Chapter 7
Staining Protocols

Due to the inherent nature of different types of histological tissues composing the body as well as the complexity of tissue antigens, there are no universal "one-size-fits-all" staining protocols in IHC: the approach that works for a particular type of tissue and "to-be detected" antigens may be completely ineffective for other types of tissue and antigens. That is why the most critical step in IHC is the optimization of a staining protocol allowing for the best detection of tissue antigens. Usually, the first step in designing an IHC experiment is determining which antigen detection strategy should be used: fluorescence or chromogenic. As soon as this is done, basic IHC protocol is taken as the foundation and tweaked to suit the experimental needs. To save reagents, slides with tissue sections are stained in a horizontal position with tissue sections facing up: unlike incubating slides vertically in a jar requiring 50 mLs or more of IHC reagents, staining them horizontally requires only 50–200 microliters per slide.

Generally, a workflow of IHC staining looks like this:

a. *Hydrophobic tissue protection*: Using a special pen (called a PapPen), a hydrophobic barrier line is drawn around the tissue section to prevent leakage of reagents from the slide during incubation;
b. *Blocking*: Tissue sections are treated with reagents to block endogenous sources of nonspecific staining such as (i) enzymes, (ii) endogenous peroxidase, (iii) free aldehyde groups, (iv) immunoglobulins, and other irrelevant molecules that can mimic specific staining;
c. *Permeabilization (this step is not mandatory and only used as needed)*: Tissue sections are incubated with permeabilization buffer to facilitate penetration of antibodies and other staining reagents into the tissue;
d. *Incubation with primary (often depicted as 1°Ab) antibody*: This incubation is done for 1–24 h either at room temperature or in a cold room at 6–8 °C depending on the affinity of antibodies and abundance of tissue target;

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e. *Rinsing with wash buffer*: This step is usually done as 3–5 short, 5–15 min repetitive cycles using fresh wash buffer and, for example, can be depicted in the scientific literature as "3 × 15 min washes";
f. *Incubation with secondary (may be depicted as 2°Ab) antibody*: This is usually done for 1–2 h at room temperature;
g. *Rinsing with wash buffer*: This is a repeat of step "e"; and
h. *Incubations with detection reagents, mingled with rinsing with wash buffer*: This is done when applicable (for example, in chromogenic detection).

Let us take a look at basic IHC protocols and discuss their advantages and limitations.

7.1 Fluorescence Protocols

Fluorescence (also frequently referred to as immunofluorescence) detection is a widely used IHC technique that can be either direct or indirect: direct indicates that primary antibodies are labeled with fluorescent probes and implies that there is no need to use fluorescent secondary antibodies, while indirect means that primary antibodies are unlabeled and secondary antibodies conjugated to fluorescent dyes have to be used for detection. Which technique is better, direct or indirect? There is no simple answer to this question as each technique has its advantages and limitations, which are discussed below.

7.1.1 Direct Fluorescence Detection

Direct detection was developed by Dr. Coons and is considered as the ancestor of all other fluorescence protocols used in IHC. It is the fastest and shortest IHC protocol, requiring incubation of tissue sections with only a primary antibody conjugated to the fluorophore of choice.

The advantage of direct fluorescence detection is that tissue staining can be obtained quickly.

Unfortunately, this technique sometimes does not work because after the conjugation of fluorescent dye to the primary antibody, its antigen-recognition capacity may be either impaired or lost, which results in weak or no IHC staining. That is why direct detection is better suited for the detection of strong, highly expressed tissue antigens. Direct detection is the technique of choice when, due to the host species of the primary antibodies and the histological nature of tissue, use of secondary detection antibodies may cause strong nonspecific staining. For example, a researcher has to use primary mouse monoclonal antibodies for staining mouse

lymph nodes because anti-mouse secondary antibodies will not only interact with mouse primary antibodies bound to the tissue target, but also to endogenous immunoglobulins abundantly present in the lymph node. This would result in strong nonspecific staining, and therefore the better option would be to employ a direct detection technique using mouse primary antibodies conjugated to a fluorophore.

7.1.2 Indirect Fluorescent Detection

Indirect detection is more sensitive than direct, which is why it is the more popular technique. The higher sensitivity of indirect detection is the result of the possibility of two secondary antibodies labeled with fluorophores interacting with a single molecule of primary antibody bound to its tissue target.

