

2. Elute in 2 ml 50 mM Tris, pH 8.0, 10 mM glutathione.
3. Concentrate the protein in Centricon (Amicon, Danvers, MA) devices to 100–300 μ l. Add 1.9 ml of the final desired buffer. Repeat the concentration and buffer dilution twice. Concentrate to final desired protein concentration and drop freeze in small aliquots into liquid nitrogen.

General Comments

We have not had any problems using this method to purify proteins that are lethal on overexpression, including Abp1p and Duo1p. Although cells are dying during the induction, significant amounts of recombinant protein are produced. Because some proteins are sensitive to longer (>4 hr) induction times, it may be important to test protein expression at different time points during the induction. The expression level varies from approximately 0.01 to 0.2 mg recombinant protein/ml high-speed supernatant, and all proteins tested have been soluble. The yield from these preparations is approximately 10% of the expressed protein; most of the loss is at the affinity purification step. We have not been successful in dialyzing out the putative inhibitor of binding to glutathione resins. We have successfully cleaved GST from GST-Aip1p bound to glutathione agarose using the thrombin site present in pEG(KT) and standard techniques,⁵ but this approach has not worked for other more thrombin-sensitive proteins. To request plasmids or yeast strains described here, please contact Dr. Bruce Goode at goode@brandeis.edu.

⁵ A. A. Rodal, J. Tetrault, P. Lappalainen, D. G. Drubin, and D. C. Amberg, *J. Cell Biol.* **146**, 1251 (1999).

[10] Protein- and Immunoaffinity Purification of Multiprotein Complexes

By DOUGLAS R. KELLOGG and DANESH MOAZED

Introduction

Virtually all proteins must interact with other proteins to carry out their activities within the cell. Protein affinity chromatography allows one to exploit this fundamental property to identify proteins that function together as components of multiprotein complexes. Affinity chromatography is similar to genetics in that it offers a highly specific and powerful means of identifying proteins that participate in a biological activity. Affinity chromatography offers an advantage over genetics

in that it can identify proteins that would be difficult or impossible to identify by genetic screens (e.g., proteins that carry out redundant functions or proteins that play a broad role in cellular activities that can be missed in specific genetic screens). In addition, genetic approaches to identifying interacting proteins can be biased by particular mutant alleles or preconceived ideas about the functions of proteins. Affinity chromatography is not biased by function and can therefore lead one in new and interesting directions.

This chapter discusses two methods for identifying interacting proteins by affinity chromatography. In the first method, a protein affinity column is made by linking relatively large amounts of a purified protein to a column matrix. A crude extract is then passed over the column and specifically bound proteins are eluted with a buffer containing high salt or denaturants such as urea. In the second approach, endogenous protein complexes are purified using an immunoaffinity column made by linking antibodies that recognize a specific protein to a column matrix.

Purification of Interacting Proteins Using Protein Affinity Chromatography

Protein affinity chromatography using proteins that are expressed in bacteria provides a powerful method for the identification of interacting proteins. This method has been used to isolate proteins that bind to the gene N transcription antiterminator of bacteriophage lambda (λ),¹ bacteriophages T4 proteins essential for DNA replication,² actin and microtubule-binding proteins,³⁻⁵ RNA polymerase C-terminal tail-binding proteins,⁶ Sir2- and Sir4-binding proteins,^{7,8} and proteins that associate with cell cycle control proteins.⁹⁻¹¹ One primary advantage of affinity chromatography is that the high concentration of purified protein immobilized on the column will allow the isolation of proteins that have a weak affinity for the protein of interest. A second advantage of this approach is that column chromatography can be performed using buffer conditions that are independent of initial cell

¹ J. Greenblatt, P. Malnoe, and J. Li, *J. Biol. Chem.* **255**, 1465 (1980).

² T. Formosa, R. L. Burke, and B. M. Alberts, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2442 (1983).

³ K. G. Miller and B. M. Alberts, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4808 (1989).

⁴ D. R. Kellogg, C. M. Field, and B. M. Alberts, *J. Cell Biol.* **109**, 2977 (1989).

⁵ R. V. Aroian, C. Field, G. Pruliere, C. Kenyon, and B. M. Alberts, *EMBO J.* **16**, 1541 (1997).

⁶ C. M. Thompson, A. J. Koleske, D. M. Chao, and R. A. Young, *Cell* **73**, 1361 (1993).

