

Topic Introduction

Protein Complex Purification by Affinity Capture

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Affinity capture has become a powerful technique for consistently purifying endogenous protein complexes, facilitating biochemical and biophysical assays on otherwise inaccessible biological assemblies, and enabling broader interactomic exploration. For this procedure, cells are broken and their contents separated and extracted into a solvent, permitting access to target macromolecular complexes thus released in solution. The complexes are specifically enriched from the extract onto a solid medium coupled with an affinity reagent—usually an antibody—that recognizes the target either directly or through an appended affinity tag, allowing subsequent characterization of the complex. Here, we discuss approaches and considerations for purifying endogenous yeast protein complexes by affinity capture.

BACKGROUND

Two interacting molecules form the cognate groups of an affinity capture system. These groups may include antibody–antigen interactions and other protein–protein and protein–ligand interactions. Typically, one group is a proteinaceous moiety (“tag”) that is fused to a protein of interest via genetic engineering, resulting in the expression of the tagged fusion protein of interest within a model organism. The second group is commonly covalently immobilized on an insoluble resin, gel, or paramagnetic medium and may be any molecule capable of interacting with the tag at high specificity and affinity. When extracts from cells expressing the affinity-tagged protein of interest are exposed to the medium coupled to the affinity capture reagent, the tagged protein becomes immobilized on the medium through interaction with its cognate binding partner, also bringing along its stably associated endogenous interacting proteins (for review, see Urh et al. 2009).

The yeast *Saccharomyces cerevisiae* is readily amenable to homologous recombination–based genomic tagging, resulting in the tagged protein being expressed normally from its endogenous genomic locus. For this reason, together with other useful traits, *S. cerevisiae* has been the leading model organism used for genome-wide tagging and affinity capture (e.g., Ho et al. 2002; Gavin et al. 2006; Krogan et al. 2006). As a result, yeast strains expressing nearly any endogenous protein as a carboxy-terminally tagged fusion protein are commercially available (Ghaemmaghami et al. 2003; Huh et al. 2003; Gavin et al. 2006), and custom strains can be constructed and validated at the bench within ~2 wk. Two commonly used tags are SpA (*Staphylococcus aureus* protein A) and GFP (*Aequorea victoria* green fluorescent protein). SpA interacts with IgG via the constant (Fc) region (Lindmark et al. 1983; Moks et al. 1986) and therefore does not require an antigen-specific antibody for affinity capture, making the affinity medium comparatively inexpensive to produce. Tandem repeats of an artificial domain derived from SpA are included in the TAP-tag (Nilsson et al. 1987; Puig et al. 2001). The use of

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GFP as an affinity tag requires a high-quality anti-GFP antibody preparation for producing the affinity capture medium; such preparations are available commercially.

The end point of an affinity capture experiment typically includes mass spectrometric analyses to define the sample composition (e.g., Cristea et al. 2005; Alber et al. 2007; Oeffinger et al. 2007). The copurifying proteins, together with the tagged protein of interest, may constitute one or more functional complexes (or parts thereof) *in vivo*—providing information about the specific constituents of particular biological machinery. Keep in mind that false positives may vary in affinity capture experiments, depending on the conditions of capture and other handling procedures (Bell et al. 2007; Devos and Russell 2007; Mellacheruvu et al. 2013) (discussed below). When the purified complexes are eluted natively from the affinity medium, they may be further fractionated (e.g., by rate-zonal centrifugation), examined physically (e.g., by electron microscopy to get size and shape information), and assayed functionally (e.g., *in vitro* enzymatic assays). Affinity-purified protein complexes serve as an important starting material for experimental programs aimed at mapping the interface between the composition, the form, and the function of biological macromolecules (e.g., Alber et al. 2007; Fernandez-Martinez et al. 2012; Lasker et al. 2012).

MAKING AFFINITY CAPTURE WORK

Although affinity capture is a conceptually straightforward approach to protein complex purification, optimizing affinity capture experiments often requires attention to a broad range of variables including pH and buffer type, overall ionic strength, salt type(s) and concentration, detergent type(s) and concentration, and temperature (Fig. 1). These factors, as well as the mechanism of cell breakage and the time required to complete the capture, can have a profound effect on protein interactions (Ugwu and Apte 2004; Oeffinger 2012). A short list of extraction solvent constituents, which may serve as a basic starting point for optimization, is given in Table 1. It is difficult to know *a priori* the appropriate

