History of IP

Using antibodies to precipitate target proteins has its origins in the 1960s, when Barrett et al. referred to **immunoprecipitation** as a tool for measuring the concentration of gamma globulins in human serum, offering more precision than other techniques available at the time such as electrophoresis.[1,2] It was further developed as an alternative to traditional column affinity chromatography, in which samples are passed across a column of resin, typically agarose, onto which an antibody has been immobilized in order to capture the target antigen. **IP uses the same principle but in a scaled-down, batch-wise format**, whereby individual tubes contain the agarose resin, making it a convenient way to purify protein from multiple small samples. The discovery and application of Protein A[3] and Protein G[4] as a way of consistently immobilizing any capture antibody to the solid support improved the efficiency and versatility of IPs. Further advances in beads, such as magnetic beads, have enhanced reproducibility and capacity for automation, while integration with other techniques such as WB and MS continue to expand its utility.

IP Formats

IPs are not limited to a single protein target, with expansions to the original IP format enhancing its versatility and enabling the study of **protein-protein (Co-IP)**, **protein-DNA (ChIP)** and **protein-RNA (RIP)** interactions.

Individual **Protein Immunoprecipitation (IP)**

IP is conventionally used to isolate **a single protein of interest from a heterogeneous mixture**. There are two standard approaches when performing an IP (Figure 1). In the pre-immobilized antibody method, also known as the direct method, an antibody specific for the target antigen is first immobilized onto a bead support. Once immobilized, the bead-antibody complex is added to the sample in order to capture the antigen. In the free antibody, or indirect, method, the antibody is added to the sample first, allowing antigen-antibody complexes to form. Then the beads are added to capture the immune complex.

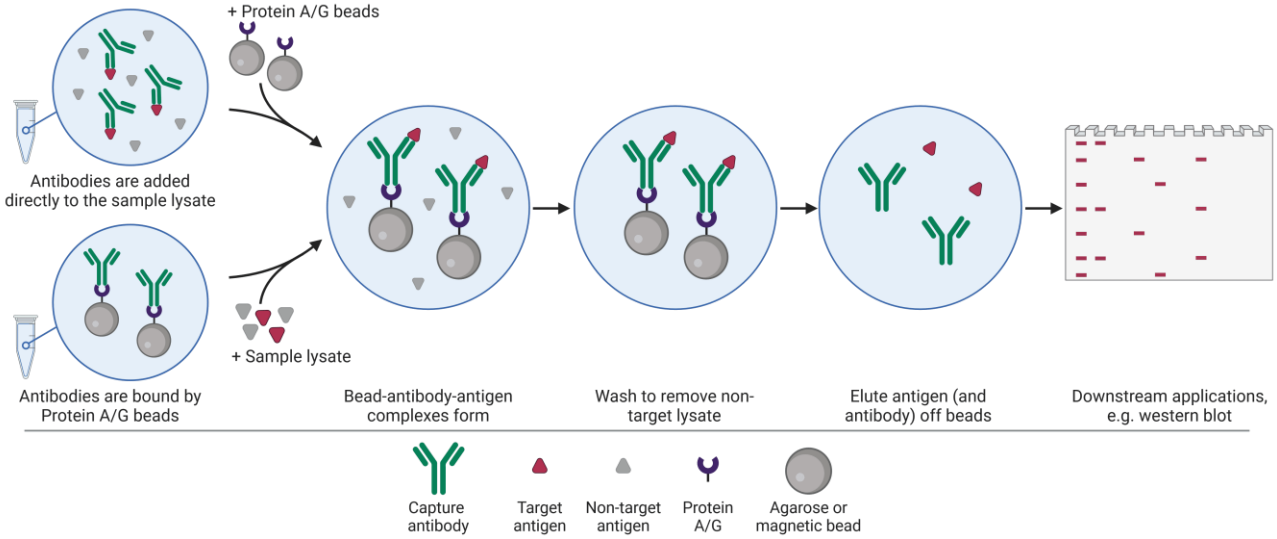

Figure 1: Schematic of individual protein immunoprecipitation (IP) workflow. IP can be performed in one of two ways, in which the antibody is first added to the lysate to capture the target before the beads are introduced, or the antibody is first immobilized to the beads.