Another advantage of indirect detection is the ability to choose secondary antibodies with fluorophores of different colors, Stokes shifts, quantum yield, and fade resistance. For example, if the tissue has a strong endogenous red autofluorescence, the researcher can use secondary antibodies conjugated to a fluorophore emitting green light. The major disadvantage of indirect detection is the risk of nonspecific staining due to the interaction of secondary antibodies with irrelevant tissue targets: if nonspecific staining is observed, tissue sections have to be treated with blocking solutions, thereby adding time and expense to the IHC experiment. Usually it is assumed that secondary antibodies have a tendency to bind nonspecifically, and therefore incubation with blocking buffers is generally done before applying primary antibodies.

7.1.3 Multi-Color Fluorescence Detection

Fluorescence detection allows for simultaneous detection of multiple overlapping and nonoverlapping tissue targets. Multicolor detection can be done using either direct or indirect detection, or a combination of both techniques (Fig. 7.1).

Usually in doing multicolor fluorescence IHC researchers use primary antibodies raised in different host species to avoid cross-reactivity of secondary antibodies with irrelevant primary antibodies.

Unfortunately, quite often the selection of the hosts of primary antibodies is limited and the researcher has only primary antibodies raised in the same host species. One approach to avoid cross-reactivity of secondary antibodies with primary antibodies is to modify the primary antibodies by conjugating them to Biotin and Digoxigenin followed by detection using Streptavidin and anti-Digoxigenin antibodies conjugated to fluorescent dyes, respectively. There are other approaches

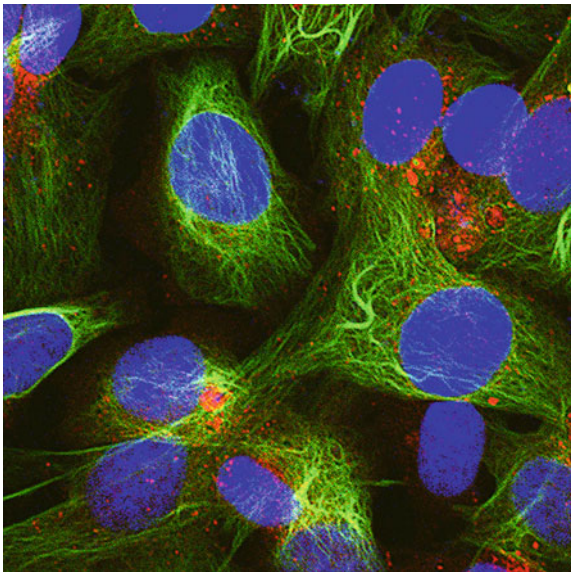

Fig. 7.1 Example of 3-color fluorescence IHC image. Nuclei were labeled *blue*, the cytoskeletal network *green*, and the cytoplasmic proteins *red*

for using primary antibodies raised in the same host species for multicolor fluorescence as well, but they are quite complicated and involve advanced IHC techniques detailed in the references listed at the end of this book.

7.1.4 Fluorescent Counterstaining

Counterstaining is the staining of tissue sections with dyes that allow one to see the entire "landscape" of the tissue section and serve as a reference for the main color used for the detection of tissue targets. Such dyes can stain cell nuclei, the cell membrane, or the entire cell. The key to tissue counterstaining is choosing a counterstain color that is distinctly different from the main, antigen-detecting color. Most often, researchers counterstain cell nuclei using these dyes:

a. DAPI, which binds to nuclear DNA and emits strong blue light;
b. Hoechst blue stain, which binds to nuclear DNA and emits strong blue light; and
c. Propidium iodide, which binds to nuclear DNA and emits strong red light.

Counterstaining of the intracellular cytoskeletal network can be done using Phalloidin conjugated to fluorescent dyes. Phalloidin is a toxin that tightly binds to Actin filaments in a cell's cytoplasm, which then become clearly visible under the microscope (Fig. 7.2).