⁷ D. Moazed and A. D. Johnson, *Cell* **86**, 667 (1996).

⁸ A. F. Straight, W. Shou, G. J. Dowd, C. W. Turck, R. J. Deshaies, A. D. Johnson, and D. Moazed, *Cell* **97**, 245 (1999).

⁹ D. R. Kellogg, A. Kikuchi, T. Fujii-Nakata, C. W. Turck, and A. W. Murray, *J. Cell Biol.* **130**, 661 (1995).

¹⁰ R. Altman and D. Kellogg, *J. Cell Biol.* **138**, 119 (1997).

¹¹ C. W. Carroll, R. Altman, D. Schieltz, J. R. Yates, and D. Kellogg, *J. Cell Biol.* **143**, 709 (1998).

lysis conditions. Thus, lysis may be carried out in the presence of high concentrations of salt that may be required for the efficient solubilization of proteins of interest, which can be removed from the extract prior to chromatography. The overall experiment involves three steps. First, the protein chosen as an affinity probe (and a suitable control protein) must be purified and immobilized on a solid matrix to construct an affinity column. Second, a whole cell extract is prepared and passed over the columns. Third, specifically bound proteins are identified by mass spectroscopy analysis. These steps must be followed by experiments designed to test the *in vivo* significance of the identified interactions (discussed later).

Choosing Proteins for Construction of Affinity Matrix

Affinity chromatography experiments generally begin with a protein that one would like to learn more about. This is usually a protein that has a known function in a biological process but its mode of action is not fully understood. The most important factor is the ability to purify relatively large quantities of the protein. An easy way to do this is by overexpression of the protein as a fusion with glutathione *S*-transferase (GST) in *Escherichia coli*.¹² We have successfully used many different GST fusions expressed in *E. coli* for our experiments, but in principle, any source that can produce milligram quantities of the protein can be used. In general, 3–6 mg of protein is required for construction of an affinity column. A general method for the purification of GST fusion proteins is described later.

Controls

In order to distinguish proteins that are specifically retained on affinity columns, a control column is always run in parallel with the column containing the protein of interest. In the case of GST fusion proteins, any other GST fusion protein that is not functionally related to the protein of interest could be used as a control. We usually try to use GST fusion proteins of similar size: one as the control column and one as the experimental column. GST alone is not an ideal control because it is not very sticky and elution profiles of GST columns are not an accurate representation of nonspecifically bound proteins. If available, an ideal control is the same protein carrying a point mutation or a truncation that disrupts a specific function *in vivo*.

Purification of GST Fusion Proteins

GST fusion proteins are purified based on the method described by Smith and Johnson.¹² The following is an adaptation of this protocol that has worked well in our laboratories for the purification of several yeast proteins involved in cell cycle regulation or gene silencing.^{7,11}

¹² D. B. Smith and K. S. Johnson, *Gene* 67, 31 (1988).

1. Inoculate 4 liters of 2× YT media¹³ containing 75 μg/ml ampicillin with 100–200 ml of overnight culture. We use three 2.8-liter flasks containing 1.3 liters each.

2. Grow the cultures at room temperature until the OD reaches 0.8 to 1, and then add isopropylthiogalactoside (IPTG) to 0.1 mM and continue incubating the cultures at room temperature for another 2–3 hr. Many proteins are more soluble when expressed at room temperature. To speed things up, we sometimes start the cultures at 37°, and by the time they reach an OD of 1.0 they usually have cooled to room temperature.

3. Pellet the cells at room temperature by spinning at 5000 rpm for 20 min (Sorvall) and rapidly scrape them out of the bottle and freeze in liquid nitrogen. The frozen chunks of cell paste can be stored at –80°.

4. We have found that the degradation of many proteins can be prevented by using a rapid lysis procedure that gets the extract into a buffer that prevents proteolysis as rapidly as possible. To do this, grind the frozen cells under liquid nitrogen in a mortar and pestle until you have a fine powder (5–10 min). Transfer the cell powder to a cold beaker and allow the cell powder to warm at room temperature for about 5 min or until the powder is just starting to thaw around the edges of the beaker. (This avoids the formation of ice crystals when the buffer is added.) Add approximately 5 volumes of room temperature phosphate-buffered saline (PBS) that contains 0.5% (v/v) Tween 20, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 M NaCl. Immediately resuspend the cell powder by stirring with a spatula and then add a stir bar and stir in a cold room for a few minutes. (For proteins where degradation is not a problem, skip the grinding step and resuspend the frozen cell pellet in the aforementioned lysis buffer containing 200 μg/ml lysozyme.) Sonicate for about a minute to reduce viscosity. The PMSF is added immediately before resuspending the cells because it is unstable in water. All of the following purification steps are carried out at 4°.

