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Discovery of host-viral protein complexes during infection

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Summary

Viruses have co-evolved with their hosts, developing effective approaches for hijacking and manipulating host cellular processes. Therefore, for their efficient replication and spread, viruses depend on dynamic and temporally-regulated interactions with host proteins. The rapid identification of host proteins targeted by viral proteins during infection provides significant insights into mechanisms of viral protein function. The resulting discoveries often lead to unique and innovative hypotheses on viral protein function. Here, we describe a robust method for identifying virus-host protein interactions and protein complexes, which we have successfully utilized to characterize spatial-temporal protein interactions during infections with either DNA or RNA viruses, including human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1), pseudorabies virus (PRV), human immunodeficiency virus (HIV-1), Sindbis, and West Nile virus (WNV). This approach involves cryogenic cell lysis, rapid immunoaffinity purification targeting a virus or host protein, followed by identification of associated proteins using mass spectrometry. Like most proteomic approaches, this methodology has evolved over the past few years and continues to evolve. We are presenting here the updated approaches for each step, and discuss alternative strategies allowing for the protocol to be optimized for different biological systems.

Keywords

virus-host interactions; infection; epitope tag; immunoaffinity purification; cryogenic cell lysis; proteomics; protein complex; protein-protein interactions; mass spectrometry

1. Introduction

The ability to identify host targets of viral proteins during the progression of an infection is invaluable for determining the functions of different viral proteins and identifying targets for therapeutic intervention. With the development of effective approaches to isolate protein complexes and identify and quantify co-isolated proteins using highly sensitive and accurate mass spectrometers, we are now able to quickly determine viral protein targets. Cristea *et al.* (1) have previously developed a rapid immunoaffinity purification approach of protein

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complexes that has been used successfully during infections with either DNA or RNA viruses. Furthermore, this approach has been used to isolate viral and cellular proteins as baits during infection, allowing the study of protein-protein interactions from either the virus or host perspective. Here, we present this strategy that, in our hands, has proven to be effective and robust for isolating and identifying protein complexes (Figure 1). Numerous approaches are available for isolating protein complexes, and selecting the appropriate approach is dependent upon your biological system and available resources. Ultimately, discovering the targets of viral proteins within infected cells provides significant insights into viral protein function.

Using proteins within the yeast nuclear pore complex as baits, Cristea *et al.* (1) initially developed the method discussed in this chapter following extensive optimization to integrate information regarding protein localization with the rapid isolation of native protein complexes from eukaryotic cells. Optimizations included determining effective cell lysis conditions, the composition of lysis buffer for protein complex extraction, the amount of resin used for immunoaffinity purification, and the incubation time of the antibody with the lysate. The method was later successfully applied to study virus-host protein interactions using the Sindbis viral protein nsP3 as bait during virus replication in mammalian cells (2). Efficient isolation of protein complexes is achieved by combining cryogenic disruption of the infected cells into a frozen powder, see section ***3.2.3***, with optimized lysis buffer conditions for each target protein complex, see section ***3.4.3*** (3). The method is also optimized using magnetic resin (beads) to which antibodies are covalently attached (4). These steps reduce the amount of nonspecific proteins and IgG contamination, and are discussed in section ***3.3.*** Finally, and central to the success of this method, is the time of incubation of the antibody-conjugated beads with the cell lysate. Incubation times longer than 60 min resulted in increased isolation of non-specific proteins with no significant increase in specific interactions (1). We discuss incubation times within section ***3.4.1***. In addition to optimizing lysis conditions and the efficiency of isolation, the ability to interpret the results and determine the specificity of identified interactions requires the use of appropriate controls. Although the method has been optimized to reduce non-specific interactions, these interactions still occur, and we discuss the various sources of non-specific interactions in section ***3.5***. The use of appropriate controls will help distinguish between specific and nonspecific binding proteins.

Over the last few years, the method presented in this chapter or aspects of it have been applied to isolating several viral proteins using different epitope and antibody combinations. We discuss these in section ***3.1.*** These included antibodies to GFP (green fluorescent protein) (2, 5–7) or FLAG epitope tags (8, 9), protein-specific antibodies (10), as well as single-step isolations using IgG to target the Protein A component of a TAP (tandem affinity purification) tag (6, 11) (Table 1). In all the studies in which we have utilized the approach described in this chapter, identifying viral-host protein interactions provided significant mechanistic insight into viral protein activity during infection (Table 2). For example, using a full-length replication-competent Sindbis virus (SINV) expressing the viral nonstructural protein nsP3 tagged with GFP, Cristea *et al.* (2) were able to track viral protein localization during infection using fluorescence microscopy, as well as identify changes in nsP3

interactions at different time points of SINV infection. Using a Protein A tag, Moorman *et al.* (11) identified tuberin (TSC2), a negative regulator of mTOR (mammalian target of rapamycin), as the target of the human cytomegalovirus (HCMV) protein, pUL38. Expression of pUL38 was demonstrated to be both necessary and sufficient to maintain mTOR kinase activity during conditions of nutrient stress (11). Similarly, building spatial-temporal interaction networks for several GFP-tagged HCMV proteins has provided insights into processes involved in virion assembly, indicating the presence of multiple intermediate virion structures that traffic independently from each other prior to merging into larger vesicles (5). Using a FLAG epitope, Reitsma *et al.* (9) determined the target of the HCMV protein, pUL27, to be the cellular acetyltransferase complex, Tip60. Expression of pUL27 was shown to be necessary and sufficient to degrade Tip60 resulting in expression of the CDK inhibitor, p21CIP1 and a transient cell cycle arrest (9). And, most recently, Youn *et al.* (10) used a specific antibody against West Nile virus (WNV) NS1 protein to determine an interaction with WNV NS4B. These studies identified a possible mechanism linking NS1 to regulating WNV RNA replication (10). Beyond viral proteins, immunoaffinity purification of protein complexes using the approach described in this chapter has also been used for identifying targets of cellular proteins including histone deacetylase 1 (12) and 5 (13), sirtuin 7 (14), metadherin (15), and interferon-inducible protein 16 (16) (Table 3). For these applications, GFP epitope or antibody against the endogenous protein was used. This chapter describes a protocol used to immunoaffinity purify protein complexes from infected cells and identify protein composition (Figure 1).

2. Materials

2.1 Epitope selection

1. TAP sequence containing Protein A (17) using IgG.
2. GFP sequence (18) or variants using an anti-GFP antibody.
3. Multiple repeats of the FLAG sequence (19) using anti-FLAG antibody.

2.2 Selection, preparation, and lysis of infected cells

2.2.1 Preparing infected cells

1. Infected mammalian cells in tissue culture dishes or flasks.
2. DPBS pH 7.4 Dulbecco's Phosphate-Buffered Saline 1x (Invitrogen).
3. Rubber policeman or a plastic cell scraper.
4. Centrifuge at 4°C.
5. 250 mL cone tipped centrifuge bottle or 50 mL conical tubes.
6. Cryo Buffer: 20 mM Na-HEPES, 1.2% (w/v) polyvinylpyrrolidone, pH 7.4, 1/100 (v/v), protease inhibitor cocktail (Sigma) added immediately before use.

2.2.2 Freezing cell pellet

1. Metal tube rack.

2. Ice bucket or liquid nitrogen safe container (e.g. Styrofoam).
3. Liquid nitrogen.
4. 50 mL screw cap tube.
5. 18-gauge needle.
6. 10 mL syringe.

