

## Chapter 2

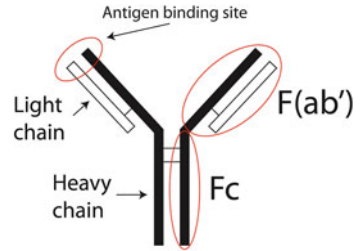
# Primary Antibodies

To a certain extent, IHC can be compared to GPS navigation: to find the place of interest, we must enter its address—which is a unique identifier—and the electronic navigator will bring us there. In IHC, the unique identifier is the structure of the molecule we want to detect, while primary antibodies serve as the chemical rather than electronic GPS navigator. The most critical reagent in IHC protocol is a primary antibody. What is the antibody and why is it primary? As we learned before, the major task of IHC is to detect and visualize proteins of interest in thin sections of animal and human tissues. In order to be detected, the protein in the tissue has to be recognized by another molecule that binds to tissue protein. Recognition by the detecting reagent should be very accurate and selective, so that thousands of other irrelevant proteins composing the tissue remain undetected. Unfortunately, there are no efficient man-made reagents allowing for selective detection of a specific protein among thousands of others. But the good news is that Mother Nature has already designed such a tool and has let us borrow it for our research needs. The tool is called the antibody (Fig. 2.1).

Antibodies are made by the immune system to defend against numerous foreign substances, antigens, which enter the living organism. This process is called adaptive immunity. Antibodies are produced by B lymphocytes (B cells) during a complex cascade of cellular interactions between different types of immune system cells and belong to the class of molecules called immunoglobulins, or Ig. Based on their chemical structure, Ig molecules belong to G, M, A, D, and E types depicted as IgG, IgM, IgA, IgD, and IgE respectively. Most primary antibodies used in IHC belong to IgG type, which, in turn, is represented by four classes, or isotypes: IgG1, IgG2, IgG3, and IgG4.

Antibodies are very selective in recognizing their antigens, which makes antibodies a perfect tool for IHC. Since antigens induce production of antibodies under natural conditions it is possible to imitate the same process by synthesizing antigens resembling the structure of a naturally occurring protein and injecting antigens into host animals like sheep, goats, rabbits, etc.

**Fig. 2.1** Simplified schematic overview of antibody molecule. Interactions of antibodies with their antigens occurs only at their antigen binding sites



For example, if we want to study the distribution of cells producing insulin in tissue sections of a pancreas, we either use a full size insulin molecule or just its shorter fragment unique to the insulin molecule as the antigens. Antigens used for injection into host animals to stimulate the production of antibodies are also called immunogens. Antibodies made by B cells are then secreted into the bloodstream and can be found in blood serum, which immunohistochemists refer to as immune serum. Immune serum can be used for IHC staining either without any further processing or after being subjected to antibody purification. Immune serum can be used for IHC as is, but the problem is that immune serum is a cocktail of antibodies: in addition to antibodies against the injected immunogen, it also contains antibodies to other unknown antigens to which the animal was exposed during its life. Such contaminating antibodies may bind to irrelevant tissue proteins and produce unwanted background staining, which can obscure a specific staining generated by the antibodies of interest. To overcome such a problem, specific antibodies should be isolated from the immune serum. One of the very efficient ways to purify specific antibodies is to run immune serum through a column (called the purification column) filled with beads of a special resin coated with immunogen: only specific antibodies will bind to the immunogen and become retained in the resin matrix, while contaminating antibodies will pass through and get discarded. Retained specific antibodies attached to the resin beads can be isolated by flushing the column with a very acidic elution buffer, and the collected eluate will only contain specific antibodies. This process is called antibody affinity purification, and purified antibodies are referred to as affinity-purified antibodies.

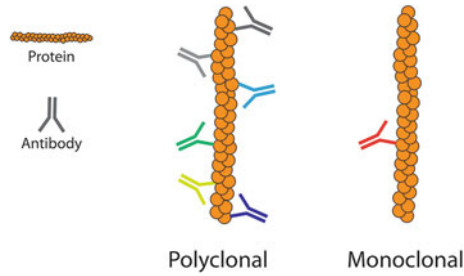
When affinity-purified antibodies are added to tissue sections they bind to tissue protein that resembles the immunogen. Binding of antibodies to its specific tissue antigen is the primary step in tissue antigen detection, and therefore such antibodies are designated primary antibodies. Primary antibodies can be generated by immunizing animals of different host species, including small laboratory rodents (such as mice, rats, rabbits, or guinea pigs) as well as large farm animals (such as goats, sheep, or llamas). In addition, the antibodies can be also made in chicken. What are the reasons for choosing different host species? Are antibodies raised in rabbits better than those raised in sheep? There are several reasons why antibodies are made in different host species. First of all, it is a matter of convenience: handling small laboratory rodents is much easier than injecting immunogens into large farm animals and collecting their blood. Another reason is the cost: handling large farm

