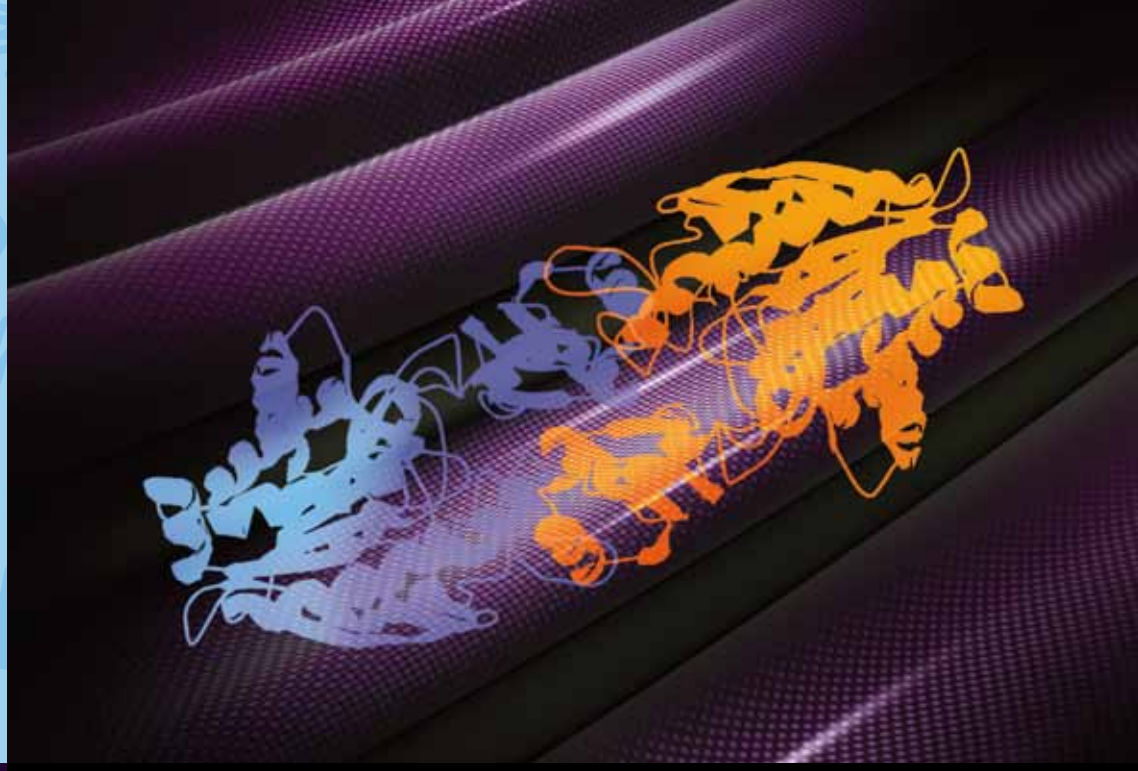

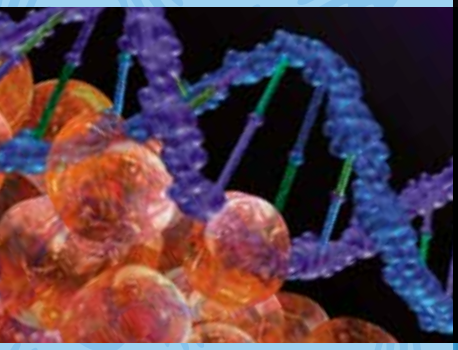

Thermo Scientific Pierce Protein Interaction Technical Handbook

Version 2

Part of Thermo Fisher Scientific

Thermo SCIENTIFIC

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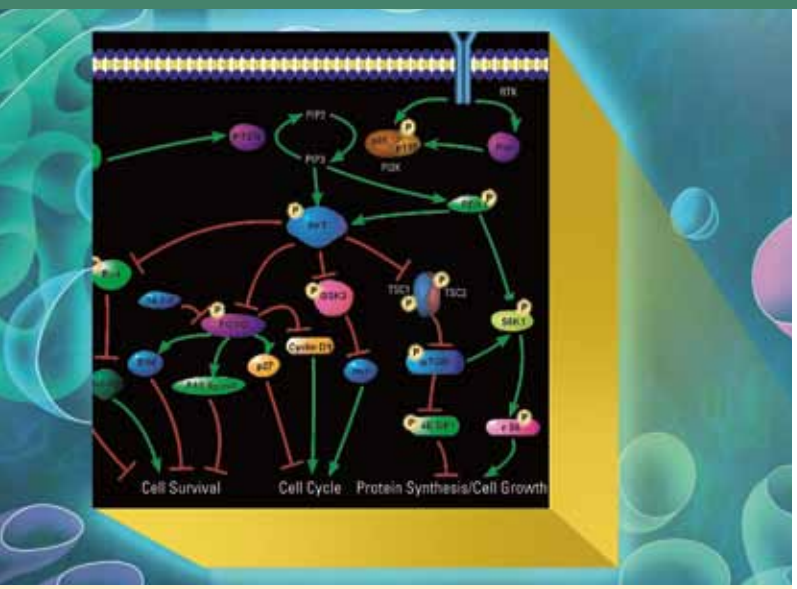
Introduction to Protein Interactions



Importance of Protein Interactions

The study of protein interactions is vital to the understanding of how proteins function within the cell. Publication 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 x 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 References on page 7). 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 crosslinking or label transfer methods.

In Vivo Methods for Protein Interaction Analysis

***In vivo* methods for protein interaction analysis.**

In Vivo Methods	Description
Yeast Two-Hybrid System	Monitor complex formation through transcriptional activation of reporter genes.
Crosslinking Reagents	Incorporating functional groups into proteins which can react, trapping a protein complex.
Immunofluorescence/FRET	Detect co-localized signal from two different proteins or monitor complex formation thought fluorescent resonance energy transfer.

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.

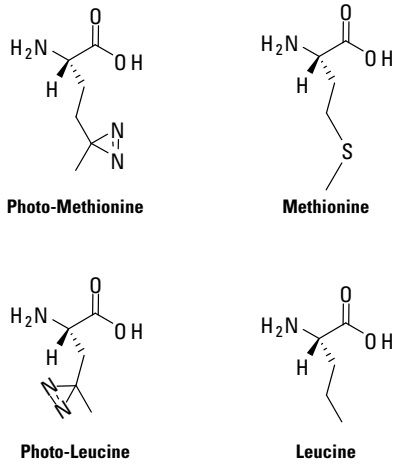
Many interactions currently known were first indicated by the yeast two-hybrid method. *In vitro* methods for protein interaction analysis are often employed to confirm interactions indicated by the yeast two-hybrid strategy described above.

In Vivo Crosslinking with Photo-Reactive Amino Acid Analogs

Rapidly crosslinking protein complexes in live cells is a powerful method to capture both stable and transient protein interactions in their native environment. The novel photo-reactive amino acid derivatives, L-photo-methionine and L-photo-leucine, can substitute for their respective amino acids in endogenously expressed proteins. These proteins are then activated by ultraviolet (UV) light to covalently photo-crosslink proteins within protein interaction domains containing methionine and/or leucine.

Photo-leucine and photo-methionine are amino acid derivatives that contain diazirine rings for UV photo-crosslinking of proteins. When used in combination with specially formulated limiting media, these photo-activatable derivatives of leucine and methionine are treated like the naturally occurring amino acids by the protein synthesis machinery within the cell. As a result, they can be substituted for leucine or methionine in the primary sequence of proteins during synthesis.

Naturally associating binding partners within the cell can be instantly trapped by photoactivation of the diazirine-containing proteins in the cultured cells. Crosslinked protein complexes can be detected by decreased mobility on SDS-PAGE followed by Western blotting. Alternative methods of crosslink detection include size exclusion chromatography, sucrose density gradient sedimentation and mass spectrometry.



Structures of photo-reactive amino acids compared to their naturally occurring counterparts.

Highlights:

- ***In vivo* labeling** – incorporate photo-reactive group into proteins using normal cellular machinery
- ***In vivo* crosslinking** – find interacting proteins in the native cellular environment
- **Increased specificity** – crosslink interacting proteins correctly positioned at their interfaces within protein interaction domains
- **Efficient recovery** – greater than 90% protein recovery in cell lysates after crosslinking
- **Compatible** – crosslink proteins expressed in a wide variety of cell lines including HeLa, 293T. COS7, U2OS, A549, A431, HepG2, NIH 3T3 and C6
- **Easy-to-use** – reagents are photo-stable under normal laboratory lighting so there is no need to work in the dark

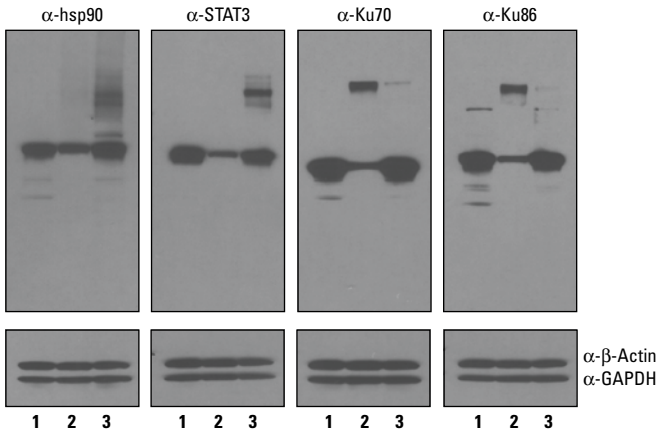


Photo-reactive amino acid crosslinking and formaldehyde crosslinking are complementary techniques for protein interaction analysis. HeLa cells were mock-treated **(Lane 1),** treated with 1% formaldehyde for 10 minutes **(Lane 2),** or treated with Thermo Scientific Pierce Photo-Methionine and Photo-Leucine followed by UV treatment **(Lane 3).** Cells were then lysed and 10 µg of each was analyzed by SDS-PAGE and Western blotting with antibodies against hsp90, Ku70 and Ku86. β-actin and GAPDH were blotted as loading controls.

Reference

1. Suchanek, M., *et al.* (2005). Photo-leucine and photo-methionine allow identification of protein-protein interactions in living cells. *Nature Methods*, **2(4)**, 261-268.

Recommended UV light source for photo-reactive amino acids.

UV Lamp	Bulb Wavelength, Power	UVA Output at 1 cm	Photo AA Halflife	Recommended Exposure
UVP-3UV	365 nm, 8W	2800 µW/cm²	4 minutes	16 minutes
Stratalinker® 2400	365 nm, 15W	3000 µW/cm²	3 minutes	12 minutes
Spectroline® XX-15A	365 nm, 15W	3500 µW/cm²	2 minutes	8 minutes
UVP UVGL-58	365, 6W	2300 µW/cm²	5 minutes	20 minutes

Flexible 3UV Photo-Crosslinking Lamp

Ideal for use with protein and nucleic acid-based methods.

One lamp that can accommodate longwave, midrange and shortwave UV applications with just the turn of a knob. The new 3UV Lamp from UVP includes an innovative design that allows you to dial-in the appropriate wavelength for the task at hand. This lamp is compatible with our extensive line of photo-reactive reagents including the new Thermo Scientific Pierce Photo-reactive Amino Acids for discovering in-cell protein interactions.

Highlights:

- Three (254 nm, 302 nm, 365 nm) UV wavelengths with one UV lamp
- Unique reflector surface behind each UV tube maximizes light projection for all applications, including fluorescence
- Lightweight and ergonomically designed for hand-held or stationary use
- Small size occupies minimal space on a lab bench and stores conveniently in laboratory drawers
- Sturdy aluminum construction and scratch-resistant finish
- Ideal for applications that require a wide area of illumination (e.g., culture plates) because each lamp has 3 x 8-watt tubes
- Two models available (Product # 95034: 115 V, 60 Hz) and (Product # 95035: 230 V, 50 Hz) to meet varied voltage requirements
- Cycles per second (Hz) may vary

Formaldehyde Ampules

Formaldehyde crosslinking may serve as a complement to in-cell crosslinking with Thermo Scientific Pierce Photo-reactive Amino Acids. Formaldehyde is a membrane-soluble crosslinker that penetrates cells, producing reversible crosslinks between proteins and/or nucleic acids.

A fresh formaldehyde solution must be prepared and used quickly since formaldehyde readily oxidizes when exposed to air and polymerizes over time. For this reason, stock solutions of formaldehyde must be prepared and sealed immediately under inert atmosphere to ensure stability. Thermo Scientific Pierce Formaldehyde (16% HCHO) is available in conveniently sealed ampules that yield consistently "fresh" material when opened.

Ordering Information

Product #	Description	Pkg. Size
22610	L-Photo-Leucine† (L -2-Amino-4,4-azi-pentanoic acid)	100 mg
22615	L-Photo-Methionine† (L -2-Amino-5,5-azi-hexanoic acid)	100 mg
30030	Dulbecco's Modified Eagle's Limiting Medium (DMEM-LM), sterile filtered (-)L-leucine, (-)L-methionine wit 4.5 g/L glucose, 4.0 mM L-glutamine, sodium pyruvate and Phenol Red	500 ml
28906	16% Formaldehyde Solution	10 x 1 ml
28908	16% Formaldehyde Solution	10 x 10 ml
95034	3UV Lamp; 115 Volts, 60 Hz	1 each
95035	3UV Lamp; 230 Volts, 60 Hz	1 each

† See patent information.

Recommended dilution and working concentrations of a 16% formaldehyde solution.

Application	Common Working Concentration	Dilution of 16% Formaldehyde Solution	Description
Cell and Tissue Fixing for Imaging (Immunofluorescence and Electron Microscopy)	4%	1:4	• Imaging of a specific factor in cells or tissues using a specific antibody conjugated with a fluorescent dye
In-cell or In-organelle Crosslinking (Protein Interaction Analysis)	1%	1:16	• Cultured cells are crosslinked to stabilize protein interactions and the interactions are analyzed by Western blotting or mass spectrometry
Chromatin Immunoprecipitation (ChIP) Assays	1%	1:16	• Reversible crosslinking-based method used to identify histone and non-histone proteins bound to specific genome sequences
Flow Cytometry (FACS Analysis)	2%	1:8	• Cells labeled with fluorescent antibodies specific to cell types are counted and sorted as they pass a tuned laser • Fixing stabilizes cells before analysis and substantially reduces any potential pathogenicity of the sample

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.

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:

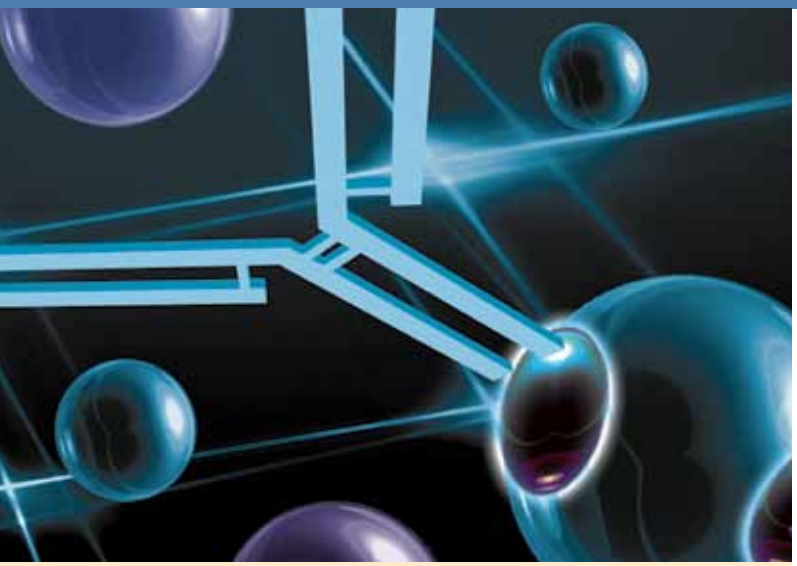
- **Purity of the tagged or labeled bait protein:** For example, using pure bait protein in a pull-down assay eliminates the possibility of selecting an interacting protein as a result of binding to a contaminant in the bait preparation.
- **Modification requirement of the bait or prey protein:** Interaction 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 technical handbook 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.

Table 1. *In vitro* methods for protein interaction analysis.

***In vitro* Methods**	**Description**
Immunoprecipitation (IP)/ 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.
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.
Crosslinking Reagents	Strategies involve homo- or heterobifunctional reagents whose chemical crosslinks may or may not be reversed. Nearest neighbors (suspected to interact) *in vivo* or *in vitro* can be trapped in their complexes for further study. Deuterated crosslinkers combined with mass spec analysis can be used to identify interacting proteins and help to localize the binding interface.
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 crosslinking agent with several important features. These include hetero-bifunctionality for stepwise crosslinking, a detectable label and reversibility of the crosslink between binding partners. Upon reduction of the crosslinked 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 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.
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.



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.

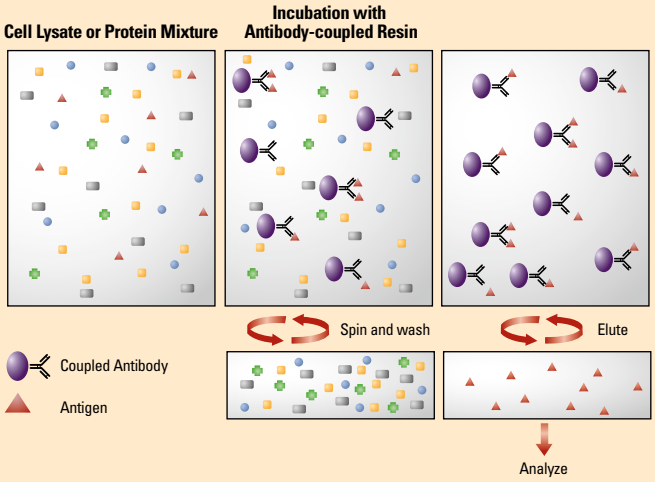


Figure 1. Summary of a traditional immunoprecipitation procedure.

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

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 resin. The assumption 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.

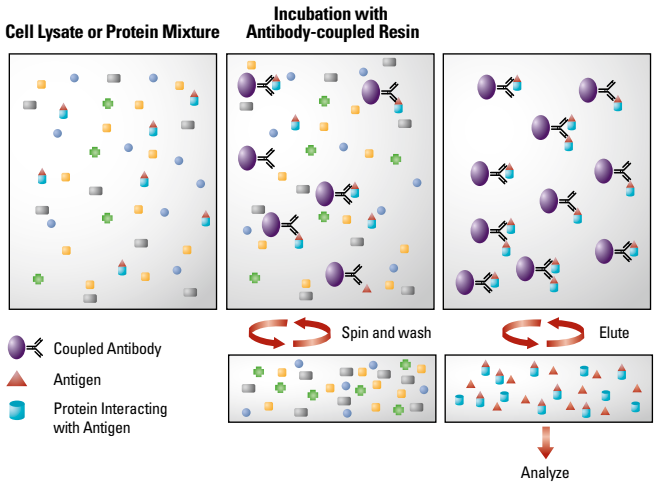


Figure 2. Summary of a traditional co-immunoprecipitation procedure.

Traditional Methods vs. Thermo Scientific 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 our solutions are summarized in Table 2.

Table 2. Comparison of traditional Co-IP and Thermo Scientific Pierce Co-IP Products.

Traditional Co-IP Problems	Thermo Scientific 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, which lowers protein yields	**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 significantly higher protein recoveries and 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 **Eliminate detection of antibody used for immunoprecipitation:** contaminating antibody fragments become denatured during SDS-PAGE. In contrast, the primary antibody used during Western blotting is still native. Use CleanBlot HRP Detection Reagent (Product # 21230) to only detect native antibody and not contaminants.
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 recycled 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 (Thermo Scientific AminoLink Plus Coupling Resin). 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. When using the Direct Strategy, the antibody source should be free of carrier protein, which can also be immobilized. Products such as Thermo Scientific Melon Gel IgG Purification Kits (Product # 45206) can easily clean-up an antibody to remove carrier proteins. The direct immobilization of antibodies is not species dependent, which allows for the use on non-traditional antibody sources that don't bind globulin binding proteins (Protein A or Protein G). Thermo Scientific Pierce Co-Immunoprecipitation Kits (Product # 26149) and Thermo Scientific Pierce Direct IP Kit (Product # 26148) use this direct immobilization approach. 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 Protein A or Protein G agarose combined with chemical crosslinking 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. The Indirect Strategy is compatible with antibody samples that contain carrier proteins, provided the carrier proteins are washed away prior to crosslinking. 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 crosslinked to the Protein A or Protein G with the bifunctional reagent disuccinimidyl suberate (DSS). Thermo Scientific Pierce Crosslink IP Kit (Product # 26147) incorporates this strategy.

Use of the Streptavidin:Biotin Interaction

This direct coupling approach incorporates the binding association between streptavidin and biotin. Streptavidin immobilized to beaded agarose resin 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. Thermo Scientific Pierce Streptavidin Agarose Resin (e.g., Product # 20347) and kit products that use this support, as well as the Thermo Scientific Pierce Streptavidin Coated Plate IP Kit (Product # 45360), provide high-capacity biotin-binding matrices suitable for IP and co-IP applications.

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.

Our research indicates that pre-binding or -coupling the antibody to the support matrix 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 Thermo Scientific Pierce Coated Plate IP Kits that use 96-well microplates coated with streptavidin or Protein A/G (Products # 45360, 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 References listed 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.

References

Adams, P.D., *et al.* (2002). Identification of associated proteins by co-immunoprecipitation, *In Protein-Protein Interactions – A Molecular Cloning Manual.* Golemis, E., Ed., Cold Spring Harbor Laboratory Press, pp 59-74.

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.

Direct, Crosslink and Classic IP and Co-IP Kits

The Thermo Scientific Pierce Classic and Crosslink IP Kits are ideal when using antibodies that bind to Protein A or Protein G. The Direct and Crosslink IP Kits are highly effective for eliminating co-elution of IgG heavy and light chain with the antigen, which interferes with downstream applications such as mass spectrometry analysis or protein sequencing.

Traditional co-IP methods result in detection of the antibody with the target proteins. Because the antibody heavy and light chains may co-migrate with one of the relevant bands, important results can be masked. The Pierce Co-IP Kit circumvents issues with co-migration of antibody chains with target proteins by retaining the antibody on the resin. The new kit is optimized for using smaller amounts of sample and offers a single lysis/wash buffer eliminating the need for a separate lysis reagent.

All four IP Kits Highlights:

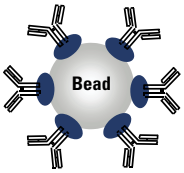
- Require minimal antibody (2-10 µg)
- Are highly effective and efficient in capturing antigens
- Use optimized protocols and buffers for efficient IPs and antigen elution
- Use common lysis/binding/wash buffer
- Include spin columns and collection tubes that shorten the protocol by minimizing handling and mixing
- Use a new elution buffer that provides milder and less denaturing recovery of antibody:antigen complexes
- Are compatible with specialized downstream applications; e.g., mass spectrometry, enzyme assays and antibody production
- Are able to scale up as needed using our flexible protocol

Comparison of immunoprecipitation methods. The following table compares the key features of traditional "do-it-yourself" immunoprecipitation techniques to the Thermo Scientific Pierce IP Kits. Consideration of these features can help to determine which method is most appropriate for the available reagents and downstream application.

Feature	Traditional IP method	Pierce Classic IP Kit	Pierce Crosslink IP Kit	Pierce Direct IP Kit	Pierce Co-IP Kit
Uses high binding capacity resin	Variable[1]	Yes (Protein A/G Plus)	Yes (Protein A/G Plus)	Yes (AminoLink Plus)	Yes (AminoLink Plus)
Crosslinker mediated immobilization	No	No	Yes (DSS)	No	No
Requires purified antibody in amine-free and protein-free storage solution	No	No	No	Yes	Yes
Antibody is covalently attached to agarose resin	No	No	Yes	Yes	Yes
Antibody is oriented	Yes	Yes	Yes	No	No
Antibody elutes with antigen	Yes	Yes	No	No	No
Antigen recovery method	Boiling w/SDS (Low pH)	Low pH elution (Boiling w/SDS)	Low pH elution	Low pH elution	Low pH elution
Relative antigen recovery[2]	Variable	Highest	Medium	High	High
Immobilized antibody can be reused	No	No	Possible[3]	Possible[3]	Possible[3]
Co-purification of interacting proteins		Possible	Possible	Possible	Optimal

[1] Commercially available resins vary in binding capacity and performance for IP assays.
[2] Antigen yield depends on the activity of the antibody, specific binding conditions and the immobilization method used.
[3] It is possible to reuse the prepared antibody affinity resin if the antibody remains functional following low pH elution.

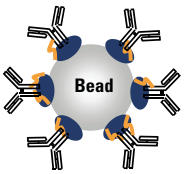
The Classic IP Kit:



Product # 26146

- High binding-capacity recombinant Protein A/G Plus Agarose Resin results in higher antigen yields
- Recombinant Protein A/G Plus Resin offers compatibility with a wider range of mammalian IgG species for IP reactions (e.g., mouse, rabbit, human and goat IgG subclasses)

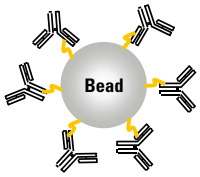
The Crosslink IP Kit:



Product # 26147

- Able to purify target protein without contamination by the antibody in the eluate
- Improved crosslinking protocol optimized for maximum antibody functionality
- Bound immunoglobulins oriented for optimal antigen-binding sites are more accessible
- High-capacity Protein A/G Plus Resin allows for better purification and immobilization of antibodies
- Offers compatibility with a wider range of mammalian IgG species

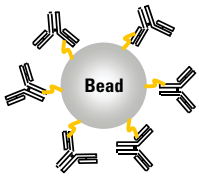
The Direct IP Kit:



Product # 26148

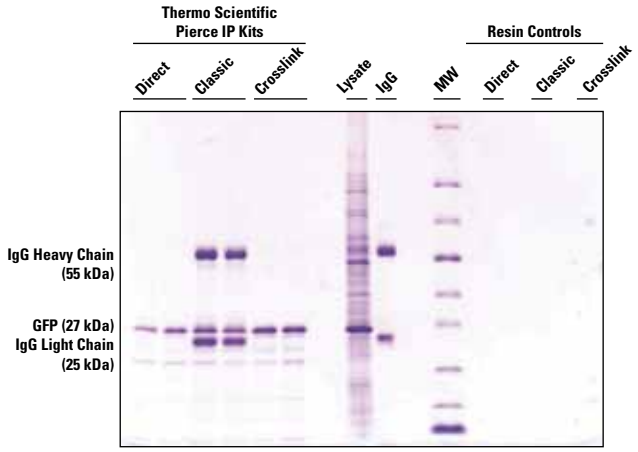
- Immobilize any antibodies independent of isotype or species
- Improved antibody coupling protocol
- Purify target protein without antibody contamination
- Activated resin for directly coupling antibodies to the support resin
- Eliminate antibody contamination in the eluate

The Co-IP Kit:

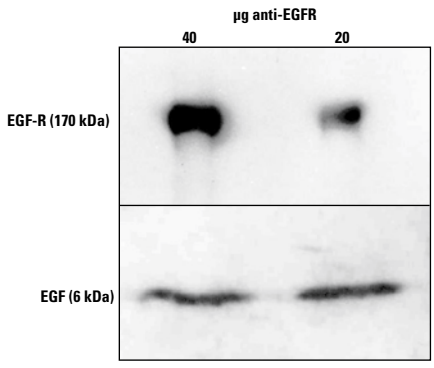


Product # 26149

- Minimal antibody requirements for co-IP reactions
- Shorter antibody coupling protocol (< 2 hours)
- Compatible with any antibody species and subclass
- Requires a purified antibody in a solution free of amines and stabilizing proteins
- Optimized protocols and buffers for efficient co-IP and antigen elution
- Allows for selective purification of target protein
- Includes spin columns and collection tubes that shorten the protocol by minimizing handling and mixing
- Compatible with specialized downstream applications, e.g., mass spectrometry
- Allows for scale up



Comparison of three different Thermo Scientific Pierce IP Kits. Immunoprecipitations were performed according to the product instructions using 10 µg of affinity-purified goat anti-GFP antibody and the Pierce Direct, Classic and Crosslink IP Kits. The cell lysate was prepared using IP Lysis/Wash Buffer and pre-cleared using the Pierce Control Agarose Resin supplied in the kits. The immune complex was formed by incubating the antibody, resin and lysate overnight. The resin was washed with IP Lysis/Wash Buffer, 1X Conditioning Buffer and eluted with Elution Buffer. For analysis, 4-20% Tris-glycine gels were loaded with 20% of the eluted sample, 5% of the cell lysate load (Lysate) and 10% of the antibody load (IgG) and stained with Thermo Scientific Imperial Protein Stain (Product # 24615). For the resin controls, the immunoprecipitation was performed without adding the antibody. MW: Thermo Scientific DyLight 549/649 Fluorescent Protein Molecular Weight Markers (Product # 26665).



