How to Prepare Protein Complexes for Mass Spectrometry: **Success is in the Details**

Protein complexes are fundamental to the accurate 'inner workings' of a correctly-functioning cell and, it follows, of correctly-functioning tissues, organs and entire organisms. Protein complexes are involved in every aspect of an organism from signaling pathways to regulatory processes and cellular function. Without this constant protein complex 'chatter', living organisms would simply be unviable. Such 'chatter' must not only continuously occur but must also correctly occur. 'Chinese whispers' amongst the proteins in a cell leads to malformed protein complexes and ultimately the development of diseases that have major repercussions on the higher organism. The study of the intricate nature of protein-protein interactions has become significantly simpler and easier over the last two decades with improvements in both biochemical techniques and analytical instrumentation.

The aim of this guide is to aid the experimental scientist in preparing samples for successful analysis by mass spectrometry and identification of the protein components of the complex. The guide should be used in conjunction with references (*1-2*). The guide addresses how to avoid some common mistakes and not overlook or disregard details that may at first glance appear insignificant and trivial but will have a major impact on the quality of the data generated.

Getting going: where and how to start?

The first obvious question in the experimental design is: how can a protein complex be retrieved from a cell in the same state as it exists within the cell? Once the complex of interest has been carefully and gently removed from a cell and kept intact, how can the nature of the proteins that constitute that complex be determined? Key to a successful protein complex purification procedure is to understand, and be aware of, all the small details. If ignored and/or overlooked these can have quite significant ramifications on the quality of the downstream process including loss of proteins, and ultimately identification of the proteins by mass spectrometry. One of the greatest problems that affect data quality is sample contamination. Sources of contamination are both chemical and biological, e.g. polymer from detergents that are necessary to release the protein complex from the cell, using the wrong type of plastics that leach polymer into the sample, and other proteins such as naturally-occurring, abundant carboxylases that are co-purified in the first step of the affinity purification procedure[1].

Keep those complexes intact: cell lysate preparation is key

When preparing the cell lysate that contains the intact, non-covalently-interacting protein complexes it is crucial to minimize protein degradation. Otherwise, the protein complexes that are affinity purified from the cells will not truly reflect the *in vivo* situation. Therefore, it is imperative that all steps are performed on ice with pre-cooled reagents and materials. This will increase complex yield and reduce the chances of degrading any of the associated proteins. In addition, to further ensure the integrity of the non-covalently interacting protein complexes, it is highly advisable to perform the purification from freshly-lysed cells.

Protein concentration determination: which assay/standard should be used?

It is vital for protein complex isolation and subsequent analysis by liquid chromatography mass spectrometry (LC/MS) to not only know the initial quantity of total protein from the cell lysate that is used; but also if the protein lysate obtained is sufficient to affinity purify an appropriate quantity of the protein complex for analysis. A latter advantage of determining the total protein content of the lysate is that normalization of protein input can aid downstream quantitative comparison of the same complex under, e.g. a perturbed or stimulated setting. Interestingly, not all protein concentration assays are the same, nor are these assays sufficiently reproducible to give consistent and accurate readings. Similarly, the protein standard chosen to generate the calibration curve to then determine the amount of protein in the cell lysate can affect the final value obtained, e.g., bovine serum albumin standards will give a different reading with some protein assays compared to using another protein such as γ-globin. Our recommendation for the minimum quantity of protein required per protein complex purification is 40 mg. This amount of protein can be harvested by lysing five plates of HEK293 cells grown to 80% confluency in 15 cm diameter cell culture dishes, determining the amount of protein with a Bradford assay and using bovine serum albumin as the protein standard. Key here is to understand what effect your standard, assay and quantity of protein used for isolating the protein complex of interest will have on the downstream analysis. Note too, with ever-increasing sensitivity of available mass spectrometers, the quantity of protein lysate can be decreased.

Isolating those protein complexes: remember the basics!

Regardless of the number of -20 and -80°C freezers available to scientists, space is often at a premium and usually never sufficient in any laboratory. Additionally, most scientists tend to unnecessarily hoard leftovers of experiments indefinitely. Nevertheless, it is important during the

How to Prepare Protein Complexes for Mass Spectrometry: **Success is in the Details**

isolation of protein complexes to keep everything that is generated throughout the experiment. Be a hoarder! This may irritate other members of the laboratory and consume valuable space in the freezer, but always take aliquots of the experiment throughout and safely store until the final results of the experiment have been generated. This will ease troubleshooting at a later time point and it will be considerably easier to locate where the error may have occurred by tracing back the steps and checking the stored aliquots by immunoblotting.

