Affinity proteomics to study endogenous protein complexes: Pointers, pitfalls, preferences and perspectives

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Dissecting and studying cellular systems requires the ability to specifically isolate distinct proteins along with the co-assembled constituents of their associated complexes. Affinity capture techniques leverage high affinity, high specificity reagents to target and capture proteins of interest along with specifically associated proteins from cell extracts. Affinity capture coupled to mass spectrometry (MS)-based proteomic analyses has enabled the isolation and characterization of a wide range of endogenous protein complexes. Here, we outline effective procedures for the affinity capture of protein complexes, highlighting best practices and common pitfalls.

I. Affinity capture: Principles

Two interacting molecules form the cognate groups of an affinity capture system (1,2). One group is the protein of interest or an affinity tag appended to the protein of interest via genetic engineering, resulting in expression of a tagged fusion protein within a model organism. The other group is usually an antibody recognizing the target protein directly or through an affinity tag, although any molecule that exhibits high affinity and specificity for the target protein may be used; when that molecule is coupled to an insoluble medium, the resulting reagent (affinity medium) can bind and immobilize the target protein. Hence, for affinity capture an affinity medium is used to specifically enrich a target protein from bulk cell extract, and under appropriate conditions endogenous interacting partners are co-purified. Such samples can then be subjected to mass spectrometry (MS)-based analyses, forming the bases of physical and functional interactomic hypotheses (Figure 1) (reviewed in References 3 and 4).

Generating unique antibodies for significant numbers of targets remains prohibitively costly and laborious. However, production of custom antibodies raised against native target proteins, on a case-by-case basis, has grown increasingly feasible (5–7). While such reagents can circumvent the need for genetic engineering and protein tagging, there may be complications: Antibodies can cross-react with related or unrelated proteins, confounding analysis; they can bind epitopes the protein uses for functional interactions; and, in many cases, one may wish to selectively enrich the product of a transgene (carrying a tag) from the product of the endogenous gene, particularly when the transgene product is mutated, to further explore its interactome. Although many commercial suppliers advertise antibodies purported to be competent for affinity capture (e.g., immunoprecipitation or IP), it is widely recognized that many are not well-tested, do not bind specifically enough to their target, and/or are too expensive to reasonably use for frequent experimentation (8–11). A major benefit of the commonly used tags is the availability of high quality, high specificity, widely validated antibodies for affinity capture, independent of the target protein. Additionally, the epitope is usually known, providing the potential for competitive native elution of protein complexes from the affinity medium, and generally heterologous, so it is not involved in the target protein's interactome. Hence, tagging is often advantageous even when so-called IP-competent antibodies are available due to cost savings, practicality, improvement in the quality of obtained results, and consistency between different tagged proteins. However, antibodies against the native protein are useful for validating tagged expression constructs versus their endogenous counterparts. This includes verification of size and titration of expression level by Western blotting (WB) and co-localization by immunofluorescence (IF) (References 12–15, Reference 163, and articles cited therein).

Affinity tags

A wide range of affinity tags are currently available (16–19). For affinity capture, tag choice should be guided first and foremost by the quality of cognate affinity reagents available. Dissociation constants (K_d) of ~10 nM or less between the cognate components are desirable for rapid and robust purification of modest to low abundance proteins of interest (as is common when expressed at the endogenous level) (7). However, it should be noted that reported

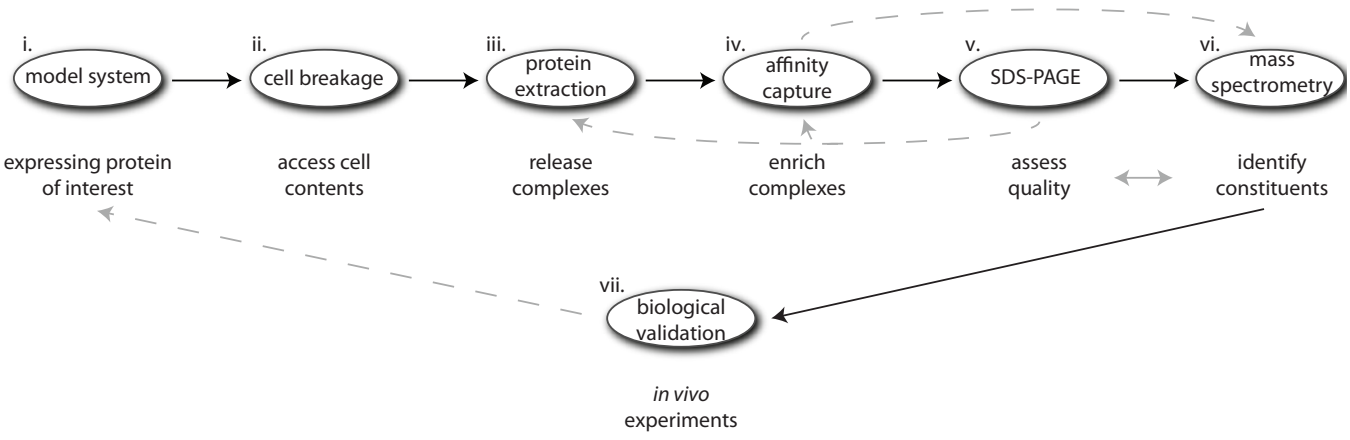

Figure 1. Chart of an affinity proteomics workflow. (i) A model organism expressing a tagged transgene is a common starting point to study the interactome of a protein of interest. (ii) Cells are broken to allow access to their contents; cryomilling allows cell breakage and macromolecule extraction to be separated. (iii) Protein complexes are extracted into solution. Optimization of the conditions to preserve endogenous interactions is empirical. (iv) Complexes associated with the tagged protein are enriched upon affinity media. Optimization of the amount of media used and time of incubation during batch binding is empirical. (v) SDS-PAGE profiles (see Figures 2 and 3) in conjunction with (vi) MS analysis of excised gel bands can reveal a significant amount of information regarding the likely quality of the prepared sample; based on results obtained in (v) and (vi), procedures (iii) and (iv) can be iteratively fine-tuned. Once optimized conditions are established, sensitive direct to MS analyses provide thorough proteomic characterization of the sample. (vii) Putative interactors should be functionally validated by orthogonal means in vivo and/or by a subsequent round of affinity proteomics starting with the putative interactor (a process sometimes called "reverse IP").

K_d measurements are typically carried out in vitro and are influenced by the experimental conditions used—therefore results may vary from application to application even when a reagent is reported to be high affinity. It is also critical to choose the best position for a tag to avoid altering protein function and native intracellular localization (14,16,20). Available evidence supports the prevailing notion that C-terminal tagging tends to be less disruptive, but other positions must be tested if functionality seems compromised. Recently, we observed a case where a C-terminal tag disrupted known interactions, inhibiting successful affinity capture of the expected complex along with the tagged protein; moving the same tag to the N terminus restored interactions and facilitated capture of the complex.