Co-immunoprecipitation of interacting proteins using the Thermo Scientific Pierce Co-IP Kit. Epidermal Growth Factor (EGF) was co-immunoprecipitated from 2,000 µg of HeLa lysate with 40 and 20 µg anti-Epidermal Growth Factor Receptor (EGFR) mouse monoclonal IgG_1 + IgG_{2a} (Thermo Scientific) using the Pierce Co-IP Kit. Co-immunoprecipitations were performed according to product instructions. Two identical Western blots were probed with anti-EGFR sheep polyclonal IgG (Millipore) or anti-EGF rabbit polyclonal IgG (Santa Cruz). The Pierce Co-IP Kit co-immunoprecipitated EGF with the EGF-R.

Classic IP Kit

A convenient kit for a new spin on traditional immunoprecipitation.

The Thermo Scientific Pierce Classic IP Kit provides all the necessary reagents, spin cups and collection tubes to perform successful immunoprecipitation (IP) experiments with ease. The kit uses high-capacity Protein A/G agarose affinity resin for efficient binding of most species and subclasses of IP antibodies. The included IgG elution buffer provides milder and less denaturing recovery of antibody:antigen complexes than the traditional method of boiling in reducing sample buffer for SDS-PAGE, facilitating a greater variety of methods for subsequent analysis. The microcentrifuge spin column format helps to ensure effective washing and separation of samples from the beaded agarose affinity resin.

Like traditional IP methods, the Pierce Classic IP Kit procedure involves formation of antibody:antigen complexes in a sample solution and then capture of that complex to an IgG-binding protein that is covalently bound to beaded agarose resin (Protein A/G Agarose). After washing to remove nonbound (presumably undesired) components of the sample, the antigen and antibody are recovered from the beaded resin with elution buffer supplied in the kit. The entire procedure is performed in a microcentrifuge spin column, allowing solutions to be fully separated from the agarose resin upon brief centrifugation.

Ordering Information

Product #	Description	Pkg. Size
26146	**Pierce Classic IP Kit** *Sufficient reagents to perform 50 reactions.*	Kit
	Includes: Pierce Protein A/G Plus Agarose	0.55 ml
	IP Lysis/Wash Buffer	2 x 50 ml
	100X Conditioning Buffer	5 ml
	20X Tris-Buffered Saline	25 ml
	Elution Buffer	50 ml
	5X Lane Marker Sample Buffer, Non-reducing	5 ml
	Pierce Spin Columns – Screw Cap	50 each
	Microcentrifuge Collection Tubes	2 ml, 100 each
	Microcentrifuge Sample Tubes	1.5 ml, 50 each
	Pierce Control Agarose Resin	2 ml

Crosslink IP Kit

Purify target protein complexes without antibody interference!

The Thermo Scientific Crosslink IP Kit extends the functionality of traditional immunoprecipitation (IP) methods by adding crosslinking technology and microcentrifuge spin column sample handling to the procedure. The primary benefits resulting from these features are the ability to purify target protein without contamination by the antibody and the ability to more effectively wash and separate samples from the beaded agarose resin.

The Pierce Crosslink IP Kit method involves capturing the IP antibody to Protein A/G Agarose resin and covalently immobilizing it to the support by crosslinking with disuccinmidyl suberate (DSS). The antibody resin is then incubated with the sample that contains the protein antigen of interest, allowing the antibody:antigen complex to form. After washing to remove nonbound (presumably undesired) components of the sample, the antigen is recovered by dissociation from the antibody with elution buffer supplied in the kit. The entire procedure is performed in a microcentrifuge spin cup, allowing solutions to be fully separated from the agarose resin upon brief centrifugation. Only antigen is eluted by the procedure, enabling it to be identified and further analyzed without interference from antibody fragments. Furthermore, the antibody resin often can be reused for additional rounds of immunoprecipitation.

Ordering Information

Product #	Description	Pkg. Size
26147	**Pierce Crosslink IP Kit** *Sufficient reagents to perform 50 reactions.*	Kit
	Includes: Pierce Protein A/G Plus Agarose	0.55 ml
	20X Coupling Buffer	25 ml
	DSS Crosslinker, No-Weigh™ Format	8 x 2 mg
	IP Lysis/Wash Buffer	2 x 50 ml
	100X Conditioning Buffer	5 ml
	20X Tris-Buffered Saline	25 ml
	Elution Buffer	50 ml
	Lane Marker Sample Buffer, Non-reducing, (5X)	5 ml
	Pierce Spin Columns - Screw Cap	50 each
	Microcentrifuge Collection Tubes	2 ml, 100 each
	Microcentrifuge Sample Tubes	1.5 ml, 50 each
	Pierce Control Agarose Resin	2 ml

Direct IP Kit

Immunoprecipitate using any antibody species or subclass! Eliminate antibody band contamination of IP products.

The Thermo Scientific Pierce Direct IP Kit represents a significant advancement in immunoprecipitation (IP) technology by replacing the use of immobilized Protein A or Protein G with a method for direct covalent attachment of antibodies to the beaded agarose resin.

The primary benefits resulting from this method are the opportunity to use any species or subclass of purified antibody (not just types that bind to Protein A or G) and the ability to purify target protein without contamination by the antibody. The method also makes it possible to immunoprecipitate antigens from serum samples without co-purifying non-target immunoglobulins. Finally, the kit uses microcentrifuge spin cups to effectively wash and separate samples from the beaded agarose resin.

Ordering Information

Product #	Description	Pkg. Size
26148	**Pierce Direct IP Kit** *Sufficient reagents to perform 50 reactions.*	Kit
	Includes: AminoLink® Plus Coupling Resin	2 ml
	20X Coupling Buffer	25 ml
	Quenching Buffer	50 ml
	Wash Solution	50 ml
	5M Sodium Cyanoborohydride Solution	0.5 ml
	IP Lysis/Wash Buffer	2 x 50 ml
	100X Conditioning Buffer	5 ml
	20X Tris-Buffered Saline	25 ml
	Elution Buffer	50 ml
	5X Lane Marker Sample Buffer	5 ml
	Pierce Spin Columns – Screw Cap	50 each
	Microcentrifuge Collection Tubes	2 ml, 100 each
	Microcentrifuge Sample Tubes	1.5 ml, 50 each
	Pierce Control Agarose Resin	2 ml

Co-Immunoprecipitation Kit

Perform co-immunoprecipitation experiments without antibody interference.

The Thermo Scientific Pierce Co-Immunoprecipitation (Co-IP) Kit enables isolation of native protein complexes from a lysate or other complex mixture by directly immobilizing purified antibodies onto an agarose support.

Co-IP is a common approach to study protein:protein interactions that uses an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins). Traditional co-IP methods that use Protein A or G result in co-elution of the antibody heavy and light chains that may co-migrate with relevant bands, masking important results. The Pierce Co-IP Kit resolves this issue by covalently coupling antibodies onto an amine-reactive resin. The kit includes optimized buffers for protein binding and recovery, reagents to perform control experiments and efficient spin columns and collection tubes, which shorten the protocol and minimize handling and mixing.

Ordering Information

Product #	Description	Pkg. Size
26149	**Pierce Co-Immunoprecipitation Kit***	Kit
	Sufficient reagents to perform 50 reactions.	
	Includes: AminoLink Plus Coupling Resin	2 ml
	20X Coupling Buffer	25 ml
	5M Sodium Cyanoborohydride Solution	0.5 ml
	Quenching Buffer	50 ml
	Wash Solution	60 ml
	IP Lysis/Wash Buffer	2 x 50 ml
	100X Conditioning Buffer	5 ml
	Elution Buffer	50 ml
	5X Lane Marker Sample Buffer, Non-reducing	5 ml
	Pierce Control Agarose Resin	2 ml
	20X Modified Dulbecco's PBS Buffer	25 ml
	Pierce Spin Columns – Screw Cap	50 each
	Microcentrifuge Collection Tubes	100 each
	Microcentrifuge Sample Tubes	50 each

** The Thermo Scientific Pierce Co-IP Kit (Product # 23600) and the Pierce Mammalian Co-IP Kit (Product # 23605) were discontinued on December 31, 2009. The next-generation Pierce Co-IP Kit (Product # 26149) will replace these old kits. The new co-IP kit is optimized for using smaller amounts of sample and offers a common lysis/wash buffer eliminating the need for a separate lysis reagent (Thermo Scientific M-PER Mammalian Protein Extraction Reagent, Product # 78503) as was offered with the Mammalian Co-IP Kit.*

Coated Plate Immunoprecipitation Kits

Pre-coated 96-well plates are easier to use and faster than traditional microcentrifuge tube methods.

Thermo Scientific Pierce Coated Plate IP Kits enable rapid immunoprecipitation of multiple samples without the usual tedium of pipetting, centrifuging and separating beaded affinity resin in individual microcentrifuge tubes. Immunoprecipitation is accomplished using coated 96-well microplates rather than beaded agarose resin. The plate format allows fast processing of multiple samples. Select from Protein A/G-, Protein G- or streptavidin-coated plates.

Highlights:

- Ready-to-use, high-quality coated plates provide high capacity and consistency
- Plate format best suited for simultaneously processing multiple samples and their control conditions
- Faster, easier and more thorough washing than with traditional tube/resin IP methods
- Uses familiar and convenient ELISA tools (multichannel pipettors and plate washing); no tedious separation of supernatant from pelleted resin beads, and no tubes to open, close and centrifuge
- Coated plates are 96-well strip plates, convenient for experiments requiring only a partial plate
- Easy-to-follow instructions, including detailed explanation of appropriate controls
- Three kits available, suitable for most common antibody types (mouse, rabbit, human and goat IgG subclasses) or any biotinylated antibody or "bait" protein

Thermo Scientific Pierce Protein A/G Coated Plate IP Kit (Product # 45350) immunoprecipitation of CD71 (transferring receptor) from human serum using and a goat anti-CD71 polyclonal antibody. Eluted products for the experimental and control samples were mixed with nonreducing sample loading buffer, separated by SDS-PAGE, transferred to nitrocellulose membrane and detected by Western blotting with the IP antibody, Goat-anti-mouse-HRP conjugated secondary antibody (Product # 31432) and Thermo Scientific SuperSignal West Dura Chemiluminescent Substrate (Product # 34076).
Lane 1: Experiment (immunoprecipitation product)
Lane 2: Antibody-only control (no sample)
Lane 3: Human serum sample control (no antibody)
Lane 4: Plate control (no antibody or human sample)
Lane 5: Pure target protein (CD71) for size reference

Choosing between Protein A/G and Streptavidin Coated Plate Kits

Streptavidin is a protein that binds specifically and strongly to biotin; therefore, the Streptavidin Coated Plate IP Kit (Product # 45360) is appropriate for immunoprecipitation when using a biotin-labeled (biotinylated) antibody. This kit can be used to affinity-purify a binding partner to any antibody species or subclass or any other protein or molecule that is biotinylated. Because the streptavidin-biotin affinity interaction is so strong, the elution step generally will dissociate only the antigen (binding partner), not the biotinylated antibody or "bait" protein.

Protein A and Protein G are different proteins that bind to immunoglobulins (primarily IgG). Typically, Protein A is preferred for use with rabbit polyclonal antibodies, while Protein G is preferred for use with mouse antibodies (especially monoclonals of the IgG_1 subclass). Protein A/G is a recombinant of Protein A and Protein G that has the additive binding properties of both proteins.

Reference

Desai, S. and Hermanson, G. (1997). *Previews* **1(3)**, 2-7.

Ordering Information

Product #	Description	Pkg. Size
45350	**Pierce Protein A/G Coated Plate IP Kit** *Antibody binding capacity/well: 2.5 µg. Sufficient capacity for downstream analysis of IP or co-IP proteins in-gel or by Western blot.* Includes: Protein A/G Coated 12 x 8-well strip plates Phosphate buffered saline Surfact-Amps® X-100 (10% Triton® X-100) Elution buffer Neutralization buffer Uncoated 96-well strip plates (white), (for sample collection and neutralization) Plate sealers	Kit 2 plates 2 packs 6 x 10 ml 50 ml 7 ml 2 ea. 18 sheets
45360	**Pierce Streptavidin Coated Plate IP Kit** *Antibody binding capacity/well: 5 µg. Sufficient capacity for downstream analysis of IP or co-IP proteins in-gel or by Western blot.* Includes: High Binding Capacity Streptavidin Coated Plates Biotin blocking buffer Phosphate buffered saline Surfact-Amps X-100 (10% Triton X-100) Elution buffer Neutralization buffer Uncoated 96-well strip plates (white), (for sample collection and neutralization) Plate sealers	Kit 2 plates 30 ml 2 packs 6 x 10 ml 50 ml 7 ml 2 ea. 18 sheets

HA- or c-Myc Tag IP/Co-Immunoprecipitation Kits

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 Thermo Scientific Pierce IP/Co-IP Kits 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 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. 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. Our 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.

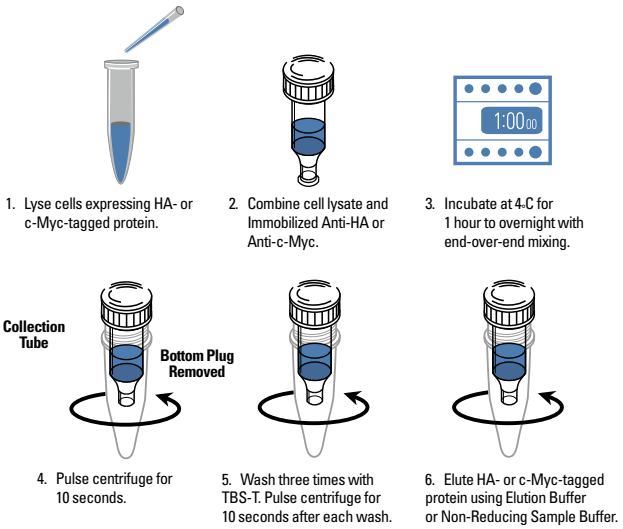
- Complete kit format offers optimum convenience in both the IP and co-IP modes.
- Eliminate Protein A or Protein G, reducing nonspecific binding and shortening the IP procedure.
- System demonstrates excellent flexibility with respect to the amount of antibody or amount of lysate used, enabling isolation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

Spin Columns.

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

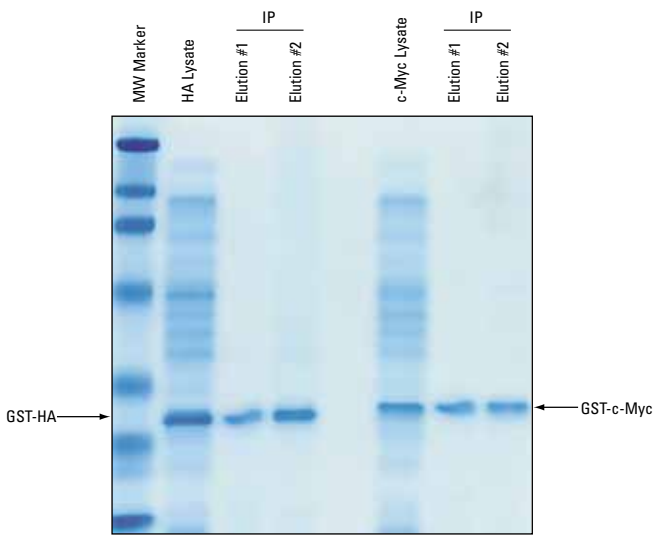
HA- or c-Myc Tag IP/Co-IP Kit Descriptions

Each Thermo Scientific Pierce 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 Thermo Scientific 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.



1. Lyse cells expressing HA- or c-Myc-tagged protein.
2. Combine cell lysate and Immobilized Anti-HA or Anti-c-Myc.
3. Incubate at 4-C for 1 hour to overnight with end-over-end mixing.
4. Pulse centrifuge for 10 seconds.
5. Wash three times with TBS-T. Pulse centrifuge for 10 seconds after each wash.
6. Elute HA- or c-Myc-tagged protein using Elution Buffer or Non-Reducing Sample Buffer.

Thermo Scientific Pierce HA- or c-Myc IP/Co-IP Kit Protocol summary.

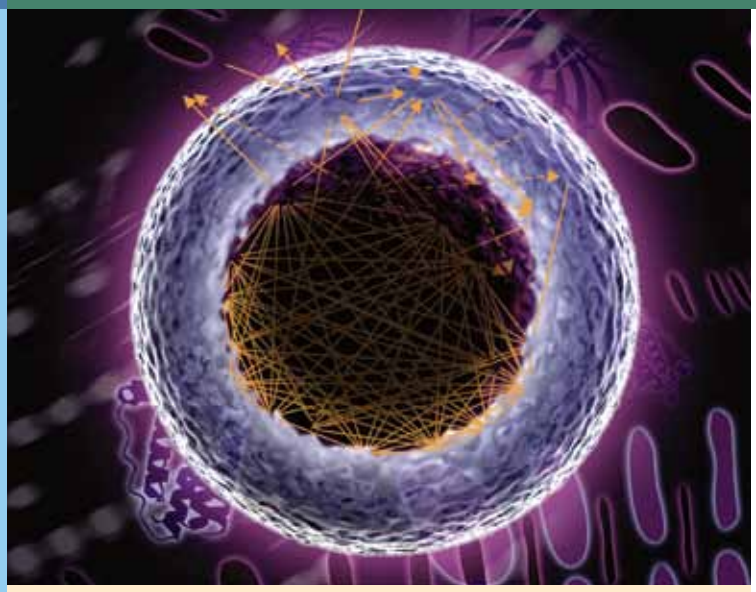


Effectiveness of elution options in the IP of GST-HA and GST-c-Myc from bacterial lysates. IP results achieved with the Thermo Scientific Pierce HA and c-Myc IP/Co-IP Kits using the appropriate positive control lysate provided and suggested elution options for GST-HA and GST-cMyc, respectively. The elution 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	**HA-Tag IP/Co-IP Kit**	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.	
	Includes: Immobilized Anti-HA (agarose resin)	150 µl
	BupH™ Tris Buffered Saline Pack	1 pack
	Elution Buffer, pH 2.8	50 ml
	Lane Marker Non-Reducing Sample Buffer (5X)	5 ml
	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
23615	**Mammalian HA-Tag IP/Co-IP Kit**	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.	
	Includes: Immobilized Anti-HA (agarose resin)	150 µl
	BupH Tris Buffered Saline Pack	1 pack
	Elution Buffer, pH 2.8	50 ml
	Lane Marker Non-Reducing Sample Buffer (5X)	5 ml
	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
23620	**c-Myc-Tag IP/Co-IP Kit**	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.	
	Includes: Immobilized Anti-c-Myc (agarose resin)	250 µl
	BupH Tris Buffered Saline Pack	1 pack
	Elution Buffer, pH 2.8	50 ml
	Lane Marker Non-Reducing Sample Buffer (5X)	5 ml
	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
23625	**Mammalian c-Myc Tag IP/Co-IP Kit**	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.	
	Includes: Immobilized Anti-c-Myc (agarose resin)	250 µl
	BupH Tris Buffered Saline Pack	1 pack
	Elution Buffer, pH 2.8	50 ml
	Lane Marker Non-Reducing Sample Buffer (5X)	5 ml
	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

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.

Pull-down assays can provide higher resolution and selectivity than some other antibody-based assays. For example, anti-phospho-tyrosine antibodies traditionally have shown high background in immunoprecipitation assay. As an alternative, the Thermo Scientific Pierce SH2 Pull-Down Assays selectively bind specific phospho-tyrosine residues found on receptor tyrosine kinases (see page 20).

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., Thermo Scientific 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 Thermo Scientific Pierce Protein Stains (e.g., Product # 24594, 24612), Western blotting detection with SuperSignal® Chemiluminescent Substrates (e.g., Product # 34080) and ^{35}S 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.

Phosphoprotein Pull-Down Assays: Introduction to SH2 Domains

SH2 domains and receptor tyrosine kinase signaling

Because of their affinity to specific targets involved in receptor tyrosine kinase signaling, SH2 domains can be used to selectively monitor receptor activity and effector protein binding. Tyrosine phosphorylation levels of specific cellular proteins can be analyzed by Western blotting and mass spectrometry (MS). Furthermore, MS can be used to identify SH2 domain interaction networks and receptor:cytosolic protein interactions.

The most widely studied post-translational modification is phosphorylation of serine, threonine and tyrosine residues in response to a biological signal. Approximately 30% of the phospho-proteome is transiently phosphorylated at any given time. Although phosphorylation of tyrosine accounts for only 0.1-1% of all phosphorylation events, it is the most intensely studied because deregulation of phosphotyrosine signaling is linked to many human diseases.

Src Homology 2 (SH2) domain-containing proteins are critical for mediating cellular signaling from receptor tyrosine kinases. The modular SH2 protein interaction domains function by recognizing and binding to specific phosphotyrosine (pY or p-Tyr) residues present in many signaling proteins. Consequently, SH2-pY interactions are crucial for signal transmission from receptor tyrosine kinases to downstream targets, resulting in regulation of multiple biological processes. SH2 domains have been identified in numerous human proteins that have specific biological functions, such as adaptor proteins, kinases, phophatases, cytoskeletal regulation, transcription, ubiquitination and phospholipid second messenger signaling. Deregulation of SH2 domain-containining proteins has been implicated in human disease including multiple cancers, diabetes and human immunodeficiency.

SH2 domains are comprised of approximately 100 amino acids that recognize binding partners in a highly specific manner. Binding of an SH2 domain-containing protein is dependent upon phosphorylation of a specific tyrosine residue in a particular amino acid consensus sequence (e.g., Src recognition sequence pYEEI). SH2 domains are essential for signal transmission and downstream biological processes and are present in many different protein classes including kinases, phosphatases, adaptor proteins, phospholipid second messengers and cytoskeletal regulators.

Accurately measuring phosphorylation states and signaling events is challenging because the events are transient and there are few specific detection reagents (e.g., antibodies) available. An affinity system based on recombinant SH2 domains overcomes these challenges by using the critical mediators or regulators of cell signaling events to capture specific tyrosine phosphorylation interactions from mammalian cell lysates. The improved specificity of the pull-down method based on specific SH2 domains means less non-specific binding and no antibody contamination compared with traditional immunoprecipitation experiments using anti-pY antibodies.

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. Thermo Scientific Pierce Pull-Down 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 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.

GST- and PolyHis-Tagged Pull-Down Assay Kits

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

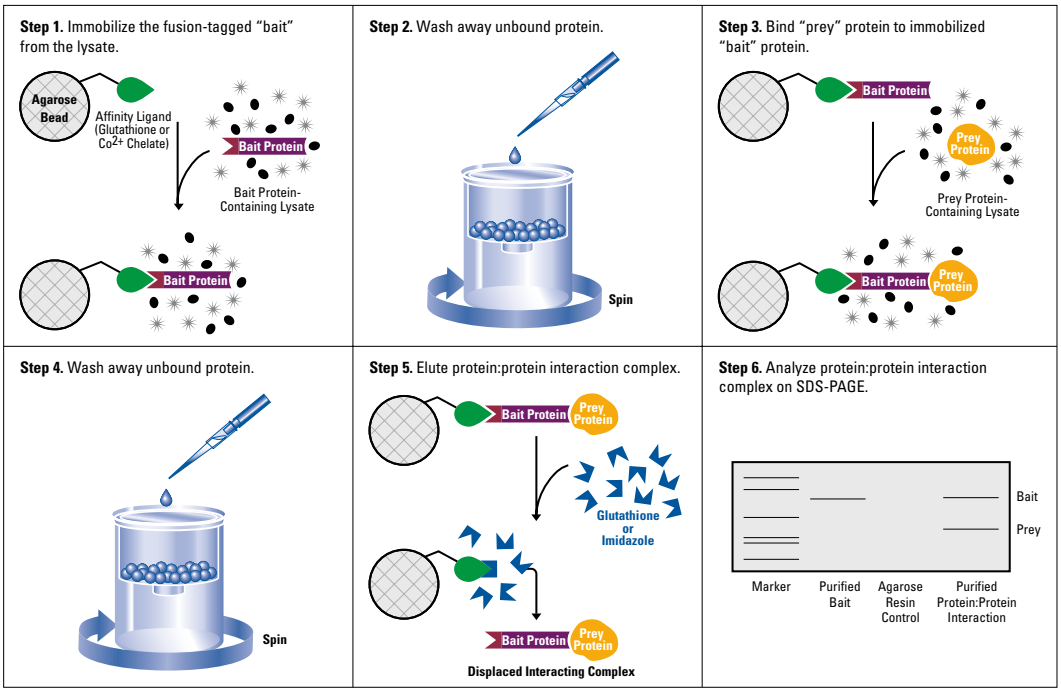
Identifying and characterizing the interactions of a given protein has emerged as the most valuable information that can be developed in the post-genomic era. Thermo Scientific Pierce Pull-Down Kits contain the necessary components to capture interacting proteins using the popular pull-down method. The only item you provide is an appropriately tagged fusion protein as the "bait." Our 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

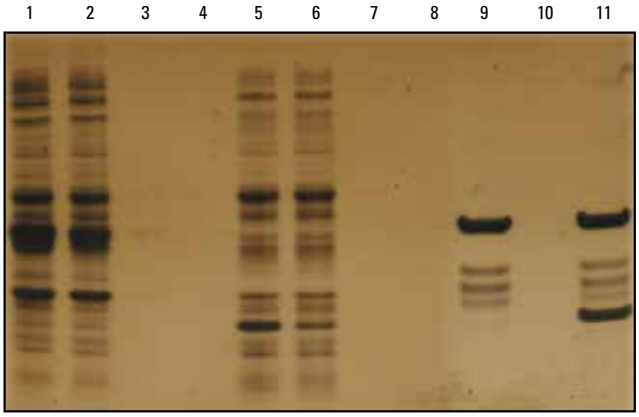


= Fusion Tag (GST or polyHis)

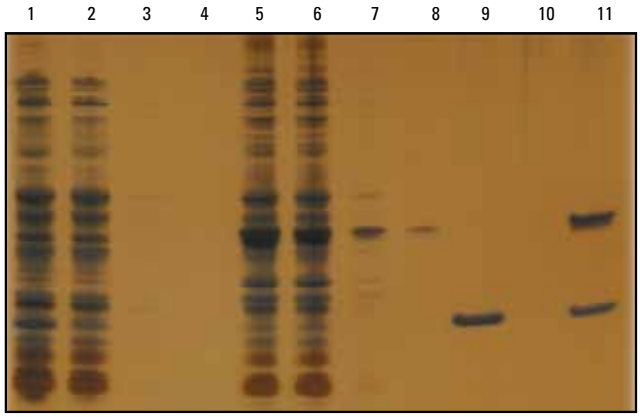
Generalized scheme for use of a Thermo Scientific Pierce Pull-Down Protein:Protein Interaction Kit using a GST-tagged or PolyHis-tagged protein as the "bait."

Pull-Down Assays

A.



B.



Lane #	A. GST-Tag Pull-Down	B. PolyHis-Tag Pull-Down
1	Lysate from *E. coli* expressing GST-tagged BIR2 **(bait protein)**.	Lysate from *E. coli* expressing 9xHis-tagged wild-type Smac **(bait protein)**.
2	Flow-through from the lysate in Lane 1 bound to an immobilized reduced glutathione support for 1 hour at 4°C.	Flow-through from the lysate in Lane 1 bound to an immobilized cobalt chelate support for 1 hour at 4°C.
3	Wash #1 of the support.	Wash #1 of the support.
4	Wash #2 of the support. (Washes 3-5 not shown.)	Wash #2 of the support. (Washes 3-5 not shown.)
5	Lysate from *E. coli* expressing 9xHis-tagged wild-type Smac **(prey protein)**.	Lysate from *E. coli* expressing GST-tagged BIR2 **(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.	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.	Wash #1 of the support.
8	Wash #2 of the support. (Washes 3-5 not shown.)	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.	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.	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.	Elution of bait:prey complex (prepared in Lanes 1-8) from the support with 250 mM imidazole.

Validation of the Thermo Scientific Pierce Pull-Down Protein Interaction Kits using a known interacting pair.

Ordering Information

Product #	Description	Pkg. Size
21516	**Pierce Pull-Down GST Protein Interaction Kit** *Sufficient materials for performing 25 pull-down assays using a GST-tagged protein as the bait.* Includes: Immobilized Glutathione Lysis Buffer Glutathione BupH Tris Buffered Saline Spin 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
We gratefully acknowledge 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	**Pierce Pull-Down PolyHis Protein Interaction Kit** *Sufficient materials for performing 25 pull-down assays using a polyhistidine-tagged protein as the bait.* Includes: Immobilized Glutathione Lysis Buffer 4 M Imidazole Stock Solution BupH Tris Buffered Saline Spin 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
1. Chai, J., *et al.* (2000). *Nature* **406**, 855-862.
2. Kaelin, W.G., *et al.* (1991). *Cell* **64**, 521-532.
3. 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.
4. Soutoglou, E., *et al.* (2000). *Mol. Cell* **5**, 745-751.

