Chapter 5

Use of Antibodies in the Research on Muscarinic Receptor Subtypes

Wisuit Pradidarcheep and Martin C. Michel

Abstract

Antibodies can be a powerful tool to detect receptor expression at the protein level. Their main advantage is the potential of good spatial resolution in immunohistochemistry, whereas their main limitation is that they yield less quantitative results as compared to radioligand binding. However, most available antibodies against muscarinic acetylcholine receptor subtypes have shown poor target selectivity when tested stringently, e.g., often yielded similar staining patterns in wild-type and knockout animals or in cells transfected with the target as compared to a closely related receptor subtype. On the other hand, a small number of antibodies have been validated to some degree for selectivity for a muscarinic receptor subtype. Protocols for their use in immunohistochemistry are discussed. However, it remains a key learning that each investigator should carefully establish whether the intended antibody is indeed selective for the target under investigation under the assay conditions being applied.

Key words Muscarinic receptor, Antibody, Validation, Immunoblot, Immunohistochemistry

1 Introduction

Determination of the number and/or subtype distribution of muscarinic receptors is relevant for the understanding of physiology and pathophysiology. Radioligands are a good tool for the quantification of total muscarinic receptor density in a tissue and its possible regulation by gender, ageing, or pathophysiology [1]. However, the use of radioligands has two limitations in the research field of muscarinic receptors. Firstly, standard muscarinic receptor radioligands such as N-methylscopolamine or quinuclidinylbenzylate are available only in tritiated forms; the associated low specific radioactivity causes a limited sensitivity, i.e. requires large samples and/or a high expression density. As a consequence of this, morphological studies based on autoradiography with the radioligands require long exposure times. Second, N-methylscopolamine or quinuclidinylbenzylate binds with similar affinity to all five muscarinic receptor subtypes. Accordingly, the

Jaromir Myslivecek and Jan Jakubik (eds.), *Muscarinic Receptor: From Structure to Animal Models*, Neuromethods, vol. 107,
DOI 10.1007/978-1-4939-2858-3_5,

relative contribution of any subtype can only be derived from experiments with subtype-selective competitors. However, most muscarinic receptor ligands exhibit only moderate subtype selectivity, which makes robust quantitative analysis of subtypes difficult, particularly within the $M_1/M_3/M_5$ or the M_2/M_4 subfamily of muscarinic receptors [2].

Receptor subtype-selective antibodies could potentially address several of these challenges. Due to their high affinity they can be very sensitive and in immunohistochemical experiments can be an excellent tool for morphological studies. On the other hand, they have the intrinsic disadvantage that the results are difficult to quantify. Most importantly, however, in practical experience most antibodies against (individual) subtypes of muscarinic receptors have proven to lack selectivity for their cognate receptor. Against this background, this chapter initially discusses the selectivity problems with commonly available antibodies against muscarinic receptor subtypes. Thereafter, we discuss protocols which can be used for immunohistochemical detection of muscarinic receptors subtypes for the limited number of cases where antibodies possess the required specificity.

2 Selectivity Problems with Muscarinic Receptor Antibodies

It had been widely assumed that presence of a single band in an immunoblot could be considered as a proof of antibody selectivity. However, the number of bands in an immunoblot can be tweaked in various ways. For example it is influenced by the choice of exposure time and image contrast, which may enhance the visibility of some bands relative to others. This becomes particularly relevant, if the validation immunoblot is generated with a cell line or tissue expressing a very high density of the target protein, for instance with a cell line transfected with the cognate receptor. Such overexpression can enhance target over background signal and may lead to false positive estimates of selectivity when applied to native tissues with a lower expression density.

Another potentially misleading criterion for target selectivity of an antibody can be the disappearance of signal upon co-incubation with a blocking peptide, mostly identical in amino acid sequence to the peptide which had been used to generate the antibody. While it appears obvious that the peptide used for immunization will absorb the antibody, the reasoning for accepting this as specificity evidence ignores the fact that a small peptide in solution may be much more flexible and hence present a very different three-dimensional epitope than a receptor with multiple membrane-spanning domains. Both of these potential problems are worsened by the fact that many commercial suppliers of receptor antibodies provide only limited technical information on the

specific experimental conditions which had been used in their validation experiments. Moreover, the frequent absence of cautionary notes that the "representative" immunohistochemical image or immunoblot in a catalog is limited to very specific receptor sources and/or experimental conditions raise doubts about how representative it really is.

