protein array follows, except for aptamer and carbohydrate arrays, which are outside the scope of this discussion.

Types of Protein Arrays

Antibody-Pair Protein Arrays

Antibody microarrays fall into one of two subtypes: those using matched antibody pairs for sandwich-type assays and those using single antibodies and a sample-labeling methodology. Several published manuscripts demonstrate the utility and effectiveness of the sandwich immunoassay microarray.¹⁻⁴ This type of microarray consists of arrayed capture antibodies and appropriate control and orientation elements. Assays are performed by adding an antigen standard or test sample, followed by a detector antibody (Figure 13). The detector antibody is either modified with a directly detectable label (enzyme, fluorescent molecule, isotope, etc.), as illustrated in Figure 13, or it is biotinylated for detection after subsequent probing with labeled streptavidin.

Antibody-pair microarrays essentially are multiplexed ELISAs. In fact, standard commercially available ELISA pairs are readily adapted for microarray use once the pairs have been screened for cross-reactivity when multiplexed. Sandwich-style antibody pair microarrays can be used for qualitative or comparative (i.e., treated versus non-treated) detection of protein analytes or for protein quantification when appropriate standards are used to assemble calibration curves.

Single Antibody/Labeled Sample Protein Arrays

When matched antibody pairs are not available, single-antibody protein microarray protocols involving labeled samples can be used. As in sandwich antibody pair arrays, the array platform consists of arrayed antibody (or antibodies). In this assay format, however, the captured protein analytes are themselves labeled for direct detection, obviating use of a detector antibody (Figure 14). The method requires that protein samples be labeled beforehand (e.g., with fluorescent molecule, isotope or biotin). The label enables detection of any proteins in the sample that interact with the microarrayed antibody and associated elements.

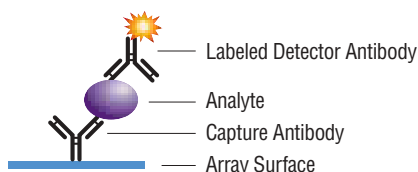


Figure 13. Architecture of sandwich-style antibody-pair microarray assays.

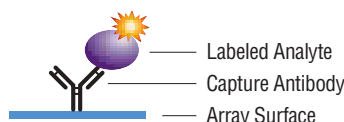


Figure 14. Architecture of single antibody/labeled sample microarray assays.

Protein Interactions Protein Arrays

Single Antibody/Labeled Sample Protein Arrays (continued)

As stated previously, this technique is useful for examining protein targets, such as poorly characterized cell-signaling proteins, for which paired antibodies do not yet exist. The main drawback to this method is its lack of antibody redundancy, which helps to ensure specific antigen recognition. Additionally, since all sample constituents are labeled (i.e., the target as well as other proteins in the sample), nonspecific background signal increases. This technique is primarily used for comparative and qualitative studies.

Cellular Lysate Protein Arrays

Microarrays of cellular proteins are either arrayed as complex protein mixtures, or arrayed as purified or overexpressed proteins. Complex protein mixture arrays are essentially dot blots of cellular lysates. Investigations performed by Paweletz, *et al.*⁵ demonstrate the utility of arraying cellular lysates and probing these arrayed elements with a variety of antibodies recognizing various intracellular proteins. Creating microarrays consisting of libraries of purified or overexpressed proteins allows for screens for protein:protein interactions and kinase activities. A representation of this type of array is the yeast kinase microarray produced by Zhu, *et al.*; this array consisted of 119 of the 122 known yeast kinases.⁶

Peptide Arrays

The final type of protein microarray to be discussed is the protein fragment or peptide microarray. An example of a peptide microarray and its uses can be found in work performed by Pellois, *et al.*⁷ and Houseman, *et al.*⁸ These papers demonstrate the use of peptide chips for profiling p53 and kinase activities, respectively.

This diverse set of applications clearly demonstrates that the protein microarray is a powerful tool. Unfortunately, costs for equipment such as arraying robots and slide scanners limit the number of researchers able to take advantage of this technology.

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Protein Interactions *Creating Your Own Arrays*

Protocol: How to attach an antibody or other sulfhydryl-containing protein onto glass, silica or quartz surface

Researchers are increasingly engaged in assay development and affinity purification methods for specialized applications and instrumentation. A versatile platform for affinity assays or purification involves immobilizing antibodies or other proteins onto glass surfaces. This Tech Tip describes a simple and flexible four-step method for covalently attaching an antibody to glass through native or added sulfhydryl (-SH) groups (Figure 15). A glass surface is derivatized with primary amines (-NH₂) using an aminosilane reagent. These amines are subsequently reacted to the heterobifunctional cross-linker Sulfo-SMCC, resulting in a maleimide-activated surface able to react with sulfhydryl groups on antibodies and other proteins.

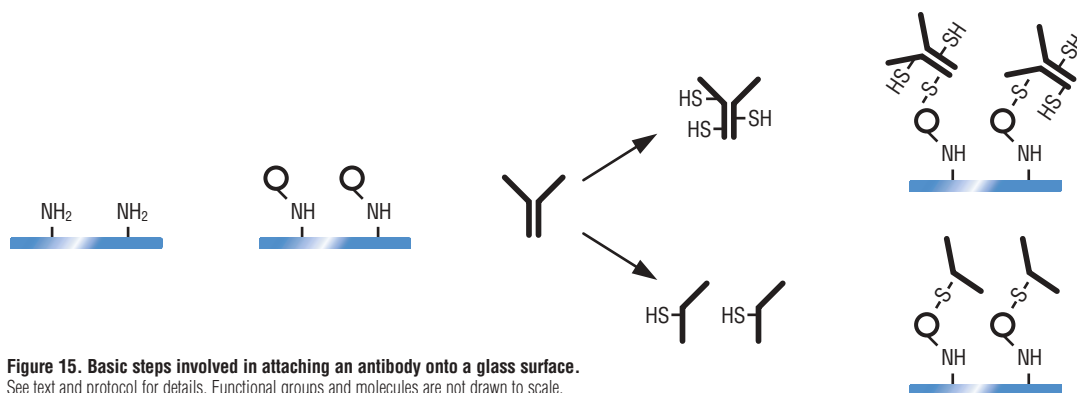


Figure 15. Basic steps involved in attaching an antibody onto a glass surface.
See text and protocol for details. Functional groups and molecules are not drawn to scale.

One of two alternative strategies may be used to ensure that sulfhydryl groups are made available on an antibody for this immobilization method. Most proteins contain cysteines, whose sulfur atoms exist as sulfhydryls (-SH) or paired in disulfide bonds (-S-S-). Sulfur atoms must be in their reduced (-SH) state for covalent coupling to the maleimide-activated surface.

One strategy is to reduce native disulfide bonds in the antibody molecule. These disulfide bonds join heavy and light polypeptide chains together in a manner that ensures proper antibody structure and antigen-binding function. Therefore, complete reduction of antibody disulfides by treatment with reducing agents will usually inactivate the antibody. However, with the proper conditions, it is possible to selectively reduce only the more labile disulfides between heavy chains in the hinge region of IgG molecules; the result is functional half-antibodies with sulfhydryls available for reaction to the activated glass surface (Figure 15). Such partial reduction of antibody disulfides usually results in sulfhydryl group attachment points that will not sterically hinder antigen binding.

A second strategy for creating the necessary sulfhydryl groups is to add them to the antibody with specific reagents. Traut's Reagent and SATA are sulfhydryl-containing modification reagents that react with primary amines (-NH₂), which are present in the side-chain of lysine residues in antibodies and other proteins. Traut's Reagent creates sulfhydryl groups that are available for immediate reaction to the maleimide-activated surface. SATA creates protected sulfhydryl groups that are then exposed upon treatment with hydroxylamine to yield sulfhydryl groups for coupling to the activated surface. With sulfhydryl-addition methods, there is no risk of completely reducing and fragmenting an antibody; nevertheless, disruption of antigen-binding capability remains possible as a result of modification of the antigen-binding sites.

The following protocol is divided into four discrete sections. Section 3 presents the two alternative options for creating sulfhydryl groups on the antibody; choose one or the other of these two options. Read through all four sections before starting, and ensure that the activated surface (Section 2) and the sulfhydryl-modified antibody (Section 3) are ready at the same time for the final conjugation reaction (Section 4).

Finally, plan reaction volumes and procedures in advance so as not to waste valuable antibody. Most importantly, notice in Section 4 that the final reaction requires antibody solution at a concentration greater than 10 µg/ml in sufficient volume to cover the glass surface, while Section 3 describes default conditions for preparing exactly 4 mg of antibody. Depending on the amount of antibody needed for the final coupling reaction, one may prepare more or less total antibody than assumed in Section 3. However, avoid altering the absolute concentrations of reagents (including antibody); otherwise molar ratios and incubation times will require optimization. Also, ensure that appropriate desalting columns are available for efficiently processing the reaction volumes used.

Materials Required

- Amino-silane Reagent: 3-Aminopropyltriethoxysilane (Product # 80370)
- Acetone: solvent/diluent for Amino-silane Reagent
- Coupling Buffer: PBS-EDTA (50 mM Phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2).
Use BupH™ Phosphate Buffered Saline Packs (Product # 28372) and add EDTA to a concentration of 10 mM.
- Cross-linker: Sulfo-SMCC (Product # 22322)
- Reagent to expose or add sulfhydryl groups — choose one of the following methods (see Introduction):
- Reducing Agent: 2-Mercaptoethylamine (2-MEA) (Product # 20408)
- Sulfhydryl Addition Reagent — choose one of the following sets of reagents:
 - Traut's Reagent (2-Iminoethiolane•HCl, Product # 26101)
 - SATA (Product # 26102) and Hydroxylamine•HCl (Product # 26103) and DMSO (Product # 20688)
 - Protein-Coupling Handle Addition Kit (Product # 23460)
- Desalting Column: D-Salt™ Polyacrylamide Desalting Columns (Product # 43240) or D-Salt™ Dextran Desalting Columns (Product # 43230)

Section 1: Aminosilylate the Glass Surface

1. Thoroughly wash and dry the glass, silica or quartz surface to be coated.
- Note:** Perform Steps 2 and 3 in a fume hood.
2. Prepare a 2% solution of the Amino-silane Reagent (3-Aminopropyltriethoxysilane) in acetone.
For example, mix 1 part Amino-silane Reagent with 49 parts dry (i.e., water-free) acetone.
Prepare a volume sufficient to immerse or cover the surface material.
 3. Immerse surface in the diluted reagent for 30 seconds.
 4. Rinse surface with dry acetone.
 5. Allow surface to air-dry.
- Note:** The dried silylated surface may be stored for later use.

Section 2: Maleimide-Activate the Amino-Modified Surface

1. Add 2 mg Cross-linker (Sulfo-SMCC) to 1 ml Coupling Buffer. This solution may be scaled as needed, and must be used immediately to avoid hydrolysis.
 2. Cover silylated surface with the Cross-linker solution.
 3. Incubate for 1 hour at room temperature (RT).
 4. Rinse the modified surface with Coupling Buffer.
- Note:** The maleimide-activated surface may be dried and stored desiccated at 4°C for later use.

Section 3, Option 1: Partially Reduce Antibody to Produce Sulfhydryls for Coupling

Note: Perform either Option 1 or Option 2 of this section (see Introduction).

