I. Introduction

Information about the regulation of protein expression, protein modification, protein:protein interactions and protein function during different stages of cell development is needed to understand the development and physiology of organisms. This complex analysis of protein function is a major task facing scientists today. Although the field of proteomics was first described only as the study of proteins encoded by the genome, it has now expanded to include the function of all expressed proteins. Thus it is not just the study of all proteins expressed in a cell but also all protein isoforms and modifications, interactions, structure and high-order complexes (Tyers and Mann, 2003).

A fundamental step for studying individual proteins is the purification of the protein of interest. A variety of strategies have been developed for purifying proteins. These strategies address different requirements of downstream applications including scale and throughput. There are four basic steps required for protein purification: 1) cell lysis; 2) binding to a matrix; 3) washing; and 4) elution. Cell lysis can be accomplished in a number of ways, including nonenzymatic methods (e.g., sonication or French press) or use of hydrolytic enzymes such as lysozyme or a detergent reagent such as FastBreak™ Cell Lysis Reagent (Cat. # V8571). FastBreak™ Cell Lysis Reagent offers a convenient format for the in-media lysis of E. coli cells expressing recombinant proteins without interfering with downstream purification of tagged proteins (Stevens and Kobs, 2004). In addition, the FastBreak™ Reagent requires only minor modifications to be used with mammalian and insect cell lines (Betz, 2004).

Affinity purification tags can be fused to any recombinant protein of interest, allowing fast, easy purification using the affinity properties of the tag (Nilsson et al. 1997). Certain tags are used because they encode an epitope that can be purified or detected by a specific antibody or because they enable simplified purification of a desired protein.

Since protein is directly involved in biological function, a great deal of emphasis has been placed on developing new tools for proteomic studies (Zhu et al. 2003). A number of methods are available for functional protein interaction studies. These include protein pull-down assays, yeast two-hybrid systems (Fields and Song, 1989; Chien et al. 1991) and mammalian two-hybrid systems (Ginger et al. 1985; Lin et al. 1988) to identify protein:protein interactions, as well as protein-chip technology, mass spectrometry and traditional one- or two-dimensional gel electrophoresis for protein identification.

II. Affinity Tags

Researchers often need to purify a single protein for further study. One method for isolating a specific protein is the use of affinity tags. Affinity purification tags can be fused to any recombinant protein of interest, allowing fast and easy purification following a procedure that is based on the affinity properties of the fusion tag (Nilsson et al. 1997). Many different affinity tags have been developed to simplify protein purification (Terpe, 2002). Fusion tags are polyepitides, small proteins or enzymes added to the N- or C-terminus of a recombinant protein. The biochemical features of different tags influence the stability, solubility and expression of proteins to which they are attached (Stevens et al. 2001). Using expression vectors that include a fusion tag facilitates recombinant protein purification.

A. Polyhistidine

The most commonly used tag to purify and detect recombinant expressed proteins is the polyhistidine tag (Yip et al. 1989). Protein purification using polyhistidine tags relies on the affinity of histidine residues for immobilized metal such as nickel, which allows selective protein purification (Yip et al. 1989; Hutchens and Yip, 1990). This affinity interaction is believed to be a result of coordination of a nitrogen on the imidazole moiety of polyhistidine with a vacant coordination site on the metal. The metal is immobilized to a support through complex formation with a chelate that is covalently attached to the support.

Polyhistidine tags offer several advantages for protein purification. The small size of the polyhistidine tag renders it less immunogenic than other larger tags. Therefore, the tag usually does not need to be removed for downstream applications following purification. A large number of commercial expression vectors that contain polyhistidine are available. The polyhistidine tag may be placed on either the N- or C-terminus of the protein of interest. And finally, the interaction of the polyhistidine tag with the metal does not depend on the tertiary structure of the tag, making it possible to purify otherwise insoluble proteins using denaturing conditions.

B. Glutathione-S-Transferase

The use of the affinity tag glutathione-S-transferase (GST) is based on the strong affinity of GST for immobilized glutathione-covered matrices (Smith and Johnson, 1988). Glutathione-S-transferases are a family of multifunctional cytosolic proteins that are present in eukaryotic organisms (Mannervik and Danielson, 1988; Armstrong, 1997). GST isoforms are not normally found in bacteria; thus endogenous bacterial proteins don’t compete with the GST-fusion proteins for binding to purification resin. The 26kDa GST affinity tag enhances the solubility of many eukaryotic proteins expressed in bacteria.

III. Purification of Polyhistidine-Tagged Proteins

A. Rapid Purification of Polyhistidine-Tagged Proteins Using Magnetic Resins

There is a growing need for high-throughput protein purification methods. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein. The MagneHis™ Protein Purification System meets these criteria, enabling
purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from crude lysates to be performed in a single tube. In addition, the system can be used on automated liquid-handling platforms for high-throughput applications.

**MagneHis™ Protein Purification System**

The MagneHis™ Protein Purification System (Cat.# V8500, V8550) uses paramagnetic precharged nickel particles (MagneHis™ Ni-Particles) to isolate polyhistidine-tagged protein directly from a crude cell lysate. Figure 11.1 shows a schematic diagram of the MagneHis™ Protein Purification System protocol. Using a tube format, polyhistidine-tagged protein can be purified on a small scale using less than 1ml of culture or on a large scale using more than 1 liter of culture. Samples can be processed in a high-throughput manner using a robotic platform such as the Beckman Coulter Biomek® 2000 or Biomek® FX or Tecan Freedom EVO® instrument. Polyhistidine-tagged proteins can be purified under native or denaturing (2–8M urea or guanidine-HCl) conditions. The presence of serum in mammalian and insect cell culture medium does not interfere with purification. For more information and a detailed protocol, see Technical Manual #TM060.

![Diagram of the MagneHis™ Protein Purification System protocol.](image-url)
1. Add 110µl FastBreak™ Cell Lysis Reagent, 10X, to 1ml of fresh bacterial culture.
2. Resuspend DNase I as indicated on the vial. Add 1µl to the lysed culture.
3. Incubate with shaking for 10–20 minutes at room temperature.
4. Vortex the MagneHis™ Ni-Particles to a uniform suspension.
5. Add 30µl MagneHis™ Ni-Particles to 1.1ml of cell lysate.
6. Pipet to mix, and incubate for 2 minutes at room temperature.
7. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Carefully remove supernatant.
8. Remove the tube from the magnet. Add 150µl of MagneHis™ Binding/Wash Buffer to the MagneHis™ Ni-Particles, and pipet to mix. Make sure that particles are resuspended well.
9. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Carefully remove supernatant. Repeat Steps 8 and 9 for a total of three washes.
10. Add 100µl of MagneHis™ Elution Buffer, and pipet to mix. Incubate for 1–2 minutes at room temperature. Place the tube in the appropriate magnetic stand, and capture the particles. Carefully remove the supernatant, which now contains the fusion protein.

**Purification using Denaturing Conditions.** Proteins expressed in bacterial cells may be present in insoluble inclusion bodies. To determine if your protein is located in an inclusion body, perform the lysis step using FastBreak™ Cell Lysis Reagent, 10X, as described in Technical Manual #TM060. Pellet cellular debris by centrifugation, and check the supernatant and pellet for the polyhistidine-tagged protein by gel analysis. Insoluble proteins need to be purified under denaturing conditions. Since the interaction of polyhistidine-tagged fusion proteins and MagneHis™ Ni-Particles does not depend on tertiary structure, fusion proteins can be captured and purified using denaturing conditions by adding a strong denaturant such as 2–8M guanidine hydrochloride or urea to the cells. Denaturing conditions need to be used throughout the procedure; otherwise the proteins may aggregate. We recommend preparing denaturing buffers by adding solid guanidine-HCl or urea directly to the MagneHis™ Binding/Wash and Elution Buffers. For more detail, see Technical Manual #TM060.

**Note:** Do not combine FastBreak™ Cell Lysis Reagent and denaturants. Cells can be lysed directly using denaturants such as urea or guanidine-HCl.

**Purification from Insect and Mammalian Cells.** Process cells at a cell density of 2 × 10^6 cells/ml of culture. Adherent cells may be removed from the tissue culture vessel by scraping and resuspending in culture medium to this density. Cells may be processed in culture medium containing up to 10% serum. Processing more than the indicated number of cells per milliliter of sample may result in reduced protein yield and increased nonspecific binding. For proteins that are secreted into the cell culture medium, cells should be removed from the medium prior to purification. For more detail, see Technical Manual #TM060.