Regardless of which approach is taken, the sample is then centrifuged in order to collect the beads, which are bound to the antibody and antigen, in a pellet at the bottom of the tube, thus precipitating out the target antigen. This pellet is washed and re-pelleted several times to remove as much non-specifically bound material as possible. The target antigen is then eluted, dissociating the antigen (and often the capture antibody) from the bead, ready to be used for downstream application such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and WB.

Protein Complex Immunoprecipitation (Co-IP)

Co-immunoprecipitation (Co-IP) follows a similar workflow to the individual IP (Figure 2), but instead of purifying a single known protein (the “bait”), **the aim is to co-precipitate unknown proteins (the “prey”)** that are bound to the target antigen, on the basis that binding partners are likely related to, and may be required for, the function of the antigen. These prey proteins may include activators or inhibitors, kinases and other mediators of post-translational modifications (PTMs), ligands, and so on. Thus, protein complexes are precipitated and can be studied in similar ways to IP products, facilitating protein-protein interaction discovery.

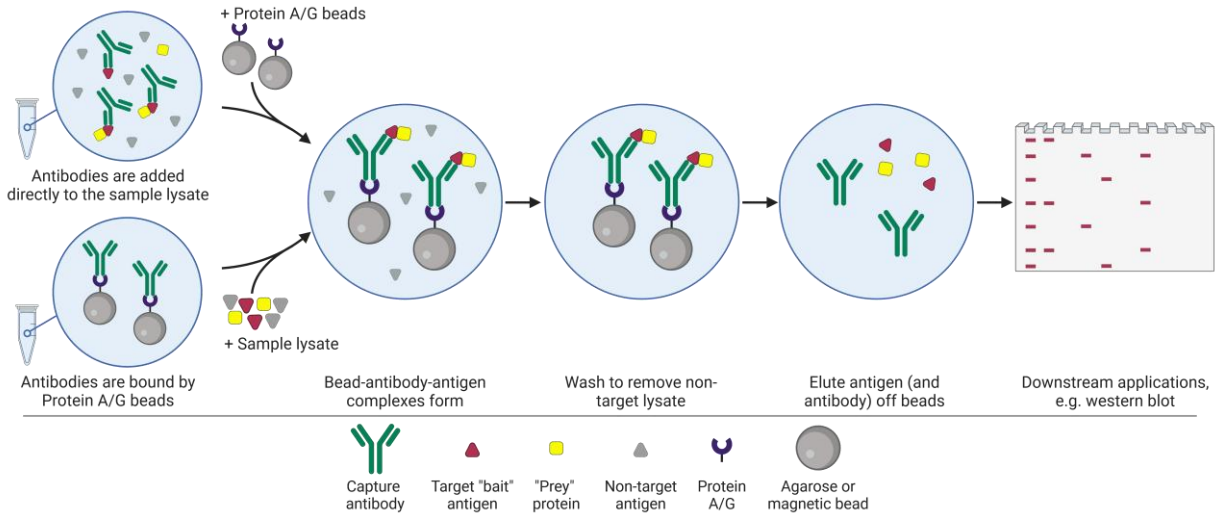

Figure 2: Schematic of co-immunoprecipitation (co-IP) workflow.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a format of immunoprecipitation used to investigate protein interactions with DNA (Figure 3), typically with the aim of identifying regions of the genome associated with a specific DNA-binding protein, such as transcription factors and histones.

The workflow of ChIP differs slightly from that of IP and co-IP because it begins by cross-linking proteins to DNA, usually by formaldehyde treatment, preserving the state of the cell (for example, after a certain treatment). Cells are then lysed and sonicated to shear DNA, resulting in small pieces of DNA attached to DNA-binding proteins. The proteins can then be captured as in regular IP, using specific antibodies immobilized on beads. Following dissociation of the DNA from the protein, the DNA is analyzed by PCR, microarray or sequencing.