1. Dissolve 4 mg antibody (IgG) in 450 µl Coupling Buffer.
2. Dissolve 6 mg Reducing Agent (2-MEA) in 100 µl Coupling Buffer (results in 0.5 M stock solution).
3. Add 50 µl of Reducing Agent solution to the 450 µl antibody solution and mix.
4. Incubate for 90 minutes at 37°C.
5. Purify the reduced antibody from the Reducing Agent using a Desalting Column equilibrated with Coupling Buffer.
Collect 500 µl fractions.
6. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
7. Pool the fractions that contain antibody (now with sulfhydryls). Proceed immediately to Section 4 to minimize disulfide formation, which will require having to repeat this section of the procedure.

Section 3, Option 2: Add Sulfhydryl Groups to Antibody for Coupling

Note: Choose one of the following two methods for adding sulfhydryl groups to the antibody (see Introduction).

Method 1: Sulfhydryl Addition with Traut's Reagent

1. Adjust about 10 ml of prepared Coupling Buffer (PBS-EDTA, pH 7.2) to pH 8.0 with concentrated NaOH.
2. Dissolve 4 mg antibody (IgG) in 475 µl the pH-adjusted Coupling Buffer.
3. Dissolve 2 mg Traut's Reagent in 1 ml of the pH-adjusted Coupling Buffer (results in 14.5 mM stock solution).
4. Immediately add 25 µl Traut's Reagent solution to the antibody solution (results in a 12-fold molar excess of reagent).
5. Incubate for 45 minutes at room temperature.
6. Purify the modified antibody from excess Traut's Reagent using a Desalting Column equilibrated with Coupling Buffer (pH 7.2).
Collect 500 µl fractions.
7. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
8. Pool the fractions that contain antibody (now with sulfhydryls).
9. Proceed immediately to Section 4.

Method 2: Sulfhydryl Addition with SATA

1. Dissolve 4 mg antibody (IgG) in 475 µl of Coupling Buffer.
2. Dissolve 3 mg SATA in 1 ml of DMSO (results in 13 mM stock solution).
3. Immediately add 25 µl SATA solution to the antibody solution (results in a 25-fold molar excess of reagent).
4. Incubate for 30 minutes at room temperature.
5. Purify the modified antibody from excess SATA and other reaction by-products using a Desalting Column equilibrated with Coupling Buffer (pH 7.2). Collect 500 µl fractions.
6. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
7. Pool the fractions that contain antibody.

Note: At this point, the modified antibody may be stored indefinitely; however, once the following steps for deacylating (deprotecting) the sulfhydryl groups are performed, the antibody must be desalted and used immediately for coupling.

8. Dissolve 348 mg of Hydroxylamine•HCl in 9 ml Coupling Buffer and then adjust to pH 7.2 with NaOH.
Finally adjust the volume to 10 ml with additional Coupling Buffer (results in 0.5 M Hydroxylamine•HCl).
9. Add 100 µl Hydroxylamine solution to each 1 ml of SATA-modified antibody solution.
10. Incubate for 2 hours at room temperature.
11. Purify the antibody from the Hydroxylamine using a Desalting Column equilibrated with Coupling Buffer (pH 7.2).
Collect 500 µl fractions.
12. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
13. Pool the fractions that contain antibody.
14. Proceed immediately to Section 4.

Section 4: Cross-link Sulfhydryl-containing Antibody to Activated Surface

1. Cover the maleimide-activated surface material with the antibody solution. The antibody solution may be diluted in Coupling Buffer to a volume sufficient to cover the surface material. For optimal results, ensure that the final protein concentration is greater than 10 µg/ml.
2. Incubate for 2-4 hours at room temperature.
3. Remove the reaction solution, which contains any antibody that did not attach to the surface.
4. Thoroughly rinse the surface with Coupling Buffer to ensure that only covalently attached antibody molecules remain.
5. The surface is now ready to use for detection assays and other applications. Depending on stability of the particular antibody, the surface material may be dried for storage or kept covered in buffer containing 0.02% sodium azide.

Ordering Information

Product #	Description	Pkg. Size
80370	3-Aminopropyltriethoxysilane	100 g
28372	BupH™ Phosphate Buffered Saline Packs	40 x 500 ml packs
22322	Sulfo-SMCC	50 mg
20408	2-Mercaptoethylamine•HCl	6 x 6 mg
43240	D-Salt™ Polyacrylamide Desalting Columns, 6K MWCO	5 x 5 ml
43230	D-Salt™ Dextran Desalting Columns, 6K MWCO	5 x 5 ml
26101	Traut's Reagent	500 mg
26102	SATA	50 mg
26103	Hydroxylamine•HCl	25 ml
20688	DMSO	950 ml
23460	Protein-Coupling Handle Addition Kit	Kit

Protein Interactions Protein Interaction Mapping

Protein Interaction Mapping Using Chemical Cleavage Reagents

Various experimental techniques have been used to identify or map the interaction site(s) between two or more proteins. These include proteolytic cleavage, site-directed mutagenesis, cross-linking, microscopy, X-ray crystallography and NMR-based methods.

Proteolytic Mapping Methods

Proteolytic cleavage strategies offer several advantages to the study of protein interactions. They can be performed *in vitro* under physiologic conditions using only small amounts of any size native protein and can be used to map the entire surface of a protein without the need for *in vivo* genetic manipulation. With site-directed mutagenesis, multiple mutations are required to achieve the same level of surface coverage. This high level of modification could result in protein conformational changes that affect the interaction.

Proteolytic methods use either enzymatic or chemical cleavage reagents. These reagents can be used to produce peptide cleavage site standards (described further below).

Guide to Pierce Proteases for Protein Analysis

Product #	Available Enzyme	Available Immobilized	Soluble	Specificity
20238, 20214	Aminopeptidase M	X	X	• Cleaves amino acids sequentially from the amino terminus
20236, 20212	Carboxypeptidase Y	X	X	• Cleaves amino acids sequentially from the carboxy terminus
32520, 32521	Factor Xa		X	• Cleaves after the carboxyl side of the Ile-Glu-Gly-Arg sequence
20341	Papain	X		• Nonspecific protein digestion
20343	Pepsin	X		• Nonspecific protein digestion
20195	Staphylococcus aureus Protease	X	X	• Cleaves at the carboxyl side of aspartic and glutamic acid
20199	Submaxillaris Protease		X	• Cleaves at the carboxyl side of arginine
20230, 20233	TPCK Trypsin	X	X	• Cleaves on the carboxyl side of arginine and lysine
20151	V-8 Protease	X		• Cleaves on the carboxyl side of glutamic and aspartic acids

Metal Chelate Chemical Cleavage Reagents

Soluble Fe-EDTA has been used with proteins by addition after the protein:protein interaction has occurred. When proteins interact, the binding site of one molecule is hidden or protected from proteolytic cleavage by the other molecule. The Fe-EDTA cleavage products of the protein:protein complex and the unbound proteins are characterized by SDS-PAGE and the patterns compared to determine the sites of interaction. A less intense or missing band (cleavage product) in the interaction sample indicates a protected area of the molecule and putative binding site. However, the utility of soluble metal chelate reagents for protein mapping is somewhat limited. The complicated cleavage pattern produced from the interaction of large proteins may be too difficult to interpret on SDS-polyacrylamide gels.

FeBABE — Artificial Protease

“Tethered” Metal Chelate Chemical Cleavage

A better approach is to conjugate or tether a metal chelate reagent like iron (S)-1-(*p*-Bromoacetamidobenzyl) ethylenediamine-tetraacetate (FeBABE) to selected sites on one of the interacting proteins to create a “cutting protein.”¹ FeBABE is a labeling reagent consisting of a chelated iron atom linked to a sulfhydryl-reactive moiety (Br-acetyl) (Figure 16). This reagent allows conjugation of the iron chelating moiety through available –SH groups of the cutting protein. The FeBABE-protein conjugate is allowed to form a macromolecular complex with its interacting partner the “target” or prey protein. The protein complex is then incubated with ascorbate and peroxide to activate the chelated iron. This active iron forms oxidative and/or hydrolytic species that cleave the polypeptide backbone of the target protein within reach of its spacer arm near the binding site (Figure 17).

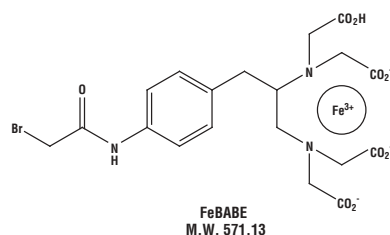


Figure 16. Structure of FeBABE.

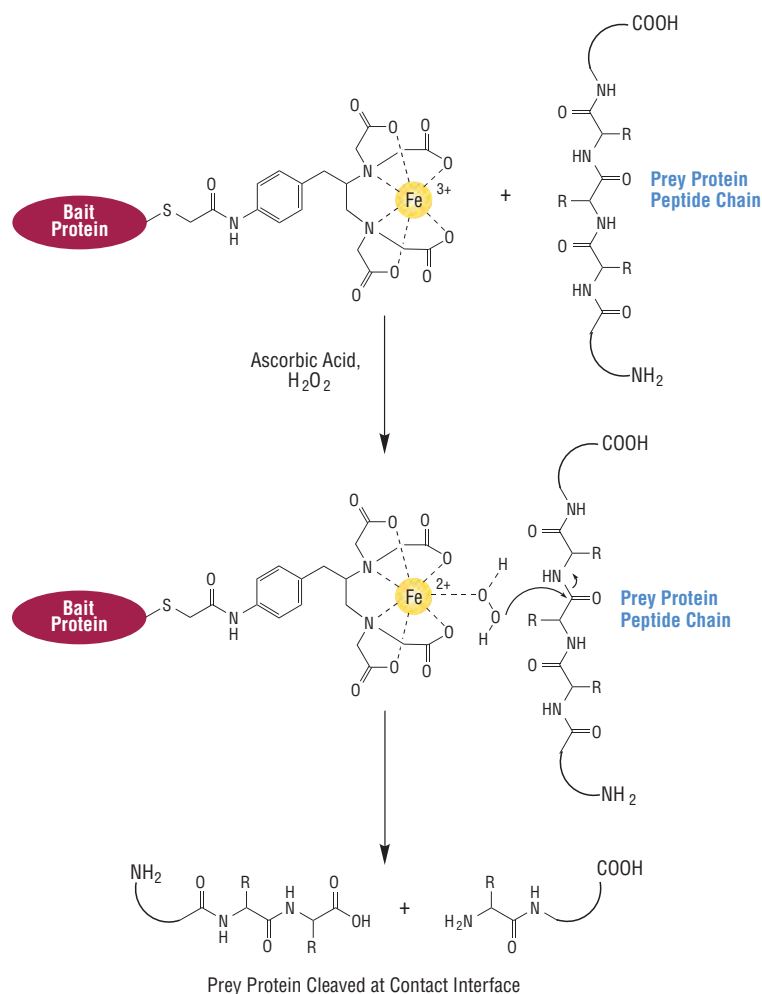


Figure 17. Peptide bond-cutting mechanism of FeBABE-modified bait protein.