**Additional Resources for the MagneHis™ Protein Purification System**

<table>
<thead>
<tr>
<th>Technical Bulletins and Manuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM060  MagneHis™ Protein Purification System Technical Manual</td>
</tr>
</tbody>
</table>

**Promega Publications**

- Efficient purification of his-tagged proteins from insect and mammalian cells
- Technically speaking: Choosing the right protein purification system
- Purifying his-tagged proteins from insect and mammalian cells
- Rapid detection and quantitation of his-tagged proteins purified by MagneHis™ Ni-Particles
- MagneHis™ Protein Purification System: Purification of his-tagged proteins in multiple formats
- Automated polyhistidine-tagged protein purification using the MagneHis™ Protein Purification System

**Citations**


Researchers used MagneHis™ Ni-Particles to purify polyhistidine-tagged peptidoglycan recognition protein-1 (PGRP1 and PGRP2) that had been excreted into medium supernatants. The polyhistidine-tagged proteins were created by making fusion-protein expression vectors from isolated *H. diomphalia* larvae cDNA and the pMT/Bip/V5-His vector (Invitrogen). The construct was then stably transfected into *Drosophila* Schneider S2 cells, and the medium was monitored for secreted protein by Western blot analysis.

**PubMed Number:** 14583608

**MagZ™ Protein Purification System for Purification of Proteins Expressed in Rabbit Reticulocyte Lysate**

Purification of a polyhistidine-tagged protein that has been expressed in rabbit reticulocyte lysate is complicated by hemoglobin in the lysate copurifying with the protein of interest. Hemoglobin copurification limits downstream applications (e.g., fluorescence-based functional assays, protein:protein interaction studies) and reduces the amount of protein purified. The MagZ™ Protein Purification System provides a simple, rapid and reliable method to purify...
expressed polyhistidine-tagged protein from rabbit reticulocyte lysate. Paramagnetic precharged particles can be used to isolate polyhistidine-tagged protein from 50–500µl of TNT® Rabbit Reticulocyte Lysate with minimal copurification of hemoglobin. These polyhistidine-tagged proteins are 99% free of contaminating hemoglobin.

The MagZ™ System is flexible enough to be used with different labeling and detection methods. Polyhistidine-tagged proteins expressed in rabbit reticulocyte lysate can be labeled with [35S]methionine or the FluoroTect™ GreenLys in vitro Translation Labeling System. FluoroTect™-labeled polyhistidine-tagged proteins can be visualized by gel analysis and analyzed using a FluorImager® instrument. Figure 11.2 shows a schematic diagram of the MagZ™ Protein Purification System protocol. For more detail, see Technical Bulletin #TB336.

Materials Required:
(see Composition of Solutions section)
• MagZ™ Protein Purification System (Cat.# V8830) and protocol
• platform shaker or rocker, rotary platform or rotator
• MagneSphere® Technology Magnetic Separation Stand (Cat.# Z5331, Z5332, Z5341, Z5342)

Additional Resources for the MagZ™ Protein Purification System
Technical Bulletins and Manuals
TB336 MagZ™ Protein Purification System Technical Bulletin
Promega Publications
The MagZ™ System: His-tagged protein purification without hemoglobin contamination
In vitro his-tag pull-down assay using MagZ™ Particles

B. Medium- to Large-Scale Purification of Polyhistidine-Tagged Proteins In Column or Batch Formats

The two most common support materials for resin-based, affinity-tagged protein purification are agarose and silica gel. As a chromatographic support, silica is advantageous because it has a rigid mechanical structure that is not vulnerable to swelling and can withstand large changes in pressure and flow rate without disintegrating or deforming. Silica is available in a wide range of pore and particle sizes.
Protein Purification and Analysis

including macroporous silica, providing a higher capacity for large biomolecules such as proteins. However, two of the drawbacks of silica as a solid support for affinity purification are the limited reagent chemistry that is available and the relatively low efficiency of surface modification.

The HisLink™ Protein Purification Resin (Cat. # V8821) and HisLink™ 96 Protein Purification System (Cat. # V3680, V3681) overcome these limitations using a new modification process for silica surfaces that provides a tetradeutate metal-chelated solid support with a high binding capacity and concomitantly eliminates the nonspecific binding that is characteristic of unmodified silica. HisLink™ Resin is a macroporous silica resin modified to contain a high level of tetradeutate-chelated nickel (>20mmol Ni/ml settled resin). Figure 11.3 shows a schematic diagram of HisLink™ Resin and polyhistidine-tag interaction. The HisLink™ Resin has a pore size that results in binding capacities as high as 35mg of polyhistidine-tagged protein per milliliter of resin.

The HisLink™ Resin enables efficient capture and purification of bacterially expressed polyhistidine-tagged proteins. This resin may also be used for general applications that require an immobilized metal affinity chromatography (IMAC) matrix (Porath et al. 1975; Lonnerdal and Keen, 1982). HisLink™ Resin may be used in either column or batch purification formats. For a detailed protocol see Technical Bulletin #TB327.

**Figure 11.3. Schematic diagram of HisLink™ Resin and polyhistidine interaction.** Two sites are available for polyhistidine-tag binding and are rapidly coordinated with histidine in the presence of a polyhistidine-tagged polypeptide.

**Column-Based Purification using HisLink™ Resin**

The HisLink™ Resin provides a conventional means to purify polyhistidine-tagged proteins and requires only a column that can be packed to the appropriate bed volume. If packed to 1ml under gravity-driven flow, HisLink™ Resin shows an average flow rate of approximately 1ml/minute. In general a flow rate of 1–2ml/minute per milliliter of resin is optimal for efficient capture of polyhistidine-tagged protein. Gravity flow of a cleared lysate over a HisLink™ column will result in complete capture and efficient elution of polyhistidine-tagged proteins; however, the resin may also be used with vacuum filtration devices (e.g., Vac-Man® Vacuum Manifold, Cat.# A7231) to allow simultaneous processing of multiple columns. HisLink™ Resin is also an excellent choice for affinity purification using low- to medium-pressure liquid chromatography systems such as fast performance liquid chromatography (FPLC).

**Example Protocol Using the HisLink™ Resin to Purify Proteins from Cleared Lysate by Gravity-Flow Column Chromatography**

**Materials Required:**

(see Composition of Solutions section)

- HisLink™ Protein Purification Resin (Cat.# V8821)
- HEPES buffer (pH 7.5)
- imidazole
- binding buffer
- wash buffer
- elution buffer
- column [e.g., Fisher PrepSep Extraction Column (Cat.# P446) or Bio-Rad Poly-Prep® Chromatography Column (Cat.# 731-1550)]

**Cell Lysis:** Cells may be lysed using any number of methods including sonication, French press, bead milling, treatment with lytic enzymes (e.g., lysozyme) or use of a commercially available cell lysis reagent such as the FastBreak™ Cell Lysis Reagent (Cat.# V8571). If lysozyme is used to prepare a lysate, add salt (>300mM NaCl) to the binding and wash buffers to prevent the lysozyme binding to the resin. Finally, adding protease inhibitors such as 1mM PMSF to cell lysates does not inhibit binding or elution of polyhistidine-tagged proteins with the HisLink™ Resin and is highly recommended. When preparing cell lysates from high-density cultures, adding DNase and RNase (concentrations up to 20µg/ml) will reduce the lysate viscosity and aid in purification.

**Example Protocol**

1. Prepare the binding, wash and elution buffers.

2. Determine the column volume required to purify the protein of interest. In most cases 1ml of settled resin is sufficient to purify the amount of protein typically found in up to 1 liter of culture (cell density of O.D. 600 < 6.0). In cases of very high expression levels (e.g., 50mg protein/liter), up to 2ml of resin per liter of culture may be needed.

3. Once you have determined the volume of settled resin required, precalibrate this amount directly in the column by pipetting the equivalent volume of water into the column and marking the column to indicate the top of the water. This mark indicates the top of the settled resin bed. Remove the water before adding resin to the column.

4. Make sure that the resin is fully suspended; fill the column with resin to the line marked on the column by transferring the resin with a pipette. Allow the resin to settle, and adjust the level of the resin by adding or removing resin as necessary.
Note: If the resin cannot be pipetted within 10–15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended. Alternatively, a magnetic stir bar may be used to keep the resin in suspension during transfer. To avoid fracturing the resin, do not leave the resin stirring any longer than the time required to pipet and transfer the resin.

5. Allow the column to drain, and equilibrate the resin with five column volumes of binding buffer, allowing the buffer to completely enter the resin bed.

6. Gently add the cleared lysate to the resin until the lysate has completely entered the column. The rate of flow through the column should not exceed 1–2ml/minute for every 1ml of column volume. Under normal gravity flow conditions the rate is typically about 1ml/minute. The actual flow rate will depend on the type of column used and the extent to which the lysate was cleared and filtered. Do not let the resin dry out after you have applied the lysate to the column.

7. Wash unbound proteins from the resin using at least 10–20 column volumes of wash buffer. Divide the total volume of wash buffer into two or three aliquots, and allow each aliquot to completely enter the resin bed before adding the next aliquot.

8. Once the wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution profiles are protein-dependent, but polyhistidine-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer have been collected per 1.0ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest.

Batch Purification Using HisLink™ Resin
One of the primary advantages of the HisLink™ Resin is its use in batch purification. In batch mode, the protein of interest is bound to the resin by mixing lysate with the resin for approximately 30 minutes at a temperature range of 4–22°C. Once bound with protein, the resin is allowed to settle to the bottom of the container, and the spent lysate is poured off. Washing only requires resuspension of the resin in an appropriate wash buffer followed by a brief period to allow the resin to settle. The wash buffer is then carefully poured off. This process is repeated as many times as desired. Final elution is best achieved by transferring the HisLink™ Resin to a column to elute the protein in fractions. The advantages of batch purification are: 1) less time is required to perform the purification; 2) large amounts of lysate can be processed; and 3) clearing the lysate prior to purification is not required.

