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IHC Made Affordable

Instructions for Universal HRP Immunostaining Kit (For Mouse and Rabbit Primary Antibodies)

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Intended Use
For In Vitro Diagnostic Use

Summary and Explanation
This product is intended for qualitative immunohistochemistry with normal and neoplastic formalin-fixed, paraffin-embedded tissue sections, to be viewed by light microscopy. Clinical interpretation of staining results should be accompanied by histological studies with proper controls. Patients' clinical histories and other relevant diagnostic tests should be utilized by a qualified person (s) when evaluating and interpreting results.

This kit is designed to label specific primary antibodies immunohistochemically on tissue sections. The immunohistochemical protocol defined in this brochure is just a guideline. Depending upon tissue fixation conditions, the primary antibody employed, and the user's experience, we encourage individual laboratory to optimize its own protocol. These reagents were tested and quality controlled using tissue sections, however, they can also be optimized for cell smears and cytospin preparations. Sufficient reagents are provided to run 50 to 100 tests.

Principles of the Procedure
The high affinity of the non covalent interaction between biotin and streptavidin (1x1015) forms the basis for this immunostaining kit. It requires the formation of an irreversible and specific linkage between biological macromolecules. The immunohistochemical applications of the interaction between avidin and biotin were introduced by Bayer et al. (1979), who described techniques for generating active biotinyl compounds such as biotin-N-hydroxysuccinimide and biotin hydrazine and for conjugating them to various organic compounds, including immunoglobulins and horseradish peroxidase (HRP). Streptavidin (SA) is a tetrameric protein (mol. wt. 4x15,000), isolated from the actinobacterium Streptomyces avidinii (Chaiet & wolf, 1964). Streptavidin can bind to four molecules of biotin. Streptavidin gives superior results as compared to avidin because its isoelectric point is closer to a neutral pH, whereas avidin is positively charged at a physiological pH. Streptavidin does not carry any carbohydrate side chain, whereas avidin is composed of 70% of carbohydrate. Because of these reasons, SA does not have the tendency to bind non-specifically. Primary antibodies bind to target antigens in the tissue sections. The conjugated secondary antibody binds specifically to these receptor antibodies. Biotin conjugated second antibody, in turn, is traced by a streptavidin conjugated enzyme and can be visualized by an appropriate substrate.

Introduction
Immunoperoxidase techniques are spreading rapidly and the practice of anatomic pathology has undergone a revolutionary change since the development of these procedures (Nadji & Morales, 1983). Because of their versatility, sensitivity, and specificity, immunoperoxidase stains are invariably the best stains when and if appropriate antibodies are available. With the ever increasing number of antibodies against cellular antigens, immunoperoxidase techniques now provide a powerful tool to resolve a wide array of diagnostic pathology. All immunohistochemical techniques require the specific antibody employed to be so labeled that they can be easily seen when attached to cellular antigens. At the same time the sensitivity of immunoperoxidase techniques are central to wide variety of specific antigen localization. Various investigators (Petrusz et al., 1983; Nagle et al. 1983; Giorno et al., 1984) have shown that direct SA-HRP conjugate technique is 4 to 8 times more sensitive than the avidin-biotin complex described by Hsu et al. (1981). Our kit is based on direct SA-HRP conjugate technology. The linker reagent is a cocktail of biotinylated anti-mouse and anti-rabbit, capable of labeling primary antibodies raised in mouse and rabbit.

Reagents Supplied

Bottle 1	Linker Reagent: 110mL clear yellow ready to use solution of biotinylated anti-mouse and anti-rabbit immunoglobulins.
Bottle 2	Tracer Reagent: 110mL clear brick red ready to use solution of conjugated streptavidin-horseradish peroxidase.

Precautions

Diagnostic BioSystems
6616 Owens Drive
Pleasanton, CA, 94588
Tel: (925) 484 3350
www.dbiosys.com

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Emergo Europe
Prinsessegracht 20
2514 AP, The Hague
The Netherlands
Tel: (31) (0) 70 345 8570

i) These reagents contain sodium azide. Follow instruction provided by local authorities for disposal. If disposed in the sink, flush the drain pipe to avoid a reaction of sodium azide with the plumbing system.
ii) Once the immunostaining process is started, don't let tissue sections dry because it can cause undesirable background and artifacts.
iii) Interpretation of the results will be the sole responsibility of the user.

Storage
All the reagents should be stored at 2-8°C. Do not freeze. Do not use beyond the expiration date stated on the label.