2.2.3 Cryogenic disruption of the cell pellets

1. Retsch MM 301 Mixer Mill.
2. Two 25 mL or two 10 mL stainless steel jars with tungsten carbide grinding balls (Retsch, Newtown, PA).
3. Liquid nitrogen in an open container.
4. Long forceps.
5. Metal spatula.
6. Windex, methanol, and ultrapure H_2O for cleaning.
7. 50 mL conical tubes.
8. Alternative method (see ***3.2.5***): 2.0 mL adaptor (Retch), 3 mm grinding balls (Retch), and 2.0 mL Safelock tubes (Eppendorf).

2.3 Antibody Conjugation

1. Dynabeads M-270 Epoxy (Invitrogen).
2. Magnetic tube rack.
3. Round bottomed microcentrifuge tubes (Eppendorf).
4. Rotating tube rack in 30°C incubator or equivalent environment.
5. Tube shaker (e.g. Tomy shaker).
6. Immunoglobin G (IgG) or high-affinity purified antibodies against the selected tag or bait protein.
7. Sodium Phosphate Buffer: (0.1 M, pH 7.4): 19 mM NaH_2PO_4, 81 mM Na_2HPO_4 in water.
8. Ammonium sulfate (3M): Prepared in 0.1 M Sodium Phosphate Buffer
9. Glycine-HCl (100 mM, pH 2.5): Prepared in water and pH adjusted with HCl. Filter sterilize with 0.2 μm filter.
10. Phosphate Buffered Saline (PBS)
11. 0.02% sodium azide (NaN_3): dissolved in PBS.
12. 0.5% Triton X-100: dissolved in PBS.
13. Tris-HCl (10 mM, pH 8.8): Prepared in water and pH adjusted with HCl.

14. Triethylamine (100 mM): Prepared immediately before use in water.

2.4 Immunoaffinity purification: Strategy, optimization, and assessment of efficiency

2.4.1 Immunoaffinity purification of protein complexes

1. Polytron homogenizer (e.g. PT 10-35 Polytron from Kinematica).
2. Centrifuge and rotor rated to spin 8000xg at 4°C (rotor must fit a 50 mL conical tube).
3. Rotating tube rack at 4°C.
4. Round bottomed microcentrifuge 1.5 mL tubes.
5. Axygen microcentrifuge tube (to reduce polymer contamination).
6. Heat block at 70°C.
7. Tube shaker (e.g. Tomy shaker).
8. Magnetic tube rack for 1.5 mL tubes and bar magnet for conical tubes.
9. Vacuum evaporator (e.g. speedvac).
10. Frozen cell powder (see ***3.2.3***).
11. Optimized Lysis Buffer and wash buffer (see ***3.4.3***).
12. Antibody conjugated magnetic beads (see ***3.3***).
13. Ammonium Hydroxide (14.8 M): (Sigma) Stored at 4°C.
14. Base Elution Buffer: 5 μL of 0.5 M EDTA, pH 8.0, and 169 μL of Ammonium Hydroxide diluted in 4.826 mL of ultrapure H_2O, prepared fresh before use.
15. 4x LDS elution buffer: 0.666 g of Tris-HCl, 0.682 g of Tris-Base, 0.8 g of LDS, and 0.006 g of EDTA (free acid) in ultrapure dH_2O to a final volume of 10 mL. Aliquot and store at −20°C.
16. 1x NuPAGE Sample Buffer (Invitrogen).
17. 10x Reducing Agent (Invitrogen).
18. 1M Iodoacetamide (IAA): (Sigma) 0.185 g of Iodoacetamide dissolved in 1 mL HPLC grade water stored at −20°C.

2.4.2 Assessing immunoaffinity purification efficiency

1. Saved samples from section ***3.4.1*** (input supernatant, cell pellet, flow through, primary eluate, and secondary eluate.)
2. 1x NuPAGE LDS Sample Buffer and 1x Reducing Agent (Invitrogen).
3. 2% SDS.
4. Acetone.
5. Heat block at 70°C.

6. NuPAGE 4-12% Bis-Tris pre-cast SDS-PAGE gel (10-well) (Invitrogen).
7. Xcell SureLock Mini-Cell electrophoresis system (Invitrogen).
8. 20x NuPAGE MOPS SDS Running Buffer (Invitrogen).

2.4.3 Optimization of lysis buffer conditions

1. At least 3 Lysis Buffers at varying stringency (see ***3.4.3***).
2. Frozen cell powder (see ***3.2.3***).
3. Table top centrifuge rated to spin 20 000xg at 4°C.
4. Sonicator.
5. Tris HCl: (50 mM pH 7.4) containing 2% SDS.
6. NuPAGE 4-12% Bis-Tris pre-cast SDS-PAGE gel (10-well) (Invitrogen).
7. 1x NuPAGE LDS Sample Buffer and 1x Reducing Agent (Invitrogen).
8. Xcell SureLock Mini-Cell electrophoresis system (Invitrogen).
9. 20x NuPAGE MOPS SDS Running Buffer (Invitrogen).

3. Method

3.1 Epitope selection

Numerous protein epitope and epitope-specific antibody combinations have been successfully used in isolating protein complexes for identifying composition by mass spectrometry. Although many combinations are possible, we have limited our discussion to the tags that have been used specifically with the described method. These tags include TAP (tandem affinity purification) (6, 11), GFP (green fluorescent protein) (2, 5–7) and FLAG epitopes (8, 9). Starting from the amino terminus of the bait protein, the TAP tag (17) (Table 1) consists of a calmodulin binding peptide (CBP), a tobacco etch virus protease (TEV) cleavage site and two IgG-binding units of protein A (ProtA) from *Staphylococcus aureus*. The TAP tag was originally used for sequential purification steps with IgG followed by TEV cleavage and re-isolation by Calmodulin-coated beads (17). Our use of the TAP tag was limited to isolation with IgG to increase the identification of weak binders that might be lost following sequential purification steps (6, 11). Although the tag is effective in isolating complexes, one limitation to consider is the large size of the epitope at 20 kDa. Another epitope successfully used in immunoisolating protein complexes is the fluorescent protein GFP (Table 1). Although GFP is also relatively large, at 27 kDa, it allows for both visualization and complex isolation (1) as demonstrated by Cristea *et al.* (2) using the Sindbis viral protein, nsP3, during infection. Immunoisolation is accomplished using an antibody against GFP (2, 5–7). For several applications, GFP was inserted at the carboxyl terminus of the bait and the starting methionine of GFP was removed to avoid the possibility of internal initiation and expression GFP alone (Table 1). Finally, immunoisolation has been completed using the FLAG tag (Table 1). The FLAG epitope consists of eight hydrophilic amino acids (AspTyrLysAspAspAspAspLys) (19) and the advantage of the tag is its small size at 1 kDa. Isolation of protein complexes has been completed using three repeats of the

FLAG sequence expressed in-frame with the bait protein (8, 9). Several antibodies are available against the FLAG epitope including the monoclonal Anti-FLAG M2 antibody (Sigma Aldrich). For all three epitopes discussed, it is possible to use either amino or carboxyl terminal tags on your bait, or on an internal flexible loop of the protein, provided that it does not interfere with protein function.

Introducing a unique epitope into the bait has the potential to alter the protein and associated functions. Therefore, it is critical to evaluate the known aspects of the bait to ensure wild-type protein function. For most of the examples referenced in this chapter, epitopes were introduced into the viral genomes and protein complexes were identified during infection. These recombinant viruses were evaluated for their ability to replicate with wild-type kinetics (2, 5, 6, 8, 11). A failure to replicate efficiently suggests that the tag has disrupted an important function of your bait. For proteins which have been previously demonstrated to be nonessential during replication *in vitro*, alternative approaches will be needed to assess functionality. For example, the HCMV protein pUL27 is nonessential for replication but is necessary for the full antiviral activity of an antiviral agent (9). Therefore, the recombinant virus was evaluated for its sensitivity to the compound. When a protein-specific antibody is available, it is also possible to evaluate the tagged protein using immunofluorescence analysis to ensure wild-type protein localization during infection. In the end, the existing phenotypical data must be intact in the tagged version in order to effectively link the identified interacting proteins to viral protein function during infection.