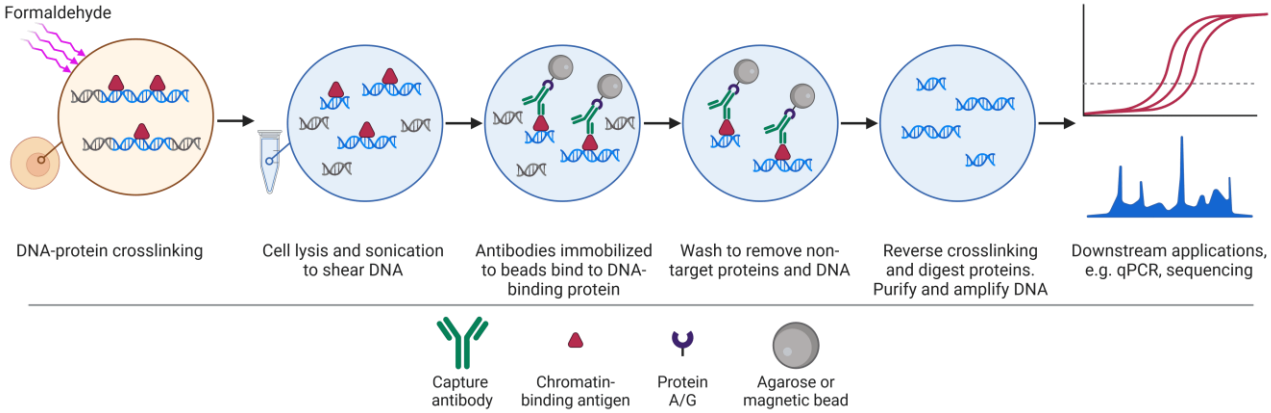

Figure 3: Schematic of chromatin immunoprecipitation (ChIP) workflow.

ChIP offers advantages over other techniques for investigating protein-DNA interactions by virtue of reflecting the native, intracellular context of protein-bound DNA, as opposed to *in vitro* assays such as the electrophoretic mobility shift assay (EMSA), and can therefore account for the effects of epigenetics as well as known and unknown binding partners.

RNA Immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) is similar to ChIP, but uses an antibody targeting **RNA-binding proteins (RBP)** of interest, which modulate the function of mRNA or noncoding RNA in ribonucleoprotein complexes (RNP).

There are two main types of RIP, referred to as native RIP and cross-linking and immunoprecipitation (CLIP).[5] Native RIP allows the identification and abundance of RNAs bound to the RBP of interest, but it misses more transient RBP-RNA interactions and can suffer from low specificity and low reproducibility due to non-stringent conditions.[6] CLIP, derived from RIP,[7] increases specificity, allows recovery of weaker protein-RNA interactions, and allows higher resolution mapping (e.g. single nucleotide resolution)[8,9] of the precise RNA binding sequence due to stringent purification conditions enabled by the UV cross-linking. UV cross-linking is advantageous when targeting RBPs because UV crosslinks are irreversible, do not form between proteins,[10] **and only form between proteins and RNAs that are in very close proximity**. CLIP has become the standard technique to assess RNA-protein interactions at genome-wide scale, though further advancements to address its limitations continue to be developed.[11,12]

The workflow for native RIP begins with cell lysis, while CLIP begins with UV cross-linking prior to lysis (Figure 4). Partial RNase digestion in both approaches results in short RNA fragments bound to RBPs, which are immunoprecipitated as described for the other formats, using specific antibodies immobilized on beads to capture the RBP-RNA complexes. 3' adaptor ligation is then performed to enable amplification downstream. Further purification via denaturing gel electrophoresis and transfer to a nitrocellulose membrane may be employed here, particularly in CLIP, allowing excision of the protein-RNA complexes while removing lone protein and nonspecific RNAs.[9,12,13] The sample is then subjected to proteases that will degrade the RBP and release the RNA for analysis by RT-PCR, hybridization, or sequencing.