Care should also be taken to preserve signal sequences or modification sites, including the host organism's N-end rule pathway that affects protein half-life (21). Small tags (~1–5 kDa) are commonly favored in affinity capture, although it is not clear that larger-sized tags offer any comparative disadvantages for most applications. When tagging in compact genomes, such as those of viruses, additional genetic sequences may not be tolerated, and therefore only small tags placed at specific locations should be used (22–24). We observed this when surveying viable tags for affinity capture of L1 retrotransposons, which have a ~6 kb genome encoding two polypeptides; inserting larger tags corresponded to reduced efficiency of transposition (25). Finally, careful consideration should be given when introducing any sequences used to physically distance a tag from the protein of interest (e.g., linkers) (26). Direct fusion of the affinity moiety may compromise the target protein or limit accessibility of the tag to its cognate antibody. A linker can be used to introduce specific protease cleavage sites, enabling native elution of the purified complexes after protease addition (27). However, the opposite can also be true; in the case of the L1 ORF1 protein C-terminally fused to green fluorescent protein, three flexible linkers all resulted in lower transposition efficiency than a shorter two amino acid linker (25). This example underscores that biological validation for preservation of function is critical (although, unfortunately, not always possible).

Three tags that we preferentially employ are SpA (*Staphylococcus aureus* Protein A), GFP (*Aequorea victoria* green fluorescent protein), and 3xFLAG. Wild-type SpA interacts with an antibody (i.e., immunoglobulin; Ig) via sites outside the antigen binding paratope regions and therefore does not require cognate antigen-specificity for affinity capture. Affinities between SpA and different Ig-types vary widely, but high affinity (K_d = 2.4 nM) has been reported for rabbit IgG (28). Therefore, effective SpA affinity medium can be produced inexpensively using bulk IgG from rabbit serum. It should be noted that different SpA-derived tags exist and different configurations of Ig binding domains yield different results in affinity-based experiments (29–33).

The tandem affinity purification (TAP)-tag (34) incorporates two tandem repeats of the synthetic SpA-derived Z-domain (35). We rely on an SpA-tag derived from a wild-type sequence (29,36), containing up to four Ig binding domains (three complete domains and a fourth nearly-complete domain that retains the two helices shown to interact with human IgG) (33,37–39). Presumably because of its greater number of Ig binding domains, our configuration outperformed the TAP-tag in affinity capture experiments using rabbit polyclonal IgG affinity medium (33). Historically, the Protein A-based TAP-tag has been most widely and successfully used in yeast, demonstrating lower efficiency in human tissue culture and thus prompting development of alternative TAP configurations for mammalian systems (4,40). It is also worth noting that improvements in sample handling practices and available reagents have largely superseded the need for tandem affinity procedures to obtain high signal, low background results; single-step affinity capture has proven sufficient, and being shorter in duration, it increases the chances of observing labile interactors (4,34). We developed native elution reagents (from previously developed SpA binding peptides) that can release SpA-tagged protein complexes from rabbit IgG coupled media—further solidifying the value of this robust and effective tag (33,41,42).

GFP or FLAG affinity tags require a high quality antibody preparation for producing the affinity capture medium. Recently, our lab produced high quality nanobody-based affinity reagents (43) for GFP, many exhibiting K_ds of <1 nM to ~30 pM, which can be cheaply produced recombinantly in *Escherichia coli* (7). High quality α-GFP affinity reagents are also available commercially, and these, along with homemade polyclonal α-GFP antibodies, can work well (13,44). Although the FLAG-tag (45) has enjoyed frequent use and is reported at low-nM K_d values in conjunction with the α-FLAG M2 antibody (46–48), the 3xFLAG version is superior in Western blotting, with sensitivity reportedly increased by over an order of magnitude (49,50). Similar improvements were also shown for immunohistochemistry (50). Our blots readily revealed over two orders of magnitude increased sensitivity for 3xFLAG (25). The only time we have observed a single FLAG-tag rival the efficacy of 3xFLAG in affinity capture is when the tagged protein is present in multiple copies within the complex being purified (e.g., ORF1p-FLAG) (25). It should be noted that 3xFLAG-tag is not three tandem repeats of the FLAG epitope, but rather includes alterations within the first two repeats (49). We have had consistent success natively eluting 3xFLAG tagged protein complexes from α-FLAG M2 antibody-coupled magnetic beads using the 3xFLAG peptide (49). Although Sigma-Aldrich (St. Louis, MO) recommends a working concentration of 0.1 mg/mL, our experiments have been more consistent and have given higher yields when eluting at 1 mg/mL; further gains were not observed at 5 mg/mL (25). A 15 min incubation with the peptide at room temperature has typically proven sufficient for thorough release—competitive, native elution is never 100% effective and varies greatly from reagent to reagent and complex to complex (25,33).

Model systems

Affinity capture can be applied to any model system for which appropriate reagents are available, and sufficient material can be obtained. Because *Saccharomyces cerevisiae* is readily amenable to plasmid-based protein expression (51), as well as homologous-recombination-based genomic tagging where the tagged protein is expressed normally from its endogenous genomic locus (52), it has been a leading model organism for genome-wide tagging and affinity capture/MS (53–55). A large collection of yeast strains expressing endogenous proteins as C-terminally tagged fusion proteins is commercially available. GFP-tagged strains (56) are currently available from Life Technologies (Grand Island, NY); TAP-tagged strains (57) are available from GE Healthcare (Pittsburg, PA) or from EUROSCARF/CellZome (Heidelberg, Germany) (58). In any model system, it is frequently desirable to maintain the tagged protein expression level at or near that exhibited by the native protein, to minimize experimental artifacts (15,59,60). One must also remain mindful that, regardless of expression level, the addition of a tag may cause changes in protein localization or function (discussed above in the "Affinity tags" subsection).

The Mammalian Gene Collection (MGC) initiative has enabled researchers to access sequence-validated, full-length protein coding cDNA clones for human, mouse, rat, and cow genes (61), and the ORFeome Collaboration provides sequence-validated, full-length human protein coding sequence (CDS) clones in Gateway entry vectors (62–64). These can be obtained from a number of commercial vendors, currently listed on www.orfeomecollaboration.org. Such sequences can be subcloned into tagging vectors and introduced into mammalian cell lines by a wide variety of stable and transient approaches (many of which are reviewed in References 15 and 65). On the other hand, full-length native gene sequences with endogenous promoters and regulatory elements can be tagged and expressed using bacterial artificial chromosome (BAC) transgenesis (66,67). BAC cloned genes contain the naturally occurring introns and can therefore give rise to cell-type-specific splicing variants. These resources have broadly facilitated implementation of affinity capture/MS strategies in mammalian tissue culture systems (66,68). Although CRISPR-based methods (69–72) are increasingly enabling tagging of endogenous proteins, numerous methods employ an additional copy of a tagged transgene. Three popular approaches for human transgene expression that we commonly use are the Flp-In T-Rex system (Life Technologies), BAC transgenesis, and episomal transfections (73,74).