Analysis of the resulting peptide fragments aids in mapping the points of contact between the two proteins. Following the cleavage reaction, fragments of the target protein are separated by electrophoresis using a denaturing gel, and the cleavage products are visualized (Figure 18). To facilitate identification of the interaction site, the target protein can be directly end-labeled using a radiolabel or fluorescent dye. Alternatively, it can be detected indirectly on a Western blot using an antibody directed to an expression tag (polyHis, FLAG, c-myc) or to an endogenous N or C terminal epitope. The resultant pattern represents only those fragments that extend from the labeled end of the protein to the point of cleavage, regardless of the end-labeling method chosen.

Use of a tethered metal chelate complex rather than free Fe/EDTA in solution results in a more limited, less ambiguous cleavage pattern that makes analysis easier (Figure 18). The binding site is indicated by the presence of a cleavage band rather than by the absence of or variation in intensity of a band that is seen with the use of a soluble metal chelate. This method provides greater signal-to-noise for better sensitivity, and it is particularly valuable for weak interactions.

Protein Interactions

Protein Interaction Mapping

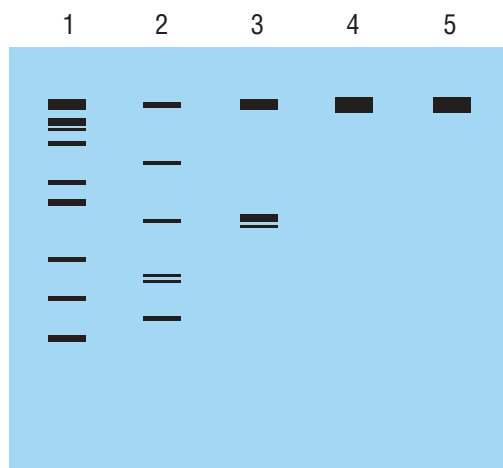


Figure 18. Analysis of peptide mapping by Western blot. Western blot using antibody directed to the N-terminal epitope of the target protein. Comparison of FeBABE cleavage of the interaction site with cleavage standards and negative reaction controls. **Lane 1.** Chemical cleavage of target protein with CNBr at methionine residues. **Lane 2.** Chemical cleavage of target protein with BNPS-skatole at tryptophan residues. **Lane 3.** Reaction mixture containing target protein, FeBABE-cutting protein conjugate and activators (ascorbate and peroxide). **Lane 4.** Reaction mixture containing target protein and FeBABE-cutting protein conjugate without activators (negative control). **Lane 5.** Reaction mixture containing target protein, unconjugated cutting protein (no FeBABE) and activators (negative control).

Mapping the Interaction

To map the interaction site, the cleavage pattern is compared with the unbound end-labeled target protein and known mobility standards (Figure 19). Molecular weight markers can be used for an initial approximation of size. More accurate determination of the residues involved in the binding site requires comparison to proteolytic fragments of the target protein (cleavage standards) that can be created using site-specific enzymatic and/or chemical cleavage. Alternatively, the target protein can be engineered with a series of truncations. The region or locus at which the cut occurred in the FeBABE-cleaved sample is assigned for each fragment by comparing it against the cleavage standards. The outcome of this method is a map (3-D if the tertiary structure is known) of residues in or near the site of interaction on the target protein.

The literature indicates that the interaction site between two proteins can be further defined by using other tethered reagents that have longer or shorter spacer arms or that are reactive with other amino acids. If desired, a multiplex format can be used that incorporates antibodies with different specificities or alternative fluorescent tags. Another option is to label both the N and C terminal amino acids with different tags or labels.

To use this protein cutter technology, a researcher must have two purified proteins of known sequence, one that can be end-labeled (target) and one that can be conjugated to FeBABE (cutting protein). If endogenous cysteines are not present in the binding site of the cutting protein, cysteine mutants can be designed to conjugate the reagent to a specific site on the molecule. Alternatively, 2-iminothiolane (Traut's Reagent, Product # 26101) can be used to randomly modify lysine residues on the cutting protein to introduce free sulfhydryl groups. Typically, each protein molecule is modified with only one to two iminothiolane/FeBABE groups. This limits possible conformational changes in the protein. The random substitution of lysines ensures that all potential binding sites are examined.

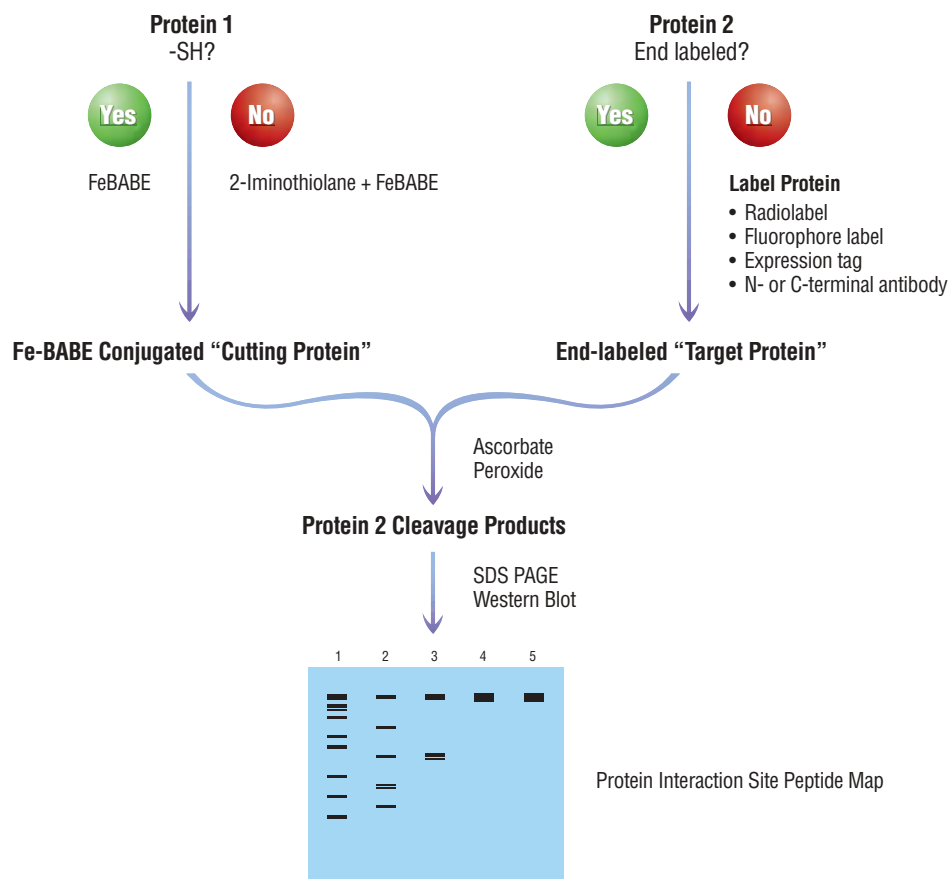


Figure 19. Analysis of peptide mapping by Western blot.

The literature reports that FeBABLE has been used to study the interaction of σ factors with RNA polymerase (RNAP).¹ In the *E. coli* system, σ^{70} was conjugated with FeBABLE and allowed to interact with the RNAP core protein. The resulting cleavage pattern of the β and β' subunits mapped the core-binding site for σ^{70} and provided data that was consistent with the results of cross-linking studies. The reagent was also used to map the binding site of σ^{54} , σ^{38} , σ^E and other RNAP regulatory proteins. The use of FeBABLE to map protein:DNA and protein:RNA interactions has also been reported in the literature. For these applications, the reagent was tethered to the protein, which was then allowed to bind to the RNA or DNA target and cleave the nucleotide backbone near the site of interaction.

Reference

1. Datwyler, S.A. and Meares, C.F. (2000). *Trends Biochem. Sci.* **25**, 408-414.

Recommended Reading

Datwyler, S.A. and Meares, C.F. (2001). Artificial Iron-Dependent Proteases. In *Metal Ions in Biological Systems*. **38**, New York: Marcel Dekker, Inc., pp. 213-253.

Hori, R. and Baichoo, N. (2002). Protease Footprinting. In *Protein-Protein Interactions. A Molecular Cloning Manual*. Cold Spring Harbor Laboratory Press, pp. 285-311. (Product # 20068)

Protein Interactions Protein Interaction Mapping

ProFound™ Protein Interaction Mapping Kit

A complete kit for protein interaction mapping featuring the FeBABE artificial protease protein-cutting reagent.

Acting as an artificial protease, FeBABE [Fe(III) (S)-1-(*p*-Bromo-acetamido-benzyl) ethylene diamine tetraacetic acid] can provide contact interface information by way of its ability to cleave peptide bonds at loci on the prey protein when the FeBABE modified bait protein and prey protein are in close proximity. (See page 41 for FeBABE structure.)

FeBABE bifunctional reagent contains a bromoacetyl functional group that can covalently couple to the bait protein through sulfhydryl (–SH) groups that occur naturally or that have been introduced by way of site-directed mutagenesis or chemical modification with 2-Iminothiolane•HCl (Traut's Reagent).

In the presence of ascorbate and peroxide, the Fe³⁺ EDTA portion of Fe(III)BABE is reduced to Fe(II). This reduction promotes the cleavage of peptide bonds by the modified bait protein in a non-sequence-specific manner (see Figure 20). When the prey protein binds to the bait, peptide bonds on the prey in proximity to the reach of the reagent (12 Å) are cleaved by the tethered Fe(II)(EDTA) portion. The resulting peptide pattern, when analyzed by electrophoresis, immunoblotting, sequencing or mass spectral techniques can provide information relating to the region of contact within the interacting complex.

Highlights:

- Conditions for conjugation of FeBABE to the bait protein are non-denaturing
- Nonspecific peptide bond cutting occurs under mild non-denaturing conditions
- Artificial protease cuts peptide bonds on target prey protein within its reach rapidly and in high yield, providing fragments for downstream deduction of cutting locus
- Kit provides all essential reagents and buffers needed to carry out the protocol offering the user better control over the reaction than preparing reagents and buffers from laboratory raw materials

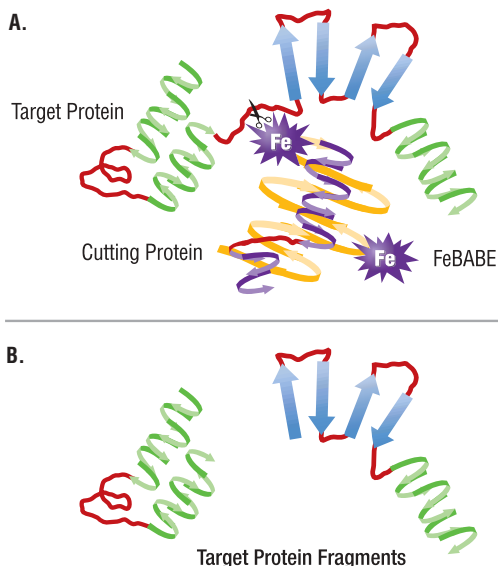


Figure 20. Diagram of FeBABE site-directed proteolysis of prey protein.
A. Cleavage occurs at contact interface between the Fe(BABE)-derivatized cutting (bait) protein and the target or prey protein. **B.** Target protein cleaved at contact interface.

Incorporation of –SH group(s) into –SH-deficient bait protein

Interaction mapping using FeBABE requires that the bait protein have available thiol groups for coupling the reagent. The addition of thiol groups may be needed when:

1. The bait protein is devoid of cysteine residues
2. Reduction of cystine disulfides results in loss of activity of the bait protein
3. Contact information is required before more targeted site directed mutagenic approaches to interaction mapping with FeBABE are undertaken.