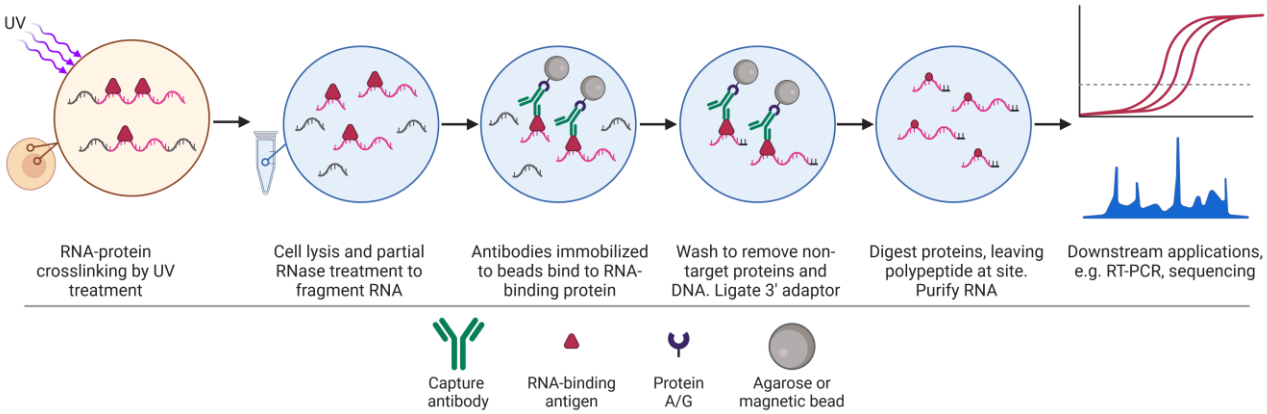

Figure 4: Schematic of crosslinking and RNA immunoprecipitation (CLIP) workflow.

Table 1:Key applications and differences between IP formats.

	IP	Co-IP	ChIP	RIP/CLIP
Target	Known protein	Protein complexes	DNA-binding proteins (histones, transcription factors)	RNA-binding proteins
Purpose	Purifying or enriching a single protein	Protein-protein interactions	Protein-DNA interactions	Protein-RNA interactions
Applications	Small scale enrichment or purification of proteins for downstream biochemical, biophysical or structural analyses Investigate PTMs Enrichment for WB analysis Epitope mapping Removal of a protein from a lysate	Discover new protein-protein interactions Prove interaction between bait and hypothesized prey Map protein interaction networks Understand dynamic protein interactions in different conditions	Understand how DNA-protein interactions change with time, treatment or cell state Identify DNA regions of high or low expression based on histone modifications Inferring the function of an unknown histone modification Identify genetic elements such as enhancers via histone modifications	Understand how RNA-protein interactions change with time, treatment or cell state Understand subcellular localization of RNA-RBP interactions Study how specific RBPs mediate RNA modifications

IP with Tagged Proteins

IPs rely on the highly specific binding of an antibody to an antigen. In the absence of an antibody that exhibits strong specificity towards a protein of interest, one solution is to instead 'tag' the protein of interest with a peptide sequence or fluorescent protein for which a high affinity antibody is available. Tagging, which involves placing the DNA sequence for the tag at either the C- or N-terminal of the target protein sequence, can be achieved via genetic modification in model organisms, or expression of a recombinant protein from a plasmid or viral vector. The disadvantage of protein tagging is that the tags themselves may affect molecular interactions. Nevertheless, it has become a standard method for protein purification.