Pull-Down Biotinylated-Protein Interaction Kit

Pull-down a binding partner with the Thermo Scientific Pierce Biotinylated-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. Our 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." Our Pull-Down Biotinylated-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

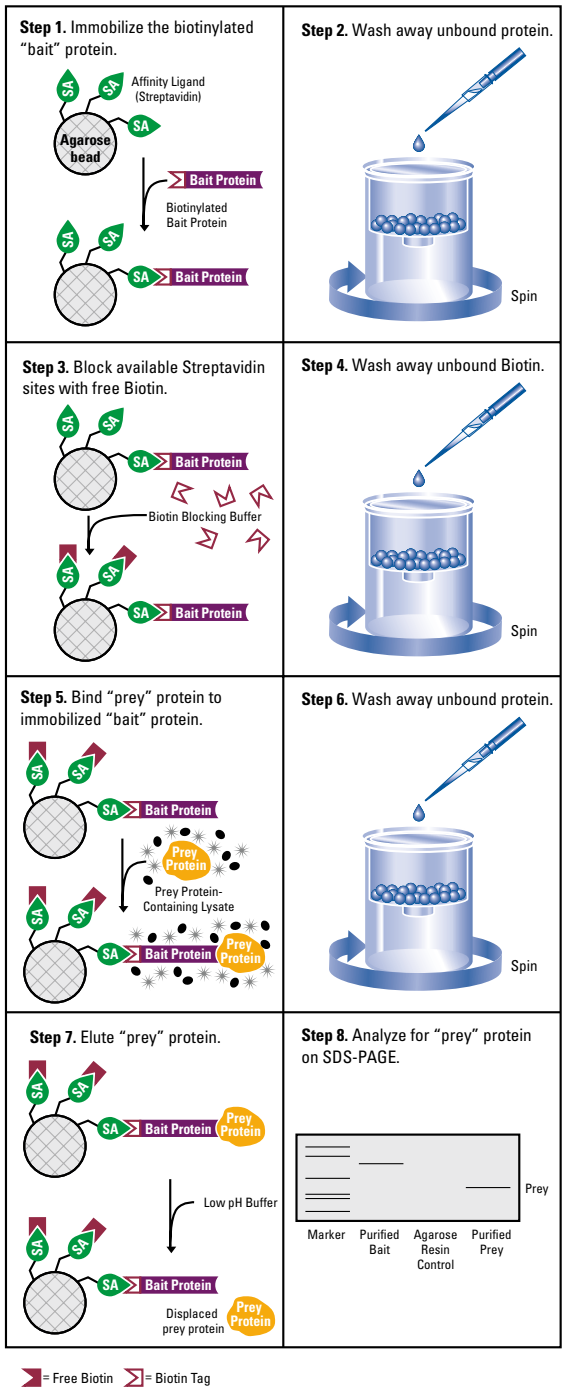
Ordering Information

Product #	Description	Pkg. Size
21115	**Pierce Pull-Down Biotinylated-Protein Interaction Kit** *Sufficient materials for performing 25 pull-down assays using a purified and biotinylated protein as the bait.* Includes: Streptavidin Agarose Resin Lysis Buffer BupH Tris Buffered Saline Biotin Blocking Buffer Wash Buffer (Acetate, pH 5.0) Elution Buffer (pH 2.8) Spin Columns Accessory Pack Collection Tubes and Caps Accessory Pack	Kit 1.5 ml settled gel 250 ml 1 pack (500 ml) 15 ml 100 ml 50 ml 27 columns 200 x 2 ml tubes, graduated

For a complete list of our biotinylation reagents, order a free Avidin-Biotin Handbook (Product # 1601676).

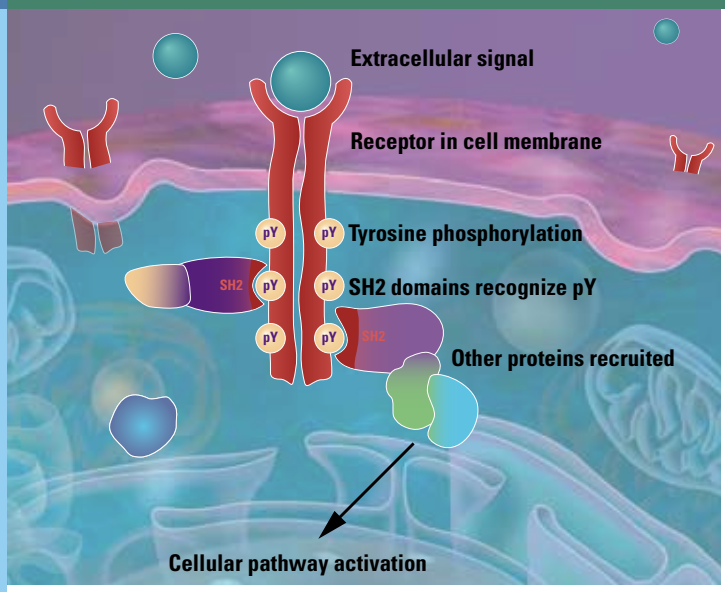
See also: GST- and PolyHis-Tagged Pull-Down Assay Kits, pages 17-18.
See also: Far-Western Biotinylated Protein Interaction Kit, pages 37-41.

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



Thermo Scientific Pierce Pull-Down Biotinylated-Protein Interaction Kit Protocol.

Phosphoprotein Pull-Down with SH2 Domains



SH2 domains – Monitoring the phospho-tyrosine state of specific proteins

SH2 domains provide an improved approach to selectively monitor receptor tyrosine kinase signaling, as well as the binding of downstream effector proteins. The interactions of SH2 domain-containing proteins represent a critical interface between extracellular stimulation of a membrane receptor and transmission of that signal to intracellular proteins.

SH2 Domain Phosphotyrosine Capture Kits

Thermo Scientific Pierce SH2 Domain Phosphotyrosine Capture Kits include optimal levels of purified GST-fused SH2 domains of various signaling proteins integral to cell biology. Each validated kit includes optimized buffers and columns to perform protein pull-downs. Using SH2 domains eliminates the background associated with low-specificity antibodies and enables analysis of receptor targets to which antibodies are not available. Using quality-tested purified GST-SH2 domains also ensures uniform results without variability.

SH2 domains specifically bind phospho-tyrosine residues.

Each SH2 domain is specific for its natural target (Figure 3). *In vivo*, the phosphatase Shp2 is recruited to tyrosine 1009 of the platelet-derived growth factor receptor (PDGFR) only when it is phosphorylated. Also, PLCγ is recruited to tyrosine 1021 on PDGFR in response to growth factor signaling. In quiescent cells both tyrosine 1009 and 1021 are not phosphorylated and, therefore, both domains are unable to bind.

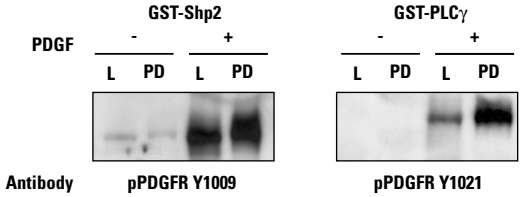


Figure 3. Site-specific interaction of GST-Shp2 and GST-PLCγ SH2 on the PDGF-receptor. NIH3T3 cells were rendered quiescent by serum withdrawal for 48 hours followed by stimulation with PDGF (50 ng/ml, Cell Signaling Technology [CST]) for 15 minutes or no stimulation. Each cell lysate (500 µg) was incubated overnight at 4°C with either 100 µg GST-PLCγ1 or GST-Shp2. Protein complexes were captured on immobilized glutathione beads and resolved by SDS-PAGE. Western blot analysis was performed using phospho-specific antibodies that detect known protein-protein interaction sequences (phospho-PDGFR Y1009 and phospho-PDGFR Y1021, CST). Lanes: L = 25 µg of total cell lysate and PD = SH2 domain pull-down.

SH2 domains provided a more efficient method of capturing site-specific phospho-tyrosine events as compared to antibody based co-immunoprecipitation.

Co-immunoprecipitation experiments are dependent on the quality and specificity of the antibody used. Antibodies against phosphoproteins are notoriously difficult to work with, yielding high background and low target protein recovery. SH2 domains provide better capture efficiency as compared to many pan- and site-specific phospho-antibodies because an SH2 domain is more selective by nature. Because of this improved specificity, SH2 domains can be used to pull-down phosphotyrosine containing proteins with improved results over traditional antibody-based methods. In Figure 4, PDGF-stimulated NIH3T3 cells were lysed and incubated with either a specific SH2 domain or a pan-antibody corresponding to the SH2 domain containing protein. Binding of each specific SH2 domain to the PDGF receptor was confirmed by Western blot using a pan-PDGFR antibody. In all cases we observed a higher capture efficiency using the SH2 domain approach as compared to the traditional antibody co-immunoprecipitation. In many cases, we were also able to detect differential binding of the SH2 domain in the presence of PDGF stimulation as compared to the unstimulated state. These differences were not apparent using the co-immunoprecipitation approach. We further tested the interaction specificity of four SH2 domains using phospho-antibodies which detect site-specific tyrosine phosphorylation events which mediate each SH2 domain interaction. We were limited to only four phospho-antibodies against specific tyrosine sites on the PDGF receptor because of commercial availability and/or antibody integrity. Using these site-specific antibodies we confirmed the selectivity and specificity of each SH2 domain tested and these interactions were much stronger as compared to the traditional antibody immunoprecipitation. Taken together these results clearly demonstrate that the SH2 domain pull-down approach represents a robust method to capture and monitor site specific tyrosine phosphorylation events which are critical for cell health.

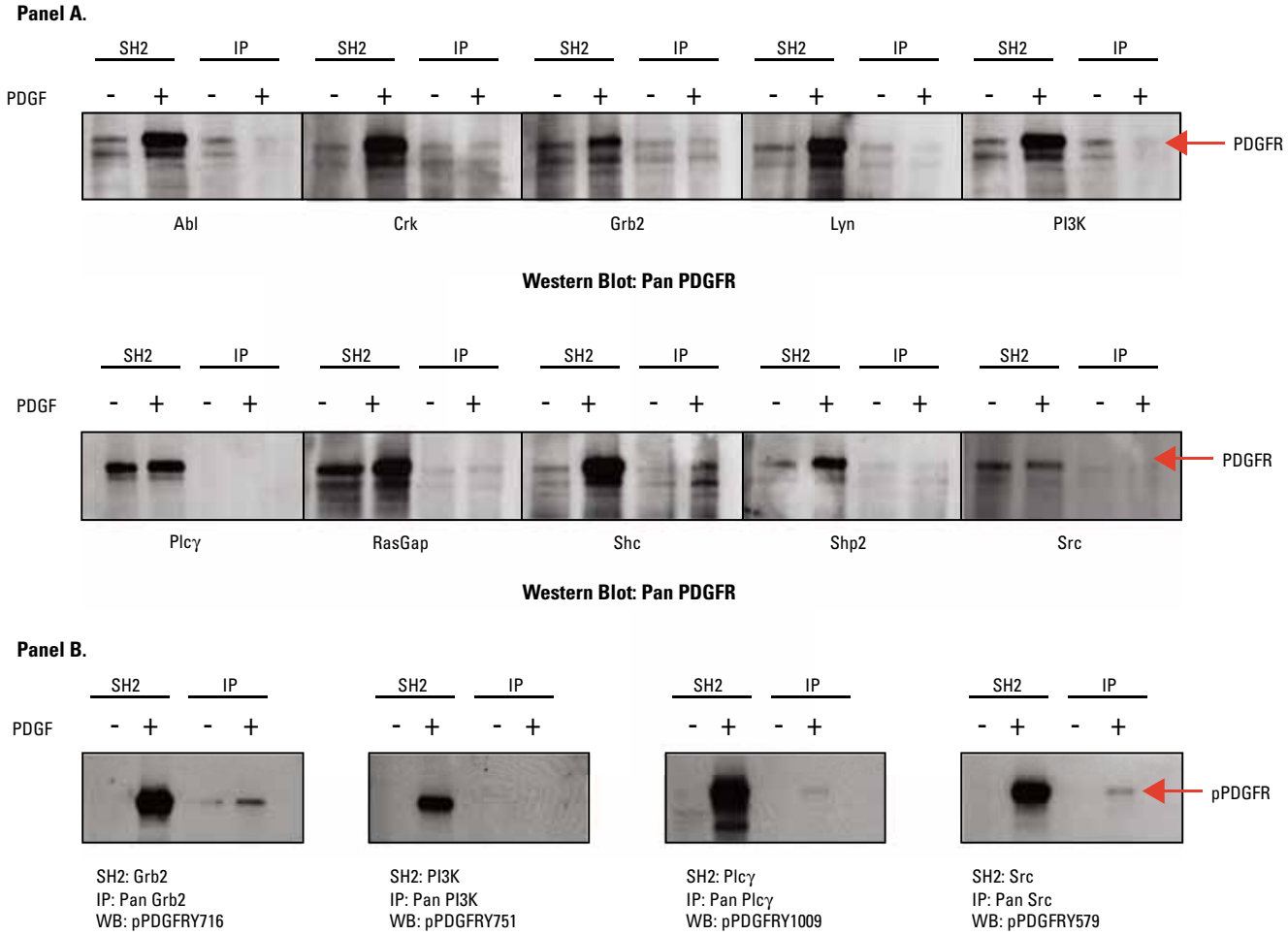


Figure 4. SH2 domains provide better enrichment of phosphorylated receptor tyrosine kinase (PDGFR) compared to antibody-based pull-down assays. Panel A. NIH3T3 cells were rendered quiescent by serum starvation for 24 hours with DMEM containing 0.1% FCS. Following starvation cells were either stimulated (100 ng/ml PDGF for 20 minutes) or left unstimulated. Cells were then lysed in NP40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 5% glycerol) containing Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail (Product # 78440) and protein concentrations determined by the Thermo Scientific Pierce 660 nm Assay. SH2 and co-immunoprecipitations were performed as follows: SH2 domain pull-downs consisted of 100 µg of SH2 domain (Abl, Crk, Grb2, Lyn, PI3K, Plc-gamma, RasGap, Shc, Shp2, Src) added to 250 µg of stimulated or unstimulated lysate. Co-immunoprecipitations were performed by incubating 10 µl of pan antibody (Abl, Crk, Grb2, Lyn, PI3K, Plc-gamma, RasGap, Shc, Shp2, Src) with 250 µg of stimulated or non-stimulated lysate. Binding was performed for 16 hours at 4°C on a rotating platform. Following binding 100 µl of a 50% slurry of immobilized glutathione was added to the SH2 domain samples and 100 µl of a 50% slurry of Protein A/G was added to the co-immunoprecipitation samples. Samples were incubated for 1 hour at 4°C on a rocking platform followed by centrifugation at 1000 x *g* for 1 minute to collect complexes bound to the resin. Resin beds were then washed 3 times with 500 µl of Thermo Scientific M-PER Wash Buffer. To elute bound proteins 25 µl of 5X DTT loading dye was added to each sample and boiled for 5 minutes. Protein complexes were then resolved by SDS-PAGE on a 4-20% Tris-glycine gel. Proteins were transferred onto PVDF membrane for 16 hours at 4°C, 30V. Membranes were blocked in 7.5% BSA in TBST and Western blots performed using a 1:2000 dilution of mouse monoclonal anti-PDGFR-beta antibody (CST 3175). Detection was performed using Thermo Scientific SuperSignal Pico Chemilumescent Detection System. **Panel B.** Membranes containing the Grb2, Plcγ, PI3K, Src, and RasGap pull-downs were stripped with Thermo Scientific Restore Plus Buffer and reprobed with phospho-antibodies which detect site-specific tyrosine phosphorylation events on the PDGF receptor which mediate the SH2 domain interaction. These antibodies include pPDGFRY716, pPDGFRY751, pPDGFRY1009, pPDGFRY579 which correspond to SH2 domain docking sites for Grb2, PI3K, Plcγ, and Src respectively. Detection was performed using SuperSignal West Pico Chemilumescent Detection System.

Phosphoprotein Pull-Down with SH2 Domains

Perform receptor phosphorylation time-course experiments.

With SH2 domain pull-downs, it is possible to monitor time-dependent binding of SH2 domains to their target. For example, PLCγ, Src and Shc bind rapidly to the PDGF receptor in response to PDGF stimulation (Figure 5). In nature, phosphorylation is rapid and transient, as evidenced by diminished binding at 10- and 20-minute post-stimulation.

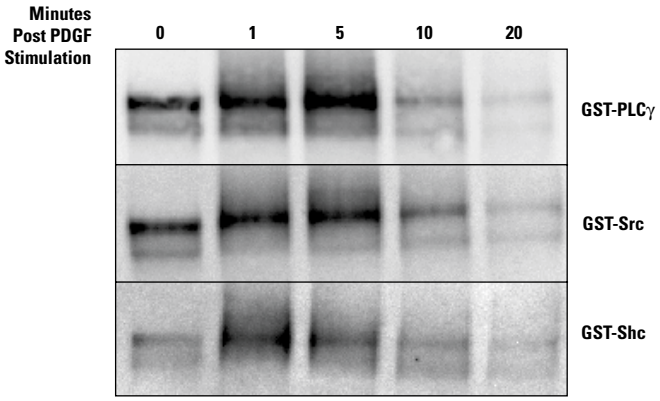


Figure 5. Time-specific binding of various SH2 domains to the PDGF receptor. NIH3T3 cells were rendered quiescent by serum withdrawal for 48 hours. Post-starvation cells were stimulated with PDGF (50 ng/ml, CST) for the indicated times or untreated. SH2 domain pull-downs were performed with 100 µg of each SH2 domain and 250 µg of each cell lysate. Protein complexes were resolved by SDS-PAGE and analyzed by Western blot using a pan antibody recognizing PDGFR (CST).

Resolve specific tyrosine phosphorylation events.

SH2 domain affinity provides a level of selectivity not previously attainable with generic anti-phosphotyrosine antibodies. Use different SH2 domains to uncover the mechanism of how signals are transduced or to screen multiple SH2 domains against a particular target at one time. For example, different naturally occurring SH2 domains efficiently bind to the epidermal growth factor receptor (EGFR) in response to EGF stimulation (Figure 6). There were strong interactions of many of the domains in the presence of epidermal growth factor (EGF) and low-level binding in quiescent, serum-starved A431 cells. RasGap binding is independent of EGF stimulation, whereas Grb2 binding is highly upregulated (Figure 6).

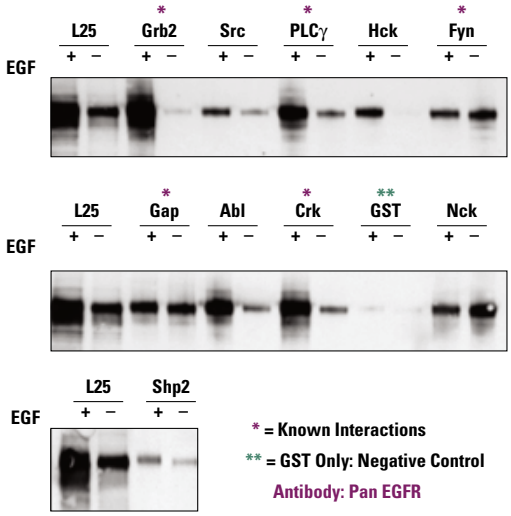


Figure 6. High efficiency binding of different naturally occurring SH2 domains to EGFR in response to EGF stimulation. A431 cells were rendered quiescent by serum withdrawal for 48 hours followed by stimulation with EGF (100 ng/ml) for 15 minutes or left untreated. Each cell lysate (500 µg) was incubated with 100 µg of each GST-SH2 domain overnight at 4°C. Protein complexes were captured on immobilized glutathione beads and resolved by SDS-PAGE. Western blot analysis was performed using a pan antibody that recognizes the EGF receptor. L25 = 25 µg total lysate load.

Identify binding interactions with MS.

Pull-down samples isolated with SH2 domains are compatible with MS analysis for identifying protein interacting partners.

Improved specificity with SH2 domains and the elimination of antibody contamination typically seen in traditional immunoprecipitation experiments deliver pure protein for MS analysis. Two different SH2 domains were used to pull down interacting proteins from NIH3T3 cell lysates, which were then analyzed by MS. Many of the interacting proteins identified are specific to either Fgr or PLCγ.

MS identification of PLCγ and Fgr SH2 domain interactions. NIH3T3 cells were rendered quiescent by serum withdrawal for 48 hours followed by stimulation with PDGF (50 ng/ml, CST) for 15 minutes. Cell lysate (500 µg) was incubated overnight at 4°C with either 100 µg GST-Fgr SH2 domain or GST-PLCγ. Protein complexes were captured on immobilized glutathione beads and resolved by SDS-PAGE. Proteins were stained with Thermo Scientific GelCode Blue Stain Reagent. Proteins of specific molecular weights were excised from the gel, digested with trypsin and analyzed with a Thermo Scientific LTQ XL Orbitrap Mass Spectrometer.

Protein(s) Identified by MS	GST-Fgr Pull-Down	GST-PLCγ Pull-Down
Proto-oncogene tyrosince-protein kinase-Yes	X	
Platelet-derived growth factor receptor-PDGFRα		X
Proto-oncogene tyrosince-protein kinase-ROS	X	
Forkhead box protein E1-FOXE1	X	
Heat shock protein-HSP90α	X	
E3 ubiquiin protein ligase-LNX		X
Breast cancer type 2 susceptibility protein homolog-BRCA2	X	X
Myc-proto-oncogene protein		X
Myb-binding protein 1A		X
Nuclear factor NF-κB p105 subunit		X
Nucleolin	X	X
Phosphatidylinoisitol-3,4,5 triphosphate 5-phosphatase 2-SHIP2		X
Protein tyrosine kinase 2beta-FAK2		X
Ras and Rab interactor 1-RIN1	X	
Ras GTPase-activating like protein IQGAP1		X
Vimentin	X	
Proto-oncogene tyrosine-protein kinase-Fyn	X	

References

Hanke, S. and Mann, M. (2008). The phosphotyrosine interactome of the insulin receptor family and its substrates IRS-1 and IRS-2. *Mol Cell Proteomics* **Nov 11**.

Liu, B.A., *et al.* (2006). The human and mouse complement of SH2 domain proteins-establishing the boundaries of phosphotyrosine signaling. *Mol Cell* **22(6)**:851-68.

Machida, K., *et al.* (2007). High-throughput phosphotyrosine profiling using SH2 domains. *Mol Cell* **26(6)**:899-915.

Pawson, T. (2004). Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* **116(2)**:191-203. Review.

Online resource: SH2ome http://sh2.uchicago.edu/, information current as of 1-9-09

Ordering Information

Product #	Description	Pkg. Size
87700	**Grb2 SH2 Domain Phosphotyrosine Capture Kit** *Sufficient reagents for six pull-down reactions (100 µg/pull-down).* Includes: GST-Grb2 SH2 Domain (37 kDa) GST (negative control, 27 kDa) Immobilized Glutathione Resin (50% resin slurry) Spin Column (0.8 ml) M-PER Mammalian Protein Extraction Reagent Lane Marker Reducing Sample Buffer (5X)	6 pull-downs 600 µg at 1 mg/ml 200 µg at 1 mg/ml 400 µl 8 each 25 ml 200 µl
87701	**Src SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87702	**Abl SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87703	**Crk SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87704	**Fyn SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87705	**Lck SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs

Choose the correct SH2 domain to study your pathway.

Monitor the mechanism of how a particular receptor tyrosine kinase transmits cell signals to initiate a biological response. We offer 17 SH2 domains that associate with a myriad of phosphorylated receptors and downstream effectors. Begin to truly map the SH2 interactome.

SH2 Domain	Example Binding Targets	Pathways / Pathology
Abl	PDGFR, integrins, p130Cas	Leukemia
Cbl	PI3K, Crk, protein tyrosine kinases, 14-3-3 proteins, c-met	B-cell lymphoma; acute myeloid leukemia; E3 ubiquitin ligase activity
Crk	Cbl, paxillin, p130Cas	Cytokine and growth factor signaling
Fgr	Integrin receptors, Bcr receptors, Fc receptors	Cell membrane signaling; regulation of B-cell activation
Fyn	Fyn pY527	T cell receptor activation; cell adhesion signaling
Grb2	EGFR, IRS-1, Gab1, Shc, PDGFR, c-met	RYK* signaling; insulin pathway
Hck	Hck pY499	Acute lymphocytic leukemia; cell differentiation and proliferation
Lck	Lck pY505	Regulation of T-cell activation and differentiation; T-cell acute lymphocytic leukemia
Lyn	Lyn pY507, Bcr, CD40	Regulation of B-cell activation
Nck	Receptor tyrosine kinases	RYK signaling; cytoskeletal dynamics
PI3K (p85)	EGFR, PDGFR, IGF/IRS, c-met	RYK signaling; insulin pathway; human cancers and metabolic disorders
PLCγ	EGFR, PDGFR, c-met	RYK signaling
RasGap (Rasa)	EGFR, PDGFR, p21Ras	Small G-protein Activation (p21 Ras); basal cell carcinomas
Shc	EGFR	RYK signaling; Ras/Raf/MAPK pathway; breast cancer and high metastatic potential
Shp2 (PTNP11)	Gab1	EGFR-PI3K signaling; Noonan syndrome; LEOPARD syndrome
Src	Src pY527, PDGFR	Metastatic colon cancer
Syk	Immunoreceptor tyrosine activation motifs (ITAMs)	Hemopoietic cell signaling; T-cell lymphoma; breast cancer

** RYK= Receptor Tyrosine Kinase*

Ordering Information

Product #	Description	Pkg. Size
87706	**Nck SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87707	**Shc SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87708	**Ras-GAP SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87709	**Shp2 SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87710	**PLCγ SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87711	**Lyn SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87712	**Hck SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87713	**FGR SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87714	**Cbl SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87715	**Syk SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs
87716	**PI3K SH2 Domain Phosphotyrosine Capture Kit**	6 pull-downs

The Use of Crosslinking 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.

Crosslinking 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 crosslinkers 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 Crosslinking

The simple addition of homobifunctional or heterobifunctional crosslinkers 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 crosslinker designs were created that incorporate photo-reactive groups, which can be made to react at selected times and only in response to irradiation by UV light. Heterobifunctional crosslinkers with a thermoreactive group (spontaneously reactive) at one end and a photo-reactive 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 photo-reactive end of the modified protein to covalently link to nearby molecules, thus "freezing" in place any interacting protein as a complex.

Photo-Reactive Crosslinkers

The use of photo-reactive crosslinkers for studying protein interactions is often preferred over methods that use standard bifunctional thermoreactive crosslinkers because photo-reactive crosslinkers limit the formation of conjugate polymer artifacts. However, the downside of some photo-reactive 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 photo-reactive intermediate often exceed reactions with a desired target.

Some photo-reactive groups, such as halogen-substituted aryl azides and benzophenones, have much better conjugation yields and can efficiently capture interacting molecules. For instance, crosslinkers that incorporate a perfluoroazidobenzamido photo-reactive 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 photo-reactive reagent is Thermo Scientific SFAD (Product # 27719), which has an amine-reactive sulfo-NHS-ester at one end and the halogen-substituted phenylazide group at the other end.

Diazirine-Based Photo-Reactive Crosslinkers

We have synthesized six new photo-crosslinkers (SDA, LC-SDA, SDAD, Sulfo-SDA, Sulfo-LCSDA and Sulfo-SDAD) that combine proven amine-reactive chemistry with diazirine-based photochemistry (see page 26 for structures). Key properties of this family of crosslinkers include a variety of spacer arm lengths, different cell membrane permeabilities and cleavage of crosslinked proteins with reducing agents.

Our photo-reactive crosslinkers allow initial specific protein labeling of one protein using amine-reactive N-hydroxysuccinimide (NHS) ester followed by UV-activated crosslinking to another via diazirine moiety to any amino acid side chain or peptide backbone (Figure 7). Diazirine-based photocrosslinkers have better photostability than phenyl azide-based photo-crosslinkers and are easily activated with long-wave UV light (330-370 nm).[1-5]

The NHS-ester diazirine derivatives (SDA, LC-SDA and SDAD) lack a charged group and are membrane-permeable. This property makes them ideal for intracellular and intramembrane conjugations. By contrast, Sulfo-SDA, Sulfo-LC-SDA and Sulfo-SDAD contain negatively charged sulfate groups that improve their water solubility and reduce cell membrane permeability for extracelluar protein crosslinking. SDAD and Sulfo-SDAD also have a disulfide bond within the spacer that can be cleaved with reducing agents. The photoactivable diazirine moiety is also used in our popular photo-reactive amino acids, L-Photo-Methionine and L-Photo-Leucine, which are used for live cell crosslinking.[1,4,5]

We compared crosslinking of the heterodimeric sodium/potassium (Na/K) ATPase after treating cells with a reversible sulfonated NHS-diazirine (Sulfo-SDAD), a sulfonated phenyl azide crosslinker (Sulfo-SANPAH) or a homobifunctional sulfo-NHS ester (BS^3). After UV exposure, significantly more crosslinked product of the cell-surface Na/Kβ chain we observed by Western blotting in samples treated with Sulfo-SDAD than those treated with Sulfo-SANPAH or BS^3 (Figure 8).