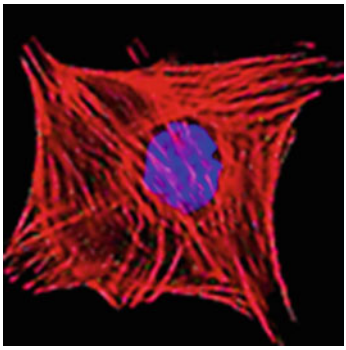

Fig. 7.2 Cell counterstained with Phalloidin conjugated to red fluorophore (labeling is in cytoskeletal filaments) and with DAPI (labeling is in the cell nucleus—*blue* color)

7.2 Chromogenic Protocols

The majority of chromogenic IHC protocols are based on the use of Avidin and Biotin molecules because detection sensitivity of a simple antigen-antibody reaction in many cases is quite low. It has been found that Avidin, which is egg white glycoprotein, has a strong binding capacity to Biotin (also known as vitamin B7, which plays an important role in maintaining healthy skin and hair). Avidin-Biotin binding serves to bridge antigen-bound antibodies with detection reagents, allowing amplification of the staining signal. Unfortunately, due to being heavily glycosylated, Avidin has a tendency to bind to tissues nonspecifically and replaced by Streptavidin (frequently abbreviated as SA) from bacterium *Streptomyces avidinii*, which is not glycosylated and therefore does not bind to tissues nonspecifically. Besides strong interactions, each molecule of Avidin and its sibling SA can bind four molecules of Biotin, which is the chemical nature of signal amplification due to the formation of large multimolecular enzyme-containing complexes. The most frequently used Biotin-based techniques include labeled SA-Biotin (LSAB) and Avidin-Biotin Complex (ABC) detection. There are also non-Biotin-based detection techniques utilizing primary antibodies either conjugated directly to enzymatic labels or to a long polymer containing multiple copies of enzymatic labels.

7.2.1 LSAB Detection

This detection technique utilizes secondary antibodies conjugated to Biotin that link primary antigen-bound antibodies to SA conjugated to an enzyme. The first step in LSAB detection is the incubation of tissue sections with primary antibodies followed by incubation with biotinylated secondary antibodies. After that, SA conjugated to the enzyme of choice (e.g., AP, HRP, etc.) is added to tissue sections followed by adding appropriate enzyme substrate. The enzyme converts substrate into colored particles precipitating at the sites of antigen localization, which can

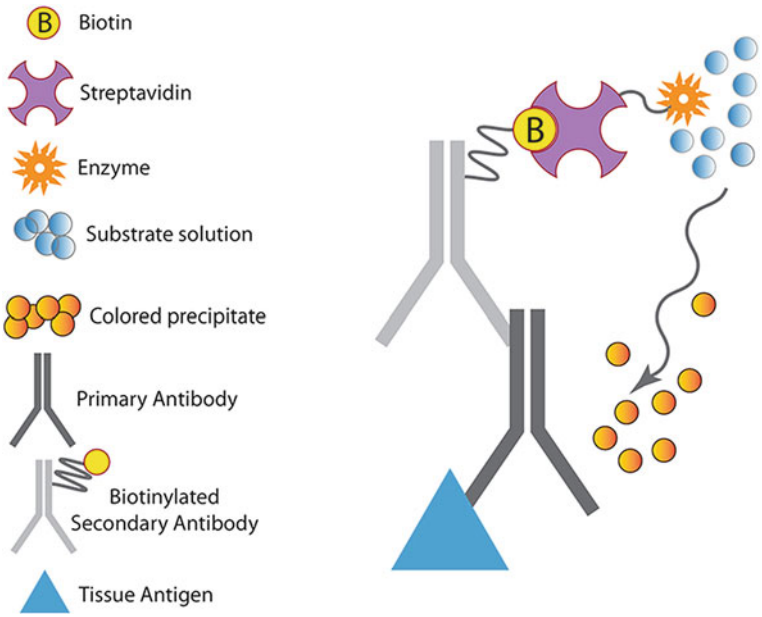

Fig. 7.3 Detection using Labeled Streptavidin Avidin Biotin (LSAB) chemistry

then be observed under the microscope. LSAB technique can be shortened using biotinylated primary antibodies, eliminating the need for incubation with biotinylated secondary antibodies (Fig. 7.3).