The Flp-In T-Rex system is based on the FLP recombinase, utilizing recombinase-mediated DNA insertion (Flp-In) (75,76). The inserted gene is placed under the control of a tetracycline (Tet) regulated CMV promoter (T-Rex) (77,78), and when an antibody against the native protein is available, expression can be titrated to the endogenous level through addition of tetracycline to the growth medium while monitoring the relative expression of the tagged and endogenous forms by Western blot (12,13,79). While some Tet-based systems respond better to tetracycline derivatives, such as doxycycline (Dox) (80), we have observed essentially identical results using both Tet and Dox as inducers of T-Rex-driven transgenes (although Dox is reported to have a longer half-life in tissue culture medium and is thus generally preferred). This approach has enabled quantitative proteomics on complexes formed with tagged proteins expressed at near-endogenous levels (79). It is important to note that traditional Tet-repressible synthetic promoters, including the T-Rex system, follow a sigmoidal dose-response curve with large changes in expression level occurring over a narrow range of inducer (81,82). Therefore careful and precise empirical titrations should be conducted and certified Tet-free serum should be used in the tissue culture medium to ensure culture-to-culture reproducibility. We typically start with a test of 1, 5, and 10 ng/mL Tet, comparing the level of the tagged protein to the endogenous protein after 16–24 h of induction, as assessed by Western blot, followed by additional rounds of fine-tuning as necessary. The Flp-In T-Rex cell lines provided by Life Technologies are isogenic, providing the benefit of roughly uniform expression across the population; however, some degree of heterogeneous expression is to be expected. It is therefore useful to assess whether the cells respond roughly uniformly to the conditions of induction by fluorescence microscopy, which also provides a chance to assess the intracellular localization of the tagged protein. Based on this assay, we have not experienced notable issues with heterogenous expression levels using the Flp-In T-Rex system. Considering the ever-decreasing price of commercial custom gene synthesis services, we recommend recoding Flp-In transgenes to be RNAi resistant. This creates the opportunity to knockdown the endogenous protein in conjunction with expression of the protein of interest, potentially increasing the yield

of complexes associated with the protein of interest (83) and allowing the functional validation of mutated or exogenous transgenes (84,85).

A popular alternative for the near-endogenous expression of a tagged protein of interest, while foregoing the need to titrate expression, is BAC transgenesis (66,67). BAC clones can encompass ~100–400 kb of genomic DNA, allowing most mammalian genes to be contained within a single clone and thus preserving endogenous-like expression context. A large number of human and mouse BAC clones are available (www.mitocheck.org/cgi-bin/BACfinder), expressible as C-terminally localization and affinity purification (LAP)-tagged transgenes; the LAP-tag used includes both the S-tag and the GFP-tag in tandem (66,86), and these have been used for quantitative affinity proteomics (79,87). While BAC transgenes are said to provide endogenous-like expression, there are a number of caveats to keep in mind. First, BAC transgenesis involves non-homologous integration of the tagged transgene at a random locus; and since BACs are very large, this occurs at a very low frequency (~1%–2%), with enrichment of integrants being achieved through, for example, G418 antibiotic selective pressure. No viral or other sequences are used to enhance integration (66). Hence, transfection with BACs results in a heterogeneous pool of cells, some expressing the transgene and others not—and those cells that express the transgene do so from distinct loci (66). However, cells transfected with LAP-tagged BAC clones can be subjected to fluorescence activated cell sorting (FACS) (88,89), using the GFP tag to isolate singly integrated, clonal cell lines from one another. We instead favor FACS to enrich the BAC transfected pool for transgene-expressing cells. While this results in a heterogenous pool of expression loci, it ensures that we maximize the yield of expressing cells (Figure 2). This approach can also be used to screen away cells that appear to express at anomalously high levels compared with the bulk population—thereby eliminating presumed multiple-integrants and/or those cells with integration loci that exaggerate the natural level of gene expression.

Finally, for tagged protein expression when native CDS or BAC resources are lacking, episome-based expression is another possibility (73,74,90). Episomal expression vectors are maintained in the nucleus in a non-integrated state, replicating extra-chromosomally, allowing rapid generation of quasi-stable cell lines, and avoiding a number of issues related to integration of stable expression systems. Because they are not integrated, episomal vectors do not damage or mutate host DNA and are not subject to regulation by host chromosomal status, rendering them resistant to the silencing that can be problematic in chromosomal overexpression. A variety of strategies can provide efficient episomal replication (reviewed in Reference 74), but we prefer EBV-derived vectors containing oriP/EBNA-1 (pCEP4, Life Technologies; and derivatives) due to their efficient replication, high transfection efficiency, and large capacity. When combined with a rapid selection marker such as puromycin, a population of selected cells can be established in under a week and maintained for a number of months. These vectors are maintained at ~5–50 copies per cell and, when combined with a Tet-inducible promoter, allow titration of expression from native levels to high levels of overexpression (with the same caveats described for the T-Rex system, above) (91,92). We have had success using this system to affinity purify LINE-1 retrotransposon protein complexes (25). In other cases, the large capacity allows reconstruction of one or more native loci in the episome. The largest disadvantage of this system is heterogeneity; each cell may have a different plasmid copy number, and this may be an issue both at a given time point and over time. This can be mitigated somewhat using antibiotic selection, and titration of puromycin levels can result in clones with higher copy number (93). Also, true native-level or sub-native expression may be difficult to achieve, as low-level induction can result in stochastic activation (94–96); in other words, there may be higher-than-native expression in a small subset of the population and no expression in the majority.

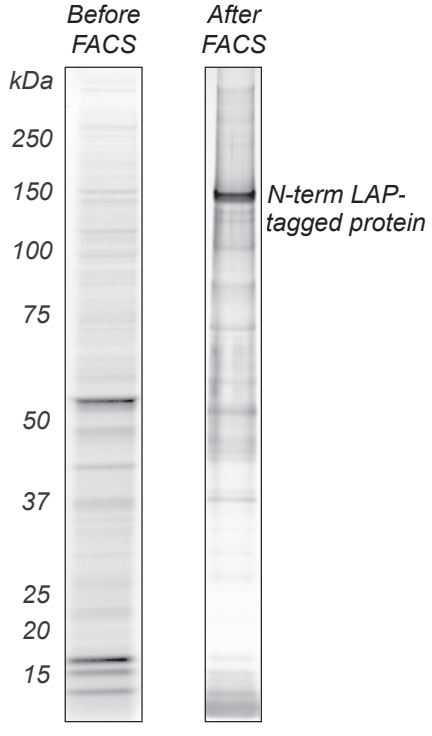

Figure 2. A stable transfected pool of HeLa cells expressing an N-terminally LAP-tagged, BAC-cloned transgene was subjected to α-GFP affinity capture, SDS-PAGE, and protein staining. (Left) Affinity capture prior to FACS-based enrichment of GFP-expressing cells—the tagged protein, running ~150 kDa, exhibits low yield, and the lane exhibits high background. (Right) Affinity capture after FACS-based enrichment of GFP-expressing cells—the tagged protein is captured readily and in abundance.

Cell breakage

Whatever expression system is used, affinity capture requires access to cellular content so that target proteins and the affinity reagents can mix. Therefore, cell breakage and protein extraction into solution are required. Detergent, enzymatic, or mechanical methods are typically employed to achieve cell breakage (97–99)—each with pros and cons to be considered in affinity capture experimental design. The main aim is to achieve a thorough, non-denaturing separation of the target protein and its specific partners from the surrounding cellular milieu. However, the precise differential partitioning of cellular constituents into soluble or insoluble phases will depend upon the method of breakage and the physicochemical environment of extraction. Having compared these, we favor the solid-state, mechanical disruption of cells at cryogenic temperature (cryomilling). We have adapted, developed, and championed this technique in the community, because it faithfully preserves protein activities and post-translational states (100,101), preserving complexes even at the highest ultrastructural level (102). By contrast, methods that utilize high levels of mechanical shear can also induce significant heating and denaturation (103). Chemical methods—such as powerful detergents—that are harsh enough to break cells, can also dissociate protein complexes (see Reference 103 and the "Protein extraction optimization" subsection below).