3.2 Selection, preparation, and lysis of infected cells

With the understanding that every viral protein is unique, successful isolation of target proteins requires optimization. Two steps requiring optimization include i) selection of an appropriate time for isolation during infection and ii) identification of an optimal lysis condition (see ***3.4.3***). The goal of this protocol is to effectively isolate and identify protein complexes containing the viral protein of interest with the long term goal of determining the functional relevance of the interactions.

Identifying the appropriate time to isolate the protein complexes during infection is critical. The time can often be determined based upon the timing of expression, as well as upon phenotypical data using viruses containing mutations in the protein of interest and loss of function. Confocal microscopy can be used to visualize the temporal expression and subcellular localization of the viral proteins of interest. If the viral protein is tagged with a fluorescent tag (e.g., GFP), time-lapse microscopy using direct fluorescence can provide an in-depth view of the dynamics of protein expression and localization. Viral protein abundance should also be considered in the decision. In general, highly abundant viral proteins are easier to isolate and analyze for identification of binding partners. Isolation of low abundant proteins can also be achieved with optimization. While the increasing sensitivity of mass spectrometric configurations allows the conclusive identification and quantification of low abundance proteins, the analysis will only be as good as the isolation. When analyzing a complex, one has to consider not only the abundance of the bait protein (i.e., the protein targeted for immunoaffinity purification), but also the stoichiometry within the isolated protein complex. Therefore, the ability to identify interactions present at low

levels increases if the isolation is effective and performed with a sufficient amount of starting material. The efficiency of isolation for low abundance proteins can be improved by increasing the multiplicity of infection, as well as by increasing the number of cells used to isolate protein complexes. Additionally, one can increase the number of cells in a stepwise manner to account for the differential expression level of viral proteins at different time points of infection. A good starting point will be to infect approximately $1x10^8$ cells, which for most viral proteins would provide sufficient amounts of affinity purified proteins for mass spectrometry analyses.

3.2.1 Preparing infected cells

1. Infect approximately $1x10^8$ cells using conditions defined by the specific virus and biological system being used. For most adherent cells, using 10x 15cm dishes of cells will provide sufficient material (*see* Note 1).
2. Harvest infected cells at the optimal time post infection by aspirating off the media and washing 2x with 5 mL of cold PBS (4°C) to remove culture serum.
3. For adherent cells, add 5 mL cold PBS and collect cells by scraping and transferring into a 250 mL cone tip centrifuge bottle on ice (*see* Note 2).
4. Wash the dish with 5 mL cold PBS to collect the residual cells and combine with the first harvest.
5. Pellet cells by centrifugation at 300xg for 10 min at 4°C and aspirate the PBS.
6. Resuspend the cell pellet in 10 mL cold PBS and transfer it to a 15 mL screw-capped tube.
7. Pellet cells by centrifugation at 300xg for 10 min at 4°C and aspirate off the PBS.
9. Determine the weight of the cell pellet by subtracting the weight of the tube from the sample. Using $1x10^8$ cells will result in approximately 1.0 g of sample material (*see* Note 3).
10. Add 100 µl Cryo Buffer to 1.0 g of sample and mix with the pipet tip. Add protease inhibitor cocktail (PIC) at a concentration of 1:100 (v/v) to the Cryo Buffer and mix by gentle inversion of the tube just before adding the buffer to the sample. The cell pellet should have the appearance of a thick paste (*see* Note 4).

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[1]The recent increase in the sensitivity of mass spectrometry instrumentation has significantly decreased the requirement for sample amount, allowing efficient isolations of viral proteins from as little as 1x 15 cm dish. However, it is still preferred to start with ~10x 15 cm dishes to allow for optimization of lysis conditions.

[2]It is also possible to collect the cells in multiple 50 ml conical tubes. If using 50 mL tubes, after the centrifugation in step 5, aspirate the PBS and combine the cell pellets into a common tube and then repeat step 5.

[3]Cell types such as HeLa and HEK293 cells tend to result in more than 1 g of sample material (~1.2 g), while cell types such as HFFs tend to result in less than 1 g (i.e., ~0.9 g).

[4]If working with smaller amounts of material, i.e. 1-3 plates/sample, it may be beneficial to add an increasing volume of the Cryo Buffer at ~200 µl Cryo Buffer per 1.0 g. In this case, step ***3.2.2*** will involve the direct slow pipetting of the resulting cell solution into the 50 mL conical tube containing liquid nitrogen. Use a 200 µL pipette to obtain an appropriate size of the frozen pellets.

3.2.2 Freezing cell pellet

1. Place a 50 mL screw-capped tube into a metal tube rack and place it in an empty ice bucket.
2. Add ~40 mL of liquid nitrogen into the 50 ml screw-capped tube and then also fill the ice bucket with liquid nitrogen to just above the rack height. Follow appropriate safety precautions when using liquid nitrogen.
3. Using a 18-gauge needle, puncture the bottom of the 15 ml sample tube and, with the plunger from a 10 mL syringe, slowly push the sample in Cryo Buffer out of the 15 mL tube and let the sample drop as pellets into the 50 mL tube containing liquid nitrogen (*see* Note 5).
4. Collect the remaining material using a wide bore pipette tip and drop the material into the liquid nitrogen filled 50 mL tube (*see* Note 6). Carefully, puncture the lid of the 50 mL tube several times with an 18 gauge needle and then cap the tube. About 4-5 holes are enough to vent the liquid nitrogen and gas (*see* Note 7).
5. Empty out liquid nitrogen from the capped 50 ml tube by reversing the tube and letting the liquid nitrogen drop in the ice bucket.
6. The remaining frozen pellets can be stored inside the 50 mL conical at −80°C until you are ready to proceed with the rest of the steps. Ensure that there is no liquid nitrogen remaining in the 50 mL conical before storing the sample in the −80°C freezer.

3.2.3 Cryogenic disruption of cell pellets—After the infected cells are frozen into small pellets, it is critical that the sample remains frozen at all times through the steps described below, all the way until the lysis step, (see ***3.4.1***. If the material partially thaws, it could cause decreased efficiency of extraction and increased non-physiological protein associations. If working with a high risk infectious virus, see section ***3.2.4***, or with very small amounts of material, see section ***3.2.5.***

1. Clean the grinding materials (spatula, Retsch Mixer mill jars, and grinding balls) stepwise with dH_2O, Windex, ultrapure dH_2O and 100% methanol, and then allow them to dry in the chemical hood. It is important that the jars are completely dry at the end of step 1, because any water remaining in the lids will freeze during steps 2 and 3 and impede the closing and opening of the jar.
2. Pre-chill the jars and balls in an ice bucket or Styrofoam container filled with liquid nitrogen. Once the liquid nitrogen no longer has a bubbling appearance, the jars and balls have reached the proper temperature. Carefully remove them from the liquid

[5]The goal during freezing the sample is to produce small ball-bearing size frozen pellets of your sample allowing for efficient cryogenic disruption.

[6]If there is still residual material remaining in the tube, an additional 100 μL of Cryo Buffer can be used to wash out remaining material to be added drop wise into the liquid nitrogen. As they freeze, the cell pellets will sink at the bottom of the liquid nitrogen filled 50 mL tube.