References

1. Suchanek, M., *et al.* (2005). Photo-leucine and photo-methionine allow identification of protein-protein interactions. *Nat. Methods.* **2(4)**:261-267.
2. Tanaka, Y. and Kohler, J. (2008). Photoactivatable crosslinking sugars for capturing glycoprotein interactions. *JACS.* **10**.1021/ja7109772
3. Hermanson, G.T. (2008). *Bioconjugate Techniques. 2nd edition.* Elsevier/Academic Press.
4. Bomgarden, R., *et al. In vivo* protein crosslinking using photo-reactive amino acids. *Previews* **11(2)**:16-18.
5. Bomgarden, R. (2008). Studying protein interactions in living cells; method for protein crosslinking utilizes photo-reactive amino acids. *GEN,* **28(7)**, 24-25.

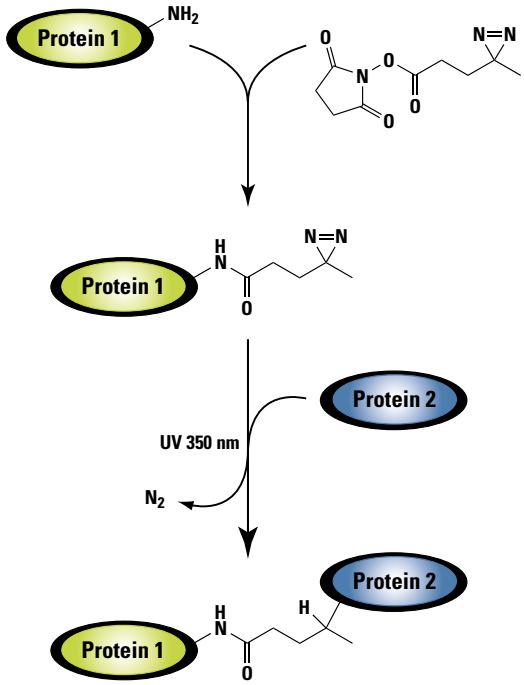


Figure 7. Mechanism of NHS-ester diazirine crosslinking. *N*-hydroxysuccinimide (NHS) esters react efficiently with primary amine groups ($–NH_2$) in pH 7-9 buffers to form stable amide bonds upon release of NHS. Photoactivation of diazirines with long-wave UV light (330-370 nm) creates reactive carbene intermediates. Such intermediates can form covalent bonds through addition reactions with any amino acid side chain or peptide backbone at distances corresponding to the spacer arm lengths.

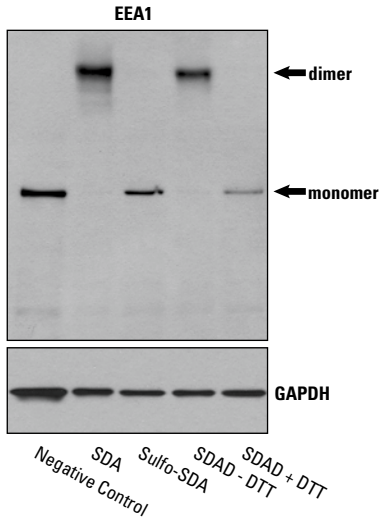
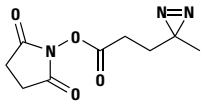
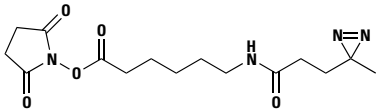
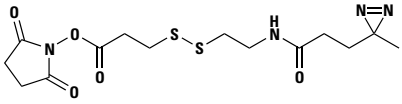
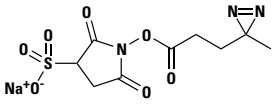
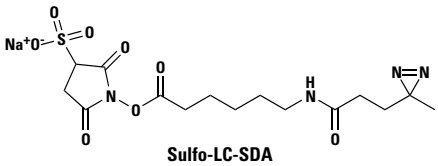
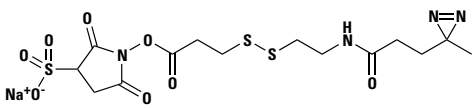


Figure 8. Comparison of extracellular crosslinking of the Na/K ATPase complex sulfonated NHS ester crosslinkers. HeLa cells (2×10^6) were labeled for 10 minutes with 1 mM Sulfo-SDAD, BS^3 or Sulfo-SANPAH in PBS. All Sulfo-samples were quenched, irradiated and lysed before SDS-PAGE analysis and Western blotting with antibodies against the Na/Kβ subunit and GAPDH.

Thermo Scientific Pierce Diazirine-Based Photo-Reactive Crosslinkers

Product #	Product Name	Structure	Key Features	Pkg. Size
26167	NHS-Diazirine (SDA) (succinimidyl 4,4-azipentanoate)	 **SDA** **NHS-Diazirine** **Spacer Arm 3.9 Å**	• Amine-reactive • Photo-reactive • Short spacer arm	50 mg
26168	NHS-LC-Diazirine (LC-SDA) (succinimidyl 6-[4,4' -azipentan-amido]hexanoate)	 **LC-SDA** **NHS-LC-Diazirine** **Spacer Arm 12.5 Å**	• Extended spacer arm • Amine-reactive • Photo-reactive	50 mg
26169	NHS-SS-Diazirine (SDAD) (succinimidyl 2-[(4,4' -azipentan-amido) ethyl]-1,3' -dithioproprionate)	 **SDAD** **NHS-SS-Diazirine** **Spacer Arm 13.5 Å**	• Reducing agent cleavable spacer arm • Amine-reactive • Photo-reactive	50 mg
26173	Sulfo-NHS-Diazirine (Sulfo-SDA) (sulfosuccinimidyl 4,4' -azipentanoate)	 **Sulfo-SDA** **Sulfo-NHS-Diazirine** **Spacer Arm 3.9 Å**	• Amine-reactive • Photo-reactive • Short spacer arm • Water-soluble	50 mg
26174	Sulfo-NHS-LC-Diazirine (Sulfo-LC-SDA) (sulfosuccinimidyl 6-[4,4' -azipentanamido]hexanoate)	 **Sulfo-LC-SDA** **Sulfo-NHS-LC-Diazirine** **Spacer Arm 12.5 Å**	• Extended spacer arm • Amine-reactive • Photo-reactive • Water-soluble	50 mg
26175	Sulfo-NHS-SS-Diazirine (Sulfo-SDAD) (sulfosuccinimidyl 2-[(4,4' -azipentanamido) ethyl] -1,3'-dithioproprionate)	 **Sulfo-SDAD** **Sulfo-NHS-SS-Diazirine** **Spacer Arm 13.5 Å**	• Reducing agent cleavable spacer arm • Amine-reactive • Photo-reactive • Water-soluble	50 mg

Bifunctional Crosslinking Reagents

The ability to selectively conjugate two or more proteins together using crosslinking 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. Thermo Scientific Pierce Bioconjugation Reagents are a critical factor in facilitating this knowledge. Additional information on label transfer and crosslinking as it pertains to the study of protein interactions follows this section.

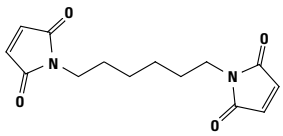
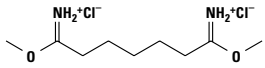
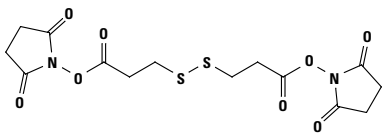
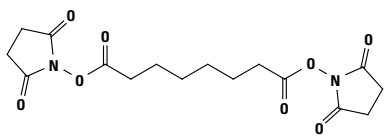
Crosslinking Reagents for Protein Interaction Capture

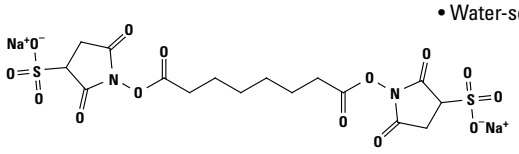
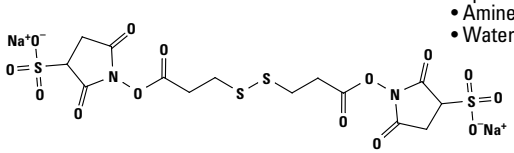
Homobifunctional crosslinking reagents and heterobifunctional photo-reactive crosslinking agents are routinely applied to protein:protein interaction studies. The following table of homobifunctional crosslinkers represents a small sampling of the Thermo Scientific Pierce Products offering in this area. 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 crosslinker. 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 photo-reactive. The photo-reactive group of the heterobifunctional reagent will ultimately crosslink a prey protein to the bait when exposed to light. Typically, this photo-reactive 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 crosslinker. Photo-reactive, heterobifunctional reagents with a cleavable disulfide linkage will allow reversal of the bait:prey crosslink and recovery of the components of the interacting pair for further analysis.

Homobifunctional Thermo Scientific Pierce Crosslinking Reagents

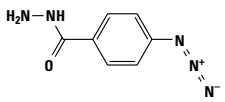
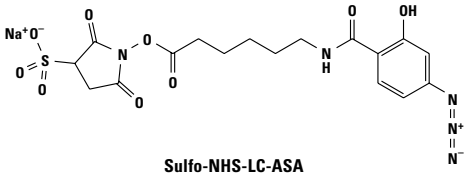
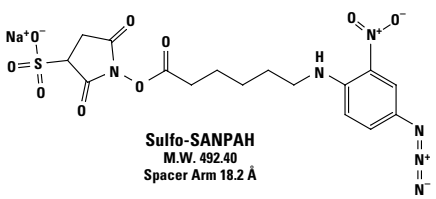
Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
22330	**BMH** Bismaleimidohexane	 **BMH** **M.W. 276.29** **Spacer Arm 16.1 Å**	• Mild, irreversible –SH to –SH crosslinking agent	1-3	50 mg
21666 21667	**DMP** Dimethyl pimelimidate•2 HCl	 **DMP** **M.W. 259.17** **Spacer Arm 9.2 Å**	• Reversible amidine bond retains net charge character of proteins • Amine-reactive	4-6	50 mg 1 g
22585 22586	**DSP** Dithiobis (succinimidyl proponate)	 **DSP** **M.W. 404.42** **Spacer Arm 12.0 Å**	• Thiol-cleavable • Amine-reactive	7-9	1 g 50 mg
21655 21555	**DSS** Disuccinimidyl suberate	 **DSS** **M.W. 368.35** **Spacer Arm 11.4 Å**	• Non-cleavable • Amine-reactive • Homobifunctional	10-12	50 mg 1 g

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
21580 21585 21586	BS[3] Bis(sulfosuccinimidlyl)suberate	 **BS[3]** **M.W. 572.43** **Spacer Arm 11.4 Å**	• Amine-reactive • Water-soluble	13-15	50 mg 8 x 2 mg 1 g
21578	DTSSP	 **DTSSP** **M.W. 608.51** **Spacer Arm 12.0 Å**	• Reducing agent cleavable spacer arm • Amine-reactive • Water-soluble	16-18	50 mg

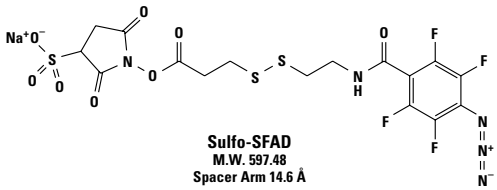
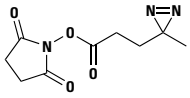
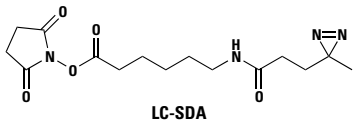
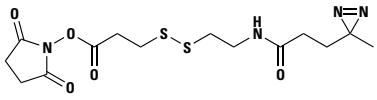
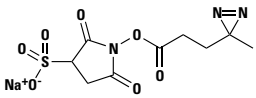
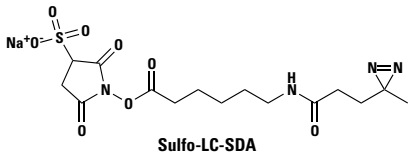
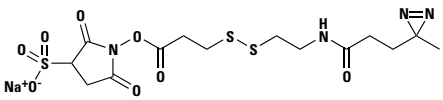
References

1. Hansen, M.S.T. and Barklis, E. (1995). *J. Virol.* **69(2)**, 1150-1159.
2. Ikushiro, S., *et al.* (1997). *Biochemistry* **36(23)**, 7154-7161.
3. Schmidt, C.J., *et al.* (1992). *J. Biol. Chem.* **267(20)**, 13807-13810.
4. Sinha, S.K. and Brew, K. (1981). *J. Biol Chem.* **256(9)**, 4193-4204.
5. Bauer, P.I., *et al.* (1990). *Biochem. J.* **270(1)**, 17-26.
6. O'Keefe, E.T., *et al.* (1980). *Biochemistry* **19(22)**, 4962-4966.
7. Joshi, S. and Burrows, R. (1990). *J. Biol Chem.* **265**, 14518-14525.
8. Adir, N. and Ohad, I. (1986). *Biochem. Biophys. Acta* **850(2)**, 264-274.
9. Leduc, M., *et al.* (1989). *Microbiol. Lett.* **51(1)**, 11-14.
10. Mathieu, M., *et al.* (1990). *Mol. Endocrinol.* **4(9)**, 1327-1335.
11. Donato, R., *et al.* (1989). *J. Neurochem.* **53(2)**, 566-571.
12. Chataway, T.K. and Barritt, G.J. (1995). *Mol. Cell. Biochem.* **145(2)**, 111-120.
13. Koller, D., *et al.* (2004) *J. Biol. Chem.* **279**, 20387-20391.
14. Partis, M.D., *et al.* (1983). *J. Protein. Chem.* **2**, 263-277.
15. Sinz, A. (2003). *J. Mass Spectrom.* **38**, 1225-1237.
16. Dihazi, G.H. and Sinz, A. (2003). *Rapid Commun. Mass Spectrom.* **17**, 2005-2014.
17. Mattson, G., *et al.* (1993). *Molecular Biology Reports* **17**, 167-183.
18. Muller, D.R., *et al.* (2001). *Anal. Chem.* **73**, 1927-1934.

Heterobifunctional Photo-Reactive Thermo Scientific Pierce Crosslinking Reagents

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
21510	ABH *p*-Azidobenzoyl hydrazide	 **ABH** **M.W. 177.16** **Spacer Arm 11.9 Å**	• Glycoprotein-reactive • Non-cleavable	1	50 mg
27735	Sulfo-NHS-LC-ASA Sulfosuccinimidyl (4-azido-salicylamido)hexanoate	 **Sulfo-NHS-LC-ASA** **M.W. 491.41** **Spacer Arm 18.0 Å**	• Can incorporate ^{125}I label before acylation step • Photolysis initiated by long-wave UV • Water-soluble • Non-cleavable • Amine-reactive	4,5	50 mg
22589	Sulfo-SANPAH Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate	 **Sulfo-SANPAH** **M.W. 492.40** **Spacer Arm 18.2 Å**	• Improved photolysis occurs at 320-350 nm limiting damage to biomolecules by irradiation • Water-soluble • Non-cleavable • Amine-reactive	6,7	50 mg

Heterobifunctional Photo-Reactive Thermo Scientific Pierce Crosslinking Reagents

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
27719	**Sulfo-SFAD** Sulfosuccinimidyl(perfluoro-azidobenzamido)ethyl-1,3′-dithiopropionate	 **Sulfo-SFAD** M.W. 597.48 Spacer Arm 14.6 Å	• Improved photoconjugation • Photolyzes at 320 nm • Coupling ~70% efficient • Water-soluble • Cleavable • Amine-reactive	8,9	50 mg
26167	**SDA** Succinimidyl 4,4-azipentanoate	 **SDA** NHS-Diazirine Spacer Arm 3.9 Å	• Amine-reactive • Photo-reactive • Short spacer arm		50 mg
26168	**LC-SDA** Succinimidyl 6-(4,4′-azipentanamido)hexanoate	 **LC-SDA** NHS-LC-Diazirine Spacer Arm 12.5 Å	• Extended arm spacer • Amine-reactive • Photo-reactive		50 mg
26169	**SDAD** Succinimidyl 2-([4,4′-azipentan-amido]ethyl)-1,3′-dithiopropri-onate	 **SDAD** NHS-SS-Diazirine Spacer Arm 13.5 Å	• Reducing agent cleavable spacer arm • Amine-reactive • Photo-reactive		50 mg
26173	**Sulfo-SDA** Sulfosuccinimidyl 4,4′-azipentanoate	 **Sulfo-SDA** Sulfo-NHS-Diazirine Spacer Arm 3.9 Å	• Amine-reactive • Photo-reactive • Short spacer arm • Water-soluble		50 mg
26174	**Sulfo-LC-SDA** Sulfosuccinimidyl 6-(4,4′-azipentanamido)hexanoate	 **Sulfo-LC-SDA** Sulfo-NHS-LC-Diazirine Spacer Arm 12.5 Å	• Extended spacer arm • Amine-reactive • Photo-reactive • Water-soluble		50 mg
26175	**Sulfo-SDAD** Sulfosuccinimidyl 2-([4,4′-azipentanamido]ethyl)-1,3′-dithioproprionate	 **Sulfo-SDAD** Sulfo-NHS-SS-Diazirine Spacer Arm 13.5 Å	• Reducing agent cleavable spacer arm • Amine-reactive • Photo-reactive • Water-soluble		50 mg

References

1. O'Shannessy, D.J. and Quarles, R.H. (1985). *J. Appl. Biochem.* **7**, 347-355.
2. Jung, S.M. and Moroi, M. (1983). *Biochim. Biophys. Acta* **761**, 152-162.
3. Vanin, E.F. and Ji, T.H. (1981). *Biochemistry* **20**, 6754-6760.
4. van der Horst, G.T.J., *et al.* (1990). *J. Biol. Chem.* **265(19)**, 10801-10804.
5. McMahan, S.A. and Burgess, R.R. (1994). *Biochemistry* **33**, 12094-12099.
6. Wood, C.L. and O'Dorisio, M.S. (1985). *J. Biol. Chem.* **260**, 1243-1287.
7. Tiberi, M., *et al.* (1996). *J. Biol. Chem.* **271(7)**, 3771-3778.
8. Pandurangi, R.S., *et al.* (1998). *Bioorganic Chem.* **26(4)**, 201-212.
9. Pandurangi, R.S., *et al.* (1997). *Photochem. Photobiol.* **65(2)**, 208-221.

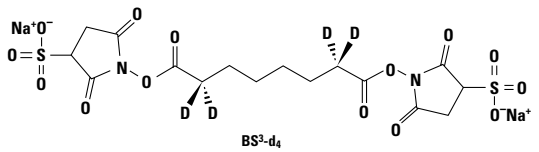
Deuterated Crosslinkers

A new strategy to study protein structure and function is rapidly emerging that integrates the proven utility of crosslinking with the power of mass spectrometry (MS) to yield insights into protein structure and protein complex formation.[1] High quality "heavy" and "light" crosslinkers analogs are now available to catalyze the potential of this method: deuterated analogs of BS^2G and BS^3 for the mass spectral study of intramolecular and intermolecular interactions.

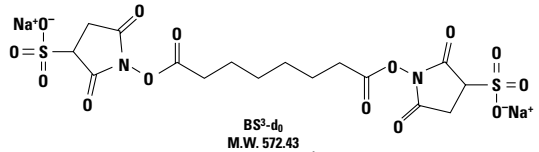
Heavy ($-d_4$) and light ($-d_0$) crosslinker analogs are reacted simultaneously with the target protein or protein complex. Incorporating deuterium at discrete positions in the crosslinking reagent allows both protein crosslinking and protein labeling to occur in a single step. Use of heavy/light crosslinker pairs in this application simplifies the MS identification of the peptides resulting from the coupling reactions. Application of a 1:1 ratio of two identical crosslinking agents differing only in the number of deuterium atoms in their chemical composition (e.g., d_4 vs d_0) is a powerful identifier of low abundant, singly crosslinked peptides in the mass spectrometer. These reagents will generate MS patterns in which the resultant crosslinked peptides will differ by four mass units after enzymatic digestion of the crosslinked protein or protein complex. Further analysis of the reaction products can yield low resolution three dimensional structure information. Intermolecular crosslinking of a protein complex and MS analysis have been successfully applied to determine the molecular contact surfaces of binding partners in protein complexes.[2-6] Crosslinker spacer arm length can be used to assign distance constraints between crosslinked peptides.

References

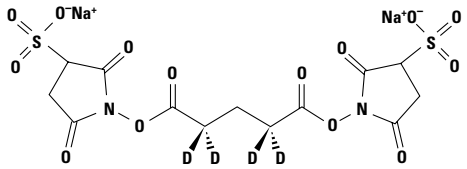
1. Sinz, A. (2003). Chemical crosslinking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. *J. Mass Spectrom.* **38**, 1225-1237.
2. Dihazi, G.H. and Sinz, A. (2003). Mapping low-resolution three-dimensional protein structure using chemical crosslinking and Fourier transform ion-cyclotron resonance mass spectrometry. *Rapid Commun. Mass Spectrom.* **17**, 2005-2014.
3. Kalkhof, S., *et al.* (2005). Chemical crosslinking and high-performance fourier transform ion cyclotron resonance mass spectrometry for protein interaction analysis: Application to a calmodulin/target peptide complex. *Anal. Chem.* **77**, 495-503.
4. Back, J.W., *et al.* (2003). Chemical crosslinking and mass spectrometry for protein structural modeling. *J. Mol. Biol.* **331**, 303-313.
5. Pearson, K.M., *et al.* (2002). Intramolecular crosslinking experiments on cytochrome c and ribonuclease A using an isotope multiplet method. *Rapid Commun. Mass Spectrom.* **16**, 149-159.
6. Muller, D.R., *et al.* (2001). Isotope-tagged crosslinking reagents. A new tool in mass spectrometric protein interaction analysis. *Anal. Chem.* **73**, 1927-1934
7. Schilling, B., *et al.* (2003). MS^2 assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J. Am. Soc. Mass Spectrom.* **14**, 834-850.
8. Peri, S., *et al.* (2001). GPMAW – a software tool for analyzing proteins and peptides. *Trends Biochem. Sci.* **26**, 687-689.



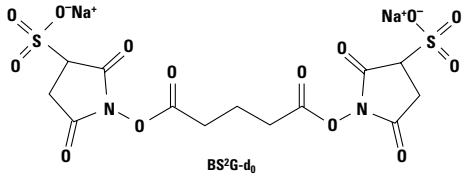
BS^3-d_4
M.W. 576.45
Spacer Arm 11.4 Å



BS^3-d_0
M.W. 572.43
Spacer Arm 11.4 Å



BS^2G-d_4
M.W. 534.38
Spacer Arm 7.7 Å



BS^2G-d_0
M.W. 530.35
Spacer Arm 7.7 Å

Structures of the heavy and light analogs of Thermo Scientific Pierce BS^3 and BS^2G developed for MS applications. Crosslinking agents with defined spacer arm lengths act as molecular rulers for estimation in spatial relationships in protein structure-function studies. Both the light analogs (BS^2G-d_0 and BS^3-d_0) and the heavy analogs (BS^2G-d_4 and BS^3-d_4) react efficiently with primary amino groups ($-NH_2$) at pH 7-9 to form stable amide bonds. All reagents are supplied as a sodium salt and are water-soluble up to 10 mM.

Ordering Information

Product #	Description	Pkg. Size
21590	BS^3-d_0 *Bis*(Sulfosuccinimidyl) suberate-d_0	10 mg
21595	BS^3-d_4 *Bis*(Sulfosuccinimidyl) 2,2,7,7-suberate-d_4	10 mg
21610	BS^2-G-d_0 *Bis*(Sulfosuccinimidyl) glutarate-d_0	10 mg
21615	BS^2-G-d_4 *Bis*(Sulfosuccinimidyl) 2,2,4,4-glutarate-d_4	10 mg

Label Transfer

Label transfer is an exciting technology that enables the study of protein interactions in new ways. It can be used to discover new interactions or to confirm putative interactions suggested by other methods, and to investigate the interface of the interacting proteins. In addition, the label transfer method is able to detect weak or transient protein interactions that often evade detection in co-immunoprecipitation and pull-down methods.

In a typical label transfer reaction, a purified bait protein is labeled with the chosen label transfer reagent. This labeled bait protein is allowed to interact with prey protein *in vitro* to form a complex. Once the complex has been formed, the reaction is exposed to UV light to activate a photo-reactive group and initiate the label transfer process by binding to the prey protein. Label transfer is completed by cleaving the spacer arm to release the bait protein, leaving the label attached to the interacting prey protein. A biotin label is especially useful in this process because it can be used both for purification and detection of the prey protein.

Traditional Label Transfer Reagents

The earliest examples of label transfer reagents incorporated a photo-reactive 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 Thermo Scientific Pierce Iodination Reagent. Iodination of the crosslinker 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 crosslinker's spacer arm.

The crosslinker 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-crosslink 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 photo-reactive 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 tri-functional reagents that more adequately segregate the reactive sites from the label. These tri-functional reagents can be designed to include non-radioisotopic labels such as biotin.

APDP: Radiolabel Transfer Reagents

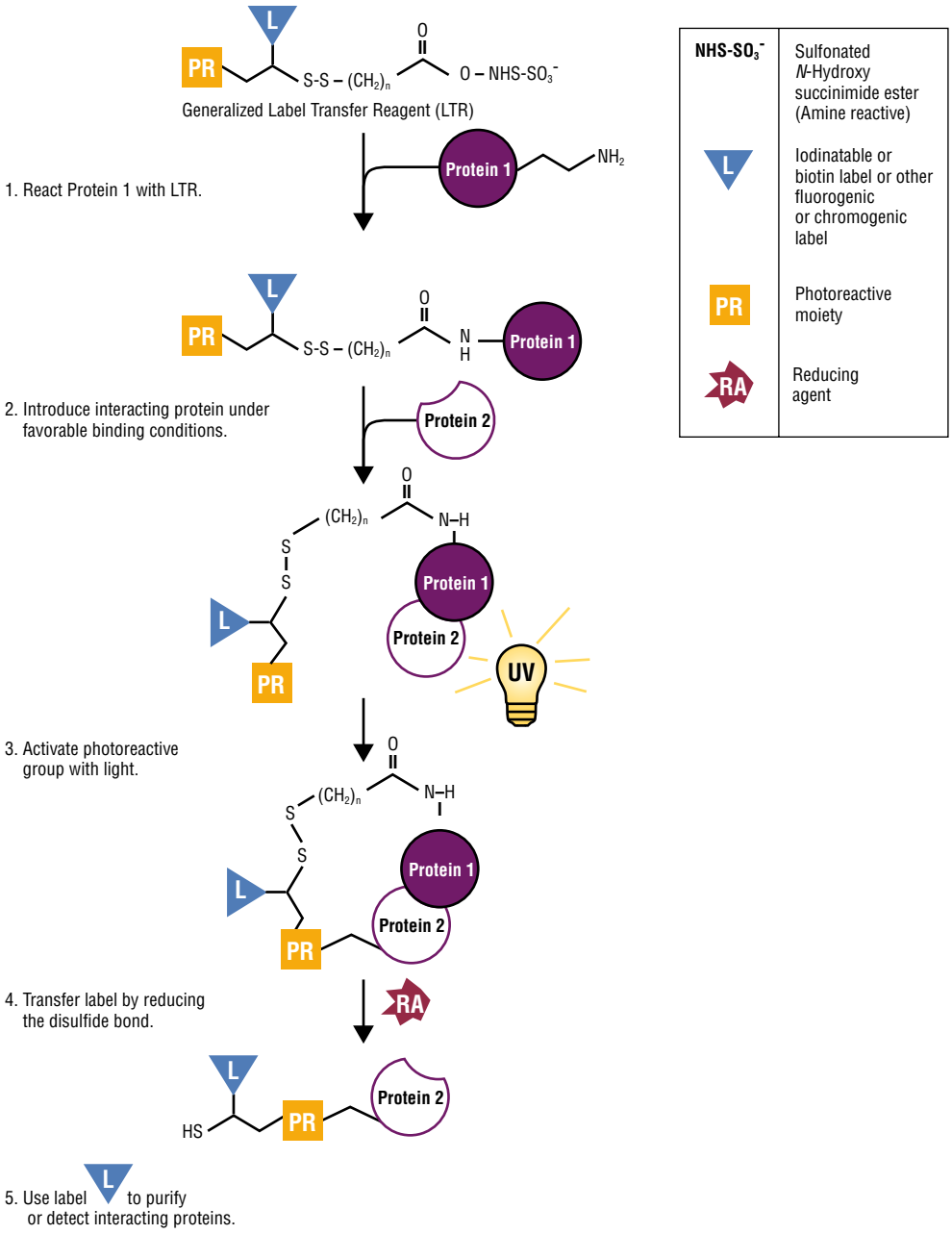
Thermo Scientific Pierce APDP (Product # 27716) is a heterobifunctional crosslinker containing a photo-reactive group that can be labeled with ^{125}I. APDP contains the sulfhydryl-reactive pyridyldithiol group which offers the advantage of allowing the course of the bait protein coupling to be monitored by following the loss of the pyridine-2-thione moiety (leaving group). The pyridine-2-thione can be detected at 343 nm (extinction coefficient: $8.08 \times 10^3\ M^{-1}cm^{-1}$).

