Protocols book

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2013/2014

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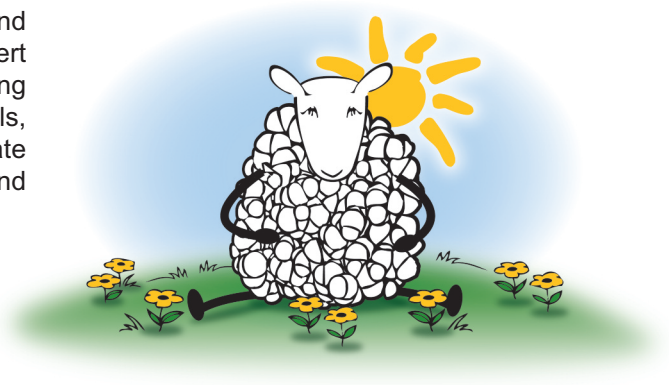

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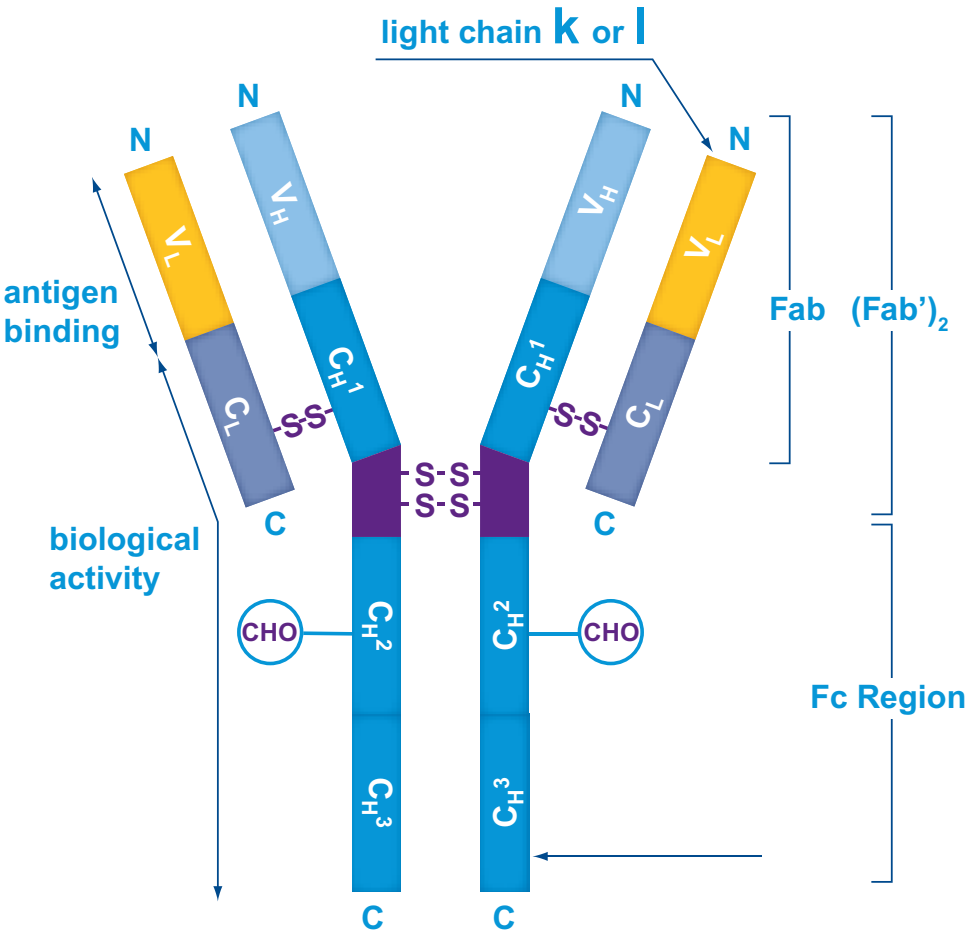
SECTION 1: Antibodies and antibody structure

Antibodies, also called immunoglobulins (Ig), are glycoproteins that are capable of specifically binding to antigens that caused their production in a susceptible animal. They are produced in response to the invasion of foreign molecules in the body. Antibodies exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each Y contains two copies of a heavy chain, and two copies of a light chain, named as such by their relative molecular weights. The top of the Y shape contains the variable region, which is the antigen binding site.

The light chains of any antibody can be classified as either a kappa (□) or lambda (□) type (based on small polypeptide structural differences); however, the heavy chain determines the class, or isotype, of each antibody.

Antibody structure:



Heavy chains

There are five types of mammalian Ig heavy chains denoted by the Greek letters: alpha (□), delta (□), epsilon (□), gamma (□), and mu (□). These chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Distinct heavy chains differ in size and composition; □ and □ contain approximately 450 amino acids, while □ and □ have approximately 550 amino acids.

Each heavy chain has two regions, the *constant region* and the *variable region*. The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. Heavy chains □, □ and □ have a constant region composed of *three* tandem (in a line) Ig domains, and a hinge region for added flexibility; heavy chains □ and □ have a constant region composed of four Ig domains. The variable region of the heavy chain differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone. The variable region of each heavy chain is approximately 110 amino acids long and is composed of a single Ig domain.

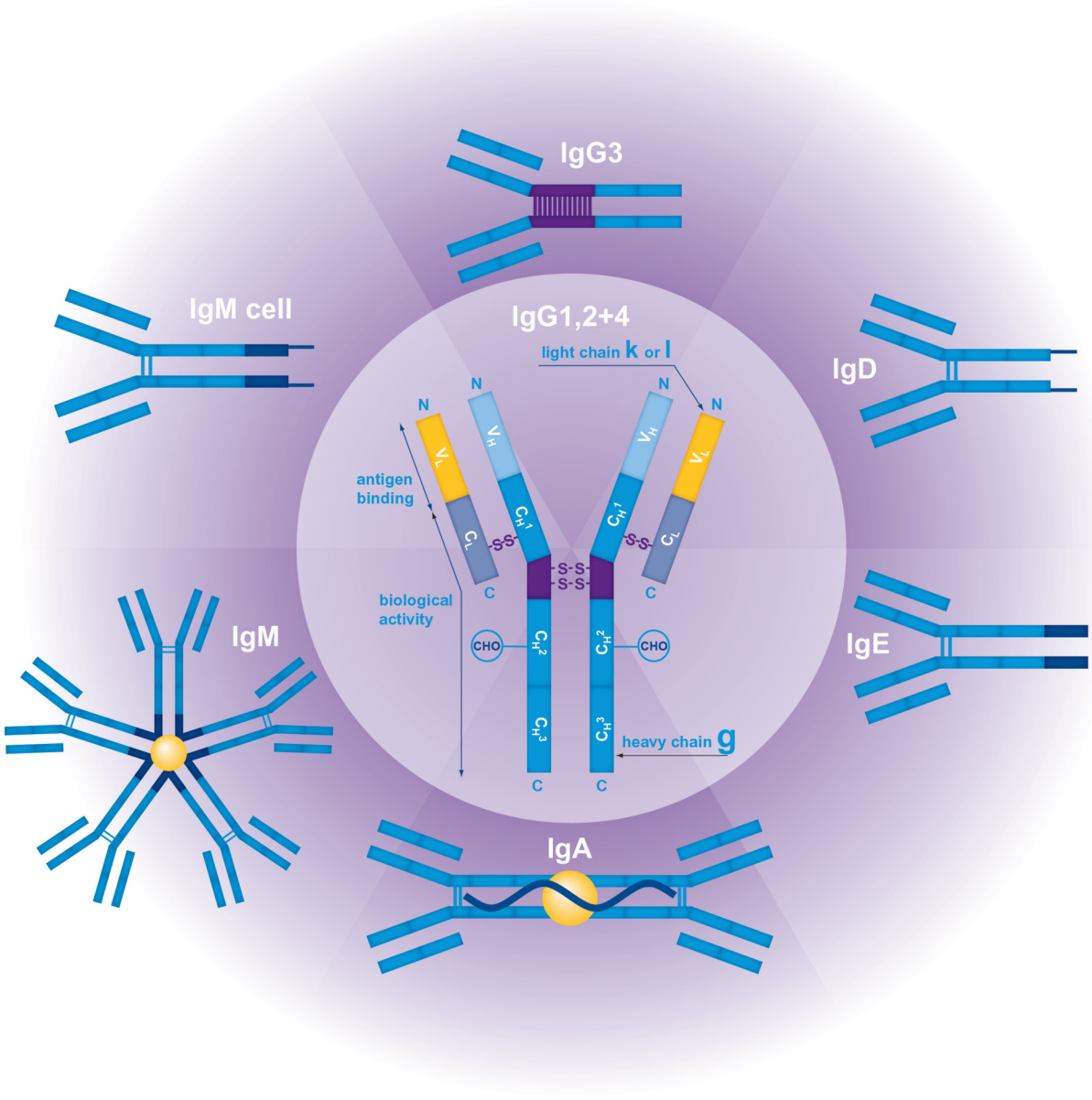
Light chains

In mammals there are only two types of **light chains**, which are called lambda (□) and kappa (□). A light chain has two successive domains: one constant domain and one variable domain. The approximate length of a light chain is 211 to 217 amino acids. Each antibody contains two light chains that are always identical; only one type of light chain, □ or □, is present per antibody in mammals. Other types of light chains, such as the iota (□) chain, are found in lower **vertebrates** like **Chondrichthyes** (cartilaginous fishes**)** and **Teleostei** (ray-finned fishes).

Fab and Fc regions

Direct-conjugated antibodies are labeled with an enzyme or fluorochrome in the Fc region. The Fc region also anchors the antibody to the plate in ELISA procedures and is also recognized by secondary antibodies in immunoprecipitation, immunoblots and immunohistochemistry. Antibodies can be cleaved into two F(ab) and one Fc fragments by the proteolytic enzyme papain, or into just two parts: one $F(ab)_2$ and one Fc at the hinge region by the proteolytic enzyme pepsin. Fragmenting IgG antibodies is sometimes useful because F(ab) fragments (1) will not precipitate the antigen; and (2) will not be bound by immune cells in live studies because of the lack of an Fc region. Often, because of their smaller size and lack of crosslinking (due to loss of the Fc region), Fab fragments are radiolabeled for use in functional studies. Interestingly, the Fc fragments are often used as blocking agents in histochemical staining.

Antibody isotypes:



In mammals, antibodies can be divided into five isotypes or classes: IgG, IgM, IgA, IgD and IgE, based on the number of Y units and the type of heavy chain. Heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as gamma (□), mu (□), alpha (□), delta (□), and epsilon (□), respectively. The isotypes differ in their biological properties, functional locations and ability to deal with different antigens, as depicted in the table below:

Class/ subclass	Heavy chain	Light chain	Molecular weight (kDa)	Structure	Function
IgA_1 IgA_2	□1 □2	□ or □	150 to 600	Monomer to tetramer	Most produced Ig. Found in mucosal areas, such as the gut, respiratory and urogenital tract, and prevents their colonization by pathogens. Resistant to digestion and is secreted in milk.
IgD	□	□ or □	150	Monomer	Function unclear; Works with IgM in B-cell development; mostly B-cell bound.
IgE	□	□ or □	190	Monomer	Binds to allergens and triggers histamine release from mast cells, and is involved in allergy. Also protects against parasitic worms.
IgG_1 IgG_{2a} IgG_{2b} IgG_3 IgG_4	□1 □2 □3 □4	□ or □	150	Monomer	Major Ig in serum. Provides the majority of antibody-based immunity against invading pathogens. Moderate complement fixer (IgG_3); can cross placenta.
IgM	μ	□ or □	900	Pentamer	First response antibody. Expressed on the surface of B cells and in a secreted form with very high avidity. Eliminates pathogens in the early stages of B cell-mediated immunity before there is sufficient IgG.

Chicken IgY

There are several advantages to choosing chickens, rather than rabbits or goats to produce polyclonal antibodies.

1. Chickens are not mammals and therefore are more able to make high-avidity antibodies to mammalian antigens (especially highly conserved mammalian proteins).

2. To our knowledge, it is the most ethical way to produce polyclonal antibodies. There is no need to bleed the chickens; simply collect the eggs. Our hens are kept in flocks of six in coops that have outside runs and our unique egg identification system allows our chickens to live freely.

3. A single chicken can produce an enormous amount of antibodies, up to 3 g of IgY per month, which is 10-20 times the amount of a rabbit. Furthermore, compared to rabbits, chickens produce antibodies more quickly. High-titer antibodies are available from eggs as early as day 25.

4. By having the IgY packaged conveniently in eggs, you can collect and store eggs over a long period of time and retroactively purify the IgY from the eggs of desired titer/avidity.

5. It is cheaper to feed and house chickens than rabbits.

6. IgY is a stable antibody sharing the following characteristics with mammalian IgG:
   - Divalent
   - Degraded by papain to yield divalent Fab fragment
   - May be enzyme-labeled, biotinylated and gold–labeled by standard procedures

7. Fc region of chicken IgY is sufficiently different from mammalian IgG:
   - Reduces background by not binding to mammalian rheumatoid factors or other naturally occurring anti-mammalian antibodies (e.g. HAMA)
   - Does not activate mammalian complement systems
   - Does not bind to mammalian Fc receptors
   - Does not bind to Staphylococcal protein A or protein G

SECTION 2: Formats of antibody and antibody purification

Centrifugation and filtration are standard laboratory techniques for sample clarification of serum, ascetic fluid and tissue culture supernatant. These techniques remove lipid and particle matter which can block chromatographic columns. For some materials buffer exchange and desalting may also be necessary. Ammonium sulphate precipitation is a further preparation step often used with ascetic fluid to concentrate the immunoglobulins.

Antiserum

Polyclonal antibodies are often available in relatively unpurified formats, described as “serum” or “antiserum”. Antiserum refers to the blood from an immunized host from which clotting proteins and red blood cells have been removed. The antiserum will contain antibodies/immunoglobulins of all classes as well as other serum proteins. In addition to antibodies that recognize the target antigen, the antiserum also contains antibodies to various non-target antigens that can sometimes react non-specifically in immunological assays. For this reason, raw antiserum is often purified to eliminate serum proteins and to enrich the fraction of immunoglobulin that specifically reacts with the target antigen.

Tissue culture supernatant

Monoclonal antibodies may be grown as hybridoma cell cultures (cells secreting antibodies) and harvested as hybridoma tissue culture supernatants.

Ascites fluid

Monoclonal antibodies can be produced by growing hybridoma cells within the peritoneal cavity of a mouse (or rat). When injected into a mouse, the hybridoma cells multiply and produce fluid (ascites) in its abdomen. This fluid contains a high concentration of antibody which can be harvested, providing higher antibody yields than hybridoma cell-culture.

Antibody purification

Polyclonal antiserum or monoclonal ascites fluid / tissue culture supernatant is commonly purified by one of three methods:

1. Protein A/G purification

Protein A/G purification makes use of the high affinity of *Staphylococcus aureus* protein A or *Streptococcus* protein G to the immunoglobulin Fc domain. Protein A/G purification eliminates serum proteins from raw antiserum, but it does not eliminate the non-specific immunoglobulin fraction. Consequently, protein A/G purified antiserum may still possess a small amount of undesirable cross reactivity.

2. Affinity purification

Affinity purification isolates a specific protein or group of proteins with similar characteristics. The technique separates proteins on the basis of a reversible interaction between the proteins and a specific ligand coupled to a chromatographic matrix.
Antigen affinity purification takes advantage of the affinity of the specific immunoglobulin fraction for the immunizing antigen against which it was generated. Antigen affinity purification results in the elimination of the bulk of the non-specific immunoglobulin fraction, while enriching the fraction of immunoglobulin that specifically reacts with the target antigen. The resulting affinity purified immunoglobulin will contain primarily the immunoglobulin of desired specificity.

3. Pre-adsorption

Polyclonal antibodies are sometimes pre-adsorbed. This means they have been adsorbed with other proteins, or serum from various species, to eliminate any antibody that may cross-react. The resulting purified antibody should be very pure and specific and any cross-reactivity should be significantly reduced.

Antibody purification at Abcam

SECTION 3: Choosing an antibody and antibody dilution

There is often more than one antibody available for any given target. To narrow the range of choice, several aspects of the experiment need to be considered:

1. Type of assay or application
2. Nature of the sample
3. Species of the sample
4. Species of the antibody host
5. Labeling and detection of the antibody

The Abcam website has a useful search function. Entering the name of the protein or other target in the search box will generate a list that can be filtered by product type, target, applications tested, species reactivity, host species, clonality, and conjugation.

Application
Antibody datasheets list the applications that have been tested and found to work. If an application is not listed, this does not mean that the antibody is not suitable. It simply means that it has not been tested and it is unknown how the antibody will perform. When an application has been tested and found not to work, this will be noted on the datasheet.

Nature of the sample
The nature of the sample will dictate which antibody will work best. At least two aspects need to be considered:

1. The region of the protein you wish to detect. Antibodies are generated by immunization of host animals with a variety of immunogenic substances including full-length proteins, protein fragments, peptides, whole organisms (for example bacteria), or cells. The immunogen is generally described on the datasheet (though in many cases an exact description of the immunogen is not available for proprietary reasons). If trying to detect a protein fragment or a specific isoform or region of the full-length protein, one needs to be sure to choose an antibody that is raised against an immunogen that is identical to or contained within the fragment or region. If trying to detect a cell surface protein on live cells by FACS, you need to choose an antibody that is raised against an extracellular domain of the protein.

2. Processing of the sample. Some antibodies require samples to be processed or treated in a specific manner. For instance, many antibodies will only recognize proteins that have been reduced and denatured, presumably because this reveals epitopes that would otherwise be obscured by secondary and tertiary folding of the proteins. On the other hand, some antibodies will only recognize epitopes on proteins in their native, folded state. (For Abcam antibodies used for western blotting, samples should be reduced and denatured unless otherwise noted on the datasheet). When searching for an antibody for immunohistochemistry, it should be noted that some antibodies are only appropriate for unfixed frozen tissue. For others, an antigen retrieval step that reverses the cross-links introduced by formalin fixation is necessary because they are incapable of binding to their targets in formalin-fixed, paraffin-embedded tissues. These restrictions on use will be noted in the applications section of datasheets.

Species of sample
The chosen antibody should have been raised against the same species you are studying, although the antibody may react with the same target protein from other species sharing sufficient amino acid sequence homology. If the sample is not from one of the species listed, this does not mean that the antibody will not detect the protein, but rather that the species has not been tested and we are reluctant to comment on its suitability. A prediction of cross-reactivity can be made based on sequence similarity: Abcam is now proud to have ExPASy and NCBI BLAST links on the datasheets to compare amino acid sequence homology among different species.

Choosing the species of primary antibody host
In general, the species of the host animal in which an antibody was raised is important when using a conjugated secondary antibody to detect an unconjugated primary. For immunohistochemistry, the primary antibody should be raised in a species as phylogenetically different as possible from the species of the sample. This is to avoid potential cross-reactivity of the secondary anti-immunoglobulin antibody with endogenous immunoglobulins in the sample. For instance, a primary antibody used to detect a protein in a sample from a mouse should not be raised in mouse or rat. A primary antibody raised in rabbit will be a more appropriate choice, followed by an anti-rabbit IgG secondary antibody conjugated to a detection molecule (enzyme, fluorochrome, biotin, etc.). This issue can be avoided if a conjugated primary antibody is available. For other techniques using samples that do not contain endogenous immunoglobulin, the choice of host species is less critical. An example would be western blotting of a cell lysate that is not expected to contain IgG. However, tissue lysates and tissue culture supernatants that contain serum will contain immunoglobulins. IgG will appear in western blots of reduced, denatured samples as bands at 50 and 25 kDa corresponding to the heavy and light chains of the IgG molecule.

Choosing a secondary antibody
Secondary antibodies should be raised against the same species as the primary antibody you are using. For example, if your primary is a mouse monoclonal, you will require an anti-mouse secondary. We recommend you check the datasheet of the secondary antibody to ensure it is tested in the application you will be using. Abcam provides a wide range of secondary antibodies conjugated to a range of fluorochromes and chromogens.
Check the bottom of the datasheet of the primary antibody you are using; there will be a list of suitable secondary antibodies.
To find your own secondary, select this product type using our navigation bar, or by using the links at the center of the homepage. Then choose the species of your primary antibody. This will then give you a range of secondary antibodies for use with that species of primary.

Choosing antibodies for dual staining
Double immunostaining of cell cultures or tissue sections using unconjugated primary antibodies requires that those antibodies are raised in different species and that the secondary antibodies recognize one of those species exclusively. Datasheets for secondary anti-immunoglubulin antibodies will state if they have been cross-adsorbed against immunoglobulins from other species to remove those reactivities.

Fluorochrome and chromogen labels
Labels are conjugated (joined) to antibodies in order to visualize the binding of the antibody. The choice of label depends on several parameters:

1. Detection method: fluorescence or colored precipitate. Fluorescent labels emit light in the visual range when excited by light of a specific wavelength. There are several available, all with their own excitation and emission characteristics. When combined with the appropriate substrate the enzymatic labels HRP and AP form a colored precipitate.

2. Available mounting media (immunohistochemistry only): AEC, Fast Red, INT or any other aqueous chromogen are alcohol soluble and require an aqueous based mounting medium. The others mentioned above are organic, so are best mounted in organic mounting media in order to take advantage of the better refractive index.
Fluorescent labels require aqueous mounting media. Phycobiliproteins (phycocyanin / phycoerythrin) require aqueous mounting media with no added glycerol, since this has a quenching effect.

3. Biotinylated antibodies are useful for amplification of signal when followed by an avidin-biotin-enzyme or fluorochrome complex (commonly abbreviated as “ABC” reagent), or avidin or streptavidin conjugated to an enzyme or fluorochrome.

Unpurified antibody suggested dilutions and concentrations

Unpurified antibody preparations vary significantly in specific antibody concentration. If the specific antibody concentration of a given unpurified antibody preparation is unknown, you may refer to the following “typical ranges” as a guideline for estimation:

	Tissue culture supernatant	Ascites	Whole antiserum	Purified antibody
WB/dot blot	1/100	1/1000	1/500	1 □g/ml
IHC/ICC	neat to 1/10	1/100	1/50 to 1/100	5 □g/ml
EIA/ELISA	1/1000	1/10000	1/500	0.1 □g/ml
FACS/Flow Cytometry	1/100	1/1000	1/500	1 □g/ml
IP	-	1/100	1/50 to 1/100	1 to 10 □g/ml
Concentration estimate	1 to 3 mg/ml	5 to 10 mg/ml	1 to 10 mg/ml	

SECTION 4: Fluorescence

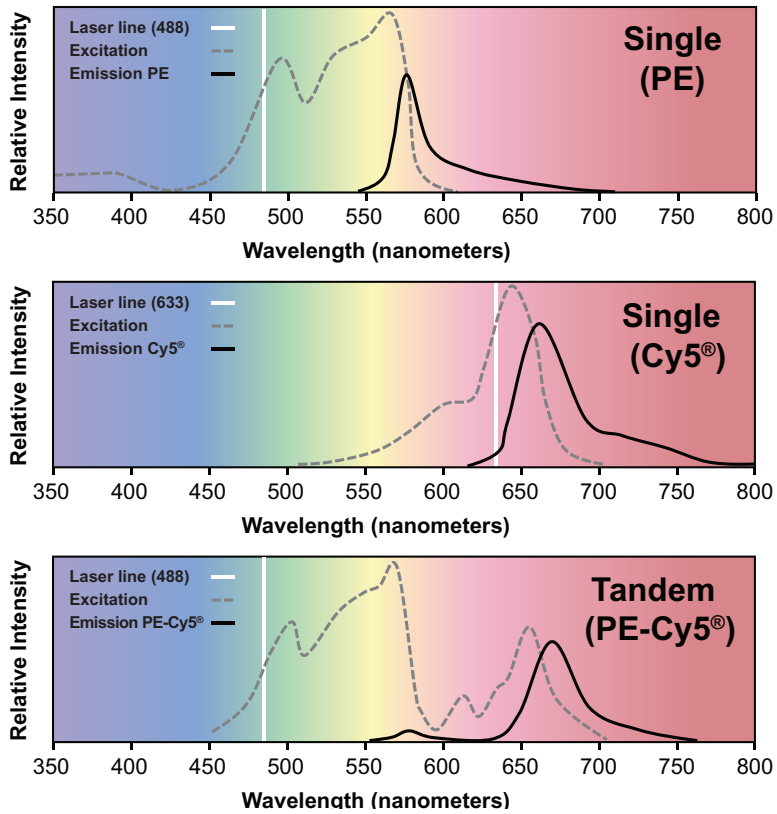
4.1 Fluorescence – how it works

Due to their novel electronic configurations, fluorochromes have unique and characteristic spectra for absorption (excitation) and emission.

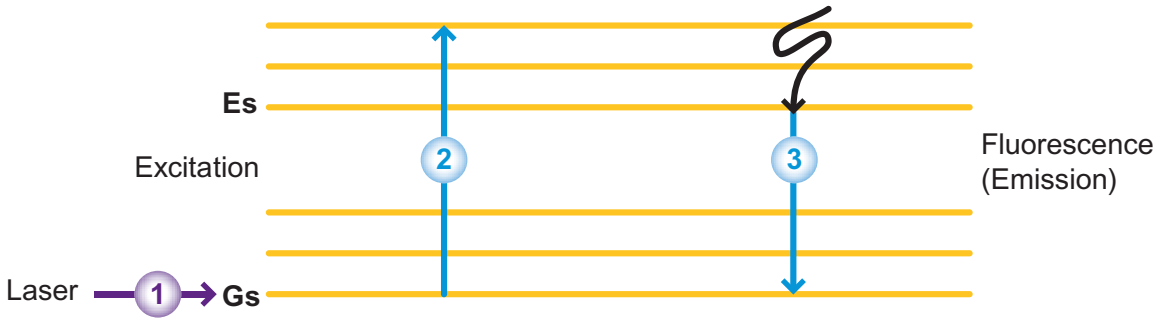
A single dye is excited at a particular wavelength and emits a photon at another wavelength.

A tandem dye consists of a donor and acceptor fluorochrome molecule, placed in close proximity, allowing for energy transfer between the two. The tandem dye is excited at the excitation wavelength of the acceptor molecule and emits a photon at the emission wavelength of the donor molecule.

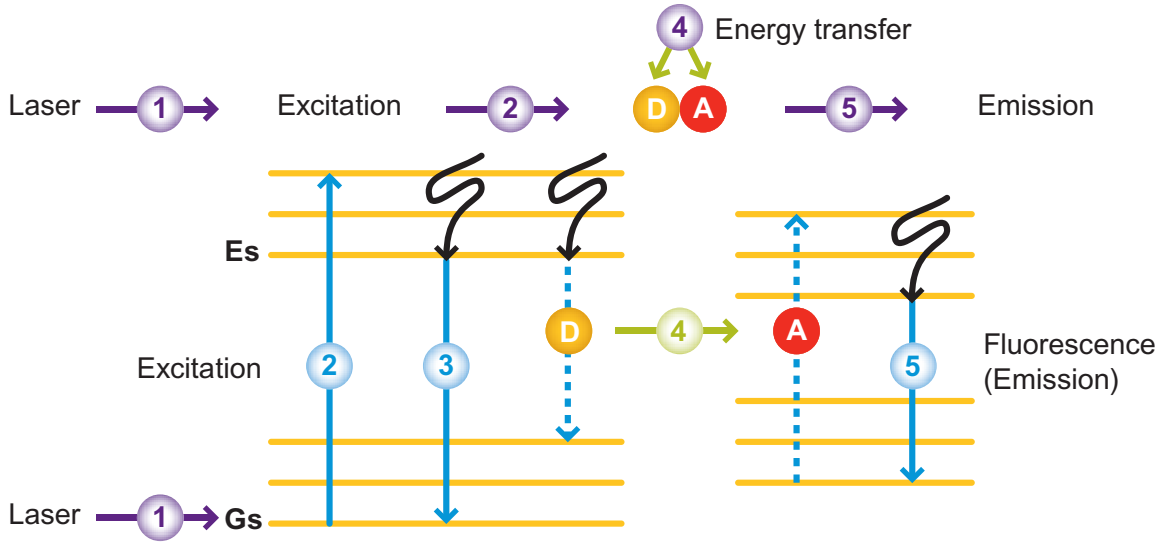
Excitation and emission spectral profiles:



Energy level diagram - Single dye:



Energy level diagram - Tandem dye:



In the case of a single fluorescent dye:

① A laser set at the signature excitation wavelength for the dye provides electromagnetic energy to an electron in that molecule.

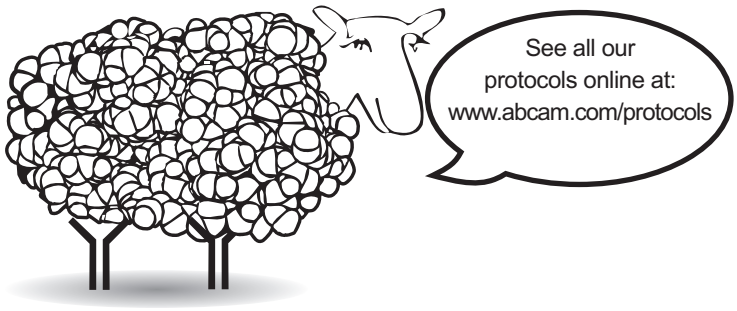
② The electron moves to an excitation state at the next energy level (Es).