5. Add dithiothreitol (DTT) to 10 mM. Spin the lysate for 60 min at 35,000 rpm in a Beckman 50.2 Ti rotor.

6. Load the supernatant onto a 5- to 10-ml glutathione agarose column (Sigma, St. Louis, MO) over a period of 2–4 hr.

7. Wash the column with 50–100 ml of wash buffer (PBS containing 0.05% Tween 20, 0.5 mM DTT, and 0.25 M KCl). Monitor the effluent from the column (Bradford assay) to make sure that there is no protein still washing off at the end of the wash step. At the end of the wash step, wash the column with 2 column volumes of wash buffer without Tween 20.

8. Elute the column with 50 mM Tris, pH 8.1, containing 0.25 M KCl and 5 mM reduced glutathione. We do the elution by pipetting 1/7 column volume aliquots

¹³ J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

of elution buffer directly onto the top of the column bed. We then let the aliquot flow through into a plastic tube and advance to another fraction. The fractions are assayed, and the peak fractions are pooled and dialyzed extensively into 50 mM HEPES, pH 7.6, 50–300 mM KCl, and 30% (v/v) glycerol (the glutathione must be removed prior to coupling to Affigel 10.) The yield of GST should be approximately 100 mg, whereas the yield of GST fusions is usually much lower.

Preparation of Affinity Columns

We usually make 1- to 2-ml affinity columns by cross-linking 3–6 mg of protein/ml of Affi-Gel 10 (Bio-Rad, Hercules, CA).

1. Protein should be at a concentration of 1–5 mg/ml in 50 mM HEPES, pH 7.6, 100–500 mM KCl, and 30% (v/v) glycerol. The amount of buffer, salt, or glycerol is not critical. What is important is that the pH is between 7.5 and 8 and that there are no primary amines or sulfhydryls in the buffer.

2. Affi-Gel 10 is stored in 2-propanol at –20. Before using Affi-Gel 10, remove the bottle from the freezer and allow it to warm to room temperature. This prevents water from condensing in the bottle (water inactivates Affi-Gel 10).

3. Transfer the desired quantity of Affi-Gel to a Buchner funnel (Pyrex, 30 ml) assembled on a vacuum line. Apply gentle or intermittent vacuum and do not allow the gel to dry. Wash the resin three times with ice-cold water (each wash is with at least two to three times the volume of the resin) and one with a small amount of coupling buffer. The active groups on Affi-Gel 10 hydrolyze in water, so the washes should be completed as rapidly as possible (20 min or less).

4. Using a spatula, transfer the moist gel to a fresh tube and add the protein solution. Mix immediately. Do the coupling in the smallest possible tube. Affi-Gel 10 sticks to surfaces and much of it will be lost if a larger than necessary tube is used for coupling. Mix using an end-over-end mixer at 4° for 2–4 hr. Monitor the rate of the coupling reaction by spinning down the beads and removing a 25- μ l sample of the supernatant. Do a Bradford assay on each time point, and when 25 μ l looks like 5 μ l of the starting reaction mix, you have reached approximately 80% coupling and the reaction should be stopped (overcoupling kills some proteins). We usually take time points at 5, 10, and 20 min and at 20-min intervals thereafter. The rate of coupling varies considerably, depending on the protein. Some proteins are completely coupled within 5 min, some take more than an hour, and some do not couple at all. Proteins that do not couple well can often be made to couple by adding 50–100 mM MgCl₂ to the reaction. This minimizes charge repulsion between negatively charged proteins and the column matrix.

5. Stop the reaction by adding 1 M ethanolamine, pH 7.5, to a final concentration of 50 mM. Leave on ice for 1 hr or overnight to block any residual active groups.

6. Pour the coupled resin into a small column. We usually use a 5- to 10-cm Bio-Rad column with a 1-cm internal diameter or the Bio-Rad Bio-Spin columns.

7. Pack and wash the columns using a peristaltic pump at a flow rate of ~ 20 ml per hour. The maximum cross-sectional flow rate for the Affi-Gel 10 resin is about 25 cm/hr. For a column with a 1-cm internal diameter, flow rate = cross-sectional flow rate (cm/hr) \times ID (cm²) = 25 \times 0.79 = 20 ml/hr.