Purification of Polyhistidine-Tagged Proteins by FPLC
The rigid particle structure of the silica base used in the HisLink™ Resin make this material an excellent choice for applications that require applied pressure to load the lysate, wash or elute protein from the resin. These applications involve both manual and automated systems that operate under positive or negative pressure (e.g., FPLC and vacuum systems, respectively). To demonstrate the use of HisLink™ Resin on an automated platform we used an AKTA explorer from GE Healthcare. Milligram quantities of polyhistidine-tagged protein were purified from one liter of culture. The culture was lysed in 20ml of binding/wash buffer and loaded onto a column containing 1ml of HisLink™ Resin. We estimate the total amount of protein recovered to be 75–90% of the protein expressed in the original lysate.

Purification under denaturing conditions: Proteins that are expressed as an inclusion body and have been solubilized with chaotrophic agents such as guanidine-HCl or urea can be purified by modifying the protocols to include the appropriate amount of denaturant (up to 6M guanidine-HCl or up to 8M urea) in the binding, wash and elution buffers.

Adjuncts for lysis or purification: The materials shown in Table 11.1 may be used without adversely affecting the ability of HisLink™ Resin to bind and elute polyhistidine-tagged proteins.
Table 11.1. Additives That Will Not Affect Binding or Elution of Polyhistidine-Tagged Proteins Using HisLink™ Resin.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES, Tris or sodium phosphate buffers</td>
<td>≤100mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>≤1M</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>≤100mM</td>
</tr>
<tr>
<td>DTT</td>
<td>≤10mM</td>
</tr>
<tr>
<td>Triton® X-100</td>
<td>≤2%</td>
</tr>
<tr>
<td>Tween®</td>
<td>≤2%</td>
</tr>
<tr>
<td>glycerol</td>
<td>≤20%</td>
</tr>
<tr>
<td>guanidine-HCl</td>
<td>≤6M</td>
</tr>
<tr>
<td>urea</td>
<td>≤8M</td>
</tr>
<tr>
<td>RQ1 RNase-Free DNase</td>
<td>≤5µl/1ml original culture</td>
</tr>
</tbody>
</table>

Additional Resources for the HisLink™ Protein Purification Resin

Technical Bulletins and Manuals

TB327 HisLink™ Protein Purification Resin
Technical Bulletin

Promega Publications

HisLink™ 96 Protein Purification System: Fast purification of polyhistidine-tagged proteins
Finding the right protein purification system
Finding the right protein purification system

C. 96-Well Format For Purification of Polyhistidine-Tagged Proteins

The HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) uses a vacuum-based method to purify polyhistidine-tagged expressed proteins directly from E. coli cultures grown in deep-well, 96-well plates. The HisLink™ 96 System is amenable to manual or automated methods for high-throughput applications. In preparation for protein purification, bacterial cells expressing a polyhistidine-tagged protein are lysed directly in culture using the provided FastBreak™ Cell Lysis Reagent. The HisLink™ Resin is added directly to the lysate and mixed, and the polyhistidine-tagged proteins bind within 30 minutes. The samples are then transferred to a filtration plate. Unbound proteins are washed away, and the target protein is recovered by elution. Figure 11.4 describes protein purification using the HisLink™ 96 System. This system requires the use of the Vac-Man® 96 Vacuum Manifold (Cat.# A2291, Figure 11.5) or a compatible vacuum manifold. For more detailed protocol information, see Technical Bulletin #TB342.

Manual Protocol

Materials Required:
(see Composition of Solutions section)

- HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) and protocol
- Nuclease-Free Water (Cat.# P1195)

Additional Resources for HisLink™ 96 Protein Purification System

Technical Bulletins and Manuals

TB342 HisLink™ 96 Protein Purification System
Technical Bulletin

Promega Publications

HisLink™ 96 Protein Purification System: Fast purification of polyhistidine-tagged proteins

Automated Purification

The manual protocol described in Section III.C can be used as a guide to develop protocols for automated workstations. The protocol may require optimization, depending on the instrument used.

- Vac-Man® 96 Vacuum Manifold (Cat.# A2291)
- plate shaker (manual) or multichannel pipette
- wide-bore tips (Racked, Sterile, Yellow Lift Top Racks; E&K Scientific Cat.# 3502-R96S)
- 96-well, deep-well plates (e.g., ABgene 2.2ml storage plate, Marsh Bio Products Cat.# AB-0932)
- 96-well sealing mats (Phenix Research Products Cat.# M-0662)
- 96-well plate adhesive sealers
- reservoir boats (Diversified Biotech Cat.# RESE-3000)
Figure 11.4. A schematic representation of the HisLink™ 96 Protein Purification protocol.
IV. Purification of GST-Tagged Proteins

A. Rapid Purification of GST-Tagged Proteins Using Magnetic Resins

There is a growing need for protein purification methods that are amenable to high-throughput screening. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein.

The MagneGST™ Protein Purification System (Cat.# V8600, V8603) meets these criteria, enabling purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from a crude lysate in a single tube. In addition, the system can be used with automated liquid-handling platforms for high-throughput applications.

MagneGST™ Protein Purification System for Purification of GST-Tagged Proteins

The MagneGST™ Protein Purification System provides a simple, rapid and reliable method to purify glutathione-S-transferase (GST) fusion proteins. Glutathione immobilized on paramagnetic particles (MagneGST™ Glutathione Particles; Cat.# V8611, V8612) is used to isolate GST-fusion proteins directly from a crude cell lysate using a manual or automated procedure. The use of paramagnetic particles eliminates several centrifugation steps and the need for multiple tubes. It also minimizes the loss of sample material. Although the MagneGST™ System is designed for manual applications, samples can also be processed using a robotic platform, such as the Beckman Coulter Biomek® 2000 or Biomek® FX workstation, for high-throughput applications. Visit the Promega web site for more information about using the MagneGST™ System in an automated format.

Bacterial cells containing a GST-fusion protein are lysed using the provided MagneGST™ Cell Lysis Reagent or an alternative lysis method, and the MagneGST™ Particles are added directly to the crude lysate. GST-fusion proteins bind to the MagneGST™ Particles. Unbound proteins are washed away, and the GST-fusion target protein is recovered by elution with 50mM glutathione. Figure 11.6 shows a schematic diagram of the MagneGST™ Protein Purification System protocol. For more detailed information about the protocol, see Technical Manual #TM240.
Figure 11.6. Schematic diagram of the MagneGST™ Protein Purification System. A bacterial culture expressing GST-fusion proteins is pelleted and lysed by enzymatic or mechanical methods. MagneGST™ Glutathione Particles are added directly to cleared or crude lysate. GST-fusion proteins bind to the particles during incubation at room temperature or 4°C, then are washed to remove unbound and nonspecifically bound proteins; three wash steps are performed. GST-fusion protein is eluted from the particles by adding 10–50mM reduced glutathione at pH 8.

Additionally, we have used the MagneGST™ Particles to purify GST-fusion protein generated in vitro using the E. coli S30 Extract System for Circular DNA (Cat. # L1020). When eluted protein was analyzed by SDS polyacrylamide gel electrophoresis, no major contaminating proteins were found to copurify with the GST-fusion proteins.

Example Protocol for the MagneGST™ Protein Purification System

Materials Required:
(see Composition of Solutions section)
- MagneGST™ Protein Purification System (Cat. # V8600, V8603) and protocol
- 1.5ml microcentrifuge tubes for small-scale protein purifications or 15ml or 50ml conical tubes for large-scale protein purifications
- magnetic separation stand
- RQ-1 RNase-Free DNase (Cat. # M6101)
- shaker or rotating platform
- centrifuge

Cell Lysis
1. Prepare cell pellets from 1ml of bacterial culture.
2. Add 200µl of MagneGST™ Cell Lysis Reagent to each fresh or frozen cell pellet. Resuspend the cell pellet at room temperature (20–25°C) by pipetting or gentle mixing.
3. Add 2µl of RQ1 RNase-Free DNase.
4. Incubate the cell suspension at room temperature for 20–30 minutes on a rotating platform or shaker.

Equilibrate Particles
1. Thoroughly resuspend the MagneGST™ Particles by inverting the bottle to obtain a uniform suspension.
2. Pipet 100µl of MagneGST™ Particles into a 1.5ml tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Magnetic capture of the particles will typically occur within a few seconds.
4. Carefully remove and discard the supernatant.
5. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and resuspend by pipetting or inverting.
6. Repeat Particle Equilibration Steps 3–5 twice for a total of three washes.