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 tri-functional reagents designed for label transfer applications. The user should be aware of the following characteristics of these reagents.

1. Photo-reactive 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 crosslinking 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.

Label Transfer



$NHS\text{-}SO_3^-$	Sulfonated *N*-Hydroxy succinimide ester (Amine reactive)
L	Iodinatable or biotin label or other fluorogenic or chromogenic label
PR	Photoreactive moiety
RA	Reducing agent

General scheme for label transfer reactions.

Sulfo-SAED Fluorescent Label Transfer Reagent

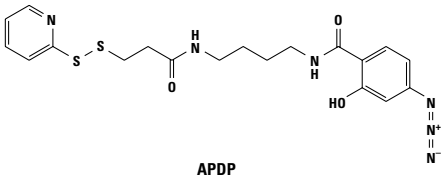
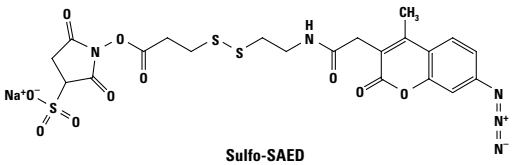
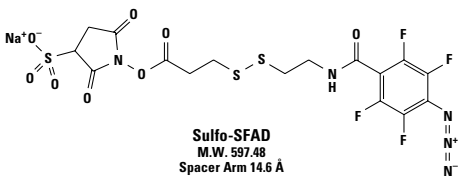
Subsequent designs of bifunctional label transfer reagents used nonradioactive labels to avoid the safety issues posed by ^{125}I. Fluorescent constituents designed into cleavable photo-reactive crosslinkers make possible transfer of a fluorescent label to an unknown interacting protein. An example of this type of reagent that incorporates a coumarin group is Thermo Scientific Sulfo-SAED (Product # 33030), which has been substituted with an azido group on the aromatic, photo-reactive ring. The reagent is non-fluorescent prior to exposure to UV light, but upon photolyzing and coupling to interacting proteins, it becomes fluorescent. The reagent also has a disulfide bond that can be reduced, resulting in cleavage of the crosslinked 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, photo-reactive, 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.

Thermo Scientific Pierce Bifunctional Label Transfer Reagents

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
27720	**APDP** *N*-[4-(*p*-Azido-salicylamido) butyl]-3´-(2´-pyridyldithio) propionamide	 **APDP** **M.W. 446.55** **Spacer Arm 21.0 Å**	• Radioiodinatable between $-N_3$ and –OH group of phenyl ring • –SH-reactive • Reaction monitored at 343 nm • Membrane permeable	1-5	50 mg
33030	**Sulfo-SAED** Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3´-dithiopropionate	 **Sulfo-SAED** **M.W. 621.60** **Spacer Arm 23.6 Å**	• Water-soluble • Amine-reactive • Photo-reactive • 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
27719	**Sulfo-SFAD** Sulfosuccinimidyl(perfluoro azidobenzamido)ethyl-1,3´-dithiopropionate	 **Sulfo-SFAD** **M.W. 597.48** **Spacer Arm 14.6 Å**	• Improved photoconjugation, efficiency • Photolyzes at 320 nm • Label transfer monitored by ^{19}F NMR • Water-soluble • Cleavable • Amine-reactive	9, 10, 14, 15	50 mg

Ordering Information

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

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

References

1. Dey, D., *et al.* (1998). *J. Biol. Chem.* **273(3)**, 1670-1676.
2. Sato, K., *et al.* (1997). *J. Biol. Chem.* **272(9)**, 5880-5886.
3. Gao, A.G. and Frazier, W.A. (1994). *J. Biol. Chem.* **269(47)**, 29650-29657.
4. Traut, R.R., *et al.* (1989). Protein Function, A Practical Approach, Oxford:IRL Press, p. 101.
5. Lam, Y.A., *et al.* (2002). *Nature* **416** (April 19), 763-766.
6. Felin, M., *et al.* (1997). *Glycobiology* **7(1)**, 23-29.
7. Lala, A., *et al.* (1996). *Pept. Res.* **9(2)**, 58-60.
8. Zhang, Q. and Mosher, D.F. (1996). *J. Biol. Chem.* **271(52)**, 33284-33292.
9. Chattopadhyay, A., *et al.* (1992). *J. Biol. Chem.* **267**, 12323-12329.
10. Smith, J.W., *et al.* (1990). *J. Biol. Chem.* **265(21)**, 12267-12271.
11. Smith, J.W. and Cheresh, D.A. (1990). *J. Biol. Chem.* **265(4)**, 2168-2172.
12. Ghinea, N., *et al.* (1989). *J. Biol. Chem.* **264(9)**, 4755-4758.
13. Shephard, E.G., *et al.* (1988). *Anal. Biochem.* **186**, 306-313.
14. Pandurangi, R.S., *et al.* (1998). *Bioorg. Chem.* **26(4)**, 201-212.
15. Pandurangi, R.S., *et al.* (1997). *Photochem. Photobiol.* **65(2)**, 208-221.
16. Thevenin, B., *et al.* (1991). *Biophys. J.* **59**, 358a.
17. Kang, J., *et al.* (1991). *Biophys. J.* **59**, 249a.

Non-Isotopic Tri-Functional Transfer

Sulfo-SBED Biotin Label Transfer 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. Thermo Scientific Pierce Sulfo-SBED (Product # 33033) is an example of such a tri-functional reagent (Figure 9). It contains an amine-reactive sulfo-NHS-ester on one arm (built off the α-carboxylate of the lysine core), a photo-reactive 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.

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) effectively couples the photo-reactive end to the nearest available C-H or N-H bond in the bait:prey complex, resulting in covalent crosslinks 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, thus facilitating isolation or identification of the unknown species using streptavidin, Thermo Scientific NeutrAvidin Biotin-Binding Protein or monomeric avidin reagents.

The architecture of this tri-functional 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 used to:

- Define interactions of complexes with activator domains[1]
- Clarify the mechanism of protein complex assembly[2]
- Convert to a sulfhydryl-reactive tri-functional reagent to map interactions[3]
- Study docking site and factor requirements for binding[4]
- Describe binding contacts of interactors[5]
- Confirm recognition of a specific phosphoepitope[6]
- Search for putative binding partners[7]
- Gain insight into chaperone-mediated refolding interactions[8]
- Investigate mechanism of protein interaction[9]
- Facilitate receptor activity-directed affinity tagging (re-tagging)[10]
- Detect low-abundance protein receptors
- Find protein:carbohydrate interactions
- Understand drug-receptor interactions[11]
- Quantitate triple helix-forming oligonucleotides[12]

Routes for prey protein identification using Sulfo-SBED are outlined schematically in Figure 10. Note that the biotin label is a purification handle for captured prey protein. In the trypsin digestion strategy, the trapped peptide(s) 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.

References

1. Neely, K.E., *et al.* (2002). *Mol. Cell. Biol.* **22(6)**, 1615-1625.
2. Ishmael, F.T., *et al.* (2002). *J. Biol. Chem.* **277(23)**, 20555-20562.
3. Alley, S.C., *et al.* (2000). *J. Am. Chem. Soc.* **122,** 6126-6127.
4. Trotman, L.C., *et al.* (2001). *Nature Cell Biology* **3,** 1092-1100.
5. Horney, M.J., *et al.* (2001). *J. Biol. Chem.* **276(4)**, 2880-2889.
6. Daum, J.R., *et al.* (2000). *Curr. Biology* **10(23)**, R850-857, S1-S2.
7. Kleene, R., *et al.* (2000). *Biochemistry* **39**, 9893-9900.
8. Minami, Y., *et al.* (2000). *J. Biol. Chem.* **275(12)**, 9055-9061.
9. Sharma, K.K., *et al.* (2000). *J. Biol. Chem.* **275(6)**, 3767-3771.
10. Ilver, D., *et al.* (1998). *Science* **279(5349)**, 373-377.
11. Jacobson, K.A., *et al.* (1995). *Life Sciences* **56(11/12)**, 823-830.
12. Geselowitz, D.A. and Neumann, R.D. (1995). *BioConjuate Chem.* **6**, 502-506.

Sulfo-SBED Label Transfer Reagent

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

A proven reagent with exceptional versatility, Thermo Scientific Pierce Sulfo-SBED combines four properties into a powerful protein interaction tool.

Highlights:

- 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	**Sulfo-SBED Label Transfer Reagent** Sulfo-SBED is the abbreviation for Sulfo-NHS-2-(6-[Biotinamido]-2-(*p*-azido benzamido)-hexanoamido) ethyl-1,3′-dithiopropionate.	10 mg
33034	**Sulfo-SBED Label Transfer Reagent** No-Weigh™ Format.	8 x 1 mg
33073	**Sulfo-SBED Label Transfer Kit-Western Blot Application** *Sufficient reagents to perform eight label transfer reactions for subsequent Western blot analysis.* Includes: Sulfo-SBED No-Weigh Format BupH Phosphate Buffered Saline Label Transfer Buffer (20X) Streptavidin-HRP Dithiothreitol (DTT), No-Weigh Format Slide-A-Lyzer® MINI Dialysis Units Plus Float, 10K MWCO, 10-100 µl	Kit 8 x 1 mg 1 pack 200 ml 0.1 mg 8 x 7.7 mg 10 units/pack

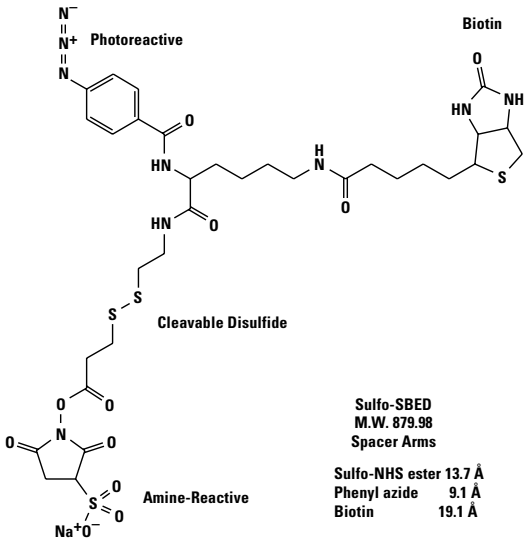


Figure 9. Structure of Thermo Scientific Pierce Sulfo-SBED.

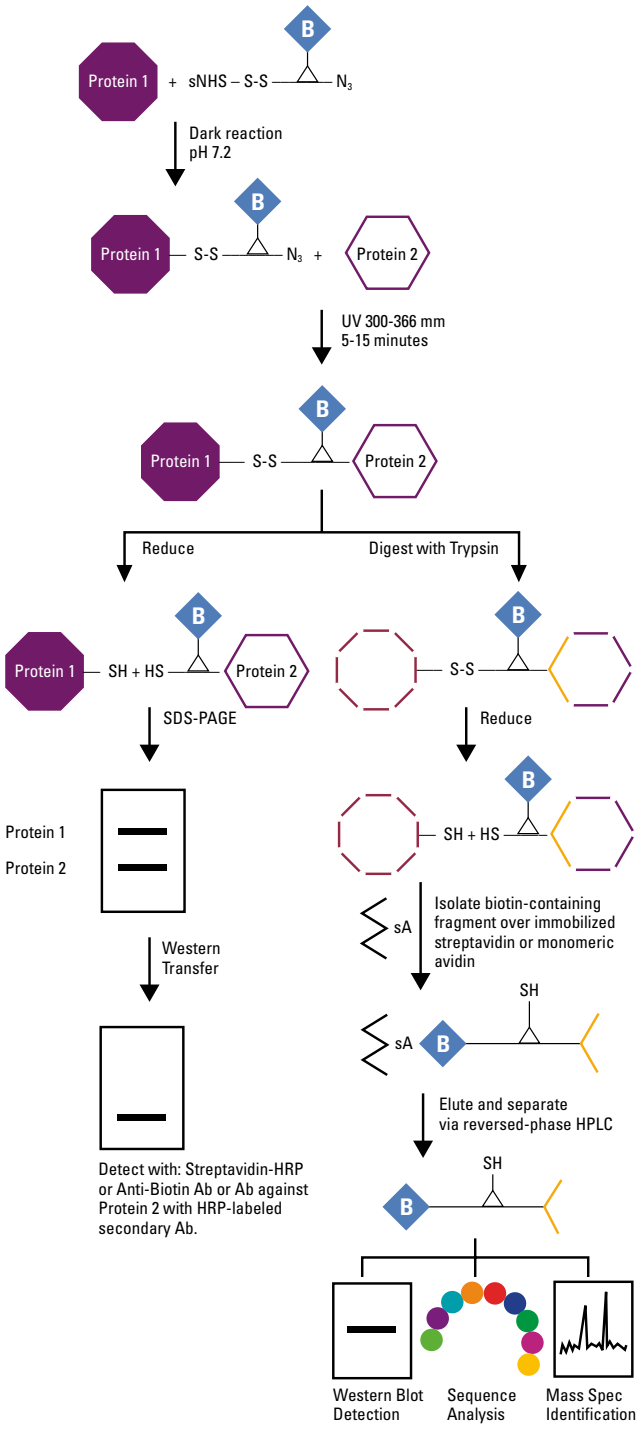


Figure 10. Applications of Thermo Scientific Pierce Sulfo-SBED in protein interaction studies.

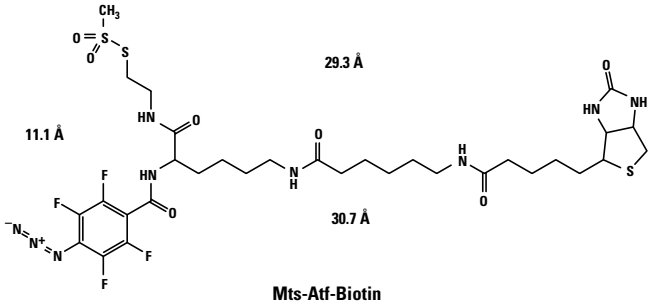
Mts-Aft-Biotin Label Transfer Reagent

Sulfhydryl-directed, photo-reactive biotin label transfer reagents.

These two Thermo Scientific Pierce 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 photo-reactive 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 photo-reactive 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:

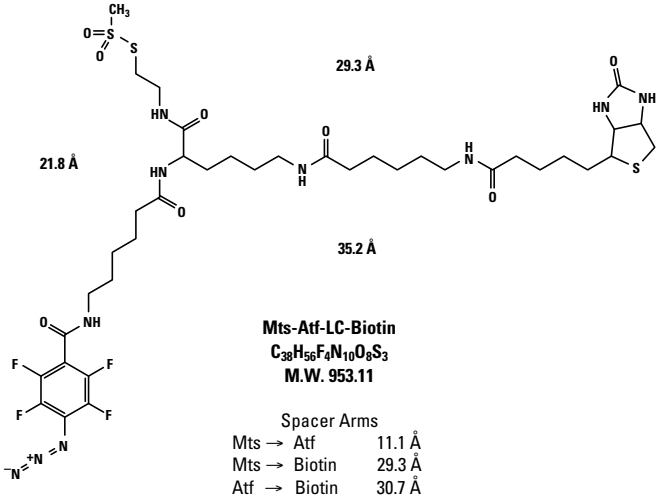
- Methanethiosulfonate (Mts) is specific for the sulfhydryl (–SH) group that occurs in reduced cysteine residues, enabling precise, rapid and quantitative labeling of the bait protein
- Tetrafluorophenyl azide group reacts 3-4 times more efficiently than regular phenyl azides moieties, increasing the likelihood of capturing detectable amounts of bait prey complex
- 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
- Disulfide bond connecting bait and prey proteins is easily cleaved with common reducing agents (e.g., DTT, 2-mercaptoethanol, TCEP)
- Mts reaction and photoreaction are compatible with physiological aqueous buffers required for most protein interactions
- Long chain (LC) and short chain versions are available to allow more precise exploration of interaction distances



Mts-Atf-Biotin
$C_{32}H_{45}F_4N_9O_7S_3$
M.W. 839.95

Spacer Arms

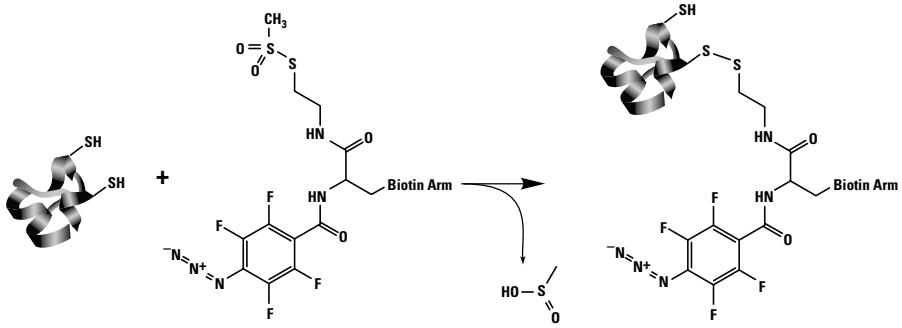
Mts →	Atf	11.1 Å
Mts →	Biotin	29.3 Å
Atf →	Biotin	30.7 Å



Mts-Atf-LC-Biotin
$C_{38}H_{56}F_4N_{10}O_8S_3$
M.W. 953.11

Spacer Arms

Mts →	Atf	11.1 Å
Mts →	Biotin	29.3 Å
Atf →	Biotin	30.7 Å



Reaction of Thermo Scientific Pierce Mts-Atf-Biotin with bait protein containing sulfhydryls. 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 (they prey) and then crosslinked together by UV-activation of the tetrafluorophyenyl 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.

Ordering Information

Product #	Description	Pkg. Size
33093	**Mts-Atf-Biotin Label Transfer Reagent** 2-[*N*2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysiny]ethyl methanethiosulfonate	5 mg

Ordering Information

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

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 probes[3] 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).

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 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.

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)* [Arrows designate sequence of steps of detection strategy]	Unlabeled primary antibody → Enzyme-labeled secondary antibody → Substrate Reagent	Unlabeled bait protein → Enzyme-labeled bait-specific antibody → Substrate Reagent
	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.*

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, electrophoresis under native conditions; i.e., nondenaturing and without reducing agent, can be used.

Transfer to Membrane

After separation on the gel, proteins are electrophoretically transferred from the gel to a membrane. 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 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 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 40). 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. By blocking all remaining binding sites on the membrane, a blocking buffer reduces nonspecific binding and aids in protein renaturation. 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 under study. 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.[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 preserve the interaction until it can be detected. This may influence the formulation of wash buffer used between probing steps.

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 with probes 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, isotopic detection methods have several disadvantages including health hazards and disposal issues.

GST-tagged or histidine-tagged recombinant bait proteins are often detected with 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. We offer 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; 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 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. SuperSignal® Chemiluminescent Substrates enable unmatched sensitivity for HRP-based conjugates.

Controls

When identifying protein:protein interactions by the far-Western technique, it is important to include appropriate controls to distinguish true protein:protein interaction bands from nonspecific artifacts. 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 as a negative control. Ideally, the control protein would be of similar size and charge to the protein under investigation and would not interact 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 our 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.

References

1. Blackwood, E.M. and Eisenman, R.N. (1991). *Science* **251**,1211-1217.
2. Kaelin, W.G., *et al.* (1992). *Cell* **70**, 351-364.
3. Edmondson, D.G. and Dent, S.Y.R. (2001). *Current Protocols in Protein Science* 19.7.1-19.7.10.
4. Burgress, R.R., *et al.* (2000). *Methods Enzymol.* **328**, 141-157.
5. Reddy, V.M. and Kumar, B. (2000). *J. Infect. Dis.* **181**, 1189-1193.
6. Golemis, E., Ed. (2002). *Protein-Protein Interactions – A Laboratory Manual*; Cold Spring Harbor Laboratory Press.

Far-Western Protein Interaction Kits

Discover your next important protein interaction with the Thermo Scientific Pierce Far-Western Kits.

The First Non-Isotopic Far-Western Protein:Protein Interaction Kits that Detect Interactions On-Membrane or In-Gel

Our Far-Western Biotinylated-Protein Interaction Kit and Far-Western GST-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.

Far-Western Biotinylated-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.

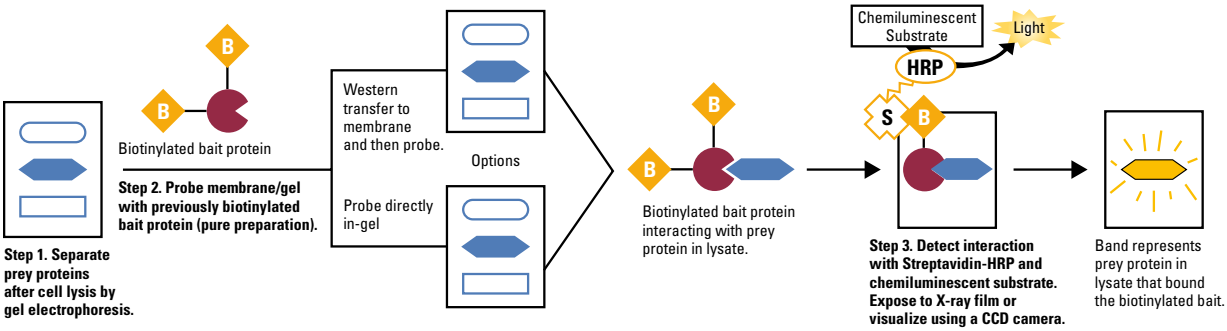
Highlights:

- 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	**Far-Western Biotinylated Protein Interaction Kit** *Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a biotinylated bait protein as the probe.*	10 mini gels
	Includes: Streptavidin-HRP	0.1 mg
	10X Dilution Buffer	50 ml
	BupH Phosphate Buffered Saline	17 packs
	10% Tween®-20	6 x 10 ampules
	Stable Peroxide	55 ml
	Luminol Enhancer	55 ml
	Cellophane Enhancer Sheets	10 pack
23505	**Far-Western GST-Protein Interaction Kit** *Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a GST-tagged bait protein as the probe.*	10 mini gels
	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
	Stable Peroxide	55 ml
	Luminol Enhancer	55 ml
	Cellophane Enhancer Sheets	10 pack

See also: Thermo Scientific Pierce Pull-down Protein Interaction Kits, pages 14-23.



General scheme for the Thermo Scientific Pierce Far-Western Kit.

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, crosslinking, 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).

Metal Chelate Chemical Cleavage Reagents

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 difficult to interpret on SDS-polyacrylamide gels.

Guide to Thermo Scientific 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
20341	Papain	X		Nonspecific protein digestion
20343	Pepsin	X		Nonspecific protein digestion
20151 **20195**	*Aureus* V-8 Protease	X	X	Cleaves at the carboxyl side of aspartic and glutamic acid
20199	Submaxillary Arg-C Protease		X	Cleaves at the carboxyl side of arginine
20230 **20233**	TPCK Trypsin	X	X	Cleaves on the carboxyl side of arginine and lysine

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) ethylene-diamin-etetraacetate (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 11). 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 12).

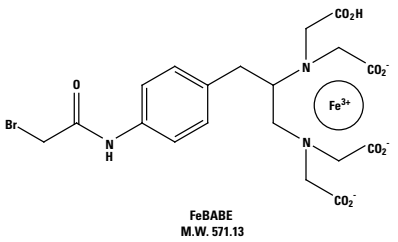


Figure 11. Structure of FeBABE.

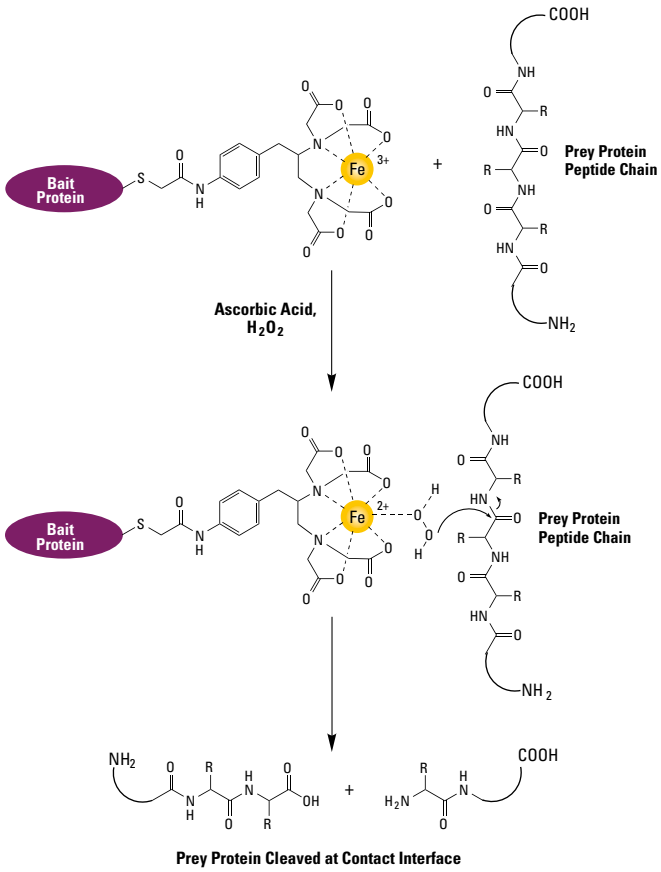


Figure 12. Peptide bond-cutting mechanism of FeBABE-modified bait protein.

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 13). 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 9). 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.

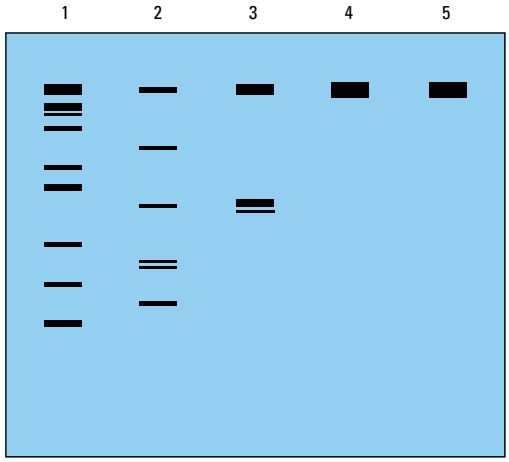


Figure 13. Analysis of peptide mapping by Western blot. Western blot using antibody directed to the N-terminal epitope of the target protein. Comparison of FeBABE cleavage of the interaction site with cleavage standards and negative reaction controls. **Lane 1.** Chemical cleavage of target protein with 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 14). 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 (3D if the tertiary structure is known) of residues in or near the site of interaction on the target protein.

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. The random substitution of lysines ensures that all potential binding sites are examined.

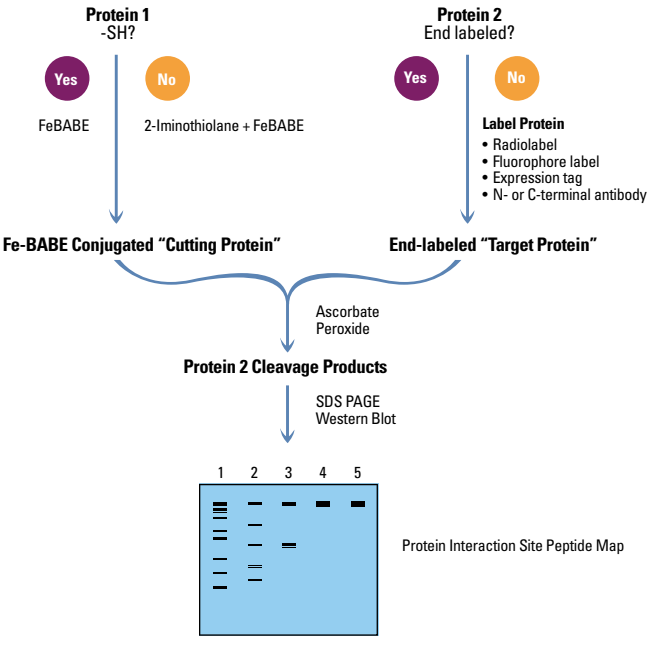


Figure 14. Analysis of peptide mapping by Western blot.