The cryogenic method of cell breakage also has the huge advantage of separating the processes of cell breakage and dispersal from the processes of lysate extraction and affinity capture. As cryomilling occurs in the solid phase, there can be no change in the relative distribution or association of component molecules, nor protease or nuclease damage during this step. This has the effect of limiting the period during which such changes can occur to the subsequent extraction and isolation stages and supports the preservation of delicate and transient states be it the changing protein phosphorylation regulating spindle assembly and mitosis (104,105), organelle biogenesis (100), or the assembly states of ribosomes and mRNPs (106–108). The milled cell powder can be stored, essentially in a state of suspended animation, at -80°C or below for long periods (≥1 year) without observable changes in attributes or experimental results, and can be conveniently distributed to many reactions tubes by mass (equivalent to wet cell weight), enabling a multitude of protein extractants to be explored in parallel during optimization. Therefore, we typically produce a large quantity of powder for the system under study and draw from this stock for a period of continuous experimental optimization.

A variety of devices have been used to achieve cryomilling of cell material (106,109–111). We primarily utilize the Retsch (Haan, Germany) PM100 planetary ball mill (13,106) because it offers a combination of attributes that make it both extremely versatile and effective for cryomilling tissue. It is able to produce a micron-scale powder with favorable properties for affinity capture that appear to be independent of the model system used (see References 13 and 33 for human cells, yeast, and bacteria—but we have also used this cryomill on trypanosomes, nematodes, flies, plants, mouse organs, and even the intact bodies of newborn mice, with equivalent results). Cryomilling has been demonstrated to be capable of producing complex cell extracts with biochemical activity (109,112) but it should be noted that higher energy and greater breakage may not always be beneficial (112) and should be optimized on a case-by-case basis. Our protocols results in essentially complete cell breakage (readily assessed by phase contrast light microscopy of the milled powder resuspended in PBS), and we have been able to obtain affinity-purified complexes exhibiting high biological activity in vitro—including the human exosome complex (Domanski et al., unpublished data) and the LINE-1 retrotransposon (25). The PM100 can be paired with a range of accessories that enable a broad array of milling regimes to be implemented on diverse tissue types while accommodating modest to large quantities of material. Moreover, by using custom-made Teflon insulators to slow the warming of the jar, milling can be done with liquid nitrogen in the milling jar without significant evaporation, ensuring uniform milling in an expedited protocol with minimal user handling (25). It is important to note that most milling apparatuses using gas-tight, closed milling jars cannot be operated with liquid nitrogen within the jar; the pressure resulting from evaporated N_2 gas could cause an explosion.

Although not a problem when working with yeast and bacteria, which can be grown cheaply and abundantly, one limitation of the PM100 is the minimum scale at which it can be practically operated (1 g wet cell weight): due to material that sticks to the jar surfaces and milling balls used in the process, ~300–500 mg of material may be lost to the process during standard cryomilling. For this reason, it is impractical to start with less than 1 g of cell material (wet cell weight), which is roughly equivalent to 10 × 150 cm^2 tissue culture plates grown to ~90% confluence for common cell lines such as HEK293 and HeLa (13). However, a recovery of ~700 mg is commonly enough material to do ~7–14 affinity capture experiments. Using 50–100 mg of cryomilled cell powder can yield captured complexes in the range of tens-to-hundreds of nanograms per protein component, provided that the complex of interest is at least moderately abundant (ca. 100s–1000s copies/cell). We are frequently asked what options exist for routine milling at scales of less than 1 g, and for this we suggest the Retsch Cryomill, which offers a range of options enabling the effective milling of very modest sample quantities.

We have used cryomilling effectively on a variety of cell- and tissue-types, and different systems can exhibit idiosyncratic properties upon protein extraction (see "Protein extraction optimization" subsection below), requiring modified handling procedures. Protein extraction from cryomilled yeast (*S. cerevisiae*) is extremely facile. When combined with a room temperature extractant, frozen yeast powder rapidly homogenizes into solution with brief (~30 s) vortex mixing (example protocol presented in Reference 33); we forego the additional mixing by Polytron seen in some protocols (111). Protein extraction from milled human cells requires a little more attention. We sometimes observe that some fraction of the powder aggregates upon combination with common extraction solutions, limiting the degree of initial homogenization. Vortexing alone is not enough to disperse these aggregates. To solve this problem we apply a very limited degree of low power microprobe sonication to the crude extract, and the aggregates are readily dispersed and homogenized (13). Additionally, human cell powders can occasionally generate viscous extracts depending on the extraction solution used (e.g., high salt or urea, due to the unfolding of chromatin) (113–115)—the same sonication treatment also resolves this problem. With *E. coli*, viscous extracts are almost always observed, further supporting our contention that while cell breakage via cryomilling is observed to be thorough, it provides an extract whose qualities parallel gentle (e.g., enzymatic and freeze-thaw) breakage methods (103). Viscosity in *E. coli* extracts can also be resolved by sonication, essentially as above. Treatment with DNase I (or other nucleases) can also be used, but is not effective in a wide range of extractants—being particularly sensitive to salt concentrations and requiring both Mg^{2+} and Ca^{2+} (116,117). Note that this low power sonication results in minimal DNA shearing—just enough to cut viscosity—and is not powerful enough for significant heating, protein complex damage or, on its own, effective cell lysis. Cryomilling in conjunction with minimal sonication nevertheless maintains a distinct advantage over high-power, sonication-only and other mechanical approaches. Cryomilling avoids heating or foaming of the materials during breakage; as it takes place in the solid state, it retains the native protein concentration up to the point of extraction and minimizes the time spent in solution (during subsequent extraction), mitigating macromolecular dissociation, deleterious enzymatic activity (e.g., nucleases and proteases), and chemical (de)modification (4,103,106,118); it provides thorough cell breakage while providing extracts with qualities comparable to gentle treatment (as assessed by viscosity) and can

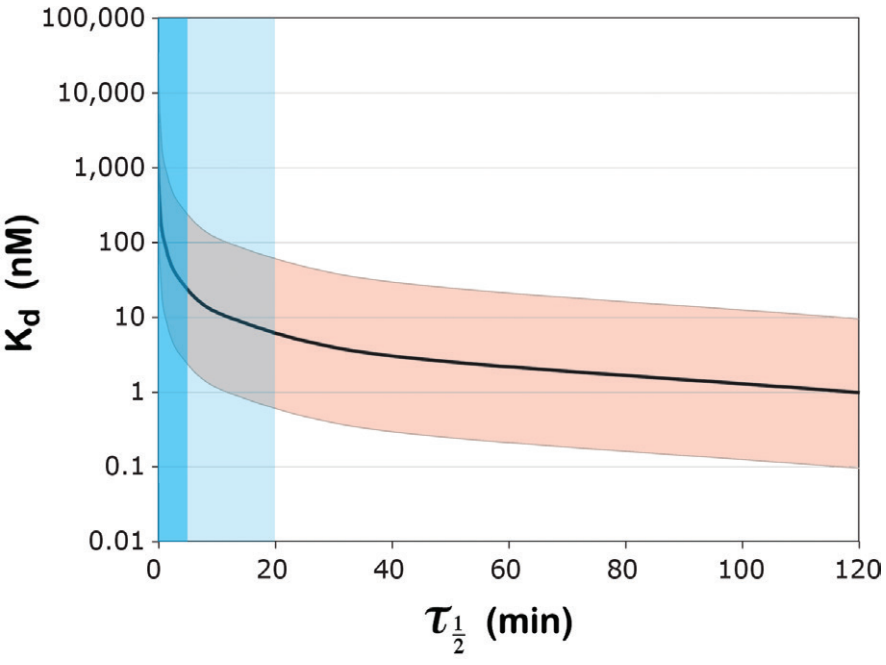

Figure 3. Half-lives of binary complexes with the indicated K_ds and a K_{on} of 10^5 $M^{-1}s^{-1}$ (black line) and between 10^4–10^6 $M^{-1}s^{-1}$ (lower–upper orange limits). Pale blue: Protein interactions exhibiting K_ds in this range have post-extraction half-lives of 5–20 min. Dark blue: Protein interactions exhibiting K_ds in this range have post-extraction half-lives of up to to 5 min. Most procedures last ~1 h or more, including extraction, centrifugation, batch binding, washing, and finally eluting. Optimizing the extractant to preserve the target complex can therefore benefit yield enormously.

decrease background binding in affinity capture applications (13).

