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# Multiple immunolabeling with antibodies from the same host species in combination with tyramide signal amplification

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ABSTRACT

A general problem in immunocytochemistry is the development of a reliable multiple immunolabeling method with primary antibodies originating from the same host species. When primary antibodies are raised in the same host species, the secondary species-specific antibodies can cross-react with each of the primary antibodies. This obstacle can however be avoided with the use of striping buffers eluting the primary/secondary antibody complex. After elution of the previous primary/secondary antibody complex, the next primary antibody from the same host species can be applied. Recently, a group from VENTANA (Tucson, AZ, USA) presented a fully automated multiplex protocol for fluorescent immunohistochemistry on the platform of VENTANA's BenchMark ULTRA slide stainer using the same species antibodies in combination with tyramide signal amplification. We adapted the automated protocol of VENTANA for the use in a routine histochemical laboratory and present here a standard procedure with a manual mode of operation for simultaneously detecting two or more antigens from the same host species.

## 1. Introduction

Methods for immunohistochemical detection of multiple tissue antigens in their simplest form make use of primary antibodies that are raised in different species and therefore can be accordingly visualized with differently labeled species-specific secondary antibodies. However, quite often the appropriate combination of primary antibodies from different host species is not available. A general problem relates to the fact that most of the available primary antibodies originate mainly from two species – either rabbit or mouse. When primary antibodies are raised in the same host species, the secondary species-specific antibodies can cross-react with each of the primary antibodies.

An intermediary elution step using "Double Stain Block" developed by DAKO avoids this obstacle. Applied prior to the use of next primary antibodies, this step serves to remove previously bound primary and secondary antibodies and leaves only the deposit of chromogen from the previous steps, thus eliminating any potential for cross-reactivity. The formulation of this "Double Stain Block" is a trade secret of DAKO. We guess that this elution technique is borrowed from protein Western Blotting methodology, whereby an intermediary elution buffer is also utilized that removes the previous pairs of primary/secondary antibodies (Harlow and Lane, 1999). In the following years, a number of buffers with different pH, osmolarity, detergent content and denaturing properties were tested to strip the bound antibody complex from previous immunostaining cycles. Pirici et al. (Pirici et al., 2009) compared the applicability of various elution buffers so as to remove previously bound primary and secondary antibodies leaving only the deposit of chromogen from the previous immunolabeling steps. It was found that the elution step with glycine-SDS buffer (25 mM glycine-HCl, 10% SDS, pH 2) for 30 min at 50 °C ensures a reliable immunoenzyme staining with water-insoluble chromogens without cross-reactivity and without loss of tissue antigenicity. Most enzyme detection substrates (chromogens), except Vector-Blue and Vector-VIP, withstand treatment in glycine SDS pH 2 buffer maintaining their properties even after multiple elution times.

As yet this approach was successfully applied for immunostaining with enzyme labels using precipitating-chromogen systems only on the level of bright-field microscopy. More recently, the authors from VENTANA Medical Systems (Tucson, AZ, USA) reported on multiple fluorescent immunolabeling using the same species antibodies in combination with tyramide signal amplification (TSA) (Zhang et al., 2017). In this method, the first on-tissue target (antigen) was detected by applying target-specific primary antibody and then anti-species secondary antibody conjugated to Horseradish Peroxidase (HRP) (Buchwalow et al., 2013). In an incubation medium containing tyramide (tyramine-fluorophore), active HRP in the presence of its substrate ($H_2O_2$) drives in-situ deposition of tyramide (Bobrow et al., 1989). The bound primary antibody/secondary antibody complex was

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then eluted with a citrate/acetate-based buffer, pH 6.0, containing 0.3% SDS (CC2 solution, available from VENTANA cat # 950-223). Thereby, the deposited tyramide is left covalently bound to tissue near the first target/antigen. The same procedure can be repeated to detect the next targets.

The automated protocol of the VENTANA group can be taken to practice only on the platform of VENTANA's BenchMark ULTRA slide stainer. Therefore we modified the protocol for simultaneous detecting two or more antigens from the same host species for a manual mode of operation used a routine histochemical laboratory.

## 2. Materials and methods

### 2.1. Case selection

Tissue samples of human breast, skin, small intestine and tonsil from patients undergoing surgery were taken at a distance of at least 3 cm from the site of the tumor. The samples were redundant clinical specimens that had been de-identified and unlinked from patient information. The range of data, in which the original archive specimens were collected for the present study and processed to paraffin block, is between 2010 and 2017. This study was conducted in accordance with the principles of World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" and approved on 10.11.2017 by the Institutional Review Board of the Institute for Hematopathology, Hamburg, Germany.

### 2.2. Immunohistochemistry

Tissue samples were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. PBS was used for all washings and dilutions. Paraffin tissue sections (1 µm thick) were deparaffinized with xylene and rehydrated with graded ethanols. For immunohistochemical assay, deparaffinized sections were subjected to antigen retrieval by heating the sections in 10 mM sodium citrate buffer, pH 6.0, at 95 °C × 30 min. Blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted, because we recently reported that endogenous Fc receptors in routinely fixed cells and tissue probes do not retain their ability to bind Fc fragments of antibodies (Buchwalow et al., 2011). After antigen retrieval and quenching endogenous peroxidase, sections were incubated with primary antibodies. The list of primary antibodies used in this study is presented in the Table 1.