The literature reports that FeBABE 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 FeBABE 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 crosslinking 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 FeBABE 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.

Protein Interaction Mapping Kit

A complete kit for protein interaction mapping featuring the FeBABE artificial protease 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 cleaving peptide bonds at loci on the prey protein when the FeBABE modified bait protein and prey protein are in close proximity. (See page 43 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 Fe^{3+} 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 15). 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 downsteam 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

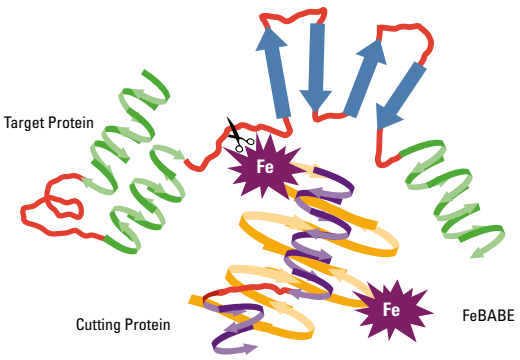


Figure 15. Diagram of FeBABE site-directed proteolysis of prey protein. Cleavage occurs at contact interface between the Fe(BABE)-derivatized cutting (bait) protein and the target or prey protein.

Incorporation of –SH Group(s) into –SH-Deficient Bait Protein

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.

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 (Thermo Scientific No-Weigh Single-Dose Packaging) and do not deteriorate by repeated sampling from a single vial.

Ordering Information

Product #	Description	Pkg. Size
32223	**Protein Interaction Mapping Kit** *Sufficient materials to perform eight protein:protein interaction mapping experiments using FeBABE.* Includes: Metal Removal Reagent	Kit
	No-Weigh FeBABE Protein Cutting Reagent	25 ml
	FeBABE Conjugation Buffer	8 x 50 mg
	FeBABE Protein Cutting Buffer	25 ml
	No-Weigh Ascorbic Acid Reducing Agent	25 ml
	Stable Peroxide Reagent	8 x 0.7 mg
	Protein Desalting Spin Columns	3 ml, 25/pkg
20332	**FeBABE Protein Cutting Reagent, No-Weigh format** *Cysteine (-SH)-reactive protein cutting reagent. Single-dose packaging ensures 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
Datwyler, S.A. and Meares, C.F. (2000). *Trends Biochem. Sci.* **25**, 408-414.
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.

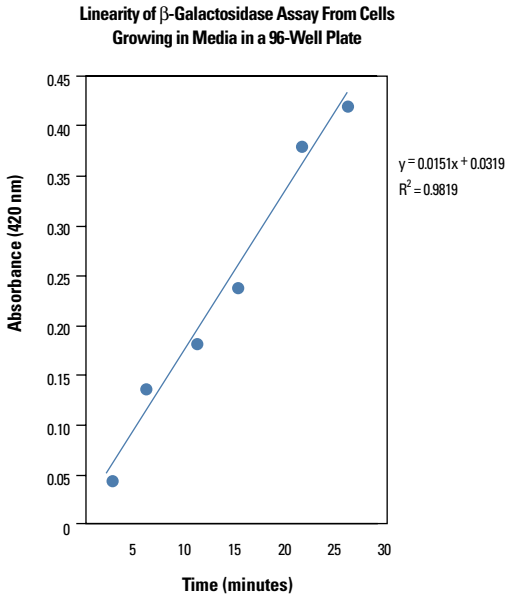
The gene encoding β-galactosidase (lacZ) of *E. coli* has been widely used as a reporter gene in many different prokaryotic and eukaryotic organisms. In particular, this gene has proven useful for studying gene expression in the yeast *Saccharomyces cerevisiae*.

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 Thermo Scientific 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 (OD_{660}).

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



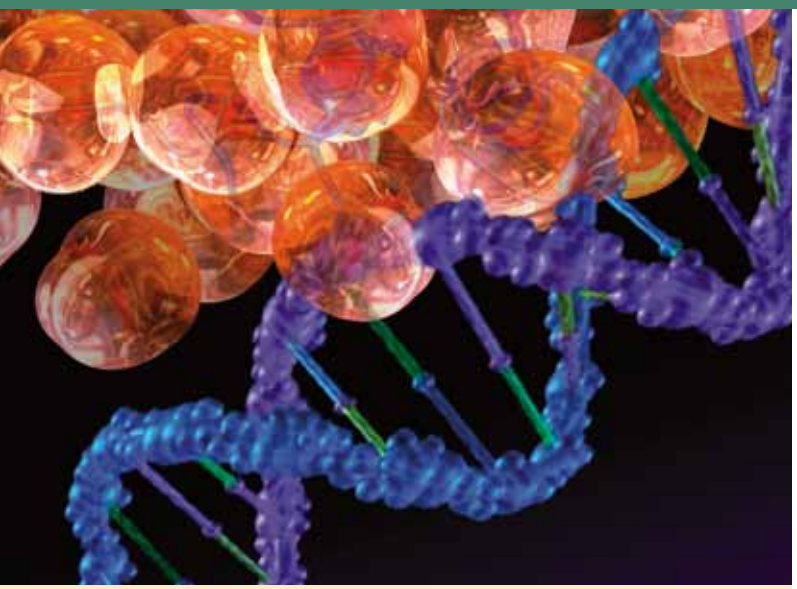
Strain BRS1002 carrying plasmid pYX122-β-Gal was grown to an OD_{600} of 1.0, then 100 µl of cells were transferred to a 96-well plate. At time = 0, 100 µl of a 1:1 mixture of lysis reagent and 2X β-Gal assay buffer were added to each well and absorbance at 405 nm was determined. Specific activity for this sample is 160 units (unit=OD_{405} x 1,000/time/OD_{660}).

Ordering Information

Product #	Description	Pkg. Size
❄ 75768	Yeast β-Galactosidase Assay Kit Protein:Protein Interaction Kit	Kit
	Includes: Y-PER® Yeast Protein Extraction Reagent	25 ml
	2X β-Galactosidase Assay Buffer	25 ml
	1M Na_2CO_3 Stop Solution	25 ml

❄ *Additional dry ice and/or freight charge.*

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.

RNA:protein interactions are critical in both the translation of mRNA into protein and in the regulatory roles of non-coding RNA. Many studies have focused on the significance of noncoding RNAs, including the 5′ and 3′ untranslated regions (UTR) of mRNA, small interfering RNA (siRNA), and microRNA (miRNA) families. More recently, some of these small RNAs have been implicated as proto-oncogenes and in various diseases, including cancer. The use of radioactivity, high background and high experimental variability have made studying protein:RNA interactions extremely difficult.

Type of Protein:Nucleic Acid Interactions

The common property of nucleic acid-binding proteins is their ability to recognize and manipulate DNA/RNA structures. Chromatin remodelling, 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.

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 products listed thereafter.

Table 4. Methods for protein:nucleic acid interaction analysis.

***In vitro* methods**	**Description**
Electrophoretic Mobility Shift Assay (EMSA)	The EMSA has been used extensively for studying protein:DNA interactions. The assay is based on the slower migration of protein:DNA complexes through a native polyacrylamide or agarose gel than unbound DNA. The individual protein:DNA complexes from discreet bands within the gel. Now, protein:RNA interactions can be detected with the first RNA-EMSA assay.
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."
Chromatin Immunoprecipitation (ChIP)	Captures protein DNA interactions via *in vivo* crosslinking. Antibodies are used to selectively precipitate a protein of interest, and the quantity of DNA bound to that protein is measured via Quantitative PCR.
Protein:DNA Crosslinking	Method for trapping protein:DNA interactions covalently under controlled conditions by labeling the protein bait and capturing the interacting DNA via coupling with a photo-reactive reagent. Excellent for capturing weak or transient interactions.
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. Binding of a protein to a specific DNA sequence protects that region of DNA from subsequent attack by DNase.
Reporter Assays	Identify gene promoter activity with reporter genes that are easily visualized. Provides real-time data in cell systems.

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 relies on the fact 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 Crothers[3] 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

Nucleic Acid Probe

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 interactions[10] have also been studied using the same electrophoretic principle. RNA secondary structure is important for RNA:protein complexes. Steric hindrance due to the probe label should be prevented to maintain this secondary structure. RNA probes can be biotinylated at the 3′ end (Product # 20160) to help avoid steric hindrance issues. Fully optimized RNA EMSA Kits are also available (Product # 20158).

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 ^{32}P by incorporating an [α-^{32}P]dNTP during a 3′ fill-in reaction using Klenow fragment or by 5′ end labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase. Non-radioactive methods are available to label and detect nucleic acid probes with biotin.

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.

Alternatively, DNA can be labeled with a biotinylated or hapten-labeled dNTP, then probed and detected using an appropriately sensitive fluorescent or chemiluminescent substrate. We offer a chemiluminescent EMSA system (Product # 20148) and a kit to facilitate labeling DNA with biotin (Product # 89818). The EMSA Kit 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 nonspecific sites to adsorb proteins that will bind to any general DNA sequence. To maximize 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 16). 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.

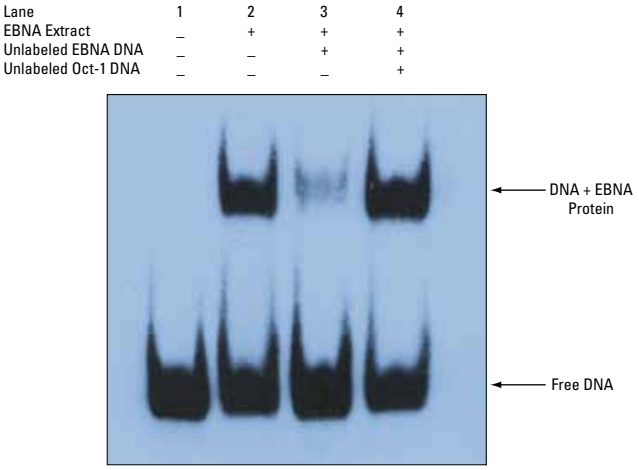


Figure 16. EMSA results using the EBNA control system. Biotin-labeled 60 bp duplex bearing the EBNA-1 binding sequence was incubated with an extract in which the EBNA-1 protein was overexpressed. The binding buffer was supplemented with 50 ng/µl poly(dI•dC), 10% glycerol and 0.05% NP-40. Exposure time was 30 seconds with X-ray film.

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.

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. 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, such as *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 ammonium persulfate to distribute/equilibrate any stabilizing factors or ions that were added to the electrophoresis buffer, and to ensure a constant gel temperature.

References

1. Hendrickson, W. (1985). *Biotechniques* **3**, 346-354.
2. Revzin, A. (1989). *Biotechniques* **7**, 346-354.
3. Fried, M. and Crothers, D.M. (1981). *Nucleic Acids Res.* **9**, 6505-6525.
4. Garner, M.M. and Revzin, A. (1981). *Nucleic Acids Res.* **9**, 3047-3059.
5. Stockley, P.G. (1994). Filter-binding assays. From Methods in Molecular Biology, Vol. 30: DNA-Protein Interactions: Principles and Protocols. G. Geoff Kneale, Ed., Humana Press Inc., Totowa, NJ.
6. Fried, M.G. and Crothers, D.M. (1984). *J. Mol. Biol.* **172**, 263-282.
7. Fried, M.G. (1989). *Electrophoresis* **10**, 366-376.
8. Allen, S.V. and Miller, E.S. (1999). *Anal. Biochem.* **269**, 32-37.
9. Park, Y.W., *et al.* (1999). *Proc. Natl. Acad. Sci. USA* **96**, 6694-6699.
10. Grunwald, M.E., *et al.* (1998). *J. Biol. Chem.* **273**, 9148-9157.

Electrophoretic Mobility Shift Assays (EMSA)

LightShift Chemiluminescent DNA EMSA Kit

Identifies regulatory sequences and determines protein:DNA binding regions and affinity.

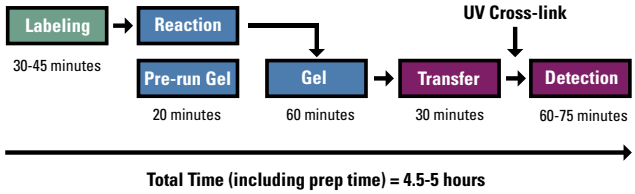
Thermo Scientific LightShift Chemiluminescent DNA EMSA Kit is an extraordinarily robust and sensitive system for performing electrophoretic mobility shift assays (EMSAs) to identify and characterize protein:DNA binding interactions. The kit includes reagents for setting up and customizing protein:DNA binding reactions, a control set of DNA and protein extract to test the kit system, stabilized streptavidin-HRP conjugate to probe for the biotin-labeled DNA target, and an exceptionally sensitive chemiluminescent substrate module for detection.

The principle for our DNA EMSA Detection Kit 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-crosslinked, probed with streptavidin-HRP conjugate and incubated with the substrate. The protocol from labeling to results can be accomplished in a single day.

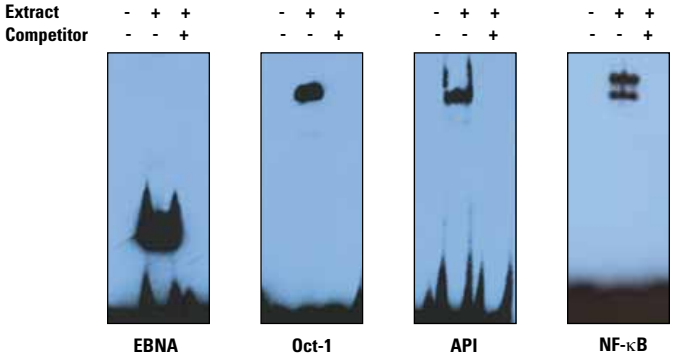
The only additional components needed to perform the assay are purified DNA target that has been end-labeled with biotin, the protein extract you wish to test, nylon membrane and basic electrophoresis equipment. DNA targets may be synthesized with 5´ or 3´ biotin labels or they may be labeled after synthesis using the Biotin 3´ End DNA Labeling Kit (see Product # 89818). Nuclear, cytosolic or whole cell protein extracts may be obtained by a variety of methods, including the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Product # 78833).

Highlights:

- Includes EBNA control system to help new users develop a working assay and understand the methods used to confirm binding interaction specificity
- Excellent for detecting low-abundance proteins in nuclear extracts
- Sensitivity that surpasses radioactive and digoxigenin methods
- Compatible with previously-established binding conditions for popular DNA:protein interactions



Timeline for the Thermo Scientific LightShift Chemiluminescent DNA EMSA Kit protocol.



Chemiluminescent DNA EMSA of four different DNA:protein complexes. Biotin-labeled target duplexes ranged in size from 21-25 bp. The EBNA reactions were supplemented with 2.5% glycerol and 0.05% NP-40, and the AP1 reactions were supplemented with 10% glycerol. The source of the Oct-1, AP1 and NF-κB transcription factors was a HeLa nuclear extract. EBNA-1 extract is provided as a control in the kit. Unlabeled specific competitor sequences (where used) were present at a 200-fold molar excess over labeled target. X-ray film exposure times for each system ranged from 2 minutes for EBNA, Oct-1 and AP1, and 5 minutes for NF-κB.

References

1. Cornelussen, R.N.M., *et al.* (2001). *J. Mol. Cell Cardiol.* **33**, 1447-1454.
2. Ishida, A., *et al.* (2002). *J. Biol. Chem.* **277(29)**, 26217-26224.
3. MacLachlan, T.K. and El-Deiry, W.S. (2002). *Proc. Natl. Acad. Sci. USA* **99(14)**, 9492-9497.
4. Matata, B.M. and Galinanes, M. (2002). *J. Biol. Chem.* **277(3)**, 2330-2335.
5. Tarumi, T., *et al.* (2002). *J. Biol. Chem.* **277(21)**, 18510-18516.
6. Sauzeau, V., *et al.* (2003). *J. Biol. Chem.* **278(11)**, 9472-9480.

Ordering Information

Product #	Description	Pkg. Size
20148	**LightShift® Chemiluminescent DNA EMSA Kit** *Sufficient components for 100 binding reactions and detection reagents for ~800 cm² of membrane.*	Kit
	Includes: 10X binding buffer	1 ml
	Biotin-EBNA control DNA	50 µl
	Unlabeled EBNA DNA	50 µl
	EBNA extract	125 µl
	Poly(dI•dC)	125 µl
	50% glycerol	500 µl
	1% NP-40	500 µl
	1 M KCl	1 ml
	100 mM $MgCl_2$	500 µl
	200 mM EDTA, pH 8.0	500 µl
	5X Loading buffer	1 ml
	Stabilized streptavidin-horseradish peroxidase conjugate	1.5 ml
	Luminol/Enhancer solution	80 ml
	Stable peroxide solution	80 ml
	Blocking buffer	500 ml
	4X Wash buffer	500 ml
	Substrate equilibration buffer	500 ml

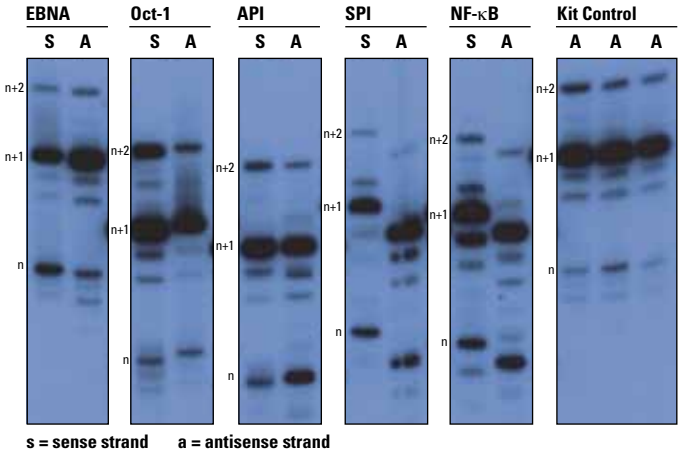
Biotin 3′ End DNA Labeling Kit

A complete kit for labeling the 3′ end of DNA with biotin.

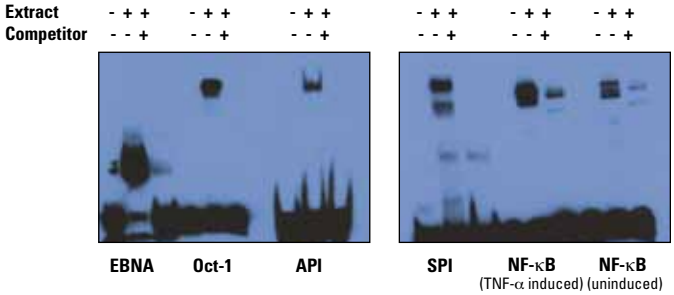
The Thermo Scientific Biotin 3′ End DNA Labeling Kit uses terminal deoxynucleotidyl transferase (TdT) to catalyze nontemplate-directed nucleotide incorporation onto the 3′-OH end of DNA.[1,2] TdT exhibits a substrate preference of single-stranded DNA, but it will label duplex DNA with 3′ overhangs and blunt duplexes, albeit with a lower efficiency.[3] The Biotin 3′ End DNA Labeling Kit has been optimized to incorporate 1-3 biotinylated ribonucleotides (biotin-11-UTP) onto the 3′ end of DNA strands. This labeling strategy has the advantage of localizing the biotin to the 3′ end of the probe where it will be less likely to interfere with hybridization or sequence-specific binding of proteins. Biotin-labeled DNA probes can be used to facilitate non-isotopic detection in a variety of applications including electrophoretic mobility shift assays (EMSA), Northern or Southern blots, colony hybridizations or *in situ* hybridizations.

Highlights:

- Non-isotopic labeling eliminates the hassle of hazardous radioactive materials or difficult-to-dispose-of waste
- 1-3 biotinylated ribonucleotides onto the 3′ end of DNA strands for less interference with hybridization or sequence-specific binding of proteins
- Biotin-labeled probes are stable for more than one year
- 30-minute labeling procedure is fast and efficient



Sequencing gel analysis of labeling efficiency. Ten different oligos (ranging in size from 21-25 nt) were labeled using the Thermo Scientific Biotin 3′ End DNA Labeling Kit. The products from the TdT reaction were then radiolabeled using T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. The PNK reactions were run on a 20% acrylamide/8 M urea/TBE. The position of the starting oligo (no biotin) is denoted by "n." Incorporation of biotin-labeled ribonucleotide by TdT is limited to one or two incorporations per strand (positions "n+1" and "n+2," respectively). Labeling efficiencies ranged from 72% (EBNA sense strand) to 94% (Oct-1 sense strand). The kit control oligo labeled with 88-94% efficiency.



EMSA results using 3′ biotin-labeled duplexes. The sense and antisense strands were labeled using the Thermo Scientific Biotin 3′ End DNA Labeling Kit and hybridized for 4 hours at room temperature to form duplexes containing the binding sites for the indicated transcription factors. Gel shift assays were performed using the Thermo Scientific LightShift Chemiluminescent EMSA Kit using 20 fmol duplex per binding reaction. The source of the transcription factors was a HeLa nuclear extract prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Product # 78833) (2 µl or 6-7 µg protein per reaction). In the case of the NF-κB system, nuclear extracts were made from HeLa cells that had been induced with TNFα or cells that were untreated. Competition reactions containing a 200-fold molar excess of unlabeled duplex were performed to illustrate the specificity of the protein:DNA interactions.

References

1. Roychoudhury, R. and Wu, R. (1980). *Methods Enzymol.* **65**, 43-62.
2. Roychoudhury, R., *et al.* (1976). *Nucleic Acids Res.* **3**, 863-877.
3. Michelson, A.M. and Orkin, S.H. (1982). *J. Biol. Chem.* **257**, 14773-14782.
4. Cornelussen, R.N.M.,*et al.* (2001). *J. Mol. Cell Cardiol.* **33**, 1447-1454.

Ordering Information

Product #	Description	Pkg. Size
89818	**Biotin 3′ End DNA Labeling Kit** *Sufficient components for 20 labeling reactions.*	Kit
	Includes: 5X TdT reaction buffer	1 ml
	Terminal deoxynucleotidyl transferase (TdT)	50 µl
	Biotin-11-UTP	100 µl
	Unlabeled control oligo	140 µl
	Biotin-control oligo	40 µl

LightShift Chemiluminescent RNA Electrophoretic Mobility Shift Assay

Fast, non-radioactive detection of RNA:protein interactions

RNA-protein interactions are critical in both the translation of mRNA into protein and in regulatory roles of non-coding RNA. Many studies have focused on the significance of non-coding RNAs, including the 5´ and 3´ untranslated regions (UTR) of mRNA, small interfering RNA (siRNA) and microRNA (miRNA) families. Recently, some of these small RNAs have been implicated as pro-to-oncogenes and in various diseases and cancer. For both coding and non-coding RNA, RNA-protein interactions are critical for cell function; however, tools for studying such interactions have been limited by the use of radioactivity and high background and high experimental variability.

The Thermo Scientific LightShift Chemiluminescent RNA EMSA Kit provides a non-radioactive solution for studying RNA-protein interactions using an electrophoretic mobility-shift assay (EMSA). An RNA EMSA is an *in vitro* technique that detects protein-RNA interactions through changes in gel electrophoresis migration patterns (Figure 17). A labeled RNA probe is incubated with a protein sample to initiate binding. Once a complex is formed, the sample is separated via non-denaturing polyacrylamide gel electrophoresis. An RNA-protein complex migrates more slowly than a free RNA probe, which shifts the migration pattern. Specificity is determined through binding competition in which excess unlabeled RNA is incubated in the binding reaction, decreasing the signal of the specific interaction.

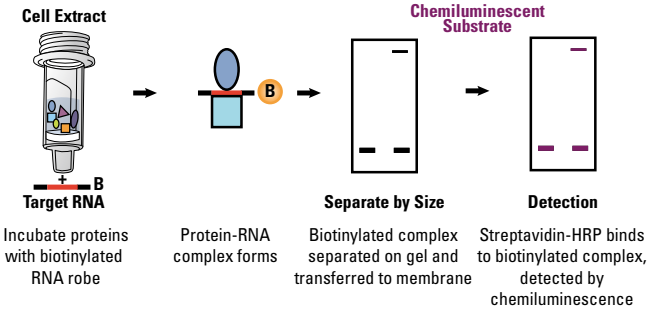


Figure 17. Schematic of the RNA electrophoretic mobility-shift assay.

Highlights:

- **Sensitive** – chemiluminescent detection is comparable to radioactive detection
- **Time-saving** – perform the assay from start to finish in less than 1 day
- **Flexible** – compatible with RNA labeled by multiple methods
- **Easy to use** – assay is compatible with cell lysates
- **Non-radioactive** – eliminate radioactive waste concerns

The chemiluminescent RNA EMSA kit contains all the reagents needed for enrichment and detection of the protein-RNA interaction. To perform an RNA EMSA, the biotinylated RNA-probe of interest and a protein source, either from a cell lysate or *in vitro* translation, are required. Biotinylated RNA probes may be acquired commercially, generated by run-off transcription with biotinylated nucleotide, or labeled with the Thermo Scientific Pierce RNA Biotin 3´ End-labeling Kit (Product # 20160). End-labeled RNA probes ensure minimal interference with RNA secondary structure and protein interactions.

This kit includes a positive control RNA-protein complex, which is formed and detected in parallel with the experimental sample. The positive control system for the RNA EMSA is the IRE (iron-responsive element)/IRP (iron-responsive protein) RNA-protein interaction. The IRP responds to cellular iron status. Under iron-starved conditions, IRP remains bound to the IRE RNA, suppressing translation of the iron-storage protein, ferritin, and transferrin iron receptor; under iron-rich conditions, IRE binding activity is lost, and ferritin and transferrin are translated. This system is ubiquitous and yields a robust band shift (Figure 18). Incubating the positive control reaction with a 200-fold molar excess of unlabeled IRE RNA reduced the band-shift signal by 70%, indicating specificity (Figure 18); however, incubating the control reaction with a similar fold excess of an unrelated RNA did not significantly reduce the band shift (Figure 18). These results demonstrate the robustness, sensitivity and specificity of the IRE/IRP positive control.

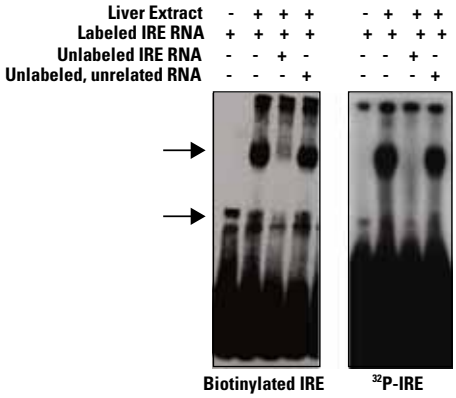


Figure 18. The IRE/IRP RNA EMSA positive control is sensitive and specific. Biotinylated IRE: For the binding buffer reaction, 5 nM (5.1 fmol) of biotinylated IRE RNA was incubated with 4 µg of cytosolic liver extract (containing IRP) in 1X REMSA Binding Buffer, 5% glycerol, and 2 µg of tRNA for 30 minutes at room temperature. Unlabeled RNA (1 µM) was added for the competition reaction, and an unrelated unlabeled RNA (telomerase RNA) was added to demonstrate specificity. Reactions were resolved on a native 6% polyacrylamide gel in 0.5X TBE and transferred to a nylon membrane. Band shifts were detected using the chemiluminescent detection module. **^{32}P-IRE:** A functional gel-shift assay was performed using ^{32}P-end-labeled IRE (5 nM), liver cell extract (5 µg) and the same biotinylated-IRE binding buffer conditions as described above. Densitometry was performed on the scanned gels.

In addition to avoiding radio-labeled nucleotides, a biotinylated RNA probe provides comparable sensitivity to radioactivity with faster detection. The sensitivity of biotinylated RNA probe was compared to a radio-labeled probe by performing RNA EMSAs with different probe concentrations. Both biotinylated and radio-labeled probe band shifts were robust and specific (Figure 19); however, the signal was amplified using the biotinylated IRE-RNA

probe after a short exposure (20 minutes) when compared to the band-shift signal and exposure (16 hours with intensifying screen) with the ^{32}P-labeled probe. Both labeling methods were sensitive to the attomole range (Figure 19).

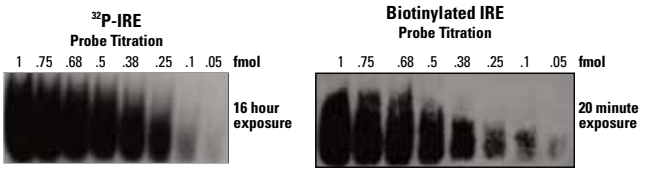


Figure 19. Chemiluminescent detection has comparable sensitivity to radioactive detection. The biotinylated IRE RNA probe was diluted to 50 amol. To generate ^{32}P-IRE, IRE (200 pmol) was labeled with γ-^{32}P ATP using T4 polynucleotide kinase, and then diluted to the same concentration as the biotinylated RNA.