Meanwhile evidence from numerous types of G-protein-coupled receptors has shown that even with the best of intentions, selectivity claims on the presence of a single band in an immunoblot and/or signal disappearance in the presence of blocking peptide in many cases provides misleading information on antibody selectivity [3, 4]. Thus, more vigorous approaches to testing antibody selectivity have more often than not failed to confirm selectivity claims based on a single immunoblot band or blocking peptide. For example, when target receptor and a closely related receptor, i.e., another subtype from the same receptor family, were expressed in the same cell line at a comparable density, a given antibody often produced almost identical band patterns in immunoblots with β-adrenoceptor [5] or dopamine receptor subtype antibodies [6]. Similarly, several galanin receptor antibodies produced similar staining patterns in both immunoblots and immunohistochemistry when comparing tissues from wild-type and knockout mice lacking the target receptor [7]. Some investigators lack access to recombinant receptors or knockout animals of the required species; in such cases use of receptor knockdown by small interfering RNA or use of tissues known to lack the receptor of interest may be alternative acceptable validation techniques [8]. The shocking finding from validation approaches using any of these hard criteria was that the vast majority of receptor antibodies failed to exhibit the promised selectivity [3].

Additional potential causes of misleading antibody-based results have been identified. These include the observation that some antibodies may have acceptable specificity in one application, e.g., immunocytochemistry, but not in another, e.g., immunoblotting [9]. Another potential cause of misleading results is the observation that a given antibody may yield acceptable target specificity in one species but not in another [8]. Finally, fixation conditions may also affect the apparent specificity of some antibodies [10]. The sum of these issues has led some investigators to refer to receptor antibodies as "reagents of mass distraction" [11]. In the following we discuss specific evidence in this regard for antibodies against muscarinic receptor subtypes.

In an heroic effort Jositsch et al. [10] have explored the target selectivity of 24 antibodies against muscarinic receptors (1–9 per subtype), with four dilutions and up to 21 different conditions tested for each antibody yielding a total of 1824 conditions being evaluated. In this study staining with several antibodies was abolished by preincubation with blocking peptide.

However, the immunohistochemical signal from M_1 receptor antibodies ABS5164, AMR-001, AS-3701S, GP20a, Rabbit 001, Rabbit 002, and sc-7471 was unaffected in dorsal root ganglia, urinary bladder, and thoracic viscera from M_1 knockout mice. Using a different validation approach, i.e., immunoblotting with membranes from human embryonic kidney (HEK) cells transfected with M_1, M_2, M_3, and M_4 receptors to yield comparable expression levels, we found that AMR-001 exhibited a similar band pattern in immunoblots from all four cell lines [12]. Actually, in these experiments we did not identify a single band that was more prominent in M_1-expressing than in other cells, and one of the bands was actually most prominent in M_2-expressing cells.

Among M_2 receptor antibodies, Jositsch et al. [10] found that immunohistochemical staining was unaffected in dorsal root ganglia, urinary bladder, and thoracic viscera from M_2 knockout mice for AS3721S and AMR-002. For the former, labeling in airways was also unaffected by M_2/M_3 double knockout, indicating that the nonspecific labeling was not due to staining of another muscarinic receptor subtype. For the latter, we have reported a similar band pattern in immunoblots with membranes from cells transfected with M_1, M_2, M_3, and M_4 receptors [12]. However, two M_2 receptor antibodies have shown at least some promise based on the work by Jositsch et al. [10]. The monoclonal antibody mAB367 labelled airway smooth muscle and the cell membrane of a subpopulation of dorsal root ganglion neurons and atrial and pulmonary vein cardiomyocytes in wild-type but not in M_2 knockout mice, particularly when a specific protocol was applied; however, signals from ciliated epithelial cells of the oviduct obtained with the same antibody were not affect in the knock-out mice (Fig. 1). The rabbit polyclonal M_2 receptor antiserum AB5166-50ULa illustrated another problem: while some batches of this antiserum produced labeling specific for wild-type vs. M_2 knockout mice, other batches from the same supplier resulted in identical labeling patterns in both strains, i.e., the producer of this batch may have been unable to deliver a consistent product across batches.