8. Wash each column with 10 column volumes of 50 mM HEPES (pH 7.6), 250 mM KCl, and 1 mM DTT. Before using the column, wash it with whatever elution conditions that you will be using. Most protein affinity columns can be stored for prolonged periods of time at -20° after they have been equilibrated with a buffer containing 50% (w/v) glycerol. For shorter storage periods of up to 2 weeks, columns can be stored at 4° with a buffer containing 0.05% azide.

Preparation of Yeast Extracts for Affinity Chromatography

Obtain 10–15 g of yeast cells from a protease-deficient strain (e.g., BJ2168)¹⁴ grown to log phase in rich medium. Resuspend cells in 50 ml of lysis buffer [buffer L: 50 mM HEPES–KOH, pH 7.6, 5% glycerol 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT] containing 500 mM KCl, 0.5% nonidet p-40 (NP-40), and the following protease inhibitors, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 μ g/ml bestatin. PMSF is added to this buffer immediately before lysis from a 100 mM stock made in 100% methanol. Break cells by grinding under liquid nitrogen in a mortar and pestle (see later) or by using a BeadBeater (Biospec).

When using a BeadBeater, leave all BeadBeater components in the cold room overnight. We use the 89-ml BeadBeater chamber filled with glass beads until the screw that holds the blade in place is covered with beads. The cell suspension is then added to the chamber containing the glass beads. A glass rod or plastic pipette is used to remove air bubbles that are trapped in the beads, and care is taken to minimize air that is trapped in the chamber when the cap is screwed on. The lysis chamber is then assembled with an ice-water chamber, and cells are broken using 12–15 pulses of bead beating each lasting 15 sec with 2 min in between pulses to allow for cooling. The crude extract is centrifuged at 20,000g for 10 min (SS34 rotor) followed by 100,000g for 1 hr. Prior to the latter spin, the salt concentration in the extract is reduced to 150–275 mM by dilution with lysis buffer lacking KCl. However, if the desired binding proteins are thought to be soluble at lower salt concentrations, the initial lysis can be performed at 150–275 mM KCl.

Running Affinity Columns and Elution of Bound Proteins

1. Load the columns in parallel at a flow rate of about 6–8 ml per hour (for 1-ml columns). It takes about 8–9 hr to load. We usually do this step overnight. To monitor how well the column is performing and also how much extract is required to saturate the binding sites on the column, take flow-through samples

¹⁴ E. W. Jones, *Methods Enzymol.* **194**, 428 (1991).

in the beginning and the end of the experiment. The first flow through equals the first flow through with the same protein concentration as the load. The last flow through equals the flow through near the end of the experiment. A bovine serum albumin (BSA) precolumn can be used to reduce the background of nonspecific proteins as described in Kellogg and Alberts.¹⁵ However, it is possible to omit this step if the binding proteins are relatively abundant.

2. Wash the columns with 5–40 column volumes of buffer L containing 0.5% NP-40 and the same KCl concentration as the extract (150–250 mM KCl). The amount of wash buffer to use is determined empirically. Proteins that interact with low affinity can be lost by extensive washing, but too little washing can sometimes give a high background. A good starting point is to wash with 10–15 column volumes.

3. Wash the columns with 5 column volumes of buffer L containing the same KCl concentration as in step 2 (but without NP-40).

4. Elute each column using a salt gradient going from 0.3 to 1.0 M KCl (see Altman and Kellogg).¹⁰ The gradient is made by pipetting 400- μ l aliquots of elution buffer directly onto the top of the column, with each aliquot increasing in KCl concentration by 50 mM. Collect 400- μ l fractions in plastic tubes. Some interacting proteins remain bound to the column after the 1 M KCl elution step. These proteins can be eluted from the column using 2.5 M urea in the elution buffer containing 150 mM KCl.^{7,8}

5. Wash columns with 10 column volumes of buffer L containing 0.05% NaN₃ and store at 4° or equilibrate with buffer L containing 50% glycerol for storage at –20°.