To demonstrate the flexibility of the optimization buffers and assay system, three known protein-RNA interactions were tested: 1) Telomerase RNA (hTR; 451 nucleotides) with telomerase reverse transcriptase (TERT); 2) Let-7 miRNA (100 nucleotides) and the Lin28 protein; and 3) RNA template for RNA polymerase (42 nucleotides) and bacterial RNA polymerase core enzyme. The hTR and Let-7 RNA were labeled using run-off transcription and biotinylated UTP. For the RNA polymerase-binding reaction, the RNA template was end-labeled using the Pierce RNA 3′ End Biotinylation Kit (Product # 20160). TERT and Lin28 proteins were obtained from over-expression lysates (OriGene Technologies). The RNA polymerase was purified from bacteria. The resulting band shifts (Figure 20) demonstrate that the binding buffer, accessory components, and detection module are suitable for various RNA-protein interactions using different RNA labeling methods and protein sources.

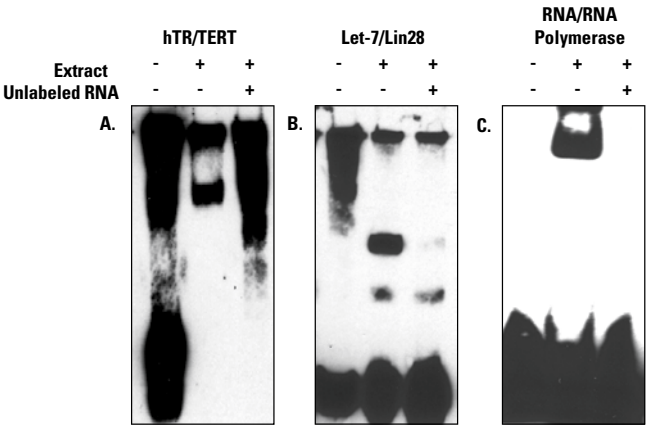


Figure 20. Chemiluminescent REMSA accommodates different RNA and protein sources. Run-off transcription: Plasmid constructs were generated, and run-off transcription was performed using biotin-11-UTP or unlabeled UTP for hTR and Let-7. **3′-end-labeling:** RNA for the RNA polymerase reaction was end-labeled using T4 RNA ligase and a modified biotinylated cytidine. RNA probes were purified and incubated with lysates (TERT, Lin28) or purified RNA polymerase in 1X Binding Buffer. For Let-7/Lin-28, additional DTT and KCl were added, and the glycerol concentration was 2.5% for hTR/TERT. A 50- to 100-fold molar excess of unlabeled RNA was used for the competition reactions. **A.** hTR/TERT, **B.** Let-7/Lin28, **C.** RNA/RNA polymerase.

The LightShift Chemiluminescent RNA EMSA Kit performed comparably to radio-labeled EMSA. The advantages of the system include the flexibility, sensitivity, specificity, and use of non-radio-labeled RNA. The kit works robustly in a variety of systems, including coding and non-coding RNA-protein interactions, various RNA lengths and labeling methods, and purified protein as well as cell lysates.

References

Leibold, E.A. and Munro, H.M. (1988). Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc Natl Acad Sci USA*. **85**:2171-5.

McKinley, B.A. and Sukhodolets, M.V. (2007). *Escherichia coli* RNA polymerase-associated SWI/SNF protein RapA: evidence for RNA-directed binding and remodeling activity. *Nucleic Acids Res* **35**:7044-60.

O'Connor, C.M., *et al.* (2005). Two purified domains of telomerase reverse transcriptase reconstitute sequence-specific interactions with RNA. *J Biol Chem* **280**:17533-9.

Piskounova, E., *et al.* (2008). Determinants of microRNA processing by the developmentally regulated RNA binding protein Lin28. *J Biol Chem* **283**:21310-4.

Sukhodolets, M.V. and Jin, D.J. (1998). RapA, a novel RNA polymerase-associated protein, is a bacterial homology of SWI2/SNF2. *J Biol Chem* **273**:7018-23.

Ordering Information

Product #	Description	Pkg. Size
20158	**LightShift Chemiluminescent RNA EMSA (REMSA)** *Sufficient components for 100 binding reactions and sufficient detection reagents for approximately 1,000 cm² of membrane* Includes: Biotinylated IRE RNA Control Unlabeled IRE RNA Control Cytosolic Liver Extract tRNA REMSA Binding Buffer (10X) Glycerol, 50% KCl, 2 M $MgCl_2$, 1 M DTT, lyophilized Nuclease-Free Water REMSA Loading Buffer (5X) Stabilized Streptavidin-Horseradish Peroxidase Conjugate Luminol/Enhancer Solution Stable Peroxide Solution Nucleic Acid Detection Blocking Buffer Wash Buffer (4X) Substrate Equilibration Buffer	Kit
89880	**Chemiluminescent Nucleic Acid Detection Module** Includes: Stabilized Streptavidin-Horseradish Peroxidase Conjugate Luminol/Enhancer Solution Stable Peroxide Solution Nucleic Acid Detection Blocking Buffer Wash Buffer (4X) Substrate Equilibration Buffer	Kit 1.5 ml 80 ml 80 ml 500 ml 500 ml 500 ml
Related Product		
20159	**tRNA, 10 mg/ml**	100 ml

RNA 3′ Biotinylation Kit

A non-radioactive, non-interfering RNA labeling method.

Regulation of cellular function is dependent on critical RNA interactions with proteins and other RNA, including miRNA. These interactions have been difficult to isolate and highly dependent on maintaining RNA secondary structure. To enrich for these interactions it is often necessary to label the RNA. The Thermo Scientific Pierce RNA 3′ End Biotinylation Kit enables rapid non-radioactive RNA labeling with minimal interference to the RNA secondary structure.

Highlights:

- **Non-radioactive** – incorporates a biotin label with detection sensitivity comparable to radioactivity
- **Fast** – RNA can be labeled in 0.5-2 hours with minimal downstream processing
- **Easy to use** – RNA ligase and optimized reaction buffer are included
- **Single label** – results in minimal disturbance of RNA secondary structure
- **Flexible** – labels synthetic and *in vitro* transcribed RNA with 22–450 nucleotides

The Pierce RNA 3′ Biotinylation Kit contains T4 RNA ligase to attach a single biotinylated nucleotide to the 3′ terminus of an RNA strand. The unique feature of this kit is the biotinylated cytidine (bis)phosphate, which contains a 3′, 5′ phosphate on the ribose ring to accommodate the ligation reaction and a biotin on the cytidine for detection (Figure 21). The kit also contains a non-labeled RNA strand as a positive control and a biotinylated-RNA probe to quantitate labeling efficiency. To enhance biotinylation efficiency and RNA stability, RNase inhibitor, glycogen, and ligation-enhancing reagents are included.

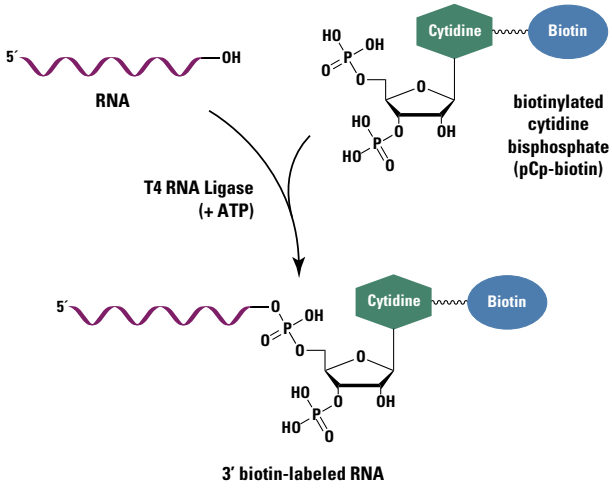


Figure 21. Scheme of T4 RNA ligation reaction. RNA ligation using T4 RNA igase requires a 3′-OH (from the desired RNA), and a 3′, 5′ nucleotide (bis)phosphate. The Pierce 3′ RNA Labeling Kit contains T4 RNA ligase and a cytidine (bis)phosphate nucleotide with a biotinylated linker for detection.

The kit provides a starting point for the ligation reaction. Typically, the reaction requires 50 pmol of RNA is ligated with a 20-fold excess of biotinylated nucleotide for 2 hours at 16°C; however, short RNAs with a minimally complicated secondary structure can be ligated in 30 minutes at 37°C (e.g., RNA polymerase RNA template). Large or structurally complex RNAs require more incubation time (e.g., Let-7 and hTR) (Table 5). Further optimization is achieved by altering the ligation ratio, increasing the incubation time, or using DMSO to relax RNA structure. Once biotinylated, the labeled RNA is easily precipitated to remove reaction byproducts. The probe can then be used in downstream applications such as RNA electrophoretic mobility shift assays (RNA REMSA, Product # 20158), RNA pull-down assays and miRNA profiling.

Table 5. Labeling efficiency of different RNA sources and lengths.

RNA	Type	RNA source	Length (bp)	Efficiency (%)§	Method Notes	Reference
IRE (iron-responsive element)	3′ or 5′ UTR element	Synthetic	28	76	2 hours at 16°C	Leibold, *et al.*
RNA polymerase template	RNA	Synthetic	42	80†	2 hours at 16°C	McKinley, *et al.*
Mir-16-1 miRNA	Mature microRNA	Synthetic	22	70	Overnight at 16°C	www.mirbase.org
TNF ARE	3′ UTR element	Synthetic	37	77	2 hours at 16°C	Hall-Pogar, *et al.*
Let-7 pre-miRNA	Pre-micro RNA	*in vitro* transcribed	~70	70	Overnight at 16°C	Piskounova, *et al.*
hTR (telomerase RNA)	Catalytic RNA	*in vitro* transcribed	350	74	Overnight at 16°C	O'Connor, *et al.*

§ *Ligation efficiency was determined by densitometry analysis on dot blots from three separate ligation reactions using serial dilutions of probe. Synthetic biotinylated RNA was used as the control, and concentrations were normalized.*

† *RNA polymerase template RNA may be ligated at 37°C for 30 minutes with > 80% efficiency.*

To monitor the effectiveness of RNA biotinylation, the control RNA labeling efficiency was assessed by two methods, dot blotting with the Thermo Scientific Chemiluminescent Nucleic Acid Detection Module (Product # 89880) or spectroscopically with the Thermo Scientific Fluorescent Biotin Quantitation Kit (Product # 46610). Biotin end-labeled IRE and RNA polymerase template RNAs have comparable sensitivity to a synthetically biotinylated RNA (Figure 22). The 50 pmol reaction provided more than enough RNA to perform an EMSA, and both RNAs could be diluted at least 25- to 50-fold (5-10 nM).

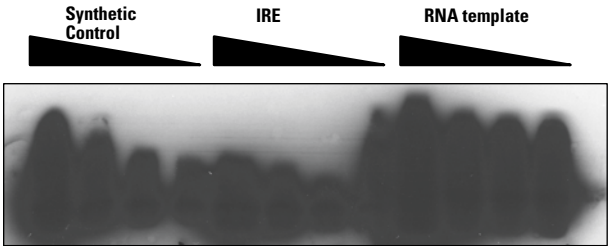


Figure 22. End-labeled RNAs have similar sensitivity to synthetically labeled RNA. Two end-labeled RNA probes and a synthetic biotinylated RNA probe were electrophoresed on a 5% acrylamide/8M urea gel, transferred to a nylon membrane, and detected using the Thermo Scientific Chemiluminescent Detection Module (Product # 89880). RNA probe concentrations were normalized and diluted 1:5, 1:20, 1:50 and 1:100. Triangles represent decreasing concentration. The membranes were exposed to film for 1.5 minutes.

RNA lengths vary from 18 to 22 nucleotides (miRNA) to hundreds of bases with a variety of secondary structures. RNA probes were derived synthetically as well as transcribed *in vitro* using T7 RNA polymerase. The different RNA used in this study had diverse secondary structure and lengths. Each of the four synthetic and both *in vitro* transcribed RNAs had ligation efficiencies > 70%, demonstrating that the kit is flexible and efficient biotinylation for RNA of different length, secondary structure, and source (Table 5).

Once labeled, a biotinylated RNA probe can be used as bait to study RNA interactions with other molecules. The biotin group enables purification and detection using biotin-binding proteins immobilized on a solid support or conjugated to enzymes such as horseradish peroxidase (HRP). To demonstrate functionality, the biotinylated RNA probes were incubated with extract containing a RNA binding protein of interest and processed with the Thermo Scientific LightShift Chemiluminescent RNA EMSA Kit (Product # 20158). Biotinylated IRE, RNA polymerase template, and Let-7 pre-miRNA probes functionally bound their respective RNA binding proteins, IRP, RNA polymerase, and Lin28 (Figure 23). RNA 3′ end biotinylation produces a stable probe that is effective for a longer time period than radiolabeled probes. Furthermore, end-labeling minimally disrupts secondary structure, can reduce background, and is much less expensive compared to synthetically labeled RNA.

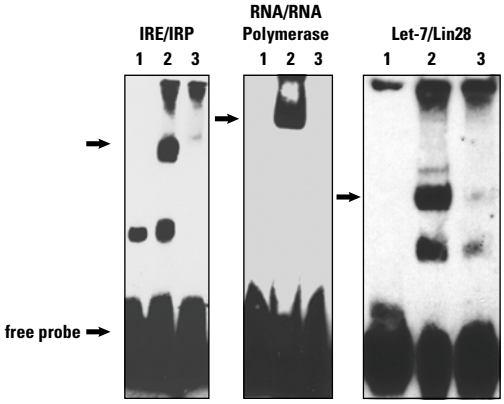


Figure 23. Biotinylated end-labeled RNA functionally binds RNA binding proteins. Probes (5-10 nM) were incubated with 4-5 µg of cell extract in the binding reaction. EMSA was performed using reagents from the LightShift Chemiluminescent RNA EMSA Kit (Product # 20158). **Exposure times**: IRE-IRP – 2 minutes; RNA-RNA polymerase – 1 minute; Let-7-Lin28 – 5 minutes. **Lane 1:** Free probe; **Lane 2:** Control binding reaction; **Lane 3:** Control binding reaction plus 100-fold excess of unlabeled probe.

References

Hall-Pogar, T., *et al.* (2007). Specific trans-acting proteins interact with auxiliary RNA polyadenylation elements in the COX-2 3′-UTR. *RNA* **13**:1103-15.

Leibold, E.A. and Munro, H.M. (1988). Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light subunit mRNAs. *Proc Natl Acad Sci* USA **85**:2171-5.

McKinley, B.A., and Sukhodolets, M.V. (2007). *Escherichia coli* RNA polymerase-associated SWI/SNF protein RapA: evidence for RNA-directed binding and remodeling activity. *Nucleic Acids Res* **35**:7044-60.

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Ueda, C.T. and Roberts, R.W. (2004). Analysis of a long range interaction between conserved domains of human telomerase RNA. *RNA* **10**:139-47.

www.mirbase.org

Ordering Information

Product #	Description	Pkg. Size
20160	Pierce RNA 3′ End Biotinylation Kit	Kit

Chemiluminescent Transcription Factor Assay Kits

Measure transcription factors by capturing them using their specific DNA binding sequences.

Thermo Scientific Chemiluminescent Transcription Factor Assay Kits are complete systems containing all the necessary components to measure activated transcription factors in a microplate format. Kits contain streptavidin-coated 96-well plates with the bound biotinylated-consensus sequence for the respective factor. The biotinylated-consensus duplexes bind only the active forms of transcription factors, producing greater signal-to-noise ratios than a traditional ELISA. The captured active transcription factor bound to the consensus sequence is incubated with specific primary antibody (NF-κB p50, NF-κB p65 or c-Fos) then with a secondary HRP-conjugated antibody. After addition of Thermo Scientific SuperSignal Substrate to the wells, a signal is detected using a luminometer or CCD camera.

Transcription factors are proteins that bind to promoter regions of genes to regulate their levels of expression. Defects in transcription factor regulation, structure and/or function have been implicated in numerous human diseases such as cancer and inflammation. The ability to screen for transcription factor activation is, therefore, important to drug discovery as well as to gene regulation studies.

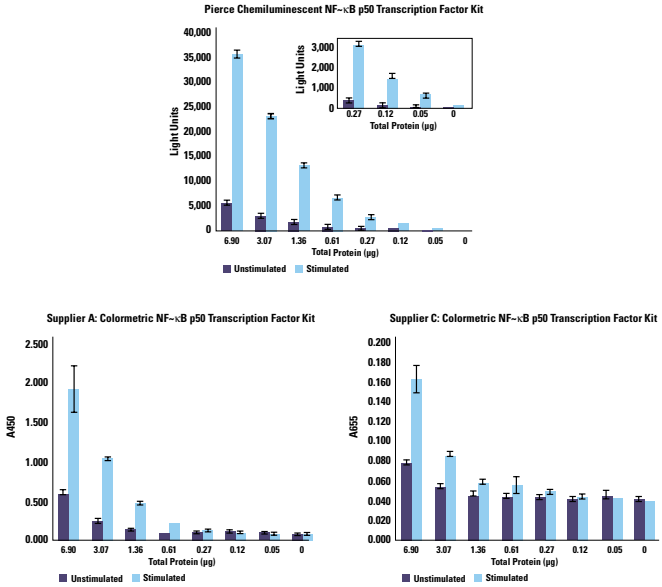
Traditional methods for measuring active transcription factors include gel-shift or electrophoretic mobility shift assays (EMSAs) and colorimetric ELISA-based assays. EMSAs may involve radioactivity and are not amenable to high-throughput applications. Colorimetric ELISA-based assays have increased speed and throughput, but they have limited sensitivity. Thermo Scientific NF-κB p50, NF-κB p65 and c-Fos Transcription Factor Kits are chemiluminescent ELISA-based assays that provide unsurpassed sensitivity and convenience over the existing methodologies.

Highlights:

- **Sensitivity** – SuperSignal Substrate yields results 20- to 140-times more sensitive than the colorimetric methods with a broader dynamic range
- **Fast** – format allows researcher to perform multiple tests in three to four hours
- **Reliable** – each kit contains a positive control lysate, and wild type and mutant competitor duplexes
- **Compatible with high-throughput systems** – 96-well format increases sample throughput over traditional EMSAs
- **Convenient** – kits contain all the reagents for two 96-well assay plates
- **Versatile** – signal is detectable using a luminometer or CCD Camera

References

1. Karin, M., *et al.* (2002). *Nature Rev. Cancer* **2(4),** 301-310.
2. Shaulian, E. and Karin, M. (2001). *Oncogene* **20(19),** 2390-2400.
3. Baldwin, A.S., Jr. (1996). *Annu. Rev. Immunol.* **17(1),** 3-9.
4. Karin, M. and Lin, A. (2002). *Science* **3(3),** 221-227.
5. Benotmane, A.M., *et al.* (1997). *Anal. Biochem.* **250(2),** 181-185.
6. McKay, I.A., *et al.* (1998). *Anal. Biochem.* **265(1),** 28-34.
7. Renard, P., *et al.* (2001). *Nucleic Acids Res.* **29(4),** e21.
8. Brivanlou, A.H. and Darnell, J.E., Jr. (2002). *Science* **295,** 813-818.



Sensitivity comparison of the Thermo Scientific Pierce NFκ B p50 Kit and other supplier's kits using nuclear extract. Various amounts of TNF-α induced and uninduced HeLa cell nuclear fractions were incubated and detected using the chemiluminescent Pierce NF-κB p50 Kit and colorimetric kits from Suppliers A and C. Chemiluminescent results were detected using a luminometer. Colorimetirc results were detected using a plate reader set to the respective wavelengths based on the manufacturer's product instructions. Error bars represent 1 standard deviation.

Ordering Information

Product #	Description	Pkg. Size
89858	**NF-κB p50 Transcription Factor Kit**	2 plates/kit
89859	**NF-κB p65 Transcription Factor Kit**	2 plates/kit

Each of the above-listed kits contains all reagents needed for two 96-well assay plates.

Includes:	
96-well assay plates	2 plates
Binding buffer (5X)	2 x 1.3 ml
Poly dI•dC (20X)	700 µl
Primary antibody	24 µl
Antibody dilution buffer	60 ml
HRP-conjugated Secondary Antibody	varies
Luminol/Enhancer solution	12 ml
Stable peroxide solution	12 ml
Plate sealers	2
Wash buffer (10X)	48 ml
Wild type and mutant duplex, 10 pmole/µl	72 µl
Activated HeLa cell nuclear extract (Positive Control)	20 µl

ChIP Products

Get highly reproducible ChIP results in 7.5 hours.

Chromatin immunoprecipitation (ChIP) is an effective method for identifying links between the genome and the proteome by monitoring transcription regulation through histone modification (epigenetics) or transcription factor-DNA binding interactions. The strength of the ChIP assay is its ability to capture a snapshot of specific protein-DNA interactions as they occur in living cells, and then quantitate the interactions using standard or quantitive PCR. The Thermo Scientific Pierce Agarose ChIP Kit provides a simple, fast and reproducible method to perform ChIP assays (Figure 24).

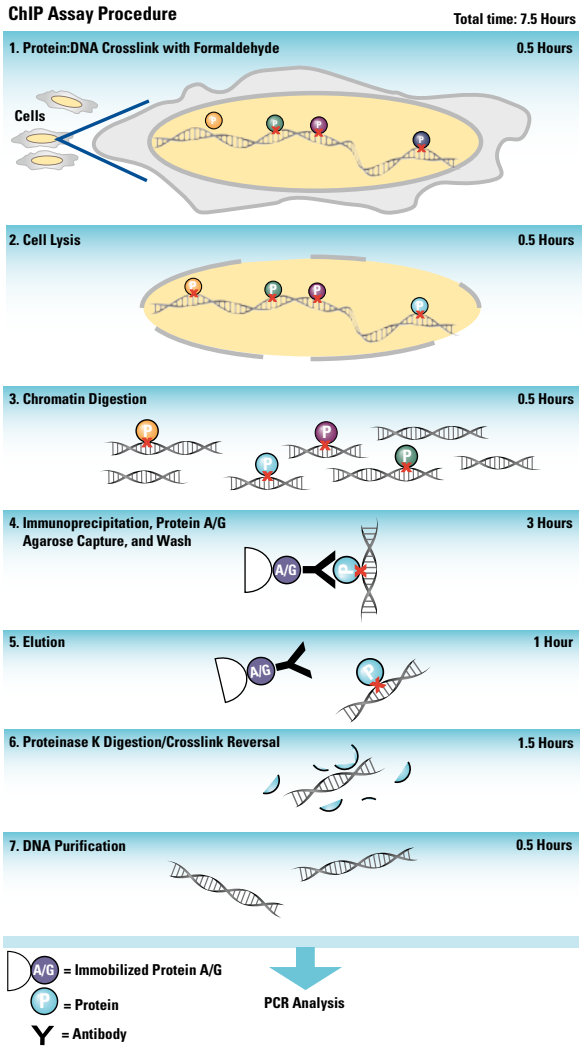


Figure 24. Schematic of the ChIP protocol.

Highlights:

- Simple and fast protocol
- Highly efficient isolation and lysis of nuclei
- Easy and reproducible enzymatic digestion
- Low-background and high-binding capacity Protein A/G agarose resin
- Highly specific RNA polymerase II antibody and GAPDH PCR primers included as positive controls
- Fast and reproducible spin-column format
- High-recovery DNA purification

To perform a ChIP assay using the Pierce Agarose ChIP Kit, protein:DNA complexes are immobilized and then extracted. *In vivo* crosslinking is achieved with formaldehyde. Crosslinking, when performed directly in cells, locks in the protein:DNA complexes, trapping these unstable and sometimes transient interactions. To lyse, extract and solubilize the crosslinked complexes, the kit includes the Chromatin Prep Module (also sold separately, Product # 78840). These reagents provide a simple, reliable and convenient means for isolating chromatin-bound DNA without a Dounce homogenizer. With less than 15% contamination from other cellular compartments, the protein of interest is efficiently enriched.

To analyze protein-binding sequences, the genomic DNA must be sheared into smaller, workable pieces, which is usually achieved by sonication or enzymatic digestion. Because sonication often produces variable and hard-to-duplicate results, the Pierce Agarose ChIP Kit includes a specially titrated and tested micrococcal nuclease to digest the DNA. The advantages of enzymatic digestion include reproducibility, reaction control and easy titration of the enzyme for specific cell types (Figure 25).

Figure 25. Micrococcal nuclease digestion is more reproducible than sonication for shearing crosslinked chromatin. HeLa cells were cultured in DMEM containing 10% FBS. Crosslinking was achieved using 1% formaldehyde in the media for 10 minutes. Nuclei were isolated using the Thermo Scientific Chromatin Prep Module and divided into four equal samples. Samples 1 and 2 were processed using a Branson Sonifier®, and enzyme digestion was per-formed on samples 3 and 4. Following nuclei lysis, crosslinking was reversed and samples were electrophoresed on a 1.2% agarose gel containing SYBR green for 1 hour. Gels were imaged with a UV light.

To isolate a specifically modified histone, transcription factor or co-factor, ChIP-validated antibodies are used to immunoprecipitate and isolate the protein:DNA complexes from other nuclear components. Antibody-antigen complexes are recovered using Protein A/G immobilized on agarose. The Pierce Agarose ChIP Kit uses a specially blocked ChIP-Grade Pierce Protein A/G Plus Agarose Resin, which provides high-binding capacity and low background. To monitor kit performance, a highly specific ChIP-grade antibody to RNA polymerase II and positive control primers to the GAPDH promoter are also provided to determine the relative binding of RNA polymerase II in the control IP.

To remove nonspecific protein and DNA binding to the resin, a series of washes is performed followed by an elution of the specific antibody-antigen-DNA complex from the resin. Traditionally, the resin is washed in a microcentrifuge tube, centrifuged and the wash buffer removed by pipetting. The Pierce Agarose ChIP Kit includes spin columns for increasing assay speed and handling convenience, while reducing sample loss.

The DNA:protein link must be reversed before quantitating the immunoprecipitated DNA. The supplied columns and optimized helps to streamline the complete crosslink reversal, protein digestion and DNA purification with minimal time and sample handling. When compared with other suppliers' kits, our kit had greater specific signal and less background (Figure 26).

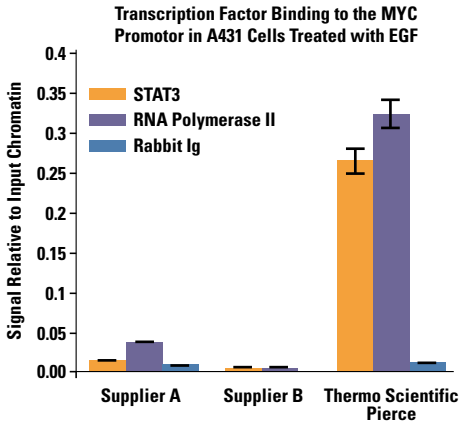


Figure 26. The Thermo Scientific Pierce Agarose ChIP Kit has greater specific signal and less background than other kits. A431 lung carcinoma cells were cultured in DMEM containing 10% FBS for 24 hours. Following a 24 hour serum withdrawal, half of the cultures plated were treated with 100 ng/ml EGF for 10 minutes. Crosslinking was achieved using 1% formaldehyde in the media for 10 minutes. ChIP assays were performed according to the manufacturers' protocols. Quantitative real-time PCR data was obtained with a Bio-Rad iQ5 Thermocycler, ABsolute™ QPCR SYBR® Green Fluorescein master mix, and primers designed to amplify a region of the human MYC promoter proximal to the transcription start site.

A hallmark of the ChIP assay is the ability to quantitate DNA binding by real-time PCR because there is a direct correlation of the immunoprecipitated complex to the bound DNA. We used the Pierce Agarose ChIP Kit to profile multiple transcription factors binding to the human MYC promoter in EGF-treated A431 lung carcinoma cells (Figure 27). In this experiment, several transcription factors differentially bound in response to growth factor stimulation. The histone acetyl-transferase CBP does not contain a DNA-binding domain; however, CBP does bind to STAT3, suggesting the increase in CBP binding to the MYC promoter is from recruitment by STAT3, which directly binds DNA. This highlights the sensitivity of the Pierce Agarose ChIP Kit, which was able to profile a difficult-to-detect, second-order protein:protein:DNA interaction *in vivo*.