Among antibodies targeted at M_3 receptors, AB9453, AMR-006, AS-3741S, GP19b, R66136, R66431, Rabbit 001, Rabbit 002, and sc-9108 yielded similar staining in immunohistochemical experiments in dorsal root ganglia, urinary bladder, and thoracic viscera from wild-type and M_3 knockout mice (Fig. 1) [10]. AS-3741S also yielded a similar band pattern in immunoblots with membranes from HEK cells transfected with M_1, M_2, M_3, and M_4 receptors [12]. Using a similar approach, another group reported that antibody sc-7474 detected a single band in immunoblots of M_3 receptor-transfected HEK cells, which was absent in non-transfected cells; the apparent molecular weight of this band, 95–100 kDa, was considerably higher that estimated based on the receptor sequence (66 kDa) but might be explained by glycosidation [13]. In a follow-up study

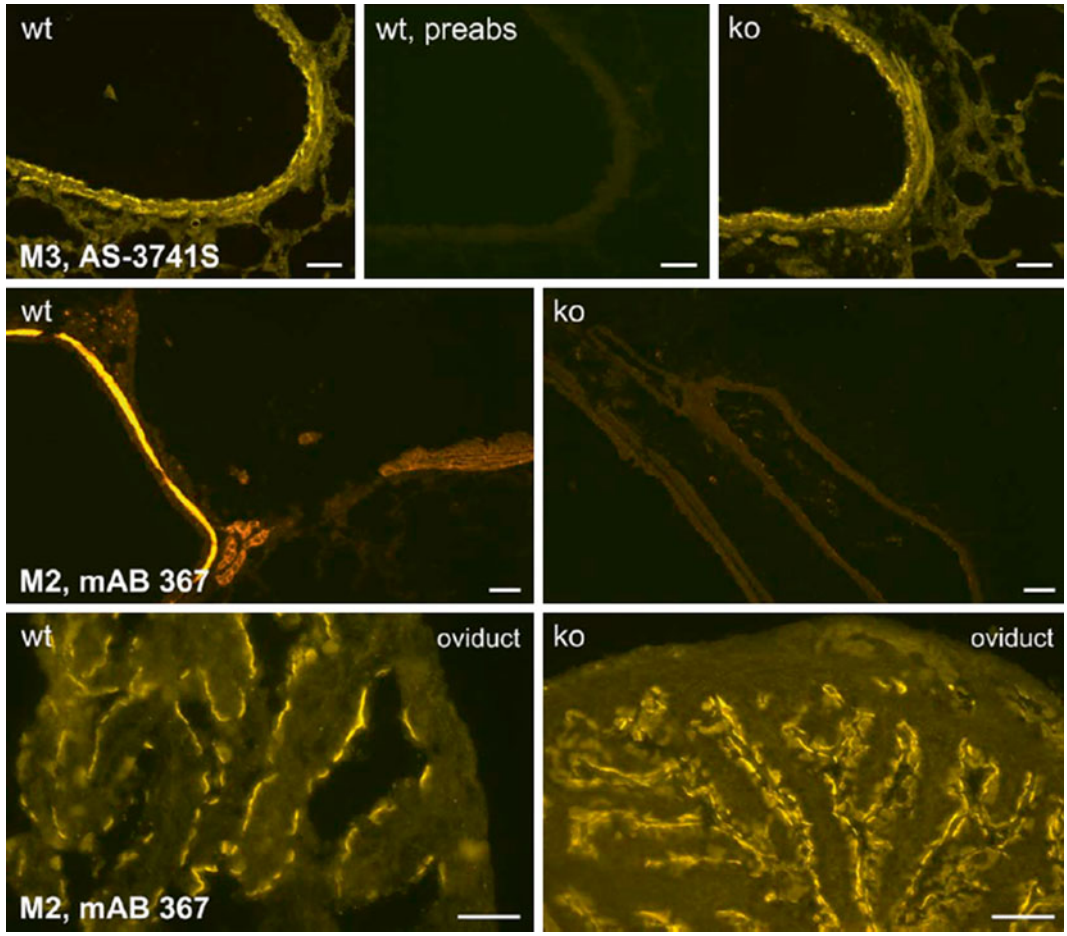

Fig. 1 An example of proven target selectivity and lack of it with antibodies against muscarinic receptor subtypes. The *upper panels* show staining of mouse bronchi with the alleged M_3 receptor antibody AS-37415. Staining appears anatomically selective for smooth muscle in wild-type mice and is prevented by liquid-phase pre-absorption with the corresponding antigen but is not affected in M_3 knockout mice. In contrast, the *middle panels* show that staining of mouse bronchial and pulmonary vein smooth muscle with the M_2 antibody mAB 367 is absent in M_2 receptor knockout mice; however, staining of ciliated epithelial cells of the oviduct was not affected in the knockout mice (*lower panels*). Adapted with permission from [10]