[7]If the mass of the cell pellet is low, i.e. <0.5 g, an alternative approach is to use a wide bore pipette tip or a cut 200 μL tip to directly withdraw the pellet and slowly add the material drop wise into the 50 mL tube containing the liquid nitrogen. If the sample freezes in the tube, thaw it out and continue the process.

nitrogen using forceps and deposit the frozen cell pellets inside the jars. For optimal grinding, the jars should be filled to no more than one third of the volume of the jar (e.g., max. of 3 g cell pellets per 10 mL jar, and 7 g cell pellets per 25 mL jar).

3. Place a chilled ball on top of the cell pellets, close the jar, and return the jar to the liquid nitrogen container for additional cooling.

4. Place the filled jars in the holders of the Retsch Mixer Mill and set the mixer for grinding at 30 Hz. If only one jar is needed, use an empty jar without the ball as a balance. Perform 20 cycles of 2.5 min each with cooling of the jars following each grinding cycle. To cool the jars between the cycles, remove the jars and chill in the liquid nitrogen container. The jars may loosen during the grinding process so check that the lids are tightly closed before cooling the jars in liquid nitrogen.

5. Once all grinding cycles are finalized, cool the jars in liquid nitrogen. Once cooled, remove the jars from the liquid nitrogen, open the jar and remove the cell powder with a chilled spatula, placing the frozen cell powder into a 50 mL conical tube on dry ice. Work in this step must be done quickly to avoid the thawing of cell powder. It is helpful to keep the liquid nitrogen on hand for chilling the spatula several times as the power is removed from the jars. Store the sample at −80°C.

3.2.4 Alternative cryogenic grinding-premixing lysis solution—The goal of this procedure is to minimize contact with the infectious cell powder.

1. Prepare frozen cell pellets by adding ~200 µl Cryo Buffer per 1 g cells, and freezing the cell pellets by slow pipetting of the cell solution into a 50 mL conical tube containing liquid nitrogen. In contrast to section ***3.2.2***, do not use needles and syringe plungers for freezing the cell pellets.

2. Prepare an appropriate Lysis Buffer for the immunoaffinity purification, optimizing its composition as described in section ***3.4.3***, allowing for 5 mL of buffer per 1 g of cells.

3. Freeze the Lysis Buffer by pipetting drop-wise into a 50 mL conical tube containing liquid nitrogen.

4. Store separately the frozen cell pellets and the frozen Lysis Buffer pellets until ready to perform the cryogenic grinding and immunoaffinity purification.

5. To perform the cryogenic disruption, combine the frozen cell pellets and the frozen Lysis Buffer pellets and carry out the grinding as described in section ***3.2.3***.

6. When the grinding is completed, place the jars in a bucket filled with ice, and allow the ground cell-lysis mixture to slowly thaw. Transfer the lysate to a 50 mL conical tube, add additional protease inhibitor cocktail (1/100 v/v) and continue directly with the immunoaffinity purification procedure section ***3.4.1.***

3.2.5 Alternative cryogenic grinding-smaller sample size—If using <0.5 g of sample as well as requiring the samples to be maintained in a sealed tube, it is possible to

grind samples within 2.0 mL tubes using adaptors for the Retsch Mixer Mill and 3 mm grinding balls.

1. Clean the grinding materials (spatula, tweezers, and grinding balls) stepwise with dH_2O, Windex, ultrapure dH_2O and 100% methanol, and then allow them dry in the chemical hood.
2. Prepare frozen cell pellets by adding ~200 µl Cryo Buffer per 1 g cells, and freezing the cell pellets by slow pipetting of the cell solution into a 50 mL conical tube containing liquid nitrogen. In contrast to section ***3.2.2***, do not use needles and syringe plungers for freezing the cell pellets.
3. Pre-chill the adaptor, 2.0 mL tubes and balls in an ice bucket or Styrofoam container filled with liquid nitrogen. Once the liquid nitrogen no longer has a bubbling appearance, the tubes and balls have reached the proper temperature.
4. Carefully remove tubes from the liquid nitrogen using forceps and empty residual liquid nitrogen from the tubes.
5. Using pre-chilled tweezers, deposit the frozen cell pellets inside the tubes and do not fill more than one third of the volume. Add one pre-chilled 3 mm grinding ball and cap tubes, returning them to liquid nitrogen.
6. Place tubes with sample into chilled adaptor and return to liquid nitrogen.
7. Place adaptor in the holders of the Retsch Mixer Mill and set the mixer for grinding at 30 Hz. If only one adaptor is needed, use an empty adaptor without tubes as a balance. Perform 6 cycles, (or until completely disrupted), of 2.5 min each with cooling of the adaptor in the liquid nitrogen following each grinding cycle. It is helpful to keep the liquid nitrogen on hand for chilling the adaptor.
8. Store the sample within the 2.0 ml tubes at −80°C. The optimized Lysis Buffer can be added directly to each 2.0 ml tube, see ***3.4.1***.

3.3 Antibody conjugation

This protocol has been optimized for use with Epoxy-coated magnetic beads of 2.8 µm diameter (Invitrogen). However, the same procedure can be utilized with other sizes of magnetic beads and other types of beads by adjusting the amount of antibodies to account for the difference in the bead surface of binding. This conjugation protocol can be utilized for conjugating antibodies against tags (e.g., GFP, FLAG), antibodies raised directly against the protein of interest, as well as IgG (for isolation of proteins tagged with Protein A or with a TAP tag containing a Protein A component) (Table 1). The antibodies utilized should be affinity purified, and the solutions in which the antibodies are stored are critical. As the binding to epoxy occurs through primary amines, ensure that the antibodies used are not stored in Tris Buffer. Also, check that the antibody solution does not contain other proteins, such as BSA, that would compete with the antibody for binding to the beads and would significantly affect the identity of co-isolated proteins. It is best to do steps 1-8 of the conjugation in the afternoon and continue steps 9-17 the following morning. Do not allow the beads to dry in between the wash steps. All steps can be performed at room temperature.

When considering the amount of beads needed, one must consider the amount of cellular material being used and the abundance of the protein of interest. A small scale pilot experiment using less than $1x10^7$ cells may require only 1-2 mg of beads, while a single isolation followed by comprehensive mass spectrometry analyses would require 5-10 mg of beads. If the protein of interest is not highly abundant, then using 10-20 mg of beads could be appropriate. A good starting point is 10 mg of beads to 1 g of ground sample.

1. Weigh out the appropriate amount of Dynabeads into a 2 mL round-bottom tube.
2. Wash the beads by adding 1 mL of Sodium Phosphate Buffer (pH 7.4) and mix using a vortex for 30 sec.
3. Place tube containing the beads in the Sodium Phosphate Buffer on a shaker for additional mixing for 15 min. A TOMY shaker or an alternative shaker with vigorous mixing (e.g., medium shaking setting on a vortex) is recommended to agitate the tube and keep the beads from settling on the bottom (*see* Note 8).
4. Place the tube on a magnetic rack to separate the beads from the solution. Once the solution becomes clear and the beads are collected on the magnet side, remove and discard the buffer using a pipette.
5. Remove the tube containing only the beads from the magnet and add 1 mL of fresh Sodium Phosphate Buffer. Mix by vortexing for 30 sec. Return the tube to the magnetic rack and remove the buffer as described above.
6. Remove the tube from the magnet and begin conjugating the antibody (*see* Note 9).
7. To conjugate 10 mg of beads, the total volume of the conjugation reaction will be 200 μL. The volume of 0.1 M Sodium Phosphate Buffer equals 200 μL – volume of antibody – 66.66 μL 3 M Ammonium Sulfate. Ammonium Sulfate solution is added last for a final concentration of 1 M (*see* Note 10).
8. Seal the tube with parafilm and rotate the slurry overnight at 30°C. Use an end-over-end rotation of the tube to help keep the beads suspended in the antibody solution throughout the conjugation period, and avoid the settling of the beads at the bottom of the tube.
9. The following morning, place the tube on the magnetic rack. Remove the supernatant using a pipette, and save it to test the efficiency of conjugation.
10. Wash the beads in 1 mL of Sodium Phosphate Buffer by gentle pipetting. Place the tube on the magnetic rack. To avoid losing beads inside the pipette tip, once the beads attach to the magnet wall, wash the tip using the buffer inside the tube, and allow again for the beads to settle on the magnet. Once the solution is clear, remove and discard the buffer.