③ Energy is then released in the form of a photon (fluorescence) and the electron moves back down to the lower energy level (Gs).

In the case of a tandem fluorescent dye:

④ After excitation of the electron by a laser (1-2 above), energy is released by an electron in the donor molecule (D) and is absorbed by an electron in the acceptor molecule (A).

⑤ The electron in the acceptor molecule moves to an excitation state at the next energy level (Es). Similar to a single dye, energy is then released in the form of a photon (fluorescence) and the electron moves back down to the lower energy level (Gs).

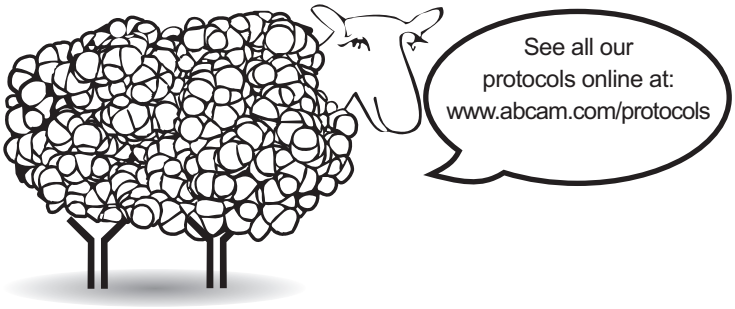


4.2 Fluorochrome table

Dye	Max. excitation wavelength (nm)	Max.emission wavelength (nm)	Excitation laser lines (nm)
Methoxycoumarin	360	410	
DyLight® 405	400	420	
Alexa Fluor® 405	402	421	
Brilliant Violet 421™	407	421	
HiLyte Fluor™ 405	404	428	
DyLight® 350	353	432	
Alexa Fluor® 350	346	442	
Aminocoumarin (AMCA)	350	445	
BD Horizon™ V450	404	448	
Pacific Blue™	404	456	360,405,407
EviTag™ quantum dots-Lake Placid Blue	470	490	
AMCyan	457	491	
BD Horizon™ V500	415	500	
Cy2®	489	506	488
Chromeo™ 488	488	517	
DyLight® 488	493	518	
Alexa Fluor® 488	495	519	488
FAM	494	519	
Fluorescein Iso-thiocyanate (FITC)	495	519	488
EviTag™ quantum dots-Adirondack Green	505	520	
Chromeo™ 505	505	526	
HiLyte Fluor™ 488	501	527	
Alexa Fluor® 514	518	540	
EviTag™ quantum dots-Catskill Green	525	540	
Alexa Fluor® 430	434	541	
Pacific Orange™	403	551	
Alexa Fluor® 532	532	554	
HEX	535	556	
EviTag™ quantum dots-Hops Yellow	545	560	
Chromeo™ 546	545	561	
Cy3®	548	561	488,514
Alexa Fluor® 555	555	565	
HiLyte Fluor™ 555	550	566	
5-TAMRA	541	568	
Alexa Fluor® 546	556	573	532
DyLight® 550	562	576	
Phycoerythrin (PE)	496,566	576	488
Tetramethyl Rhodamine Isothiocyanate (TRITC)	557	576	
EviTag™ quantum dots-Birch Yellow	560	580	
Cy3.5®	576	589	568,543
Rhodamine Red-X	570	590	
PE-Dyomics® 590	488	599	
EviTag™ quantum dots-Fort Orange	585	600	
ROX	575	602	
Alexa Fluor® 568	578	603	532
Red 613	480,565	613	
Texas Red®	595	613	568,543,514
HiLyte Fluor™ 594	593	616	
PE-Texas Red®	566	616	
Alexa Fluor® 594	590	617	
DyLight® 594	593	618	
EviTag™ quantum dots-Maple-Red Orange	600	620	
Alexa Fluor® 610	612	628	
Chromeo™ 494	494	628	
Alexa Fluor® 633	632	647	
SureLight® APC	652	657	
DyLight® 633	638	658	
Allophycocyanin (APC)	650	660	595,633,635,647
Chromeo™ 642	642	660	
Quantum Red	488	660	
SureLight® P3	614	662	
Alexa Fluor® 647	650	665	595,633,635,647
Cy5®	647	665	633,635
PE-Cy5®	565	666	488
SureLight® P1	545	666	
PE-Alexa Fluor® 647	567	669	
PE-Dyomics® 647	488	672	
DyLight® 650	654	673	
HiLyte Fluor™ 647	650	675	
Peridinin Chlorophyll (PerCP)	477	678	488
IRDye® 700DX	680	687	
Alexa Fluor® 660	663	690	
PE-Cy5.5®	565	693	488
APC-Cy5.5®	650	694	595,633,635,647
Cy5.5®	675	694	647
TruRed	490,675	695	
HiLyte Fluor™ 680	678	699	
Alexa Fluor® 680	679	702	
DyLight® 680	692	712	
Alexa Fluor® 700	702	723	
APC-Cy7®	650	774	595,633,635,647
Alexa Fluor® 750	749	775	
Cy7®	753	775	
PE-Dyomics® 747	488	776	
DyLight® 755	754	776	
HiLyte Fluor™ 750	753	778	
PE-Cy7®	566	778	488
IRDye® 800RS	770	786	
DyLight® 800	777	794	
IRDye® 800CW	778	794	
Alexa Fluor® 790	782	805	

Nucleic acid probes

Nucleic acid dyes	Max. excitation wavelength (nm)	Max. emission wavelength (nm)	Excitation laser lines (nm)
DAPI	359	461	325,360,405,407
Hoechst 33258	352	461	
Hoechst 33342	350	461	
SYTOX Blue	431	480	
YOYO-1	491	509	
SYTOX Green	504	523	
TOTO-1, TO-PRO-1	509	533	
Mithramycin	450	570	
SYTOX Orange	547	570	
Chromomycin A3	445	575	
CyTRAK Orange™*	457,488,549	615	
Ethidium Bromide	493	620	
Propidium iodide (PI)	305,540	620	325,360,488
DRAQ5™	647	681,697	
DRAQ7™	599, 644	>678 to <697	

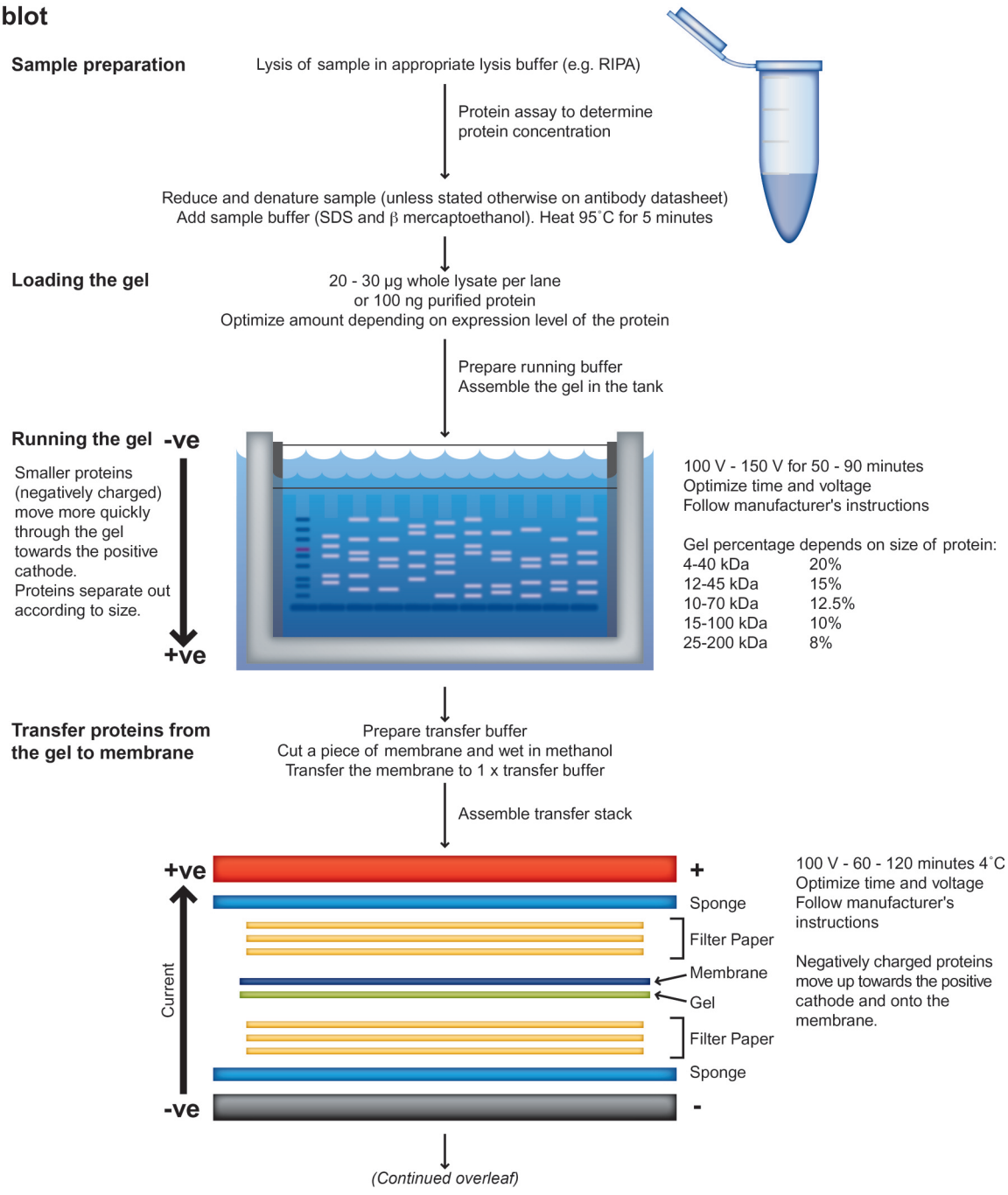


SECTION 5: Western blotting

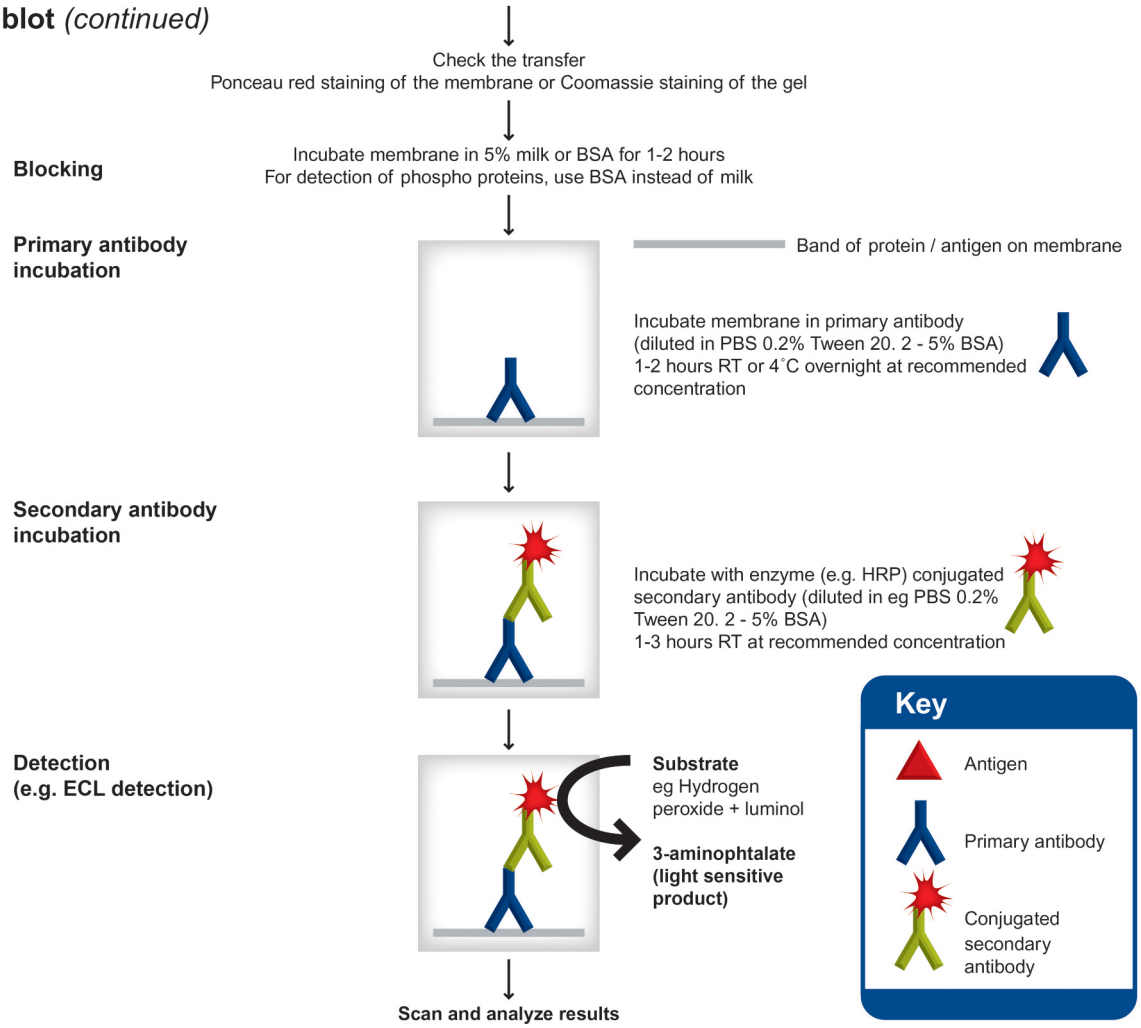
Western blotting is a technique that identifies specific proteins in a given sample or extract after their separation using polyacrylamide gel electrophoresis. The polyacrylamide gel is placed adjacent to a membrane, which is typically nitrocellulose or PVDF (polyvinylidene fluoride), and the application of an electrical current induces the proteins to migrate from the gel to the membrane on which they become immobilized. The membrane is then a replica of the gel protein and can subsequently be stained with an antibody.

The following western blotting protocol includes the process of sample preparation, gel electrophoresis, transfer from gel to membrane, and immunostaining for protein detection. Protocols may require optimization according to the electrophoresis and transfer equipment used and you are advised to consult the specific manufacturer's instructions.

Western blot



Western blot *(continued)*



Contents:

5.1. Sample preparation

Lysis buffers

To prepare samples for running on a gel, cells and tissues need to be lysed to release the proteins of interest. This solubilizes the proteins so they can migrate individually through a separating gel. There are many recipes for lysis buffers but a few will serve for most western blotting experiments. In brief, they differ in their ability to solubilize proteins, with those containing sodium dodecyl sulfate and other ionic detergents considered to be the harshest and therefore most likely to give the highest yield.

Most Abcam antibodies recognize reduced and denatured protein and should be used under reducing and denaturing conditions. It is important to note though that some antibodies will only recognize a protein in its native, non-denatured form and will not recognize a protein that has been extracted with a denaturing detergent (SDS, deoxycholate, and somewhat less denaturing, Triton X-100 and NP-40).

The main consideration when choosing a lysis buffer is whether the antibody you have chosen will recognize denatured samples. When this is not the case, it will be noted on the antibody datasheet, and buffers without detergent or with relatively mild non-ionic detergents (NP-40, Triton X-100) should be used.

Protein location and lysis buffer choice
For buffer recipes, please see the Buffers section 11 beginning on page 71.

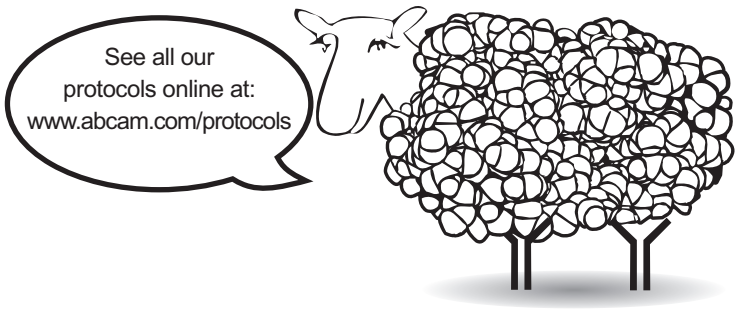
Sample type	Lysis buffer
Whole cell	NP-40 or RIPA
Cytoplasmic (soluble)	Tris-HCl
Cytoplasmic (cytoskeletal bound)	Tris-Triton
Membrane bound	NP-40 or RIPA
Nuclear	RIPA or use nuclear fraction protocol*
Mitochondria	RIPA or use mitochondrial fraction protocol*

*Proteins that are found exclusively or predominantly in a sub-cellular location can be enriched in a lysate of the sub-cellular fraction compared to whole cell or tissue lysates. This can be useful when trying to obtain a signal for a weakly-expressed protein. For instance, a nuclear protein will be a larger proportion of the total protein in a nuclear lysate than it will be in a whole-cell or whole-tissue lysate, making it possible to load more of the protein per gel lane. Another advantage is the removal of potentially cross-reactive proteins present in the unused fractions. Please consult our separate protocols for sub-cellular fractionation.

Nonidet-P40 (NP40) buffer
This is a popular buffer for studying proteins that are cytoplasmic, or membrane-bound, or for whole cell extracts. If there is concern that the protein of interest is not being completely extracted from insoluble material or aggregates, RIPA buffer may be more suitable, as it contains ionic detergents that may more readily bring the proteins into solution.

RIPA buffer (RadioImmunoPrecipitation Assay buffer)
This buffer is also useful for whole cell extracts and membrane-bound proteins, and may be preferable to NP-40 or Triton X-100-only buffers for extracting nuclear proteins than buffers containing only NP-40 or Triton X-100. It will disrupt protein-protein interactions and may therefore be problematic for immunoprecipitations/pull down assays.

In cases where it is important to preserve protein-protein interactions or to minimize denaturation (for example, when it is known that the antibody to be used will only recognize a non-denatured epitope), a buffer without ionic detergents (e.g. SDS) and ideally without non-ionic detergents (e.g. Triton X-100) should be used. Cell lysis with detergent-free buffer is achieved by mechanical shearing, often with a Dounce homogenizer or by passing cells through a syringe tip. In these cases a simple Tris buffer will suffice, but as noted above, buffers with detergents are required to release membrane- or cytoskeleton- bound proteins.



Protease and phosphatase inhibitors

As soon as lysis occurs, proteolysis, de-phosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added **fresh** to the lysis buffer.

Ready-to-use cocktails of inhibitors from various suppliers are available, but you can make your own cocktail.

Inhibitor	Protease/phosphatase inhibited	Final concentration in lysis buffer	Stock (store at -20°C)
Aprotinin	Trypsin, Chymotrypsin, Plasmin	2 μg/ml	Dilute in water, 10 mg/ml. Do not re-use once defrosted.
Leupeptin	Lysosomal	5-10 μg/ml	Dilute in water. Do not re-use once defrosted.
Pepstatin A	Aspartic proteases	1 μg/ml	Dilute in methanol, 1 mM.
PMSF	Serine, Cysteine proteases	1 mM	Dilute in ethanol. You can re-use the same aliquot.
EDTA	Metalloproteases that require Mg++ and Mn++	5 mM	Dilute in H_2O, 0.5 M. Adjust pH to 8.0.
EGTA	Metalloproteases that require Ca++	1 mM	Dilute in H_2O, 0.5 M. Adjust pH to 8.0.
Na Fluoride	Serine/Threonine phosphatases	5-10 mM	Dilute in water. Do not re-use once defrosted.
Na Orthovanadate	Tyrosine phosphatases	1 mM	Dilute in water. Do not re-use once defrosted.

Sodium orthovanadate preparation
All steps to be performed in a fume hood.

1. Prepare a 100 mM sodium orthovanadate solution in double distilled water.
2. Set pH to 9.0 by addition of HCl.
3. Boil until colorless. Minimize volume change due to evaporation by covering loosely.
4. Cool to room temperature.
5. Set pH to 9.0 again.
6. Boil again until colorless.
7. Repeat this cycle until the solution remains at pH 9.0 after boiling and cooling.
8. Bring up to the initial volume with water.
9. Store in aliquots at - 20°C. Discard if samples turn yellow.

Preparation of lysate from cell culture

1. Place the cell culture dish in ice and wash the cells with ice-cold PBS.
2. Drain the PBS, then add ice-cold lysis buffer (1 ml per 10^7 cells/100 mm^2 dish/150 cm^2 flask; 0.5ml per $5x10^6$ cells/60 mm^2 dish/75 cm^2 flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled micro centrifuge tube.
4. Maintain constant agitation for 30 minutes at 4°C.
5. Spin at 16,000 x *g* for 20 minutes in a 4°C pre-cooled micro centrifuge.
6. Gently remove the tubes from the micro centrifuge and place on ice. Transfer the supernatant to a fresh tube kept on ice, and discard the pellet.

Preparation of lysate from tissues

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round-bottom micro centrifuge tubes or Eppendorf tubes and immerse in liquid nitrogen to “snap freeze”. Store samples at -80°C for later use or keep on ice for immediate homogenization.

For a ~5 mg piece of tissue, add ~300 □l lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2 X 300 □l lysis buffer, then maintain constant agitation for 2 hours at 4°C (e.g. place on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present. Protein extract should not be too dilute, to avoid the need to load large volumes per gel lane. The minimum protein concentration is 0.1 mg/ml; optimal concentration is 1-5 mg/ml.

3. Centrifuge for 20 minutes at 16,000 rpm at 4°C in a microcentrifuge for 20 minutes. Gently remove the tubes from the centrifuge and place on ice. Transfer the supernatant to a fresh tube kept on ice; discard the pellet.

The buffer (with inhibitors) should be ice-cold prior to homogenization.

Determination of protein concentration

Perform a Bradford, Lowry, or BCA assay. Bovine serum albumin (BSA) is a frequently-used protein standard.

Once you have determined the concentration of each sample, you can freeze them at -20°C or -80°C for later use or prepare for immunoprecipitation or for loading onto a gel.

Preparation of samples for loading into gels: denatured and native, reduced and non-reduced

a) Denatured, reduced samples

Antibodies typically recognize a small portion of the protein of interest (referred to as the epitope) and this domain may reside within the 3D conformation of the protein. To enable access of the antibody to this portion it is necessary to unfold the protein, i.e. denature it.

To denature the protein, use a loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS), and boil the mixture at 95-100°C for 5 minutes. Heating at 70°C for 5-10 minutes is also acceptable and may be preferable when studying trans-membrane proteins. These tend to aggregate when boiled and the aggregates may not enter the gel efficiently.

The standard loading buffer is called 2 X Laemmli buffer, first described in Nature, 1970 Aug 15;227(5259):680-5. It can also be made at 4 X and 6 X strength to minimize dilution of the samples. The 2 X stock solution is mixed in a 1:1 ratio with the sample.

When SDS is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. SDS denatures proteins by "wrapping around" the polypeptide backbone. SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length i.e. the denatured polypeptides become "rods" of negatively charged clouds with equal charge or charge densities per unit length.

In denaturing SDS-PAGE separations, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. SDS grade is of utmost importance: a protein stained background along individual gel tracts with indistinct protein bands are indicative of old or poor quality SDS.

It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size by adding □-mercaptoethanol or dithiothreitol (DTT).

Glycerol is added to the loading buffer to increase the density of the sample to be loaded and hence maintain the sample at the bottom of the well, restricting overflow and uneven gel loading.

To enable visualization of the migration of proteins it is common to include a small anionic dye molecule (e.g. bromophenol blue) in the loading buffer. The dye will migrate the fastest of any component in the mixture to be separated and provide a migration front to monitor the separation progress.

During protein sample treatment, vortexing before and after the heating step is required for the best resolution.

b) Native and non-reduced samples

Alternatively, an antibody may recognize an epitope made up of non-contiguous amino acids in their native conformation. Although the amino acids of the epitope are separated from one another in the primary sequence, they can be closer to each other in the three-dimensional structure of the protein. The antibody will only recognize the epitope as it exists on the surface of the folded structure.

It is imperative in these circumstances to run a western blot in **non-denaturing** conditions, and this will be noted on the datasheet in the applications section. In general, a non-denaturing condition simply means leaving SDS out of the sample and migration buffers and not heating the samples.

Certain antibodies only recognize protein in its non-reduced form i.e. in an oxidized form (particularly on cysteine residues) and the reducing agents □-mercaptoethanol and DTT must be left out of the loading buffer and migration buffer (**non-reducing conditions**).

Protein state	Gel condition	Loading buffer	Migration buffer
Reduced - Denatured	Reducing & Denaturing	With β-mercaptoethanol or DTT and SDS	With SDS
Reduced - Native	Reducing & Non-Denaturing	With β-mercaptoethanol or DTT, no SDS	No SDS
Oxidized - Denatured	Non-Reducing & Denaturing	No β-mercaptoethanol or DTT, with SDS	With SDS
Oxidized - Native	Non-Reducing & Native	No β-mercaptoethanol or DTT, no SDS	No SDS

Rule of thumb: *Reduce and denature unless the datasheet specifies otherwise.*

5.2. Electrophoresis

Electrophoresis can be one dimensional (i.e. one plane of separation) or two dimensional. One-dimensional electrophoresis is used for most routine protein and nucleic acid separations. Two-dimensional separation of proteins is used for finger-printing (i.e. analysis of total protein content), and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell.

Here we will be describing techniques for one dimensional electrophoresis. We recommend Gel Electrophoresis of Proteins: A Practical Approach (3rd Edition, B.D. Hames and D. Rickwood, The Practical Approach Series, Oxford University Press, 1998) as a reference for basic understanding of 2D electrophoresis protocols.

Preparation of PAGE gels

When separated on a polyacrylamide gel, the procedure is abbreviated as SDS-PAGE (for Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). The technique is a standard means for separating proteins according to their molecular weight.

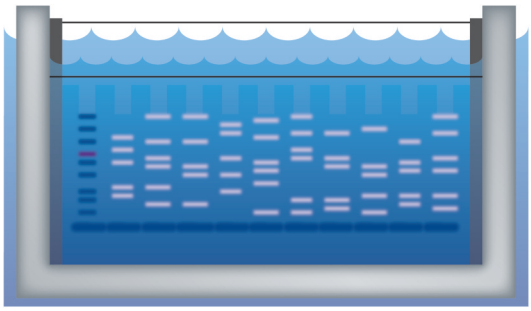
Polyacrylamide gels are formed from the polymerization of two compounds; acrylamide and N,N-methylenebis-acrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups.

The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors: the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the percentage of acrylamide increases, the pore size decreases. With cross-linking 5% C gives the smallest pore size. Any increase or decrease in %C increases or decreases the pore size. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a percentage (w/v) of the acrylamide plus the bis-acrylamide. Thus, a 7.5%T would indicate that there is a total of 7.5 g of acrylamide and bis per 100 ml of gel.

Running the gel **-ve**

Smaller proteins (negatively charged) move more quickly through the gel towards the positive cathode.
Proteins separate out according to size.

+ve



100 V - 150 V for 50 - 90 minutes
Optimize time and voltage
Follow manufacturer's instructions

Gel percentage depends on size of protein:

4-40 kDa	20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
25-200 kDa	8%

Gels can be purchased ready-made from commercial sources or produced in the laboratory (recipes can be found in laboratory handbooks). The percentage of the gel is critical to the rate of migration and degree of separation between proteins.

Rule of thumb: *The smaller the size of the protein of interest, the higher the percentage of mono/bis. The bigger the size of the protein of interest, the lower the percentage of mono/bis.*

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

Acrylamide is a potent cumulative neurotoxin: wear gloves at all times.
Place gels in the electrophoresis tank as instructed by the manufacturer and bathe in migration buffer.

Positive controls

A positive control lysate is used to demonstrate that the protocol is efficient and correct and that the antibody recognizes the target protein which may not be present in the experimental samples.

We strongly recommend the use of a positive control lysate when setting up a new experiment; this will give you immediate confidence in the protocol.

Molecular weight markers

A range of molecular weight markers will enable the determination of the protein size (see below) and allow you to monitor the progress of an electrophoretic run. There are many commercially available MW markers.

Abcam has the following molecular weight markers:

Catalog ID ab48854 (MW 70, 57, 40, 28, 18, 13.5 and 8.5 kDa)
Catalog ID ab115832, Prism Protein Ladder (10-175 kDa)
Catalog ID ab116027, Prism Ultra Protein Ladder (10-180 kDa)
Catalog ID ab116028, Prism Ultra Protein Ladder (10-245 kDa)
Catalog ID ab116029, Prism Ultra Protein Ladder (3.5-245 kDa)

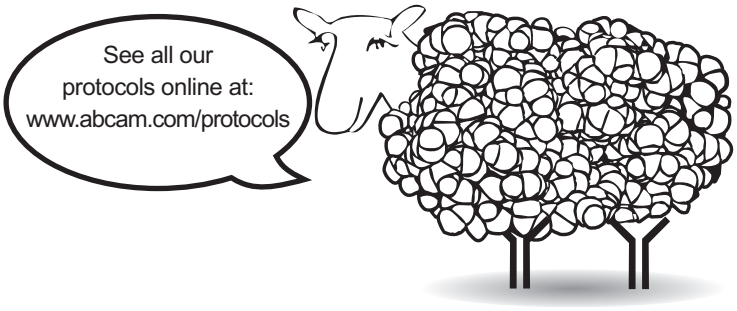
Loading samples and running the gel

Use special gel loading tips or a micro-syringe to load the complete sample in a narrow well. Take care not to pierce the base of the well with the tip as this will create a distorted band.
Never overfill wells, this could lead to poor data if samples spill into adjacent wells and poorly resolved bands.
Load 20-40 □g total protein per mini-gel well.
The gels will be submerged in migration buffer which normally contains SDS, except in native gel electrophoresis.