In such cases, Traut's Reagent (2-Iminothiolane) can be used to convert available ε-amino groups of lysines in the bait protein into –SH group-containing loci to which the FeBABE can be coupled. When this strategy is employed the resulting spacer arm reach is 18 Å. Traut's Reagent (2-Iminothiolane) is available separately.

Essential components prepared or formulated in user friendly packaging

All critical reagent components are supplied in unique single-dose packaging. All the essential buffers and solutions have been carefully formulated using low-metal salts and additives and packaged to minimize errors in use. Reagents stay fresh (No-Weigh™ Single-Dose Packaging) and do not deteriorate by repeated sampling from a single vial. The kit also includes desalting spin columns for the convenience of the user.

Ordering Information

Product #	Description	Pkg. Size
32223	ProFound™ Protein Interaction Mapping Kit Sufficient materials to perform eight protein:protein interaction mapping experiments using FeBABE as the protein cutting agent. Includes: Metal Removal Reagent No-Weigh™ FeBABE Protein Cutting Reagent FeBABE Conjugation Buffer FeBABE Protein Cutting Buffer No-Weigh™ Ascorbic Acid Reducing Agent Stable Peroxide Reagent Protein Desalting Spin Columns	Kit 25 ml 8 x 50 mg 25 ml 25 ml 8 x 0.7 mg 3 ml 25/pkg
20332	No-Weigh™ FeBABE Protein Cutting Reagent Cysteine (–SH group)-reactive protein cutting reagent. Special single-dose packaging offers user a fresh reagent for each experiment. Eight microtube strip; 50 µg (85 nMoles)/microtube	8 x 50 µg
26101	Traut's Reagent (2-Iminothiolane•HCl) M.W. 137.63 Spacer Arm 8.1 Å	500 mg

References

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 Rana, T. M. and Meares, C.F. (1990). *J. Amer. Chem. Soc.* **112**, 2458-2459.
 Rana, T.M. and Meares, C.F. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 10578-10582.

Yeast Two-hybrid Reporter Assay

Yeast β -Galactosidase Assay Kit

Ideal for identifying protein:protein interactions in vivo using two-hybrid systems.

The gene encoding β -galactosidase (lacZ) of *E. coli* has been widely used as a reporter gene in many different prokaryotic and eukaryotic organisms. In particular, this gene has proven useful for studying gene expression in the yeast *Saccharomyces cerevisiae*.

In addition to its utility in studying the regulation of gene expression, the measurement of β -galactosidase activity can be used to identify protein:protein interactions *in vivo* using two-hybrid systems. The strength of the interaction is usually verified and/or quantitated using a β -galactosidase activity assay.

In contrast to methods using 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) as a β -galactosidase substrate, this reagent system allows for the qualitative or quantitative determination of β -galactosidase activity in solution directly from colonies growing on solid medium. Part of a colony is picked from a plate and resuspended in a mixture of Y-PER[®] Yeast Protein Extraction Reagent and β -galactosidase assay buffer. After a brief incubation period, the solution turns yellow from the hydrolysis of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) to *o*-nitrophenol (ONP) and galactose in a mildly alkaline solution. The assay becomes quantitative if the quantity of cells in the assay is first determined with an absorbance reading taken at 660 nm (OD₆₆₀).

Highlights:

- Efficient lysis of yeast cells and a colorimetric detection system
- Quantitative or qualitative assay
- Allows user to test cell cultures directly with no harvesting and washing steps (ideal for screening applications)
- Assay activity from colonies growing on solid media, qualitative or quantitative, with no re-streaking involved
- Can be used with bacterial cells

Linearity of β -Galactosidase Assay From Cells Growing in Media in a 96-Well Plate

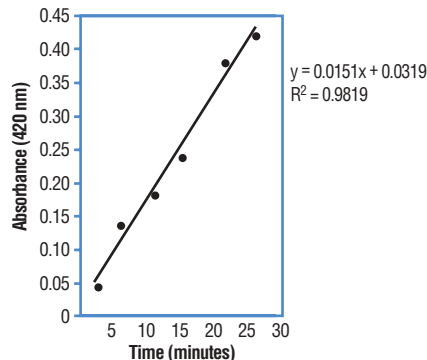


Figure 21. Strain BRS1002 carrying plasmid pYX122- β -Gal was grown to an OD₆₀₀ of 1.0, then 100 μ l of cells were transferred to a 96-well plate. At time = 0, 100 μ l of a 1:1 mixture of lysis reagent and 2X β -Gal assay buffer were added to each well and absorbance at 405 nm was determined. Specific activity for this sample is 160 units (unit=OD₄₀₅ x 1,000/time/OD₆₀₀).

Ordering Information

Product #	Description	Pkg. Size
75768	Yeast β-Galactosidase Assay Kit	Kit
	Protein:Protein Interaction Kit	
	Includes: Y-PER [®] Yeast Protein Extraction Reagent	25 ml
	2X β -Galactosidase Assay Buffer	25 ml
	1M Na ₂ CO ₃ Stop Solution	25 ml

* Additional dry ice and/or freight charge.

Protein Interactions

Protein:Nucleic Acid Interactions

Importance of Protein:Nucleic Acid Interactions

Proteins and nucleic acids do not operate within the biological system as independent entities. Protein:nucleic acid interactions (i.e., protein:RNA and protein:DNA interactions) are involved in several processes essential to normal cell function. As with protein:protein interactions, disruption of protein:nucleic acid interactions leads to serious and often catastrophic consequences within the system.

Protein:nucleic acid interactions are integrated into several key cellular processes. These processes include transcription, translation, regulation of gene expression, recognition, replication, recombination, repair, nucleic acid packaging and the formation of cellular machinery, such as ribosomes. The role of DNA as the genetic repository of information requires interaction with proteins for the extraction of this information for timely utilization within the cell.

Type of Protein:Nucleic Acid Interactions

The common property of nucleic acid-binding proteins is their ability to recognize and manipulate DNA/RNA structures. Transcription complex formation, initiation of transcription, and translation of messenger RNA to protein all involve formation of protein:nucleic acid complexes containing either DNA or RNA. These complexes by their nature play a role in the regulation of protein expression. Depending on the nature of the complex, proteins bind to nucleic acids in either a sequence-specific or secondary structure-dependent manner, often inducing drastic structural changes in the nucleic acid. Proteins can interact with nucleic acids in a variety of modes involving either major or minor groove associations. Defining sequence-specific interactions can aid in the development of high-affinity aptamers, which may be used as purification tools for DNA or RNA binding proteins. Sequence-specific interactions also have application in the study of gene regulation and drug discovery.

In vitro Methods for Protein:Nucleic Acid Interaction Analysis

Several methods for detecting and identifying protein:nucleic acid interactions are listed and defined in Table 4. These methods provide specific information as to the binding-site locus of a DNA-binding protein to a nucleic acid substrate. As with the protein:protein interaction methods, most of the techniques introduced in Table 4 are described more fully in the pages that follow, and relevant Pierce products listed thereafter.

Table 4. *In vitro* Methods for Protein:Nucleic Acid Interaction Analysis

<i>In vitro</i> Methods	Description
Electrophoretic Mobility Shift Assay (EMSA)	The EMSA has been used extensively for studying protein:DNA interactions. The assay is based on the fact that protein:DNA complexes migrate more slowly through a native polyacrylamide or agarose gel than unbound DNA. The individual protein:DNA complexes can be visualized as discreet bands within the gel using chemiluminescent or radioisotopic detection.
Supershift Assay	A variation of the EMSA that uses antibodies to identify proteins involved in the protein:DNA complex. The formation of an antibody:protein:DNA complex further reduces the mobility of the complex within the gel resulting in a "supershift." The method is described more fully in the Protein/Gene Expression section of the catalog.
Protein:DNA Cross-linking	Method for trapping protein:DNA interactions covalently under controlled conditions by labeling the protein bait and capturing the interacting DNA via coupling with a photoreactive reagent. Excellent for capturing weak or transient binding.
Affinity-based Methods	Uses labeled DNA or RNA fragments bound to an affinity support to capture or purify specific binding proteins from crude extracts.
DNA Footprinting	Method identifies the recognition site of a protein for a specific nucleic acid sequence. <i>Principle:</i> Binding of a protein to a specific DNA sequence protects that region of DNA from subsequent attack by DNase. The banding pattern observed on comparison of protected and unprotected reactions reveals the presence and position within the sequence of the protein's binding site.

Protein Interactions Electrophoretic-Mobility Shift Assays (EMSA)

Introduction to the EMSA [Gel-shift] Technique

The interaction of proteins with DNA is central to the control of many cellular processes including DNA replication, recombination and repair, transcription, and viral assembly. One technique that is central to studying gene regulation and determining protein:DNA interactions is the electrophoretic-mobility shift assay (EMSA).

The EMSA technique is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis.^{1,2} Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel-shift or gel-retardation assay. Until conception of the EMSA by Fried and Crothers³ and Garner and Revzin,⁴ protein:DNA interactions were studied primarily by nitrocellulose filter-binding assays.⁵

An advantage of studying DNA:protein interactions by an electrophoretic assay is the ability to resolve complexes of different stoichiometry or conformation. Another major advantage for many applications is that the source of the DNA-binding protein may be a crude nuclear or whole cell extract rather than a purified preparation. Gel-shift assays can be used qualitatively to identify sequence-specific DNA-binding proteins (such as transcription factors) in crude lysates and, in conjunction with mutagenesis, to identify the important binding sequences within a given gene's upstream regulatory region. EMSAs can also be used quantitatively to measure thermodynamic and kinetic parameters.^{3,4,6,7}

The ability to resolve protein:DNA complexes depends largely upon the stability of the complex during the brief time (approximately one minute) it is migrating into the gel. Sequence-specific interactions are transient and are stabilized by the relatively low ionic strength of the electrophoresis buffer used. Upon entry into the gel, protein complexes are quickly resolved from free DNA, in effect freezing the equilibrium between bound and free DNA. In the gel, the complex may be stabilized by "caging" effects of the gel matrix, meaning that if the complex dissociates, its localized concentration remains high, promoting prompt reassociation.^{3,6} Therefore, even labile complexes can often be resolved by this method.

Critical EMSA Reaction Parameters

Target DNA

Typically, linear DNA fragments containing the binding sequence(s) of interest are used in EMSAs. If the target DNA is short (20-50 bp) and well defined, complementary oligonucleotides bearing the specific sequence can be synthesized, purified by gel or HPLC, and annealed to form a duplex. Often, a protein:DNA interaction involves the formation of a multiprotein complex requiring multiple protein binding sequences. In this situation, longer DNA fragments are used to accommodate assembly of multiprotein complexes. If the sequence is larger (100-500 bp), the DNA source is usually a restriction fragment or PCR product obtained from a plasmid containing the cloned target sequence. Protein:DNA complexes formed on linear DNA fragments result in the characteristic retarded mobility in the gel. However, if circular DNA is used (e.g., minicircles of 200-400 bp), the protein:DNA complex may actually migrate faster than the free DNA. Gel-shift assays are also good for resolving altered or bent DNA conformations that result from the binding of certain protein factors. Gel-shift assays need not be limited to DNA:protein interactions. RNA:protein interactions^{8,9} as well as peptide:protein interactions¹⁰ have also been studied using the same electrophoretic principle.