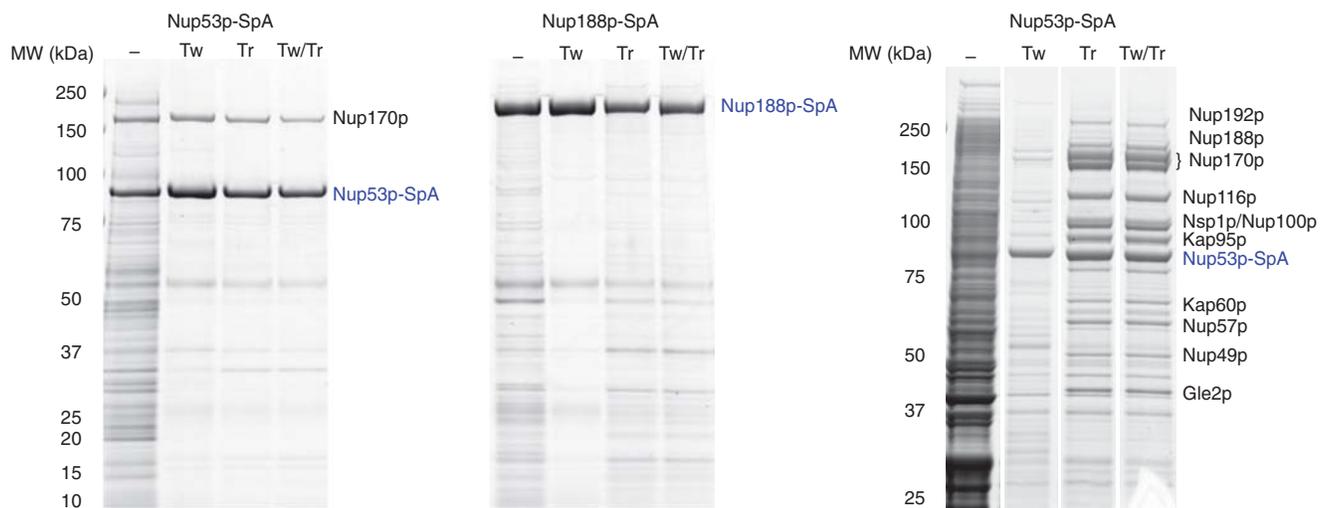


FIGURE 1. Three panels illustrating differences in protein purification patterns depending on the reagents present in the extraction solvent. In the presence of a low trisodium citrate concentration and moderate sodium chloride concentration, Tween 20 provided for comparatively enhanced recovery of the tagged protein while keeping background low: (*Left panel*) Protein A-tagged Nup53 purified by affinity capture in 40 mM Tris, pH 8.0, 50 mM trisodium citrate, 300 mM NaCl, and either no detergent [–], 0.1% (v/v) Tween 20 [Tw], 1.0% (v/v) Triton X-100 [Tr], or both [Tw/Tr]; and (*center panel*) an identical experiment is presented for an Nup188-SpA affinity capture experiment. Tween 20 does not work well in conjunction with a high trisodium citrate concentration: (*Right panel*) Nup53-SpA purified by affinity capture in the same conditions as above, but including 250 mM trisodium citrate. In all three experiments, mixtures of Tween 20 and Triton X-100 showed the pattern associated with Triton X-100 alone, regardless of the relative quantities of trisodium citrate and sodium chloride present.

TABLE 1. A nonexhaustive list of reagents we commonly use in extraction solvents for purifying protein complexes

Reagent	Suggested concentration	Notes
Sodium chloride	0.05–2 M	Higher concentrations improve extraction of total protein and keep background low but may strip away some otherwise stable interactors.
Trisodium citrate	0.05–0.25 M	At alkaline pH (when fully deprotonated in solution), higher concentrations stabilize some protein complexes. Salting-out of total protein can be observed at ~400 mM and above. Can be used alone, or combined with sodium chloride to improve extraction.
Ammonium acetate	0.1–2 M	A salt, consisting of two buffers, that yields a neutral pH solution. Higher concentrations stabilize some protein complexes. Acidic solutions can result from old, improperly stored crystalline stocks on account of ammonia loss. No additional buffer or salt are required in solvents containing ammonium acetate.
Urea	1–3 M	Can strip off background as well as stable core complex components, potentially revealing binary connectivity. Can be used in combination with NaCl.
Tween 20	0.01%–0.1% (v/v)	A nonionic detergent that works well in conjunction with high sodium chloride concentrations for protein extraction (see Fig. 1).
Triton X-100	0.1%–1% (v/v)	A nonionic detergent that works well in conjunction with high sodium citrate concentrations for protein extraction (see Fig 1).
CHAPS	2–5 mM	A zwitterionic detergent. Especially useful for membrane protein complex extraction.
Sarkosyl	0.5–5 mM	An anionic detergent that can strip off stable core complex components, potentially revealing binary connectivity, and keep background low.
DTT	1 mM	A reducing agent effective at alkaline pH. In some cases, this agent may improve protein stability and enzyme activity. High concentrations of DTT and elevated temperature can unlink the chains of the affinity antibody.
EDTA	0.1–1 mM	A chelator of divalent cations typically used to limit the activity of metalloproteases and nucleases. Not necessary in the presence of high concentrations of citrate, which also chelates divalent cations.
Tris–Cl	40 mM (pH 8.0–8.5)	pK_a of 8.8 at 4°C and 8.1 at 25°C.
Na- or K-HEPES	40 mM (pH 7.4–7.6)	pK_a of 7.8 at 4°C and 7.5 at 25°C. NaOH or KOH used for pH equilibration depending on the salt (e.g., NaCl, KCl, or CH ₃ CO ₂ K) used in solvent.
Sodium phosphate	40–250 mM (pH 7.0–8.0)	Consult a chart of phosphate buffers for appropriate proportions of mono- and dibasic species required to achieve the desired pH. We often omit salts when working with concentrations greater than 100 mM.

