Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind

Preparing, purifying, analysing and identifying the constituent components of non-covalently-interacting protein complexes by mass spectrometry has become significantly simpler and easier over the last two decades, with advances in both biochemistry and analytical chemistry. Although biochemical procedures and mass spectrometry-based analytical instrumentation are quite standard and robust in many laboratories, there are still many points that are easily overlooked in the successful preparation and analysis of these complexes. This guide coalesces over 10 years of background and experience in performing and optimising protein-protein interactomics. The focus is to raise and address all those seemingly trivial, yet incredibly important details that can aid a scientist in generating samples for analysis by liquid chromatography mass spectrometry (LCMS) and identification of the protein components of the complex. These tips and tricks are an aid to following the protocols described in references 1 and 2 (*1*, *2*).

Thorough preparation minimises downstream experimental problems

Before starting an experiment to isolate and prepare protein complexes for analysis by MS, there are some general considerations to always keep in mind:

- Depending on the number of purifications, seriously consider running the entire experiment in a 4˚C cold room. If this is too humanely uncomfortable, it is of course possible to perform the experiments on the bench in a standard laboratory. Just ensure that all buffers etc. are cooled on ice at 4˚C.
- Consider aliquoting buffers for daily use and discarding leftovers. This prevents cross contamination, buffers becoming old and 'stale', and ultimately saves money for the laboratory.
- Prepare buffers and solutions in non-autoclaved glassware. Avoid using glassware that has been washed with detergents. Check for columns, beads and racks the day before the experiments.
- Prepare and clearly label tubes for immunoblot aliquots.
- Avoid keratin contamination as much as possible by ensuring a dust-free work bench, do not leave tips and buffers open to the environment.
- Prepare all solutions with extreme care to minimise contamination with keratin.

Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind

- Use gloves at all times - change if keratin contamination is suspected. Always use filter tips to prevent cross contamination of samples (autoclaved tips are a source of contamination).
- Save and store all column flow-through or supernatants, washes etc., save all beads, and save columns. This can be key in troubleshooting and tracking back a problem if the LCMS data does not identify many proteins.

Critical points for mass spectrometry

One of the greatest problems that affects the quality of MS-generated data is sample contamination. This is perhaps still one of the most overlooked points in modern-day mass spectrometry. Sources of contamination can be chemical or 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 abundant proteins that are present at high levels. These include the notorious keratins and naturally-occurring carboxylases that are co-purified in the first step of the affinity purification procedure (*1*).

Avoiding keratin and other protein contamination

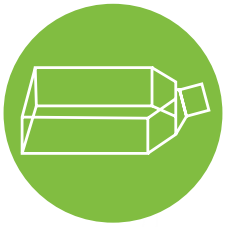
Never forget! Keratin is a protein and exists in abundance anywhere where a human being has been or resided. When preparing cell lysates and affinity-purifying protein complexes, a very important point that is often misconstrued during sample preparation for mass spectrometry is to work cleanly. In this context, clean refers to the absence of dirt and dust and is not a reference to working under sterile conditions. Of course, gloves are essential, as is maintaining the sample preparation and all associated 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 woollen jumpers) that end up in the experiment. Simply put, avoid keratin contamination at all steps of the experiment by consistently using dust-free, not sterile/autoclaved plastic tubes, pipette tips *etc.* In addition, use pipette tips that contain a filter throughout the sample preparation. This 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, thus contaminating your next sample with proteins from the previous.

Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind

Minimising detergent and polymer contamination

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. Reagent and consumable quality is very important. Avoid unstable plasticware: polymers can leach from the plastic into your sample. Any plastics used should be acid, or low pH, stable. Major polymer contamination of the sample is readily observed by mass spectrometry and affects the quality of your protein complex identification. Ideally, after purification the protein sample should be as free of detergent as possible.