Labeling and Detection

If large quantities of DNA are used in EMSA reactions, the DNA bands can be visualized by ethidium bromide staining. However, it is usually preferable to use low concentrations of DNA, requiring the DNA to be labeled before performing the experiment. Traditionally, DNA is radiolabeled with ³²P by incorporating an [α -³²P]dNTP during a 3' fill-in reaction using Klenow fragment or by 5' end labeling using [γ -³²P]ATP and T4 polynucleotide kinase.

Protein Interactions

EMSA Applications

The Supershift Reaction

This technique can aid in the identification of the DNA-bound protein. This is accomplished by including an antibody, specific for the DNA-binding protein, to the binding reaction. If the protein of interest binds to the target DNA, the antibody will bind to that protein:DNA complex, further decreasing its mobility relative to unbound DNA in what is called a “supershift.”

In addition to antibodies, supershift reactions could include other secondary or indirectly bound proteins.

Shift-Western Blot

This application involves transferring the resolved protein:DNA complexes to stacked nitrocellulose and anion-exchange membranes. Proteins captured on the nitrocellulose membrane can be probed with a specific antibody (Western blot) while autoradiography or chemiluminescent techniques can detect the DNA on the anion-exchange membrane. For an in-depth discussion of DNA End Labeling for EMSA, see the Pierce Application Handbook and Catalog.

Alternatively, DNA can be labeled with a biotinylated or hapten-labeled dNTP, then probed and detected using an appropriately sensitive fluorescent or chemiluminescent substrate. Pierce offers a chemiluminescent EMSA system (LightShift® Chemiluminescent EMSA Kit, Product # 20148) and a kit to facilitate labeling DNA with biotin (Biotin 3' End DNA Labeling Kit, Product # 89818). The LightShift® EMSA Kit uses Pierce's patented SuperSignal® Chemiluminescent Detection Technology to offer detection levels rivaling that of isotopic-based systems.

Nonspecific Competitor

Nonspecific competitor DNA such as poly(dI•dC) or poly(dA•dT) is included in the binding reaction to minimize the binding of nonspecific proteins to the labeled target DNA. These repetitive polymers provide an excess of nonspecific sites to adsorb proteins in crude lysates that will bind to any general DNA sequence. The order of addition of reagents to the binding reaction is important in that, to maximize its effectiveness, the competitor DNA must be added to the reaction along with the extract prior to the labeled DNA target. Besides poly(dI•dC) or other nonspecific competitor DNA, a specific unlabeled competitor sequence can be added to the binding reaction. A 200-fold molar excess of unlabeled target is usually sufficient to out-compete any specific interactions. Thus, any detectable specific shift should be eliminated by the presence of excess unlabeled specific competitor (Figure 22). The addition of a mutant or unrelated sequence containing a low-affinity binding site, like poly(dI•dC), will not compete with the labeled target and the shifted band will be preserved.

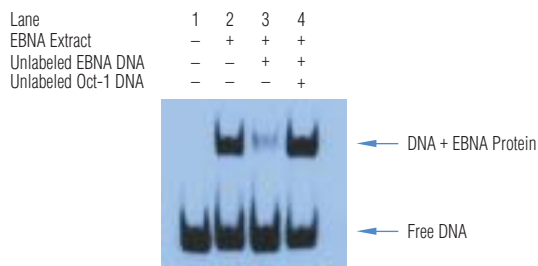


Figure 22. EMSA results using the EBNA control system. Biotin-labeled 60 bp duplex bearing the EBNA-1 binding sequence was incubated with an extract in which the EBNA-1 protein was overexpressed. The binding buffer was supplemented with 50 ng/μl poly(dI•dC), 10% glycerol and 0.05% NP-40. Exposure time was 30 seconds with X-ray film.

Protein Interactions Electrophoretic-Mobility Shift Assays [EMSA or Gel-Shifts]

Binding Reaction Components

Factors that affect the strength and specificity of the protein:DNA interactions under study include the ionic strength and pH of the binding buffer; the presence of nonionic detergents, glycerol or carrier proteins (e.g., BSA); the presence/absence of divalent cations (e.g., Mg^{2+} or Zn^{2+}); the concentration and type of competitor DNA present; and the temperature and time of the binding reaction. If a particular ion, pH or other molecule is critical to complex formation in the binding reaction, it is often included in the electrophoresis buffer to stabilize the interaction prior to its entrance into the gel matrix.

Gel Electrophoresis

Non-denaturing TBE-polyacrylamide gels or TAE-agarose gels are used to resolve protein:DNA complexes from free DNA. The gel percentage required depends on the size of the target DNA and the size, number and charge of the protein(s) that bind to it. It is important that the protein:DNA complex enters the gel and does not remain in the bottom of the well. Polyacrylamide gels in the range of 4-8% are typically used, although it is not uncommon for higher percentage gels to be used with certain systems. Agarose gels (0.7-1.2%) can be used to resolve very large complexes, as is the case with *E. coli* RNA polymerase (~460 kDa).

Gels are pre-run at a constant voltage until the current no longer varies with time. The primary reasons for pre-running gels are to remove all traces of ammonium persulfate (used to polymerize polyacrylamide gels), to distribute/equilibrate any special stabilizing factors or ions that were added to the electrophoresis buffer, and to ensure a constant gel temperature. After loading samples onto the gel, it is important to minimize the electrophoretic dead time required for the free DNA to enter the gel matrix, especially when analyzing labile complexes.

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Electrophoretic-Mobility Shift Assay (Gel-Shifts)

LightShift® Chemiluminescent EMSA Kit

Identifies regulatory sequences and determines protein:DNA binding regions and affinity – a nonradioactive chemiluminescent alternative.

- Stable, non-isotopic (biotin end-labeled) DNA probes, eliminating radioactive hazards and waste disposal
- Sub-femtomole sensitivity and low background noise, equivalent to radioactive systems
- One-day assay – progress from labeling DNA to EMSA data analysis in a single day

The LightShift® EMSA (Gel-Shift) Kit includes reagents for setting up and customizing protein-DNA binding reactions, a control DNA target and corresponding control protein extract, buffers, conjugate and luminol reagents for the detection phase of the assay. Customer target biotin-labeled DNA can be prepared using the Biotin 3' End DNA Labeling Kit (Product # 89818) or ordered directly from a custom oligo supplier.

Principle

The principle for LightShift® EMSA Detection is similar to that of a Western blot. Biotin end-labeled duplex DNA is incubated with a nuclear extract or purified factor and electrophoresed on a native gel. The DNA is then rapidly (30 minutes) transferred to a positive nylon membrane, UV-cross-linked, probed with streptavidin-HRP conjugate and incubated with the substrate. The protocol from labeling to results can be accomplished in a single day as outlined in Figure 23.

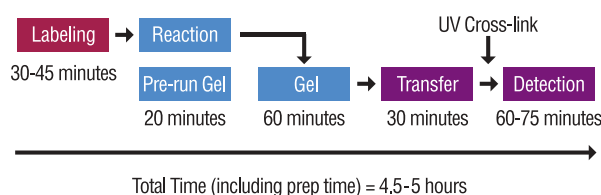


Figure 23. Timeline for the LightShift® EMSA Kit protocol.

The LightShift® EMSA Control System consists of an *E. coli* extract containing the overexpressed Epstein Barr Nuclear Antigen-1 (EBNA-1) and a 60 bp biotin-labeled target DNA duplex.

The LightShift® Chemiluminescent EMSA Kit was benchmarked against a commercial digoxigenin EMSA kit and a radioactive (³²P) "home-brew." The Pierce LightShift® Kit has been shown to be equivalent in sensitivity to the radioactive method (~37 attomoles) in a fraction of the exposure time and has a superior signal-to-noise ratio compared to the digoxigenin kit.



Numerous buffer additives can be added to the binding reaction to optimize and characterize the binding of the protein-DNA complex.⁴⁻⁸ Increasing the salt concentration helps to differentiate the higher affinity interactions from the lower affinity binding. Also, band shifts can be affected in the presence of MgCl₂, while glycerol tends to stabilize the higher affinity interactions. The LightShift® Kit contains several common reagents that can be used to optimize and tailor binding reactions for specific systems.

Ordering Information

Product #	Description	Pkg. Size
20148	LightShift® Chemiluminescent EMSA Kit Sufficient components for 100 binding reactions and detection reagents for ~800 cm ² of membrane. Includes: 10X Binding Buffer Biotin-EBNA Control DNA Unlabeled EBNA DNA EBNA Extract Poly(dI•dC) 50% Glycerol 1% NP-40 1 M KCl 100 mM MgCl ₂ 200 mM EDTA, pH 8.0 5X Loading Buffer Stabilized Streptavidin-Horseradish Peroxidase Conjugate Luminol/Enhancer Solution Stable Peroxide Solution Blocking Buffer 4X Wash Buffer Substrate Equilibration Buffer	Kit 1 ml 50 µl 50 µl 125 µl 125 µl 500 µl 500 µl 1 ml 500 µl 500 µl 1 ml 750 µl 80 ml 80 ml 500 ml 500 ml 500 ml
89818	Biotin 3' End DNA Labeling Kit	Kit

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* Additional dry ice and/or freight charges.

Protein Interactions Protein:Nucleic Acid Conjugates

Cross-linking Protein:Nucleic Acid Interactions

Heterobifunctional reagents are now available that can be applied to the study of site-specific protein:nucleic acid interactions. These reagents are designed to be deployed in a stepwise manner, enabling the capture of a protein:nucleic acid complex. Such conjugations between a protein bait and a nucleic acid prey involve use of cross-linking agents, a subject treated in greater detail as a previous topic in this handbook. Protein:nucleic acid interactions are most often stabilized by linkage with heterobifunctional cross-linkers that have a photoreactive aryl azide as one of the two reactive groups.

Generally, a purified binding protein is modified by reaction with one of the two reactive groups of the cross-linker. Most reagents target amine functions on the proteins, but other functional groups can be targeted as well. The initial reaction is carried out in the dark since the remaining reactive group of the cross-linker is photoreactive. The photoreactive group of the heterobifunctional reagent will ultimately cross-link the site at which the target protein binds when the resulting complex is exposed to light. Typically, the photoreactive group is an aryl azide-based moiety that can insert nonspecifically upon photolysis.

The modified putative binding protein is incubated with the nucleic acid sample. The complex is captured when exposed to the proper light conditions for the reagent. Band shift analysis can be used to indicate capture of the complex. Alternatively, nucleases can be used to remove those portions of the nucleic acid not protected by the protein binding, thereby isolating the sequence-specific site of interaction. Photoreactive, heterobifunctional reagents with a cleavable disulfide linkage allow reversal of the protein:nucleic acid cross-link and recovery of the components of the interacting pair for further analysis.

Preparation of Protein:Nucleic Acid Conjugates

The ability to conjugate proteins to nucleic acids, including RNA and DNA, is important in a number of life-science applications. Perhaps the most common conjugate of these molecules made using cross-linking compounds is the labeling of oligonucleotide probes with enzymes. Conjugating enzymes like horseradish peroxidase (HRP) or alkaline phosphatase (AP) to oligos that can hybridize to specific target sequences is important for detecting and quantifying target DNA or RNA. In this application, the enzyme activity is an indicator of the amount of target present similar to immunoassay detection using ELISA techniques. In this case, the oligo probe takes the place of the antibody, but the enzyme assay is detected by substrate turnover in the same manner.