Protein extraction optimization

Cryomilling facilitates convenient access to the internal contents of cells, where diverse physicochemical factors modulate the formation and maintenance of macromolecular interactions within distinct local environments (aspects of this profound and exciting topic are reviewed in References 119–122, but precise understanding of intracellular environments remains elusive). Upon extraction, the cellular constituents are instead partitioned into a uniform artificial extractant, which cannot be optimal for the preservation of all endogenous complexes (or the minimization of post-extraction artifacts), making case-by-case optimization of the extractant for each target protein and complex of immense importance to affinity capture performance (4,123,124). One of the foremost and longest-standing challenges for affinity capture studies is to establish a suitable environment for the high fidelity retention of physiological interactions of any chosen macromolecular complex from extraction to analysis (125). An ideal solvent performs three functions. First, it mimics certain desirable aspects of the native cellular milieu from which the isolated complexes are obtained, in order to prevent denaturation or dissociation of complex components. Second, it inhibits interactions that are not wanted (such as those retaining a particular complex in an insoluble fraction) or are not present in the living cell (contaminant association and aggregation). Third, it promotes selected interactions in the complex, prolonging the life of the complex—even beyond its normal cellular lifetime—such that the complex survives the isolation procedure and can be studied in vitro. The post-extraction lifespan of protein complexes is very important as the duration of most affinity capture procedures ensures that labile interactions are lost (Figure 3).

Commonly explored variables include buffer pH and type, salts, and detergents—among many other additives (123,126–128). The chemical character of the buffering agent can contribute to the extraction milieu in unpredictable ways beyond simple pH control (120,123,129,130). Salts are frequently classed as kosmotropes or chaotropes in accordance with their effects on protein structure and solubility; the mechanisms of these effects are not well characterized (120,131). Detergents, which extract membrane-anchored complexes and inhibit aggregation of all complexes, also exhibit broad, unpredictable characteristics (127,132). Therefore, many combinations of reagents need to be tested and optimized empirically in order to arrive at the best combination for the complex(es) under study. At the present time, such optimization of the affinity capture solvents remains time consuming.

After homogenization and extraction has been achieved, clarification of the extract is a typical next step. Insoluble materials will adhere to the surfaces of tubes and the affinity media, creating very high background if not first removed. Clarification can be achieved by centrifugation [e.g., 10 min at 20k RCF in an Eppendorf (Hauppauge, NY) 5417R microcentrifuge or equivalent] or by filtration, provided an appropriate stringency of filter is used. Our experience is that filtration to 0.2 µ is required to match the quality of results achievable with high-speed centrifugal clarification. Most commercially available 0.2 µ filters will rapidly clog upon exposure to crude cell extracts; therefore, a multistage filter designed to counter act this problem is recommended. We have developed such a filter that is highly effective on crude yeast extracts (Hakhverdyan et al., unpublished data), and is commercially available from Orochem Technologies (Lombard, IL).

II. Affinity capture: Practice

Variables affecting affinity capture include cell lysis, the artificial milieu of extraction, the concentration of the extract, the properties of the tag, antibody (or other reagent), interacting proteins, affinity medium, the time, temperature, and other handling conditions. We have also observed that the scale of the experiment can affect the result (discussed below). Since one cannot know in advance the optimal extraction and handling conditions in which to carry out affinity capture for the protein complex(es) of interest, the most straightforward way to discover the best practices is empirically. We advise testing many different conditions, in parallel and iteratively. Comparing different results side-by-side will aid in orienting the direction of the next round of optimization. In the initial stages of optimization. we rely heavily on SDS-PAGE and protein staining (Figure 4), with identification of bands in promising profiles by MS, which provides a rapid readout on experimental progress. The results yield information on

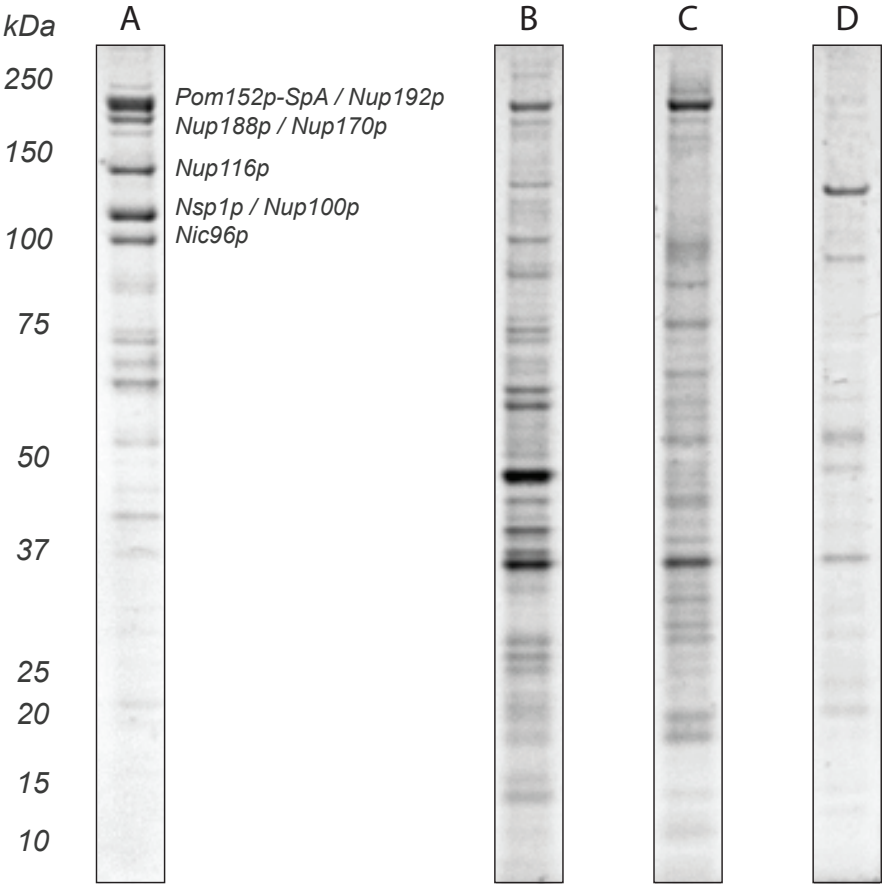

Figure 4. An example qualitative assessment of different affinity capture results by visual inspection after SDS-PAGE and Coomassie blue staining. The tagged protein (Pom152p-SpA) and a group of abundant co-purifying proteins identified by MALDI-MS are labeled. (A) An example of a qualitatively promising lane. The tagged protein is present in abundance, the bands are sharp and several bands exhibit similar intensities to one another and to the handle protein, descending with molecular mass—indicating proportionality. (B) is less promising than (A) because it exhibits a hazy background with some band smearing, and although discrete bands are present, there is a cluster between 37–50 kDa that exhibits a disproportionate increase in intensity relative to the tagged protein (or other bands). (C) is less promising still, exhibiting the same smeary haze as (B) and fewer discrete bands. (D) does not have the tagged protein present; the bands in this lane are likely artifacts.

the ability of the affinity system, as a whole, to provide a high quality preparation of the complex of interest. Since these are an enormous number of variables, a practical approach is to apply some rational limits on the experimental design. The following include some of our standard procedures arrived at on theoretical and/or practical bases.