7.2.2 ABC Detection

The initial steps—incubation with primary and biotinylated secondary antibodies—in ABC detection are the same as in LSAB, but the next steps and reagents are quite different. Avidin and biotinylated enzymes are first mixed and incubated together for about 30 min at room temperature and then added to tissue sections. During this incubation, Avidin interacts with the biotinylated enzymes, forming large complexes densely packed with enzyme molecules—far exceeding the concentration found in the LSAB detection technique—that boost the sensitivity of antigen detection. The critical drawback of ABC technique is that the large size of these formed complexes impairs their ability to penetrate inside the cells, which reduces detection sensitivity. Like LSAB, ABC protocol can be shortened using biotinylated primary antibodies (Fig. 7.4).

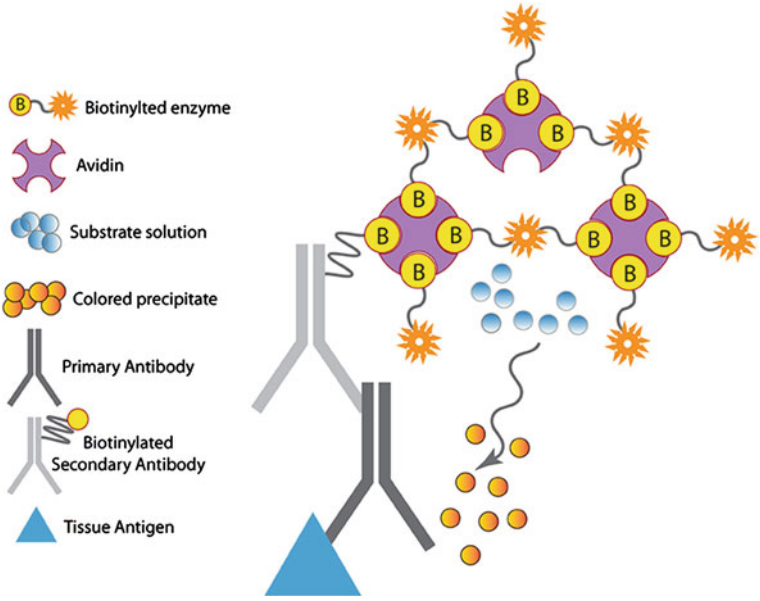

Fig. 7.4 Detection using Avidin-Biotin Complex (ABC) formation

7.2.3 Non-Biotin-Based Detection

Non-Biotin detection techniques have gained popularity because they are devoid of such limitations of Avidin-Biotin detection as nonspecific background staining due to the endogenous biotin that is abundant in different types of animal tissues, including kidney, brain, and placenta.

Non-Biotin protocols are shorter and simpler because they do not require the blocking of endogenous biotin or incubation of tissue sections with biotinylated secondary antibodies and either Avidin or SA enzyme conjugates. Detection can be done either using primary/secondary antibodies conjugated directly to enzymes like AP or HRP, or by employing polymer-enzyme chemistry, which offers much stronger sensitivity compared to direct enzyme conjugates of antibodies and LSAB and ABC detection (Fig. 7.5).

7.2.4 Counterstaining in Chromogenic Detection

In chromogenic IHC, tissue counterstaining serves the same purpose as it does in fluorescence detection: to visualize the entire layout of the tissue section and label organelles of the same type. Usually counterstaining is done to label cell nuclei that should not be of the same color as the main color depicting antigens of interest. For example, if the main color is red (AEC chromogen) or brown (DAB chromogen),