Conjugation to 5'-Phosphate Groups

Using chemical reagents to effect the conjugation of nucleic acids to enzymes can be done using different strategies. A convenient functional group that can be chemically modified to allow the coupling of protein molecules on oligos is the 5'-phosphate group. Using the 5' end of the oligo as the conjugation point has an advantage of keeping the rest of the nucleic acid sequence unmodified and free so it can easily hybridize to a complementary target. For oligos that have been synthesized, a 5'-phosphate group may be put on the end of the molecule to facilitate this type of conjugation. The alkyl phosphate is reactive with the water-soluble carbodiimide EDC (Product # 22980, 22981), which forms a phosphate ester similar to the reaction of EDC with a carboxylate group. Subsequent coupling to an amine-containing molecule (i.e., nearly any protein or unmodified peptide) can be done to form a stable phosphoramidate linkage (Figure 24).

If a diamine molecule is used to modify the DNA 5'-phosphate, then the resultant amine-modified oligo can be coupled to enzyme molecules using a heterobifunctional reagent. Using a diamine compound that contains a disulfide (e.g., cystamine) and then reducing the disulfide group results in a sulfhydryl that may be conjugated with proteins rendered sulfhydryl-reactive using the hetero-bifunctional reagent Sulfo-SMCC (Product # 22322). Pierce offers HRP and AP enzymes that have been made sulfhydryl-reactive (i.e., maleimide-activated) by this mechanism (Product # 31485 and # 31486, respectively).

Protein Interactions

Protein:Nucleic Acid Conjugates

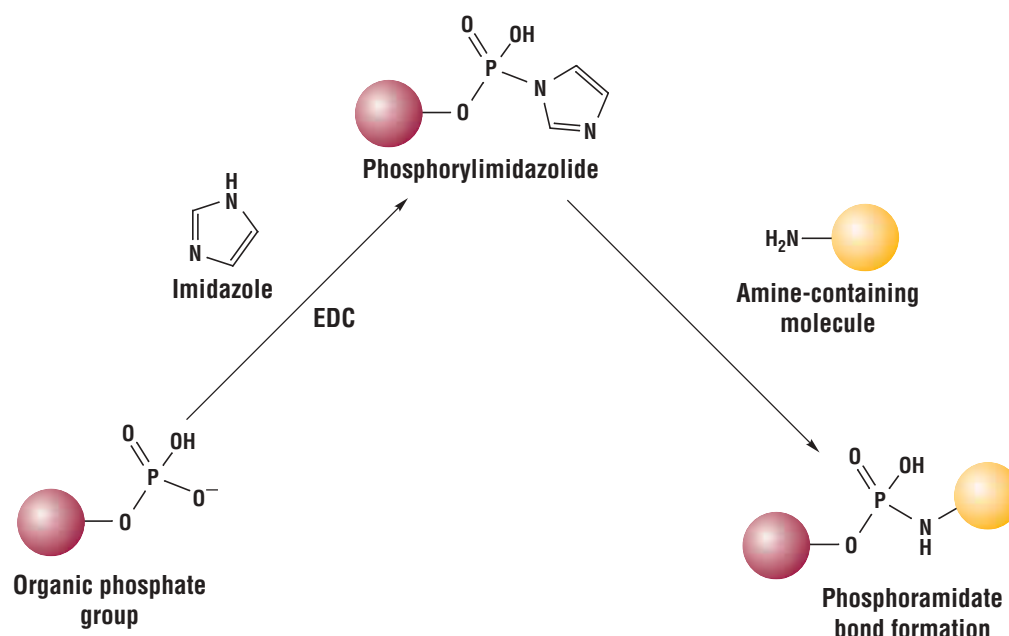


Figure 24. Reaction scheme showing typical route to conjugating an amine-containing biomolecule to a 5' terminal phosphate group.

Conjugation to the 3' End of RNA

Alternatively, the 3' end of RNA molecules may be chemically modified to allow coupling with amine-containing molecules or proteins. The diol on the 3'-ribose residue may be oxidized to result in two aldehyde groups using Sodium *meta*-Periodate (Product # 20504). The aldehydes then can be conjugated to the amine groups on a protein using reductive amination with Sodium Cyanoborohydride (Product # 44892). The aldehyde and the amine first form a Schiff base that is reduced to a secondary amine linkage with the cyanoborohydride reductant.

Biotinylation of Nucleic Acids

Nucleic acid molecules also can be biotinylated by a number of chemical methods. Using the strategies previously described to modify the 5' or 3' ends of oligos with a diamine (e.g., Product # 23031) will provide a functional group that can be reacted with any amine-reactive biotinylation compound, such as Sulfo-NHS-LC-Biotin (Product # 21335). This modification method would provide a biotin group at the end of an oligo probe, thus allowing streptavidin reagents to be used to detect a hybridization event with a target.

In addition, biotinylation of oligonucleotides can be done using photoreactive reagents. There are two main options commonly used to add one or more biotin residues to nucleic acid probes. Photoactivatable Biotin (Product # 29987) contains a phenyl azide group at the end of a spacer arm with the biotin group at the other end. Photolyzing a solution of the biotin compound together with an oligo in solution results in biotin being nonselectively inserted into the nucleic acid structure. Alternatively, Psoralen-PEO-Biotin (Product # 29986) can be used to label double-stranded DNA or RNA. The psoralen ring structure effectively intercalates into the double-stranded portions, and exposure to UV light causes a cyclo-addition product to be formed with the 5,6-double bond in thymine residues. The poly(ethylene oxide) spacer in Psoralen-PEO-Biotin contributes excellent water solubility, thus assuring that the resultant derivative will have accessibility to streptavidin-containing detection reagents.

Other Nucleic Acid-Labeling Methods

Other methods of end-labeling nucleic acids are described fully in the Pierce Applications Handbook and Catalog.

Protein:Nucleic Acid Interaction Cross-linking Agents

Cross-linking Agents

This section covers reagents that can be applied to the preparation of nucleic acid-based conjugates, including biotinylation and the study of protein:nucleic acid interactions.^{1,2,3} Heterobifunctional reagents can be applied to the study of site-specific protein:nucleic acid interactions. These reagents are designed to be deployed in a stepwise manner enabling the capture of a protein:nucleic acid complex. For a discussion of how these reagents are used, see the Pierce Applications Handbook and Catalog. Reagents that have been applied or with potential application to the study of protein:nucleic acid interactions are listed below.

Product #	Double Agents™ Product Name	Structure	Key Features	Ref.	Pkg. Size
27720	APDP <i>N</i> -(4-[<i>p</i> -Azido-salicylamido]-butyl)-3'-(2'-pyridyldithio)-propionamide M.W. 446.55 Spacer Arm 21.0 Å		<ul style="list-style-type: none"> • -SH group-reactive • Iodinatable • Cleavable • Reaction with nucleic acid binding protein monitored at 343 nm 	4	50 mg
27719	SFAD Sulfosuccinimidyl(perfluoro-azidobenzamido)ethyl-1,3'-dithiopropionate M.W. 597.48 Spacer Arm 14.6 Å		<ul style="list-style-type: none"> • Improved photoconjugation efficiency • Photolyzes at 320 nm • Water-soluble • Cleavable • Amine-reactive 	5,6	50 mg
23013	SPB Succinimidyl-(4-[psoralen-8-yloxy])-butyrate M.W. 385.32 Spacer Arm 8.9 Å		<ul style="list-style-type: none"> • Amine-reactive • DNA intercalating agent • Psoralen couples via 5 or 6 member end rings 	7,8,9	50 mg
27735	Sulfo-NHS-LC-ASA* Sulfosuccinimidyl(4-azido-salicylamido)hexanoate M.W. 491.41 Spacer Arm 18.0 Å *See also: NHS-ASA (Product # 27714)		<ul style="list-style-type: none"> • Can incorporate ¹²⁵I label before acylation step • Photolysis initiated by long-wave UV • Water-soluble • Non-cleavable • Amine-reactive 	10	50 mg
21553	Sulfo-SADP Sulfosuccinimidyl(4-azido-phenyldithio)propionate M.W. 454.45 Spacer Arm 13.9 Å		<ul style="list-style-type: none"> • Cleavable by 50 mM DTT, 100 mM BME or 1% NaBH₄ • Photolyzes at 265-275 nm • Amine-reactive 	11	50 mg
22589	Sulfo-SANPAH* Sulfosuccinimidyl 6-(4-azido-2'-nitrophenylamino)hexanoate M.W. 492.39 Spacer Arm 18.2 Å **See also: SANPAH (Product # 22600)		<ul style="list-style-type: none"> • Optimal photolysis occurs at 320-350 nm limiting damage to biomolecules by irradiation • Water-soluble • Non-cleavable • Amine-reactive 	12	50 mg

References

1. DNA-Protein Interactions; Principles and Protocols, (1994). G. Geoff Kneale, Ed. Humana Press, p. 427 (Product # 20012).
2. Rhodes, D. and Fairall, L. (1997). In Protein Function, 2nd edition, T.W. Creighton, Ed., The Practical Approach Series, IRL Press, pp. 215-244.
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9. Elsner, H.I. and Mouritsen, S. (1994). *Bioconj. Chem.* **5**, 463-467.
10. McMahan, S.A. and Burgess, R.R. (1994). *Biochemistry* **33**, 12092-12099.
11. Reeves, R. and Missen, M.S. (1993). *J. Biol. Chem.* **268**(28), 21137-21146.
12. Prossnitz, E. (1991). *J. Biol. Chem.* **266**(15), 9673-9677.

Protein:Nucleic Acid Conjugate Formation

Alkaline Phosphatase

Ready-to-conjugate preparation.

Highlight:

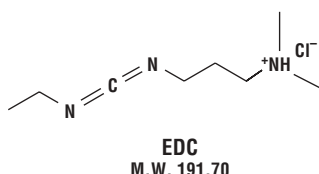
- Specific activity 2,000 units/mg

Ordering Information

Product #	Description	Pkg. Size
31391	ImmunoPure® Alkaline Phosphatase	20 mg
31392	ImmunoPure® Alkaline Phosphatase	100 mg

EDC

Activates phosphate groups on oligos.



Ordering Information

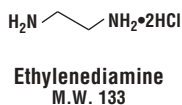
Product #	Description	Pkg. Size
22980	EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride)	5 g
22981	EDC	25 g

Highlight:

- Water-soluble carbodiimide that activates phosphate groups on oligos and carboxylate groups on proteins similarly. Coupling occurs via amine groups on enzymes such as HRP or AP, forming a stable phosphoramidate linkage.

Ethylenediamine Dihydrochloride

Used in 3' or 5' end labeling strategies for oligos.



Ordering Information

Product #	Description	Pkg. Size
23031	Ethylenediamine Dihydrochloride	10 g

Horseradish Peroxidase

High-specific activity preparation ideal for protein:nucleic acid conjugate preparations.

Highlights:

- Small high-turnover rate enzyme of 40K MW
- Conjugates compatible with a number of substrates

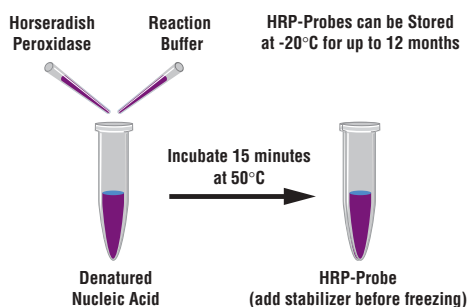
Ordering Information

Product #	Description	Pkg. Size
31490	ImmunoPure® Horseradish Peroxidase	10 mg
31491	ImmunoPure® Horseradish Peroxidase	100 mg

* Additional dry ice and/or freight charges.