A standard migration buffer (also called running buffer) for PAGE is 1 X Tris-glycine (See Buffers section beginning on page 71).

Run the gel for the recommended time as instructed by the manufacturer. This can vary from machine to machine (1 hour to overnight depending on the voltage).

When the dye molecule (the “migration front”) reaches the bottom of the gel, the power is turned off. Proteins will slowly elute from the gel at this point, so do not store the gel and proceed immediately to transfer.



Loading controls

Loading controls are required to check that the lanes in your gel have been evenly loaded with sample. This is important especially when a comparison must be made between the expression levels of a protein in different samples. They are also useful to check for even transfer from the gel to the membrane across the whole gel. Where even loading or transfer have not occurred, the loading control bands can be used to quantify the protein amounts in each lane. For publication-quality work, use of a loading control is absolutely essential.

Loading control	Sample type	Molecular weight (kDa)	Caution
Beta Actin	Whole cell / cytoplasmic	43	Not suitable for skeletal muscle samples. Changes in cell-growth conditions and interactions with extracellular matrix components may alter actin protein synthesis (Farmer *et al*, 1983).
GAPDH	Whole cell / cytoplasmic	30-40	Some physiological factors, such as hypoxia and diabetes, increase GAPDH expression in certain cell types.
Tubulin	Whole cell / cytoplasmic	55	Tubulin expression may vary according to resistance to antimicrobial and antimitotic drugs (Sangrajrang S. *et al*, 1998, Prasad V *et al*, 2000).
VDCA1/Porin	Mitochondrial	31	-
COXIV	Mitochondrial	16	Many proteins run at the same 16 kDa size as COXIV.
Lamin B1	Nuclear	66	Not suitable for samples where the nuclear envelope is removed.
TATA binding protein TBP	Nuclear	38	Not suitable for samples where DNA is removed.

5.3. Transfer of proteins and staining

Visualization of proteins in gels

The visualization of protein at this stage is useful to determine if proteins have migrated uniformly and evenly. Use the copper stain if you plan to transfer the separated proteins to a membrane, as the Coomassie stain is not reversible. Only use the Coomassie stain on gels post-transfer to check the efficiency of the transfer, or if you have no plans to transfer and just want to observe the results of the SDS-PAGE separation.

a) Coomassie stain

As soon as the power is turned off the separated protein bands will begin to diffuse as they are freely soluble in aqueous solution. To prevent diffusion, treat the gel with a 40% distilled water, 10% acetic acid, and 50% methanol solution which causes almost all proteins to precipitate (become insoluble). To visualize the fixed proteins, place the gel in the same mixture but with the addition of 0.25% by weight Coomassie Brilliant Blue R-250. Incubate 4 hours to overnight at room temperature on a shaker. Transfer the gel (save the dye mixture; it can be re-used many times) to a mixture of 67.5% distilled water, 7.5% acetic acid, and 25% methanol, place on shaker, and replace with fresh rinse mixture until the excess dye has been removed. The stain will not bind to the acrylamide, and will wash out to leave a clear gel. The bands of proteins in the gel take on a deep blue color from the stain.

b) Copper Stain

Briefly rinse freshly electrophoresed gels in distilled water (30 sec maximum) and then transfer to 0.3 M $CuCl_2$ for 5 to 15 min. Wash the gels for a short time in de-ionized water, and view them against a dark-field background. Proteins come up as clear zones in a translucent blue background.

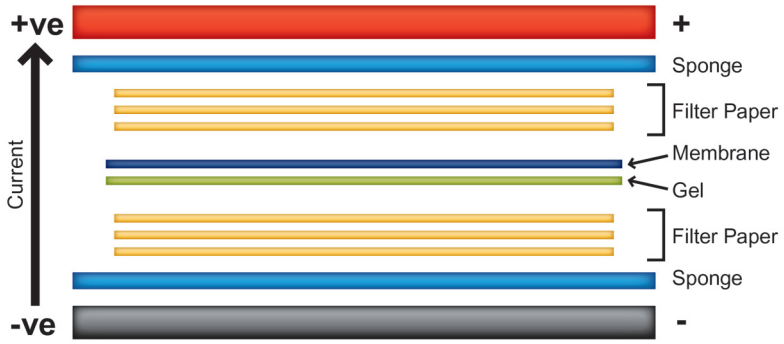
Gels may be destained completely by repeated washing in 0.1- 0.25 M Tris/0.25 M EDTA pH 8.0. Move the gel to a dish of transfer buffer before proceeding with transfer according to the transfer apparatus manufacturer's instructions.

Transfer

1. Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus, and will vary depending on the system. The principle for transfer is the migration of proteins upon application of an electrical charge from the gel onto a membrane.

2. Transfer can be done in wet or semi-dry conditions. Semi-dry transfer is generally faster but wet transfer is less prone to failure due to drying of the membrane and is especially recommended for large proteins, >100 kDa. For both kinds of transfer the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and clamped between solid supports to maintain tight contact between the gel and membrane.

3. In wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring no air bubbles have formed between the gel and membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on. As a guideline, the gel should be run for 1 to 2 hours at 100V. However, this time and voltage may require some optimization. We recommend that you follow manufacturer's instructions.



100 V 60 -120 minutes at 4c
Optimize time and voltage
Follow manufacturer's instructions

Negatively charged proteins move up towards the positive cathode and onto the membrane.

4. A standard buffer for wet transfer is the same as the 1 X Tris-glycine buffer used for the migration/running buffer without SDS but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kDa, it is recommended that SDS is included at a final concentration of 0.1%.

5. In semi-dry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode respectively). As for wet transfer, it is important that the membrane is closest to the positive electrode and the gel closest to the negative electrode. The proportion of Tris and glycine in the transfer buffer is not necessarily the same as for wet transfer; consult the apparatus manufacturer's protocol. A standard recipe is 48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol.

6. Two types of membranes are available: nitrocellulose and PVDF (positively-charged nylon). Both work well. PVDF membranes require careful pre-treatment: cut the membrane to the appropriate size then soak it in methanol for 1-2 minutes. Incubate in ice cold transfer buffer for 5 minutes. The gel needs to equilibrate for 3-5 minutes in ice cold transfer buffer. Failure to do so will cause shrinking while transferring, and a distorted pattern of transfer.

Note on transfer of large and small proteins

The balance of SDS and methanol in the transfer buffer, protein size, and gel percentage can affect transfer efficiency. The following modifications will encourage efficient transfer.

Large proteins (>100 kDa)

1. For large proteins, transfer out of the gel may be very slow, just as they run slowly within the gel during separation. If blotting a large protein, be sure to run your samples in a low-concentration gel, 8% or less. These will be very fragile, so handle carefully.

2. Large proteins will tend to precipitate in the gel, hindering transfer. Adding SDS to a final concentration of 0.1% in the transfer buffer will discourage this. Methanol tends to remove SDS from proteins, so reducing the methanol percentage to 10% or less will also guard against precipitation.

3. Lowering methanol in the transfer buffer also promotes swelling of the gel, allowing large proteins to transfer more easily.

4. Methanol is only necessary if using nitrocellulose. If using PVDF, methanol can be removed from the transfer buffer altogether, and is only needed to activate the PVDF before assembling the gel/membrane sandwich.

5. Choose wet transfer overnight at 4°C instead of semi-dry transfer.

Small proteins (<100 kDa)

1. All proteins are hindered from binding to membranes by SDS, but small proteins more so than large proteins. If your protein of interest is small, consider removing SDS from the transfer buffer.

2. Keep the methanol concentration at 20%.

The following reference discusses a gel and buffer system that allows transfer of proteins as large as 500 kDa:

Bolt and Mahoney, High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate–polyacrylamide, gel electrophoresis. Analytical Biochemistry ***247,*** *185–192 (1997).*

More transfer tips:

Avoid touching the membrane with your fingers; use tweezers instead. Oils and proteins present on fingers will block efficient transfer and create dirty blots.

After sandwiching the gel and membrane between paper, air bubbles between the gel and membrane can be removed by rolling them out with a pipet or 15 ml tube, or by assembling the sandwich in a dish of transfer buffer to prevent formation of bubbles in the first place. Wear gloves!

Make sure the paper and membrane are cut to the same size as the gel. Large overhangs may prevent a current from passing through the membrane in semi-dry transfers.

Chicken antibodies tend to bind PVDF and other nylon-based membranes, leading to high background. Switching to a nitrocellulose membrane should help reduce background staining.

Visualization of proteins in membranes: Ponceau Red

To check the success of transfer, wash the membrane in TBST (for a TBST recipe, see buffers section 11 page 71). Prepare a stock of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid. Incubate on an agitator for 5 minutes. Then dilute the stock 1:10.

Wash the membrane extensively in water until the water is clear and the protein bands are well-defined.

The membrane may be destained completely by repeated washing in TBST or water. When using a PVDF membrane, re-activate the membrane with methanol then wash again in TBST.

Blocking the membrane

Two blocking solutions are traditionally used: non-fat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phospho-proteins (milk contains casein which is a phospho-protein; it causes high background because the phospho-specific antibody detects the casein present in the milk).

Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane.

Some antibodies give a stronger signal on membranes blocked with BSA as opposed to milk. Check the application notes on the datasheet in case there are specific instructions on how to block the membrane.

Incubate in blocking buffer for 1 hour at 4°C with agitation. Rinse for 5 seconds in TBST after the incubation.

Incubation with the primary antibody

Incubation buffer

Dilute the antibody in TBST at the suggested dilution. If the datasheet does not have a recommended dilution test a range (1:100-1:3000) and choose one based on the results. Too much antibody will result in non-specific bands.

It is traditional in certain laboratories to incubate the antibody in blocking buffer, while other laboratories incubate the antibody in TBST without a blocking agent. The results are variable from antibody to antibody and you may find it makes a difference to either use no blocking agent in the antibody buffer or the same agent as the blocking buffer.

If high background is not an issue, some antibodies produce a much stronger signal if diluted in buffer with low concentrations (0.5 – 0.25%) of milk or BSA, or none at all.

Incubation time

The time can vary between a few hours and overnight (rarely more than 18 hours), and is dependent on the binding affinity of the antibody for the protein and its abundance. We recommend a more dilute antibody and a prolonged incubation to ensure specific binding.

Incubation temperature

If incubating in blocking buffer overnight, it is imperative to incubate at 4°C or contamination will cause degradation of the protein (especially phospho groups). Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

Incubation with the secondary antibody

Wash the membrane several times in TBST while agitating for 5 minutes or more per wash to remove residual primary antibody.

Incubation buffer and dilution

Dilute the antibody in TBST at the suggested dilution. If the datasheet does not have a recommended dilution, try a range (1:1000- 1:20,000) and optimize according to the results. Too much antibody will result in non-specific bands.

You may incubate the secondary antibody (and primary antibody) in blocking buffer, but a reduction in background may come at the cost of a weaker specific signal, presumably because the blocking protein hinders binding of the antibody to the target protein.

Incubation time and temperature
1-2 hours at room temperature with agitation.

Which conjugate? We recommend HRP-conjugated secondary antibodies. ALP-conjugated secondary antibodies (alkaline phosphatase) are not recommended as they are less sensitive.

Development methods

Detection kits
For HRP-conjugated antibodies: ECL and ECL+ (home made or commercially available) are the traditional kits used and we recommend ECL+. For the new generation detection machines such as Genegnome, use the detection kit recommended by the manufacturer of the machine.

We do not recommend ECL or BCIP/NBT detection kits as they are not as sensitive.

X-ray films
Manual film development is traditionally used and enables the scientist to control the incubation time of the x-ray film in the developing agent and fixation agent. Automated x-ray film developers are also widely used and easy to use.

Remember that an over-exposed film is not suitable for analysis as determination of the relative amount of protein is not possible. Overexposed films show totally black bands with no contrast, and/or numerous non-specific bands.

Digital images
The new generation of film developers are units with a camera inside an enclosure, removing the need for a darkroom. The camera detects the chemiluminescence emanating from the membrane, transforming the signal into a digital image for rapid analysis with software.

A range of machines are now commercially available. At the front of the next generation are systems which do not use HRP-conjugated antibodies (i.e., chemiluminescence). For example, STORM analyzers detect fluorescence from fluorochrome-conjugated secondary antibodies. The Odyssey Infrared Imaging System detects infrared fluorescence.

5.4. References

Harlow, Ed, and David Lane. Using Antibodies. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1999.

B.D. Hames and D. Rickwood. Gel Electrophoresis of Proteins: A Practical Approach 3rd Edition, The Practical Approach Series, Oxford University Press, 1998.

Bolt and Mahoney, High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate–polyacrylamide, gel electrophoresis. Analytical Biochemistry **247,** 185–192 (1997).

5.5. Troubleshooting tips – Western blotting

No signal

The primary antibody and the secondary antibody are not compatible.
Use secondary antibody that was raised against the species in which the primary was raised (e.g. primary is raised in rabbit, use anti-rabbit secondary).

Not enough primary or secondary antibody is bound to the protein of interest.
Use more concentrated antibody. Incubate longer (e.g. overnight) at 4ºC.

Cross-reaction between blocking agent and primary or secondary antibody.
Use a mild detergent such as Tween 20 or switch blocking reagent (i.e. commonly used blocking reagents are milk, BSA, serum or gelatin).

The primary antibody does not recognize the protein in the species being tested.
Check the datasheet or perform an alignment of the immunogen sequence with the sequence of the protein you are trying to detect to ensure your antibody should react with the target protein. Run the recommended positive control.

Insufficient antigen.
Load at least 20-30 µg protein per lane. Use protease inhibitors. Run the recommended positive control.

The protein of interest is not abundantly present in the tissue.
Use an enrichment step to maximize the signal (e.g. prepare nuclear lysates for a nuclear protein, etc.).

Poor transfer of protein to membrane.
Check the transfer with a reversible stain such as Ponceau S. Check that the transfer was not performed the wrong way. Lift using PVDF membrane making sure you pre-soak the membrane in MeOH then in transfer buffer.

Excessive washing of the membrane.
Do not over wash the membrane.

Too much blocking does not allow you to visualize your protein of interest.
Instead of using 5% milk in the antibody buffers try removing the milk or using 0.5%. Switch blocking reagents or block for less time.

Over-use of the primary antibody.
Use fresh antibody as the effective concentration is lowered upon each re-use of the diluted working solution.

Secondary antibody inhibited by sodium azide.
Do not use sodium azide together with HRP-conjugated antibodies.

Detection kit is old and substrate is inactive.
Use fresh substrate.

High background

Blocking of non-specific binding might be absent or insufficient.
Increase the blocking incubation period and consider changing blocking agent. Abcam recommends 5% non-fat dry milk, 3% BSA, or normal serum for 30 minutes. These can be included in the antibody buffers as well.

The primary antibody concentration may be too high.
Titrate the antibody to the optimal concentration. Incubate for longer but in more dilute antibody (a slow but targeted binding is best).

Incubation temperature may be too high.
Incubate blot at 4°C.

The secondary antibody may be binding non-specifically or reacting with the blocking reagent.
Run a secondary control without primary antibody.

Cross-reaction between blocking agent and primary or secondary.
Add a mild detergent such as Tween 20 to the incubation and washing buffer. Milk contains casein which is a phosphoprotein; this is why it causes high background because the phospho-specific antibody detects the casein present in the milk. Use BSA as a blocking reagent instead of milk.

Washing of unbound antibodies may be insufficient.
Increase the number of washes.

Your choice of membrane may give high background.
Nitrocellulose membrane is considered to give less background than PVDF.

The membrane has dried out.
Care should be taken to prevent the membrane from drying out during incubation. Ensure the membrane is covered with enough buffer at all stages and place on a rotator or on gentle agitation to ensure membrane is gently washed in the solution.

Multiple bands

Cell lines that have been frequently passaged gradually accumulate differences in their protein expression profiles.
Go back to the original non-passaged cell line and run the current and original cell line samples in parallel.

The protein sample has multiple modified forms *in vivo* such as acetylation, methylation, myristylation, phosphorylation, glycosylation etc.
Examine the literature and use an agent to dephosphorylate, de-glycosylate your samples to demonstrate post-translation modifications.

The target in your protein sample has been digested (more likely if the bands are of lower molecular weight).
Make sure that you incorporate sufficient protease inhibitors in your sample buffer.

Unreported novel proteins or different splice variants that share similar epitopes and could possibly be from the same protein family are being detected.
Check the literature for other reports and also perform a BLAST search. Use the cell line or tissue reported on the datasheet.

Primary antibody concentration is too high - at high concentration multiple bands are often seen.
Try decreasing the antibody concentration and/or the incubation period.

Secondary antibody concentration is too high - at high concentration secondaries will bind non-specifically.
Try decreasing the concentration. Run a secondary antibody control (without the primary).

The antibody has not been purified.
Try to use affinity purified antibody. This will often remove non-specific bands.

The bands may be non-specific.
Where possible use blocking peptides to differentiate between specific and non-specific bands. Only specific bands should be blocked and thus disappear.

The protein target may form multimers.
Try boiling in SDS-Page for 10 minutes rather than 5 minutes to disrupt multimers.

Uneven white "spots"on the blot

Air bubbles were trapped against the membrane during transfer or the antibody is not evenly spread on the membrane.
Make sure you remove bubbles when preparing the gel for transfer. Incubate antibodies under agitation.

Black dots on the blot

The antibodies are binding to the blocking agent.
Filter the blocking agent.

White bands on a black blot (negative of expected blot)

Too much primary and/or too much secondary antibody.
Dilute the antibodies more.

MW marker lane is black

The antibody is reacting with the MW marker.
Add a blank lane between the MW marker and the first sample lane.

The band of interest is very low/high on the blot

Separation is not efficient.
Change the gel percentage: a higher percentage for small protein, lower percentage for large proteins.

Smile effect of the bands

Migration was too fast or migration was too hot (changing the pH and altering the migration).
Slow down the migration or run the gel in the cold room or on ice.

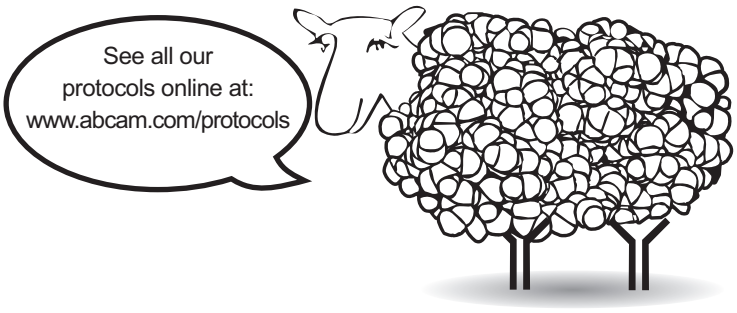
Uneven band size in lanes probed for the same protein

Gel has set too quickly while casting and the acrylamide percentage is not even along the lanes.
Review the recipe of the gel and the addition of TEMED to the gels, add a little 0.1% SDS in water to the top of the migrating gel while it sets to stop it from drying.

Uneven staining of the gel

Contamination from bacteria.
Keep antibodies at 4°C and use fresh buffers to cover the gel.

Not enough antibody.
Make sure the membrane is covered with the antibody/incubate under agitation.



Further useful western blot protocols

There are many more useful western blot procedures and guides on our protocols web pages. Please see the following URL addresses for more information:

Blocking with immunizing peptide protocol
www.abcam.com/index.html?pageconfig=resource&rid=11378

De-phosphorylation of proteins protocol
www.abcam.com/index.html?pageconfig=resource&rid=11407

Histone blotting protocol
www.abcam.com/index.html?pageconfig=resource&rid=11409

Histone extraction protocol
www.abcam.com/index.html?pageconfig=resource&rid=11410

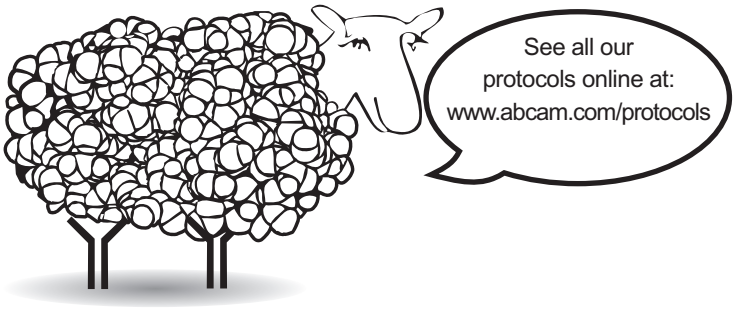
Mitochondrial purification protocol
www.abcam.com/index.html?pageconfig=resource&rid=11411

Nuclear fractionation protocol
www.abcam.com/index.html?pageconfig=resource&rid=11408

Phospho-proteins protocol
www.abcam.com/index.html?pageconfig=resource&rid=11406

Soluble (S-100) fractionation protocol
www.abcam.com/index.html?pageconfig=resource&rid=11412

Stripping for reprobing protocol
www.abcam.com/index.html?pageconfig=resource&rid=11353



SECTION 6: Immunohistochemistry and Immunocytochemistry

6.1 IHC-Paraffin Protocol (IHC-P)

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections. Though less sensitive quantitatively than immunoassays such as western blotting or ELISA, it enables the observation of processes in the context of intact tissue. This is especially useful for assessing the progression and treatment of diseases such as cancer. In general, the information gained from IHC combined with microscopy literally provides a “big picture” that can help make sense of data obtained using other methods.

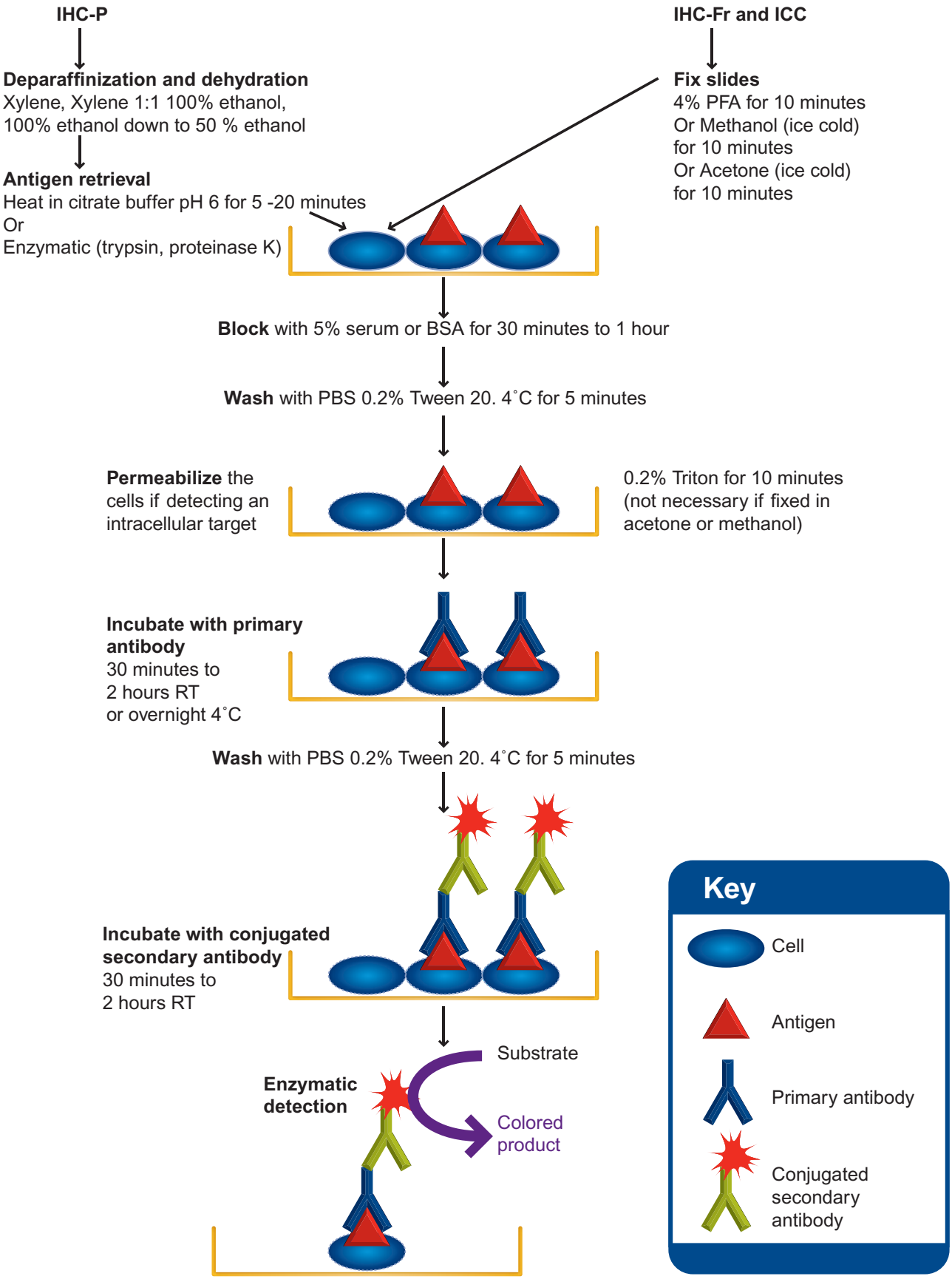
Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorochrome is conjugated to the antibody and can be visualized using fluorescence microscopy.

IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned. The basic steps of the IHC-P protocol are as follows:

1. Fixing and embedding the tissue
2. Cutting and mounting the section
3. Deparaffinizing and rehydrating the section
4. Antigen retrieval
5. Immunohistochemical staining
6. Counterstaining (if desired)
7. Dehydrating and stabilizing with mounting medium
8. Viewing the staining under the microscope

Contents:

Immunohistochemical staining



Optimization of a new antibody for IHC-P

When using a new antibody in IHC-P, the antibody must be tested to find the optimal staining conditions. Each antigen has a preferred method of antigen retrieval, and each antibody has an optimal dilution.

Antigen retrieval

Try staining without antigen retrieval, and also using the following antigen retrieval methods. Detailed protocols for these procedures are in the Antigen retrieval section on this page.

1. Heat induced: Sodium citrate 10 mM, pH 6.0
2. Heat induced: Tris/EDTA pH 9.0
3. Enzymatic: trypsin, pepsin, or other protease

Once the optimal antigen retrieval method is established, the antibody concentration can be fine tuned.

Primary antibody concentration

If the concentration of the antibody is provided, we recommend trying 0.5 µg/ml and 5 µg/ml overnight at 4°C. If the antibody is unpurified, we recommend starting with the following starting dilutions, and also testing a 20-fold higher dilution.

1. Whole antiserum: 1/50
2. Ascites: 1/100
3. Tissue culture supernatant: undiluted (also referred to as “neat”)

Detection

We recommend horseradish peroxidase (HRP) for visible light microscopy. Peroxide/DAB are the substrate and recommended chromogen for horseradish peroxidase. Various fluorochrome conjugated antibodies are available for fluorescent microscopy; choice will be dictated by the needs of the experiment.

Fixation

Proper fixation is key for the success of immunohistochemistry. Ten percent neutral buffered formalin (NBF) is most commonly used. Where Abcam’s datasheets state IHC-P as a tested application, this fixative has been used unless stated otherwise. Other fixatives such as paraformaldehyde (PFA) or Bouin solution (formalin/picric acid) are used less frequently. Recipes for these fixatives can be found in the Buffers section beginning on page 71.

The ideal fixation time will depend on the size of the tissue block and the type of tissue, but fixation between 18-24 hours seems to be ideal for most applications. Under-fixation can lead to edge staining, with strong signal on the edges of the section and no signal in the middle; over-fixation can mask the epitope. Antigen retrieval can help overcome this masking, but if the tissue has been fixed for a long period of time (i.e. over a weekend), there may be no signal even after antigen retrieval.

After fixation, the tissue block is embedded in paraffin, then cut in a microtome to the desired thickness (approximately 5 to 20 microns is ideal for IHC depending on the tissue) and affixed onto the slide. Tissue sections are best mounted on positively charged or APES (amino-propyl-tri-ethoxy-silane) coated slides. Once mounted, the slides should be dried to remove any water that may be trapped under the section. This can be done by leaving the slide at room temperature overnight. If there is a problem with the section adhering to the slide, you may also incubate the slide at 60ºC for a few hours.

Deparaffinization

Before proceeding with the staining protocol, the slides must be deparaffinized and rehydrated. Incomplete removal of paraffin can cause poor staining of the section.

Materials and reagents

- Xylene
- 100% ethanol
- 95% ethanol

Method

Place the slides in a rack, and perform the following washes:

1. Xylene: 2 X 3 minutes
2. Xylene 1:1 with 100% ethanol: 3 minutes
3. 100% ethanol: 2 X 3 minutes
4. 95% ethanol: 3 minutes
5. 70 % ethanol: 3 minutes
6. 50 % ethanol: 3 minutes
7. Running cold tap water to rinse

Keep the slides in the tap water until ready to perform antigen retrieval. At no time from this point onwards should the slides be allowed to dry. Drying out will cause non-specific antibody binding and therefore high background staining.

Antigen retrieval

Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. The two methods of antigen retrieval are heat-mediated (also known as heat-induced epitope retrieval, or HIER) and enzymatic.