Common peptide sequences are listed below:

FLAG: DYKDDDDK
c-Myc: EQKLISEEDL
Hemagglutinin (HA): YPYDVPDYA
V5: GKPIPNPLLGLDST

IP Applications

IP is an ideal technique for the small-scale enrichment or purification of proteins, as an alternative, batch-wise method to column affinity chromatography. Once the target protein has been eluted, it can be assessed by any biochemical, biophysical or structural technique, but a common next step is to analyze it using SDS-PAGE. After electrophoresis, the gel can be stained with Coomassie blue or silver stain to visualize the protein and confirm its purity and molecular weight. **More commonly**, the protein will be transferred to a nitrocellulose or PVDF membrane **and visualized by WB**. Running a WB on an immunoprecipitated protein **offers advantages over performing the WB against the original cell lysate**. Primarily, the resultant enrichment of the antigen makes it possible to detect low abundance proteins that otherwise may not have been detectable by WB.

This is particularly valuable when investigating specific PTMs on a target protein, which will be lower abundance than all forms of the protein. Thus, many studies of PTMs begin with IP, though it is important to remember that PTM modifications may block the binding site of the IP antibody. In this instance, a PTM-specific antibody can be used, followed by WB probing for the protein of interest. This offers significant flexibility, with kits often available containing PTM-specific antibodies pre-conjugated to beads, and can bypass issues arising from lacking a target protein-specific antibody validated for IP conditions.

In other cases**, MS can be used**. The efficiency of PTM detection can be low without prior enrichment (e.g. by IP) for mass spectrometric analysis of PTMs;[14] following IP, eluted proteins can be subjected to MS immediately, or can be extracted from an excised portion of a stained SDS-PAGE gel or WB membrane. MS does not rely on antibodies and can be less biased than WB, though it is far more expensive and requires specialized equipment.

Sample Types and Preparation

IP begins with separating soluble proteins from a lysate, typically from cells or tissue. Tissue usually needs to be homogenized in order to make cells fully accessible to the lysis buffer, while lysis buffer can be directly added to cell culture after washing. Brief sonication of samples can sometimes help to disrupt the nuclear membrane to release nuclear proteins, but often agitation of cells or tissue homogenate in lysis buffer for 30 minutes on ice is sufficient to release soluble proteins. Insoluble material can then be pelleted, while the supernatant will be taken forward for IP.

The amount of total protein needed for successful IP will depend on the abundance of the protein and the affinity of the antibody. For a cell culture lysate, approximately 300 µg of total protein is a useful starting point. This can be increased up to 2 mg for low abundance proteins, but more starting material may increase background. If the target protein is only present in one region of the cell, such as the nucleus, a more refined option is to perform subcellular fractionation first in order to increase the abundance of the target as a proportion of the total input pool.

Something that is essential to do in any IP protocol is to set aside 1-10% of the lysate (**before the addition of any antibody, beads etc**.). This is termed **the input**, and represents the starting sample material. The input will be run alongside the precipitate at the end of the experiment (e.g. by WB). This is a useful positive control to determine if the IP has worked: if a band is seen in the input but not the IP, then the IP did not successfully precipitate the protein. The input is also a useful control to give a sense of 1) efficiency, by comparing the strength of the target band in the IP lane to the band in the input lane, and 2) specificity, by comparing the strength of consistent non-specific bands between the lanes. Finally, it serves as quality control to ensure that the starting material is consistent across different experiments or samples.

Lysis Buffers

One of the most important technical aspects of IP is the lysis buffer, the choice of which will depend on the sample type and purpose of the experiment. Lysis buffers should stabilize native protein conformation, inhibit enzyme activity to decrease degradation and PTM modification, and rupture membranes for protein release from cells. The location of the protein in a cell (e.g. in the cytosol, nucleus) can affect how easily a protein will be released, which in turn will affect the choice of lysis buffer.