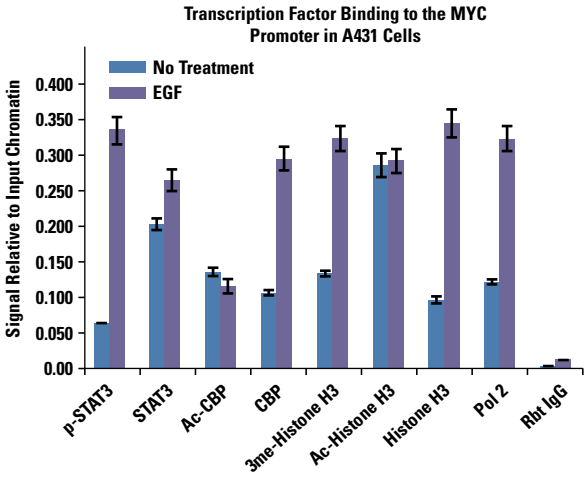


Figure 27. The Thermo Scientific Pierce Agarose ChIP Kit is effective for profiling multiple transcription factors and their binding sites. Crosslinked A431 cells were prepared as described in Figure 22. Binding of phosphorylated-STAT3 (p-STAT3), acetylated-CBP (Ac-CBP), CBP, trimethyl histone H3 (3me-Histone H3), acetylated-histone H3 (Ac-Histone H3), histone H3, and RNA polymerase II (Pol 2) to the proximal MYC promoter was determined using the Pierce Agarose ChIP Kit. Primary antibody amounts were determined empirically. Quantitative real-time PCR data was obtained as described in Figure 26.

References

Aparicio, O., *et al.* (2004). Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences *in vivo. Curr Protoc Cell Biol* UNIT **17.7**: 17.7.1-17.7.23.

Das, P.M., *et al.* (2004). Chromatin immunoprecipitation assay. *Biotechniques* **6**:961-9.

Lee, D.Y., *et al.* (2005). Role of protein methylation in regulation of transcription. *Endocr Rev* **26(2)**:147-70.

Wells, J. and Farnham, P.J. (2002). Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods* **1:48**-56.

Yuichi, Y. and Hughes, D.E. (2006). C/EBPα and HNF6 protein complex formation stimulates HNF6-dependent transcription by CBP coactivator recruitment in HepG2 cells. *Hepatology* **43(2)**:276-86.

Zeng, P.Y., *et al.* (2006). *In vivo* dual cross-linking for identification of indirect DNA-associated proteins by chromatin immunoprecipitation. *Biotechniques* **41**:694-8.

Ordering Information

Product #	Description	Pkg. Size
26156	**Pierce Agarose ChIP Kit**	Kit
	Sufficient reagents for 30 ChIP reactions	
	Includes: ChIP Grade Protein A/G Plus Agarose	0.65 ml
	IP Dilution/Wash Buffer (5X)	11 ml
	IP Wash Buffer 3 (5X)	4.5 ml
	IP Elution Buffer (2X)	4.5 ml
	Column Accessory Pack, 10 spin columns, 20 collection tubes and 10 plugs	3 each
	Microcentrifuge Tubes 75	1.5 ml
	DNA Clean-Up Columns	40
	DNA Column Binding Solution,	30 ml
	DNA Column Wash Solution	6 ml
	pH Indicator	0.8 ml
	DNA Column Elution Solution	5 ml
	Anti-RNA Polymerase II Antibody	25 µl
	Normal Rabbit IgG (1 mg/ml)	10 µl
	ChIP Positive Control Primers (GAPDH promoter)	100 µl
	Pierce Chromatin Prep Module (Product # 26158)	
26158	**Pierce Chromatin Prep Module**	Kit
	Sufficient reagents for 30 chromatin preparations	
	Includes: Membrane Extraction Buffer	15 ml
	Nuclear Extraction Buffer	15 ml
	MNase Digestion Buffer	5 ml
	MNase Stop Solution	0.5 ml
	Halt™ Protease and Phosphatase Inhibitor Cocktail EDTA-free (100X)	4 x 90 µl
	Sodium Chloride (5 M)	3 ml
	Glycine Solution (10X)	15 ml
	PBS (20X)	15 ml
	Micrococcal Nuclease (ChIP Grade) (10 U/µl)	25 ml
	Proteinase K (20 mg/ml), DTT, Lyophilized	0.25 ml 1 vial
26159	**ChIP-Grade Protein A/G Plus Agarose**	0.65 ml
26160	**Proteinase K (20 mg/ml)**	0.25 ml

Crosslinking 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 crosslinking 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 crosslinkers that have a photo-reactive 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 crosslinker. 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 crosslinker is photo-reactive. The photo-reactive group of the heterobifunctional reagent will ultimately crosslink the site at which the target protein binds when the resulting complex is exposed to light. Typically, the photo-reactive 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. Photo-reactive, heterobifunctional reagents with a cleavable disulfide linkage allow reversal of the protein:nucleic acid crosslink 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 crosslinking 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 Thermo Scientific Pierce 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 28).

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 heterobifunctional reagent Sulfo-SMCC (Product # 22322). We offer HRP and AP enzymes that have been made sulfhydryl-reactive (i.e., maleimide-activated) by this mechanism (Product # 31485 and # 31486, respectively).

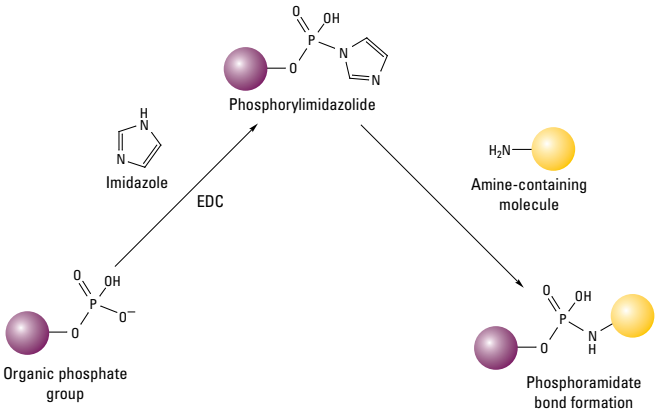


Figure 28. Reaction scheme showing typical route to conjugating an amine-containing biomolecule to a 5´ terminal phosphate group.

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 Thermo Scientific Sodium *meta*-Periodate (Product # 20504). The aldehydes then can be conjugated to the amine groups on a protein using reductive amination with Thermo Scientific 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 Thermo Scientific 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.

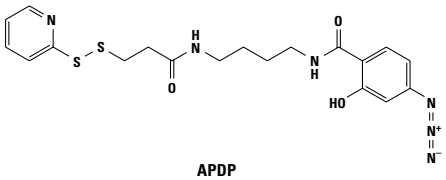
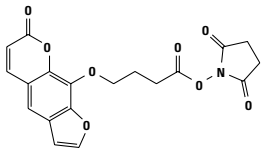
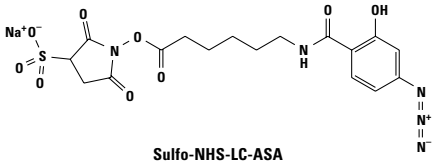
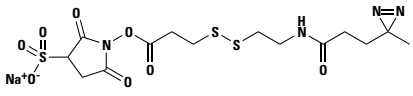
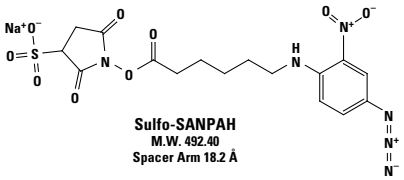
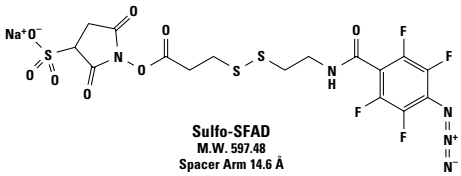
Biotinylation of oligonucleotides can also be done using photo-reactive reagents. There are two main options commonly used to add one or more biotin residues to nucleic acid probes. Thermo Scientific Photoactivatable Biotin (Product # 29987) contains a phenyl azide group at the end of a spacer arm with 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, Thermo Scientific Psoralen-PEG-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-PEG-Biotin contributes excellent water solubility, thus assuring that the resultant derivative will have accessibility to streptavidin-containing detection reagents.

Additionally, enzymatic biotinylation of DNA (Product # 89818) or RNA (Product # 20160) provides 3' end labeling. This method reduces interference of complex formation, which can occur with internal biotin labeling.

Crosslinking Agents

This section covers the Thermo Scientific Pierce Crosslinking 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. Reagents that have been applied or with potential application to the study of protein:nucleic acid interactions are listed on the next page.

Ordering Information

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
27720	**APDP** *N*-[4-(*p*-Azido-salicylamido) butyl]-3´-(2´-pyridyldithio) propionamide	 **APDP** M.W. 446.55 Spacer Arm 21.0 Å	• –SH group reactive • Iodinatable • Cleavable • Reaction with nucleic acid binding protein monitored at 343 nm	4	50 mg
23013	**SPB** Succinimidyl-(4-[psoralen-8-yloxy])-butyrate	 **SPB** M.W. 385.32 Spacer Arm 8.9 Å	• Amine-reactive • DNA intercalacting 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 **See also: NHS-ASA (Product # 27714)*	 **Sulfo-NHS-LC-ASA** M.W. 491.41 Spacer Arm 18.0 Å	• Can incorporate ^{125}I label before acylation step • Photolysis initiated by long-wave UV • Water-soluble • Non-cleavable • Amine-reactive	10	50 mg
26175	**Sulfo-SDAD** (sulfosuccinimidyl 2- [(4,4'-azipentanamido) ethyl]-1,3'-dithiopropri-onate)	 **Sulfo-SDAD** Sulfo-NHS-SS-Diazirine Spacer Arm 13.5 Å			50 mg
22589	**Sulfo-SANPAH*** Sulfosuccinimidyl 6-(4-azido-2'-nitrophenyla-mino) hexanoate **See also: SANPAH (Product # 22600)*	 **Sulfo-SANPAH** M.W. 492.40 Spacer Arm 18.2 Å	• Optimal photolysis occurs at 320-350 nm limiting damage to biomolecules by irradiation • Water-soluble • Non-cleavable • Amine-reactive	12	50 mg
27719	**Sulfo-SFAD** Sulfosuccinimidyl(perfluoro azidobenzamido)ethyl-1,3´-dithiopropionate	 **Sulfo-SFAD** M.W. 597.48 Spacer Arm 14.6 Å	• Improved photoconjugation, efficiency • Photolyzes at 320 nm • Water-soluble • Cleavable • Amine-reactive	5,6	50 mg

References

1. DNA-Protein Interactions; Principles and Protocols, (1994). G. Geoff Kneale, Ed. Humana Press, p. 427.
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.
3. Kaplan, K.B. and Sorger, P.K. (1997). In Protein Function, 2nd edition, T.W. Creighton, Ed., The Practical Approach Series, IRL Press, pp. 245-278.
4. Burgess, B.R. and Richardson, J.P. (2001). *J. Biol. Chem.* **286(6)**, 4182-4189.
5. Pandurangi, R.S., *et al.* (1998). *Bioorganic Chem.* **26(4)**, 201-212.
6. Pandurangi, R.S., *et al.* (1997). *Photochem. Photobiol.* **65(2)**, 208-221.
7. Laskin, J.D., *et al.* (1986). *Proc. Natl. Acad. Sci. USA* **83**, 8211-8215.
8. Inman, R.B. and Schnos, M. (1987). *J. Mol. Biol.* **193,** 377-384.
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.

Alkaline Phosphatase

Ready-to-conjugate preparation.

Highlight:

- Specific activity 2,000 units/mg

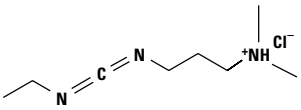
Ordering Information

Product #	Description	Pkg. Size
❄ 31391	Alkaline Phosphatease	20 mg
❄ 31392	Alkaline Phosphatease	100 mg

❄ *Additional dry ice and/or freight charge.*

EDC

Activates phosphate groups on oligos.



EDC
M.W. 191.70

Highlight:

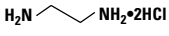
- Water-soluble carbodiimide that activates phosphate groups on oligos and carboxylate groups on proteins similarly. Coupling occurs via amine groups on enzymes such as HRP or AP, forming a stable phosphoramidate linkage.

Ordering Information

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

Ethylenediamine Dihydrochloride

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



Ethylenediamine
M.W. 133.02

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

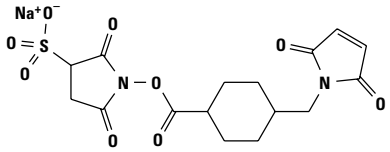
Ordering Information

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

❄ *Additional dry ice and/or freight charge.*

Sulfo-SMCC

Yields stable protein:nucleic acid conjugates.



Sulfo-SMCC
M.W. 436.37
Spacer Arm 8.3 Å

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

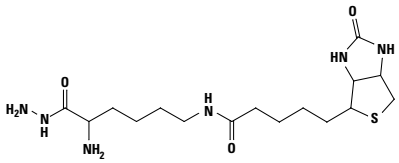
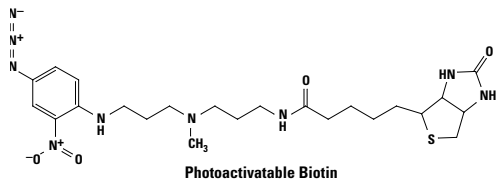
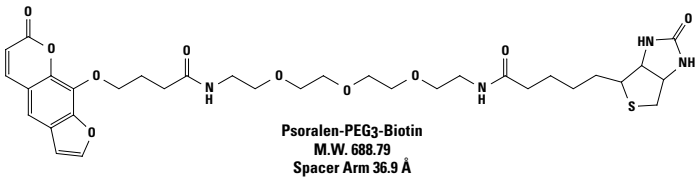
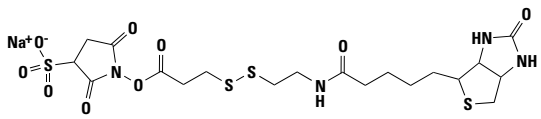
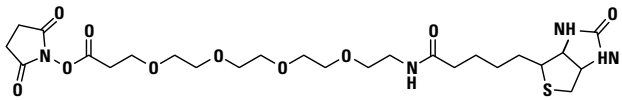
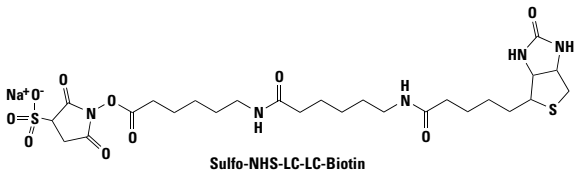
Product #	Description	Pkg. Size
✖ 20504	**Sodium *meta*-Periodate**	25 g
✖ 44892	**AminoLink Reductant** (Sodium cyanoborohydride)	2 x 1 g

✖ *Additional hazardous charge.*

Nucleic Acid-Biotin Conjugates

See the biotinylation section at www.thermo.com/pierce for our complete selection of Thermo Scientific EZ-Link Reagents.

Ordering Information

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
28020	Biocytin Hydrazide	 Biocytin-Hydrazide M.W. 386.51 Spacer Arm 19.7 Å	• Carbohydrate-reactive • Used to label DNA and RNA through cytosine • Extended spacer arm • Forms hydrazone bond • Water-soluble	1	25 mg
29987	Photoactivatable Biotin	 Photoactivatable Biotin M.W. 533.65 Spacer Arm 30.0 Å	• Water-insoluble • Membrane-permeable • Modifies DNA/RNA • Covalent nonspecific coupling occurs in presence of UV light (350-370 mn)	2	0.5 mg
29986	Psoralen-PEG_3-Biotin	 Psoralen-PEG3-Biotin M.W. 688.79 Spacer Arm 36.9 Å	• 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
21331	NHS-SS-Biotin	 Sulfo-NHS-SS-Biotin M.W. 606.69 Spacer Arm 24.3 Å	• Water-soluble • Amine-reactive, forms stable amide bond • Cleavable spacer arm	6	100 mg
21362	NHS-PEG_4-Biotin	 NHS-PEG4-Biotin M.W. 588.67 Spacer Arm 29 Å	• Water-soluble spacer arm increases solubility of conjugate • Amine-reactive, forms stable amide bond	--	50 mg
21335	Sulfo-NHS-LC-Biotin	 Sulfo-NHS-LC-LC-Biotin M.W. 669.75 Spacer Arm 30.5 Å	• Water-soluble • Amine-reactive, forms stable amide bond	7	100 mg

References

1. Reisfeld, A., *et al.* (1987). *Biochem. Biophys. Res. Comm.* **142**, 519-526.
2. Forster, A.C., *et al.* (1985). *Nucleic Acids Res.* **13**, 745-761.
3. Henriksen, U., *et al.* (1991). *J. Photochem. Photobiol. A:Chem.* **57**, 331-342.
4. Oser, A., *et al.* (1988). *Nucleic Acids Res.* **16**, 1181-1196.
5. Wassarman, D.A. (1993). *Mol. Biol. Rep.* **17**, 143-151.
6. Leary, J.J., *et al.* (1983). *Proc. Natl Acad. Sci. USA* **80**, 4045-4049.
7. Shukla, S., *et al.* (2004). *J. Biol. Chem.* **279**, 13668-13676.

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 crosslinker. 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., Thermo Scientific Pierce 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.

Avidin-Biotin Technical Handbook

This guide brings together everything needed to biotinylate cell-surface proteins, purify a biotinylated target, detect a biotinylated antibody and perform many other applications. It includes dozens of references along with protocols, troubleshooting tips, selection guides and a complete listing of available tools. (Product # 1601675)

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.

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.

Selected Thermo Scientific products for use in the study of protein:nucleic acid interactions.

Product Name	Product #	Product Description
Pierce 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.
Streptavidin Agarose Resin	**20347** (2 ml gel) **20349** (5 ml gel) **20353** (10 ml gel)	Binds 15-28 µg biotin per ml of gel (1-3 mg biotinylated BSA per ml of resin).
High Capacity Streptavidin Agarose	**20357** (2 ml gel) **20359** (5 ml gel) **20361** (10 ml gel)	Binds ≥ 10 mg biotinylated-BSA per ml of resin.
MagnaBind™ Streptavidin Beads	**21344** (5 ml)	Binds 2 µg biotin per ml of beads. Allows magnetic separation. Supporting equipment also available.
Pierce Streptavidin Magnetic Beads	**88816** (1 ml) **88817** (5 ml)	Binds 550 µg biotinylated IgG/ ml beads
Pierce 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.
Pierce 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 Plus Immobilization Kit	**44894**	Binds primary amines. Standard buffer system and 5 x 2 ml columns included.
Pierce 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.*

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.

Thermo Scientific Pierce 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 DNA[1]
- Purification of double-stranded DNA[2]

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	Avidin Agarose Resin Support: Crosslinked 6% beaded agarose Spacer: None (directly attached) Supplied: 50% aqueous slurry containing 0.02% NaN_3)	5 ml
20362	Avidin Columns	5 x 1 ml
20225	Avidin Agarose Resin	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	Monomeric Avidin Agarose	5 ml
20267	Monomeric Avidin Agarose	10 ml
20227	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	Monomeric Avidin UltraLink® Resin	5 ml
29129	Biotin *Promotes the gentle elution of biotinylated complex from an immobilized monomeric avidin support.*	1 g

Immobilized NeutrAvidin Supports

Less nonspecific binding makes these exclusive 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	NeutrAvidin™ Agarose Resin	5 ml
29201	NeutrAvidin Agarose Resin	10 ml
29202	High Capacity NeutrAvidin Agarose Resin	5 ml
29204	High Capacity NeutrAvidin Agarose Resin	10 ml
53150	NeutrAvidin UltraLink Resin Capacity: ~ 12-20 µg of biotin/ml gel	5 ml
53151	NeutrAvidin Plus UltraLink Resin 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: crosslinked 6% beaded agarose
- Capacity: approx. 1-3 mg biotinylated BSA/ml gel

Ordering Information

Product #	Description	Pkg. Size
20347	Streptavidin Agarose Resin	2 ml
20349	Streptavidin Agarose Resin	5 ml
20353	Streptavidin Agarose Resin	10 ml
20357	High Capacity Streptavidin Agarose Resin	2 ml
20359	High Capacity Streptavidin Agarose Resin	5 ml
20361	High Capacity Streptavidin Agarose Resin	10 ml
20351	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 Thermo Scientific 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 sequencing[1]

References

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

Ordering Information

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

Ordering Information

Product #	Description	Pkg. Size
87741	High Capacity NeutrAvidin Chromatography Cartridges Support: Crosslinked 6% beaded agarose Capacity: > 75 µg biotinylated p-NPE/ml resin (> 8 mg biotinylated BSA/ml resin)	2 x 1 ml
87742	High Capacity NeutrAvidin Chromatography Cartridge Support: Crosslinked 6% beaded agarose Capacity: > 75 µg biotinylated p-NPE/ml resin (> 8 mg biotinylated BSA/ml resin)	1 x 5 ml
87739	High Capacity Streptavidin Chromatography Cartridges Support: Crosslinked 6% beaded agarose Capacity: > 10 mg biotinylated BSA/ml of resin	2 x 1 ml
87740	High Capacity Streptavidin Chromatography Cartridge Support: Crosslinked 6% beaded agarose Capacity: > 10 mg biotinylated BSA/ml of resin	1 x 5 ml

Thermo Scientific Pierce Coated Plates for Capturing Protein:Nucleic Acid Interactions

Biotin-binding plates offer a convenient platform for designing assays to detect biotinylated molecules in complex samples. These plates are coated with either streptavidin or NeutrAvidin biotin-binding protein, and are pre-blocked to prevent nonspecific binding. We offer three types of biotin-binding plates: Standard Binding Capacity (SBC), High Binding Capacity (HBC) and High Sensitivity (HS) plates. Additionally, these plates are available in different sizes (8-well strips, 96-well and 384-well) and different colors (clear, black or white). Choose a plate with the right specifications to fit your assay's needs.

Comparison of characteristics of Thermo Scientific Pierce Coated Plates.

	High Sensitivity (HS)	High Binding Capacity (HBC)	Standard Binding Capacity (SBC)
Application	Detect low concentrations of biotinylated molecules	Detect high concentrations of biotinylated molecules	General ELISA screening applications
Biotinylated Molecule Minimum Size	> 26 kDa	> 8 kDa	> 8 kDa
Detection Range ** Streptavidin Plates	5-300 ng/ml	62-10,000 ng/ml	31-1,250 ng/ml
Detection Range NeutrAvidin Plates**	5-125 ng/ml	15-2,500 ng/ml	15-300 ng/ml

*** Determined using Thermo Scientific QuantaBlu Fluorogenic Peroxidase Substrate.*

Plate Characteristics for Standard Capacity Biotin-Binding Plates.

Biotin-Binding Protein	Plate Type	Coat Volume*	Block Volume*	Approximate Binding Capacity
NeutrAvidin	96-Well, 8-Well Strip	100 µl/well	200 µl/well	~15 pmol biotin/well
	384-Well	50 µl/well	100 µl/well	~10 pmol biotin/well
Streptavidin	96-Well, 8-Well Strip	100 µl/well	200 µl/well	~5 pmol biotin/well
	384-Well	50 µl/well	100 µl/well	~4 pmol biotin/well

** The coating and blocking volumes give here are typical. Additional variations are also available.*

NeutrAvidin Coated Polystryene Plates (SBC)

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	NeutrAvidin Coated 96-Well Plates with Blocker™ BSA (Clear)	5 plates
15129	NeutrAvidin Coated 96-Well Plates with SuperBlock® Blocking Buffer (Clear)	5 plates
15128	NeutrAvidin Coated Strip Plates with Blocker BSA (Clear)	5 plates
15127	NeutrAvidin Coated Strip Plates with SuperBlock Blocking Buffer (Clear)	5 plates

To view the complete line of NeutrAvidin Coated Plates, visit www.thermo.com/pierce.

Streptavidin Coated Polystyrene Plates (SBC)

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 (96-well plates)

Ordering Information

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

To view the complete line of Streptavidin Coated Plates, visit www.thermo.com/pierce.

Plate Characteristics for High Binding Capacity Biotin-Binding Plates.

Biotin-Binding Protein	Plate Type	Coat Volume	Block Volume (SuperBlock Blocking Buffer)	Approximate Binding Capacity
NeutrAvidin	96-Well, 8-Well Strip	100 µl/well	200 µl/well	~60 pmol biotin/well
	384-Well	50 µl/well	100 µl/well	~35 pmol biotin/well
Streptavidin	96-Well, 8-Well Strip	100 µl/well	200 µl/well	~125 pmol biotin/well
	384-Well	50 µl/well	100 µl/well	~60 pmol biotin/well

NeutrAvidin High Binding Capacity (HBC) Coated Plates

Unique technology for improved assay precision.

Highlights:

- Unique plate-coating technology for high binding capacity
- Improved sensitivity
- Broader dynamic range
- Pre-blocked
- Flexble assay formats (see www.thermo.com/pierce for complete listing)

Ordering Information

Product #	Description	Pkg. Size
15507	NeutrAvidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (Clear)	5 plates
15508	NeutrAvidin Coated Plates (HBC), 8-well Strips, with SuperBlock Blocking Buffer (Clear)	5 plates
15509	NeutrAvidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (White)	5 plates
15510	NeutrAvidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (Black)	5 plates

Streptavidin HBC Coated Plates

Special coating technology results in four to five times the typical binding capacity.

Highlights:

- Increased sensitivity of complex detection
- Broader dynamic range
- Pre-blocked
- Flexible assay formats (see www.thermo.com/pierce for complete listing)
- High-binding capacity (HBC)

Ordering Information

Product #	Description	Pkg. Size
15500	Streptavidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (Clear)	5 plates
15501	Streptavidin Coated Plates (HBC), 8-well strips with SuperBlock Blocking Buffer (Clear)	5 plates
15502	Streptavidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (White)	5 plates
15503	Streptavidin Coated Plates (HBC), 96-well Plates with SuperBlock Blocking Buffer (Black)	5 plates

Streptavidin and NeutrAvidin Coated Plates – High Sensitivity

High sensitivity biotin-binding plates for low-level target detection.

Highlights:

- Sensitive – detect down to 5 ng/ml of biotinylated IgG
- Specific – low background with high signal-to-noise ratios
- Versatile – use either fluorescence, chemiluminescence or colorimetric detection
- Robust – broad dynamic range
- Ready to use – plates are supplied pre-blocked to save time

Ordering Information

Product #	Description	Pkg. Size
15520	Streptavidin Coated Plates (High Sensitivity), 8-well Strips with Blocker BSA (Clear)	5 plates
15525	Streptavidin Coated Plates (High Sensitivity), 8-well Strips with Blocker BSA (Black)	5 plates
15530	NeutrAvidin Coated Plates (High Sensitivity), 8-well Strips with Blocker BSA (Clear)	5 plates
15535	NeutrAvidin Coated Plates (High Sensitivity), 8-well Strips with Blocker BSA (Black)	5 plates

Thermo Scientific Magnetic Beads for Capture of Interacting Complexes

MagnaBind Streptavidin Coated Magnetic 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 x 10^8 particles/mg

Ordering Information

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

Pierce Streptavidin Magnetic Beads

Accelerate magnetic purification of biotinylated molecules from samples.

Highlights:

- High-performance, non-aggregating, pre-blocked, iron oxide, superparamagnetic microparticles
- Stable immobilization chemistry
- Nearly three times higher binding capacity
- Low non-specific binding

Ordering Information

Product #	Description	Pkg. Size
88816	Pierce Streptavidin Magnetic Beads	1 ml
88817	Pierce Streptavidin Magnetic Beads	5 ml

Thermo Scientific Pull-Down Assay for Capture of Interacting Complexes

Pierce Pull-Down Biotinylated-Protein Interaction Kit

Pull-down a binding partner with the Thermo Scientific Pierce Biotinylated-Protein Interaction Kit.

See the complete description of this kit in the Pull-Down Assays segment of this handbook, page 19.

Ordering Information

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

Thermo Scientific Chemiluminescent Detection in Polystyrene Plates

SuperSignal ELISA Pico and Femto Chemiluminescent Substrates

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

See the complete description of this product at www.thermo.com/pierce.

Ordering Information

Product #	Description	Pkg. Size
37070	SuperSignal ELISA Pico Chemiluminescent Substrate	100 ml
	Includes: Luminol/Enhancer	50 ml
	Stable Peroxide Buffer	50 ml
37075	SuperSignal ELISA Femto Maximum Sensitivity Substrate	100 ml
	Includes: Luminol/Enhancer	50 ml
	Stable Peroxide Buffer	50 ml

Handbooks

Protein Purification Technical Handbook

We offer 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. More on these products can be found in the free Protein Purification Handbook (Product # 1601617).

Also visit our web site at www.thermo.com/pierce for more information.

Crosslinker Technical Handbook

For the complete Thermo Scientific Pierce Crosslinker Products offering, including a broad selection of heterobifunctional-photo-reactive crosslinking reagents, request a free copy of our Crosslinker Technical Handbook (Product # 1601673).

An online Crosslinker Selection Guide can also be found at www.thermo.com/pierce.

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