Gel Electrophoresis and Identification of Bound Proteins

1. Precipitate the proteins present in each fraction with trichloroacetic acid (TCA). To each 400- μ l fraction add 40 μ l 100% TCA, mix, leave on ice for 15 min, and spin in a microfuge at 4° for 15 min. Discard the supernatant, spin again for a few seconds, remove the last bit of supernatant, and resuspend in 50 μ l SDS sample buffer. Leave at room temperature for 1 hr and then heat to 85° for 10 min.

2. Load 10 μ l on an 8.5% SDS–polyacrylamide gel. Visualize proteins by silver or Coomassie blue staining.

3. Proteins that are specifically bound to the experimental column and not the control column are then excised from the gel and identified by mass spectroscopy analysis. Approximately 10–100 ng of protein is sufficient for identification by most mass spectroscopy facilities. A silver staining protocol compatible with mass spectroscopy analysis is described by Morrissey.¹⁶ However, it is advisable to

¹⁵ D. R. Kellogg and B. M. Alberts, *Mol. Biol. Cell* **3**, 1 (1992).

¹⁶ J. H. Morrissey, *Anal. Biochem.* **117**, 307 (1981).

consult the mass spectrometry facility that you intend to use for their preferred staining protocol.

Purification of Endogenous Multiprotein Complexes by Immunoaffinity Chromatography

Protein affinity chromatography utilizing proteins expressed in bacteria has provided a powerful means of identifying interacting proteins. However, many proteins can be difficult or impossible to purify from *E. coli*, and proteins purified from heterologous expression systems lack posttranslational modifications that may be necessary for protein–protein interactions. Ideally, one would like to be able to rapidly and specifically purify endogenous multiprotein complexes because these are assembled under native conditions and therefore provide information that is most relevant to the *in vivo* functions of the protein of interest.

Immunoaffinity chromatography has provided a good approach to isolating endogenous multiprotein complexes.^{15,17–19} In this approach, immunoaffinity beads are made by binding antibodies that recognize the protein of interest to protein A beads. The beads are then incubated with crude extract to allow binding of the protein complex to the antibody. After washing the beads with buffer, the protein complex is eluted. In some cases, associated proteins have been eluted with a high salt buffer, leaving the original protein still bound to the antibody on the column. In other cases, the immunoaffinity beads have been made using an antibody raised against a C-terminal or N-terminal peptide, allowing competitive elution of the protein complex with excess peptide. Competitive elution with peptide has the strong advantage of allowing highly specific elution of the entire complex under gentle conditions. This results in a considerably lower background and allows one to purify an intact complex for functional studies. In some cases, it has been possible to identify associated proteins after elution with denaturing conditions; however, this often results in a high background that can make analysis difficult or impossible.

Generally Applicable Protocol for Immunoaffinity Purification of Multiprotein Complexes

A problem with immunoaffinity chromatography is that one must generate a new antibody for each protein complex to be purified. In addition, many antibodies raised against synthetic peptides do not recognize native proteins. To make

¹⁷ Y. Zheng, M. L. Wong, B. Alberts, and T. Mitchison, *Nature* **378**, 578 (1995).

¹⁸ C. M. Field, O. al-Awar, J. Rosenblatt, M. L. Wong, B. Alberts, and T. J. Mitchison, *J. Cell Biol.* **133**, 605 (1996).

¹⁹ C. M. Field, K. Oegema, Y. Zheng, T. J. Mitchison, and C. E. Walczak, *Methods Enzymol.* **298**, 525 (1996).

immunoaffinity chromatography more generally applicable, we have developed a protocol that allows single-step immunoaffinity purification of proteins tagged with three copies of the HA epitope. Because this is a commonly used epitope tag, the same methods and reagents can be used to purify numerous different protein complexes. Proteins are eluted from the immunoaffinity column with an HA dipeptide (i.e., a synthetic peptide that includes a tandem repeat of the standard HA peptide). The use of a dipeptide is essential for obtaining quantitative elution of 3× HA-tagged proteins, due most likely to high-avidity binding of anti-HA antibodies to triple HA repeats. We have now used this method to purify six different multiprotein complexes in our laboratory, including complexes that assemble in response to cell cycle-dependent posttranslational modifications. In addition, we have analyzed all of these complexes by mass spectrometry, and we know which proteins appear in the elutions from multiple columns and are likely to represent background bands. More importantly, when we identify new proteins that have never been observed binding to other immunoaffinity columns, we have confidence that the proteins are binding due to specific interactions with the HA-tagged protein. Each multiprotein complex that is purified therefore provides an additional control for purifications of other multiprotein complexes. Similar approaches for affinity purification of protein complexes have been described elsewhere, including a two-step purification scheme that employs a combination of protein A and a calmodulin-binding peptide, called the TAP tag.²⁰