The most important lysis buffer consideration is whether the buffer used contains ionic or non-ionic detergent. Ionic detergents contain a charged head group and have a much stronger denaturing effect, which can result in altered protein conformations and protein-protein interactions. Non-ionic detergents are non-denaturing and less harsh than ionic detergents, meaning they are less likely to affect protein-protein interactions. Given that co-IP relies on protein-protein interactions, ionic detergents generally cannot be used. **RIPA** (radio-immunoprecipitation assay) buffer is commonly used in WBs and sometimes recommended for IP, but it contains sodium deoxycholate and SDS (0.01-0.5%), which are ionic and will disrupt protein-protein interactions. Nevertheless, ionic, denaturing lysis buffers can still be useful for individual protein IP as some proteins can be difficult to release with only non-denaturing buffers, or an antibody may only recognize a denatured protein form. Buffers containing NP40 or Triton X-100 (0.1-2%) are useful, non-ionic alternatives to RIPA, but may result in slightly higher background. Buffers that completely lack detergent are also available for proteins that can be released using only physical disruption, usually consisting of just EDTA in phosphate buffered saline (PBS).

Both ionic and non-ionic lysis buffers tend to contain NaCl and Tris-HCl, and usually have a slightly basic pH (7.4 to 8), though this can be optimized (between pH 6-9). Other buffer components that can be optimized include: salts (0-1 M) for maintaining ionic strength and correct tonicity for easy cell lysis; divalent cations such as Mg^{2+} (0-10 mM) which can help to prevent DNA causing the solution to become viscous; and EDTA (0-5 mM) for chelating ions such as Zn^{2+} that proteases require for proteolytic function.

Enzyme Inhibitors

The final components of lysis buffers are inhibitors of proteases and enzymes that alter protein PTMs, particularly phosphatases. Protease inhibitors prevent the degradation of target proteins, while PTMs are often necessary for protein-protein interactions and so must be maintained for co-IP. Preserving PTMs is obviously essential if the target of the IP is to understand the PTM state of target proteins.
Inhibitors should be added fresh, immediately before lysis buffer use.

Pre-clearing and Pre-blocking

Pre-clearing is an optional but often worthwhile step to reduce non-specific binding in IP. Pre-clearing refers to incubating samples with the beads (including Protein A/G or other attachment substrates), or with a nonspecific antibody from the same host species as the IP antibody immobilized to the beads, in order to remove lysate components that bind to beads or immunoglobulins non-specifically. This prevents these components from being carried through and ultimately eluted, therefore resulting in a purer final product containing predominantly the target antigen.
Reducing non-specific binding can also be achieved by pre-blocking the bead. This works in a similar way to blocking in immunostaining, western blotting and ELISA. The bead is incubated with a mild blocking buffer containing 1-5% BSA, non-fat milk, 1% gelatin or 0.1-1% Tween-20 to block sites of non-specific binding on the bead, preventing lysate components from binding.
Pre-clearing is not necessary if the target is particularly abundant, and is less important if using magnetic beads over agarose beads, because magnetic beads are less prone to non-specific binding.
The supernatant from a pre-clearing step can be kept for WB analysis to confirm that no target antigen was removed during the pre-clearing step.

Antibody Selection

Antibodies are generated by the immune system of a host organism (e.g. mouse, rabbit) that has been repeatedly immunized with a specific antigen. The antibodies recognize and specifically bind to that antigen with a high affinity, allowing the protein to be precipitated from the lysate. High specificity is essential for a successful IP, as non-specific binding can result in high background or false positive results.
To confirm the specificity of the antibody-antigen interaction, an isotype control should be included. The isotype control consists of a non-immune antibody of the same isotype as the experimental antibody. This should not have any affinity for the target antigen, but it may non-specifically bind to other factors, therefore confirming that the precipitated protein band in a WB is specifically recognized by the chosen antibody. Any bands in both the IP and isotype lanes in WB analysis is likely to represent a protein that binds immunoglobulins non-specifically.

Bead support

The type of beads chosen when performing IP is an important consideration and will dictate certain aspects of the overall protocol. Beads are usually either agarose (or Sepharose, which is a tradename for a crosslinked, beaded-form of agarose) or magnetic beads. Table 4 summarizes the key differences between agarose and magnetic beads, which are described further below.