When preparing cell lysates and affinity-purifying protein complexes, a very important point that is often misconstrued during sample preparation for mass spectrometry is to work cleanly. In this context, clean refers to the absence of dirt and dust and is not a reference to working under sterile conditions. Indeed, sometimes the process of sterilizing laboratory equipment, e.g. pipette tips, can introduce unwanted contaminants from the autoclaving procedure that ultimately appear in the samples that will be analyzed by LC/MS. It goes without saying that gloves are essential, as is maintaining the sample preparation and all areas as clean as possible and free from dirt, dust and debris. It is highly advisable to use fresh consumables and not use plasticware that has been left open to the environment and is covered by a thick layer of dust. This contains human skin particles (and often sheep proteins if laboratory colleagues wear woolen jumpers) that end up in the experiment. It's also important to avoid unstable plasticware: polymers can leach from the plastic into your sample. Any plastics used should be acid, or low pH, stable. In addition, use pipette tips that contain a filter throughout. This key point eliminates the chances of cross contamination of samples due to incorrect use of the pipette and the aspiration of sample into the barrel of the pipette.

Another basic is to avoid unnecessary exposure to detergents: do not incubate your cell lysates in any plastic columns/tubes etc. The residual detergents from preparing the cell lysate can coat the plasticware and is then efficiently removed by the acids used to elute your protein complexes from the affinity matrix. Major polymer contamination of the sample is readily observed by mass spectrometry and affects the quality of your protein complex identification. A basic that is obvious, but nevertheless worthy of a mention, is to always ensure that any tubes containing your sample are firmly closed and sealed with Parafilm. This is vital to prevent loss of the sample during any of the rotation/incubation steps.

Isolating those protein complexes: remember the basics!

Following the second step of the affinity purification of the protein complex (1), remove all the supernatant (i.e. any unbound material after incubation with anti-HA agarose) with a pipette. It is important to remove all biotinylated carboxylases, as these proteins result in a high background contamination when the samples are analyzed by mass spectrometry. When washing any beads, aim to prevent resuspension of the buffer containing detergent as this will lead to some of the detergent coating the walls and following elution with an acid, the detergent again will predominate the mass spectrometry data. Apply the wash buffers (without detergent) to the walls of the column from the 'top' (level of the previous buffer) in a circular manner to wash the detergent contamination from the walls of the column. Allow the first aliquot of wash buffer to drain before applying the second wash. Wash the outside of the column tip with wash buffer to remove any residual detergent. This step is the key to producing samples that are polymer-free for subsequent analysis by mass spectrometry. Without extreme care at this point, protein samples will be contaminated with detergent. Detergents and plasticizers are scavengers of charge. Thus, during analysis by mass spectrometry, these components are preferentially ionized at the expense of the peptide samples. Ultimately, weak signals or no peptide signals are observed. Another important tip is to never allow the columns to dry out. Elute the sample immediately after the last wash, else some of the protein complexes may remain irreversibly bound to the column material. Once again, avoid plastic consumables at this point and elute directly into a glass vial. We have seen that even short-term storage in plastic results in plasticizer extraction from the consumables. With long-term storage this problem is exacerbated.

Digesting those protein complexes

There is a plethora of protocols available for reducing protein disulphide bonds, blocking free cysteine residues with alkylating agents, and solution digestion of proteins. Basically, all are tried and tested, and you cannot really go wrong with any of these methods. Most importantly, it is key to remember that to successfully digest a protein with e.g., trypsin, then the pH should be between 7.5 and 8.5. We have found that storage of acidified proteins can result in protein degradation over time via acid hydrolysis[3], thus; we do not recommend the addition of acid at the end of the digest to quench the reaction and stop the action of the enzyme. A bonus with this approach is that if you wish to use the remains of the protein complexes for another experiment, e.g. labelling with stable isotope reagents for quantitative mass spectrometry; the samples are already in the appropriate conditions for labelling and the pH does not need to be re-adjusted.

Purifying and concentrating those protein complexes

Once the proteins from the protein complexes have been digested into peptides, the next step is to concentrate and purify the peptides from any residual salts and contaminants that can also impact the quality of the mass spectrometry data. The following is based on reference (4) but many researchers do prefer to either use alternative commercial products or omit this step completely and inject the digest directly onto an LC/MS system. Nevertheless, given below are some tips and tricks that can aid a researcher opting for the self-made STop And Go Extraction (STAGE) columns. Note that if at any point in the protocol the liquid in the STAGE tips does not flow through completely with centrifugation, then gently push the liquid through manually with a syringe.