Both antigen retrieval methods serve to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind. Some antigens prefer enzymatic to heat-mediated antigen retrieval and vice versa. Enzymatic retrieval can sometimes damage the morphology of the section, so the concentration and treatment time need to be tested. Antigen retrieval with Tris/EDTA pH 9.0 buffer is suitable for most antigens. Sodium citrate pH 6.0 is also widely used. For an explanation of why Abcam recommends using Tris/EDTA pH 9.0 buffer before sodium citrate pH 6.0. We can recommend reviewing the following useful website:

www.nordiqc.org/Techniques/Epitope_retrieval.htm

Heat-induced epitope retrieval is most often performed using a pressure cooker, a microwave, or a vegetable steamer. Additionally, some labs will use a water bath set to 60°C and incubate the slides in retrieval solution overnight. Unless the antigen retrieval method is stated on the antibody datasheet, the optimal method for each antigen must be found experimentally. Abcam recommends testing several methods to find the retrieval that gives optimal staining.

1. Buffer solutions for heat induced epitope retrieval
The following solutions are three of the more popular buffers for HIER. In the absence of advice from other researchers for a particular antibody, choice of retrieval buffer is best accomplished by experiment.

1. Sodium Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0)
2. 1 mM EDTA, adjusted to pH 8.0
3. Tris/EDTA Buffer (10mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)

2. Heat-induced epitope retrieval methods
a) Pressure cooker
Slides should be placed in a metal rack for this procedure.

Materials and reagents
• Domestic stainless steel pressure cooker
• Hot plate
• Vessel with slide rack to hold approximately 400-500 ml
• Antigen retrieval buffer (i.e. Tris/EDTA pH 9.0, sodium citrate pH 6.0)

Method
1. Add the appropriate antigen retrieval buffer to the pressure cooker. Place the pressure cooker on the hotplate and turn it on full power. Do not secure the lid of the pressure cooker at this point, simply rest it on top.
While waiting for the pressure cooker to come to a boil, deparaffinize and rehydrate the sections as described above.

2. Once boiling, transfer the slides from the tap water to the pressure cooker. TAKE CARE WITH HOT SOLUTION - USE FORCEPS! Secure the pressure cooker lid as in the manufacturer's instructions.

3. As soon as the cooker has reached full pressure (see the manufacturer's instructions), time 3 minutes (See note 2).

4. When 3 minutes has elapsed, turn off the hotplate and place the pressure cooker in an empty sink.

5. Activate the pressure release valve (see the manufacturer's instructions) and run cold water over the cooker. Once de-pressurized, open the lid and run cold water into the cooker for 10 minutes. TAKE CARE WITH HOT SOLUTION! (See note 2).

6. Continue with the immunohistochemical staining protocol.

Notes:
1. Three minutes is only suggested as a starting point antigen retrieval time. Less than 3 minutes may leave the antigens under-retrieved, leading to weak staining. More than 3 minutes may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides. A control experiment is recommended beforehand, where slides of the same tissue section are retrieved for 1, 2, 3, 4 and 5 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.

2. This is to allow the slides to cool enough so they may be handled, and to allow the antigenic site to re-form after being exposed to such high temperature.

b) Microwave
The use of a domestic microwave is inadvisable. Hot and cold spots are common, leading to uneven antigen retrieval. Antigen retrieval times are usually longer, due to the absence of a pressurized environment, nearly always leading to section dissociation. A scientific microwave is much more appropriate. Most brands have onboard pressurized vessels and can keep the temperature at a constant 98°C to avoid section dissociation. The only drawback is the expense of purchasing one!

When using this method, it is possible for the buffer to boil over, and a large amount of the retrieval buffer will evaporate. Be sure to watch the buffer level of the slide vessel, and add more buffer if necessary. Do not allow the slides to dry out.

Slides should be placed in a plastic rack and vessel for this procedure. Standard glass histology staining racks and vessels will crack when heated.

Materials and reagents
• Domestic (850W) or scientific microwave
• Microwaveable vessel with slide rack to hold approximately 400-500 ml or Coplin jar
• Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc.)

Method
1. Deparaffinize and rehydrate the sections as described above.

2. Add the appropriate antigen retrieval buffer to the microwaveable vessel (See note 1).

3. Remove the slides from the tap water and place them in the microwaveable vessel. Place the vessel inside the microwave. If using a domestic microwave, set to full power and wait until the solution comes to the boil. Boil for 20 minutes from this point. If using a scientific microwave, program so that antigens are retrieved for 20 minutes once the temperature has reached 98°C. (See note 2).

4. When 20 minutes has elapsed, remove the vessel and run cold tap water into it for 10 minutes. TAKE CARE WITH HOT SOLUTION! (See note 3).

5. Continue with the immunohistochemical staining protocol.

Notes:
1. Use a sufficient volume of antigen retrieval solution in order to cover the slides by at least a few centimeters if using a non-sealed vessel to allow for evaporation during the boil. Be sure to watch for evaporation and for boiling over during the procedure, and do not allow the slides to dry out!

2. Twenty minutes is only a suggested antigen retrieval time. Less than 20 minutes may leave the antigens under-retrieved, leading to weak staining. More than 20 minutes may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides. A control experiment is recommended beforehand, where slides of the same tissue section are retrieved for 5, 10, 15, 20, 25 and 30 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.

3. This allows the slides to cool enough so they may be handled, and allows the antigenic site to reform after being exposed to high temperature.

c) Vegetable steamer
Many labs use a vegetable steamer or rice cooker for heat-mediated antigen retrieval. The procedure is similar to microwaving in that it maintains the temperature of the buffer at 100°C, but without the vigorous boiling of the microwave method. This method may be adapted to a water bath set at 100°C in place of the steamer.

Slides should be placed in a plastic or metal rack and vessel for this procedure. Standard glass histology staining racks and vessels will crack when heated.

Materials and reagents
• Vegetable steamer
• Vessel with slide rack to hold approximately 400-500 ml (or 250 ml if using Tissue –Tek containers)
• Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc.)

Method
1. Deparaffinize and rehydrate the sections as described above.

2. Set up the vegetable steamer according to the manufacturer's instructions and pre-heat.

3. Pre-heat the appropriate antigen retrieval buffer to boiling in a flask (a microwave is handy for this).

4. Put the container that will hold the rack of slides into the vegetable steamer.

5. Carefully add the hot buffer to the container, followed by the rack of slides. If more convenient, add the buffer to the container before placing the container in the steamer.

6. Close the lid of the steamer. The container of buffer should also have a lid. The rack of slides will initially bring the temperature of the antigen retrieval solution down but it will return to 95-100°C within several minutes.

7. Keep the container in the steamer for 20 minutes from this point. (See note 2 for the microwave method).

8. When 20 minutes has elapsed, remove the vessel and run cold tap water into it for 10 minutes. TAKE CARE WITH HOT SOLUTION! (See note 3 for the microwave method).

9. Continue with the immunohistochemical staining protocol.

3. Enzymatic antigen retrieval
Choice of enzyme will be indicated on the datasheet for the antibody. If not, trypsin has been shown to be useful for a wide range of antigens that require retrieval post formalin/PFA fixation.

There are at least two methods for applying the enzyme solution to the tissue: directly pipetting the solution onto the tissue on the slide, or placing a rack of tissue slides into a container of enzyme solution. The first method uses less reagent, but since each slide needs to be handled individually, the incubation time needs to be monitored carefully to ensure all slides are receiving the same treatment. For this reason, it is easier to treat large batches of slides (e.g. > 5) by immersing them in a container of enzyme solution. If using an automated staining system (e.g. Ventana), consult the manufacturer for an appropriate enzymatic retrieval protocol.

a) Pipetting method
Materials and reagents
• 37°C incubator
• Humidified chamber (either the incubator itself or a container with a wet paper towel)
• Two slide rack containers of TBS (See Buffers section beginning on page 71 for TBS recipe)
• Enzymatic antigen retrieval solution (For trypsin, see below. For pepsin and proteinase K, see buffers section on page 71)

The following method uses trypsin. There are commercially available trypsin preparations optimized for IHC (Abcam has a convenient trypsin product, catalogue ID ab970), or it can be prepared as described in the Buffers section beginning on page 71.

Method
1. Prepare the trypsin and preheat to 37°C. Carefully blot excess water from around the tissue section and pipette the enzyme solution (generally 50-100 µl will suffice) onto the section. It may be necessary to spread the solution around the section with the pipette tip; be careful not to damage the tissue.

2. Place the slides in a humidified container and then into the 37°C incubator. Avoid placing the slides directly on the incubator shelves as there will be variations in temperature that could affect staining intensity. Ideally, the container holding the slides is pre-heated in the incubator.

3. After 10-20 minutes (this will need to be optimized), remove the slides from the incubator and transfer to a rack in a container of tap water. Rinse by running tap water for 3 minutes.

4. Continue with the immunohistochemical staining protocol.

b) Immersion method
Materials and reagents
• 37°C water bath
• Slide racks and slide rack containers
• Enzymatic antigen retrieval solution (For trypsin, see pipetting method. For pepsin and proteinase K, see Buffers section on page 71).

Method
1. Set water bath to the optimal temperature for the enzyme you are using. Add ultrapure water to two containers that can hold slide racks. Place the containers into the water bath to warm. (See note 2).

2. Deparaffinize and rehydrate sections described as above. Place slides in one water container to warm (See note 3).

3. Prepare the enzymatic antigen retrieval buffer from the warm water in the other container, and then return the container to the water bath to allow the solution to reheat (See note 4).

4. Transfer the warmed slides into the enzyme solution for 10-20 minutes (See note 5) with intermittent gentle agitation, then remove the slides and place them in running tap water for 3 minutes to rinse off the enzyme.

5. Continue with the immunohistochemical staining protocol.

Notes:
1. Be sure to read the manufacturer's literature for the enzyme you choose, as some enzymes require specific buffers and cofactors for activity.

2. Use a sufficient volume of water or buffer to cover the slides.

3. Placing cold slides into the enzyme solution will lower the temperature of the solution, reducing enzyme activity and leading to under-retrieval of the antigenic site.

4. Prepare the enzymatic antigen retrieval solution as quickly as possible to avoid impairing the activity of the enzyme. Allow this solution to return to required temperature before introducing the slides.

5. Ten to twenty minutes is only suggested as a starting point incubation time. Less than 10 minutes may leave the antigens under-retrieved,

leading to weak staining. More than 20 minutes may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides or damage to the morphology of the tissue. A control experiment is recommended beforehand, where slides of the same tissue section are incubated in the enzyme solution for 10, 15, 20, 25, and 30 minutes before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.

Immunohistochemical staining

1. General guidelines

The following protocol assumes the laboratory does not have an automated stainer or other capillary gap system that allows rapid application and rinsing of reagents (e.g. Shandon Sequenza). Reagents can be applied manually by pipette or protocol can be adapted to automated and semi-automated systems if these are available.

All incubations should be carried out in a humidified chamber to avoid drying of the tissue. Drying at any stage will lead to non-specific binding and ultimately high background staining. A shallow, plastic box with a sealed lid and wet tissue paper in the bottom is an adequate chamber, as long as the slides are kept off the paper and can lay flat so that the reagents don't drain off! A good solution is to cut a plastic serological pipette into lengths to fit your incubation chamber. Glue them in pairs to the bottom of the chamber, with the two individual pipette tubes of each pair being placed about 4 cm apart. This provides a level and raised surface for the slides to rest on away from the wet tissue paper.

Dilutions of the primary and secondary antibody are listed on the datasheets or are determined by testing a range. Adjust dilutions appropriately from the results obtained. Adhere strictly to all incubation times in the protocol.

For enzymatic methods, horseradish peroxidase (HRP) or alkaline phosphatase (AP) are the most commonly used enzymes. There are a number of chromogens used with these enzymes.

2. Protocol

Please refer to Buffers section 11 on page 71 for recipes. If necessary, perform antigen retrieval before commencing with the following protocols.

Day 1

1. If using an HRP conjugate for detection, blocking of endogenous peroxidase can be performed here but we recommend waiting until after the primary antibody incubation.

2. Wash the slides 2 X 5 minutes in TBS plus 0.025% Triton X-100 with gentle agitation.

3. Block in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper.

4. Apply primary antibody diluted in TBS with 1% BSA.

5. Incubate overnight at 4°C.

Day 2

1. Rinse 2 X 5 minutes in TBS 0.025% Triton X-100 with gentle agitation.

2. If using an HRP conjugate for detection, incubate the slides in 0.3% H_2O_2 in TBS for 15 minutes to block endogenous peroxidase.

3. For enzymatic detection (HRP or AP secondary conjugates):

Apply enzyme-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubate for 1 hour at room temperature.

For fluorescent detection:

Apply fluorochrome-conjugated secondary antibody to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubate for 1 hour at room temperature.

This step should be done in the dark to avoid photobleaching.

4. Rinse 3 X 5 minutes in TBS.
If using fluorescent detection, end at this step and use a mounting medium to mount the tissue with a cover slip.
If visualizing the protein with a chromogen, continue with the following steps.

5. Develop with chromogen for 10 minutes at room temperature.

6. Rinse in running tap water for 5 minutes.

7. Counterstain (if required).

8. Dehydrate, clear and mount.

3. Controls
To estimate the contribution of the non-specific interaction and Fc receptor binding, staining protocols using an antibody directed to an irrelevant antigen (e.g. BrdU) having the same isotype as the antibody of interest may be analyzed in parallel with the antibody of interest. The antibody directed to the irrelevant antigen is known as the isotype control. For whole serum antibodies, use normal serum from a non-immunized animal of the same species as the primary antibody.

If an isotype control is not available, a negative antibody control is recommended. Simply replace the primary antibody with antibody diluent.
A positive tissue control is strongly recommended to ensure that the antibody is performing as expected. Depending on the experiment, it may also be useful to include a negative tissue control; a tissue in which the protein of interest is not expected to be found.

The use of 0.025% Triton X-100 in the TBS helps reduce surface tension, allowing reagents to cover the whole tissue section with ease. It is also believed to dissolve Fc receptors, therefore reducing non specific binding. Abcam recommends TBS to give a cleaner background than PBS.

Notes:
1. The secondary antibody may cross react with endogenous immunoglobulins in the tissue. This is minimized by pre-treating the tissue with normal serum from the species in which the secondary was raised. The use of normal serum before the application of the primary antibody also eliminates Fc receptor binding of both the primary and secondary antibodies. BSA is included in the antibody dilution buffer to reduce non-specific binding caused by hydrophobic interactions.

If the tissue samples are fixed with an aldehyde fixative such as formalin, paraformaldehyde or glutaraldehyde and immunofluorescence (IF) is the detection method, consider including 0.3 M glycine in the blocking buffer before applying the primary antibody. Glycine will bind free aldehyde groups that would otherwise bind the primary and secondary antibodies and lead to high background. Background staining due to free aldehyde groups is more likely to occur when the fixative is glutaraldehyde or paraformaldehyde.

2. The primary antibody should be diluted to the manufacturer's recommendations or to a previously optimized dilution. If there is no suggested starting point, we recommend following the recommendations. Most antibodies will be used in IHC-P at a concentration between 0.5 and 10 µg/ml. Make sure the primary antibody is raised in a species different from the tissue being stained.
If, for example, you had mouse tissue and your primary antibody was raised in a mouse, an anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue, leading to high background. Use of mouse monoclonals on mouse tissue is discussed in our mouse-on-mouse protocol.

3. Overnight incubation allows antibodies of lower affinity more time to bind to the antigen. However, regardless of the antibody's affinity for its target, once the binding has reached saturation point or equilibrium, no more binding can take place. Overnight incubation ensures that binding reaches equilibrium.

4. Peroxide (H_2O_2) suppresses endogenous peroxidase activity and therefore reduces background staining. To check for the presence of endogenous peroxidases, incubate a tissue slide after rehydration in a solution of DAB (3,3' Diaminobenzidine substrate). If areas of the section appear brown under the microscope, peroxidase is present, and a blocking step should be included. Some epitopes are modified by peroxide, leading to reduced antibody:antigen binding. Incubating sections with peroxide after the primary incubation avoids this problem.

Peroxide can be diluted in TBS or water. Some laboratories use methanol which is useful for blood smears or other peroxidase rich tissues such as liver. Peroxide diluted in methanol tends to reduce damage to the tissue caused by the reaction in aqueous solutions. For other tissue though, we recommend diluting in TBS or water. Reduced binding of some antibody:antigen pairs, in particular cell surface proteins, has been observed after methanol/peroxide incubation.

Blocking of endogenous peroxidases is only required for peroxidase conjugates such as HRP.

5. Develop the colored product of the enzyme with the appropriate chromogen. The choice depends on which enzyme label you are using, the colored end product you prefer and whether you are using aqueous or organic mounting media. Some commonly used substrates are listed below:

Enzyme	Substrate	Color	Advantages	Disadvantages	Abcam ID
Horseradish peroxidase (HRP)	3,3'-Diaminobenzidine (DAB)	Brown	Intense color; permanent	Endogenous peroxidase activity in the tissue can lead to false positive staining. AEC is alcohol soluble and incompatible with organic mounting media.	ab675
	3-Amino-9-ethyl carbazol (AEC)	Red	Intense color; contrasts well with blue for double staining		
Alkaline phosphatase (AP)	5-bromo-4-chloro-3-indoyl phosphate; Nitroblue tetrazolium (BCIP/NBT)	Blue	Intense color	Endogenous alkaline phosphatase activity in the tissue can lead to false positive staining.	ab7413 ab7468
	Vector Blue	Blue	Less intense color, but better for double staining		
Glucose oxidase	Nitroblue tetrazolium (NBT)	Blue	No endogenous enzyme activity	Low staining intensity (high concentration of primary and secondary antibodies required for effectiveness)	

6. Some commonly used counterstains to observe tissue and cell morphology are hematoxylin (blue), nuclear fast red, or methyl green. When using fluorescent detection, DAPI (blue) or propidium iodide/PI (red) can be used to counterstain.

7. Don't forget that DAB is a suspected carcinogen. Wear the appropriate protective clothing. Deactivate it with sodium hypochlorite in a sealed container overnight (it produces noxious fumes) and dispose of it according to laboratory guidelines.

If using AP, add 0.24 mg/ml Levamisole to the chromogen solution. Levamisole suppresses endogenous phosphatase activity and therefore reduces background staining.

8. If using AEC, Fast Red, INT or any other aqueous chromogen then don't forget that they are alcohol soluble. Use a suitable aqueous mounting media. Don't dehydrate and clear as in step 9!

9. Dehydrate and clear DAB, New Fuchsin, Vega Red, NBT, TNBT or any other organic chromogen developed sections by sending them through the rehydration protocol listed in the opposite order.

1. Place slides in 50% ethanol for 3 minutes.
2. 70% ethanol for 3 minutes.
3. 95% ethanol for 3 minutes.
4. 100% ethanol for 3 minutes.
5. Xylene 1:1 with 100% ethanol for 3 minutes.
6. Xylene 2 X 3 minutes.

Mount sections in a suitable organic mounting media. Sections mounted in organic mounting media have a better refractive index than those mounted in aqueous mounting media. This means that the image seen under the microscope will be sharper and clearer.

4. Signal amplification
To achieve a stronger signal, various strategies have been developed to add more enzyme or fluorochrome to the target of interest.

a) Avidin-biotin complex (ABC)
This technique, developed by Su-Ming Hsu and colleagues (J Histochem Cytochem. 1981 Apr 29 (4):577-80), utilizes the high affinity of avidin, a protein found in chicken egg white, for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible.

In brief, the primary antibody is bound to the protein of interest. A biotinylated secondary antibody is then bound to the primary antibody. In a separate reaction, a complex of avidin and biotinylated enzyme is formed by mixing the two in a ratio that leaves some of the binding sites on avidin unoccupied. This complex is then incubated with the tissue section after the antibody incubations. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody. The result is more enzyme attached to the target than is possible using an enzyme-conjugated secondary or primary antibody.

The components of the avidin-biotin complex are commercially available in kits that provide the two reagents and instructions for combining them in the optimal ratio. The complex can be used with any of Abcam's biotinylated antibodies. One concern is the presence of endogenous biotin in tissues such as kidney, liver, brain, prostate, colon, intestines, and testes, which can bind the avidin-biotin complex leading to background staining. (Wang and Pevsner, Cell Tissue Res. 1999 Jun;296(3):511-6.) To block binding to endogenous biotin, Abcam offers a blocking kit, ab3387.

b) Labeled streptavidin biotin (LSAB)
This method is similar to ABC in that it utilizes the interaction of streptavidin (similar to avidin in binding affinity) and biotin. The primary antibody is followed by a biotinylated anti-Ig secondary antibody, followed by streptavidin conjugated to an enzyme or fluorochrome. Abcam offers a streptavidin – HRP conjugate, ab7403.

Streptavidin produces less non-specific background staining than avidin since it is non glycosylated (unlike avidin). Consequently, it does not interact with lectins or other carbohydrate binding proteins. LSAB was shown to be 4 to 8 times more sensitive than ABC in the following publication: see Giorno R, Diagno Immunol. 1984;2(3):161-6.

c) HRP polymer
Both avidin-biotin methods (ABC and LSAB) are losing favor to new polymer-enzyme-antibody products that consist of a secondary antibody (e.g. anti-mouse and/or rabbit IgG) attached to a polymer-enzyme complex. One step is eliminated compared to the avidin-biotin methods and the issue of endogenous biotin is avoided.

d) Tyramide signal enhancing (TSE)
One of the most effective amplification procedures is the patented and licensed method, TSE (also known as TSA or CSA, depending on the manufacturer of the commercially available kits). It is particularly useful for detection of relatively sparse antigens that other systems have difficulty detecting, and for improving results obtained with poorly performing antibodies.

The method relies on a peroxidase-catalyzed reaction to covalently attach the tyramide portion of tyramine-protein conjugates to the antibody, after first applying a primary antibody and secondary HRP conjugate. The covalently attached protein cannot be washed off, even if the slides are treated to remove the antibodies, since the tyramide bond is covalent. To obtain a signal, an antibody enzyme or fluorochrome conjugate is directed against the protein portion of the tyramine protein conjugate. In one commercially available version of the method, the protein is biotin and a streptavidin enzyme conjugate is applied instead of an antibody conjugate. The disadvantages of the procedure are the expense of the kits and the time required to perform the multiple steps.

Resources
IHC World (www.ihcworld.com) has a wealth of information on antigen retrieval, positive controls, and troubleshooting the immunohistochemical procedure.

Histonet (www.histosearch.com/histonet.html) searches archived messages posted to the Histonet list server from scientists around the world.

Nordic immunohistochemical Quality Control (www.nordiqc.org) has information regarding appropriate positive controls and antigen retrieval steps for many target proteins.

6.2 Immunohistochemistry (IHC-Fr)-Frozen sections protocol
Frozen sections: Once mounted on APES coated slides, frozen sections are best kept at -80°C until needed.

1. When required, leave to warm at room temperature for 5 minutes.

2. Pre-cool the fixative (acetone, methanol or ethanol) at -20°C for 30 minutes. (Abcam recommends starting with acetone).

3. Fix with the pre cooled fixative for 5-10 minutes, at room temperature.

4. Rinse 3-4 X in PBS.

5. Continue with the immunohistochemical staining protocol.

The absence of a formaldehyde based fixative eliminates the need for an antigen retrieval step. However, if frozen tissue or cytological specimens have been fixed in formalin, antigen retrieval can be attempted, although the friable nature of the specimens, in particular brain tissue, may compromise the success. The following reference describes a protocol in which slides are treated with 3-aminopropyltriethoxysilane (APES) before placing sections on the slides. This treatment improved adhesion, allowing heat mediated antigen retrieval with minimal damage to the tissue morphology:

Warembourg M, Leroy D., Microwave pretreatment of sections to improve the immunocytochemical detection of progesterone receptors in the guinea pig hypothalamus. J Neurosci Methods. 2000 Dec 15;104(1):27-34.

A more thorough discussion of antigen retrieval applied to frozen tissue sections is found in the following reference:

Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde fixed fresh frozen sections. J Histochem Cytochem. 2005 Nov;53(11):1421-32.

If the tissue samples are fixed with an aldehyde fixative such as formalin, paraformaldehyde or glutaraldehyde and immunofluorescence (IF) is the detection method, consider including 0.3 M glycine in the blocking buffer, before applying the primary antibody. Glycine will bind free aldehyde groups that would otherwise bind the primary and secondary antibodies, leading to high background. Background due to free aldehyde groups is more likely to occur when the fixative is glutaraldehyde or paraformaldehyde.

See IHC-Paraffin protocol (IHC-P) for detailed protocols for chromogenic and fluorescent detection.

6.3 Immunocytochemistry (ICC) protocol

General procedure

1. Coat coverslips with polyethylineimine or poly-L-lysine for 1 hour at room temperature.

2. Rinse coverslips well with sterile H_2O_2 (3 X 5 minutes each).

3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 hours.

4. Grow cells on glass coverslips or prepare cytospin or smear preparation.

5. Rinse briefly in phosphate buffered saline (PBS).

Fixation

1. Fix the samples either in ice-cold methanol, acetone (1-10 minutes). Or fix in 3-4% paraformaldehyde in PBS pH 7.4 for 15 minutes at room temperature.

2. Wash the samples twice with ice cold PBS.

Permeabilization

If the target protein is expressed intracellularly, it is very important to permeabilize the cells. Note: acetone fixed samples do not require permeabilization.

3. Incubate the samples for 10 minutes in PBS containing 0.25% Triton X-100 (or 100 □M digitonin or 0.5% saponin). Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for the use of membrane-associated antigens since it destroys membranes.

4. Wash cells in PBS 3 X 5 minutes.

Blocking and incubation

5. Incubate cells with 1% BSA in PBST for 30 minutes to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species in which the secondary antibody was raised).

If the tissue samples are fixed with an aldehyde fixative such as formalin, paraformaldehyde or glutaraldehyde and immunofluorescence (IF) is the detection method, consider including 0.3 M glycine in the blocking buffer. Glycine will bind free aldehyde groups that would otherwise bind the primary and secondary antibodies, leading to high background. Background staining due to free aldehyde groups is more likely to occur when the fixative is glutaraldehyde or paraformaldehyde.

6. Incubate cells with the antibody (diluted in 1% BSA in PBST) in a humidified chamber for 1 hour at room temperature or overnight at 4°C.

7. Decant the solution and wash the cells with PBS 3 X 5 minutes.

8. Incubate cells with the secondary antibody in 1% BSA for 1 hour at room temperature in the dark.

9. Decant the secondary antibody solution and wash with PBS 3 X 5 minutes in the dark.

Counter staining

10. Incubate cells with 0.1-1 □g/ml Hoechst or DAPI (DNA stain) for 1 minute.

11. Rinse with PBS.

Mounting

12. Mount coverslip with a drop of mounting medium.

13. Seal coverslip with nail polish to prevent drying and movement under microscope.

14. Store in the dark at -20°C or +4°C.

6.4 Fixation and permeabilization in IHC/ICC

Fixation
Fixation should immobilize antigens while retaining cellular and subcellular structure. It should also allow for access of antibodies to all cells and subcellular compartments. The fixation and permeabilization method used will depend on the sensitivity of the epitope and the antibodies themselves, and may require some optimization.

Fixation can be done using crosslinking reagents, such as paraformaldehyde. These are better at preserving cell structure, but may reduce the antigenicity of some cell components as the crosslinking can obstruct antibody binding. For this reason, antigen retrieval techniques may be required, particularly if there is a long fixation incubation or if a high percentage of crosslinking fixative is used. Another option is to use organic solvents. These remove lipids while dehydrating the cells. They also precipitate proteins on the cellular architecture.

1. 4% Paraformaldehyde
Add 4% paraformaldehye to slides for 10 minutes only.

Wash with PBS or PBS with 1% BSA.

Note: *Fixing in paraformaldehyde for more than 10-15 minutes will cross-link the proteins to the point where antigen retrieval may be required to ensure the antibody has free access to bind and detect the protein.*

2. Ethanol
Add 100-200 µl per slide of cooled 95% ethanol, 5% glacial acetic acid for 5-10 minutes.
Wash with PBS or PBS with 1% BSA.

3. Methanol
Add 100-200 µl per slide of ice cold methanol.
Place at -20°C for 10 minutes.
Wash with PBS or PBS with 1% BSA.

Note: *Methanol will also permeabilize. Some epitopes are very sensitive to methanol as it can disrupt epitope structure. Can try acetone instead for permeabilization if required.*

4. Acetone
Add 100-200 µl per slide ice cold acetone.
Place at -20°C for 5 to 10 minutes.
Wash with PBS or PBS with 1% BSA.

Note: *acetone will also permeabilize. Consequently, no further permeabilization step is required.*

Permeabilization
Permeabilization is only required when the antibody needs access to the inside of the cells to detect the protein. These include intracellular proteins and transmembrane proteins whose epitopes are in the cytoplasmic region.

Solvents
1. Acetone fixation will also permeabilize.
2. Methanol fixation can be used to permeablize but is not always suitable.

These reagents can be used to fix and permeabilize, or can be used after fixation with a crosslinking agent such as paraformaldehyde.

Detergents
1. Triton or NP-40
Use 0.1 to 0.2% in PBS for 10 minutes only.

These will also partially dissolve the nuclear membrane and are therefore very suitable for nuclear antigen staining.