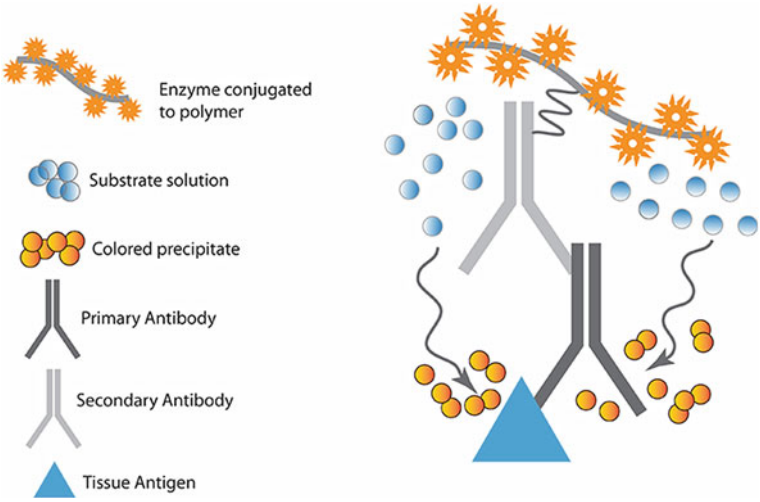

Fig. 7.5 Detection using enzyme-conjugated polymer linked to secondary antibody. A polymer packed with a large number of enzyme molecules is attached to secondary antibodies. The high density of the enzyme results in fast conversion of the substrate into a dense colored precipitate marking the sites of antigen localization. The polymer complex is small, which allows good intracellular penetration and increases the detection sensitivity

nuclei may be stained using Hematoxylin, which produces a blue color, or Methyl Green, which produces a green color. If the main color is blue (BCIP/NBT chromogen), then nuclei may be counterstained red using Nuclear Fast Red dye. In cases when tissue antigen is localized in cell nuclei, the duration of their counterstaining may be either shortened to make them barely visible or even skipped to avoid masking the main IHC color.

7.3 IHC Controls

Running controls side-by-side with stained tissue sections is a critical part of IHC experiments. The ultimate purpose of IHC controls is to confirm the specificity and relevance of detected tissue antigens. Usually, it is recommended to perform (i) tissue antigen, (ii) endogenous tissue condition, and (iii) reagents specificity controls.

7.3.1 Tissue Antigen Controls

There are two types of tissue antigen controls: positive and negative. Positive tissue control is running IHC staining on tissues that are known to express the antigen of

interest, whereas negative tissue control is staining tissues known to lack the antigen. Positive controls are a must when investigating the expression of a known antigen in a new type of tissue. For example, when studying the localization of antigen "X" in the brain, which is known to be expressed in the liver, the latter will serve as a positive control. But if antigen "X" is known not to be expressed in the kidney, then kidney tissue sections can be used as a negative control. Seeing staining in a positive control and a lack of staining in a negative control indicates that the primary antibodies are specific and therefore suitable for IHC experiments. However, if negative control tissue becomes stained, this can indicate that either primary antibodies are nonspecific or further optimization of staining protocol is required.

7.3.2 Endogenous Tissue Condition Controls

Endogenous tissue condition controls need to be done to ensure that there are no endogenous substances in the tissue that can either obscure or mimic specific staining. Usually these controls are essential for fluorescence but not for chromogenic enzymatic detection, because it is important to make sure that after IHC staining is complete, fluorescent staining observed under the microscope depicts targets of interest rather than artefacts. Nonspecific staining may be due to endogenous tissue autofluorescence, nonspecific binding of secondary antibodies, or cross-reactivity of primary antibodies with irrelevant tissue targets.

Special IHC controls need to be employed to rule out the interference of nonspecific staining with specific staining. For example, endogenous tissue autofluorescence can be easily ascertained by analyzing unstained tissue sections under the fluorescence microscope. If a tissue's autofluorescence has a green color, the use of fluorescent labels other than green is recommended. It is always advisable to quench endogenous autofluorescence using treatment strategies tailored to its nature. Autofluorescence may be inherent to a particular type of tissue or it can be induced by tissue processing. Tissues may naturally autofluoresce due to the presence of organic compounds called porphyrins (which are abundant, for example, in red blood cells) and flavins (a component of vitamin B_2 or Riboflavin involved in energy production and the oxidation of fatty acids). Porphyrins and flavins are not stable in organic solvents and therefore do not represent a serious problem when doing IHC on paraffin-embedded tissue sections processed using xylene and alcohol. The presence of Porphyrins and Flavins is more of an issue when staining frozen tissue sections, which do not require organic solvents for their processing. Another compound with a strong autofluorescence is Lipofuscin, nicknamed an "aging pigment," which is a product of degraded cellular material accumulated in cytoplasmic organelles lysosomes. The biggest problem with Lipofuscin is that it can be excited by the light within a wide range (360–647 nm), emitting orange, green, yellow, and red light that can resemble fluorescent signals emitted by the most frequently used fluorophores conjugated to primary and

secondary antibodies. One efficient anti-Lipofuscin remedy is the use of Sudan Black B, a histological dye that binds to lipids in lysosomes to mask them: after finishing IHC staining, tissue sections are incubated in a solution of Sudan Black B, which blocks Lipofuscin fluorescence but does not affect specific fluorescent signals emitted by specific probes. Unfortunately, in some tissues treated with Sudan Black B the level of endogenous autofluorescence remains strong, and therefore the only option left would be to switch to chromogenic detection.