How to Prepare Protein Complexes for Mass Spectrometry: **Success is in the Details**

To acidify the sample, pipette 30 µL of the tryptically-digested proteins (equivalent to 5% (v/v) of the digest) (2) into a plug of the STAGE tip solution (0.4% formic acid, 2% TFA) added to each STAGE tip. Look for bubbles indicating the release of CO_2 and acidification of the sample. It is imperative that the digested sample is acidified to ensure efficient binding to the C18 material. To load larger volumes of the buffered digest onto a STAGE tip, it is necessary to add additional volumes of 30% (v/v) trifluoroacetic acid (TFA) to ensure complete acidification of the sample, e.g. 50 µL of STAGE tip solution plus 180 µL of digested sample plus 10 µL of 30% (v/v) TFA gives a volume of 240 µL and a final TFA concentration of ~1% (v/v). When centrifuging the STAGE tips, do not centrifuge too rapidly as the peptides will not bind efficiently to the C18 material and will be lost in the flow-through. Note again, that if the liquid does not flow through completely, push through manually with a syringe. Although plasticware is used to create the STAGE tips, rapid washing and elution decreases the chance of extracting polymer.

Elute the bound peptides with 50 µL STAGE tip elution buffer (0.4% formic acid, 90% acetonitrile) into clear HPLC vials with a glass V-shaped insert, and pool three STAGE tip extractions per affinity purification into the same glass vial. This will give sufficient sample for technical replicate injections plus a 'back-up' injection in case one of the injections is lost due to instrument problems. Remove the acetonitrile from the STAGE tip eluted sample in a vacuum concentrator (e.g. 30°C for 15 min) until ~2 µL final volume remains. Avoid drying to completeness as loss of peptides via adsorption to the glass surface is increased. Finally, reconstitute the samples with ~8 µL 5% (v/v) formic acid to a final volume of ~10 µL. Add multiples of 8 µL 5% (v/v) formic acid depending on how many repeat analyses will be performed by LC/MS. Volumes are dependent on the HPLC sample loop and can be adjusted accordingly.

What quantity of digested protein complex to inject?

The affinity purification of a tagged protein complex is different for every protein, thus, prior to analysis of the tryptically-digested proteins by mass spectrometry it is not possible to determine whether the sample quantity suggested above is suitable for all protein complexes. If weak signals are observed by mass spectrometry, the researcher can return to the original, stored digested sample and use a larger volume. If strong signals consistent with analytical column overloading are observed by mass spectrometry, a lower volume of the digested proteins can be re-purified and analyzed or the purified sample diluted. With time, one trick that can alleviate the guesswork is to always have a standard protein such as tagged green fluorescent protein (GFP). Analyzing an affinity purification of GFP in parallel with the protein complex of interest by immunoblotting against the tag will give a result indicating the relative strength of the tag present in the purifications. For example, consider 5% (v/v) of the digest from affinity-purified GFP. This quantity consistently achieves a maximal signal by mass spectrometry, but without overloading the analytical column. If on the immunoblot, the protein of interest has approximately half the intensity of the GFP experiment; then double the quantity of the digest from the protein of interest should be purified and injected (i.e. 10% v/v). Again, approximations and ideas are provided to give an idea of how to normalize between experiments and different proteins whilst still maximizing analytical depth. Such streamlining must be customized for each individual laboratory and work flow.

Success really is in the details

With this guide, we hope to provide researchers with a series of tips and tricks that can circumvent many basic, yet common errors that can and do frequently occur during the preparation and affinity purification of non-covalently-interacting protein complexes from cells. From preparing the cell lysate through to determining the appropriate quantity of a digested protein complex to inject onto an LC/MS system, these details can vastly improve the quality of the data that is ultimately generated.

References

1. T. Glatter et al., *Mol. Syst. Biol*. **5**, 237 (2009).
2. E. L. Rudashevskaya et al., *Nat. Protoc*. **8**, 75-97 (2013).
3. M. Planyavsky et al., *Anal. Biochem*. **473**, 11-13 (2015).
4. J. Rappsilber et al., *Anal. Chem*. **75**, 663-670 (2003).