Note: *as these are harsh detergents, they will disrupt proteins when used at higher concentrations or for longer amounts of time, affecting staining results.*

2. Tween 20, Saponin, Digitonin and Leucoperm
Use 0.2 to 0.5% for 10 to 30 minutes.

These are much milder membrane solubilizers. They will give large enough pores for antibodies to go through without dissolving the plasma membrane. They are suitable for antigens in the cytoplasm or the cytoplasmic face of the plasma membrane and for soluble nuclear antigens.

Special recommendations
Cytoskeletal, viral and some enzyme antigens usually give optimal results when fixed with acetone, ethanol or formaldehyde (high concentration).

Antigens in cytoplasmic organelles and granules will require a fixation and permeabilization method depending on the antigen. The epitope needs to remain accessible.

6.5 Perfusion fixation

For many purposes, adequate fixation is obtained by simple immersion of small tissue pieces into the fixative solution, and this is the only mode of fixation possible for many tissues. However, a more rapid and uniform fixation is usually obtained if the fixative solution is perfused via the vascular system, either through the heart or through the abdominal aorta. The following procedures provide fixation of most rat organs with 4% paraformaldehyde.

Materials
- Anaesthetic
- Scissors, forceps, and clamps for surgical procedures
- Small forceps with fine claws
- Scalpel
- Vials (5-10 ml) with lids for specimens
- 0.9% saline
- 500 ml beakers
- 4% paraformaldehyde, fixation solution
- Gloves, eye goggles
- Perfusion pump (or flask with fixative placed upside down about 150 cm above the operating table)
- Short syringe needle for heart perfusion of aorta, length about 50 mm, outer diameter 1.3-1.5 mm
- Perfusion set with drip chamber as used for intravenous blood infusions

Perfusion fixation through the heart
1. Set up the perfusion pump; attach perfusion set and perfusion needle. First, run about 100 ml of normal tap water through the tubing to remove any residue. Then place open end of perfusion tube in a beaker filled with cold 4% paraformaldehyde (in ice box). The volume of solution should be scaled to the size of the animal, although 200-300 ml will usually be sufficient for one animal. Open valve and adjust to a slow steady drip (20 ml/min), and then close valve.

2. Set up surgery site with scissors, forceps and clamps. Give an appropriate amount of anesthetic to the animal. Once the animal is under anesthesia, place it on the operating table with its back down. You may use some tape to hold the appendages so that the animal is securely fixed.

3. Use pinch response method to determine depth of anesthesia. Animal must be unresponsive before proceeding with the following steps.

4. Make an incision with scalpel through the abdomen the length of the diaphragm. With sharp scissors, cut through the connective tissue at the bottom of the diaphragm to allow access to the rib cage.

5. With large scissors, blunt side down, cut through ribs just left of the rib cage midline.

6. Make one center or two end horizontal cuts through the rib cage, and open the thoracic cavity. Clamp open to expose heart and provide drainage for blood and fluids.

7. While holding the heart steady with forceps (it should still be beating), insert the needle directly into the protrusion of the left ventricle and extend straight up about 5 mm. ***Be careful not to extend the needle too far in, as it can pierce an interior wall and compromise circulation of solutions!*** Secure the needle by clamping in place near the point of entry. Release the valve to allow slow, steady flow of around 20 ml/min of 0.9% saline solution.

8. Make a cut in the atrium with sharp scissors, and make sure solution is flowing freely. If fluid is not flowing freely or is coming from the animal's nostrils or mouth, reposition the needle.

9. When the blood has been cleared from body, change to 4% paraformaldehyde solution (200-300 ml).

Take care not to introduce air bubbles while transferring from one solution to the other. It is best to wear protective eye goggles during the whole perfusion process, as sometimes the connecting tubes might come undone and spurt fixation solution into your eyes!

Perfusion is almost complete when spontaneous movement (formalin “dance”) and lightened color of the liver are observed. (Note: In general, an adult rat will require around 30-60 min of perfusion time, but this may vary depending on the size of the animal and technique).

10. Stop the perfusion and excise the tissues of interest. Place them in vials containing the same fixation solution and fix for another 2 hours on ice or at 4°C before proceeding to dehydration and embedding. For better results, immersion fix overnight at 4°C.

Perfusion fixation through the abdominal aorta
1. Prepare materials and animal as stated above (steps 1-3).

2. Open the abdominal cavity by a long midline incision with lateral extension, and move the intestines gently to the left side of the animal.

3. Carefully expose the aorta below the origin of the renal arteries and very gently free the aorta from overlaying adipose and connective tissues.

4. Hold the wall of the aorta firmly with fine forceps with claws about 0.5-1.0 cm from its distal bifurcation. Insert a bent needle close to the forceps towards the heart into the lumen of the aorta.

5. In very rapid succession:
a) Cut a hole in the inferior vena cava with fine scissors.
b) Start the perfusion.
c) Clamp the aorta below the diaphragm, but above the origin of the renal arteries.

When performing these manipulations, accuracy and speed are essential and the fixation procedure is preferably carried out by two persons. It is particularly important to clamp the aorta rapidly after the perfusion has been started. This is most easily done by compressing the aorta toward the posterior wall of the peritoneal cavity with a finger (wear gloves) which is then replaced by a clamp. Finally, cut the aorta above the compression.

6. The kidney surface must blanch immediately and show a uniform, pale color. The flow rate should be at least 60-100 ml/min for an adult rat. Perfuse for 3 minutes. Stop the perfusion and excise and trim the tissues. Place the tissues in vials and fix in the same fixative (post fixation step) for 2 hr on ice or at 4°C. For better results, immersion fix overnight at 4°C.

7. The tissue is now ready for dehydration and embedding.

6.6 Mouse on Mouse staining tips

Staining of mouse tissue using mouse antibody is a complicated process as high levels of background are often observed. It is notoriously difficult to eliminate this background.
Much of the background is caused by secondary antibody binding to endogenous mouse IgG in the tissue being stained, and to Fc receptors on B cells, plasma cells and macrophages.

Abcam is unable to guarantee monoclonal mouse antibodies on mouse tissue (unless stated on the datasheet). However, there are a few tips to try and reduce this background should mouse on mouse staining be necessary:

Blocking of endogenous IgG
1. Prepare tissue sections as usual.

2. At the usual blocking step, block with serum (from same species as the secondary antibody) for 30 minutes at room temperature.

3. Wash 3 X 2 minutes with PBS Tween 20.

4. Incubate sections with an unconjugated AffiniPure Fab fragment Anti-Mouse IgG (H+L) for 1 hour at room temperature, or overnight 4°C.

Try this blocking antibody at 0.1 mg/ml although the optimal concentration will need to be assessed by the end user.

5. Proceed with antibody staining.

Blocking endogenous Fc receptors
There are kits available for this which use F(ab) monomeric secondary antibodies.

Other tips that can be used to decrease general background:
1. Incubate sections with 1% Triton (in PBS) at room temperature for 30 minutes to ‘clean’ the tissue.

2. Use TBS–Tween 20 as a washing buffer. This often gives less background than using PBS Tween 20

6.7 Troubleshooting tips – IHC/ICC

No staining

The primary antibody and the secondary antibody are not compatible.
Use a secondary antibody that was raised against the species in which the primary was raised (e.g. primary is raised in rabbit, use anti-rabbit secondary).

Not enough primary antibody is bound to the protein of interest.
Use less dilute antibody. Incubate longer (e.g. overnight) at 4°C.

The antibody may not be suitable for IHC procedures which reveal the protein in its native (3D form).
Test the antibody in a native (non-denatured) WB to make sure it is not damaged.

The primary/secondary antibody/amplification kit may have lost its activity due to improper storage, improper dilution or extensive freezing/thawing.
Run positive controls to ensure that the primary/secondary antibody is working properly.

The protein is not present in the tissue of interest.
Run a positive control recommended by the supplier of the antibody.

The protein of interest is not abundantly present in the tissue.
Use an amplification step to maximize the signal.

The secondary antibody was not stored in the dark.
Always prevent the secondary antibody from exposure to light.

Deparaffinization may be insufficient.
Deparaffinize sections longer, change the xylene.

Fixation procedures (using formalin and paraformaldehyde fixatives) may be modifying the epitope the antibody recognizes.
Use antigen retrieval methods to unmask the epitope. Fix for less time.

The protein is located in the nucleus (nuclear protein) and the antibody cannot penetrate the nucleus.
Add a permeabilizing agent to the blocking buffer and antibody dilution buffer.

The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest.
Add 0.01% azide to the PBS antibody storage buffer or use fresh sterile PBS.

High background

Blocking of non-specific binding might be absent or insufficient.
Increase the blocking incubation period and consider changing the blocking agent. Abcam recommends blocking with 10% normal serum for 1 hour for sections or with 1-5% BSA for 30 minutes for cells in culture.

The primary antibody concentration may be too high.
Titrate the antibody to the optimal concentration, incubate for longer but in more dilute antibody (a slow but targeted binding is best).

Incubation temperature may be too high.
Incubate sections or cells at 4°C.

The secondary antibody may be binding non-specifically (damaged).
Run a secondary control without primary antibody.

Tissue not washed enough, fixative still present.
Wash extensively with PBS between all steps.

Endogenous peroxidases are active.
Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H_2O_2 (0.3% v/v) for peroxidase. (See IHC protocol).

Fixation procedures (using formalin and paraformaldehyde fixatives) are too strong and modified the epitope the antibody recognizes.
Change antigen retrieval method, decrease the incubation time with the antigen unmasking solution.

Too much amplification (amplification technique).
Reduce amplification incubation time and dilute the amplification kit.

Too much substrate was applied (enzymatic detection).
Reduce substrate incubation time.

The chromogen reacts with the PBS present in the cells/tissue (enzymatic detection).
Use Tris buffer to wash sections prior to incubating with the substrate. Then wash sections/cells in Tris buffer again after substrate incubation.

Pemeabilization has damaged the membrane and removed the membrane protein.
Remove permeabilizing agent from your buffers.

Non-specific staining

Primary/secondary antibody concentration may be too high.
Try decreasing the antibody concentration and/or the incubation period. Compare signal intensity against cells that do not express the target.

Endogenous peroxidases are active.
Use enzyme inhibitors i.e. Levamisol (2 mM) for alkaline phosphatase or H_2O_2 (0.3% v/v) for peroxidase. (See IHC protocol).

The primary antibody is raised against the same species as the tissue stained (e.g. mouse primary antibody tested on mouse tissue). When the secondary antibody is applied, it binds to all the tissue as it is raised against that species.
Use a primary antibody raised against a different species than your tissue.

The sections/cells have dried out.
Keep sections/cells at high humidity and do not let them dry out.

Further useful immunohistochemistry/immunocytochemistry protocols.

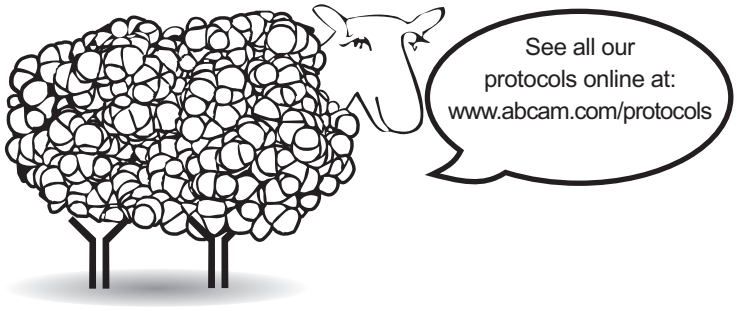
There are many more useful immunohistochemistry/immunocytochemistry procedures and guides on our protocols webpages. Please see the following URL addresses for more information:

Double immunofluorescence: sequential protocol
www.abcam.com/index.html?pageconfig=resource&rid=11458

Double immunofluorescence: simultaneous protocol
www.abcam.com/index.html?pageconfig=resource&rid=11459

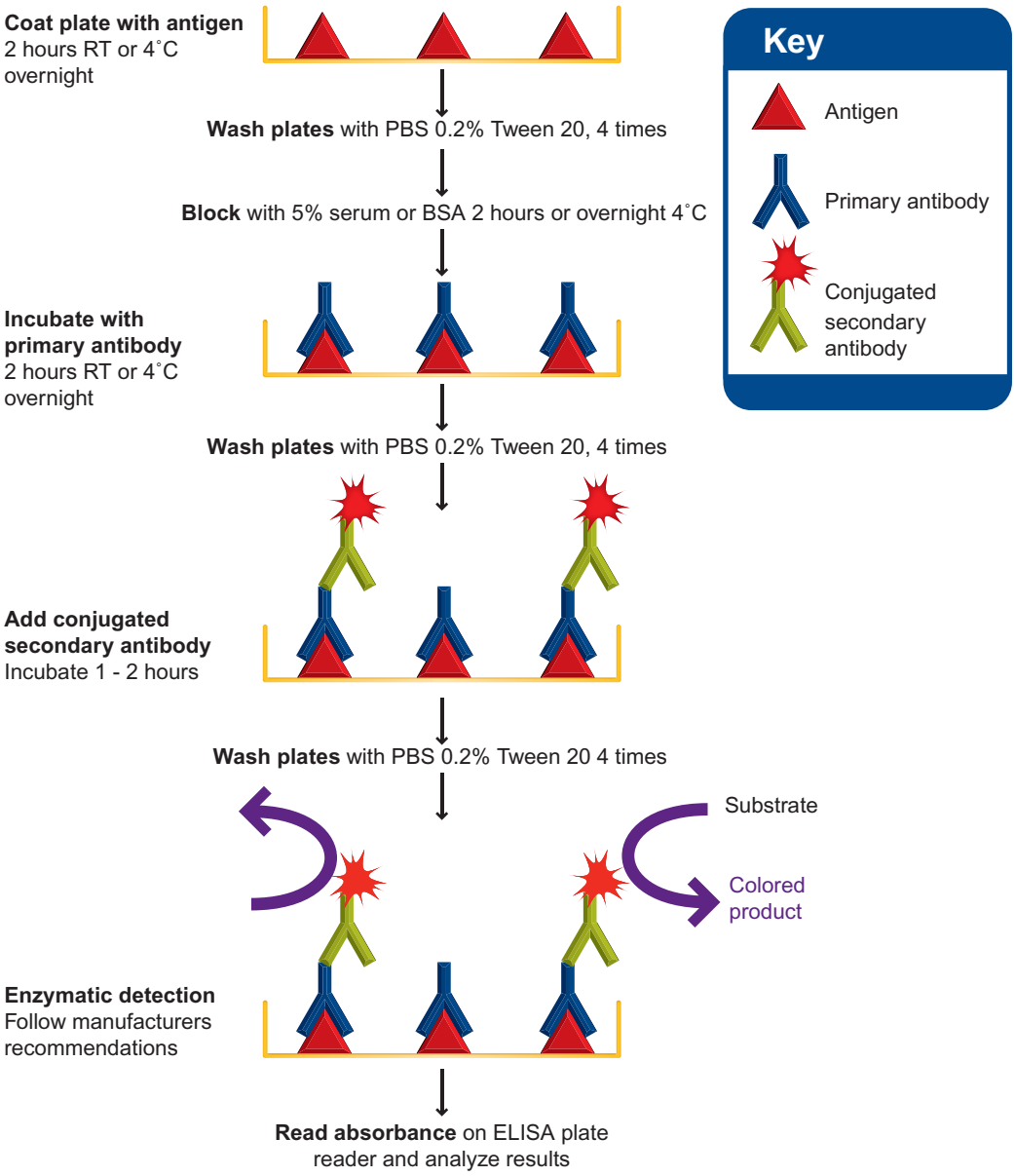
Preparing cells and sections for BrdU immunostaining
www.abcam.com/index.html?pageconfig=resource&rid=11533

Whole mount staining protocols
www.abcam.com/index.html?pageconfig=resource&rid=11330



SECTION 7: ELISA

7.1 Indirect ELISA



Buffers and reagents

For accurate quantitative results, always compare signals of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blanks must be run with each plate to ensure accuracy.

General procedure

Coating antigen to microplate

1. Dilute the antigen to a final concentration of 20 □g/ml in PBS or other carbonate buffer. Coat the wells of a PVC microtiter plate with the antigen by pipetting 50 □l of the antigen dilution in the top wells of the plate. Dilute down the plate as required.

Test samples containing pure antigen are usually pipetted onto the plate at less than 2 µg/ml.
Pure solutions are not essential, but as a guideline, over 3% of the protein in the test sample should be the target protein (antigen).
Antigen protein concentration should not be over 20 µg/ml as this will saturate most of the available sites on the microtitre plate.

Ensure the samples contain the antigen at a concentration that is within the detection range of the antibody.

2. Cover the plate with an adhesive plastic and incubate for 2 hours at room temperature, or 4°C overnight. The coating incubation time may require some optimization.

3. Remove the coating solution and wash the plate 3 X by filling the wells with 200 µl PBS. Remove the wash solutions by flicking the plate gently over a sink. Remove any remaining drops by patting the plate on a paper towel.

Blocking
4. Block the remaining protein binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk or 5% serum in PBS. Alternative blocking reagents include BlockACE or BSA.

5. Cover the plate with an adhesive plastic and incubate for at least 2 hours at room temperature or if more convenient, overnight at 4°C.

6. Wash the plate twice with PBS.

Incubation with primary and secondary antibody
7. Add 100 µl of diluted primary antibody to each well.

8. Cover the plate with an adhesive plastic and incubate for 2 hours at room temperature. This incubation time may require optimization. Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often be observed when incubated overnight at 4°C.

9. Wash the plate 4 X with PBS.

10. Add 100 µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use.

11. Cover the plate with an adhesive plastic and incubate for 1-2 hours at room temperature.

12. Wash the plate 4 X with PBS.

Detection
Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the most widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells) and this may result in non-specific signal. If necessary, perform an additional blocking treatment with Levamisol (for ALP) or with a 0.3% solution of H_2O_2 in methanol.

ALP substrate
For most applications **pNPP** (p-Nitrophenyl-phosphate) is the most widely used substrate. The yellow color of nitrophenol can be measured at 405 nm after 15-30 min incubation at room temperature (this reaction can be stopped by adding equal volume of 0.75 M NaOH).

HRP chromogens
The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of ahydrogen donor which changes color during reaction.

TMB (3,3',5,5'-tetramethylbenzidine)
Add TMB solution to each well, incubate for 15-30 min, add equal volume of stopping solution (2 M H_2SO_4) and read the optical density at 450 nm.

OPD (o-phenylenediamine dihydrochloride)
The end product is measured at 492 nm. Be aware that the substrate is light sensitive so keep and store it in the dark.

ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt)
The end product is green and the optical density can be measured at 416 nm.

Note*: *some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.

13. Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipette or a multipipette.

14. After sufficient color development add 100 □l of stop solution to the wells (if necessary).

15. Read the absorbance (optical density) of each well with a plate reader.

Analysis of data
Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs. absorbance on the y axis (linear). Interpolate the concentration of the sample from this standard curve.

7.2 Direct ELISA

General Procedure

Coating antigen to microplate
1. Dilute the antigen to a final concentration of 20 □g/ml in PBS or other carbonate buffer. Coat the wells of a PVC microtiter plate with the antigen by pipetting 50 □l of the antigen dilution in the top wells of the plate. Dilute down the plate as required.

Test samples containing pure antigen are usually pipetted onto the plate at less than 2 µg/ml.
Pure solutions are not essential, but as a guideline, over 3% of the protein in the test sample should be the target protein (antigen).
Antigen protein concentration should not be over 20 µg/ml as this will saturate most of the available sites on the microtitre plate.

Ensure the samples contain the antigen at a concentration that is within the detection range of the antibody.

2. Cover the plate with an adhesive plastic and incubate for 2 hours at room temperature, or 4°C overnight. The coating incubation time may require some optimization.

3. Remove the coating solution and wash the plate twice by filling the wells with 200 □l PBS. Remove the wash solutions by flicking the plate gently over a sink. Remove any remaining drops by patting the plate on a paper towel.

Blocking
4. Block the remaining protein binding sites in the coated wells by adding 200 □l blocking buffer, 5% non fat dry milk in PBS per well. Alternative blocking reagents include BlockACE or BSA.

5. Cover the plate with an adhesive plastic and incubate for at least 2 hours at room temperature or if more convenient, overnight at 4°C.

6. Wash the plate twice with PBS.

Incubation with the antibody
7. Add 100 □l of the antibody, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use.

8. Cover the plate with an adhesive plastic and incubate for 2 hours at room temperature. This incubation time may require optimization. Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often be observed when incubated overnight at 4°C.

9. Wash the plate 4 X with PBS.

Detection
10. Dispense 100 □l (or 50 □l) of the substrate solution per well with a multichannel pipette or a multipipette.

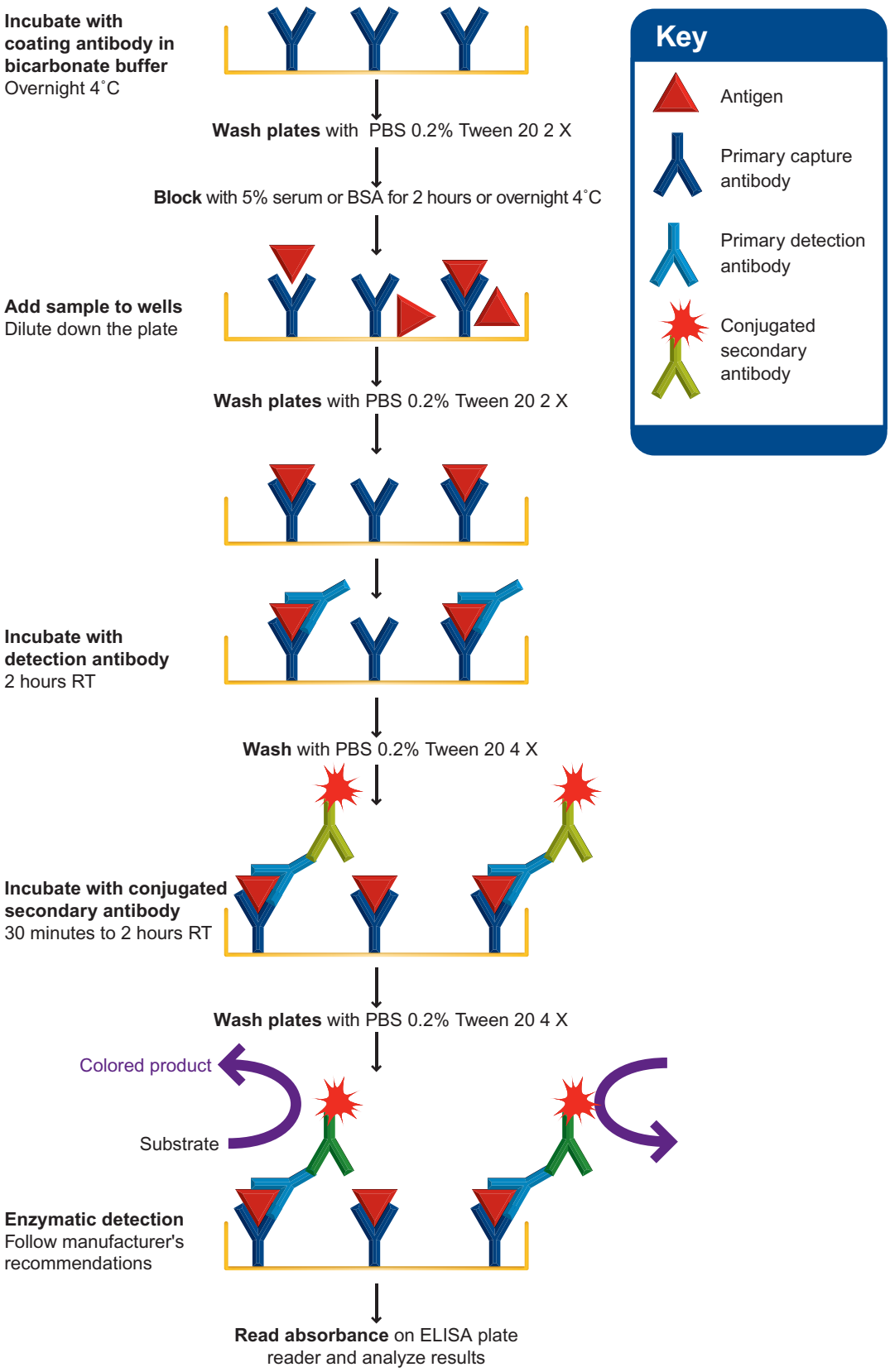
11. After sufficient color development add 100 □l of stop solution to the wells (if it is necessary).

Read the absorbance (optical density) of each well with a plate reader.

Note: *some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.*

Analysis of data
Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs. absorbance on the y axis (linear). Interpolate the concentration of the sample from this standard curve.

7.3 Sandwich ELISA



The sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich.

Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible.

The advantage of sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to two to five times more sensitive than direct or indirect).

General note:
Sandwich ELISA procedures can be difficult to optimise and tested match pair antibodies should be used. This ensures the antibodies are detecting different epitopes on the target protein so they do not interfere with the other antibody binding. Therefore, we are unable to guarantee our antibodies in sandwich ELISA unless they have been specifically tested for sandwich ELISA. Please review antibody datasheets for information on tested applications.

General procedure
Coating with capture antibody
1. Coat the wells of a PVC microtiter plate with the capture antibody at a concentration of 1-10 □g/ml in carbonate/bicarbonate buffer (pH 7.4).

If an unpurified antibody is used (e.g. ascites fluid or antiserum), you may need to compensate for the lower amount of specific antibody by increasing the concentration of the sample protein (try 10 µg/ml).

2. Cover the plate with an adhesive plastic and incubate overnight at 4°C.

3. Remove the coating solution and wash the plate twice by filling the wells with 200 □l PBS. Remove the wash solutions by flicking the plate gently over a sink. Remove any remaining drops by patting the plate on a paper towel.

Blocking and adding samples
4. Block the remaining protein binding sites in the coated wells by adding 200 □l blocking buffer, 5% non fat dry milk in PBS, per well.

5. Cover the plate with an adhesive plastic and incubate for at least 1-2 hours at room temperature or if more convenient, overnight at 4°C.

6. Add 100 □l of appropriately diluted samples to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy. Incubate for 90 minutes at 37°C.

For quantification, the concentration of the standard used should span the most dynamic detection range of antibody binding. You may need to optimize the concentration range to ensure you obtain a suitable standard curve. For accurate quantfication, always run samples and standards in duplicate or triplicate.

7. Remove the samples and wash the plate twice by filling the wells with 200 □l PBS.

Incubation with detection antibody and then secondary antibody
8. Add 100 □l of diluted detection antibody to each well.

Ensure the secondary detection antibody recognizes a different epitope on the target protein than the coating antibody. This prevents interference with the antibody binding and ensures the epitope for the second antibody is available for binding. Use a tested matched pair whenever possible.

9. Cover the plate with an adhesive plastic and incubate for 2 hours at room temperature.

10. Wash the plate 4 X with PBS.

11. Add 100 □l of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use.

12. Cover the plate with an adhesive plastic and incubate for 1-2 hours at room temperature.

13. Wash the plate 4 X with PBS.

Detection
Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (AP) are the two most widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high AP in alveolar cells, high peroxidase in red blood cells) and this may result in a non-specific signal. If necessary, perform an additional blocking treatment with Levamisol (for AP) or with 0.3% solution of H_2O_2 in methanol (for peroxidase).

ALP substrate
For most applications pNPP (p-Nitrophenyl-phosphate) is the most widely used substrate. The yellow color of nitrophenol can be measured at 405 nm after 15-30 minutes incubation at room temperature (this reaction can be stopped by adding equal volume of 0.75 M NaOH).

HRP chromogenes
The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during reaction.

TMB (3,3',5,5'-tetramethylbenzidine)
Add TMB solution to each well, incubate for 15-30 min, add an equal volume of stopping solution (2 M H_2SO_4) and read the optical density at 450 nm.

OPD (o-phenylenediamine dihydrochloride)
The end product is measured at 492 nm. Be aware that thesubstrate is light sensitive so keep and store it in the dark.

ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt)
The end product is green and the optical density can be measured at 416 nm.

Note: *some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.*

14. Dispense 100 □l (or 50 □l) of the substrate solution per well with a multichannel pipette or a multipipette.

Analysis of data
Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs. absorbance on the y axis (linear). Interpolate the concentration of the sample from this standard curve.

7.4 Troubleshooting tips - ELISA

Positive results in negative control

Contamination of reagents/samples.
May be contamination of reagents or samples, or cross contamination from splashing between wells. Use fresh reagents and pipette carefully.

Sandwich ELISA – detection antibody is detecting coating antibody.
Check the correct coating antibody and detection antibodies are being used and that they will not detect each other.

Insufficient washing of plates.
Ensure wells are washed adequately by filling them with wash buffer. Ensure all residual antibody solutions are removed before washing.

Too much antibody used leading to non-specific binding.
Check the recommended amount of antibody suggested. Try using less antibody.

High background across entire plate

Conjugate too strong or left on too long.
Check dilution of conjugate, use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for absorbance readings.

Substrate solution or stop solution is not fresh.
Use fresh substrate solution. Stop solution should be clear (if it has gone yellow, this is a sign of contamination and it should be replaced).

Reaction not stopped.
Color will keep developing if the substrate reaction is not stopped.

Plate left too long before reading on the plate reader.
Color will keep developing (though at a slower rate if stop solution has been added).

Contaminants from laboratory glassware.
Ensure reagents are fresh and prepared in clean glassware.

Substrate incubation carried out in the light.
Substrate incubation should be carried out in the dark.