[8]The round-bottom tube further prevents the beads from settling and allows maximum exposure of the bead surface to the solution.
[9]To calculate the necessary volume of antibody solution to use, for purified high affinity antibodies (e.g., in-house generated anti-GFP antibodies) use 3-5 μg Ab/mg beads. For commercially available antibodies that may not have as high affinity for binding, use 5-8 μg Ab/mg beads, and for conjugation of IgG use 8-10 μg IgG/mg beads.
[10]In certain situations, the total volume of ~20 μL/mg beads may not be feasible because of diluted concentrations of available antibodies. For these circumstances, a larger total volume of conjugation can be used, but it is recommended that this total volume is not increased beyond ~60 μL/mg beads, as the efficiency of conjugation can be affected.

11. Wash the beads with 1 mL 100 mM Glycine-HCl, pH 2.5 using the same procedure and cleaning of the tip introduced in the step above. However, it is critical that this is a FAST WASH (*see* Note 11).
12. Wash the beads with 1 mL of 10 mM Tris-HCl, pH 8.8 using the same procedure as the one described in step 10.
13. Wash the beads in 100 mM Triethylamine. This is a FAST WASH, so, remove the solution as soon as the beads are attached to the magnet.
14. Wash the beads 4 times with DPBS using 1 mL for each wash. Remove the buffer following each wash.
15. Add DPBS containing 0.5% Triton X-100 to the beads and place the tube on a shaker (e.g., a TOMY shaker) for 15 min.
16. Wash the beads twice with DPBS using 1 mL for each wash. Remove the buffer following each wash.
17. Suspend the beads in DPBS with 0.02% NaN_3 and store at 4°C until their use in ***3.4.1*** (*see* Note 12).

3.4 Immunoaffinity purification: Strategy, optimization, and assessment of efficiency

3.4.1 Immunoaffinity purification of protein complexes

1. Prepare two Lysis Buffers: i) IP Buffer for suspending the cell powder, and ii) Wash Buffer for washing the magnetic beads prior to and after the immunoaffinity purification. The composition of the IP Buffer is optimized as described in section ***3.4.3***. Prepare 5-8 mL of IP Buffer per gram of frozen cell powder (*see* Note 13).
2. Bring the frozen cell powder from the −80°C storage, and keep the cells on dry ice until ready to proceed with the experiment.
3. Place the tube containing the cell powder in a 4°C ice bucket, and wait 20-30 sec (*see* Note 14). Slowly add the IP Buffer to the frozen cell powder and periodically swirl the tube until the powder becomes solubilized (*see* Note 15).
4. Prepare a Polytron for the homogenization of the cell lysate. First, rinse the Polytron with ultrapure dH_2O, then fill a beaker with ultrapure dH_2O and allow the Polytron to run inside the beaker for 10 sec.

[11]Do not leave the beads in contact with this solution for an extended period of time. Remove the solution as soon as the beads are secured on the magnet.
[12]It is recommended to use the beads within 2 weeks of conjugation, as their isolation efficiency will decrease significantly if the beads are stored for extensive periods of time (e.g., ~40% decrease after one month storage).
[13]In most cases the Wash buffer is the same as the Lysis Buffer, without the inclusion of protease and phosphatase inhibitors, or DNase. Prepare 10 mL of wash buffer per sample, and keep both IP and Wash Buffers on ice (4°C).
[14]The 20-30 sec waiting step prior to the addition of the Lysis Buffer will ensure that the buffer does not freeze on contact with the sample.
[15]If using a lysis buffer containing DNase, first incubate the sample on a rotor at room temperature for 5-10 min. If no DNase is used, place the tube containing the cell lysate on a rotor at 4°C, and allow for gentle mixing for 5-10 min, until the cell powder is fully suspended in the buffer. This step is not necessary if the entire cell powder was already dissolved in the buffer in step 3, which is the case when working with small amounts of material.

5. To homogenize the cell lysate, run the Polytron for 2x 15 sec at a speed of 22.5k with cooling on ice between the two steps. For this homogenization step, the cell lysate can be present in either a 15 mL or 50 mL conical tube. As foam is formed during this step, ensure that the volume of the cell lysate does not occupy more than 1/3 of the tube to avoid over flow.

6. If processing more than one sample, perform a rinse and a wash step in ultrapure dH_2O (see step 4) to clean the Polytron and avoid cross-contamination of samples (*see* Note 16).

7. Place the homogenized cell lysate on a slow rotor at 4°C for 5 min.

8. To separate the insoluble fraction, subject the homogenized cell lysate to centrifugation at 8,000xg at 4°C for 10 minutes.

9. While waiting for the completion of the centrifugation step, place the tube containing the conjugated magnetic beads on a magnetic rack to remove and discard the storage solution. Wash the beads 3 times with 1 mL cold Wash Buffer by gentle pipetting. To avoid drying of the beads, suspend the washed beads in 100-200 μL of Wash buffer and store them in the 4°C ice bucket.

10. Once the centrifugation (step 8) is completed, retain the pellet and 40 μL of the supernatant for later analysis (see section ***3.4.2***).

11. Move the clarified cell supernatant to a new conical tube. If using a centrifuge with swinging buckets, pour the cell supernatant into the new conical tube. If using a centrifuge with a fixed-angle container, then move the clarified cell supernatant to the new conical tube by pipetting. It is recommended to use a 50 mL conical tube if the cell supernatant is >10 mL, a 15 mL conical tube for cell lysates of 4-10 mL, and a 6 mL tube if the supernatant is <4 mL.

12. Carefully analyze the appearance of the cell supernatant, and ensure that there are no floating or mixed insoluble particles in the supernatant. If such particles are observed, remove them by carefully pipetting before proceeding. Particles may clog the beads and interfere with the affinity purification.

13. Add the necessary amounts of washed beads to the cell supernatant by pipetting (see section ***3.3).***

14. Place the tube containing the cell lysate and the beads on a rotor at 4°C and allow for 1 h of slow mixing (*see* Note 17).

15. Once the 1 h incubation is completed, separate the beads from the cell lysate by placing the tube into contact with a magnet (large bar magnet); secure the tube on the magnet using a rubber band. Wait 2-5 min (as necessary) for the beads to be fully captured on the magnet. Remove the flow through supernatant by pipetting, and save it into a new conical tube for later analysis (see section ***3.4.2***).

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[16] When finished with the last sample, rinse and wash the Polytron in ultrapure dH_2O, followed by a rinse with methanol. Use a tissue to tap off the excess methanol and let the Polytron to dry.

[17] Avoid using more than 1 hour of mixing, as the increase in incubation time will lead to an increase in accumulation of non-specific proteins and loss of weak binding partners (1).

16. Add 1 mL Wash Buffer to the beads in the large conical tube and transfer them to a 1.5 mL tube. Check that no beads are left in the large conical tube. If beads are still remaining in the conical tube, use some additional Wash buffer to transfer them to a 2.0 mL tube.