Material required but not provided
Some of the reagents and materials required for IHC are not provided. Pretreatment reagents, detection systems, control reagents and other ancillary reagents are available from Diagnostic BioSystems. Please refer to the Diagnostic BioSystems website at www.dbiosys.com

Specimen Collection and Preparation
Tissues fixed in 10% formalin are suitable for use prior to paraffin embedding. Consult references (Kiernan, 1981: Sheehan & Hrapchak, 1980) for further details on specimen preparation.

The user is advised to validate the use of the products with their tissue specimens prepared and handled in accordance with their laboratory practices.

Reagents Preparation
All of the reagents in this kit are provided in a ready to use format.

Preparation of working Stable DAB/Plus Substrate/Chromogen solution (DBS catalog # K 047)
Aliquot 1mL of Stable DAB/Plus Buffer into the mixing bottle. Add 20µL (one drop) of the Stable DAB/Plus Chromogen. Replace tip and mix.

Note: The working Stable DAB/Plus solution is stable for at least 2 weeks and should be prepared in an opaque bottle. Store at 2-8°C when not in use. Any solution not used after this period should be discarded.

Preparation of Mono AEC Plus Substrate/Chromogen (Cat # K 050)
The Mono AEC Plus substrate/chromogen solution is provided in a ready-to-use format.

Positive and Negative controls
Each immunostaining run should include a known positive and negative control to assure proper functioning of staining system and valid interpretation of the results.
Positive control
A tissue which is known to contain the desired antigen and has given positive staining.

Negative control
One of the following should be used as negative control:
i) Instead of primary antibody, use the normal nonimmune serum from the same species of animal in which the primary antibody was raised.
ii) Instead of the primary antibody, use the buffer in which the primary antibody was diluted.
iii) Use a tissue known not to contain the desired antigen.
iv) Absorb the primary antibody with the appropriate antigen and use it instead of primary antibody.

Staining Protocol

Step I Removal of paraffin wax: Deparaffinize the tissue sections according to the established procedure in your lab and bring tissues to wash buffer.

Step II Peroxidase Blocking: Apply enough drops of Peroxidase Block to cover the tissue. Incubate for 5 minutes at room temperature. Use 0.3% H_2O_2 (dilute the Peroxidase Block 1:10) for the frozen tissue sections, cell smears, and cytospin preparations.

Step III Washing: Drain off excess reagent. Rinse with wash buffer three times for 1 minute each time. Drain off excess buffer and carefully wipe slide around the tissue to remove excess buffer from the glass. Leave the tissue wet.

Immunostaining Protocol I

This protocol is recommended for optimally fixed tissues with abundant antigens in the tissue and for high affinity primary antibodies.

Step I Primary antibody: Apply enough drops of the primary antibody to cover the tissue section. Incubate according to the manufacture's recommended conditions. Wash and wipe slides as described above.

Step II Linker: Apply enough drops of linker reagent to cover the tissue section. Incubate for 10 minutes at room temperature. Wash and wipe slides as described previously.

Step III Tracer: Apply enough drops of tracer reagent to cover the tissue section. Incubate for 10 minutes at room temperature. Wash and wipe as described previously.

Step IV Substrate/Chromogen: Apply working substrate/chromogen solution for 5-8 minutes at room temperature for color development. For best results, look under the microscope for signal development. Once desired signal to noise ratio is achieved, stop the reaction by washing slides in wash buffer. Note evelopment time and follow it during subsequent incubations.

Immunostaining Protocol II

This protocol is recommended for less than optimally fixed tissues with low antigenic density in the tissue and for low affinity primary antibodies.

Step I Primary antibody: Apply enough drops of primary antibody to cover the tissue section. Incubate according to the manufacture's recommended conditions. Wash and wipe slides as described previously.

Step II Linker: Apply enough drops of linker reagent to cover the tissue section. Incubate for 20 minutes at room temperature. Wash and wipe slides as described previously.

Step III Tracer: Apply enough drops of tracer reagent to cover the tissue section. Incubate for 20 minutes at room temperature. Wash and wipe slides as described previously.

Step IV Substrate/Chromogen: Apply working substrate/chromogen solution for 2-5 minutes at room temperature for color development. For best results, look under the microscope for signal development. Once desired signal to noise ratio is achieved, stop the reaction by washing slides in wash buffer. Note development time and follow it during subsequent incubations.

Step V Wash slides and counter stain them with AN appropriate counter stain. Mount and observe staining under the microscope.

References

(i) Bayer et al., Method Enzymol. 62, 308 (1979).

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(iv) Hsu et al., J Histo. Cyto. 29, 577 (1981).
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