Incubation temperature too high.
Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and working. Incubation temperature may require some optimization.

Non-specific binding of antibody.
Ensure a block step is included and a suitable blocking buffer is being used. We recommend using 5 to 10% serum from the same species as the secondary antibody, or bovine serum.
Ensure wells are pre-processed to prevent non specific attachment. Use an affinity purified antibody, preferably pre-absorbed.

Also check suggestions listed under 'Positive results in negative control'.

Low absorbance values

Target protein not expressed in sample used or low level of target protein expression in sample used.
Check the expression profile of the target protein to ensure it will be expressed in your samples. If there is low level of target protein expression, increase the amount of sample used or you may need to change to a more sensitive assay. Ensure you are using a positive control within the detection range of the assay.

Insufficient antibody.
Check the recommended amount of antibody is being used. The concentration of antibody may require increasing for optimization of results.

Substrate solutions not fresh or combined incorrectly.
Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed at the correct concentration.

Reagents not fresh or not at the correct pH.
Ensure reagents have been prepared correctly and are in date.

Incubation time not long enough.
Ensure you are incubating the antibody for the recommended amount of time if an incubation time is suggested. The incubation time may require increasing for optimization of results.

Incubation temperature too low.
Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and are working. Incubation temperature may require some optimization. Ensure all reagents are at room temperature before proceeding.

Stop solution not added.
Addition of stop solution increases the intensity of color reaction and stabilizes the final color reaction.

High absorbance values

High absorbance values for samples and/or positive control. Absorbance is not reduced as the sample is diluted down the plate
The concentration of samples or positive control is too high and out of range for the sensitivity of the assay. Re-assess the assay you are using or reduce the concentration of samples and control by dilution before adding to the plate. Consider the dilution when calculating the resulting concentrations.

Inconsistent absorbances across the plate

Plates stacked during incubations.
Stacking of plates does not allow even distribution of temperature across the wells of the plates. Avoid stacking.

Pipetting inconsistent.
Ensure pipettes are working correctly and are calibrated. Ensure pipette tips are pushed on far enough to create a good seal. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of liquid. This will greatly affect consistency of results between duplicates.

Antibody dilutions/reagents not well mixed.
To ensure a consistent concentration across all wells, ensure all reagents and samples are mixed before pipetting onto the plate.

Wells allowed to dry out.
Ensure lids are left on the plates at all times when incubating. Place a humidifying water tray (bottled clean, sterile water) in the bottom of the incubator.

Inadequate washing.
This will lead to some wells not being washed as well as others, leaving different amounts of unbound antibody behind which will give inconsistent results.

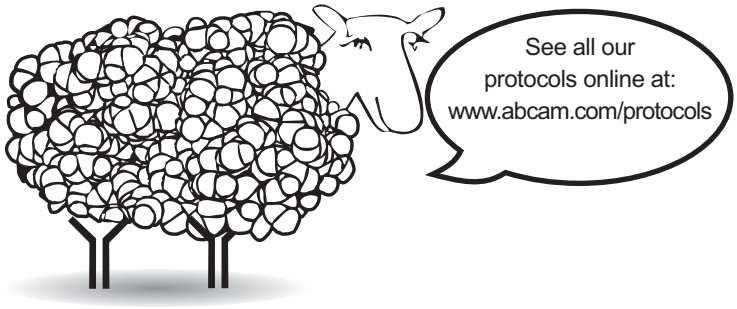
Bottom of the plate is dirty affecting absorbance readings.
Clean the bottom of the plate carefully before re-reading the plate.

Color developing slowly

Plates are not at the correct temperature.
Ensure plates are at room temperature and that the reagents are at room temperature before use.

Conjugate too weak.
Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed, at the correct concentration.

Contamination of solutions.
Presence of contaminants, such as sodium azide and peroxidases can affect the substrate reaction. Avoid using reagents containing these preservatives.



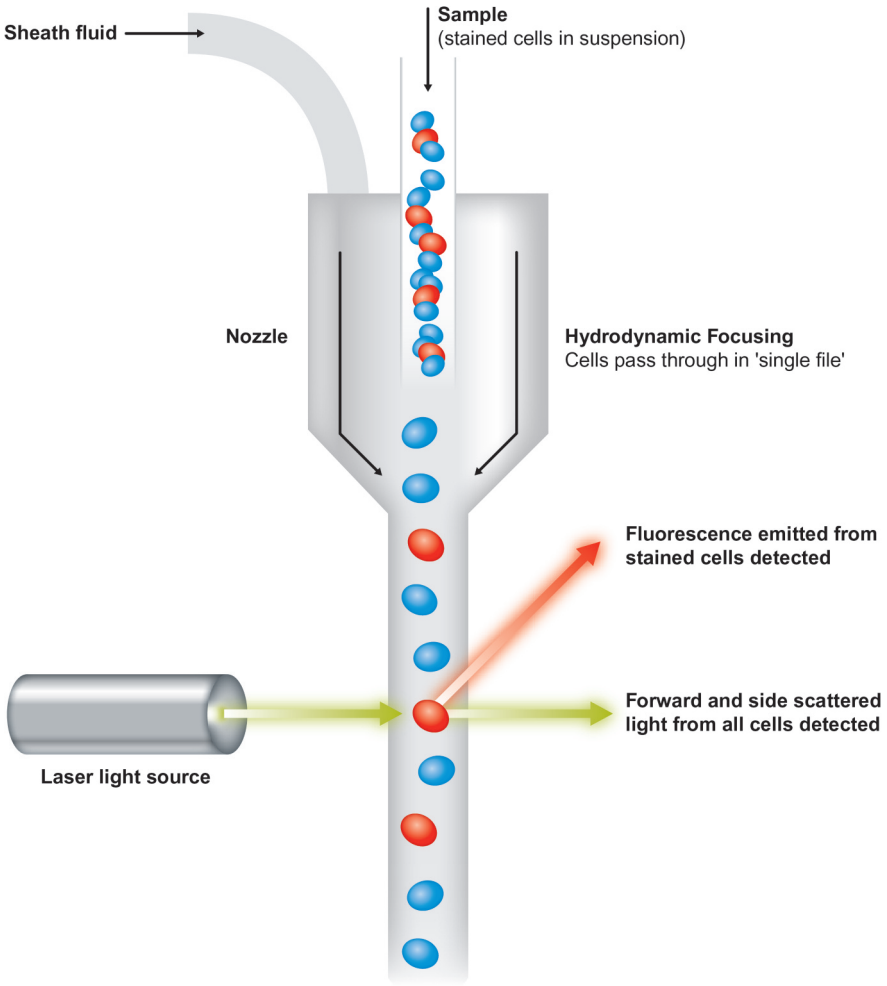
SECTION 8: Flow cytometry

Flow cytometry is now a widely used method for analyzing expression of cell surface and intracellular molecules, characterizing and defining different cell types in heterogeneous cell populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. It allows simultaneous multi-parameter analysis of single cells. It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins or ligands that bind to specific cell-associated molecules, such as DNA binding by propidium iodide.

The staining procedure involves making a single-cell suspension from cell culture or tissue samples. The cells are then incubated in tubes or microtiter plates with unlabeled or fluorochrome-labeled antibodies. Cells are then analyzed on the flow cytometer.

Contents

8.1 The Flow Cytometer: Fluidics



When the stained cell sample in suspension buffer is run through the cytometer, it is hydrodynamically focused, using sheath fluid, through a very small nozzle. The tiny 'stream' of fluid takes the cells past the laser light one cell at a time. There are a number of detectors to detect the light scattered from the cells/particles as they go through the beam. There is one in front of the light beam (Forward Scatter or FS) and several to the side (Side Scatter or SS). Fluorescent detectors are used for the detection of fluorescence emitted from positively stained cells/particles.

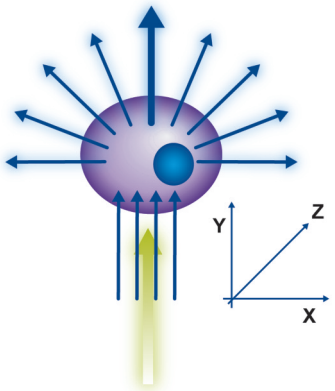
8.2 The Flow Cytometer: Measurement of forward and side scatter of light

Particles/cells passing through the beam will scatter light, which is detected as forward scatter (FS) and side scatter (SS). The combination of scattered and fluorescent light is detected and analyzed. FS correlates with the cell size and SS depends on the density of the particle/cell (i.e. number of cytoplasmic granules, membrane size), and in this manner cell populations can often be distinguished based on differences in their size and density.

The direction of light scattered by the cell correlates to:

- Cell size (Forward Scatter; FS)
- Granularity (Side Scatter; SS)

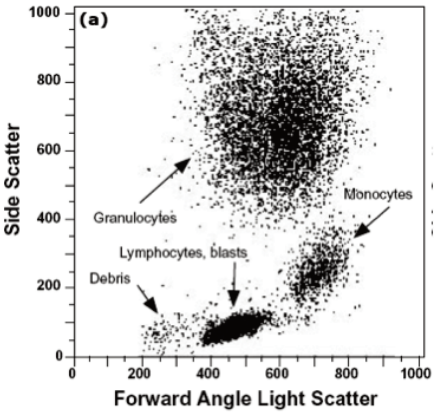
Diagram showing light scatter as the green laser interrogates the cell:



A useful example of this is when running blood samples on the flow cytometer.

- Larger and more granular granulocyte cells produce a large population with high SS and FS.
- Monocytes are large cells, but not so granular, so these produce a separate population with high FS but lower SS.
- Smaller lymphocytes and lymphoblasts produce a separate population with less FS. They are not granular cells, so also have low SS.

Therefore, these cells can be separated into different populations based on their FS and SS alone:



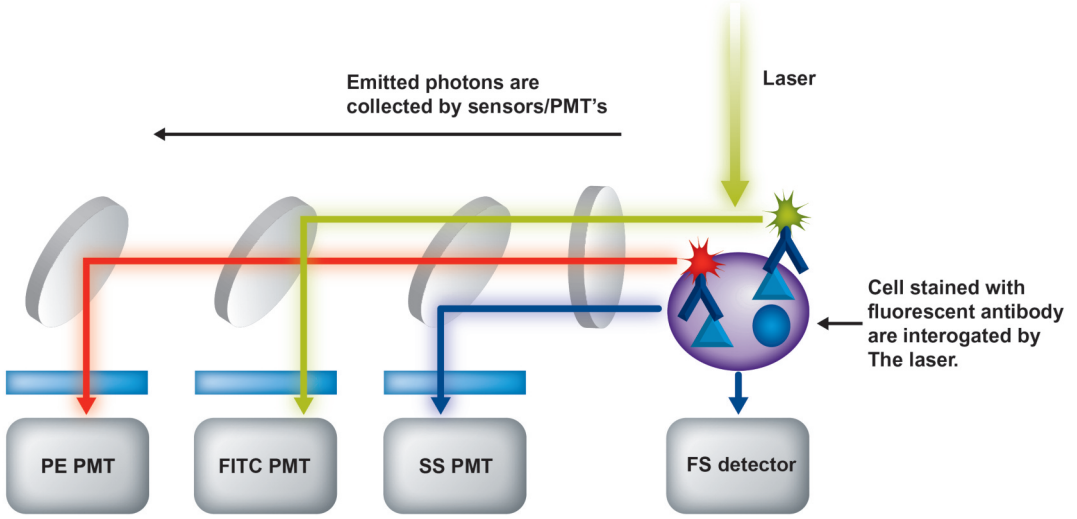
Data: Roger S. Riley et al.

8.3 The Flow Cytometer: Measurement of scattered light and fluorescence

Fluorochromes used for detection/staining of target proteins will emit light when excited by a laser with the corresponding excitation wavelength. These fluorescent stained particles or cells can be detected individually and the data analyzed.

Forward and side scattered light and fluorescence from stained cells are split into defined wavelengths and channeled by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered so each sensor will detect fluorescence only at a specified wavelength. These sensors are called photomultiplying tubes (PMT's).

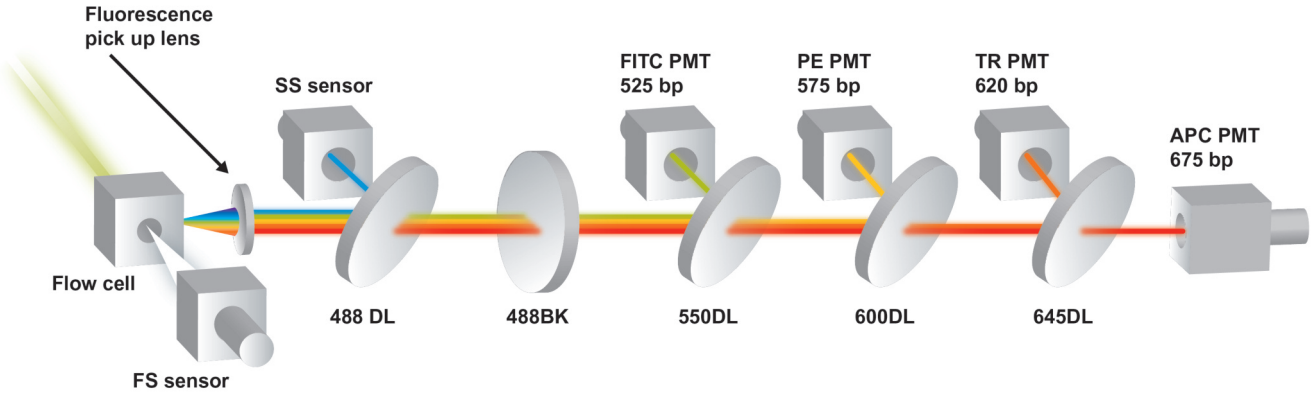
The PMT's convert the energy of a photon into an electronic signal = **A voltage.**



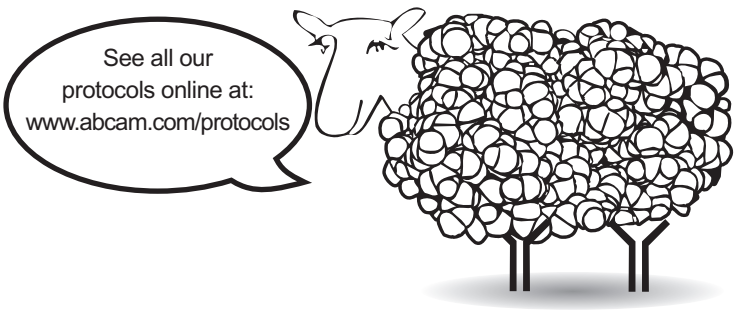
For example:

The FITC channel PMT will detect light emitted from FITC at approximately 519 nm wavelength. (It will also detect any other fluorochromes emitting at similar wavelength).
The PE channel PMT will detect light emitted from PE at 575 nm wavelength (each PMT will detect any other fluorochromes emitting at similar wavelength).

The following diagram shows an example of flow cytometer optics:

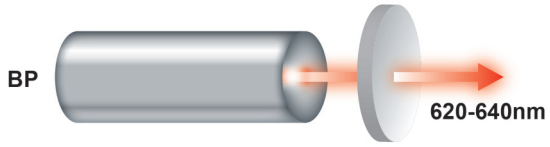


BP = band pass filter; DL= dichroic long pass filter/mirror;

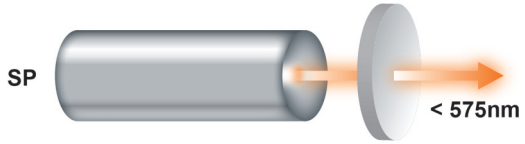


Various filters are used in the flow cytometer to direct the photons of the correct wavelength to each PMT:

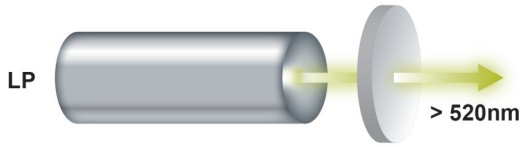
Band Pass (BP) filters allow transmission of photons that have wavelengths within a narrow range.



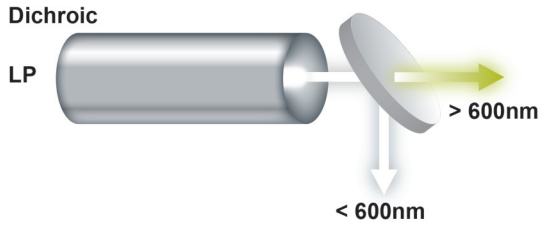
Short Pass (SP) filters allow transmission of photons below a specified wavelength.



Long Pass (LP) filters allow transmission of photons above a specified wavelength.



Dichroic filters/mirrors (such as dichroic LP long pass mirrors) are positioned at a 45° angle to the light beam. In a long pass dichroic filter, photons above a specific wavelength are transmitted straight ahead, whilst photons below the specific wavelength are reflected at a 90° angle.

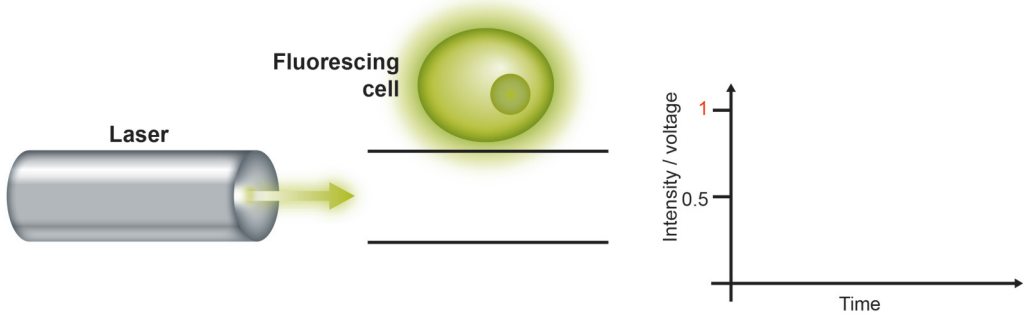


Measurement of a signal:

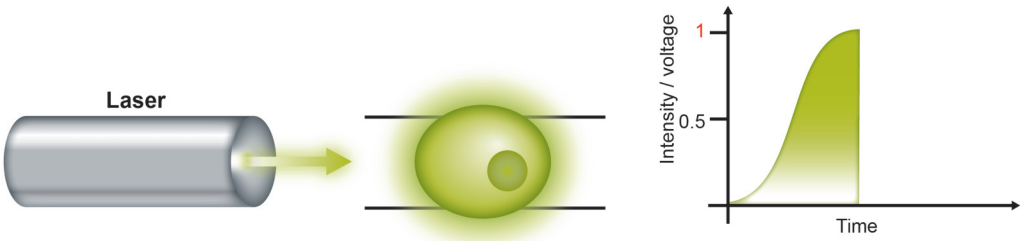
The PMT measures the pulse area of the voltage created each time a fluorescent cell provides photons:

As the fluorescing cell passes through the laser beam, it creates a peak or pulse over time in the number of photons. These are detected by the PMT and converted to a voltage pulse. Each pulse for each cell is known as an event. The measured voltage pulse area will correlate directly to the intensity of fluorescence for that event.

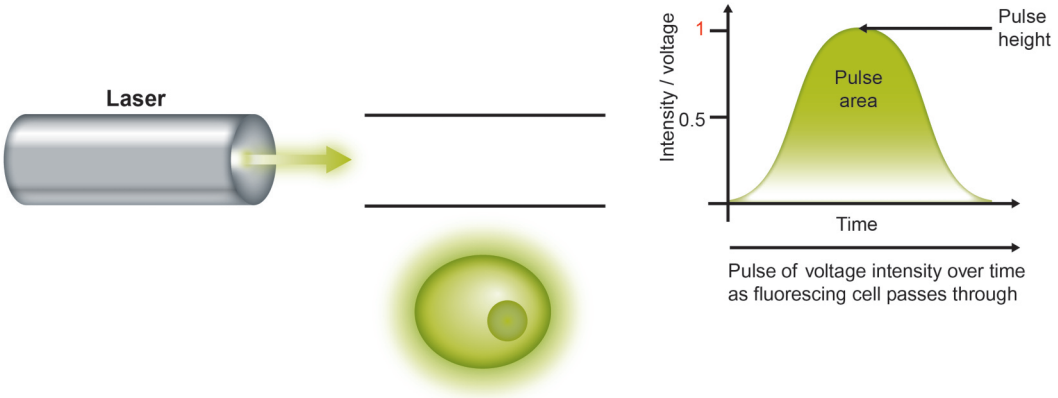
1. When there are no fluorescing cells passing through the optics, there are no photons emitted and no signal is detected.



2. As the fluorescent labeled cell passes through the optics and is interrogated by the laser, the number of photons emitted increases, and so the intensity of the voltage measured increases.

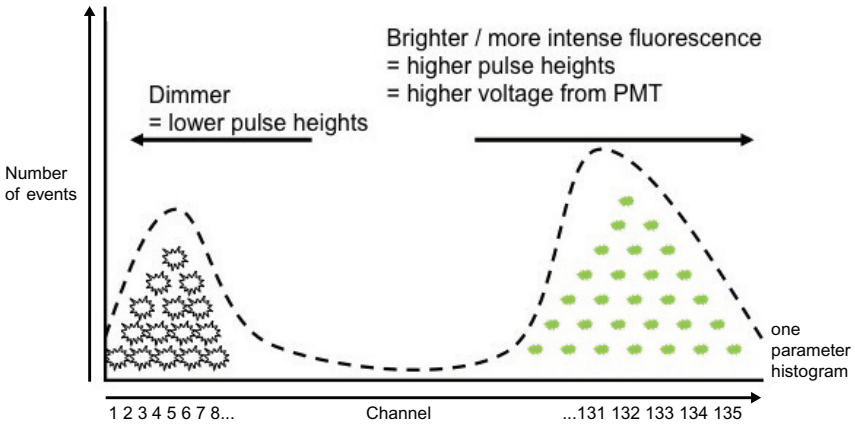


3. As each fluorescing cell completes its path through the laser beam, this leaves a pulse of voltage over time. The total pulse height and area is measured by the flow cytometer. This pulse is known as an event. Each event will have an intensity assigned to it depending on the pulse area obtained.



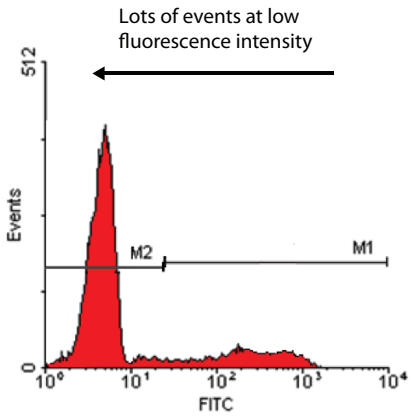
The pulse area is determined by adding the height values for each time slice of the pulse (which is determined by the speed of the ADC) which is 10 MHz i.e. 10 million per second or 10 per microsecond. The area is a better representation of the total amount of fluorescence.

These events are assigned channels based on the PULSE INTENSITY (pulse area).
This signal can be amplified by turning up the voltage going through the PMT.

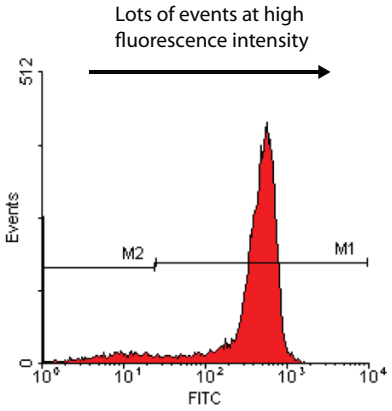


Each event is given a channel number depending on its measured intensity. There are 1,024 channels. The more intense the fluorescence, the higher the channel number the event is assigned. The one parameter histogram is a plot of channel number vs. number of events. The channels are usually viewed on a log scale on the x axis.

Negative result with no staining



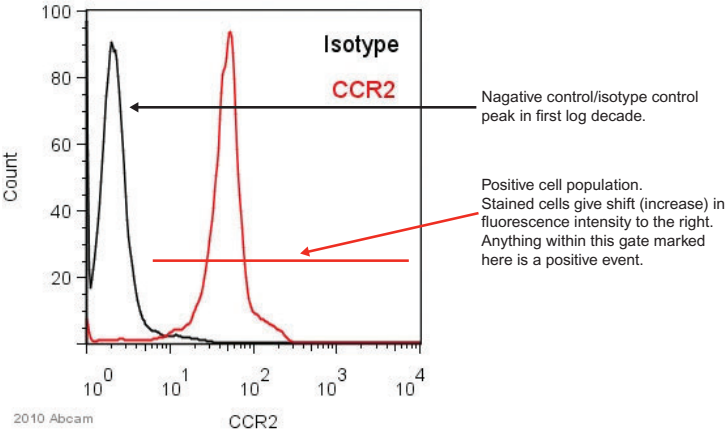
A positive result



For a positive result, you are looking for the shift in intensity between negative control and a positive sample.

Example: ab21667 CCR2 antibody staining human PBMC gated on monocytes.

Data: Anonymous Abreview



For more information regarding the use of the flow cytometer and data analysis, view our flow cytometry protocol.

8.4 Antibody staining

Direct staining

In direct immunofluorescence staining, cells are incubated with an antibody directly conjugated to a fluorochrome (e.g. FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody. It is particularly useful for intracellular staining, where large antibody-fluorochrome complexes including secondary antibodies can become trapped causing non-specific binding, or even fail to enter the cell and prevent primary antibody detection.

Indirect staining
In indirect staining, the primary antibody is not fluorochrome labeled but is detected by a fluorochrome-labeled secondary antibody. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used, whereby an antibody is conjugated to biotin and detected with fluorochrome-labeled avidin. With the wide range of conjugated secondary antibodies now available, this method means that unconjugated primary antibodies raised against many different targets can be used in conjunction with a labeled secondary antibody for FACS analysis. This widens the choice of target proteins for the researcher.

Intracellular staining
Staining of intracellular antigens for flow cytometry depends on various fixation and permeabilization methods to allow access of antibodies to internal cellular proteins. A successful staining procedure in all cases is dependent on optimization of experimental conditions through titering of antibodies, use of appropriate controls to set up the flow cytometer correctly, and optimized fixation and permeabilization procedures.

Detection of secreted proteins
Detection of secreted proteins is difficult as the protein will be released from the cell before detection, or may degrade rapidly. A Golgi-Block such as Brefaldin A can be used. Cells are incubated with Brefaldin A which prevents proteins being released from the golgi. Any expressed protein is retained in the golgi and can then be detected within the cell. The intracellular staining method is therefore used for detection of the target protein in this case.

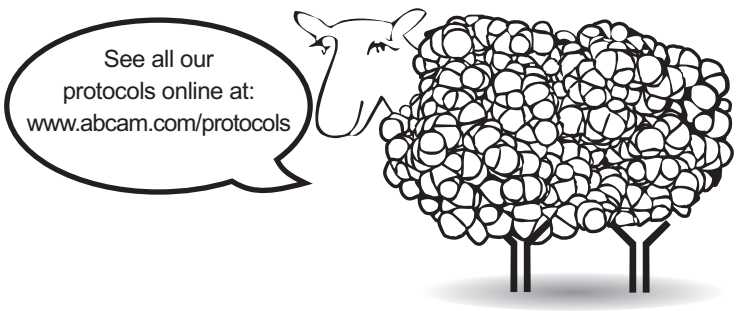
8.5 Selecting a fluorochrome conjugate

The ability of a given antibody to resolve a positive signal from a negative signal often depends on which fluorochrome conjugate is used.

A general guideline for the relative intensities of the various fluorochromes is, from brightest to dimmest: PE, PE-Cy7, PE-Cy5, APC > APC-Cy7, Alexa Fluor 647, Alexa Fluor 700 > FITC, Pacific Blue, Alexa Fluor 488. This is a general pattern; some differences in the relative intensities are seen for individual antibodies.

A highly expressed antigen will usually be detected and resolved from the negative control with almost any fluorochrome. An antigen expressed at lower density might require the higher signal/background ratio provided by a brighter PE or APC conjugate to separate the positive cells adequately from the unstained cells.

The relative fluorochrome intensity depends on the instrument. This is because of differences in the laser and filter combinations used on the different instruments. Be sure to use the appropriate FACS instrument.



SECTION 9: Immunoprecipitation (IP) protocol

Immunoprecipitation procedure

Immunoprecipitation is a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a cell extract enabling the antibody to bind to the protein in solution. The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled agarose beads. This physically isolates the protein of interest from the rest of the sample. The sample can then be separated by SDS-PAGE for western blot analysis.

9.1a Lysis buffers

The ideal lysis buffer will leave proteins in their native conformation, minimizing denaturation of antibody binding sites while at the same time releasing adequate amounts of protein from the sample for subsequent analysis. Non-ionic detergents such as NP-40 and Triton X-100 are less harsh than ionic detergents such as SDS and sodium deoxycholate. Other variables that can affect the success of IP include salt concentration, divalent cation concentration, and pH. Therefore to optimize these variables they should be tested within the following ranges (From Harlow and Lane, page 231; see references page 67):

- 0-1 M Salts
- 0.1-2% Detergent, non-ionic
- 0.01-0.5% Detergent, ionic
- 0-10 mM Divalent cations
- 0-5 mM EDTA
- pH: 6-9

The recipe for various lysis buffers can be found in the Buffers section 11, page 71.

Denaturing lysis buffer
Use for antigens that are detergent soluble and can be recognized in native form by the antibody.