In addition to natural occurrence, autofluorescence can be also induced by tissue processing. For example, fixation with formaldehyde and glutaraldehyde fixatives, in addition to cross-linking proteins, leads to formation of free aldehyde groups that can cause autofluorescence. In order to block free aldehyde groups, tissue sections can be treated with sodium borohydrate, which converts CHO aldehyde groups to OH hydroxyl groups. Another way of blocking free aldehyde groups is to treat tissues sections with reagents containing NH_2 amino groups such as the amino acids glycine and lysine: the chemical reaction between NH_2 amino and CHO aldehyde groups leads to the reduction of the number of free aldehyde groups.

To check whether staining is produced by the nonspecific binding of fluorescent secondary antibodies to tissue sections, instead of incubating tissue sections with primary antibodies it is recommended to incubate just with antibody diluent lacking primary antibodies followed by incubation with secondary antibodies. If it turns out that secondary antibodies bind to tissue sections nonspecifically, then it is recommended to optimize their working dilution and also prolong the incubation of tissue sections with blocking buffers. If none of these solutions help, then it is advisable to switch vendors of secondary antibodies.

Although it is tempting to run endogenous peroxidase and enzyme controls in advance of a chromogenic detection IHC experiment, they may be skipped because it is assumed that tissues have such problems and therefore it is more practical to simply employ peroxidase and enzyme blocking steps.

7.3.3 Reagents Controls

In order to make sure that primary antibodies do not cross-react with irrelevant tissue targets, it is recommended to employ what is called an absorption control. Primary antibodies are mixed with the immunogen that was used to immunize host animals. It is expected that the binding of immunogen to antibodies will neutralize, or absorb them and adding such absorbed primary antibodies to tissue should not generate any specific staining. If staining is produced by immunogen-absorbed primary antibodies, then such staining is most likely nonspecific. If optimization of the working dilution of primary antibodies does not help, then it may be recommended to use a different antibody (made in-house or by a different commercial vendor) against the same tissue target.

When using primary monoclonal antibodies, it is recommended to run an isotype control by incubating tissue sections with nonimmune antibodies of the same

isotype as the primary antibodies (e.g., IgG1, IgG2b, or IgG2a). If there is staining in the isotype control, then it may be necessary to find antibodies of a different isotype that do not bind nonspecifically to tissue sections.

7.4 Selection and Optimizaion of Primary Antibodies

When planning IHC experiments, one the most frequently questions asked is that of which primary antibodies to use: polyclonal or monoclonal? As we already know, monoclonal antibodies are made by a single B-cell clone and therefore represent a homogeneous population of antibodies that have the same affinity and specificity toward a single epitope of the tissue antigen. Such consistency is particularly useful when the purpose of an IHC experiment is the detection of a single member of a large protein family when its other members have similar amino acid sequences. However, due to tissue fixation, the conformation of the target protein may change in such a way that its antibody-interacting epitope may become inaccessible to the antibody, which results in a lack of tissue staining. Unlike monoclonal, polyclonal antibodies are the mixture of antibodies produced by different B-cell clones that can bind to multiple different epitopes: it is unlikely that all epitopes become inaccessible during the changes in target protein conformation. In other words, "back-up" epitopes always remain that are recognized by polyclonal antibodies. Because of this, polyclonal primary antibodies are thought to be a better choice for IHC experiments.