the same group found a similar immunohistochemical staining pattern in the guinea pig urinary bladder for three antibodies, sc-7474, sc-9108, and Ab-13063 [7]. When tested in immunoblots with membranes from HEK cells transfected or non-transfected with M_3 receptors, sc-7474 again yielded a single band of 102 kDa. In contrast, Ab-13063 did not yield any band, and sc-9108 yielded two bands of 45 and 65 kDa. Of note, staining with sc-9108 was abolished by blocking peptide [14] but has not been affected by M_3 receptor knock-out in immunohistochemistry experiments by other investigators [10].

A smaller number of M_4 receptor antibodies have been evaluated by hard criteria. Among these AS-3761S, MAB1576 and sc-9109 yielded similar staining in immunohistochemical experiments in dorsal root ganglia, urinary bladder, and thoracic viscera from wild-type and M_4 knockout mice [10]. MAB1576 also yielded a similar band pattern in immunoblots with membranes from HEK cells transfected with M_1, M_2, M_3, and M_4 receptors [12]. AS-3781S, claimed to be an M_5 receptor antibody, exhibited staining in immunohistochemical experiments in dorsal root ganglia, urinary bladder, and thoracic viscera from wild-type and M_5 knockout mice [10].

Taken together, with the possible exception of few M_2 and M_3 receptor antibodies, most antibodies with claimed selectivity for individual subtypes of muscarinic receptors fail to exhibit target selectivity when tested under stringent conditions. However, even antibodies which exhibit target selectivity under some experimental conditions may not do so under others (Fig. 1). Therefore, the key message from the above is that receptor antibodies need to be validated as carefully as possible and that data based on such antibodies always need to be interpreted very cautiously.

3 Methods for Immunohistochemical Detection of Muscarinic Receptors

Immunostaining is a widely used technique that combines biochemistry and immunology. The concept of immunostaining was developed from the antigen-antibody binding reaction and visualizes the distribution and localization of specific antigens or cellular components (in this case muscarinic receptors) in tissue sections (immunohistochemistry) or isolated cells (immunocytochemistry). Compared to other techniques that are based on the antigen-antibody reaction, such as immunoprecipitation and Western blotting, which provide material for further biochemical analyses and provide information on molecular weight of the antigens (and contaminations), immunostaining provides topographical information.

Immunostaining can be divided into (1) direct staining (one-step staining) and (2) indirect staining (two or more step staining) procedures. The indirect staining technique is more popular, because it allows the amplification of the signals at the site of antigen-antibody binding by different hapten-conjugated antibodies. According to the type of labeling of the antibodies, immunostaining methods can be classified as immunogold, immunofluorescence, and immunoenzyme stainings. In immunogold staining, colloidal gold is bound to the antibodies and visualized. Colloidal gold is the hydrosol form of gold and can bind proteins rapidly and stably. Moreover, colloidal gold has little effect on the biological activity of natural proteins. Therefore, colloidal gold can

be conjugated with both primary and secondary antibodies. Due to the high electronic density of colloidal gold, immunogold technique is also suitable for antigen detection with an electron microscope. In immunofluorescence staining methods, the antigens are visualized with fluorescent dyes conjugated to antibodies. Because the exciting and emitted light have to be separated, dedicated fluorescence microscopes are necessary. Due to ease, high sensitivity, and convenience, the immunofluorescence staining method is widely used in biomedical sciences. In immunoenzyme staining, enzymes are coupled to antibodies that are used to bind to specific antigens in tissue samples or cultured cells. After adding substrate, the enzyme generates insoluble or electron-dense particles that can be localized under a light or electron microscope. Compared to immunofluorescence staining, immunoenzyme-stained samples can be stored longer. Two major enzymes covalently linked with secondary antibodies that are commercially available are horse radish peroxidase (HRP) and alkaline phosphatase (AP). These enzymes catalyze reactions that produce stained products that are easily detectable by light microscopy. Binding reaction between the HRP and its substrate yields the products in brown. However, adding metals to HRP changes color. Reaction between AP and its substrate gives rise to products which stain blue (if substrate used is nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate) or stain red (if aminocarbazol is used as the substrate).