animals requires assistance from a trained veterinarian and therefore increases the cost of antibody production. Because of this, low cost mice, rats, rabbits, and guinea pigs are favorite species in academic laboratories, whereas more expensive sheep, goats, and llamas are mostly utilized by commercial organizations that make money by selling their antibodies to customers: much more immune serum can be produced by a goat compared to a guinea pig, which results in a much higher yield of antibodies for sale. However, due to recent advances in antibody production technologies, small animal sizes are no longer an issue as researchers have learned how to generate an almost endless supply of antibodies from mice, rats, and rabbits. As mentioned before, antibodies are produced by B cells of the immune system and there are slight differences in antibodies made by different B cells. These differences include the specificity of antibodies. It has been found that antibodies secreted from myriad B cells have different specificities toward the antigen: antibody specificity is defined as its capacity to bind with only a certain part of the antigen but not to others or to irrelevant proteins. Specificity also determines whether antibodies bind exclusively to their target (high specificity) or cross-react with proteins bearing resemblance to the specific target. The higher the potency of the antibody to cross-react with irrelevant proteins, the lower its specificity.

Another important feature is the affinity of the antibody. Affinity is defined as the strength of the antibody binding to its antigen and is determined by the chemical structure of the antigen and the binding site on the antibody. Antibodies may have a high or low affinity, and the higher the affinity the better the detection of the antigen in tissue sections, which is always required in IHC.

Immunologists have discovered that B cells exist as clones and a single B cell clone divides to produce a large number of descendant B cells, which produce antibodies with the same specificity. However, the immune response to the injected immunogen activates multiple B cell clones, which produce antibodies with high and low specificities. Antibodies combined from different B cell clones into a common pool are called polyclonal antibodies. But what if we want to produce antibodies with the highest specificity to the antigen? Is there a way to isolate the corresponding B cells from a single clone, culture them in a dish (called *in vitro*—outside the body of a host animal), and collect the antibodies secreted by these cells into a culture media where they live? The answer to all these questions is “yes,” and antibodies produced by a single B cell clone are called monoclonal antibodies. Unfortunately, B cells cultured *in vitro* have a very short life that is not long enough for the production of antibodies in quantities required for IHC staining. This problem was solved in 1975 by Georges Köhler and César Milstein in Cambridge, UK, when they found a way to immortalize B cells by fusing them with cancer myeloma cells. Such immortalized B cells can be cultured long enough to produce large quantities of monoclonal antibodies. Köhler and Milstein received the Nobel Prize in Physiology or Medicine in 1984 for their discovery.

Which antibodies are better for IHC, polyclonal or monoclonal? Each type has its pros and cons (Fig. 2.2). Cost-wise, making polyclonal antibodies is less



**Fig. 2.2** Polyclonal antibodies can be considered as a mixture of monoclonal antibodies which, due to variations in their antigen specificity, bind to different regions of the same protein molecule. Monoclonal are the antibodies of the same specificity that bind to the same region of a protein molecule

expensive. Theoretical considerations suggest that due to the presence of antibodies with different specificities that bind to different regions of tissue antigen, IHC staining using polyclonal antibodies should be much stronger compared to monoclonal antibodies. Unfortunately, in practice this does not always hold true and researchers have reported it going both ways. The commercial drawback in making polyclonal antibodies is that their supply is limited and depends on the quantity of serum blood collected from the immunized animals (not much can be collected from guinea pigs and rabbits), whereas monoclonal antibodies can be supplied in unlimited quantities. The major advantage of monoclonal antibodies is the consistency in their specificity between different production batches. That is why monoclonal antibodies are widely used for IHC diagnostics when high accuracy in detecting the pathological tissue changes is of critical importance. For IHC research it appears that monoclonal antibodies tend to produce staining with less of a background compared to their polyclonal siblings. It appears that mice and rats are the most frequently used animals for the production of monoclonal antibodies, but recently new technologies have emerged for making rabbit monoclonal antibodies. Buying monoclonal antibodies for IHC research costs more than buying polyclonal ones, and therefore due to budget constraints some academic laboratories prefer choosing polyclonal antibodies at the expense of the quality of tissue staining.

Currently, many small and large companies manufacture antibodies for sale and reading the labels on the antibody vials may be challenging. First of all, the species of antibodies must be abbreviated due to small printing area of the label: goat—Gt, sheep—Sh, rabbit—Rb, guinea pig—Gp, mouse—Ms, rat—Rt. Antibodies are usually made to stain tissues of a particular species like human, rat, mouse, etc., which are shortened to just the first letter: human—h, mouse—m, rat—r. The typical label on the vial may look like this (except that lines are not numbered, which has been done for sake of explaining their meaning in point-by-point manner below):

- (1) anti-hmrActivin
- (2) Purified rat monoclonal
- (3) Clone: 123765
- (4) Catalog Number: M1534
- (5) Lot Number: B12
- (6) 0.05 mg
- (7) For Research Use Only
- (8) Best Antibodies, Inc.

What does this label say?

*Line 1*—Antibodies were made to cross-react with Activin molecules in human, mouse, and rat tissues. In other words, such antibodies are suitable for IHC staining of human, mouse, and rat tissue sections.

*Line 2*—Antibodies are monoclonal and were raised in rats.