North2South® Direct HRP Labeling and Detection Kit

Fast, easy-to-use, two-hour nonradioactive blotting kit that is as sensitive as radioactivity.



This ready-to-use kit includes all of the reagent required to label a probe with HRP and detect with Pierce ultrasensitive (single-gene copy detection) chemiluminescent substrate, and all necessary hybridization and wash buffers. The user supplies the nucleic acid (DNA, RNA or oligonucleotide, 50 bp) and water.

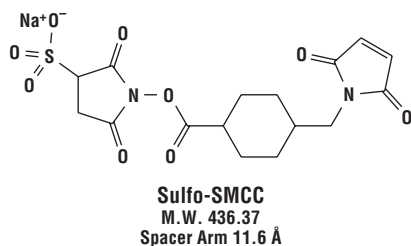
Ordering Information

Product #	Description	Pkg. Size
17195	North2South® Direct HRP Labeling and Detection Kit	Kit

For a list of kit components, visit our web site or see the Pierce Applications Handbook and Catalog.

Sulfo-SMCC

Yields stable protein:nucleic acid conjugates.



Highlights:

- Couples readily to an amine-derivatized oligo by EDC coupling of a diamine to the 5' phosphate group
- Nucleic acid can be sequentially coupled to an available -SH group on a protein/enzyme

Ordering Information

Product #	Description	Pkg. Size
22322	Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate)	50 mg

Sodium meta-Periodate

An oxidation agent of choice for creating active aldehydes from 3' ribose diols in RNA.

Highlight:

- Aldehydes can be conjugated to amine groups on a protein or enzyme using reductive amination with sodium cyanoborohydride

Ordering Information

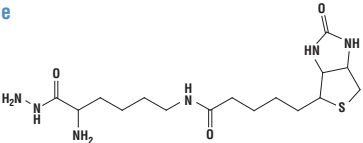
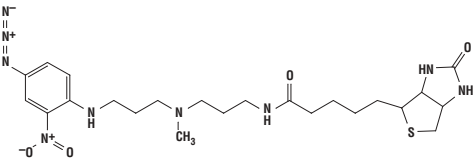
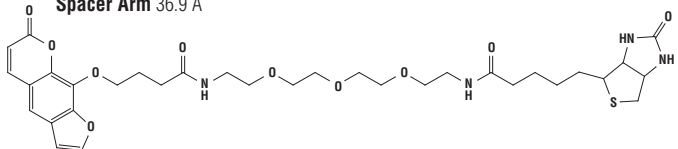
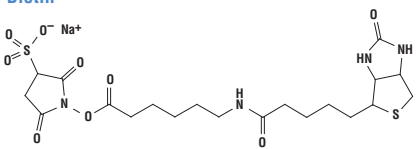
Product #	Description	Pkg. Size
✗ 20504	Sodium meta-Periodate	25 g
✗ 44892	AminoLink® Reductant (Sodium cyanoborohydride)	2 x 1 g

✗ Additional hazardous charge.

Protein:Nucleic Acid Conjugate Formation

Nucleic Acid-Biotin Conjugates

See the biotinylation section of the Pierce web site for our complete selection of reagents.

Product #	Biotinylation Agent Product Name	Structure	Key Features	Ref.	Pkg. Size
28020	EZ-Link® Biotin Hydrizide M.W. 386.51 Spacer Arm 19.7 Å		<ul style="list-style-type: none"> Carbohydrate-reactive Used to label DNA and RNA through cytosine Extended spacer arm Forms hydrazone bond Water-soluble 	1	25 mg
29987	EZ-Link® Photoactivatable Biotin M.W. 533.65 Spacer Arm 30 Å		<ul style="list-style-type: none"> Water-insoluble Membrane-permeable Modifies DNA/RNA Covalent nonspecific coupling occurs in presence of UV light (350-370 nm) 	2	0.5 mg
29986	EZ-Link® Psoralen-PEO-Biotin M.W. 688.79 Spacer Arm 36.9 Å		<ul style="list-style-type: none"> One-step labeling Photoactivate at ~350 nm Labels DNA/RNA probes/PCR products/oligos or cDNA inserts Water-soluble Promotes conjugate solubility Does not interfere with hybridization 	3-5	5 mg
21335	EZ-Link® Sulfo-NHS-LC-Biotin* M.W. 556.59 Spacer Arm 22.4 Å		<ul style="list-style-type: none"> Water-soluble Amine-reactive 	6	100 mg

*See also: EZ-Link® Sulfo-NHS-SS-Biotin (Product # 21331)

References

1. Reisfeld, A., et al. (1987). *Biochem. Biophys. Res. Comm.* **142**, 519-526.
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Protein Interactions Affinity Methods for Protein:Nucleic Interactions

Affinity-Capture Methods for Protein:Nucleic Acid Interactions

Access to the latest nucleic acid-sequencing and labeling technologies has been a great asset to *in vitro* affinity methods of verifying and characterizing the interaction of protein with specific nucleic acid sequence motifs. Short nucleic acid oligos (DNA or RNA) encoding the sequence under study are most popularly labeled with amine or biotin tags linked to the 5' end via a cross-linker. These biotin- or amine-labeled oligos are then amenable to immobilization and detection strategies that allow *in vitro* protein:nucleic acid interaction studies.

Plate Capture Methods

In the literature, there are several ways to immobilize DNA or RNA (bait) and analyze the interaction of specific proteins (prey) with the bait. One popular method uses 96- or 384-well microplates coated with streptavidin to bind biotinylated DNA/RNA baits. A cellular extract is prepared in binding buffer and added for a sufficient amount of time to allow the putative binding protein to come in contact and “dock” onto the immobilized oligonucleotide. The extract is then removed and each well is washed several times to remove nonspecifically bound proteins. Finally, the protein is detected using a specific antibody labeled for detection. This method can be extremely sensitive because the antibody is usually labeled with an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), that amplifies the signal over time according to the label's enzyme activity. Coupling enzymatic amplification of signal with a chemiluminescent substrate suited to ELISA-based applications (e.g., Product # 37070) can lead to detection of less than 0.2 pg of the protein of interest per well. This same ELISA-based method may also be used for amine-labeled oligos using microplates coated with an amine-reactive surface chemistry (e.g., Reacti-Bind™ Maleic Anhydride Plates, Product # 15110).

Pull-down (Gel Support) Methods

Another popular affinity-based format for studying protein:nucleic acid interactions *in vitro* is the pull-down method. In this case, as in the ELISA method, the amine- or biotin-labeled nucleic acid is immobilized on either an amine-reactive or immobilized streptavidin gel surface. The gel may be prepared in a spin cup, column or batch format, depending on individual requirements. After the nucleic acid bait has been immobilized, a cellular extract containing the putative prey protein is prepared in binding buffer and incubated for a sufficient time with the immobilized oligonucleotide. Once the gel has been washed thoroughly, the purified protein prey may be eluted from the nucleic acid bait by a stepwise salt gradient or other buffer condition sufficient to disrupt the interaction. After the prey has been eluted, it is amenable to virtually any characterization technique. SDS-PAGE may be performed with the eluted sample, allowing sizing relative to molecular weight standards. It also may be transferred to membrane for more thorough identification by Western blotting. Depending on the method of detection and abundance of the putative DNA or RNA binding protein in the cellular extract, the pull-down technique may require a greater amount of starting material.

Flexible Methods

There are many variations to the ELISA and pull-down methods that do not significantly alter the basic premise of each. For example, the labeled DNA or RNA oligo may be first incubated with the cellular extract and then the entire protein:nucleic acid complex immobilized on the plate surface or gel. In contrast to the sequential binding and washing of the oligo, and the addition of the cellular extract, adding the oligo directly to the cellular extract before binding the nucleic acid:protein complex on the gel surface may solve logistical problems, especially when steric hindrance is suspected.

Protein Interactions

Affinity Methods for Protein:Nucleic Interactions [continued]

Current options in gel format selection may also be chosen to fit the requirements of each experimental system, though the assay basics remain similar. For instance, the amine-reactive or streptavidin-coated gel may be placed in a column for standard column chromatography. Additionally, the gel may be processed in a spin cup for use with a microcentrifuge, vacuum manifold or syringe. Alternatively, derivatized magnetic beads may be used to achieve magnetic separation. The list of options is quite extensive and is limited only by the imagination of the researcher and/or the logistics of a particular experiment.

Table 5. Selected Pierce Products for use in the Study of Protein:Nucleic Acid Interactions

Product Name	Product #	Product Description
Profound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit	21115	Each kit allows 25 pull-down assays in a spin column format, standard buffer system included. Successfully used for nucleic acid:protein interactions.
ImmunoPure® Immobilized Streptavidin Gel	20347 (2 ml gel) 20349 (5 ml gel) 20351 (5 x 1 ml columns)	Binds 15-28 µg biotin per ml of gel (1-3 mg biotinylated BSA per ml of gel).
MagnaBind™ Streptavidin Beads	21344 (5 ml)	Binds 2 µg biotin per ml of beads. Allows magnetic separation. Supporting equipment also available.
Reacti-Bind™ Streptavidin Coated Plates	15118-15122, 15124-15126 (various 96-well plate packages) 15405-15407 (various 384-well plate packages)	Binds 5 pmoles biotin per well. Large variety of plate formats. Custom-made plates available upon request.
Reacti-Bind™ Streptavidin High Binding Capacity Coated Plates	15500-15503 (various 96-well plate packages) 15504-15506 (various 384-well plate packages)	Binds 60 pmoles biotin per well, large variety of plate formats. Custom-made plates available upon request.
AminoLink® Immobilization Kit	44890	Binds primary amines. Standard buffer system and 5 x 2 ml columns included.
Reacti-Bind™ Maleic Anhydride Activated Polystyrene Plates	15100, 15102, 15110, 15112 (various 96-well plate packages)	Binds primary amines. Custom-made plates available upon request.

References

- Kadonaga, J. and Tjian, R. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 5889-5893.
 Kneale, G. (1994). *Methods in Molecular Biology*, Volume 30: DNA-Protein Interactions: Principles and Protocols. (Product # 20012).

Practical Considerations

Several steps may be taken to reduce the chances of anomalous data generation when working with proteins and nucleic acids in the context of cellular extracts.

1. Remember to always include protease and nuclease inhibitors to decrease the chances of protein and oligo degradation.
2. Take appropriate measures to reduce nonspecific binding of proteins to either the oligo or gel surface. For example, poly(dI•dC) is often included in the cellular extract as a weak competitor to the oligo, and can significantly decrease nonspecific binding events.
3. Make sure to include all cofactors and conditions required for the protein to bind the DNA or RNA. Also, some proteins may require the nucleic acid to be double- or single-stranded before binding can occur.
4. Consider the length of the carbon chain between the biotin or amine label and the oligo. It can make a significant difference, reducing the steric hindrance of the bound oligo.
5. Proper use of controls will be essential to successful execution of any experiment.