Cell culture steps

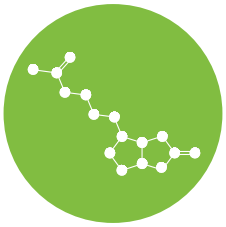
When it comes to cell culture practices for the cells containing the tagged, non-covalently-interacting protein complexes, abide by the rules of the laboratory where you are working and follow standard procedures. For the protocol in reference 2 (*2*), 5 × 15 cm dishes or 2 × 10^8 cells (suspension), *i.e.*, a minimum of 50 mg total protein per protein complex purification, is recommended. Ideally, use fresh lysates for the purifications, however, if not feasible snap freeze the washed cell pellets in liquid nitrogen and store at -80°C until required.

Preparing the cell lysate

When preparing the cell lysate that contains the intact, non-covalently-interacting protein complexes it is crucial to minimise 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.

Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind



Purifying the protein complexes: the first step

Follow the protocol for this first step according to reference 2 (*2*). Important at this point is to freshly-prepare the biotin solution and not use stored aliquots. Biotin is sensitive to degradation by ultra-violet light and will lose efficiency with time. Slowly apply the protein lysate to the prepared column and allow the lysate to enter the resin by gravity flow. No incubation time is necessary. After collecting aliquots of the flow-through for immunoblotting and potential troubleshooting, wash the column and then elute the bound proteins with 3 × 300 µL of the freshly-prepared 2.5 mM biotin into a 1.5 mL plastic tube. Save aliquots (30 µL) of the biotin elution for immunoblot analysis and save and store the remaining beads and columns.

Purifying the protein complexes: the second step

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-haemagglutinin (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 analysed 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 very important to produce samples that are polymer-free for analysis by mass spectrometry. Without extreme care at this point, protein samples will be contaminated with detergent. Detergents and plasticisers 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 not let the columns dry out. Elute the sample immediately after the last wash otherwise there is the potential that some of the protein complexes may remain irreversibly bound to the column material. Once again, avoid plastic consumables and elute directly into a glass vial. We have seen that even short-term storage in plastic results in plasticiser extraction from the consumables. With long-term storage this problem is exacerbated.

Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind

Eluting protein complexes

The proteins from the purified protein complex can be eluted from the column directly into a high-performance liquid chromatography (HPLC) glass vial (containing 125 µL 1 M triethylammonium bicarbonate (TEAB) buffer) with 500 µL 100 mM formic acid stored in a glass vial (final concentration TEAB buffer approx. 200 mM). If the acid does not elute from the column, it might be necessary to apply a small amount of pressure to initiate the flow of liquid. It might also be necessary to flush out all remaining eluate. Once the proteins have been eluted, gently mix to neutralise the acid and remove 200 µL of the eluate. This can be used for *e.g.*, running a silver-stained gel (100 µL) and/or an immunoblot (2 × 50 µL). These samples can be aliquoted into 1.5 mL plastic tubes and lyophilised. Importantly, remove this volume of eluate even if it may not be required. The remaining 425 µL can be snap frozen in liquid nitrogen or stored in a -20°C freezer. Do not lyophilse the samples!! Protein loss can occur, and it may be difficult to fully re-solubilise the proteins for the digestion step. Save all beads and columns, potentially boiling the beads after acid elution in Laemmli buffer to determine if any bait remained on the column.

Digesting protein complexes

There are a plethora of protocols available for reducing protein disulphide bonds, blocking free cysteine residues with alkylating agents, and solution digestion of proteins. Basically, all work well and you cannot really go wrong with any of these methods. Most importantly, remember that to successfully digest a protein with *e.g.*, trypsin, then the pH should be between 7.5 and 8.5. Storage of acidified proteins can result in protein degradation over time via acid hydrolysis (*3*), thus; addition of acid at the end of the digest to quench the reaction and stop the action of the enzyme is not recommended. A bonus with this approach is that if you wish to use the rest 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.

Preparing Perfect Non-Covalent Protein Complexes for Analysis by Mass Spectrometry

12 Key Thoughts to Keep in Mind

Purifying and concentrating protein complexes

After the proteins from the complexes have been digested into peptides, the next step is to concentrate and purify these 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 prefer to either use alternative commercial products or omit this step completely and inject the digest directly onto an LCMS 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.

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% trifluoroacetic acid (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 LCMS. Volumes are dependent on the HPLC sample loop and can be adjusted accordingly.

12 Key Thoughts to Keep in Mind

Calculating the 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 analysed 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). Analysing 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.

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).