These are suggested reagents and concentrations; conditions outside of these suggestions and reagents not presented in this list may be necessary for success in capturing a particular protein complex. As a general rule, when formulating extraction solvents, we use the minimum number of different additives needed to achieve success in capturing protein complexes—this is determined on an empirical, complex-by-complex basis, and informed by previous successes on related complexes.

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

artificial milieu that will best stabilize the constituents of a given protein complex, while minimizing postextraction artifacts at the same time. For this reason, optimized solvents for producing cell extracts typically need to be determined empirically on a complex-by-complex basis. We commonly use Tris–chloride, sodium phosphate, and ammonium acetate as pH buffer systems in our extraction solvents. This may appear to run contrary to the sound logic of Good et al. (1966), who set out several excellent criteria in selecting biological pH buffer systems as alternatives to these, although we commonly use the Good buffer, sodium/potassium-HEPES (see Table 1). We should point out, however, that our overarching objective is the structural preservation of macromolecular protein complexes in an artificial milieu. For this, the reagents listed in Table 1 have all proven effective in our hands, and particular agents such as ammonium acetate, sodium phosphate, and trisodium citrate (not “Good” buffers) have showed effects in protein stabilization distinct from pH buffering (Kunz et al. 2004; Bostrom et al. 2005; Lo Nostro and Ninham 2012). The environment that offers the best in vitro enzymatic activity is not necessarily the same one that best preserves a particular subset of a

protein interaction network. All that being said, the criteria set out by Good et al. (1966) in biological buffer selection remain as valid today as ever.

Despite the daunting number of variables, there are some practical bounds to the “optimization space” to be explored for a given affinity capture. It is important to explore within a range of milieus that permit the affinity capture system to function with high specificity and high affinity; and indeed there will be some conditions that directly promote the affinity interaction. For example, the SpA/IgG interaction is promoted by alkaline pH and the presence of, for example, citrate or sulfate (Brown et al. 1998; Schwarz 2000; Ngo and Narinesingh 2008). It is wise to use affinity capture systems that are functionally robust across a wide range of variables and to titrate the quantity of the affinity medium to the minimum needed to extensively deplete the target protein from the cell extracts; excess unbound antibody can contribute to nonspecific experimental noise.

We have also often found the best results, including higher stability of bona fide constituents and fewer false positives, occur when working with concentrated cell extracts of not less than one part wet cell weight of yeast to four parts extraction solvent (w:v). This may be due to the greater stability of protein interactions at high concentration and a more cell-like resulting milieu (Ellis 2001). Concentrated yeast cell extracts tend to run acidic because of the breakage of acidified organelles such as vacuoles; therefore it is important to include the pH-buffering component of the extraction solvent at an appropriately high concentration and check that buffering is achieved. We have found that, for example, 40 mM for Tris at pH 8.0 or HEPES at pH 7.4 possess sufficient strength to buffer a 1:4 (w:v) extract at the expected pH of the buffered extraction solvent.

Affinity capture is typically conducted at 4°C, which retards the disintegration of most protein complexes and helps reduce proteolysis and other enzyme activities within the cell extract. An appropriate cocktail of protease inhibitors should be included at least during protein extraction and binding to the affinity medium. Other kinds of enzyme inhibitors may be important on a case-by-case basis—for example, for preserving posttranslational modifications such as protein phosphorylation or protecting other kinds of macromolecular complex constituents such as nucleic acids. Solvents for extracting protein complexes from cells typically use a near-physiological pH (~7.0–8.0 based on the pH of the cytosol), although there is good reason to vary this parameter for complexes believed to reside in cellular compartments of differing physiological pH.

For a description of a general affinity isolation protocol, including extraction and capture procedures, followed by a denaturing elution in, for example, SDS-PAGE sample buffer for gel-based proteomic analyses, see Protocol: **Optimized Affinity Capture of Yeast Protein Complexes** (LaCava et al. 2015a). For a description of a procedure for nondenaturing elution by specific protease cleavage or competitive displacement from the affinity medium, see Protocol: **Native Elution of Yeast Protein Complexes Obtained by Affinity Capture** (LaCava et al. 2015b). Natively eluted samples may be processed by rate-zonal centrifugation or size-exclusion chromatography for further enrichment of the affinity-purified fraction. Such fractions are typically suitable for electron microscopy studies of protein complex structure. For an approach for rate-zonal centrifugation of natively eluted samples in a sucrose density gradient, see Protocol: **Density Gradient Ultracentrifugation to Isolate Endogenous Protein Complexes after Affinity Capture** (Fernandez-Martinez et al. 2015).

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Cold Spring Harb Protoc; doi: 10.1101/pdb.top077545

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