Bind Proteins
1. After the final wash, gently resuspend the particles in 100µl of MagneGST™ Binding/Wash Buffer.
2. Add 200µl of cell lysate, prepared as described above, to the particles.
3. Mix gently by pipetting or inverting. If the combined volume of cell lysate and MagneGST™ Particles is less than 300µl, add additional MagneGST™ Binding/Wash Buffer so that the final volume is 300µl.
4. Incubate with gentle mixing on a rotating platform or shaker for 30 minutes at 4°C or room temperature.
Wash
1. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
2. Carefully remove the supernatant. Save the supernatant (flowthrough) for SDS-PAGE analysis, if desired.
3. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by pipetting or inverting. Incubate at room temperature or 4°C for 5 minutes. Occasionally mix by inverting the tube.
4. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
5. Carefully remove the supernatant. Save the supernatant if analysis of wash solution is desired.
6. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by pipetting or inverting. Incubation is not necessary at this step.
7. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
8. Carefully remove the supernatant. Save the supernatant if analysis of wash solution is desired.
9. Repeat Wash Steps 6–8 once for a total of three washes.

Elution
1. After the final wash, add 200µl of elution buffer.
2. Incubate at room temperature or 4°C for 15 minutes with gentle mixing.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
4. Carefully remove the supernatant, and transfer it to a clean tube. The supernatant contains the eluted GST-fusion protein.
5. If a second elution is desired, repeat Elution Steps 1–4.

Compatibility with Common Buffer Components: The MagneGST™ Particles have been shown to be compatible with many common buffer components (Table 11.2).

Table 11.2. Buffer Components Compatible with the MagneGST™ Particles.

<table>
<thead>
<tr>
<th>Buffer Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>≤10mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>≤0.64M</td>
</tr>
<tr>
<td>Tris, HEPES, sodium phosphate</td>
<td>≤100mM</td>
</tr>
<tr>
<td>Triton® X-100</td>
<td>≤1%</td>
</tr>
<tr>
<td>Tween®</td>
<td>≤1%</td>
</tr>
<tr>
<td>MAZU</td>
<td>≤1%</td>
</tr>
<tr>
<td>cetyltrimethylammonium bromide (CTAB)</td>
<td>≤1%</td>
</tr>
<tr>
<td>ethanol</td>
<td>20%</td>
</tr>
<tr>
<td>protease inhibitor cocktail (Roche Molecular Systems, Inc. Cat.# 1836170)</td>
<td>1X</td>
</tr>
</tbody>
</table>

Additional Resources for the MagneGST™ Protein Purification System

Technical Bulletins and Manuals
- TM240 MagneGST™ Protein Purification System Technical Manual

Promega Publications
- Purification of GST-fusion proteins by magnetic resin-based MagneGST™ Particles
- Finding the right protein purification system

V. Purification of Biotinylated Proteins

A. PinPoint™ Xa System and SoftLink™ Resin for Purification of Biotinylated Protein

Biotinylated fusion proteins such as those produced with the PinPoint™ Xa Protein Purification System (Cat.# V2020) can be affinity-purified using the SoftLink™ Soft Release Avidin Resin (Cat.# V2011). This proprietary resin allows elution of a fusion protein under native conditions by adding exogenous biotin. The TetraLink™ Tetrameric Avidin Resin (Cat.# V2591) can be used for irreversible capture of biotinylated proteins to generate affinity resins to purify other proteins that interact with the fusion protein. This approach is particularly useful in the affinity purification of antibodies.

The PinPoint™ Xa Protein Purification System is designed to produce and purify fusion proteins that are biotinylated in vivo. The biotinylation reaction in *E. coli* is catalyzed by biotin ligase holoenzyme and results in a fusion purification tag that carries a single biotin specifically on one lysine residue (Wilson *et al.* 1992; Xu and Beckett, 1994; Cronan, 1990). The biotin moiety is accessible to avidin or streptavidin, as demonstrated by binding to resins containing either molecule, and serves as a tag for detection and purification. *E. coli* produces a single endogenous biotinylated protein that, in its native conformation, does not bind to avidin, rendering the affinity purification highly specific for the recombinant fusion protein.
The system contains vectors in all possible reading frames, an avidin-conjugated resin, Streptavidin-Alkaline Phosphatase, a purification column and biotin. The PinPoint™ Xa Control Vector contains the chloramphenicol acetyltransferase (CAT) gene and is provided as a means of monitoring protein expression, purification and processing conditions. The PinPoint™ Vectors feature the encoded endoproteinase Factor Xa proteolytic site that provides a way to separate the purification tag from the native protein. These vectors also carry a convenient multiple cloning region for ease in construction of fusion proteins.

Biotinylated proteins synthesized using the PinPoint™ Xa System can be affinity-purified using the SoftLink™ Soft Release Avidin Resin. Avidin-biotin interactions are so strong that elution of biotin-tagged proteins from avidin-conjugated resins usually requires denaturing conditions. In contrast, the SoftLink™ Soft Release Avidin Resin, which uses monomeric avidin, allows the protein to be eluted with a nondenaturing 5mM biotin solution. The rate of dissociation of the monomeric avidin-biotin complex is sufficiently fast to effectively allow recovery of all bound protein in neutral pH and low salt conditions. The diagram in Figure 11.7 outlines the expression and purification system procedure.

The SoftLink™ Soft Release Avidin Resin is highly resistant to many chemical reagents (e.g., 0.1N NaOH, 50mM acetic acid and nonionic detergents), permitting stringent wash conditions. The TetraLink™ Tetrameric Avidin Resin (Cat.# V2591) can be used in place of the SoftLink™ Soft Release Avidin Resin to create affinity resins for purifying antibodies that recognize the antigenic portion of a fusion protein. Therefore, the TetraLink™ Resin is useful for immobilization and not necessarily the recovery of the fusion protein.

Figure 11.7. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

Additional Resources for the PinPoint™ Xa Protein Purification System

Technical Bulletins and Manuals

TM028  PinPoint™ Xa Protein Purification System Technical Manual
VI. Protein:Protein Interaction Analysis: In Vivo and In Vitro Methods

Determining the protein:protein interaction map (“interactome”) of the whole proteome is one major focus of functional proteomics (Li et al. 2004; Huzbin et al. 2003). Various methods have been used for studying protein:protein interactions, including yeast, bacterial and mammalian two- and three-hybrid systems, immunoaffinity purifications, affinity tag-based methods and mass spectrometry (reviewed in Li et al. 2004; Huzbin et al. 2003; Zhu et al. 2003). Moreover, in vitro pull-down-based techniques such as tandem affinity purification (TAP) are being widely used for isolating protein complexes (Forler et al. 2003).

In vitro protein pull-down assays can be performed using cell lysates, cell-free lysates, tissue samples, etc. These options are not possible with two-hybrid approaches. There are several reports describing the use of in vitro pull-down assays for analyzing protein:protein interactions using proteins translated in vitro using cell-free expression systems such as rabbit reticulocyte lysate-based expression systems (Charron et al. 1999; Wang et al. 2001; Pfleger et al. 2001). Cell-free expression is a powerful method for expressing cDNA libraries. This technique is also amenable to high-throughput protein expression and identification. Cell-free expression systems, especially rabbit reticulocyte lysate-based methods, have been extensively used for in vitro pull-down assays because of the ease of performing these experiments (Charron et al. 1999; Wang et al. 2001; Pfleger et al. 2001). There are also reports describing high-throughput identification of protein:protein interactions using TNT® Rabbit Reticulocyte Lysate (Pfleger et al. 2001).

A. Mammalian Two-Hybrid Systems

Two-hybrid systems are powerful methods to detect protein:protein interactions in vivo. The basis of two-hybrid systems is the modular nature of some transcription factor domains: a DNA-binding domain, which binds to a specific DNA sequence, and a transcriptional activation domain, which interacts with the basal transcriptional machinery (Sadowski et al. 1988). A transcriptional activation domain in association with a DNA-binding domain promotes the assembly of RNA polymerase II complexes at the TATA box and increases transcription. In the CheckMateTM Mammalian Two-Hybrid System (Cat. # E2440), the DNA-binding domain and transcriptional activation domain, produced by separate plasmids, are closely associated when one protein (“X”) fused to a DNA-binding domain interacts with a second protein (“Y”) fused to a transcriptional activation domain. In this system, interaction between proteins X and Y results in transcription of a reporter gene or selectable marker gene (Figure 11.8).

Originally developed in yeast (Fields and Song, 1989; Chien et al. 1991), the two-hybrid system has been adapted for use in mammalian cells (Dang et al. 1991; Fearon et al. 1992). One major advantage of the CheckMate™ Mammalian Two-Hybrid System over yeast systems is that the protein:protein interaction can be studied in the cell line of choice. The CheckMate™ System also uses the Dual-Luciferase® Reporter Assay System for rapid and easy quantitation of luciferase reporter gene expression.

Application of the CheckMate™ Mammalian Two-Hybrid System confirms suspected interactions between two proteins and identifies residues or domains involved in protein:protein interactions. When identifying residues or domains involved in an interaction, the GeneEditor™ in vitro Site-Directed Mutagenesis System (Cat.# Q9280) for making site-directed mutants and Erase-a-Base® Systems for deletion analysis are useful tools. These products are fully compatible with the CheckMate™ Mammalian Two-Hybrid System. Detailed protocol information is available in Technical Manual #TM049.