A detailed protocol for immunoaffinity purification of 3× HA-tagged protein complexes from yeast cells is presented. This protocol is based on previous work using antipeptide antibodies to purify protein complexes from *Drosophila* and *Xenopus*.^{17,18}

Generation of Anti-HA Peptide Antibodies

Anti-HA antibodies are generated by immunizing rabbits with an HA peptide conjugated to keyhole limpet hemocyanin (KLH). We initially use a peptide that includes the last half of the standard HA peptide followed by a full peptide to ensure that we obtain antibodies that recognize the juncture between HA peptides in tandem repeats (peptide sequence: CPDYAGYPYDVPDYAG, the cysteine is included to allow coupling to KLH via the sulfhydryl group). It should also work to use an HA dipeptide so that one can use the same peptide to generate the antibody and to elute proteins from the antibody. The antibody is affinity purified using a column constructed with a purified GST-2× HA fusion protein. We generally obtain approximately 20 mg of purified antibody from each rabbit. Commercially available monoclonal anti-HA antibodies (12CA5 and HA.11) can also be used for

²⁰ G. Rigaut, A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin, *Nature Biotech.* **17**, 1030 (1999).

affinity purification experiments. However, we have found that the commercially available antibodies are prohibitively expensive.

Preparation of Immunoaffinity Beads

Immunoaffinity beads are prepared by binding 0.45 mg of antibody to 0.45 ml of protein A beads (Bio-Rad). Binding is carried out in the presence of PBS containing 0.05% Tween 20 for 1–3 hr at room temperature or overnight at 4°. Binding is carried out in a 1.6-ml tube. The presence of Tween 20 minimizes sticking of the beads to plastic or glass surfaces. After binding, the beads are washed twice with extract buffer (see later).

Controls

The ideal control for these experiments is an identical strain that does not carry an HA-tagged protein. We have also used a column made from a nonspecific antibody as a control. The use of a column constructed with a nonspecific antibody has several advantages. First, one needs to grow only one strain for each experiment. Second, one reduces the amount of anti-HA antibody needed. However, we have found that there can be background bands that bind to anti-HA affinity columns, but not to control columns constructed with anti-GST antibodies, depending on the batch of antibody. In our experience, these include Ydj1 and Cdc48. All binding interactions should therefore be verified in experiments using identical columns and strains carrying untagged proteins as a control.

Choice of Extract and Wash Buffers

The choice of the extract and wash buffers used for affinity chromatography experiments is critical and often must be determined empirically. We generally start by using buffers of physiological ionic strength (150–200 mM) containing 50 mM HEPES–KOH, pH 7.6, and 100–150 mM KCl. In many cases, the salt concentration can be increased without disrupting specific protein interactions, resulting in a lower nonspecific background. Yeast extracts contain highly active phosphatases that rapidly dephosphorylate proteins, which will disrupt protein interactions that are dependent on phosphorylation. In recent experiments, therefore, we have replaced the KCl in the extract and wash buffers with 100 mM β -glycerophosphate and 50 mM NaF, which act as good phosphatase inhibitors. This has proven to be crucial for the purification of multiprotein complexes that are dependent on cell cycle-specific phosphorylations.

Immunoaffinity Purification of 3 \times HA-Tagged Proteins from Yeast Cells

1. Obtain approximately 10–15 g of cells carrying the 3 \times HA-tagged protein. We usually grow 4–6 liters of culture to an OD of 1.0. The cells are pelleted,

resuspended in 50 mM HEPES–KOH, pH 7.6, and then pelleted again in a 50-ml conical tube. The cells are either frozen directly in the tube on liquid nitrogen or a hole is made in the bottom of the tube with an 18-gauge syringe needle and a stream of cell paste is extruded directly into the liquid nitrogen using a plunger from a 60-ml syringe. The latter method makes a “spaghetti” that is easier to grind. Frozen cells may be stored indefinitely at -80° .

2. Break open cells by grinding for 30 min in a mortar and pestle under liquid nitrogen to obtain a fine powder the consistency of flour. An initial grinding can be done for several minutes in a coffee mill, which reduces the total grinding time in the mortar and pestle by approximately 10 min. The coffee mill should be prechilled by grinding dry ice. A motorized mortar and pestle can also be used.