17. Place the tube in the magnetic rack and remove the Wash Buffer by pipetting.

18. Wash the beads 3 times with 1 mL Wash Buffer (*see* Note 18). After the third wash, move the bead slurry to a new eppendorf tube.

19. Once the beads are transferred to the new tube, wash the beads 2 more times with 1 mL Wash Buffer, and remove the Wash solution by pipetting.

20. Add 1 mL of PBS and transfer the beads to a new 1.5 mL round bottom tube. Wash once more with 1 mL PBS to remove any residual detergent and improve the efficiency of elution.

21. Add 750 μL base Elution Buffer to the beads and place the tube on a shaker with vigorous shaking that precludes the beads from settling for 20 mins at room temperature (*see* Note 19).

22. Place the tube on the magnetic rack and, once the beads are separated, transfer the eluate to a microcentrifuge tube (e.g., Axygen).

23. Flash freeze the eluate by dipping the tube in liquid nitrogen.

24. Place the tube in a vacuum centrifuge and evaporate the liquid overnight.

25. To test for the efficiency of elution, perform a second elution by adding 40 μL of a solution containing 1x LDS and 50 mM DTT to the beads remaining from step 22. Incubate at 70°C for 10 min, then place the tube on a shaker at room temperature for another 10 min. Remove this second elution and reserve 10% (i.e., 4 μL) for future analysis. Freeze the remainder of the second elution and store at −20°C. Store the remaining beads at 4°C to test the efficiency of the elution (see section ***3.4.2***).

26. The next day, as step 24 is completed, the primary eluate should be completely dry and can be prepared for analysis by mass spectrometry. One of the following steps should be performed depending on which of these bottom-up strategies is selected for preparing the samples for mass spectrometry analysis.

    27.1 For in-gel digestion, resolve the co-isolated proteins by SDS-PAGE. Suspend the dried primary eluate in 40 μL of 1x NuPAGE Sample Buffer containing 1x Reducing Agent and heat for 10 min at 70°C. Set

[18]For each wash, use gentle pipetting with the Wash Buffer on top of the beads until they are fully suspended, and place the beads back on the magnet support.

[19]Alternative Elution: The base Elution Buffer (see step 21) is preferred because it leads to less background protein and IgG contamination. However, if low recovery of bait protein is observed, this alternative procedure can be used in place of the base elution. i) Add 40 μl of 1x LDS Elution Buffer to beads and incubate for 10 min at 70°C, then for 10 min at room temperature with shaking. ii) Remove the primary eluate and repeat the elution with 1×LDS Elution Buffer to obtain a second elution. iii) Add 2.0 μL 1 M DTT to each eluate and heat to 70°C for 10 min. Set aside 10% of both eluates for future analysis. iv) If performing in-gel digestion, add 4 μL of 1 M Iodoacetamide to remaining 90% of eluates and incubate at RT for 30 min in the dark. v) Freeze remaining 90% of eluates in liquid nitrogen and store at −20°C or proceed immediately to proteomic analysis.

aside 10% (4 μl) of the eluate in a clean microcentrifuge tube for future analysis (see section ***3.4.2***). Add 4 μL of 1 M Iodoacetamide to the remaining 90% of primary eluate, and then incubate 30 min at room temperature in the dark. If not continuing to sample preparation for mass spectrometry, freeze in liquid nitrogen and store at −20°C.

27.2 For in-solution digestion, suspend the dried primary eluate in 40 μL of 1x LDS Elution Buffer containing 50 mM DTT and heat at 70°C for 10 min. Set aside 10% (4 μl) of the eluate in a clean microcentrifuge tube for future analysis (see section ***3.4.2***). If not continuing to sample preparation for mass spectrometry, freeze remaining 90% of primary eluate in liquid nitrogen and store at −20°C.

3.4.2 Assessing the efficiency of immunoaffinity purification

1. To assess the efficiency of isolation of the targeted protein, it is necessary to compare, using Western blot, equivalent amounts (5–10%) of the cell pellet (remaining after the centrifugation), the flow through (remaining after the immunoaffinity purification), the primary elution (obtained after the first elution), and the secondary elution (obtained after the second elution). A fraction of the input supernatant can also be compared to provide a point of reference for the molecular mass of the targeted protein.
2. For the input supernatant, dilute 40 μL of the saved supernatant (see section ***3.4.1***, step 10) to a final volume of 60 μL containing 1x NuPAGE LDS Sample Buffer and 1x Reducing Agent.
3. To prepare the cell pellet (see section ***3.4.1***, step 10): i) homogenize the pellet in 1 mL of 2% SDS, ii) heat the mixture at 70°C for 10 min, and then iii) subject it to centrifugation for 5 min max speed at room temperature (if the sample is viscous, the solubilization can be aided by sonication), iv) from the solubilized pellet, remove an aliquot that corresponds to the equivalent amount of the utilized input, and v) dilute the aliquot in 1x NuPAGE LDS Sample Buffer and 1x Reducing Agent.
4. To prepare the flow through (see section ***3.4.1***, step 15), i) add 4 volumes of ice cold acetone to a volume equivalent of 10% of the total flow through, ii) mix briefly by vortexing, and iii) incubate on ice for at least 1 hour, iv) separate the precipitated fraction by centrifugation at 3000×g for 10 min at 4°C, v) discard the supernatant and wash the remaining precipitated pellet with 4 volumes of 80% acetone/20% dH_2O, and vi) allow the pellet to air dry and then resuspend it in 40 μL of 1x NuPAGE LDS Sample Buffer and 1x Reducing Agent.
5. For the primary eluate (see section ***3.4.1***, step 21), dilute the equivalent of 10% of the elution (i.e. 4 μL) into a final volume of 40 μL 1x NuPAGE Sample Buffer and 1x Reducing Agent. If performing the “alternative elution” (*see* Note 19), the primary eluate can be prepared the same day as opposed to the base elution that must be prepared on the next day.

6. Prepare the secondary eluate (see section ***3.4.1***, step 25) similarly to the first eluate, by diluting the equivalent of 10% (i.e. 4 μL) into a final volume of 40 μL 1x NuPAGE Sample Buffer and 1x Reducing Agent.
7. Heat all samples (prepared in steps 1-5 above) for 10 min at 70°C, and either directly proceed with the comparison by Western blotting or store them at −20°C.
8. The comparison of these fractions using Western blot analysis should show the presence of the targeted protein significantly enriched in the primary eluate.

3.4.3 Optimization of lysis buffer conditions

A. Optimization prior to the immunoaffinity purification: Multiple factors can contribute to the properties of the targeted protein, such as subcellular localization and the presence of posttranslational modifications. It is recommended to perform optimization experiments utilizing small scale sample amounts, such as ~$1x10^7$ cells or one 15 cm cell culture plate, prior to proceeding with large scale proteomics experiments. Different combinations and concentrations of salts and detergents (such as NaCl and Triton X-100) determine the stringency of the buffer, which must be stringent enough to allow for an efficient solubilization and isolation of the protein of interest, and mild enough to prevent the loss of interactions. It is recommended that at least three lysis buffer conditions with varying degrees of stringency are tested. Table 2 and Table 3 list examples of Lysis Buffer conditions that have been successfully used to isolate proteins from various viral infections and cell types. The following steps are recommended for optimizing the Lysis Buffer.