Assessing Protein:Protein Interactions

cDNA sequences encoding the polypeptides of interest are subcloned into pBIND and pACT Vectors. The insert in each vector must be in the correct orientation and reading frame. See the CheckMate™ System Technical Manual #TM049 for the multiple cloning region following the 3’ end of the GAL4 fragment for pBIND Vector and for the multiple cloning region following the 3’ end of the VP16 fragment for pACT Vector. All vectors in the CheckMate™ Mammalian Two-Hybrid System confer ampicillin resistance and are compatible with E. coli strains such as JM109. We strongly recommend sequencing the 5’ junction between the insert and vector to ensure that the insert is subcloned properly. The T7 EEV Promoter Primer (Cat.# Q6700) can be used for sequence verification.

Certain inserts appear to show vector “directionality” (or preference) in which the interaction between a pair of proteins is fusion vector-dependent (Finkel et al. 1993).
Figure 11.8. Schematic representation of the CheckMate™ Mammalian Two-Hybrid System. The pG5 luc Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from the pBIND Vector) and VP16 (from the pACT Vector). Interaction between the two test proteins, as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls.

Protein:protein interactions may appear stronger given a particular vector context for the inserts. Because of this phenomenon, we advise subcloning each cDNA of interest into both the pBIND and pACT Vectors and testing the two possible fusion protein interactions.

Following the successful subcloning of the test cDNAs into the pBIND and pACT Vectors, the resultant plasmids should be purified such that the DNA is free of protein, RNA and chemical contamination. Before completing any experiments with the CheckMate™ System, optimize the transfection method for the cell type being transfected. The optimization process is easier using a reporter gene and assay system. Many DNA delivery agents exist for transfecting mammalian cells. Transfection of DNA into mammalian cells may be mediated by cationic lipids, calcium phosphate, DEAE-dextran or electroporation. Transfection systems based on cationic lipids (TransFast™ Transfection Reagent, Transfectam® Reagent, Tfx™-20, Tfx™-50 Reagents) and calcium phosphate (ProFec® Mammalian Transfection System) are available from Promega. The efficiency of each transfection method is highly dependent upon the cell type. When optimizing a transfection method for a particular cell type, use a reporter gene such as the firefly luciferase gene whose activity is easily and rapidly assayed. The pGL3-Control Vector (Cat.# E1741) expresses the firefly luciferase gene from the SV40 early promoter.

Table 11.3 presents the recommended combinations of vectors to properly control an experiment when using the CheckMate™ System to determine the extent to which two proteins interact in a two-hybrid assay.

The amount of vector DNA to use will depend upon the method of transfection. However, we recommend that the molar ratio of pBIND:pACT Vector constructs be 1:1. We have varied the amount of pG5 luc Vector in the positive control experiment and have found that the signal-to-noise ratio of firefly luciferase expression does not differ significantly. We routinely use a molar ratio of 1:1:1 for pBIND:pACT:pG5 luc Vector in the CheckMate™ Mammalian Two-Hybrid System. Maintain a constant amount of DNA for each transfection reaction within an experiment by adding plasmid DNA such as pGEM®-3Zf(+) Vector (Cat.# P2271).

We recommend testing a specific cell line with positive and negative control transfection reactions before initiating test experiments. The pBIND Vector encodes the Renilla luciferase gene to normalize for transfection efficiency. Replication of pBIND and pACT Vectors and their recombinants is expected in COS cells or other types of cells.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>pBIND Vector</th>
<th>pACT Vector</th>
<th>pG5 luc Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pBIND Vector</td>
<td>pACT Vector</td>
<td>pG5 luc Vector</td>
</tr>
<tr>
<td>2</td>
<td>pBIND-Id Control Vector</td>
<td>pACT-MyoD Control Vector</td>
<td>pG5 luc Vector</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>pBIND-X Vector</td>
<td>pACT Vector</td>
<td>pG5 luc Vector</td>
</tr>
<tr>
<td>5</td>
<td>pBIND Vector;</td>
<td>pACT-Y Vector</td>
<td>pG5 luc Vector</td>
</tr>
<tr>
<td>6</td>
<td>pBIND-X Vector;</td>
<td>pACT-Y Vector</td>
<td>pG5 luc Vector</td>
</tr>
</tbody>
</table>
B. In Vitro Pull-Down Assays

**Glutathione-S-Transferase (GST) Pull-Down Assays**

The glutathione-S-transferase (GST) pull-down assay (Kaelin et al. 1991) is an important tool to validate suspected protein:protein interactions and identify new interacting partners (Benard and Bokoch, 2002; Wang et al. 2000; Wada et al. 1998; Malloy et al. 2001). GST pull-down assays use a GST-fusion protein (bait) bound to glutathione (GST)-coupled particles to affinity purify any proteins that interact with the bait from a pool of proteins (prey) in solution. Bait and prey proteins can be obtained from multiple sources, including cell lysates, purified proteins, and in vitro transcription/translation systems.

The MagneGST™ Pull-Down System (Cat.# V8870) is optimized for detection of protein:protein interactions where the bait protein is prepared from an *E. coli* lysate and mixed with prey protein synthesized in the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). The magnetic nature of the MagneGST™ GSH-linked particles in this system offers significant advantages over traditional resins, which require lengthy preparation and equilibration and are hard to dispense accurately in small amounts. The MagneGST™ Particles are easy to dispense in volumes less than 5µl, and equilibration is quick and easy and does not require any centrifugation steps. Another advantage of this system is that the pull-down reaction is performed in one tube. The particles are easily and efficiently separated from supernatants using a magnetic stand without centrifugation, increasing reproducibility and reducing sample loss. The flexible format of the MagneGST™ Pull-Down System allows optimization of experimental conditions, including modification of particle volume, to fit specific requirements of each unique protein:protein interaction. Additionally, the system allows easy processing of multiple samples at once.

The MagneGST™ Pull-Down System provides GST-linked magnetic particles that enable simple immobilization of bait proteins from bacterial lysates and an in vitro transcription/translation system for expressing prey proteins. The MagneGST™ Pull-Down protocol can be divided into three phases: 1) the prey protein is expressed in the TNT® T7 Quick Coupled System; 2) bait protein present in crude *E. coli* lysate is immobilized on the MagneGST™ Particles; and 3) the prey protein is mixed with MagneGST™ Particles carrying the bait protein and captured through bait:prey interaction. Nonspecifically bound proteins are washed away, and the prey and bait proteins are eluted with SDS loading buffer. Prey proteins can be analyzed by SDS-PAGE and autoradiography if the prey protein was radioactively labeled during synthesis.

The transcription/translation component of the MagneGST™ Pull-Down System is the TNT® T7 Quick Master Mix, which allows convenient, single-tube, coupled transcription/translation of genes cloned downstream from a T7 RNA polymerase promoter. The TNT® System is compatible with circular (plasmid) or linear (plasmid or PCR product) templates. For more information on the TNT® T7 Quick Coupled Transcription/Translation System, refer to Technical Manual #TM045. An overview of the MagneGST™ Pull-Down System is depicted in Figure 11.9. An animated presentation (requires Flash plug in) of the MagneGST™ pull-down process using the TNT® T7 Quick Coupled System is available. More information and detailed protocol information is available in Technical Manual #TM249.

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**Additional Resources for the CheckMate™ Mammalian Two-Hybrid System**

**Technical Bulletins and Manuals**

**TM049** CheckMate™ Mammalian Two-Hybrid System Technical Manual

**Promega Publications**

The CheckMate™ Mammalian Two-Hybrid System

**Vector Maps**

pACT Vector and pACT-MyoD Control Vector

pBIND Vector and pBIND-Id Control Vector

pGSluc Vector

**Citations**


The authors investigated the role of the promyelocytic leukemia (PML) nuclear body in transactivation of myeloid elf-1-like factor (MEF), a transcription factor that upregulates lysozyme transcription. To determine if the nuclear factors affected MEF, HeLa cells were cotransfected with 0.2µg of a pGL2 Vector construct with a lysozyme promoter and various combinations of 0.1µg of MEF, 0.5µg of PML and 1µg of Sp100 (another nuclear body factor) plasmids. Expression was normalized to 10ng of phRG-TK Vector. Forty-eight hours post-transfection, the cells were harvested and luciferase activity measured using the Dual-Luciferase® Reporter Assay System. In addition, MEF mutants were made and tested in the same dual-reporter system to determine if transactivation was affected by the various deletion mutations. These MEF mutants were also cloned into a vector with the yeast GAL4 DNA-binding domain to help determine which domain of MEF was interacting with PML nuclear body in a mammalian two-hybrid system. This was done using the CheckMate™ Mammalian Two-Hybrid System.