Binding to and releasing from affinity media

We use batch binding for our affinity capture experiments. Batch binding promotes thorough mixing of the affinity medium with the clarified cell extract, maximizing binding in minimal time. We also use spherical, non-porous, micron-scale, paramagnetic beads, rather than traditional porous resins; because the affinity binding occurs on the bead surface, there are no size exclusion limitations that can bias the capture of large complexes assembled with the protein of interest, as can occur with traditional chromatographic media (2,106,133,134). We were unable to release bound LINE-1 RNPs from anti-FLAG-agarose beads using native elution by competitive displacement with 3xFLAG peptide (and comparable outcomes have been seen by us and others with protease cleavage and Sepharose) (135), although the complexes were readily released from magnetic anti-FLAG medium by this approach (25). The paramagnetic aspect of the beads also increases the practicality of the medium: it is rapidly cleared from solutions by placement on a neodymium-iron-boron magnet, and the resulting pellets are tightly packed and strongly retained at the side of the tube—minimizing retained volumes and allowing thorough removal of solutions without risking accidental loss of media during pipetting. Our choice medium has been Dynabeads M-270 Epoxy (Life Technologies), which can be coupled to antibodies via epoxy reactive groups (13,136,137). We have generally obtained higher quality purifications with Dynabeads as compared with agarose and Sepharose (13,136). We have also used carboxylic acid functionalized paramagnetic beads from Spherotech (Lake Forest, IL) with results frequently comparable to those obtained with Dynabeads, and at a significant savings in cost.

Whatever solid medium is ultimately selected, keep in mind that it may exhibit some distinctive, sample-dependent properties depending upon its own chemical character. The amount of beads used in an affinity capture should be titrated such that the yield of protein is maximized for the time of incubation, while the background is simultaneously minimized. This can be achieved by monitoring the percentage of protein depleted from a given cell extract with increasing quantities of beads and increasing length of incubation by Western blotting or protein staining, and this can be compared with the accumulation of common contaminants by MS; known interactors can also be monitored by Western blot or MS (25,44). These results will vary widely by protein, tag, antibody, and extraction solution (as well as other parameters)—but the protein-to-protein variation in these effects is somewhat mitigated when a common tag and antibody combination is used. We consider an affinity capture regime to be within an acceptable range if better than ~70% of the soluble pool of the protein of interest can be depleted by a given amount of affinity reagent in less than 1 h, with the above-mentioned considerations in mind. Hitting or exceeding this target can be important to avoid bias in assessing the typical steady state interactors of the protein of interest. For many proteins and complexes, there may be a soluble pool that remains inaccessible to the affinity medium regardless of the amount of medium used or time of incubation—this population will typically be discovered during the initial titration and is presumed to be sterically blocked from access to the medium. Use of different extraction conditions (below) should provide access to such pools. It is important to

always monitor the pellets produced during centrifugal clarification of the extracts to determine what percentage of your protein of interest partitions into the soluble fraction; this may vary considerably depending on the extraction conditions used. Due to difficulty in handling the pellets, it is often more practical to compare the total extract before clarification with the final clarified extract. Additionally, when comparing the purification of a tagged protein to that of a mock purification, it always better to use control cells expressing the tag alone or an orthogonal but equivalently-tagged control protein. Alternatively, purified tag or tagged-control protein can be added to untagged cell extracts. This is because antibodies can exhibit substantial off-target binding in the absence of their epitope, even when they exhibit exquisite affinity and specificity in the presence of the epitope. This will vary from antibody-to-antibody, and condition-to-condition.

Initial conditions of capture: Where to start?

As discussed above in the "Protein extraction optimization" subsection, finding excellent conditions for affinity capture requires time-consuming optimization. Although there are no guarantees, learning what has been successfully used for the purification of your protein or structurally/functionally related proteins may save you some time—these conditions can serve as an initial seed, and possibly a basic positive control to facilitate your further optimizations (126). When prior knowledge is lacking, we suggest starting with a small collection of extractants—on the basis of our experience we may start with a collection containing ammonium acetate (pH 7.0), HEPES-Na (pH 7.4), or TRIS-Cl (pH 8.0) combined with NaCl at 100, 300, and 500 mM, and Triton X-100 (1% v/v) or Tween 20 (0.1% v/v) (123,138,139). From there we frequently swap out the detergent, testing, respectively, zwitterionic (e.g., CHAPS) and anionic (e.g., sarkosyl) detergents (139,140); as well as swapping out, or adding, an additional salt such as potassium acetate and trisodium citrate (further discussed in Reference 141). Some common starting parameters that we use are as follows: We start out with 50–200 mg of cell powder in initial affinity capture tests, and our extracts are produced at 1:4–1:5 w:v (i.e., 400–500 µL extractant is added to 100 mg cell powder). Working concentrated in the specified range in most cases improves results over more dilute solutions, but ensure that enough buffering capacity has been included to equilibrate and maintain the intended final pH; at the above stated proportions, 20–50 mM is typically sufficient for most commonly used buffers at near physiological pH (i.e., ~6–8). In cases where very large quantities of lipid and/or pellet are observed, a 1:9 (w:v) extraction may also be tested. We commonly use 5–10 µL of Dynabeads slurry per 100 mg of cell material. Our slurries are prepared by adding 2 mL of storage solution to 300 mg equivalent (dry weight) of coupled beads (136). We have observed that small working volumes positively affect yield and signal-to-noise in affinity capture. Therefore, we advise that multiple smaller reactions be carried out and then pooled prior to subsequent manipulations when greater yield is needed, as opposed to direct scale-up within a single experiment. We typically try to limit routine experiments to a maximum 250 mg cell powder per affinity capture. This can be stretched as far as 500 mg per capture with only limited deterioration in the quality of the result, and this is practical due to the inconvenience of working with many tubes when large-scale is needed. Above 500 mg per capture, a significant increase in non-specific background relative to yield of the protein of interest can frequently be observed. We combine affinity medium, pre-washed in the extraction solution, with the extracts (treated with appropriate protease and/or other enzyme inhibitors) and incubate 30 min at 4°C with rotary mixing. The beads are typically washed 3 times with 1 mL of the extraction solution and then eluted natively (by competing peptide or protease cleavage) or in denaturing conditions (commonly 1x SDS-PAGE loading buffer, without reducing agent). A seemingly minor detail that we have found can make a large difference in the final purity of the eluted sample (especially if denaturing conditions are used) is to switch the beads to a fresh tube before elution—we commonly do this during the second wash. Macromolecules frequently adsorb nonspecifically to the surfaces of plastics used during handling procedures, hence the tube used for batch binding the medium with cell extract becomes coated with abundant contaminants—many of which will be washed off of the tube, along with your complex of interest, during elution. We switch to a fresh tube prior to elution in order to minimize this effect.

