Background Suppression In Immunoperoxidase Cytochemistry Using AURION BSA-c™

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Introduction

Background is an unintended reaction in immuno-cytochemical localization studies which is caused by non-specific binding of one or more of the essential compounds used during incubation. In immunoperoxidase cytochemistry these are the primary (and secondary) antibody and the peroxidase conjugate. In addition, the presence of endogenous peroxidase-like activity may interfere. The antibody and peroxidase conjugate based background are governed by physico-chemical interactions between these reagents and the specimen such as London-van der Waals interactions, hydrophobicity and charge determined interactions. Background can be controlled by paying special attention to the blocking step used prior to the primary antibody incubation step and to the incubation buffer.

The Blocking Step Prevents Background Based On Hydrophobic Interactions.

As a rule, any protein including antibodies possesses hydrophobic domains. In albumin for example 40% of the amino acids are hydrophobic. The Fc-region of rabbit antibodies is also enriched in hydrophobic amino acids. Pretreatment of rabbit primary antibodies with protein A or recombinant protein A has been shown to give significantly reduced background levels (1). Since protein A binds to the Fc-region, it is not unlikely that the sticky hydrophobic areas become masked. In specimens hydrophobic areas are represented by membranes, lipid droplets, embedding media and hydrophobic substrata like nitrocellulose. The hydrophobic interaction between antibodies and conjugates can be diminished by sealing the hydrophobic specimen domains with a blocking protein such as serum albumin or casein, the main compound of milk powder. In a hydrophilic environment (as in incubation media) the blocking protein/substrate interaction is most effective at a pH-value close to the isoëlectric point of the blocking protein (2). The interaction is strong and will not easily dissociate during incubation.

The Incubation Buffer Composition Prevents Background Based On Charge Determined Interactions.

In the prevailing pH domain of incubation (between 7.2 and 8.5) many antibody molecules and conjugates have a net negative charge. This may lead to background at those sites in the specimen where positive charges are abundant (for instance some proteins of the cytoskeletal network, collagen compounds and the nuclear histone proteins). Such areas are not easily blocked in a more or less permanent way as is the case for hydrophobic interactions. Charge determined background can to some extent be dealt with by suppressing the charges using high salt buffers, but most efficiently by adding proteins to the incubation mix which have a strong net *negative* charge at the pH of incubation. Such proteins compete with immunocytochemical compounds for non-specific binding to positively charged sites. AURION developed a chemically modified BSA with an increased net negative charge by acetylation of amino groups of basic amino acids (AURION BSA-c™). A 0.1 - 0.2% solution of AURION BSA-c™ in PBS pH 7.2-7.4 has proven to be a most efficient incubation buffer for ImmunoGold cytochemistry (2). Here we report on the effectivity of AURION BSA-c™ to eliminate background in Immunoperoxidase cytochemistry.

Experimental Set-up

The research at the Department of Functional Morphology involves the labeling of TGF-β1 in feline tissues and of TNF-α and TGF-α in rat tissues (3). These components are detected in cryostat and paraffin sections using specific rabbit antibodies and the Vectastain® elite kit. Especially in paraffin sections using an incubation buffer with 1% bovine serum albumin, an overall brown background staining is found over the entire section. Since this background is not present when the primary antibody is omitted it can be concluded that the background can be attributed entirely to stickiness of the primary rabbit antibodies. The labeling of TNF-α on cryostat sections of rat tissue did not give background staining with the standard incubation protocol.
In order to diminish background, the 1% bovine serum albumin in the incubation buffer was replaced with 0.05 - 0.5% AURION BSA-c™.

Incubation Procedure

Tissue was perfusion fixed with 4% formaldehyde in TBS buffer (0.01 M Tris, 0.15 M NaCl, pH 7.6) for 6-8 hours, followed by an overnight fixation in Bouins modified in the sense that 0.9% picric acid was used instead of a saturated solution.

Sections are deparaffinized with xylene for 2 x 10 minutes; next the xylene was removed by washing in ethanol 100% for 2 x 10 minutes.

Endogenous peroxidase was inactivated by a 30 minutes treatment with 1% hydrogen peroxide in methanol 100%. The hydrogen peroxide used should not be older than 12 months.

Rinse 3x in TBS for 5 minutes each.

Inactivate residual formaldehyde by treatment with 0.1 M Glycin in TBS for 30 minutes.

Rinse 3x in TBS for 5 minutes each.

Blocking is performed with 5% Normal Goat Serum in TBS for 30 minutes.

Incubate with an appropriate concentration of primary rabbit antibody in incubation buffer (TBS with 0.05 to 0.5% AURION BSA-c™). The pH of the incubation buffer should optimally be between 7.2 and 7.4. The anti-TGF-β1 incubation was done overnight in a humid atmosphere.

Rinse 6x in TBS for 5 minutes each.

Incubate with the biotinylated secondary antibody Goat-anti-Rabbit at a concentration of 10-15 μg/ml in incubation buffer for 60 minutes.

Rinse 6x in TBS for 5 minutes each.

Prepare the Avidin-Biotin complex according to the recommendations of the manufacturer in incubation buffer and incubate for 60 minutes.

Rinse 6x in TBS for 5 minutes each and 1x 5 minutes in Tris/HCl 0.05 M pH 7.6.

The substratum for the peroxidase consists of 0.6 mg/ml of Diaminobenzidine and 0.03% H_2O_2 and is applied for 2-10 minutes.

Finally sections are rinsed with distilled or ultra pure water and counterstained with Haematoxylin.

Results

By changing the composition of the incubation buffer, using 0.05%-0.5% AURION BSA-c™, the background induced by the rabbit anti-TGF-β1 antibodies was easily controlled and no longer detectable, whereas the specific signal was not influenced.

References

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2. Jan L.M. Leunissen. *Background suppression using AURION BSA-c™ and/or Tween-20®.* AURION Newsletter 1.

3. Katja J. Teerds and Jennifer H. Dorrington. *Immunolocalization of transforming growth factor-α and luteinizing hormone receptor in healthy and artretic follicles of the adult rat ovary.* Biology of Reproduction. In press.