**PubMed Number:** 14976184
Example Protein Pull-Down Protocol Using the MagneGST™ Pull-Down System

Materials Required:
(see Composition of Solutions section)
- MagneGST™ Pull-Down System (Cat.# V8870) and protocol
- Magnetic Separation Stand (Cat.# Z5342, Z5343, Z5332, Z5333 or A2231)
- radiolabeled methionine (e.g., ³⁵S]Met, 10–40µCi per T7 reaction) for radioactive detection of prey protein or specific antibodies for detection using Western blot analysis
- RQ1 RNase-Free DNase (Cat.# M6101)
- NANOpure® or double-distilled water
- SDS loading buffer
- BSA (Cat.# W3841) or IGEPAL® CA-630 (Sigma Cat.# I3021)

Express Prey Protein using a T7 Quick Coupled Transcription/Translation Reaction

1. Remove the reagents from storage at –70°C. (Store the RQ1 DNase at –20°C after first use.) Thaw the T7 Quick Master Mix by hand-warming or on ice. The other components can be thawed at room temperature and stored on ice.

2. Assemble the reaction components as shown in the table below using template DNA encoding your prey protein of interest. Incubate the reaction at 30°C for 60–90 minutes. During this incubation period, prepare the MagneGST™ Particles.

Figure 11.9. Schematic diagram of the MagneGST™ Pull-Down System protocol. P = prey protein, M = MagneGST™ Particle.
Example of a TNT® T7 Quick Reaction Using Plasmid DNA.

**Components** | **Reaction Using [³⁵S]Methionine** | **Reaction Using Unlabeled Methionine**
--- | --- | ---
TNT® T7 Quick Master Mix | 40µl | 40µl
Methionine, 1mM | – | 1µl
[³⁵S]methionine (1,000Ci/mmoll at 10mCi/ml) | 2µl | –
plasmid DNA template(s) (0.5µg/µl) | 2µl | 2µl
Nuclease-Free Water to a final volume | 50µl | 50µl

Immobilize GST-Fusion Proteins onto MagneGST™ Particles

**Lyse Cells**
1. Harvest cells from 1ml of bacterial culture.
2. Add 200µl of MagneGST™ Cell Lysis Reagent to each fresh or frozen cell pellet. Resuspend the cell pellet at room temperature (20–25°C) by pipetting or gentle mixing.
3. Add 2µl of RQ1 RNase-Free DNase.
   **Note:** Addition of DNase reduces viscosity and can increase the purity of GST-fusion proteins. Up to 5µl of RQ1 RNase-Free DNase can be added to reduce viscosity. The DNase can be omitted, if desired.
4. Incubate the cell suspension at room temperature for 20–30 minutes on a rotating platform or shaker. During this incubation, begin the particle equilibration procedure.

**Equilibrate Particles**
1. Thoroughly resuspend the MagneGST™ Particles by inverting the bottle several times to obtain a uniform suspension.
2. Pipet 20µl of MagneGST™ Particles into a 1.5ml tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Magnetic capture will typically occur within a few seconds.
4. Carefully remove and discard the supernatant.
5. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and resuspend by pipetting or inverting.
6. Repeat Steps 3–5 two more times for a total of three washes.

**Bind Protein**
1. After the final wash, resuspend the particles in 100µl of MagneGST™ Binding/Wash Buffer.
   **Note:** Adding up to 1% BSA may reduce nonspecific binding and potential problems with background. IGEPAL® CA-630 (NP40 analog) at final concentration 0.5% may have the same effect. The amount of BSA used may need to be optimized for your particular protein.
2. Add 200µl of cell lysate containing the GST-fusion protein or GST control to the MagneGST™ Particles.
3. Incubate (with constant gentle mixing) for 30 minutes at room temperature on a rotating platform.
   **Note:** Do not allow the MagneGST™ Particles to settle for more than a few minutes during capture of the bait protein as this will reduce binding efficiency.

**Wash**
1. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and save for gel analysis (optional).
2. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and gently mix. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and discard (or save if analysis of wash is desired).
4. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)
5. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and discard (or save if analysis of wash is desired).
6. Repeat Steps 4–5 for a total of three washes.
7. After the last wash, resuspend the particles in 20µl of MagneGST™ Binding/Wash Buffer.
8. We recommend using 5µl of the immobilized GST-fusion or GST control for the pull-down assay. Thus, 20µl of particles will provide sufficient material for more than one set of pull-down reactions. However, in some cases more than 5µl may be required for one pull-down reaction.

**Capture, Wash and Analysis of Prey Protein**
Capture
1. Add 20µl of the TNT® T7 Quick coupled transcription/translation reaction from Phase 1 to each 5µl aliquot of particles carrying GST-fusion protein (or GST control).

2. Add 155µl MagneGST™ Binding/Wash Buffer and 20µl 10% BSA (or 175µl MagneGST™ Binding/Wash Buffer if BSA is omitted) to a final volume of 200µl for each pull-down reaction.

Note: MagneGST™ Binding/Wash Buffer is a neutral PBS buffer, allowing the user to optimize buffer conditions for each specific protein:protein interaction. Some protein interactions will require the presence of various cofactors, salts and detergents.

3. Incubate for 1 hour (with gentle mixing) at room temperature on a rotating platform.

Note: Do not allow the MagneGST™ Particles to settle for more than a few minutes during capture of the prey protein, as this will reduce binding efficiency.

4. Place the tube in a magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.

Washing
1. Add 400µl of MagneGST™ Binding/Wash Buffer, and mix gently by inverting the tube.

2. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.

3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Remove the supernatant, and save for analysis (it is especially important to keep this fraction during initial optimization).

4. Add 400µl of MagneGST™ Binding/Wash Buffer, and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)

5. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.

6. Repeat Steps 4 and 5 three more times for a total of five washes.

Elution
1. Add 20µl of 1X SDS loading buffer.

2. Incubate for 5 minutes at room temperature with mixing.

3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Remove the eluate for analysis.

Analysis
Prepare samples for SDS-PAGE analysis. For radioactively labeled prey proteins, we recommend loading 1–2% of each sample volume.

Additional Resources for the MagneGST™ Pull-Down System

Technical Bulletins and Manuals
TM249 MagneGST™ Pull-Down System Technical Manual

Promega Publications
Detection of protein:protein interactions using the MagneGST™ Pull-Down System

VII. Analysis of DNA:Protein Interactions

Regulation of chromatin structure and gene expression is essential for normal development and cellular growth. Transcriptional events are tightly controlled both spatially and temporally by specific protein:DNA interactions. Currently there is a rapidly growing trend toward genome-wide identification of protein-binding sites on chromatin to characterize regulatory protein:DNA interactions that govern the transcriptome. Common methods to examine protein:DNA interactions include the electrophoretic mobility shift assay, also known as the gel shift assay, and chromatin immunoprecipitation (Solomon et al. 1985; Solomon et al. 1988) coupled with DNA microarray or ultrahigh-throughput sequencing analysis.

A. Gel Shift Assays

Electrophoretic mobility shift assays (EMSA) or gel shift assays can be used to analyze protein:DNA complexes expressed in vitro. The proteins are incubated with an oligonucleotide containing a target consensus sequence site, and DNA binding is detected by gel shift. An animated presentation (requires Flash plug in) of protein:DNA interaction detection using the TNT® Systems and Gel Shift Assay is available. The gel shift assay provides a simple and rapid method to detect DNA-binding proteins (Ausubel et al. 1989). This method is used widely in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell extract preparations), with a 32P end-labeled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

Promega gel shift assay systems contain target oligonucleotides, a control extract containing DNA-binding proteins, binding buffer and reagents for phosphorylating...


**B. Chromatin Immunoprecipitation**

Chromatin immunoprecipitation (ChIP) is an experimental method used to determine whether DNA-binding proteins, such as transcription factors, associate with a specific genomic region in living cells or tissues. Cells are treated with formaldehyde to form covalent crosslinks between interacting proteins and DNA. Following crosslinking, cells are lysed, and the crude cell extracts are sonicated to shear the DNA. The DNA:protein complex is immunoprecipitated using an antibody that recognizes the protein of interest. The isolated complexes are washed, then eluted. The DNA:protein crosslinks are reversed by heating and the proteins removed by proteinase K treatment. The remaining DNA is purified and analyzed by various ways, including PCR, microarray analysis or direct sequencing.

**Antibody-Based ChIP**

The standard ChIP assay requires 3–4 days for completion (Figure 11.10). The procedure requires antibodies highly specific to the protein of interest to immunoprecipitate the DNA:protein complex. The success of the procedure relies on the ability of the antibody to bind to the target protein after crosslinking, cell lysis and sonication, all of which can negatively affect epitope recognition by the antibody.

**HaloCHIP™ System—an Antibody-Free Approach**

To address the difficulties that arise when performing ChIP, a novel method that does not require the use of antibodies, the HaloCHIP™ System, has been devised for the covalent capture of protein:DNA complexes. DNA-binding proteins of interest are expressed in cells as HaloTag® fusion proteins, crosslinked to DNA, then captured on the HaloLink™ Resin, which forms a highly specific, covalent interaction with HaloTag® proteins. Due to the covalent linkage between the resin and crosslinked protein:DNA complexes, the resin can be stringently washed to remove nonspecifically bound DNA and protein more efficiently than co-immunoprecipitation. The crosslinks are reversed to release purified DNA fragments from the resin. By improving specificity and reducing background during the isolation of protein:DNA complexes, the HaloCHIP™ approach effectively increases the signal-to-noise ratio to permit detection of small changes in protein binding within a genome. The HaloCHIP™ System (Cat.# C9410) is currently available. An animation of this procedure (requires Flash plug in) is available.