*Line 3*—Clone number designated by the manufacturer. There may be different clones of the antibody against Activin molecule that work in different applications: one clone works for IHC, another for Flow Cytometry, and a third for Western Blotting.

*Line 4*—Catalog number assigned by the manufacturer, which helps to find the antibody on the vendor's web site.

*Line 5*—Production lot number assigned by the manufacturer, usually to a group of vials with the same antibodies, that were produced on the same date from the same bulk material. Sometimes different lots of the same antibody do not produce the same IHC staining due to so-called lot-to-lot variations. Customers can request that they be sent antibodies from the best-performing lot for IHC.

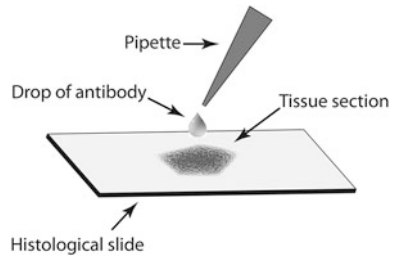
*Line 6*—The quantity of antibody in a vial. If antibodies are supplied in a dry (lyophilized) format the quantity is given in milligrams, (mg) or in micrograms (can be written either as  $\mu\text{g}$  or  $\text{ug}$ ). When antibodies are supplied in liquid form their volume is given in milliliters (mL) or in microliters (can be written either as  $\mu\text{L}$  or  $\text{uL}$ ). Microgram and microliter are 1/1000 parts of a milligram and a milliliter respectively. When antibodies are sold as a liquid, the label will also indicate their concentration, for example, 1, 0.5 mg/mL, etc. Knowing the original concentration of primary antibody is very important in order to make correct working dilutions.

*Line 7*—Intended use of the antibody. When antibodies are made for research rather than diagnostic purposes the manufacturer must state this on the label. Unlike antibodies for research, IHC diagnostic antibodies must be subjected to much more rigorous quality testing according to the rules set forth by the United States Food and Drug Administration (FDA).

*Line 8*—The name of the antibody vendor. No matter how much information is put on the label, it is always a good idea to visit the antibody vendor's web site and carefully read the actual antibody datasheet, which, in addition to the antibody details, may also provide examples of IHC images as well as the tissue staining protocol and troubleshooting recommendations.

Antibodies are supplied by commercial vendors in a concentrated form and need to be further diluted, defined as preparing antibody working dilutions. Typical

**Fig. 2.3** Application of primary antibody solution to tissue section on the histological slide



working dilution of antibodies in IHC ranges from 1 to 20 micrograms per 1 mL of solution. To make a working dilution of immune serum in which the concentration of specific antibodies is not known, it is usually diluted at 1:100–1:2000. One important thing to remember: the higher the concentration, the higher the risk of getting nonspecific background staining.

Antibodies can be supplied either as liquid or dry (lyophilized) concentrated stock reagents, which should be further diluted according to the manufacturer's instructions to make working dilution that can be applied to tissue sections (Fig. 2.3). Liquid antibody concentrates usually need to be stored between 4 and 8 °C and are usually stable for up to a year. If vial with antibodies cannot be used within its shelf life term due to low volume of IHC experiments, it is recommended to make aliquots. For example, a single 0.5 mL stock solution can be aliquoted into 50 vials containing 10 uL. Aliquots should be stored frozen and used to prepare a working solution immediately before use. Original stock solution vials can be also stored frozen, but have to be taken out from the freezer and thawed to make working solutions and then transferred back to the freezer. Repetitive freezing and thawing of antibodies is the worst enemy, which destroys their staining capacity. Unopened vials of dry, lyophilized, antibodies can be stored for years at  $-20\text{ }^{\circ}\text{C}$  and lower, but after reconstitution with the diluents, they have to be treated like liquid stock solutions. To extend the shelf life of antibody stock solutions, some vendors recommend adding glycerol and storing them in freezers at  $-20$  to  $-80\text{ }^{\circ}\text{C}$  as glycerol prevents the solution from freezing and therefore eliminates the risks of freeze–thaw cycles.

## 2.1 Summary

The key reagent in IHC technique is primary antibodies. Primary antibodies are made by B cells in response to natural pathogens, but can be also produced for IHC needs artificially by immunizing host animals with antigens of a known chemical structure. Primary antibodies can be applied as immune serum, affinity purified polyclonal and monoclonal antibodies. Polyclonal antibodies may produce unwanted background staining in addition to specific staining, whereas monoclonal antibodies produce cleaner and more consistent IHC staining.

## 2.2 Quiz (True or False)

1. Antibodies are called “primary” because they are made by B cells: **T F**
2. Primary antibodies are not an essential reagent for IHC and there are many other reagents that can be used in place of primary antibodies: **T F**
3. Immune serum is the source of primary antibodies: **T F**
4. Antigens used for immunization are not the same as immunogens: **T F**
5. Polyclonal antibodies are a pool of antibodies with identical specificities: **T F**
6. The major advantage of monoclonal antibodies is consistency in their specificity between different production batches: **T F**
7. Monoclonal antibodies are preferred over polyclonal in IHC diagnostics: **T F**