From our own experience with immunostaining of muscarinic receptors in tissue sections and cultured cells, we prefer the AP- to HRP-based method. This is because the staining intensity of properly diluted AP-coupled antisera increases linearly with time for 1–2 h, whereas the product inhibition of peroxidase coupled antisera yields their maximal staining in a few minutes and does, hence, not differentiate very well between locally differing concentrations of antigens. The localization of antigen when stained with AP is adequate but HRP-based staining with diaminobenzidine is superior in this respect. However, the main advantage of staining with AP is that the intensity develops linearly with time for several hours and can also be intensified by developing at a higher temperature [15].

Three major steps in a complete immunoenzyme staining session are the following:

1. Binding of primary antibody to specific antigen (e.g., muscarinic receptors).
2. Forming the antibody-antigen complex by incubation with an (enzyme-conjugated) secondary antibody.
3. Generating a colored precipitate at the sites of antibody-antigen binding by exposing the section to the chromogenic substrate.

3.1 Tips in Immunoenzyme Staining

Tissue preparation or fixation is essential for the preservation of cell morphology and tissue architecture. Inappropriate or prolonged fixation may significantly diminish the accessibility of the antigen to the antibody. Fixatives that are suitable for immunostaining should at least preserve antigenic sites and should not destroy antigenicity by acting as a very strong protein cross-linker. In our experience, 4 % formaldehyde in phosphate-buffered saline (PBS) or an ice-cold mixture of methanol:acetone:water (MAW; 2:2:1 (v/v)) are a proper protein cross-linker and precipitating fixative, respectively. These fixatives are able to successfully preserve antigens in paraffin-embedded tissue sections or in cultured cells. However, some antigens will not survive even moderate amounts of aldehyde fixation. Under such conditions, tissues should be rapidly fresh frozen in liquid nitrogen and cut with a cryostat. The disadvantages of frozen sections include poor morphology, poor resolution at higher magnifications, difficulty in cutting relative to paraffin sections, and the need for a cryotome [16].

If 4 % formaldehyde/PBS is used as fixative, the detection of antigens can be dramatically improved by antigen retrieval. This method breaks up some of the protein cross-links formed by fixation to uncover hidden antigenic sites. This can be accomplished by heating in citrate or EDTA-based solution (e.g., autoclave or microwave) for varying lengths of times [17–19]. However, if MAW is employed as fixative agent, the step of antigen retrieval is not required.

One of the main difficulties with immunostaining is reducing non-specific background. Optimization of fixation methods and times, pre-treatment with blocking agents, incubating antibodies diluted in a high-salt solution (e.g., 500 mM Na-acetate, pH 8), and optimizing post-antibody washing buffers and washing times are all important for obtaining high-quality immunostaining. In addition, the presence of positive and negative controls for staining is essential for determining specificity.

The following and Table 1 reflect the protocols that we have used to successfully reveal antigens in the paraffin-embedded tissue sections [20].

4 Protocol: Indirect Immunoalkaline Phosphatase Staining on Paraffin Sections

1. Deparaffinize sections in three changes of xylene, 3 min each.
2. Hydrate the sections in a graded descending series of ethanol: 100 %, 96 %, 90 %, 80 %, 70 %, and 50 % for 1 min each.
3. Rinse in water.
4. If the sections were fixed with formaldehyde, it is necessary to perform an antigen retrieval step. If the sections were fixed by ice-cold mixture of methanol:acetone:water

Table 1
Chemicals used in [20]

Chemical and material	Manufacturer	Product No.
Paraformaldehyde	VWR	4005
Tween-20	VWR	822184.0500
Normal goat serum (NGS)	Gibco	16210-072
Fetal calf serum (FCS)	Gibco	
NBT/BCIP	Roche	1681 451
GAM-AP	Sigma	A3562
GAR-AP	Dako	D0487
RAG-AP	Sigma	A4187
Super PAP-pen	Beckman	IM3850

(MAW; 2:2:1 (v/v)) (or other alcohol fixative) antigen retrieval is not necessary.
The steps for antigen retrieval are as follows:

(a) Prepare 10 mM sodium citrate from a stock solution of 1 M sodium citrate; adjust the pH to 6.0 with 1 M citric acid.