**Additional Resources for the HaloCHIP™ System**

**Technical Bulletins and Manuals**

- TM075  
  HaloCHIP™ System Technical Manual

**Promega Publications**

- HaloCHIP™ System: Mapping intracellular protein:DNA interactions using HaloTag® technology

**Additional Resources for the HaloTag® Technology**

**Technical Bulletins and Manuals**

- TM260  
  HaloTag® Technology: Focus on Imaging

**Promega Publications**

- HaloTag™ technology: Cell imaging and protein analysis
- Perform multicolor live- and fixed-cell imaging applications with the HaloTag™ interchangeable labeling technology
- HaloTag™ interchangeable labeling technology for cell imaging and protein capture
- HaloTag® protein: A novel reporter protein for human neural stem cells
- Cell surface HaloTag® technology: Spatial separation and bidirectional trafficking of proteins
- HaloLink™ Resin: High capacity, specificity and scalable throughput for protein analysis
- HaloTag™ interchangeable labeling technology for cell imaging, protein capture and immobilization
Figure 11.10. Overview of chromatin immunoprecipitation using antibodies. Cells are grown using the appropriate conditions to form an interaction between the transcription factor (TF) of interest and DNA. To preserve the DNA:protein association during cell lysis, formaldehyde is added, resulting in crosslinks between the DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. A polyclonal or monoclonal antibody that recognizes the target protein is added, then incubated overnight. Protein A or Protein G agarose beads are added to capture the complex, then washed. The antibody must specifically and tightly bind its target protein under the wash conditions used. Finally, reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of the immunoprecipitated DNA.

VIII. Proteomics Approaches for the Analysis of Complex Mixtures of Proteins

Mass spectrometry is a powerful tool for protein analysis and the major technique used to study proteins in the field of proteomics (Mann et al. 2001). Mass spectrometry can be used to characterize recombinant proteins, identify proteins and detect and characterize posttranslational modifications.

One method of protein identification uses enzymatic digestion followed by mass spectrometry analysis. In this procedure, complex protein mixtures such as cell extracts are resolved by gel electrophoresis, and the band or spot of interest is excised from the gel and digested with trypsin. Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The distribution of Lys and Arg residues in proteins is such that trypsin digestion yields peptides of molecular weights that can be analyzed by mass spectrometry. The pattern of peptides obtained is used to identify the protein. Database searches can then be performed, using the mass of the peptides to identify the protein(s) resolved on the gel (Mann et al. 2001).

The stringent specificity of trypsin is essential for protein identification. Native trypsin is subject to autolysis, generating pseudotrypsin, which exhibits a broadened specificity, including a chymotrypsin-like activity (Keil-Dlouha et al. 1971). Such autolysis products would result in additional peptide fragments that could interfere with database analysis of the mass of fragments detected by mass spectrometry.

Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280), provides maximum specificity. Lysine residues in the porcine trypsin are modified by reductive methylation, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion (Rice et al. 1977). The specificity of the purified trypsin is further improved by TPCK treatment, which inactivates chymotrypsin. The treated trypsin is then purified by affinity chromatography and lyophilized to yield Trypsin Gold, Mass Spectrometry Grade. More information and a detailed protocol are available in Technical Bulletin TB309.
Figure 11.11. Capture of DNA:protein interactions using the HaloTag® technology. The protein-coding sequence of a transcription factor (TF) is cloned into a HaloTag® (HT) mammalian expression vector. This recombinant vector is transfected into mammalian cells, and the cells are grown under the appropriate conditions to allow formation of DNA:protein interactions. To preserve the DNA:protein association, formaldehyde is added, resulting in crosslinks between DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. The complex is then immobilized by adding the HaloLink™ Resin, followed by a short incubation. Reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of immunoprecipitated DNA.

A. In-Gel Trypsin Digestion of Proteins

Numerous protocols for in-gel protein digestion have been described (Flannery et al. 1989; Shevchenko et al. 1996; Rosenfeld et al. 1992). The following procedure has been used successfully by Promega scientists.

Materials Required:
(see Composition of Solutions section)
- Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280) and protocol
- SimplyBlue™ SafeStain (Invitrogen Cat.# LC6060)
- trifluoroacetic acid (TFA)
- acetonitrile (ACN)
- 200mM NH₄HCO₃ buffer (pH 7.8)
- NANOpure® water
- ZipTip® scx pipette tips (Millipore Cat.# ZTSCXS096)
- α-cyano-4-hydroxycinnamic acid (CHCA)
- MALDI target
- ZipTip® C18 pipette tips (Millipore Cat.# ZTC18S096)
1. Separate protein samples by electrophoresis on an SDS-Tris-Glycine gel.
   **Note:** Other gel systems and staining reagents can be used for in-gel digestions but should be tested to ensure compatibility with the protein of interest and detection system being used.

2. Rinse the gel three times, for 5 minutes each rinse, in NANOpure® water. Stain for 1 hour in SimplyBlue™ SafeStain (Invitrogen Corporation) at room temperature with gentle agitation. When staining is complete, discard the staining solution.

3. Destain the gel for 1 hour in NANOpure® water at room temperature with gentle agitation. When destaining is complete, discard the solution.

4. Using a clean razor blade, cut the protein bands of interest from the gel, eliminating as much polyacrylamide as possible. Place the gel slices into a 0.5ml microcentrifuge tube that has been prewashed twice with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA).

5. Destain the gel slices twice with 0.2ml of 100mM NH₄HCO₃/50% ACN for 45 minutes each treatment, at 37°C to remove the SimplyBlue™ SafeStain.

6. Dehydrate the gel slices for 5 minutes at room temperature in 100µl of 100% ACN. At this point the gel slices will be much smaller than their original size and will be whitish or opaque in appearance.

7. Dry the gel slices in a Speed Vac® concentrator for 10–15 minutes at room temperature to remove the ACN.

8. Resuspend the Trypsin Gold at 1µg/µl in 50mM acetic acid, then dilute in 40mM NH₄HCO₃/10% ACN to 20µg/ml. Preincubate the gel slices in a minimal volume (10–20µl) of the trypsin solution at room temperature (do not exceed 30°C) for 1 hour. The slices will rehydrate during this time. If the gel slices appear white or opaque after one hour, add an additional 10–20µl of trypsin and incubate for another hour at room temperature.

9. Add enough digestion buffer (40mM NH₄HCO₃/10% ACN) to completely cover the gel slices. Cap the tubes tightly to avoid evaporation. Incubate overnight at 37°C.

10. Incubate the gel slice digestes with 150µl of NANOpure® water for 10 minutes, with frequent vortex mixing. Remove and save the liquid in a new microcentrifuge tube.

11. Extract the gel slice digestes twice, with 50µl of 50% ACN/5% TFA (with mixing) for 60 minutes each time, at room temperature.

12. Pool all extracts (from Steps 10 and 11), and dry in a Speed Vac® concentrator at room temperature for 2–4 hours (do not exceed 30°C).

13. Purify and concentrate the extracted peptides using ZipTip® pipette tips (Millipore Corporation) following the manufacturer’s directions.

14. The peptides eluted from the ZipTip® tips are now ready for mass spectrometric analysis.

### B. In-Solution Trypsin Digestion of Proteins

In general, proteins require denaturation and disulfide bond cleavage for enzymatic digestion to reach completion (Wilkinson, 1986). If partial digestion of a native protein is desired, begin this protocol at Step 3.

1. Dissolve the target protein in 6M guanidine HCl (or 6–8M urea or 0.1% SDS), 50mM Tris-HCl (pH 8), 2–5mM DTT (or β-mercaptoethanol).

2. Heat at 95°C for 15–20 minutes or at 60°C for 45–60 minutes. Allow the reaction to cool.

3. For denatured proteins, add 50mM NH₄HCO₃ (pH 7.8) or 50mM Tris-HCl, 1mM CaCl₂ (pH 7.6), until the guanidine HCl or urea concentration is less than 1M. If SDS is used, dilution is not necessary. For digestion of native proteins, dissolve in buffer with a pH between 7 and 9.

4. Add Trypsin Gold to a final protease:protein ratio of 1:100 to 1:20 (w/w). Incubate at 37°C for at least 1 hour. Remove an aliquot, and chill the remainder of the reaction on ice or freeze at –20°C.

5. Terminate the protease activity in the aliquot from Step 4 by adding an inhibitor. Alternatively, precipitate the aliquot by adding TCA to 10% final concentration. The reaction can also be terminated by freezing at –20°C. Trypsin can also be inactivated by lowering the pH of the reaction below pH 4. Trypsin will regain activity as the pH is raised above pH 4 (Wilkinson, 1986).
   **Note:** The following are general trypsin inhibitors: Antipain (50µg/ml), antithrombin (1unit/ml), APMSF (0.01–0.04mg/ml), aprotinin (0.06–2µg/ml), leupeptin (0.5µg/ml), PMSF (17–170µg/ml), TLCK (37–50µg/ml), trypsin inhibitors (10–100µg/ml).