1. RIPA (Radioimmunoprecipitation Assay) buffer
More denaturing than NP-40 or Triton X-100 lysis buffer, RIPA buffer contains the ionic detergents SDS and sodium deoxycholate as active constituents and is particularly useful for nuclear membrane disruption for nuclear extracts. RIPA buffer gives low background but can denature some proteins, including kinases.

2. Detergent-free soluble protein lysis buffer
Use PBS containing EDTA and sodium azide. Some soluble proteins may not require use of detergents. Use this buffer with mechanical breakage of cells, e.g. repeated passage through a syringe or homogenization with a Dounce homogenizer.

3. Denaturing lysis buffer or buffer for non-detergent soluble antigens
Epitopes of native proteins are not always accessible to antibodies that only recognize denatured proteins. When harvesting and lysing the cells, heat the cells in denaturing lysis buffer. This method can also be used for antigens that cannot be extracted from the cell with non-ionic detergents. Use of DNase1 will aid extraction of proteins from chromatin.

9.1b Other reagents

Protease inhibitors
As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer. Mixtures (“cocktails”) of protease and phosphatase inhibitors are commercially available. If not using a cocktail, two of the most commonly used protease inhibitors for IP are PMSF (50 ug/ml) and aprotinin (1 □g/ml). For more details of protease and phosphatase inhibitors, please see our western blot guide.

Other reagents required:
- Sterile PBS pH 7.4
- Sterile PBS-BSA 1% w/v (filtered)
- TBST buffer
- Loading/sample buffer for western blotting
- 100 mM EDTA stock solution is made with 1.86 g EDTA dissolved into 40 ml H_2O. Add NaOH to adjust the pH to 7.4. Finally, adjust the total volume to 50 ml.

9.2 Preparation of lysates

Lysates from cell culture
Non-denaturing
1. Place the cell culture dish on ice and wash the cells with ice cold PBS.

2. Drain the PBS, then add ice cold lysis buffer (1ml per 10^7 cells/100 mm^2 dish/150 cm^2 flask; 0.5 ml per $5x10^6$ cells/60 mm^2 dish or 75cm^2 flask).

3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled micro centrifuge tube.

4. Maintain constant agitation for 30 minutes at 4°C.

5. Centrifuge in a micro centrifuge at 4°C.

You may have to vary the centrifugation force and time depending on the cell type. A guideline is 20 minutes at 12,000 rpm but this must be determined by the end user (e.g. leukocytes need a very light centrifugation).

6. Gently remove the tubes from the centrifuge and place on ice. Aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

Denaturing
1. Add 100 µl denaturing lysis buffer to 0.5 to 2 x 10^7 cells.

2. Mix well by vortexing vigorously for 2 to 3 seconds at maximum speed. Transfer the cell suspension to a micro centrifuge tube.

The solution can be viscous at this stage due to release of DNA.

3. Heat samples to 95°C for 5 minutes to denature.

4. Dilute the suspension with 0.9 ml non denaturing lysis buffer. Mix gently. (The excess 1% Triton X-100 in the non denaturing lysis buffer quenches the SDS in the original denaturing buffer).

5. Fragment the DNA by passing the lysed suspension 5 to 10 times through a needle attached to a 1 ml syringe.

Repeat mechanical disruption until the viscosity is reduced to manageable levels. If the DNA is not fully digested and fragmented, it can interfere with the separation of the pellet and supernatant following centrifugation.

6. Incubate on ice for 5 minutes.

7. Proceed with the immunoprecipitation.

Lysates from tissue
1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.

2. Place the tissue in round bottom micro centrifuge tubes and immerse in liquid nitrogen to “snap freeze”. Store samples at -80°C for later use or keep on ice for immediate homogenization.

3. For a ~5 mg piece of tissue, add ~300 µl lysis buffer rapidly to the tube and homogenize with an electric homogenizer.

4. Rinse the blade twice with another 300 µl lysis buffer per rinse and then maintain constant agitation for 2 hours at 4°C (e.g. place on an orbital shaker in the refrigerator).

Volumes of lysis buffer must be determined in relation to the amount of tissue present. Protein extract should not be too dilute in order to avoid loss of protein and to minimize the volume of samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml; optimal concentration is 1-5 mg/ml.

Note: *If denatured samples are required, use denaturing lysis buffer and perform steps 2 to 5 from the denaturing protocol above.*

5. Centrifuge for 20 minutes at 12,000 rpm at 4°C in a micro centrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice. Discard the pellet.

9.3 Pre-clearing the lysates

Pre-clearing the lysate can help reduce non-specific binding of proteins to agarose or sepharose beads. Pre-clearing with an irrelevant antibody or serum will remove proteins that bind immunoglobulins non-specifically. The end result will have a lower level of background and an improved signal to noise ratio. However, if the final detection of the protein is by western blotting, pre-clearing may not be necessary, unless a contaminating protein is interfering with visualization of the protein of interest.

1. Add either 50 µl of irrelevant antibody of the same species and isotype as the IP antibody or normal serum (rabbit is preferred by some researchers, see Harlow and Lane, page 243) to 1 ml of lysate. Incubate for 1 hour on ice.

2 Add 100 µl of bead slurry to the lysate.

3. Incubate for 10 to 30 minutes at 4°C with gentle agitation.

4. Spin in micro centrifuge at 14,000 x *g* at 4°C for 10 minutes.

5. Discard bead pellet and keep supernatant for immunoprecipitation.

To increase the yield, the beads can be washed 1 or 2 more times in lysis buffer, and the supernatants collected together.

It is important to make sure that as much of the normal serum is removed as possible as this will compete with the specific antibody against the antigen of interest. To check for this, a test can be done with lysis buffer instead of sample, performing all pre-clearing steps as above. A coomassie stain of a gel, in which the resulting supernatant is run, will reveal if the serum Ig is being removed effectively. If serum has not been sufficiently removed, bands will be present at 50 and 25 kDa for heavy and light chains; its presence may contribute to a weak IP. Consider either decreasing the amount of serum or increasing the amount of beads incubated with your samples in the pre-clearing step.

9.4 Immunoprecipitation

1. On ice, in a micro centrifuge tube add 10-50 □g cell lysate plus the recommended amount of antibody. These amounts will be chosen depending on the abundance of the protein and the affinity of the antibody for the protein. Typically in a pilot experiment a fixed amount of protein is precipitated by increasing amounts of antibody.

You can check the antibody datasheet for recommended antibody concentration. As a guideline use:

- 1-5 µl polyclonal antiserum
- 1 µg affinity purified polyclonal antibody
- 0.2-1 µl ascites fluid (monoclonal antibody)
- 20-100 µl culture supernatant (monoclonal antibody)

2. Incubate the sample with the antibody for a fixed time between 1-12 hours (overnight) at 4°C, preferably under gentle agitation or rotation. The length of the incubation period depends on the amount of protein and affinity properties of the antibody.

3. Meanwhile prepare the sepharose beads. If using a monoclonal antibody choose protein G coupled sepharose beads. If using a polyclonal antibody, protein A-coupled sepharose beads are usually suitable (please refer to 'Choosing the protein beads,' table 9.1.5. below). If the beads come as a powder, incubate 100 mg of beads in 1 ml 0.1 M PBS, wash for 1 hour so they swell up, then centrifuge, remove the supernatant and discard. Add 1 ml PBS 0.1% BSA, mix for 1 hour and rinse in PBS 2 X. Remove the supernatant and add 400 □l of buffer made with protease inhibitors (can be the same as the lysis buffer). The slurry is now ready for use. It can be stored at 4°C for a few days; for longer periods keep the beads in PBS with 0.02% azide (rinse the beads extensively on the day of use and make up in fresh lysis buffer). You can also buy pre-swollen beads as slurry ready for use.

It is advisable to use pipette tips with the end cut off to prevent damage to the beads.

IgM antibody: Do not use protein-A or protein-G conjugated beads. Use Goat anti Mouse IgM (or polyvalent Ig, or anti-heavy chain) beads.

4. Mix the slurry well and add 70-100 □l of the beads to each sample. Always keep samples on ice. Beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette and use a tip cut 5 mm from the top.

5. Incubate the lysate beads mixture at 4°C under rotary agitation for 4 hours (the optimal incubation time can be determined in a preliminary experiment).

6. When the incubation period is over, centrifuge the tubes, remove the supernatant and wash the beads in lysis buffer 3 X (each time centrifuging at 4°C and removing the supernatant).

7. Finally, remove the last supernatant and add 25-50 □l of 2 X loading buffer. Boil at 95-100°C for 5 minutes to denature the protein and separate it from the protein-A/G beads, then centrifuge and keep the supernatant which contains the protein. You can then freeze the samples or run them on a SDS-PAGE gel.

Using loading buffer is the harshest elution method, and will also elute any non covalently bound antibodies and antibody fragments, which will appear on western blot gels. Antigens can be gently eluted with a glycine gradient (up to 1 M) to reduce the amount of eluted antibody. Please also see separate procedure for cross-linking antibody to Sepharose.

9.5 Choosing the correct beads- summary table

Species immunoglobulin isotype	Protein A	Protein G
Human IgG1	+++	+++
Human IgG2	+++	+++
Human IgG3	-	+++
Human IgG4	+++	+++
Human IgM	Use anti Human IgM	
Human IgE	-	+
Human IgA	-	+
Mouse IgG_1	+	+++
Mouse $IgG2_a$	+++	+++
Mouse $IgG2_b$	++	++
Mouse IgG_3	+	+
Mouse IgM	Use anti Mouse IgM	
Rat IgG	-	+
Rat IgG_{2a}	-	+++
Rat IgG_{2b}	-	++
Rat IgG_{2c}	+	++
Chicken all isotypes	-	++
Cow all isotypes	++	+++
Goat all isotypes	-	++
Guinea Pig all isotypes	+++	++
Hamster all isotypes	+	++
Horse all isotypes	++	+++
Pig all isotypes	+	++
Rabbit all isotypes	+++	++
Sheep all isotypes	-	++

Key: +++ = Strong binding, ++ = Medium binding, + = Weak binding, - = No binding

References

Harlow, Ed, and David Lane. Using Antibodies. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1999.

Bonifacino, Juan S. et al. Current Protocols in Immunology 8.3.1-8.3.28, New York: John Wiley, 2001.

9.6 Procedure for cross-linking the antibody to the beads

Reducing the amount of antibody contamination in eluted protein solution

To enable elution of protein with little antibody contamination, for cleaner protein preparation and cleaner western blots, cross-linking the antibody to the beads is recommended. An example procedure for this is shown below (there is much information on this procedure on several websites). The target protein should then be eluted with a mild eluent, such as glycine buffer. More detailed buffer recipes can be found in the Buffers section on page 71.

Reagents

Cross linking reagent

Dimethyl pimelimidate (DMP)
Stock concentration 13 mg/ml

Elution reagent
1 M glycine (Add conc. HCl to adjust to pH 3)

Dilution buffer
PBS + 1 mg BSA per 1 ml PBS

Wash buffer
0.2 M triethanolamine in PBS (3.04 ml triethanolamine per 100 ml buffer)

Quenching buffer
50 mM ethanolamine in PBS (311.7 µl per 100 ml)

Preparation
1. Wash beads 2 X in PBS. The end concentration should be 50% bead slurry.

2. Mix well with PBS and rotate overnight at 4°C.

Cross-linking
1. Wash the beads by micro centrifuging (14,000 rpm for 1 minute) into a pellet. Aspirate out the PBS supernatant.

2. Add dilution buffer at 1:1 ratio, mix gently and rotate for 10 minutes at 4°C. Micro centrifuge and aspirate/discard the supernatant as before.

3. Prepare the antibody solution in dilution buffer at the required concentration (see antibody datasheet for suggested concentration). Add diluted antibody at 1:1 ratio to the beads. Mix gently and rotate 1 hour at 4°C.

3. Centrifuge and aspirate/discard the supernatant.

4. Add dilution buffer to beads at 1:1 ratio. Rotate for 5 minutes at 4°C. Centrifuge and aspirate/discard the supernatant.

5. Add PBS to beads at 1:1 ratio. Centrifuge and aspirate/discard the supernatant.

6. Cross-linking
Dissolve 1 ml of prepared 13 mg/ml stock of DMP with 1 ml wash buffer. Vortex immediately to mix. Add DMP solution to beads at 1:1 ratio. Rotate for 30 minutes at room temperature (RT).

DMP is unstable in aqueous solution. Prepare solution immediately prior to use.

***Note:** You will need to verify pH of DMP is between 8-9 before and after addition to beads (cross-linking efficiency is greatly reduced outside this pH range).*

Wash the beads with wash buffer (rotate 5 minutes at RT, then spin and aspirate).
Add DMP for second time at 1:1 ratio, rotate 30 minutes at RT, wash as before.
Add DMP for third time at 1:1 ratio, rotate 30 minutes at RT, wash as before.

7. Quench and wash
Add Quench buffer at 1:1 ratio, rotate 5 minutes at RT, spin and aspirate; repeat.
Wash with PBS.

8. Remove excess (unlinked) antibody
Wash with 1 M glycine pH 3. Rotate 10 minutes RT. Repeat.

9. Storage washes.
Wash with buffer to be used for immunoprecipitation (usually PBS + Tween). Rotate 5 minutes at RT.
Wash three times and store in final wash (after rotation). Beads can be stored at 4°C for a few days. Sodium azide 0.02 – 0.05% w/v can be added to prevent bacterial growth.

Immunoprecipitation
The antibody bound beads can now be used in a normal IP procedure, as above.
To prevent elution of antibody with the target protein, use a gentle glycine elution gradient (up to 1 M).

9.7 Using IgM antibodies for immunoprecipitation

IgM antibodies are difficult to use in immunoprecipitation due to its pentameric structure. IgM antibodies also do not bind well to protein A or protein G. There are two alternative methods available:

9.8a Protein L beads

Protein L is a 36 kDa immunoglobulin binding protein that can be purified from *Peptostreptococcus magnus*. Protein L binds antibodies through light chain interactions (whereas protein A and G bind the Fc heavy chain region of antibodies). Protein L binds a wider range of antibody classes than protein A or G because no part of the heavy chain is involved in the binding interaction. It is able to bind all antibody classes, including IgG, IgM, IgA, IgE and IgD. Single chain variable fragments (ScFv) and Fab fragments also bind to protein L.

Binding of protein L to immunoglobulins is restricted to those that contain kappa (□) light chains (i.e □ □chain of the VL domain). In humans and mice, kappa (□) light chains predominate. The remaining immunoglobulins have lambda (□) light chains, which will not bind Protein L. Furthermore, protein L is effective in binding only certain subtypes of kappa light chains. For example, it binds human V□I, V□III and V□IV subtypes but does not bind the V□II subtype. Binding of mouse immunoglobulins is restricted to those having V□I light chains.

There are several advantages to using protein L:

1. Protein L does not bind to bovine, sheep or goat antibodies, making it ideal for purification of mouse IgM from cell culture media containing bovine serum.

2. Protein L binds to kappa light chains on antibodies from a wide range of species without interfering with antigen binding sites, making it very suitable for immunoprecipitation using IgM antibodies.

3. It binds to all classes of Ig (IgG, IgM, IgA, IgE and IgD) making it particularly useful for IgM purification which cannot be purified using protein A or G.

9.8b Use an IgG anti-IgM antibody on protein A or protein G beads

This method involves coupling an IgG anti-IgM antibody to protein A or G beads (see method below). These beads can then be used in the normal immunopreciptiation procedure using the IgM antibody. The IgM will bind indirectly to the beads by binding to the anti-IgM antibody.

The procedure below describes how to couple the anti-IgM antibody to protein A or G coated beads using dimethylpimelimidate (DMP):

Reagents

- 200 mM Borate buffer + 3M NaCl pH 9.0
- 20 mM Dimethylpimelimidate (DMP) in 200 mM borate buffer + 3 M NaCl, pH 9.0
- 200 mM Ethanolamine pH8 (1:80 dilution of stock ethanolamine)
- Phosphate buffered saline (PBS)
- PBS + 0.1% sodium azide
- 200 mM Glycine pH 2.5
- Protein A or G beads (Sepharose)

Method

We recommend using 2 mg of antibody per ml wet beads (use appropriate antibody/protein A or G combination).

1. Add 250 mg Protein A or G Sepharose beads (beads) to 2 ml PBS + 0.1% sodium azide, for 1 hour before use. This allows the beads to swell.

2. Mix the beads with an appropriate concentration of antibody for 2 hours at room temperature (using a rotator or roller).

3. Centrifuge at 2500 rpm for 5 minutes.

4. Wash the beads twice in 10 X volume of 200 mM borate buffer + 3 M NaCl.

5. Resuspend the beads in 20 mM DMP.

6. Rotate/agitate the beads for 30 minutes at room temperature.

7. Centrifuge the beads at 2,500 rpm for 5 minutes.

8. Wash the beads 2 X in 200 mM borate buffer + 3M NaCl.

9. Wash the beads 1 X in 20 mM ethanolamine. This stops the coupling reaction.

10. Resuspend the beads in 20 mM ethanolamine and rotate for 2 hours at room temperature.

11. Centrifuge the beads at 2,500 rpm for 5 minutes.

12. Wash the beads 2 X in PBS.

13. Wash the beads 2 X in 200 mM glycine.

14. Wash the beads 2 X in PBS.

15. The beads can be stored at 4°C in PBS + 0.1% sodium azide (ratio of 1:1 PBS:beads).

16. These beads are now ready for use in immunoprecipitation with IgM antibody. From this point, proceed with the immunoprecipitation as described on our immunoprecipitation protocol, as on page 60.

9.9 Troubleshooting tips - IP

High background

Carry over of proteins that are not detergent soluble.
Remove supernatant immediately after centrifugations. This should leave insoluble proteins in the pellet. If resuspension occurs, centrifuge again.

Incomplete washing.
Wash well at relevant stages by placing a lid on the tube and inverting several times before centrifuging.

Non specific proteins are binding to the beads.
Beads are not pre-blocked enough with BSA. Make sure the BSA (fraction V) is fresh and incubate fresh beads 1 hour with 1% BSA in PBS. Wash 3-4 times in PBS before using them.

Antibody used contains antibodies that are not specific enough.
Use an affinity purified antibody, preferably pre-absorbed.

Too much antibody used giving non-specific binding.
Check the recommended amount of antibody suggested. Try using less antibody.

Too many cells or too much protein in lysate, leading to a lot of additional (false positive) proteins in the eluate.
Reduce the number of cells/lysate used. We recommend using 10-500 □g cell lysate.

Non-specific binding of proteins to antibody.
If there are many proteins binding non-specifically, then try reducing the amount of sample loaded onto the beads. You can also pre-clear the lysate by pre-incubating the prepared lysate with the beads before commencing with the immunoprecipitation (please see the protocol). This should clear the lysate of any proteins that are binding non-specifically to the beads. Some researchers also use an irrelevant antibody of the same species of origin and same Ig subclass to pre-clear the lysate.

Antigen degrading during immunoprecipitation.
Ensure fresh protease inhibitors are added when sample is lysed.

High amount of antibody eluting

To much antibody eluting with the target protein.
Try reducing the amount of antibody. Cross-linking the antibody to the beads before the immunoprecipitation and eluting using a gentle glycine buffer gradient should significantly reduce the amount of antibody eluted.

No eluted target protein detected

Target protein not expressed in sample used or low level of target protein expression in sample used.
Check the expression profile of the target protein to ensure it will be expressed in the cells of your samples. If there is low level of target protein expression, increase the amount of lysate used. However, this may result in increased non-specific binding so it would be advisable to pre-clear the lysate before commencing with the IP procedure.

Insufficient antibody for capture of the target protein.
Check that the recommended amount of antibody is being used. The concentration of antibody may need to be increased for optimization of results.

Target protein has not eluted from the beads.
Ensure you are using the correct elution buffer and that it is at the correct strength and pH for elution of the protein.

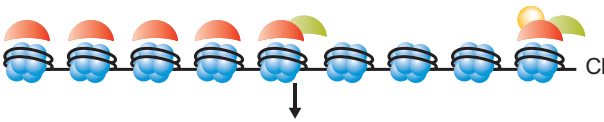
Antibody has not bound to immunoadsorbent beads.
Ensure you are using the correct beads for the antibody isotype used.

Incorrect lysis buffer used.
Check datasheet to see if the antibody detects denatured or native protein and ensure the correct lysis buffer is used.

SECTION 10: ChIP

10.1 Cross-linking Chromatin Immunoprecipitation (X-ChIP) Protocol

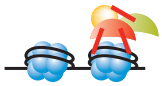
Map proteins/histone modifications to genomic loci



Chromatin and associated proteins.

Cross-link

Start with 2 X 150 cm^2 dishes of confluent cells
(1 X 10^7 - 5 X 10^7 cells per dish)
Add formaldehyde to a final conc of 0.75%
Incubate 2 - 30 min RT
Add glycine to a final conc of 125 mM
Shake gently for 5 min



Cross-linking fixes proteins to DNA.

Cell collection

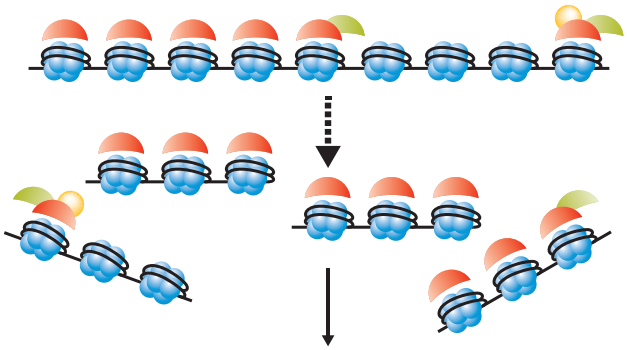
Wash cells with ice cold PBS
Scrape and collect cells into 5 ml ice cold PBS and transfer to a new tube
Wash dishes with 3 ml PBS to ensure all cells collected

Cell lysis

Centrifuge 5 min 1 000 g and remove supernatant
Add FA lysis buffer to cell pellet (750 µl per 1 X 10^7 cells)

Sonication

Sonicate to give a fragment size of 500 - 1000 bp



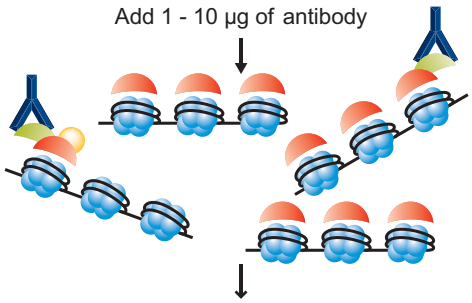
Sonication generates sheared, soluble chromatin. Optimize by performing a time course and purify DNA. Analyze fragment size on 1.5 % agarose gel.

Centrifuge 30 sec 4˚C 8 000 g to pellet cell debris
Transfer supernatant containing chromatin to a new tube

Remove 50 µl (INPUT) and purify DNA to calculate the DNA concentration

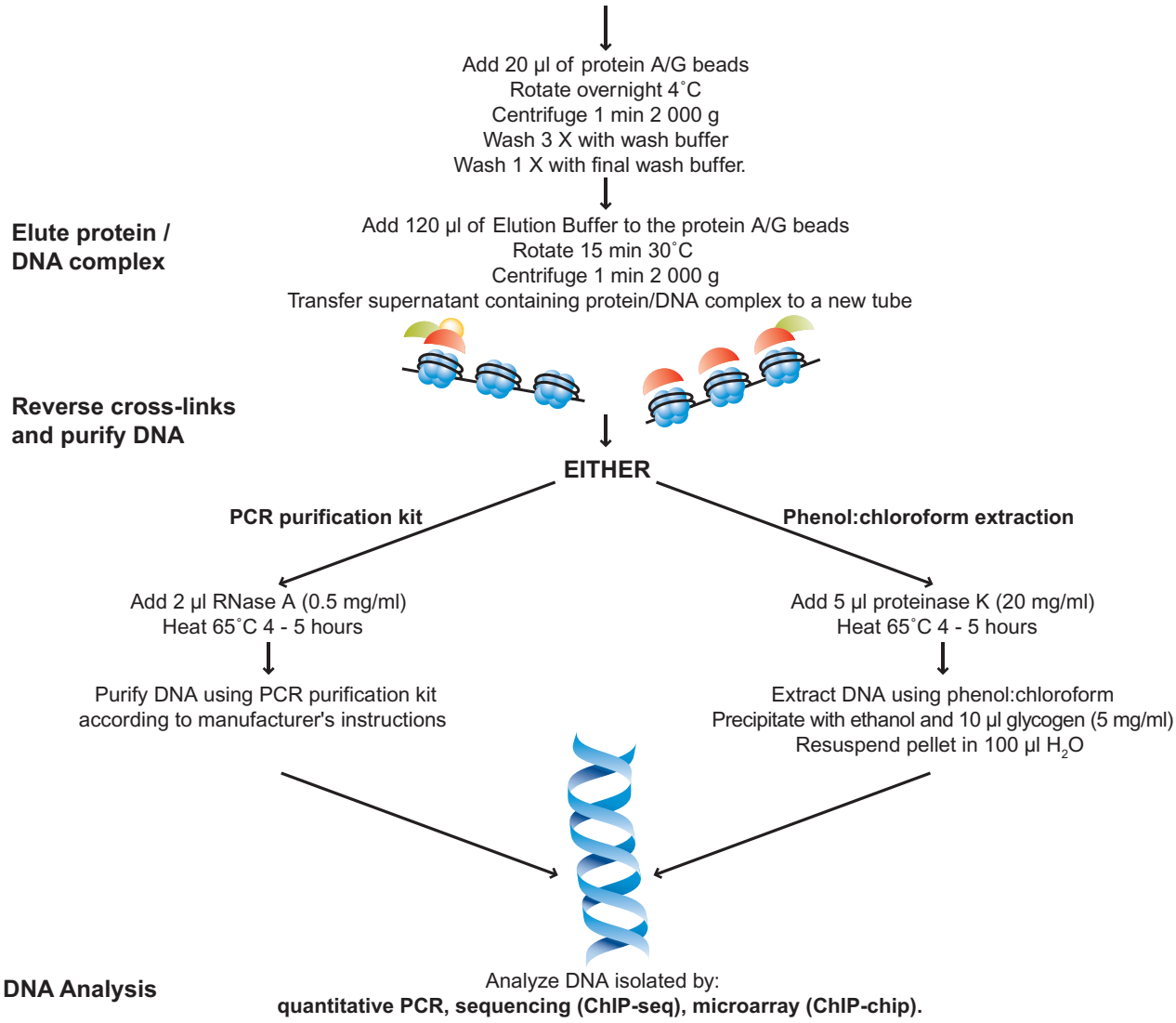
Immunoprecipitation

Use an amount of chromatin equivalent to approximately 25 µg of DNA per IP
Dilute 1:10 with RIPA buffer
Add 1 - 10 µg of antibody



Antibody binds to target and associated DNA is isolated. DNA fragments not associated are removed during washes.

(Continued Overleaf)



ChIP is a powerful tool that specifically correlates the localization of proteins or their modifications to regions of the genome. Chromatin is isolated and antibodies to the antigen of interest are used to determine whether the target binds to a specific DNA sequence. ChIP can also be used to map the distribution of the target across the genome in a spatial and/or temporal manner (using a microarray or DNA sequencing). This protocol provides specific details of how a cross-linking ChIP (X-ChIP) experiment can be performed.

1. Cross-linking and cell harvesting

Formaldehyde is used to cross-link the proteins to the DNA. Cross-linking is a time dependent procedure and optimization will be required. We would suggest cross-linking the samples for 2-30 minutes. Excessive cross-linking reduces antigen accessibility and sonication efficiency. Epitopes may also be masked. Glycine is added to quench the formaldehyde and terminate the cross-linking reaction.

1.1 Start with 2 confluent 150 cm^2 dishes ($1x10^7$- $5x10^7$ cells per dish). Cross-link proteins to DNA by adding formaldehyde drop wise directly to the media to a final concentration of 0.75 % v/v and rotate gently at room temperature (RT) for 10 minutes.

1.2 Add glycine to a final concentration of 125 mM to the media and incubate with shaking for 5 minutes at RT.

1.3 Rinse cells 2 X with 10 ml cold PBS.

1.4 Scrape cells into 5 ml cold PBS and transfer into 50 ml tube.

1.5 Add 3 ml PBS to dishes and transfer the remainder of the cells to the 50 ml tube.

1.6 Centrifuge for 5 minutes, 1,000 g.

1.7 Carefully aspirate off supernatant and resuspend pellet in FA Lysis Buffer (750 µl per $1x10^7$ cells).

When using suspension cells, start with $1x10^7$- $5x10^7$ cells and treat with both 0.75 % v/v formaldehyde and glycine as described above (Section 1). Pellet cells by centrifugation (5 minutes, 1000 g). Wash 3 X with cold PBS and resuspend pellet in FA Lysis Buffer (750 µl per $1x10^7$ cells). Proceed to Step 2.1.

2. Sonication

When using tissue as a starting material (See section 10.2), generate a unicellular suspension and start the protocol here at the sonication stage.

2.1 Sonicate lysate to shear DNA to an average fragment size of 500-1000 bp. This will need optimizing as different cell lines require different sonication times.

The cross-linked lysate should be sonicated over a time course to identify optimal conditions. Samples should be removed over the time course and DNA isolated as described in Section 3. The fragment size should be analyzed on a 1.5 % w/v agarose gel as demonstrated in Figure 1.