1. Prepare cryogenically ground cell powder as described in section ***3.2.3***. Split the frozen cell powder equally into pre-chilled tubes.
2. Add Lysis Buffer to each sample using 5 mL of buffer per 1 g of cells. The volume of Lysis Buffer per sample is usually ~0.5-1 mL if using adherent cells grown in a 15 cm culture dish.
3. Instead of the Polytron used to homogenize the larger scale purification (see section ***3.4.1***, steps 4-6), perform the homogenization of the cell powder in the Lysis Buffers by vortexing 2x 30 sec with cooling on ice in between the two steps.
4. Separate the insoluble fraction by centrifugation at 8,000xg for 10 min at 4°C. Remove and save the supernatant as the soluble fraction.
5. To extract the pellet, add 50 mM Tris-HCl, pH 7.4, containing 2% SDS. Sonicate, then boil at 95°C for 5 min. Centrifuge at 20,000xg for 10 min and save the supernatant as representing the “pellet” fraction.
6. Compare the relative amounts of bait protein in the soluble and pellet fractions by loading equivalent amounts (5–10%) for analysis by Western blot, probing with antibodies against the tag or the endogenous protein.
7. Select the lysis condition that yields the highest amount of bait protein in the soluble fraction (see Note 20).

B. Optimization following the immunoaffinity purification: The comparison performed in the assessment of the efficiency of immunoaffinity purification (see section ***3.4.2***) can inform on how to proceed with further optimizing the Lysis Buffer conditions.

1. The presence of the targeted protein in the cell pellet may suggest the need for a more stringent lysis buffer condition.
2. The presence of the protein in the flow through may indicate that i) the amount of beads utilized is not sufficient for capturing all of the target protein, or ii) the tag or the epitope of the endogenous protein is not folded in an accessible manner or hindered by other interactions in this lysis condition.
3. The presence of the protein in the secondary elution suggests that the efficiency of primary elution is not sufficient, and that either a larger elution volume or the alternative elution approach should be utilized.

The isolation conditions can be further optimized once the co-isolated proteins are analyzed by mass spectrometry. If a balance between an efficient isolation and reduced presence of non-specific associations is difficult to achieve, the composition of the Wash Buffer (i.e., the buffer used to wash the beads following the immunoaffinity purification) can also be optimized. An experiment worth trying in this situation is to use a less stringent IP Buffer and a more stringent Wash Buffer (see section ***3.4.1***, step 1).

Once the bait (targeted protein) and its interacting proteins are eluted from the beads, the resulting mixture of co-isolated proteins can be prepared for mass spectrometry analysis using several approaches. For example, the co-isolated protein mixtures can be digested either using an in-gel or an in-solution approach, as described in (14, 20, 21). The in-gel approach involves the separation of proteins by SDS-PAGE prior to the enzymatic digestion, while the in-solution digestion involves the direct digestion of proteins while maintaining them in solution, and is usually followed by a chromatographic separation prior to mass spectrometry analysis. Since the in-gel digestion strategy already resolves the sample by 1-D or 2-D SDS-PAGE, this step may be directly followed by analysis by mass spectrometry. However, this will depend on the complexity of the sample (i.e., number of co-isolated proteins and posttranslational modifications). Therefore, it is common that either the in-gel or in-solution prepared samples are analyzed using a chromatographic separation (nLC, nano liquid chromatography) coupled online with the mass spectrometer.

3.5 Selecting controls

For every immunoaffinity purification experiment, it is important to select and perform the appropriate controls that will allow interpreting the results and distinguishing specific from non-specific associations. Among the possible sources for non-specific associations that have to be considered, the most prominent ones are non-specific associations to i) the tag and antibody utilized, ii) the resin selected for immunoaffinity purification (e.g., magnetic beads, agarose), or iii) the isolated proteins (i.e., the protein complex of interest).

[20]If the yields are equivalent across several conditions, select the condition with the lowest stringency. This will allow for an efficient extraction of the bait protein, while helping to preserve interacting partners.

3.5.1 Controlling for non-specific association to the tag, antibody, and resin

1. If a tag is being used to isolate the protein of interest, control samples are needed to ensure that the tag or the utilized antibodies are not the source of the identified protein interactors. It is recommended that the same system used to generate the tagged viral protein be used to generate a virus that expresses the free tag. For example, a control virus triggering the pan-cellular expression of GFP should be used in parallel experiments to immunoaffinity purifications of GFP-tagged viral proteins (1, 2). These control viral infections should be analyzed at the same time points of infection as those used for the isolation of the viral protein of interest.
2. To rule out the possibility that proteins are interacting with the antibody or the beads, empty beads or antibody (or purified IgG)-conjugated beads can be used for parallel experiments.
3. When compared side by side, proteins identified by mass spectrometry in these controls but not in the experimental samples may correspond to non-specific associations.
4. In some cases, an interacting protein may be identified in both the experimental sample and the control. It is possible that these interactions are still specific if they show a significant enrichment relative to the control. To help determine the specificity of interaction, the nLS (nano-liquid chromatography)-MS/MS data obtained from the mass spectrometry analysis of the co-isolated proteins can be used in conjunction with a computation program called SAINT (Significance Analysis of INTeractome) (22) to assign confidence scores to the protein interaction data.

3.5.2 Controlling for non-specific associations to the isolated protein complex of interest

1. The non-specific associations that are most difficult to control are those that occur with the isolated protein complex of interest. Once a viral protein is isolated, it's true virus and host interacting partners can act as binding sites for non-specific associations with other proteins in the cell lysate. These types of interactions frequently occur with highly abundant, "sticky" proteins. For example, the solubilization of a protein complex may trigger some unfolding that can result in the association with heat shock proteins. Strategies involving metabolic labeling with stable isotopes can be utilized to test for the presence of such non-specific interactions.
2. When using a tagged protein, the I-DIRT (isotopic differentiation of interactions as random or targeted) approach can be utilized (14, 23). Briefly, the cells infected with the tagged virus are grown in regular (light) media, and the cells infected with the wild type virus are grown with media containing heavy amino acid isotopes. The cells are harvested and frozen as described above, and the frozen cell pellets are mixed prior to the cryogenic cell lysis. The immunoaffinity purification from the mixed cell population is performed as described above, and the co-isolated proteins are analyzed by mass spectrometry. Upon analysis by mass spectrometry,

the presence of single peaks representing peptides containing only the light amino acid isotopes would represent specific interactions. In contrast, the presence of doublet peaks representing peptides containing both the heavy and light isotopes will signify a non-specific association or a fast-exchanging association.

3. A similar approach can be used when isolating endogenous proteins. shRNA-mediated knockdown can be performed on the protein of interest prior to the differential metabolic labeling.

3.5.3 Controlling for spatial preference of non-specific interactions

1. In many circumstances, the protein of interest is localized to a distinct sub-cellular compartment, such as the nucleus or mitochondria. Upon lysis, the protein of interest and its interacting partners will be brought into contact with other subcellular compartments (e.g., the cytoplasmic fraction), exposing the isolated protein to interactions previously prevented by spatial restriction. These associations can be assessed using the two approaches shown above (see sections ***3.5.1*** and ***3.5.2***). However, if these associations are numerous, these proteins can substantially increase the complexity of the sample and hinder the identification of real interactions by mass spectrometry. To prevent these non-physiological interactions, sub-cellular fractions can be performed prior to the cryogenic lysis and immunoaffinity purification.