(b) Put your slides in the solution-containing box and cover with aluminum foil.

(c) Put the slides in the autoclave.

(d) Set the autoclave at 10 min and 120 °C.

(e) Wait until the pressure is off and take the sections out.

(f) Let them cool down to room temperature, wash shortly in distilled water and then continue with the procedure.

5. Wash in PBS, pH 7.4, for a minimum of 5 min on a shaking platform at room temperature.
6. Draw circle around the sections on the glass slides with a Pap-pen to prevent mixing of the different antibodies between adjacent sections.
7. Incubate the sections in 1× TENG-T/10 % serum (normal goat serum (NGS) or fetal calf serum (FCS)) for a minimum of 30 min to reduce nonspecific background staining.
8. Remove the TENG-T + 10 % serum by suction and apply the primary antibody. The appropriate dilution(s) of primary antibody are made in TENG-T/10 % serum. The incubation is done overnight at room temperature in a humidity chamber.

It is noteworthy that the volume applied on each section should not be too big to avoid intermingling with adjacent incubations.

9. Remove the unbound first antibody by gentle suction and drop PBS directly on the sections.
10. Wash the sections in three changes of PBS, 5 min each on a shaking platform at room temperature.
11. Incubate the sections with the AP-conjugated secondary antibody for at least 2 h at room temperature. The optimal dilution of AP-conjugated secondary antibody is dependent on the first antibody used:
    (a) If the first antibody was raised in mouse, use GAM-AP: **GAM**-**AP**: **G**oat-**a**nti-**M**ouse IgG conjugated with **A**lkaline **P**hosphatase (1:100 in TENG-T/10 % serum).
    (b) If the first antibody was raised in rabbit, use GAR-AP: **GAR**-**AP**: **G**oat-**a**nti-**R**abbit IgG conjugated with **A**lkaline **P**hosphatase (1:200 in TENG-T/10 % serum).
    (c) If the first antibody was raised in goat, use RAG-AP: **RAG**-**AP**: **R**abbit-**a**nti-**G**oat IgG conjugated with **A**lkaline **P**hosphatase (1:50 in TENG-T/10 % serum). In this case the serum should *not* be a normal goat serum.
12. Wash the sections in three changes of PBS, 5 min each on a shaking platform at room temperature.
13. Incubate the sections in NBT/BCIP (a substrate of alkaline phosphatase) diluted in NTM at room temperature.

    Note

    (a) NBT/BCIP: nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; Dako).
    (b) NTM contains: (1) 100 mM NaCl, (2) 100 mM Tris pH 9.5, and (3) 50 mM $MgCl_2$.
    (c) $MgCl_2$ should be added to the solution just before use.
    (d) Dilute the NBT/BCIP 1:50 in NTM just before use. Make it fresh, do not store.
14. Stop the reaction in distilled water, after the staining is satisfactory when viewing under light microscope (it can be 30 min, but up to 2 h is permitted).
15. Dehydrate the sections by dipping quickly through a graded ascending series of ethanol (50 %, 70 %, 80 %, 90 %, 96 %, and 100 %). If you do not go quickly through the ethanol, the staining will become faint.
16. Dip the sections in three changes of xylene, 7 min each. This step makes the color in the tissues clearer.
17. Mount the sections in Enthallan (a mounting media).

18. Let the sections dry in fume hood and then overnight in an incubator at 37 °C.
19. Observe and photograph under light microscope.

5 Notes

1. Prepare stock solution of 10× TENG-T containing:
   - 100 mM **T**ris–HCl.
   - 50 mM **E**DTA (pH 8.0).
   - 1.5 M **N**aCl.
   - 2.5 % **G**elatin.
   - 0.5 % v/v **T**ween-20.

   Mix well and store at 4 °C.
2. In order to prepare 1× TENG-T:
   Put 10× TENG-T in warm water and allow the content to melt. Shake gently and dilute it to 1× TENG-T with bidistilled water. Adjust the pH with HCl or NaOH to 8.0.
3. Prepare 1× TENG-T + 10 % serum:
   Add 1 ml of serum to 9 ml of 1× TENG-T (NGS, FCS, or another serum can be used, but it must not be the serum from the animal in which the first antibody was raised (e.g., do not use rabbit serum when your first antibody was raised in rabbits)). Ideally primary and secondary antibody should be from different orders of mammals; if this is not feasible, non-specific binding can be tested on a Western blot.