	Agarose	Magnetic
Size	50-150 µm	1-4 µm
Uniformity	Low	High
Binding capacity	High	Medium
Diffusion	Slow	Fast
Washing	Extensive washing Easier to pick up some of the pellet when removing supernatant	Washing steps reduced Less chance of proteolytic damage
Yield	Medium	High
Time required	60-90 minutes	30-60 minutes
Automation	No	Yes
Reproducibility	Medium	High

Table 4: Key features of agarose and magnetic beads in IP.

Agarose Beads
Agarose beads tend to be 50-150 µm in size, and they have a very high binding capacity because they are porous, creating a very high surface area per bead that is available for binding to antibodies. However, this can create a requirement to use a large amount of antibody to ensure that every surface of the agarose beads is covered in antibody, lest any unoccupied agarose non-specifically bind to lysate components **and increase background signal**. To solve this issue, it is possible to back-calculate from the amount of expected analyte to the amount of antibody needed for detection, to the amount of agarose needed to hold the antibody. The downside is that this approach requires significant knowledge or prior experience to estimate such quantities. An alternative would be to simply saturate the beads with excess antibody, but antibody is typically expensive and limiting. The best option is therefore to pre-clear the lysate to remove anything that will non-specifically bind to the agarose.

Magnetic Beads
Magnetic beads lack the porosity of agarose because they **are solid spheres,** resulting in only the external surface being available for binding. While this may at first glance suggest that magnetic beads have much lower binding capacity, they are **much smaller** than agarose particles at 1-4 µm, meaning that there can be far more beads per unit volume, so the ultimate binding capacities of the two are similar, with agarose maintaining a small advantage.
Magnetic beads offer **several other advantages** over agarose for use in IP, however. They are **highly uniform in size** and have a consistent binding capacity, which can make experiments more consistent between runs. Magnetic beads are manipulated using powerful magnets that pull the beads to one side of a tube so that the supernatant can be removed, obviating the need for centrifugation. Centrifugation can be stressful for protein complexes and can lead to a loss of yield compared to the gentler magnet-based washing steps, thus final yield can be higher with magnetic beads. Magnet-based manipulation is faster and makes IPs more amenable to higher throughput applications.

Immobilizing Antibodies to Beads
Immobilizing antibodies to the solid phase (beads) is essential for IP, enabling the precipitation of the target protein. While the most common method is to use Protein A or G to capture the IP antibody, various approaches can be used, each of which has its own benefits and applications. These approaches are illustrated in Figure 5 and explained in more detail below.