III. Readout: SDS-PAGE, protein staining, mass spectrometry

In the early stages of an investigation, some or all of the constituents of the affinity-enriched mixture are typically unknown and MS-based identification may be applied to map the complement of proteins present in a sample (3,4). MS analyses can produce a dizzying quantity of data, with lists of identified proteins in an affinity-captured protein mixture easily running into the 100s or more. On the other hand, one must also consider the limitations of MS when deciding whether samples prepared by affinity capture are suitable for this technique: Identification of protein complex components requires that they are present in sufficient absolute amounts as well as relative amounts. While the most modern instruments can measure masses over an intra-scan intensity range of at least a few thousand (142), the more limited dynamic range of some mass spectrometers and the limited loading capacity of small volume liquid chromatography columns may preclude the detection of bona fide complex components among an excess of non-specific contaminant proteins. Thus, detection by Western blot does not guarantee detection by MS. Moreover, as a number of interfering species can reduce sensitivity and compromise protein identification by MS, proper sample preparation is critical. Because different MS-based analytical approaches may require different sample workup procedures, we recommend adopting appropriate practices based on the preferences of your proteomics core facility or collaborator—our preferred approaches are described below.

Begin with SDS-PAGE

Regardless of which MS-based proteomic technique will be utilized to characterize the protein constituents of the affinity captured fraction, we almost always analyze our samples first by SDS-PAGE and protein staining, which provides a proven, rapid, robust, parallel, and semiquantitative assay revealing the approximate number, size, and amount of each protein in the sample as a collection of bands on the gel (143). The

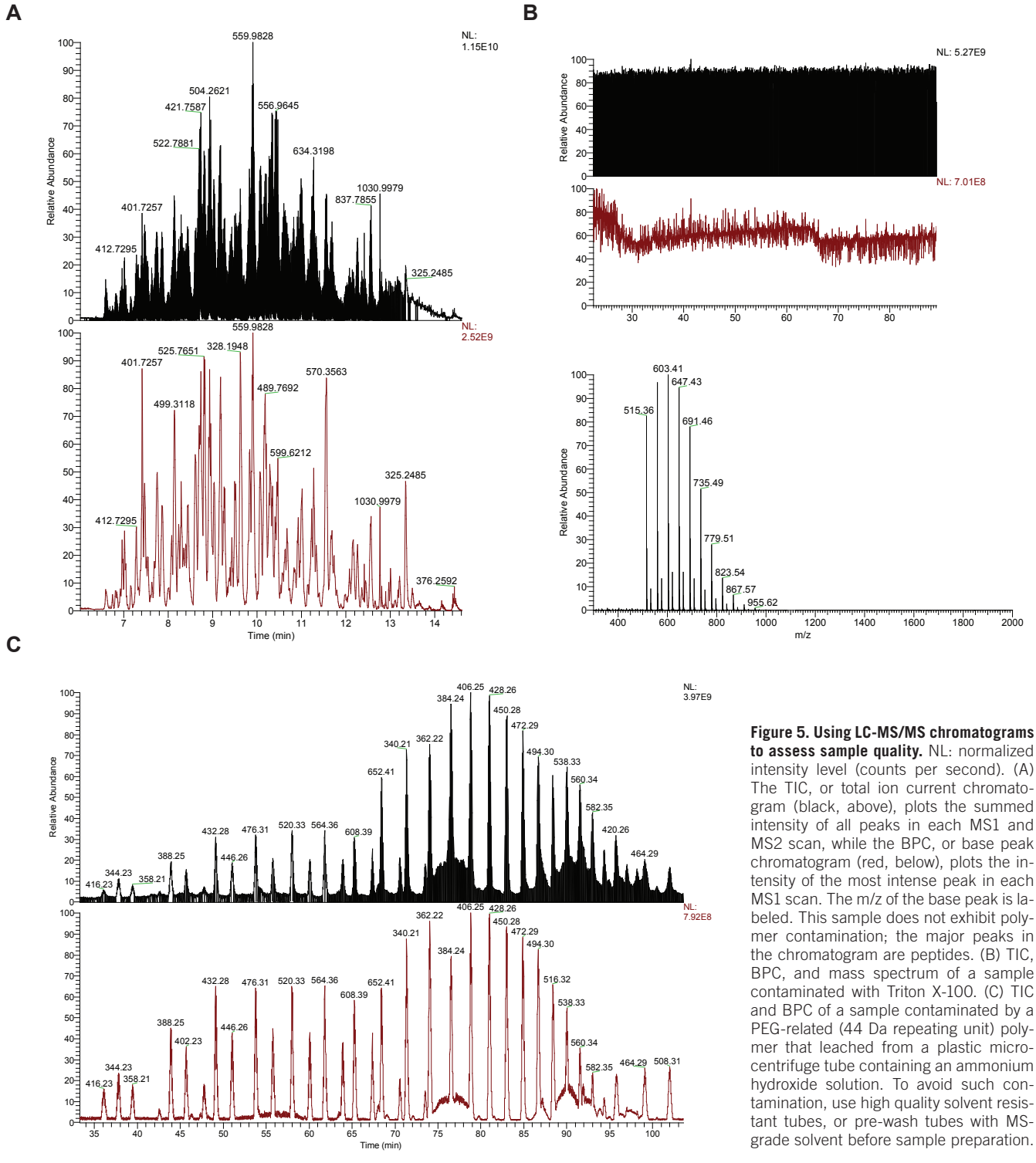

Figure 5. Using LC-MS/MS chromatograms to assess sample quality. NL: normalized intensity level (counts per second). (A) The TIC, or total ion current chromatogram (black, above), plots the summed intensity of all peaks in each MS1 and MS2 scan, while the BPC, or base peak chromatogram (red, below), plots the intensity of the most intense peak in each MS1 scan. The m/z of the base peak is labeled. This sample does not exhibit polymer contamination; the major peaks in the chromatogram are peptides. (B) TIC, BPC, and mass spectrum of a sample contaminated with Triton X-100. (C) TIC and BPC of a sample contaminated by a PEG-related (44 Da repeating unit) polymer that leached from a plastic microcentrifuge tube containing an ammonium hydroxide solution. To avoid such contamination, use high quality solvent resistant tubes, or pre-wash tubes with MS-grade solvent before sample preparation.

specificity and physiological relevance of co-purifying protein bands cannot be predicted a priori. However, in our experience, high quality affinity capture experiments—those enriched for specific and physiological co-purifying proteins—are typified in SDS-PAGE profiles by a discrete pattern of sharp, abundant, and roughly stoichiometric bands as well as a paucity of background staining from other fainter bands (Figure 4) (34,136, 44)—making this readout highly informative regarding the overall quality of the sample, and providing a basis for the judicious use of time-consuming MS analyses as well as complementary information to MS alone. Furthermore, SDS-PAGE provides the opportunity to excise individual bands (or regions) from the gel for analysis by MALDI-MS (matrix-assisted laser desorption/ionization) with peptide mass fingerprinting and/or liquid chromatography electrospray ionization with tandem MS peptide sequencing (LC-MS/MS) (125,145,146) in order to catalog the identities and gel migration behavior of the most abundant proteins

in the fraction, comprising the discrete stained bands observed.