Protein:Nucleic Acid Interaction **Complex Isolation**

Immobilized avidin and other biotin-binding supports can be used to isolate protein:nucleic acid complexes in which either the protein or the end-labeled nucleic acid is biotinylated. Several different formats such as solid supports, coated plates, magnetic beads and pull-down assays can be applied to this application.

Affinity Supports for Capturing Protein:Nucleic Acid Interactions

Immobilized Avidin

Strong biotin interaction creates a nearly irreversible bond.

Immobilized avidin can be used in a variety of applications for the affinity purification of biotinylated macromolecules.

Highlights:

- Hybridization of biotinylated RNA to its complementary DNA and binding to immobilized avidin, with subsequent elution of the single-stranded DNA¹
- Purification of double-stranded DNA²

References

1. Manning, J., *et al.* (1977). *Biochemistry* **16**, 1364-1370.
2. Pellegrini, M., *et al.* (1977). *Nucleic Acids Res.* **4**, 2961-2973.

Ordering Information

Product #	Description	Pkg. Size
20219	Immobilized Avidin Gel Support: Cross-linked 6% beaded agarose Spacer: None (directly attached) Supplied: 50% aqueous slurry containing 0.02% NaN ₃	5 ml
20362	AffinityPak™ Immobilized Avidin Columns	5 x 1 ml
20225	Immobilized Avidin Gel	5 x 5 ml

Immobilized Monomeric Avidin

Ideal affinity support for gentle, reversible binding of biotinylated macromolecules.

Highlights:

- Retains biotin specificity with reduced binding affinity (kDa ~ 10⁻⁸ M)
- Purifies biotinylated products under mild elution conditions (2 mM free biotin)
- Can be regenerated and reused at least 10 times
- Exhibits little nonspecific binding (3% or less)

Ordering Information

Product #	Description	Pkg. Size
20228	Immobilized Monomeric Avidin Gel	5 ml
20227	Immobilized Monomeric Avidin Kit Includes: Monomeric Avidin Column BupH™ Phosphate Buffered Saline Pack (yields 500 ml) Biotin Blocking and Elution Buffer Regeneration Buffer Column Extender	Kit 2 ml 1 pack 200 ml 250 ml
53146	UltraLink® Immobilized Monomeric Avidin	5 ml
29129	ImmunoPure® D-Biotin <i>Promotes the gentle elution of biotinylated complex from an immobilized monomeric avidin support.</i>	1 g

Protein:Nucleic Acid Interaction Complex Isolation

Immobilized NeutrAvidin™ Supports

Less nonspecific binding makes these exclusive Pierce supports well-suited for capturing interacting complexes.

Highlights:

- Carbohydrate-free
- No interaction with cell-surface molecules
- Neutral pI (6.3) eliminates electrostatic interaction that contributes to nonspecific binding

Ordering Information

Product #	Description	Pkg. Size
29200	Immobilized NeutrAvidin™ on Agarose	5 ml
53150	UltraLink® Immobilized NeutrAvidin™ Biotin-Binding Protein Capacity: ~ 12-20 µg of biotin/ml gel	5 ml
53151	UltraLink® Immobilized NeutrAvidin™ Biotin-Binding Protein Plus Capacity: ≥ 30 µg of biotin/ml gel	5 ml

Immobilized Streptavidin

High biotin-binding affinity and low nonspecific binding offer advantages for interaction capture.

Highlights:

- Purified recombinant streptavidin
- Stable leach-resistant linkage of streptavidin to the support
- Support: cross-linked 6% beaded agarose
- Capacity: approx. 1-3 mg biotinylated BSA/ml gel

Ordering Information

Product #	Description	Pkg. Size
20347	Immobilized Streptavidin Gel	2 ml
20349	Immobilized Streptavidin Gel	5 ml
20351	AffinityPak™ Immobilized Streptavidin Columns	5 x 1 ml

UltraLink® Immobilized Streptavidin

A high-performance support offering faster flow rates and overall superior performance in affinity applications.

If using immobilized streptavidin for purifying proteins that bind to a biotinylated ligand (DNA or peptides), UltraLink® Products are recommended. The UltraLink® support comes in a "Plus" version, with twice the amount of streptavidin loaded per ml of gel.

Application:

- Recovery of single-stranded DNA for dideoxy sequencing¹

Ordering Information

Product #	Description	Pkg. Size
53113	UltraLink® Immobilized Streptavidin Gel Capacity: ≥ 2 mg of biotinylated BSA/ml gel	2 ml
53114	UltraLink® Immobilized Streptavidin Gel	5 ml
53116	UltraLink® Immobilized Streptavidin Plus Gel Capacity: ≥ 4 mg of biotinylated BSA/ml gel	2 ml
53117	UltraLink® Immobilized Streptavidin Plus Gel	5 ml

Reference

1. Mitchell, L.G. and Merril, C.R. (1989). *Anal. Biochem.* **178**, 239-242.

Coated Plates for Capturing Protein:Nucleic Acid Interactions

Reacti-Bind™ NeutrAvidin™ Coated Polystyrene Plates

The high affinity of avidin for biotin, without the nonspecific binding problems.

Highlights:

- Lowest nonspecific binding properties of all biotin-binding proteins
- NeutrAvidin™ Biotin-Binding Protein has no carbohydrate and an isoelectric point of 6.3
- Pre-blocked
- Binding capacity: 15 pmoles of biotin/ 100 µl coat volume

Ordering Information

Product #	Description	Pkg. Size
15123	Reacti-Bind™ NeutrAvidin™ Coated 96-Well Plates with Blocker™ BSA (Clear)	5 plates
15129	Reacti-Bind™ NeutrAvidin™ Coated 96-Well Plates with SuperBlock® Blocking Buffer (Clear)	5 plates
15128	Reacti-Bind™ NeutrAvidin™ Coated Strip Plates with Blocker™ BSA (Clear)	5 plates
15127	Reacti-Bind™ NeutrAvidin™ Coated Strip Plates with SuperBlock® Blocking Buffer (Clear)	5 plates

Reacti-Bind™ Streptavidin Coated Polystyrene Plates

The specific binding affinity of streptavidin for biotin – in a microplate.

Highlights:

- Gentle immobilization of biotinylated complexes
- Low nonspecific binding
- Pre-blocked
- Binding capacity: 5 pmoles of biotin/ 100 µl coat volume

Ordering Information

Product #	Description	Pkg. Size
15120	Reacti-Bind™ Streptavidin Coated Strip Plates with SuperBlock® Blocking Buffer (Clear)	5 plates
15124	Reacti-Bind™ Streptavidin Coated 96-Well Plates with SuperBlock® Blocking Buffer (Clear)	5 plates
15125	Reacti-Bind™ Streptavidin Coated 96-Well Plates with Blocker™ BSA (Clear)	5 plates
15126	Reacti-Bind™ Streptavidin Coated 96-Well Plates with SuperBlock® Blocking Buffer (Clear)	25 plates

Reacti-Bind™ Streptavidin HBC Coated Plates

New coating technology results in improved binding capacity.

Highlights:

- Increased sensitivity of complex detection
- Broader dynamic range
- Pre-blocked
- High-binding capacity (HBC): 125 pmoles of biotin/ 100 µl coat volume

Ordering Information

Product #	Description	Pkg. Size
15500	Reacti-Bind™ Streptavidin Coated Plates (HBC), 96-Well Plates with SuperBlock® Blocking Buffer (Clear)	5 plates
15501	Reacti-Bind™ Streptavidin Coated Plates (HBC), 8-Well Strip Plates with SuperBlock® Blocking Buffer (Clear)	5 plates

Protein:Nucleic Acid Interaction **Complex Isolation**

Magnetic Beads for Capture of Interacting Complexes

Streptavidin Coated Beads

A convenient method for isolating biomolecules using affinity binding, while retaining biological activity.

Highlights:

- Fast capture and separation of complexes
- Beads respond well to weak magnetic fields
- 1–4 μm diameter
- 1×10^8 particles/mg

Ordering Information

Product #	Description	Pkg. Size
21344	MagnaBind™ Streptavidin Beads	5 ml
21357	MagnaBind™ Magnet for 1.5 ml Microcentrifuge Tube	1 magnet
21359	MagnaBind™ Magnet for 6 Microcentrifuge Tubes	1 magnet

Pull-down Assay for Capture of Interacting Complexes

ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit

Pull-down a binding partner with the new Pierce Biotinylated-Protein:Protein Interaction Kit.

See the complete description of this kit in the Pull-Down Assays segment of this handbook, pages 15–20.

Ordering Information

Product #	Description	Pkg. Size
21115	ProFound™ Pull-Down Biotinylated-Protein:Protein Interaction Kit	Kit

Chemiluminescent Detection in Polystyrene Plates

SuperSignal® ELISA Pico Chemiluminescent Substrate

Experience the same sensitivity in your luminometer that you've come to expect from all Pierce SuperSignal® Products.

See the complete description of this product in the Pierce Applications Handbook and Catalog or visit the Pierce web site.

Ordering Information

Product #	Description	Pkg. Size
37070	SuperSignal® ELISA Pico Chemiluminescent Substrate Includes: Luminol/Enhancer Stable Peroxide Buffer	100 ml 50 ml 50 ml

Activated Supports for Coupling via Primary Amine Groups

Pierce offers a number of activated supports incorporating innovative chemistries for the coupling of proteins and custom terminal amine group-containing oligos for use in a variety of applications. These activated supports are offered under the AminoLink®, Reacti-Gel®, Reacti-Bind™ and MagnaBind™ trademarks and can be found in the free Affinity Purification Handbook (Product # 1600975), or visit the Pierce web site.



Grasp the Proteome™

Protein:Protein Interaction

Discover

Confirm

Characterize

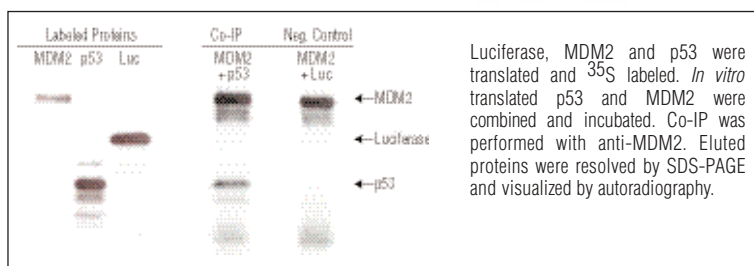
Perfect Co-IPs are within reach

Pierce Introduces ProFound™ Co-Immunoprecipitation Kits

- Couple any antibody with the universal kit or use the pre-conjugated anti-HA or c-Myc kits
- Spin columns provide higher recovery of target proteins and more consistent, reproducible results
- Kit supplied complete with resin controls



- No antibody contamination of Co-IP results because antibodies are covalently attached
- Reusable (up to 10 times) immobilized support
- Can be easily scaled up or down (10-200 µl)



Product #	Description	Product #	Description
23600	ProFound™ Co-Immunoprecipitation Kit	23615	ProFound™ Mammalian HA-Tag IP/Co-IP Kit
23605	ProFound™ Mammalian Co-Immunoprecipitation Kit	23620	ProFound™ c-Myc-Tag IP/Co-IP Kit
23610	ProFound™ HA-Tag IP/Co-IP Kit	23625	ProFound™ Mammalian c-Myc Tag IP/Co-IP Kit

FREE! Protein:Protein Interaction Guide
Request a FREE copy from Pierce, your Perbio Science branch office or distributor.

www.piercenet.com

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