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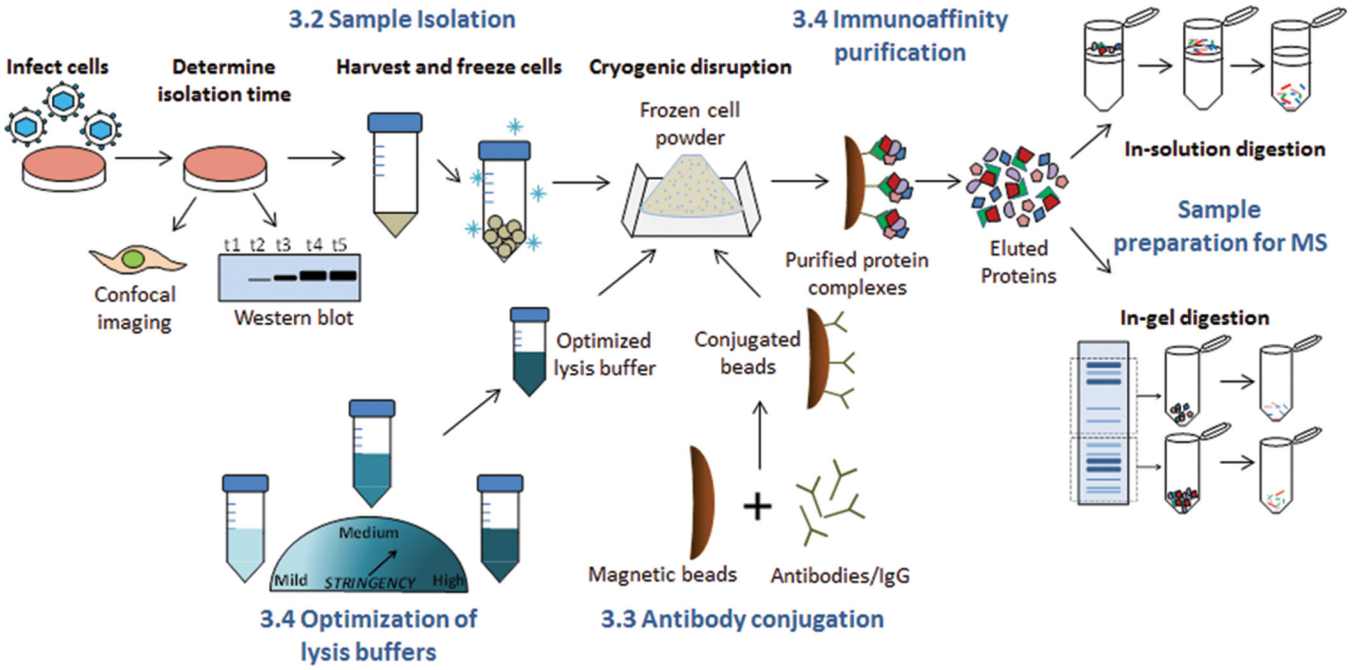

Figure 1.

Table 1

Examples of epitope and epitope-specific antibodies that we have utilized for studying virus-host protein interactions.

[a]Epitope Tag		Mass	Antibody	Ref.
TAP		20 kDa	IgG	(6, 11)
GFP		27 kDa	anti-GFP	(1, 2, 5–7, 12–14, 24, 25)
FLAG		1 kDa/FLAG	anti-FLAG	(8, 9)

[a]Epitopes can be introduced at either the amino or carboxyl terminus of the bait protein, or on an internal flexible loop of the protein, provided that it does not interfere with protein function.

Table 2

Examples of lysis buffers that in our hands have yielded successful isolations of different viral proteins with a range of properties.

Bait	Description & Localization	Tag	Virus	Cell type	[a]Optimized Lysis Buffer	Ref
nsP3	Non-structural protein 3 (cytoplasm)	GFP	Sindbis Virus	Rat2 cells	1% Triton, 0.5% deoxycholate, 500 mM NaCl, 25 units/mL DNase	(2)
pUL38	Early phase non-structural protein(dynamic localization in nucleus and cytoplasm)	ProtA	HCMV	HFF	1% Triton X-100, 250 mM NaCl, 4 μg/mL DNase, 20 mg/ml phenylmethylsulfony fluoride, 0.4 mg/mL pepstatin A	(11)
pUL99, pUL32	Both involved in assembly (cytoplasm)	GFP	HCMV	HFF	1 μM $ZnCl_2$, 1 μM $CaCl_2$, 1% Triton, 250 mM NaCl	(5)
pUL83	Tegument protein; (nuclear and cytoplasmic)	GFP ProtA	HCMV	HFF	1% Triton X-100, 250 mM NaCl, 4 μg/mL DNase, 20 mg/ml phenylmethylsulfony fluoride , 0.4 mg/mL pepstatin A	(6)
pUL27	Early phase protein (nuclear)	3xFLAG	HCMV	HFF	1% Triton X-100, 250 mM NaCl, 4 μg/mL DNase	(9)
nsP4	Non-structural RNA-dependent RNA polymerase (cytoplasmic)	3xFLAG	Sindbis Virus	Rat2 cells	1% Triton X-100, 0.5% deoxycholate, 500 mM NaCl, 25 U DNase and RNase/mL	(8)
NS1	Non-structural glycoprotein (cell surface and secreted)	No tag	West Nile Virus	BHK2 1-15	1 μM $ZnCl_2$, 1 μM $CaCl_2$, 1% Triton, 0.5% sodium deoxycholate, 0.3% sodium N-lauroylsarcosine, 0.1 M NaCl, and phosphatase inhibitor cocktail	(10)

[a]Alllysis buffers contained 20 mM K-HEPES pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% Tween 20, protease inhibitor mixture in addition to optimized lysis buffer components listed.

Table 3

Examples of lysis buffer conditions that we previously used to isolate mammalian host proteins from both cultured cells and tissue.

Bait	Description & Localization	Tag	Cell type	Optimized Lysis Buffer	Ref
Nup37 Nup43	Member of nuclear pore complex and subunit of Nup107-160 subcomplex (nuclear membrane)	GFP	HeLa	0.5% Triton, 200 mM NaCl, 20 mg/mL PMSF, 0.4 mg/mL pepstatin A	(1)
GluRδ2	postsynaptic densities; (cerebellar excitatory synapses)	GFP	Mouse tissue	[b] 10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 132 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 0.5% Triton X-100, 1/100 (v/v/) protease inhibitor cocktail	(25)
HDAC1	Histone deacetylase 1 (nucleus)	GFP	HFF	1μM $ZnCl_2$, 1 μM $CaCl_2$, 0.5% Triton X-100, 250 mM NaCl	(12)
H3	Histone 3 isoforms (nucleus)	YFP	mouse ES cells	0.5% Triton, 300 mM NaCl	(24)
HDAC5	Histone deacetylase 5 (nucleus and cytoplasm)	GFP	HEK293	1 μM $ZnCl_2$, 1 μM $CaCl_2$, 0.5% Triton X-100, 250 mM NaCl, 4 μg/mL DNase, and phosphatase inhibitor cocktails	(13)
SIRT7	Sirtuin 7 (nucleoli)	GFP	HEK293	1 μm $ZnCl_2$, 1 μm $CaCl_2$, 0.5% Triton X-100, 250 mm NaCl, 4 μg/ml DNase, and phosphatase inhibitor cocktails	(14)
IFI16	Interferon inducible protein 16 (nucleus and cytoplasm)	No tag	CEM T	1 μM $ZnCl_2$, 1 μM $CaCl_2$, 0.6% Triton X-100, 200mM NaCl, 10 μg/mL DNase I, phosphatase inhibitor cocktail	(16)

[a] Alllysis buffers contained 20 mM K-HEPES, pH 7.4, 110 mM KOAc, 2 mM MgCl2, 0.1% Tween 20, 1/100 protease inhibitor mixture in addition to optimized lysis buffer components listed.

[b] This buffer is the complete buffer composition without core components listed above.