6. Determine the extent of digestion by subjecting the aliquot in Steps 4 and 5 to reverse phase HPLC or SDS-PAGE.

7. If no inhibitors were added to the remainder of the reaction and further proteolysis is required, incubate at 37°C until the desired digestion is obtained (Sheer, 1994). Reducing the temperature will decrease the digestion rate. Incubations of up to 24 hours may be
required, depending on the nature of the protein. With long incubations, take precautions to avoid bacterial contamination.

Additional Literature for Trypsin Gold, Mass Spectometry Grade
Technical Bulletins and Manuals
TB309  Trypsin Gold, Mass Spectometry Grade, Technical bBulletin

C. Affinity Tag In Vitro Pull-Down Assay with Trypsin Digestion and Protein Analysis

Markillie and associates describe a simple exogenous protein complex purification and identification method that can be easily automated (Markillie et al. 2005). The method uses MagneHis™ Ni Particles (Cat.# V8560, V8565) to pull down target proteins, followed by denaturing elution, trypsin digestion and mass spectrometry analysis (Figure 11.12).

SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on a polyacrylamide gel, and high voltage is applied, causing the proteins to migrate toward the positive electrode (anode).

Since the proteins have a net negative charge that is proportional to their size, proteins are separated solely on the basis of their molecular mass—a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated by comparing the gel mobility of a band with those of protein standards. Sharp protein bands are achieved by using a discontinuous gel system, having stacking and separating gel layers that differ in either salt concentration or pH or both (Hanes, 1981).

Materials Required:
(see Composition of Solutions section)
• acrylamide solution, 40%
• upper gel 4X buffer
• lower gel 4X buffer
• ammonium persulfate, 10%
• TEMED
• SDS-polyacrylamide gel running 1X buffer
• loading 2X buffer
• trichloroacetic acid (TCA) (optional)
• acetone, ice-cold (optional)

This gel system uses the method described by Laemmli (Laemmli, 1970). Formulations for preparing resolving and stacking minigels are provided in Tables 11.4 and 11.5. The amounts of reagents indicated in Tables 11.4 and 11.5 are sufficient to prepare two 7 × 10cm gels, 0.75–1.00mm thick. Add ammonium persulfate and TEMED just prior to pouring the gel, as these reagents promote and catalyze polymerization of acrylamide. Pour the resolving gel mix into assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. Gently overlay the gel mix with 0.1% SDS, and allow the gel to polymerize for at least 15–30 minutes. After polymerization, remove the SDS overlay, and rinse the surface of the resolving gel with water to remove any unpolymerized acrylamide. Rinse one more time with a small volume of stacking gel buffer. Fill the remaining space with the stacking gel solution, and insert the comb immediately. After the stacking gel has polymerized, remove the comb, and rinse the wells with water to remove unpolymerized acrylamide. At least 1cm of stacking gel should be present between the bottom of the loading wells and the resolving gel.

<table>
<thead>
<tr>
<th>Table 11.4. Formulation for Stacking Gel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>upper gel 4X buffer</td>
</tr>
<tr>
<td>water</td>
</tr>
<tr>
<td>acrylamide solution, 40%</td>
</tr>
<tr>
<td>APS, 10% 1</td>
</tr>
<tr>
<td>TEMED 2</td>
</tr>
</tbody>
</table>

1 ammonium persulfate (always prepare fresh)
2N,N,N′,N′-tetramethylethylenediamine

Prepare Samples
1. Add an equal volume of loading 2X buffer to the sample.
2. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

Optional
1. If the sample is very dilute or contains salts that may interfere with gel analysis, add TCA to a final concentration of 10% (w/v).
2. Place the sample on ice for 5 minutes, centrifuge at 4°C for 2 minutes at 12,000 × g in a microcentrifuge and discard the supernatant.
3. Wash the protein pellet with ice-cold acetone, and resuspend it in a suitable volume (generally <20µl) of loading 1X buffer (prepared by adding an equal volume of water to loading 2X buffer).
4. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

X. Composition of Solutions

MagneHis™ Binding/Wash Buffer (pH 7.5)
100mM HEPES
10mM imidazole

MagneHis™ Elution Buffer (pH 7.5)
100mM HEPES
500mM imidazole

MagneHis™ Binding/Wash Buffer for Denaturing Conditions (pH 7.5)
100mM HEPES
10mM imidazole
2–8M guanidine-HCl or urea

MagneHis™ Elution Buffer for Denaturing Conditions (pH 7.5)
100mM HEPES
500mM imidazole
2–8M guanidine-HCl or urea

4X SDS gel-loading buffer
0.24M Tris-HCl (pH 6.8)
2% SDS
3mM bromophenol blue
50.4% glycerol
0.4M dithiothreitol

SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

MagZ™ Binding/Wash Buffer (pH 7.4)
20mM sodium phosphate
500mM NaCl

MagZ™ Elution Buffer
1M imidazole (pH 7.5)

Binding Buffer (HisLink™)
100mM HEPES (pH 7.5)
10mM imidazole

Wash Buffer (HisLink™)
100mM HEPES (pH 7.5)
10–100mM imidazole

Elution Buffer (pH 7.5) (HisLink™)
100mM HEPES
500mM imidazole

MagneGST™ Binding/Wash Buffer
4.2mM Na₂HPO₄
2mM K₂HPO₄
140mM NaCl
10mM KCl

Elution Buffer (MagneGST™ System)
50mM glutathione (pH 7.0–8.0)
50mM Tris-HCl (pH 8.1)
The glutathione provided has a pH value between 7.0 and 8.0. If a different source of glutathione is being used, adjust the pH to 7.0–8.0 before adding the Tris-HCl (pH 8.1). The glutathione solution has little buffering capacity at pH 7.0–8.0, so take care when adjusting the pH. Failure to adjust the pH of glutathione will decrease the pH of the elution buffer, especially when final glutathione concentrations are ≥50mM.

Table 11.5. Formulation for Resolving Gel.

<table>
<thead>
<tr>
<th>Component</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>lower gel 4X buffer</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>water</td>
<td>5.4ml</td>
<td>4.9ml</td>
<td>4.4ml</td>
<td>3.65ml</td>
<td>2.4ml</td>
</tr>
<tr>
<td>acrylamide solution, 40%</td>
<td>2.0ml</td>
<td>2.5ml</td>
<td>3.0ml</td>
<td>3.75ml</td>
<td>5.0ml</td>
</tr>
<tr>
<td>APS, 10%;¹</td>
<td>50.0µl</td>
<td>50.0µl</td>
<td>50.0µl</td>
<td>50.0µl</td>
<td>50.0µl</td>
</tr>
<tr>
<td>TEMED ²</td>
<td>5.0µl</td>
<td>5.0µl</td>
<td>5.0µl</td>
<td>5.0µl</td>
<td>5.0µl</td>
</tr>
</tbody>
</table>

¹ammonium persulfate (always prepare fresh)
²N,N,N′,N′-tetramethylethylenediamine
1X SDS gel-loading buffer
50mM Tris-HCl (pH 6.8)
2% SDS
0.1% bromophenol blue
10% glycerol
10mM dithiothreitol
SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

acrylamide solution, 40%
38.9g acrylamide
1.1g bisacrylamide
Dissolve in 100ml of water.

upper gel 4X buffer
0.5M Tris-HCl (pH 6.8)
0.4% SDS

lower gel 4X buffer
1.5M Tris-HCl (pH 8.8)
0.4% SDS

gel running buffer
25mM Tris base
192mM glycine
0.1% SDS
Adjust pH to 8.3.

TBE 10X buffer (1L)
107.80g Tris base
~55g boric acid
7.44g disodium EDTA•2H₂O
Add components in the order listed above to ~800ml of distilled water. Add slightly less than the total amount of boric acid. Mix until completely dissolved, check pH and adjust to 8.3 with boric acid. Bring final volume to 1L with distilled water.

TE buffer
10mM Tris-HCl (pH 8.0)
1mM EDTA

T4 Polynucleotide Kinase 10X Buffer
700mM Tris-HCl (pH 7.6)
100mM MgCl₂
50mM DTT

Coomassie® Blue staining solution
50% (v/v) methanol
10% (v/v) acetic acid
0.25% (w/v) Coomassie® Blue R-250

destaining solution
10% (v/v) methanol
5% acetic acid

gel loading 10X buffer
250mM Tris-HCl (pH 7.5)
0.2% bromophenol blue
40% glycerol

Gel Shift Binding 5X Buffer
20% glycerol
5mM MgCl₂
2.5mM EDTA
2.5mM DTT
250mM NaCl
50mM Tris-HCl (pH 7.5)
0.25mg/ml poly(dI-dC)•poly(dI-dC)

XI. References
11Protein Purification and Analysis


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