3. Transfer the powder to a 50-ml beaker prechilled with liquid nitrogen. Allow the powder to warm for approximately 5 min until the powder around the edges of the beaker is just beginning to thaw. This avoids the formation of ice crystals when the extract buffer is added. Resuspend the powder in 25 ml of extract buffer at room temperature.

50 mM HEPES–KOH, pH 7.6

100 mM β -glycerolphosphate

50 mM NaF

1 mM $MgCl_2$

1 mM EGTA

5% Glycerol

0.25% Tween 20

1 mM PMSF

The PMSF is added from a 100 mM stock made in 100% ethanol and is added to the extract buffer immediately before resuspending the cells. After adding the extract buffer, immediately stir the powder into solution with a spatula. Stir for another 5–10 min with a magnetic stir bar in a cold room, taking care to avoid foaming. Spin the extract at 10,000g for 5 min, followed by 100,000g for 1 hr. Take 10- μ l samples of the crude extract and the supernatants from both spins. Store on ice. Carry out all of the following steps at 4° .

4. Add the supernatants from tagged and untagged strains to 0.45 ml of protein A beads that have 0.45 mg of polyclonal anti-HA bound in 15-ml conical tubes. Prepare the beads by prebinding the antibody to the beads for at least 1 hr at room temperature in 1.5 ml PBS containing 0.1% Tween 20.

5. Mix for 2–3 hr on a rotator. Pellet beads and take a 10- μ l sample of each supernatant.

6. Wash twice with 15 ml of extract buffer with 25 mM NaF and without PMSF.

7. Transfer to a column and wash with 5 ml of extract buffer. Washes are carried out by pipetting 1-ml aliquots of buffer onto the column and letting them wash through by gravity. Before eluting, wash with 1 ml of extract buffer without

Tween 20. If there is a possibility that you will be sequencing the purified proteins, wear gloves for all of the following steps.

8. Make 1 ml of elution buffer: 50 mM HEPES–KOH, pH 7.6, 100 mM β -glycerophosphate, 1 mM $MgCl_2$, 1 mM EGTA, 5% glycerol, and 0.5 mg/ml HA dipeptide. Add 0.25 ml of elution buffer and collect the flow through in a 1.6-ml tube. Incubate for 30 min and then add another 0.25 ml of elution buffer. Allow to incubate overnight. Elute with two more aliquots of elution buffer, allowing each aliquot to incubate on the column for 30 min. Wash the last aliquot of elution buffer off the column with 0.25 ml of elution buffer without peptide. You should have a total of five fractions for each column.

9. Take 10 μ l of each fraction and dilute into 90 μ l of 1 \times sample buffer. In addition, dilute the crude extract and supernatant fractions into 90 μ l of sample buffer. Heat all of the samples at 100° for 5 min and load 15 μ l of each on an SDS–polyacrylamide gel. Use this gel for a Western blot and probe with an antibody against the protein or against HA.

10. Pool fractions 2–5 and precipitate proteins by the addition of trichloroacetic acid to 10%. Resuspend the precipitates from each column in 50 μ l of sample buffer and load 15 μ l/lane for a Coomassie blue-stained gel. Also load samples of the crude extract and supernatants for comparison.

Determining *in Vitro* Protein Interactions Relevant *in Vivo*

Once interacting proteins have been identified by affinity chromatography, an important next step is to gain additional information to help confirm that the proteins functionally interact *in vivo*. False positives may arise, for example, from the mixing of proteins in whole cell extracts, which are localized to separate compartments *in vivo*. Protein interactions that can only be disrupted with high salt (e.g., >0.5 M) or denaturing conditions are most likely to interact *in vivo*, and it is perhaps best to focus efforts on these first. Genetic interactions can provide perhaps the strongest evidence that two proteins that interact *in vitro* also interact *in vivo*. For example, if loss of function of an interacting protein causes a mutant phenotype that is similar or identical to the mutant phenotype caused by loss of function of the protein used for affinity chromatography, it is likely that the two proteins functionally interact *in vivo*. Similarly, unique genetic interactions between the two interacting proteins would provide strong evidence for an *in vivo* interaction. In the case of proteins isolated by standard protein affinity chromatography, it is important to show that the two proteins coimmunoprecipitate with each other in crude extracts. Finally, another criteria that can be used is to determine whether interacting proteins are colocalized within the cell.