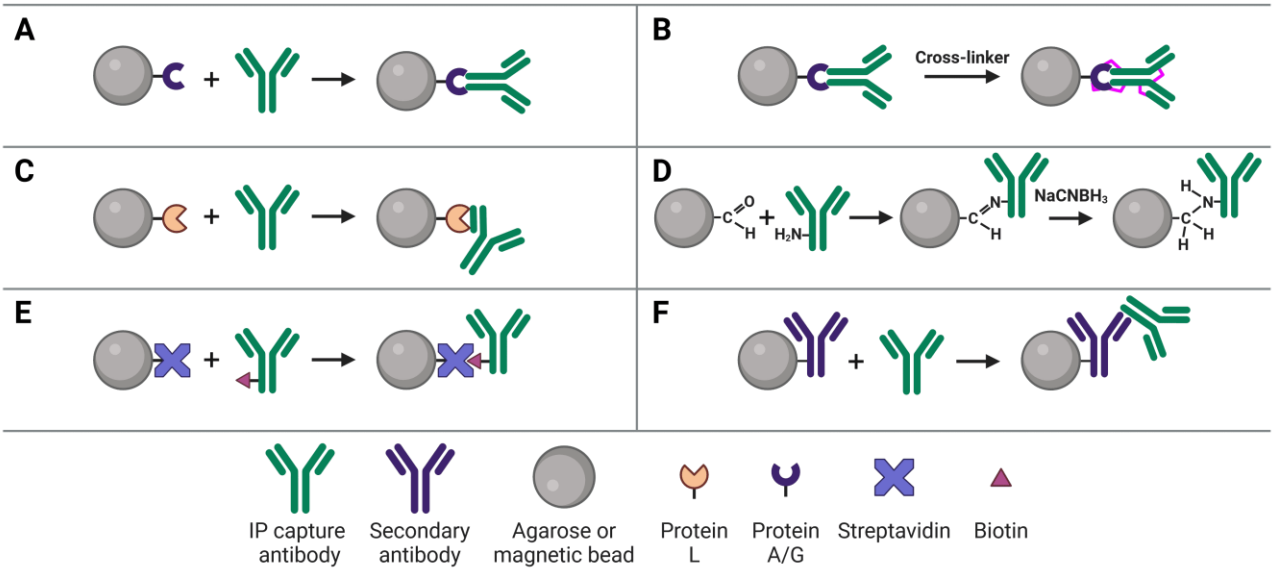

Figure 5: Approaches to IP antibody immobilization on beads.**A**, Protein A, G or A/G bind to the Fc region of antibodies. **B**, Antibodies can be crosslinked to Protein A, G or A/G to prevent eluting the antibodies during antigen elution. **C**, Protein L binds to the light chain of antibodies. **D**, Direct immobilization of antibodies to beads obviates the need for Protein A, G or L, and prevents antibody elution. **E**, Streptavidin-conjugated beads capture biotinylated IP antibodies with very high affinity. **F**, Secondary antibodies directly bound to beads can be used to capture IP antibodies from a specific host or of a specific isotype, but offer less flexibility than Protein A or G.

Protein A and Protein G

The most common approach to immobilize the antibody to the chosen bead is with Protein A[3] or Protein G,[4] which are covalently bound to the beads following bead activation with a coupling agent such as cyanogen bromide or N-hydroxysuccinimide (NHS). Protein A and Protein G are derived from bacteria, and bind to the heavy chains of the antibody's Fc region (Figure 5a). Binding to this site has the advantage of orienting the antibody such that the Fab region is clear and directed away from the bead to bind to the target protein.

Due to their promiscuity in generally binding immunoglobulin Fc regions, care must be taken when performing IP on a sample that contains immunoglobulins besides the added capture antibody, such as serum. In this instance, the capture antibody would need to be added to the beads first (pre-immobilized/direct method) and could be covalently bound to Protein A or G (see Covalently Linking Capture Antibodies to Protein A/G below).[15]

Biotin-avidin binding

Avidin is a protein found in egg whites, while streptavidin is from purified from the bacterium Streptomyces avidinii, but they both have an extremely high and specific affinity for biotin. Biotin is a small 244 Da vitamin that is easy to covalently bond to protein (the carboxyl group in biotin can be modified with reactive groups such as NHS esters, maleimides or hydrazides that target amines ($-NH_2$), sulfhydryls (-SH) or aldehydes (>C=O), respectively, on proteins).

The affinity between biotin and avidin is extremely strong and specific, making it an ideal system for affinity purification, in which a biotinylated antibody is bound by streptavidin-conjugated beads (Figure 5e). Only harsh buffers can dissociate biotin-avidin, such as 8 M guanidine-HCl at pH 1.5, meaning the association will remain throughout all wash steps and not be eluted at the end.

Because any protein can be biotinylated, this system is also adaptable to general pull-downs in which antibodies are not used. The extremely high affinity of biotin-avidin offers a distinct advantage in pull-downs whereby prey proteins can be eluted in a regular elution buffer, but the bait protein will remain attached to the beads. The disadvantage when using biotin-streptavidin for co-IPs is that antibodies are not necessarily oriented in the optimal direction with the Fab region accessible, but this has only a minor effect on co-IP efficiency.

References

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