Primary antibodies were applied in concentration from 1 to 5 µg/ml and developed using AmpliStain™ HRP conjugates (SDT GmbH, Baesweiler, Germany) according to the manufacturers' instructions (Buchwalow et al., 2013). The HRP label was amplified with TSA Plus Fluorescein and TSA Plus Cyanine 3 systems purchased from PerkinElmer (Rodgau Germany). In this study, we used also fluorophore-conjugated tyramines synthetized in our laboratory. FITC- and Cy3-tyramine conjugates were synthesized from FITC-succinimidyl ester (FITC-NHS, Molecular Probes) and Cyanine3-succinimidyl ester (Cy3-NHS, AbCam), respectively, with tyramine-HCl (Sigma) in DMF (Sigma) (Table 2) according to our protocols (Buchwalow and Boecker, 2010). Sections were incubated for 3–8 min at room temperature with fluorophore-tyramines dissolved 1:500 in amplification diluent (0.02% $H_2O_2$ in PBS) and washed in PBS for 3 × 3 min.

### 2.3. Controls

Control incubations were: omission of primary antibodies and substitution of primary antibodies by the same IgG species (Dianova, Hamburg, Germany) at the same final concentration as the primary antibodies. The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, substitution of primary antibodies with the corresponding IgG at the same final concentration resulted in lack of immunostaining. The TSA step alone did not contribute to any specific immunostaining that might have influenced the analysis. Moreover, the specific and selective staining of different cells or cell compartments (cytoplasm vs. nuclei) with the use of primary antibodies from the same species on the same preparation is by itself a sufficient control of the specificity of immunostaining.

**Table 1**
Primary antibodies used in this study.

| Antibodies | Source | Dilution |
|---|---|---|
| α Smooth Muscle Actin (mouse Ab) Catalog No: MSK030 | ZYTOMED | 1/50 |
| α Smooth Muscle Actin (rabbit Ab) Catalog No: ab5694 | Abcam | 1/200 |
| Cytokeratins 5 (rabbit Ab) Catalog No: 305R-16 | Medac | 1/100 |
| Cytokeratin 5/6 (mouse Ab) Catalog No: M 7237 | DAKO | 1/50 |
| Cytokeratin 10 (mouse Ab) Catalog No: M7002 | DAKO | 1/50 |
| Cytokeratin 8/18 (mouse Ab) Catalog No: Mob189 | Zytomed | 1/50 |
| Estrogen Receptors (rabbit Ab) Catalog No: RM-9101 - S0 | Thermo Fisher Scientific | 1/100 |
| Ki67 (rabbit Ab) Catalog No: RM-9106 | Thermo Fisher Scientific | 1/100 |
| Ki67/MIB1 (mouse Ab) Catalog No: M7240 | DAKO | 1/25 |
| p63 (mouse Ab) Catalog No: CM163B | BioCare Medical | 1/50 |
| PD-L1 (rabbit monoclonal Ab) Catalog No: SP263 | Ventana/Roche, Germany | Ready-to-use |
| CD3 (rabbit Ab) Catalog No: 790-4341 | Ventana/Roche, Germany | Ready-to-use |

### 2.4. Image acquisition

For fluorescence detection we used filter sets for GFP, Cy3, and Cy5. Tissue sections were observed on a Zeiss Axio Imager Z1 configured for fluorescence microscopy and equipped with a microscope camera (Zeiss Axio Cam HRm, monochrome CCD) for recording microscopic images. Images shown are representative of 3 independent experiments, which gave similar results.

## 3. Results

In our study, we compared home-made (Hopman et al., 1998; Buchwalow and Boecker, 2010) and commercially available tyramine-fluorophore conjugates. All tested home-made and commercially available TSA kits showed similar results.

Next, we assessed the efficacy of the proposed elution methods to remove the primary/secondary antibody complex after immunostaining. Pirici et al (2009) reported the efficiency of the eluting regime in a glycine-SDS buffer (25 mM glycine-HCl, 10% SDS, pH 2) for 30 min at 50 °C (Pirici et al., 2009). Zhang et al (2017) recognized a citrate/acetate-based buffer, pH 6.0, containing 0.3% SDS, as the most effective deactivation condition at 100 °C. The length of the heat treatment on the VENTANA BenchMark ULTRA slide stainer was not however specified (Zhang et al., 2017). Comparing the efficacy of these two elution buffers (Pirici et al., 2009; Zhang et al., 2017) applied for 10, 20 and 30 min at 50 °C and at 95 °C, we found that the treatment in CC2 solution (available from VENTANA cat # 950-223) at 95 °C for 20 min ensures the optimal immunostaining without cross-reactivity and without loss of tissue antigenicity. The immunofluorescence staining remained stable after the heat elution treatment only in combination with TSA, whereas the immunolabeling with fluorophore-conjugated secondary antibodies without TSA was completely lost after the heat treatment in the elution buffers.

Establishing that the heat treatment with VENTANA striping buffer