We usually favor 4%–12% NuPAGE (Life Technologies) Bis-Tris gels with MOPS running buffer and colloidal Coomassie blue G250 stain (147) for most of our routine analyses. We frequently use Sypro Ruby (Life Technologies) stain instead of silver if greater sensitivity is required, owing to Sypro's superior inter-protein linearity, but we find silver staining still proves convenient in some cases when highly sensitive and rapid readout are needed (143,148,149). The existence of increasingly sensitive general protein stains provides gel-based visualization options even for very low abundance samples (150).

Characterize an optimized sample by MS

Once we are confident that the affinity capture procedure has been well optimized on the basis of SDS-PAGE results and protein IDs obtained for individual bands, we take that sample forward for LC-MS/MS—often referred to as shotgun proteomics (151). This provides a sensitive readout on the entire composition of the sample, including many proteins that are present at levels far below what is detectible by common protein staining methods. Most of the top protein ID assignments obtained in a shotgun experiment should correspond to the IDs determined separately for the abundant proteins present in excised gel bands, followed by less abundant proteins also present in the mixture—but which are often not observed in the stained gel. High-level, constitutively expressed gene products frequently contaminate affinity-captured fractions. The precise composition of contaminant binding will vary with experimental conditions (124,152), but regardless, their levels should generally be low relative to abundant putative interactors in an optimized sample. For high fidelity discrimination of signal from noise, we have used I-DIRT (25,153), a technique that implements metabolic labeling (154,155) to differentiate proteins that form interactions with the tagged protein of interest in vivo from those that occur after extraction from the cell. This technique is able to identify high confidence candidates for biological validation (25), but for reliable outcomes the method of expression should not be artifact prone in vivo.

In any case, identification of proteins by shotgun proteomics requires their digestion into peptides prior to MS analysis. Proteins must be made susceptible to trypsin activity (e.g., by denaturation) while ultimately remaining compatible with downstream reverse phase chromatography and MS. Once affinity capture is complete, and the samples have been eluted from the medium, commonly by denaturation in SDS or natively by competitive elution (in a buffer containing salts, detergents, etc.), proteins must be separated from these interfering species—for which there is a range of options. Some examples of the detrimental effects of interfering species on MS analysis are presented in Figure 5. Methods to digest protein complexes while still immobilized on the affinity medium have been utilized (156), but the necessity of removing interfering species remains.

One popular method, filter-aided sample preparation (FASP) (157), combines the solubilization of proteins with SDS and their release from the SDS-protein complex with the strong chaotropic reagent urea, facilitating tryptic digestion and subsequent MS analysis. The procedure is performed on the membrane surface of an ultrafiltration device. Our colleagues have reported that removal of SDS is not always complete following the FASP protocol precisely as presented—indicating that some user-to-user or sample-to-sample variations may affect the outcome. We therefore advise users to empirically determine the number of washes required to prevent SDS (or other detergent) carryover in their hands before executing this procedure on precious samples—which will depend on (at least) the detergent type, its CMC in the washing condition, the number of washes, and the membrane pore size. We adapted this method for label-free quantitative MS characterization of protein complexes engaged with the human exosome and mRNA cap binding complexes (79). The affinity-captured complexes were eluted using low pH (acetic acid), avoiding SDS, in conjunction with a modified version of the FASP protocol (158) utilizing PEG, which allows for higher recovery of low abundance proteins. However, PEG can also lead to pollution of mass spectra acquired during subsequent analyses, so care must be taken to avoid carryover in protocols utilizing this reagent. We must also note that, on several occasions, we and colleagues have lost valuable samples during processing due to O-ring failures in ultrafiltration devices; we have not experienced failures using the ultrafiltration devices from Millipore (159). For all of the above reasons, we favor gel-plugs (see below) over FASP, but we believe that FASP is of utility in the analysis of affinity-captured samples when carefully executed.

In our hands, gel-plugs have proven most robust, providing the beneficial qualities of gel-based sample preparation and allowing thorough removal of interfering substances, while foregoing the usual full separation across the gel. To produce a gel-plug, the whole sample is loaded on an SDS polyacrylamide gel and run only 4–6 mm into the gel, stained, and then excised—producing a small region of gel containing all the proteins present in the fraction and facilitating typical processing by in-gel digestion (146). A similar procedure, gel-aided sample preparation, has recently been described (160). It is desirable to keep the quantity of gel matrix at a minimum, to avoid limiting the efficiency of downstream peptide extraction (161). If the sample volume is large, it is therefore advantageous to load twice into the same well (rather than load into a larger well). The first aliquot of the sample is loaded and run into the gel and once it has fully entered the gel (as assessed by the loading dye), the second aliquot is loaded into the well, and the run is resumed until the dye front proceeds ~6 mm into the gel. Approximately 60 µL can easily be loaded in 2 stages into a 1 mm thick 10-well NuPAGE gel (Life Technologies), and up to ~80 µL can be run with very careful loading. Comparable volumes are achievable with most standard 1 mm thick PAGE 10-well combs. Upon loading the second aliquot, it is important to insert the gel tip to the bottom of the well (use a gel loading tip), to ensure the sample is stably deposited, because the well will contain residual glycerol from the previous loading that will be displaced during the subsequent loading.

IV. Concluding remarks

Because of the complexity and effort involved, the degree of optimization performed on many affinity capture/MS studies has been limited, resulting in a concomitant limitation in biological discovery potential (162). This common one-size-fits-all strategy continues to limit the scope of physiological complexes that can be accurately characterized by affinity

capture procedures and contributes to the high error rates (both false positive and negative) contaminating current databases (59,124,164–166).

The problem of affinity capture optimization is analogous to that which hindered protein crystallographic efforts: namely, empirically determining solvents that permit efficient crystal growth. The structural biology field responded by developing massively parallel crystallization screens (167,168). For crystallographers, such screening led to a revolution—the design and implementation of 96-well format plates that allow hundreds of conditions to be simultaneously screened, enabling high throughput Protein Structure Initiative centers to produce ~10 structures per week (169). The need for similar tools for interactomics has not gone unnoticed by the field (4,125,170).

However, the needs of crystallography differ from affinity capture in a practically and conceptually important way. Generally, a crystal screen aims to find solvent conditions that are conducive to protein crystal formation for the protein or protein complex under study. Although subsequent validations are required to ensure that the structure obtained is physiologically accurate—generally speaking, one, "right" structure is sought. However, in vivo protein interactions comprise a dynamic continuum of inclusive and exclusive constituent compositions. For a given protein of interest, this continuum of complexes may consist of different sets of interaction partners reflecting, for example, different stages of a metabolic pathway or localization to different cellular compartments. The interacting partners of a protein found both in the cytoplasm and the nucleus may be distinct within the two compartments—their cellular milieus are distinct. Therefore, the extraction conditions conducive to high fidelity purification of the interactors of the nuclear form of a protein of interest may be very different from those required to obtain the cytoplasmic interactors. And of course, the interactome is dynamic, with different interactions occurring at different times. Hence, there will likely never be one "correct" extractant for a given target, but instead several, differentially optimized to purify the local interactome of a complex given its numerous functional and compartmental states. There is simply no good substitute for old-fashioned elbow grease; for the most rewarding results, one must toil away for as along as it takes to develop high quality sample preparation conditions.

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Competing interests

The authors declare no competing interests.

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