6 Conclusions

The most important conclusion from this data is that investigators must apply great care in their choice of antibody for immunological detection of muscarinic receptor subtypes. As target selectivity may depend on the assay, i.e., immunoblotting vs. immunohistochemistry, each antibody must be carefully validated for the intended use. Choice of fixation protocols and other steps may critically affect signal strength in immunohistochemistry studies.

Acknowledgment

We thank Prof. Wouter H. Lamers, Academisch Medisch Centrum, Universiteit van Amsterdam, for his continued advice and support.

References

1. Schneider T, Hein P, Michel-Reher M et al (2005) Effects of ageing on muscarinic receptor subtypes and function in rat urinary bladder. Naunyn Schmiedebergs Arch Pharmacol 372: 71–78
2. Caulfield MP, Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290
3. Michel MC, Wieland T, Tsujimoto G (2009) How reliable are G-protein-coupled receptor antibodies? Naunyn Schmiedebergs Arch Pharmacol 377:385–388
4. Kirkpatrick P (2009) Specificity concerns with antibodies for receptor mapping. Nat Rev Drug Discov 8:278
5. Hamdani N, van der Velden J (2009) Lack of specificity of antibodies directed against human beta-adrenergic receptors. Naunyn Schmiedebergs Arch Pharmacol 379:403–407
6. Bodei S, Arrighi N, Sigala S (2009) Should we be cautious on the use of commercially available antibodies to dopamine receptors? Naunyn Schmiedebergs Arch Pharmacol 379:413–415
7. Lu X, Bartfai T (2009) Analyzing the validity of GalR1 and GalR2 antibodies using knockout mice. Naunyn Schmiedebergs Arch Pharmacol 379:417–420
8. Cernecka H, Pradidarcheep W, Lamers WH et al (2014) Rat β_3-adrenoceptor protein expression: antibody validation and distribution in rat gastrointestinal and urogenital tissues. Naunyn Schmiedebergs Arch Pharmacol 387:1117–1127
9. Cernecka H, Ochodnicky P, Lamers WH et al (2012) Specificity evaluation of antibodies against human β_3-adrenoceptors. Naunyn Schmiedebergs Arch Pharmacol 385:875–882
10. Jositsch G, Papadakis T, Haberberger RV et al (2009) Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice. Naunyn Schmiedebergs Arch Pharmacol 379:389–395
11. Rhodes KJ, Trimmer JS (2006) Antibodies as valuable neuroscience research tools versus reagents of mass distraction. J Neurosci 26: 8017–8020
12. Pradidarcheep W, Stallen J, Labruyere WT et al (2009) Lack of specificity of commercially available antisera against muscarinic and adrenergic receptors. Naunyn Schmiedebergs Arch Pharmacol 379:397–402
13. Grol S, Essers PBM, van Koeveringe GA et al (2009) M_3 muscarinic receptor expression on suburothelial interstitial cells. BJU Int 104: 398–405
14. Grol S, Nile CJ, Martinez-Martinez P et al (2011) M_3 muscarinic receptor like immunoreactivity (M_3-IR) in sham-operated and obstructed guinea pig bladders. J Urol 185: 1959–1966
15. van Straaten HWM, He Y, van Duist MM et al (2006) Cellular concentrations of glutamine synthetase in murine organs. Biochem Cell Biol 84:215–231
16. Fischer AH, Jacobson KA, Rose J et al (2008) Cryosectioning tissues. CSH Protoc 2:1–2
17. Yamashita S (2007) Heat-induced antigen retrieval: mechanisms and application to histochemistry. Prog Histochem Cytochem 41: 141–200
18. Syrbu SI, Cohen MB (2011) An enhanced antigen-retrieval protocol for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues. Methods Mol Biol 717: 101–110
19. Gadd VL (2014) Combining immunodetection with histochemical techniques: the effect of heat-induced antigen-retrieval on picro-Sirius red staining. J Histochem Cytochem 62:902–906
20. Pradidarcheep W, Labruyere WT, Dabhoiwala NF et al (2008) Lack of specificity of commercially available antisera: better specifications needed. J Histochem Cytochem 56: 1099–1111