Primary antibodies are the most critical IHC reagents, and their optimization should be the first step in developing IHC protocol. Optimization usually means determining the appropriate concentration of primary antibodies, their incubation time with tissue sections, and the incubation temperature. To find the optimal concentration, tissue sections are usually incubated with three-fold serial working dilutions of primary antibodies starting with the highest concentration. For example, the highest concentration of polyclonal antibodies in IHC does not exceed 10–20 μg/mL and for monoclonal antibodies, it does not exceed 20–30 μg/mL. For immune serum, the highest antibody concentration is usually obtained when it is diluted 1:100. Serial working dilutions of polyclonal antibodies may be, for example, as follows: 18 , 6, 2, and 0.7 μg/mL, and for immune serum – 1:100, 1:300, 1:900, 1:2,700. When doing IHC with a new antibody for the first time, it is recommended to do a pilot study that tests a broad range of antibody concentrations. The purpose of this task is to determine the concentration that produces the strongest specific and most minimal nonspecific, or background, staining. As a common starting point in determining incubation time and temperature, it is recommended first to run overnight incubations at a low (4–8 °C) rather than room temperature. If the specific signal is strong and there is no background staining, then incubation time may be shortened to 2–3 h and done at room temperature. When comparing tissue sections stained with different antibody concentrations, it is critical to use a consistent time and temperature for the incubation step.

Antibody optimization is tissue dependent, which means that the optimal conditions established for one type of tissue (e.g., brain) cannot be blindly applied when staining another type of tissue (e.g., liver): antibodies will need to be re-optimized. Re-optimization may also be required when staining tissue of the same type that is sectioned at different thicknesses. For example, in staining thicker, 15 um tissue sections, the concentration of primary antibodies needs to be higher than with Sects. 5 um thick in order to allow the primary antibodies to better penetrate and interact with tissue targets localized deeper in the tissue section. The incubation time for thicker sections may need to be longer than for thinner sections. Primary antibodies raised against the same tissue antigens in the same host species (e.g., rabbit, mouse, goat, etc.) may be supplied by different vendors or by the same vendor but made in different host species (for example, rabbit antihuman Insulin versus mouse antihuman Insulin). Although it is tempting to assume that such antibodies are the same based on their antigen specificity, they may, and in many instances do, have different affinities to the same target: high-affinity antibodies require a shorter incubation time and working dilutions of a lower concentration, whereas low-affinity antibodies require higher concentrations and a longer incubation time. In other words, antibodies against the same targets originating from different sources (e.g., suppliers or/and host species) need to be treated as completely different antibodies requiring optimization.

7.5 Summary

Commonly, basic IHC protocol is used as a foundation for designing a specific IHC protocol to suit experimental needs. Incubation of tissue sections with antibodies and staining reagents is alternated with buffer washes to avoid cross-contamination of IHC solution. Direct fluorescence IHC detection is less sensitive than indirect detection, but the latter protocol is longer and more expensive. Direct fluorescence detection can be combined with indirect when doing multicolor IHC detection. Most chromogenic IHC protocols utilize Avidin-Biotin detection chemistry such as LSAB and ABC techniques. Non-biotin, HRP polymer detection chemistry is gaining popularity due to its high sensitivity and the lack of interference from tissue endogenous biotin. In addition to the main IHC staining, tissues are also counterstained to allow researchers better orientation when analyzing stained tissue sections under the microscope. IHC controls are an essential part of staining protocols, which must be done to rule out artefacts that can mimic specific staining signals. Primary antibodies are critical IHC reagents, which must be optimized to obtain a strong specific signal with as little background staining as possible. Primary polyclonal antibodies are preferred over monoclonal for IHC staining.

7.6 Quiz (True or False)

1. There are no universal "one-size-fits-all" staining IHC protocols: **T F**
2. To save staining reagents, slides with tissue sections are incubated vertically in jars: **T F**
3. Direct and indirect fluorescence detection can be combined in a single IHC protocol: **T F**
4. The majority of chromogenic IHC protocols are based on Avidin-Biotin detection chemistry: **T F**
5. LSAB is the abbreviation of "Low Sensitivity Avidin Binding": **T F**
6. Biotin-free HRP polymer detection chemistry does not have the advantage over Avidin-Biotin detection: **T F**
7. Tissue counterstaining is done to enhance nonspecific staining: **T F**
8. IHC controls are essential to every IHC experiment: **T F**
9. Background staining can occur as a result of endogenous tissue conditions or it can be introduced by tissue processing: **T F**
10. Primary antibody optimization is only required when doing fluorescence detection IHC: **T F**
11. Primary polyclonal antibodies are generally preferred over monoclonal for IHC: **T F**