2.2 After sonication, pellet cell debris by centrifugation for 30 seconds, 4°C, 8,000 g. Transfer supernatant to a new tube. This chromatin preparation will be used for the immunoprecipitation (IP) in Step 4.

2.3 Remove 50 µl of each sonicated sample. This sample is the INPUT and is used to quantify the DNA concentration (see step 3) and as a control in the PCR step.

The sonicated chromatin can be snap frozen in liquid nitrogen and stored at -80°C for up to 2 months. Avoid multiple freeze thawing.

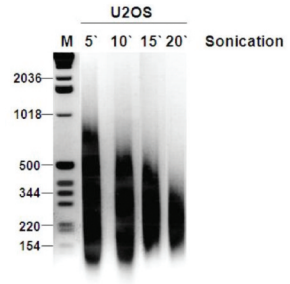


Figure 1. U2OS cells were sonicated for 5, 10, 15 and 20 minutes. The fragment size decreases during the time course. The optimal fragment size is observed 15 minutes after sonication. Note: sonicating for too long will disrupt nucleosome-DNA interactions so the band size should not be smaller than 200bp.

3. Determination of DNA concentration

3.1 The INPUT samples are used to calculate the DNA concentration for subsequent IPs. Purify the DNA using either a PCR purification kit (add 70 µl of Elution Buffer and proceed to step 3.2a) or phenol:chloroform (add 350 µl of Elution Buffer and proceed to Step 3.2b).

3.2a Add 2 µl RNase A (0.5 mg/ml). Heat with shaking at 65°C for 4-5 hours (or overnight) to reverse the cross-links. Purify the DNA using a PCR purification kit according to the manufacturer's instructions. The samples can be frozen and stored at -20°C.

Samples are treated with RNase A as high levels of RNA will interfere with DNA purification when using the PCR purification kit. Yields can be severely reduced as the columns become saturated.

3.2b Add 5 µl proteinase K (20 mg/ml). Heat with shaking at 65°C for 4-5 hours (or overnight) to reverse the cross-links. Extract the DNA with phenol:chloroform followed by ethanol precipitation in the presence of 10 µl glycogen (5 mg/ml). Resuspend in 100 µl H_2O. The samples can be frozen and stored at -20 °C.

Samples are treated with proteinase K, which cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids. Cross-links between proteins and DNA are disrupted to aid DNA purification.

3.3 To determine the DNA concentration, transfer 5 µl of the purified DNA into a tube containing 995 µl TE to give a 200 fold dilution and measure the OD_{260}. Calculate the DNA concentration of the chromatin preparation in µg/ml. Concentration of DNA in µg/ml = OD_{260} x 10,000.

4. Immunoprecipitation

4.1 Use the chromatin preparation from step 2.2. Chromatin containing approximately 25 µg of DNA per IP is recommended. Dilute each sample 1:10 with RIPA Buffer. You will need one sample for the specific antibody and one sample for the control (beads only).

4.2 Add primary antibody to all samples except the control. The amount of antibody to be added should be determined empirically. 1-10 µg of antibody per 25 µg of DNA often works well.

4.3 Add 20 µl of protein A/G beads (pre-adsorbed with sonicated single stranded herring sperm DNA and BSA, see Step 4.3a) to all samples and IP overnight with rotation at 4°C.

4.3a Preparation of protein A/G beads with single-stranded herring sperm DNA: if using both Protein A and Protein G beads, mix an equal volume of Protein A and Protein G beads and wash 3 X in RIPA Buffer. Aspirate RIPA Buffer and add single stranded herring sperm DNA to a final concentration of 75 ng/µl beads and BSA to a final concentration of 0.1 µg/µl beads. Add RIPA Buffer to twice the bead volume and incubate for 30 minutes with rotation at RT. Wash once with RIPA Buffer and add RIPA Buffer to twice the bead volume.

Protein A beads, protein G beads or a mix of both should be used. The table in section 9 page 61 shows the affinity of protein A and G beads to different immunoglobulin isotypes.

4.4 Centrifuge the protein A/G beads for 1 minute, 2,000 g and remove the supernatant.

4.5 Wash beads 3 X with 1 ml wash buffer. Centrifuge 1 minute, 2,000 g.

4.6 Wash beads 1 X with 1 ml Final wash buffer. Centrifuge 1 minute, 2,000 g.

If high background is observed, additional washes may be needed. Alternatively, the sonicated chromatin may also be pre-cleared by incubating with the Protein A/G beads for 1 hour prior to Step 4.2. Any non-specific binding to the beads will be removed during this additional step. Transfer the supernatant (sonicated chromatin) to a new tube and incubate with the antibody and beads as described in Step 4.2 onwards.

5. Elution and reverse cross-links

5.1 Elute DNA by adding 120 µl of elution buffer to the protein A/G beads and rotate for 15 minutes at 30 °C.

5.2 Centrifuge for 1 minute 2,000 g, and transfer the supernatant into a fresh tube. The samples can be stored at -20 °C.

5.3 The DNA can be purified using a PCR purification kit (proceed with step 2a) or phenol:chloroform (add 280 µl of Elution Buffer and proceed with Step 3.2b).

5.4 DNA levels are quantitatively measured by real-time PCR. Primers and probes are often designed using software provided with the real-time PCR apparatus. Alternatively, online design tools may be used.

6. Chromatin preparation from tissues for ChIP

This protocol describes how chromatin is prepared from tissue, which can subsequently be used for ChIP. It is recommended that 30 mg of liver tissue is used for each ChIP/antibody. However, this amount may vary for other tissues. The exact amount of tissue depends upon protein abundance, antibody affinity and the efficiency of cross-linking. The protocol was optimized using 5-15 µg chromatin for each ChIP assay. The exact chromatin concentration should be determined for each tissue type before starting the cross-link ChIP (X-ChIP) assay. Our

X-ChIP protocol should be used after the chromatin preparation detailed below. Protease inhibitors should be included in all solutions used, including PBS [PMSF (10 □l/ml), aprotinin (1 □l/ml) and leupeptin (1 □l/ml)].

This section was adapted from protocols kindly provided by Henriette O'Geen, Luis G. Acevedo and Peggy J. Farnham.

6.1 Cross-linking

Frozen tissues should be thawed on ice. (This process could take hours depending on the amount of tissue). It is important that the frozen tissue samples do not reach high temperatures, in order to prevent sample degradation by proteases. Samples should be kept on ice at all times and all steps performed quickly to minimize thawing. Tissue should be cut in a petri dish resting on a block of dry ice.

6.1.1 Chop frozen or fresh tissue into small pieces using 2 razor blades (between 1-3 mm^3).

6.1.2 Determine the weight of an empty 15 ml conical tube, transfer tissue into the tube and weigh again to calculate the amount of tissue.

6.1.3 Prepare cross-linking solution in fume hood. Use 10 ml PBS per gram of tissue. Add formaldehyde to a final concentration of 1.5 % v/v and rotate tube at RT for 15 minutes.

6.1.4 Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M. Continue to rotate at RT for 5 minutes.

6.1.5 Centrifuge tissue samples for 5 min, 720 rpm, 4°C.

6.1.6 Aspirate media and wash with 10 ml ice cold PBS. Centrifuge for 5 minutes, 720 rpm, 4°C and discard wash buffer.

The tissue may be snap frozen at this stage in liquid nitrogen and stored at -80°C. Avoid multiple freeze thaws.
If using immediately, resuspend tissue in 10 ml cold PBS per gram of starting material. Place on ice.

6.2 Tissue disaggregation
The Medimachine from Becton Dickinson may be used to obtain a single cell suspension. Use 2 medicones (50 □m) per gram of tissue to process.

6.2.1 Cut the end off a 1 ml pipette tip to make the orifice larger.

6.2.2 Add between 50-100 mg (3-4 chunks) of tissue resuspended in 1 ml of PBS.

6.2.3 Add this solution to the medicone and grind tissue for 2 minutes.

6.2.4 Collect cells from the medicone by inserting an 18 gauge blunt needle attached to a 1 ml syringe. Transfer cells to a conical tube on ice.

6.2.5 Repeat step 2 until all the tissue is processed.

6.2.6 Check the cell suspension using a microscope to ensure a unicellular suspension has been obtained. If more grinding is necessary, add more PBS to the tissue and repeat steps 2 to 5 until all tissue is ground into a homogeneous suspension.

6.2.7 Centrifuge cells for 10 minutes, 1000 rpm, 4°C. Measure/estimate cell pellet volume for next step.

6.2.8 Carefully aspirate off supernatant and resuspend pellet in FA Lysis Buffer (750 □l per $1x10^7$ cells).

6.2.9 Continue with the X-ChIP protocol from replace from stage 2 (Sonication).

10.2 Troubleshooting tips - ChIP

High background in non specific antibody controls

Non-specific binding to Protein A or G beads.
Include a pre-clearing step, whereby the lysed sample is mixed with beads alone for 1 hour and removed prior to adding the antibody.

The ChIP buffers may be contaminated.
Prepare fresh lysis and wash solutions.

Some Protein A or G beads give high background.
Some Protein A or G beads can give high background levels. Find a suitable supplier that provides the cleanest results with low background in the non-specific control.

Low resolution with high background across large regions

DNA fragment size may be too large

DNA fragmentation should be optimized when using different cell types. Both sonication times and enzyme incubation times may be varied. We would suggest a DNA fragment size of no larger than 1.5 kbp. If chromatin is being digested using enzymes, mononucleosomes (175 bp) can be obtained.

Low signal

The chromatin size may be too small.

Do not sonicate chromatin to a fragment size of less than 500 bp. Sonication to a smaller size can displace nucleosomes as intra nucleosomal DNA becomes digested. If performing N-ChIP, enzymatic digestion is generally sufficient to fragment chromatin.

If performing X-ChIP, the cells may have been cross-linked for too long.

Cross-link with formaldehyde for 10-15 minutes and wash well with PBS. Cells may need to be treated with glycine to quench the formaldehyde. Excessive cross-linking can reduce the availability of epitopes and thus decrease antibody binding.

Insufficient starting material.

We would suggest using 25 µg of chromatin per immunoprecipitation

Insufficient antibody included in the immunoprecipitation.

We would suggest using between 3-5 µg of antibody in the first instance. This could be increased to 10 µg if no signal is observed.

Specific antibody binding is being eliminated.

Do not use NaCl concentrations higher than 500 mM in the wash buffers as this may be too stringent and remove specific antibody binding.

Cells are not effectively lysed.

We would suggest using RIPA buffer to lyse cells

No antibody enrichment at region of interest.

The epitope is not found at the region of interest. Be sure to include a positive control antibody to confirm the procedure is working well e.g. a H3K4me3 antibody at active promoters or a H3K9me3 antibody at heterochromatic loci.

Some monoclonal antibodies may not be suitable for X-ChIP.

The epitope recognized by the monoclonal antibody may have become masked during cross-linking, thus preventing epitope recognition. We would suggest using polyclonal antibodies that will recognize multiple epitopes as there is an increased chance of immunoprecipitating the protein of interest.

The wrong antibody affinity beads were used

Protein A and G are bacterial proteins that bind various classes of immunoglobulins with varying affinities. Use an affinity matrix that will bind your antibody of interest. We would suggest using a mix of protein A and protein G that have been coupled to sepharose.

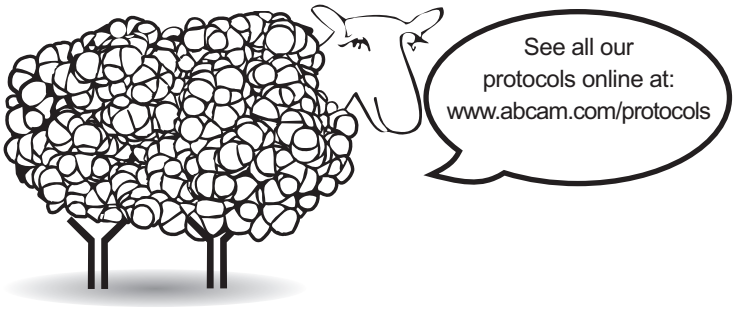
Problems with PCR amplification on immunoprecipitated DNA

High signal in all samples after PCR, including no template control.

Contamination in real-time PCR solutions. We suggest preparing new solutions from stocks

No DNA amplification in samples.

Be sure to include standard/input DNA to confirm that the primers are working well.



SECTION 11: Buffers

11.1 Standard PBS, TBS, TBS Tween

PBS (10x)
• 80 g of NaCl
• 2.0 g of KCl
• 14.4 g of Na_2HPO_4
• 2.4 g of KH_2PO_4
• Mix in 800 ml ultra pure water and adjust pH to 7.4 with pure HCl. Top up with ultra pure water to 1 L.

TBS (10x)
• 24.23 g Trizma HCl
• 80.06 g NaCl
• Mix in 800 ml ultra pure water and adjust pH to 7.6 with pure HCl. Top up with ultra pure water to 1 L.

TBST (0.1% Tween20)
For 1 L: 100 ml of TBS 10x + 890 ml ultra pure water + 10ml Tween 20 (10%)

Tween 20 is very viscous and will stick to the tip of measuring pipettes. Be sure to add the right amount of detergent to the Tris buffer. We recommend a 10% solution as is easier to dispense than undiluted Tween 20.

11.2 Western blot

Lysis buffers:
These buffers may be stored at 4°C for several weeks or for up to a year aliquotted and stored at -20°C.

Nonidet-P40 (NP40) buffer
• 150 mM NaCl
• 1.0% NP-40 (possible to substitute with 0.1% Triton X-100)
• 50 mM Tris-HCl pH 8.0
• Protease Inhibitors

RIPA buffer (RadioImmunoPrecipitation Assay) buffer
• 150 mM NaCl
• 1.0% NP-40 or 0.1% Triton X-100
• 0.5% sodium deoxycholate
• 0.1% SDS (sodium dodecyl sulphate)
• 50 mM Tris-HCl pH 8.0
• Protease inhibitors

More denaturing than NP-40 or Triton X-100 lysis buffer, RIPA buffer contains the ionic detergents SDS and sodium deoxycholate as active constituents and is particularly useful for nuclear membrane disruption for nuclear extracts. RIPA buffer gives low background but can denature kinases.

The 10% sodium deoxycholate stock solution (5 g into 50 ml) must be protected from light.

Tris-HCl buffer
• 20 mM Tris-HCl pH 7.5
• Protease inhibitors

Tris-Triton buffer (Cytoskeletal proteins):
• 10 mM Tris, pH 7.4
• 100 mM NaCl
• 1 mM EDTA
• 1 mM EGTA
• 1% Triton X-100
• 10% glycerol
• 0.1% SDS
• 0.5% sodium deoxycholate
• Protease inhibitors

The 10% sodium deoxycholate stock solution (5 g into 50 ml) must be protected from light.

Non-denaturing lysis buffer
• 20 mM Tris HCl pH 8
• 137 mM NaCl
• 10% glycerol
• 1% NP-40/Triton X-100
• 2 mM EDTA
• Protease inhibitors

Use for antigens that are detergent soluble and can be recognised in native form by the antibody. Triton X-100 can be substituted for NP-40.

Detergent-free soluble protein lysis buffer
• 5 mM EDTA
• 0.02% Sodium Azide
• In PBS
• Protease inhibitors

Some soluble proteins may not require use of detergents. Use this buffer with mechanical breakage of cells, e.g. repeated passage through a syringe or homogenization with a Dounce homogenizer.

Denaturing lysis buffer/buffer for non-detergent soluble antigens:
• 1% SDS
• 5 mM EDTA

Immediately before use add:
• 10 mM dithiothreitol or beta-mercaptoethanol
• Protease inhibitors
• 15 U/ml DNase1

Epitopes of native proteins are not accessible to antibodies that only recognise denatured proteins. When harvesting and lysing the cells, heat the cells in denaturing lysis buffer. This method can also be used for antigens that cannot be extracted from the cell with non-ionic detergents. Use of DNase1 will aid extraction of proteins from chromatin.

Running and blotting buffers
Laemmli 2X buffer / loading buffer
• 4% SDS
• 10% 2-mercaptoethanol
• 20% glycerol
• 0.004% bromophenol blue
• 0.125 M Tris-HCl

Check the pH and adjust pH to 6.8.

Running buffer
• 25 mM Tris base
• 190 mM glycine
• 0.1% SDS

Check the pH, which should be about pH 8.3. Adjust if necessary.

Transfer buffer (Wet)
• 25 mM Tris base
• 190 mM glycine
• 20% methanol

The pH should be about pH 8.3. Adjust if necessary.

For proteins larger than 80 kDa, we recommend that SDS is included at a final concentration of 0.1%.

Transfer buffer (Semi-dry)
• 48 mM Tris
• 39 mM glycine
• 20% methanol
• 0.04% SDS

Blocking buffer:
5% milk or BSA (bovine serum albumin)

Add to TBST buffer. Mix well and filter. Failure to filter can lead to “spotting” where tiny dark grains will contaminate the blot during colour development.

11.3 Immunohistochemistry/Immunocytochemistry (IHC)

Fixation buffers

Formalin Solution (10%, buffered neutral):

- 3.7-4% Formaldehyde (37-40%)
- 33 mM NaH_2PO_4
- 46 mM Na_2HPO_4 (anhydrous)

Paraformaldehyde (4%, buffered neutral):

8% Paraformaldehyde

0.2 M Phosphate Buffer (PB), pH 7.4:

- 53 mM NaH_2PO_4
- 154 mM Na_2HPO_4

Heat 8% PFA solution at 60°C while stirring (do not allow the solution to go above 60°C). Once the solution has reached 60°C and the PFA is dissolved, add 500 ml of 0.2 M phosphate buffer, to bring the solution to 4% PFA in 0.1 M phosphate. Carefully add 1 N NaOH dropwise until solution is clear (try 1-2 drops per 500 ml; if still not clear, add a few more drops. Alternatively, you can add 2 pellets of solid NaOH in 1-2 L of solution). Cool the solution and filter.

The solution should be at pH 7.4. Do not adjust the pH using acid or base! If you need to adjust the pH, make up a separate 0.2 M solution of either the monobasic or dibasic sodium phosphate (depending on how you need to adjust the pH) and add accordingly.

PFA should always be made up fresh on the same day you wish to use it. Storage overnight at 4°C is possible, but it will not fix as well the second day. It is possible possible to freeze the PFA solution at -20°C, but for consistency of results, tissue should either be fixed always with fresh PFA or always with freshly thawed PFA.

Bouin Solution (especially for preserving soft and delicate structures such as brain tissues)

- 75 ml picric acid (saturated)
- 25 ml formaldehyde (37-40%)
- 5 ml glacial acetic acid
- Mix well.

Antigen retrieval buffers

Sodium Citrate Buffer pH 6.0

- 10 mM Sodium citrate (Tri-sodium citrate (dihydrate))
- 0.05% Tween 20
- Mix to dissolve sodium citrate and adjust pH to 6.0 with 1N HCl.

Add Tween 20 and mix well.
Store at room temperature for 3 months or at 4°C for longer storage.

Tris-EDTA Buffer pH9.0

- 10 mM Tris Base
- 1 mM EDTA
- 0.05% Tween 20

Mix to dissolve and adjust pH to pH 9.0. Add Tween 20 and mix well.
Store at room temperature for 3 months or at 4°C for longer storage.

Enzymatic antigen retrieval buffers:

Trypsin Stock Solution

0.5% Trypsin

Mix to dissolve in distilled water. Store at -20°C.

Calcium Chloride Stock Solution (1%)

1% Calcium chloride

Mix well in distilled water. Store at 4°C.

Trypsin Working Solution

- 0.05% Trypsin (Use 0.5% Trypsin stock solution
- 0.1% Calcium chloride (Use 1% Calcium chloride stock solution)
- 8 ml distilled water

Mix well in distilled water and adjust pH to 7.8 with 1 M NaOH
Store at 4°C for one month or -20°C for long term storage.

11.4 ELISA

Bicarbonate/carbonate coating buffer (100 mM) pH9.6
Antigen or antibody should be diluted in coating buffer to immobilize them to the wells:
• 29 mM Na_2CO_3,
• 71 mM $NaHCO_3$

Blocking solution
Commonly used blocking agents are 1% BSA , serum, non-fat dry milk, casein, gelatin in PBS.

Wash solution
Usually PBS or Tris -buffered saline (pH 7.4) with detergent such as 0.05% (v/v) Tween 20 (TBST).

Antibody dilution buffer
Primary and secondary antibody should be diluted in 1x blocking solution to reduce Non-specific binding.

11.5 Flow cytometry

FACS buffer/antibody dilution buffer
• 10% FCS
• 1% sodium azide
• In PBS

Permeabilization
0.1-1% Triton X-100 / NP-40
In PBS. Tween 20, Saponin, Digitonin and Leucoperm may also be used and are mild membrane solubilisers. Use at 0.5% in PBS

Fixative
0.01%-0.1% paraformaldehyde In PBS

11.6 Immuno Precipitation

See western blot buffers.

RIPA (RadioImmunoPrecipitation Assay) buffer
See western blot buffers.

11.7 ChIP

FA Lysis Buffer
• 50 mM HEPES-KOH pH 7.5
• 140 mM NaCl
• 1 mM EDTA pH 8
• 1% Triton X-100
• 0.1% Sodium Deoxycholate
• 0.1% SDS
• Protease inhibitors

RIPA Buffer
• 50 mM Tris-HCl pH 8
• 150 mM NaCl
• 2 mM EDTA pH 8
• 1% NP-40
• 0.5% Sodium Deoxycholate
• 0.1% SDS
• Protease inhibitors

Wash Buffer
• 0.1% SDS
• 1% Triton X-100
• 2 mM EDTA pH 8
• 150 mM NaCl
• 20 mM Tris-HCl pH 8

Final Wash Buffer
• 0.1% SDS
• 1% Triton X-100
• 2 mM EDTA pH 8
• 500 mM NaCl
• 20 mM Tris-HCl pH 8

Elution Buffer
• 1% SDS
• 100 mM NaHCO3

Proteinase K
Dissolve in H_2O at 20 mg/ml, store at -20°C.

SECTION 12: Antibody storage guide

Guidelines for the storage of different types of antibody, avoiding contamination or damage

Please always check datasheets for specific storage recommendations. Abcam can not guarantee the performance of antibodies that have not been stored correctly. With proper storage and handling, most antibodies should retain activity for months, if not years.

Storage temperatures and conditions
For many of our antibodies, freezing at -20°C or -80°C in small aliquots is the optimal storage condition. Aliquotting minimizes damage due to freezing and thawing, as well as contamination introduced by pipetting from a single vial multiple times. Aliquots are to be frozen and thawed once, with any remainder kept at 4°C. Upon receiving the antibody, centrifuge at 10,000 x g for 20 seconds to pull down solution that is trapped in the threads of the vial, and transfer aliquots into low-protein-binding microcentrifuge tubes. The size of the aliquots will depend on how much you typically use in an experiment. Aliquots should be no smaller than 10 µl; the smaller the aliquot, the more the stock concentration is affected by evaporation and adsorption of the antibody onto the surface of the storage vial.

In most cases, with the possible exception of ascites fluids which may contain proteases and should be frozen as soon as possible, storage at 4°C upon receipt of the antibody is acceptable for one to two weeks, followed by freezing for long-term storage. Again, it is important to follow the recommendations on the datasheet.

Exceptions and other special conditions
Enzyme-conjugated antibodies, should not be frozen at all and should instead be kept at 4°C. Freezing and thawing will reduce enzymatic activity in addition to affecting the antibody binding capacity.
Conjugated antibodies, whether conjugated to fluorochromes, enzymes, or biotin, should be stored in **dark vials or wrapped in foil**. Exposure to light will compromise the activity of conjugates. Fluorescent conjugates in particular are susceptible to photo-bleaching and should be protected from light during all phases of an experiment.
IgG3 isotype antibodies are unique in their tendency to form aggregates upon thawing and should always be stored at 4°C.

Preventing contamination with sodium azide
To prevent microbial contamination, sodium azide can be added to an antibody preparation to a final concentration of 0.02% (w/v). Many Abcam antibodies already contain this preservative at concentrations ranging from 0.02 to 0.05%. This will be indicated on the datasheets in the section titled “Storage buffer”.

When NOT to use sodium azide
If staining or treating live cells with antibodies, or if using antibodies for *in vivo* studies, be sure to use preparations that do not contain sodium azide.This antimicrobial agent is toxic to most other organisms as well: it blocks the cytochrome electron transport system.
Sodium azide will interfere with any conjugation that involves an amine group, and should be removed before proceeding with the conjugation. After conjugation, antibodies can be stored in sodium azide but 0.01% thimerosal (merthiolate), which does not have a primary amine, is an acceptable alternative.

Sodium azide can be removed from antibody solutions by dialysis or gel filtration. The molecular weight of IgG is 150,000 daltons (IgM is ~ 600,000); the molecular weight of sodium azide is 65 daltons. A micro-dialysis unit with a cut off at 14,000 daltons will retain the antibody as the azide diffuses out. In a beaker on a magnetic stirrer kept at 4°C, use at least a liter of cold PBS per ml of antibody and stir the dialysis unit for 6 hours. Change the PBS twice, stirring at least 6 hours for each change. If possible, all materials should be sterilized and the resulting preparation should be handled aseptically.

Freeze/thaw damage
Repeated freeze/thaw cycles can denature an antibody, causing it to form aggregates that reduce the antibody’s binding capacity.

Storing at -20°C should be adequate for most antibodies; there is no appreciable advantage to storing at -80°C. The freezer must not be of the frost-free variety. These cycle between freezing and thawing (to reduce frost-build-up), which is exactly what should be avoided. For the same reason, antibody vials should be placed in an area of the freezer that has minimal temperature fluctuations, for instance towards the back rather than on a door shelf.

Some researchers add the cryoprotectant glycerol to a final concentration of 50% to prevent freeze/thaw damage; glycerol will lower the freezing point to below -20°C. While this may be acceptable for many antibodies, only a small percentage of the antibodies we offer have been tested for stability in this storage condition and our guarantee only applies to antibodies stored as recommended on the datasheet. Storing solutions containing glycerol at -80°C is not advised since this is below the freezing point of glycerol. Please be aware that glycerol can be contaminated with bacteria. If adding glycerol or any cryoprotectant, care should be taken to obtain a sterile preparation.
Diluting antibodies to working concentration and storing at 4°C for more than a day should be avoided. Proteins in general are less susceptible to degradation when stored at higher concentrations, ideally 1 mg/ml or higher. This is the rationale for including proteins such as BSA to the antibody solution as stabilizers. The added protein also serves to minimize loss of antibody due to binding to the vessel wall. For antibodies that one intends to conjugate, stabilizing proteins should not be added since they will compete with the antibody and reduce the efficiency of the conjugation.

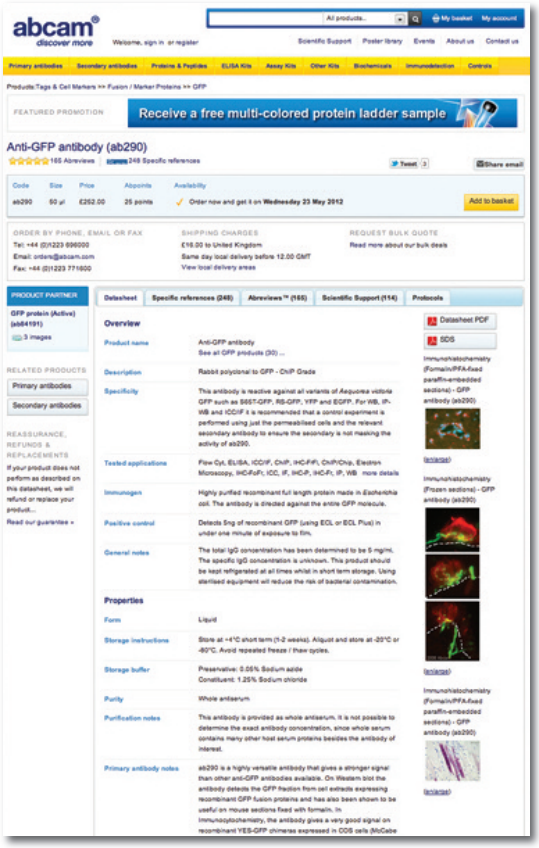
Technical help - online resources

Finding products and information online

Searching for products

Search our catalog for antibodies, reagents, lysates, kits and more...

- Type the target name into the product search field on our homepage.
- You can also search by research area or product type.
- Further filters can be applied to narrow your search, such as tested applications and reactivity etc.



Specific references

Publications using the product plus other papers for background reading and reference.

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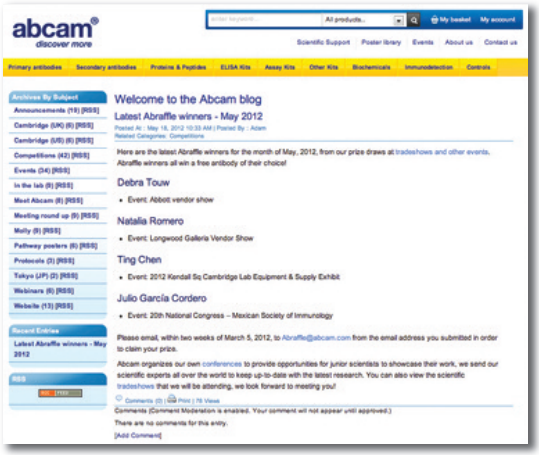

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