MILLIPORE IHC Select®

Immunoperoxidase Secondary Detection System

Cat. No. DAB150 - 150 Slide Kit
DAB500 - 500 Slide Kit

GENERAL PURPOSE REAGENTS

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Application

The **IHC Select® Immunoperoxidase Secondary Detection System** is intended for use with the **MILLIPORE IHC Select® Prediluted Primary Antibody** reagents which contain rabbit and/or mouse IgG for the qualitative identification of antigens by light microscopy in paraffin-embedded tissues. The **IHC Select® Immunoperoxidase Secondary Detection System** may also be used with other rabbit or mouse IgG primary antibodies.

General Purpose Reagents

Summary and Explanation

Immunohistochemical techniques have been in use as adjunctive methods for the recognition of cells in tissue sections for at least 50 years. The first method reported use of fluorescent labels in 1942[1]. In 1974, reports of using enzymatic labels, such as horseradish peroxidase (HRP) in routine H & E paraffin sections appeared[2,3]. The methods have since become the 'standard of care' in surgical pathology when classic methods alone fail to yield a definitive diagnosis[4-9].

Early techniques were based on the peroxidase-anti-peroxidase (PAP) reaction[28], while improvements exploited the strong affinity of avidin for biotin[10]. This technique uses HRP-labeled streptavidin. The secondary antibody is conjugated with biotin and the streptavidin-HRP complex reacts with the biotin on the secondary antibodies. The resulting biotin-avidin-HRP complex can react with the primary antibody bound to the specific epitope of the target antigen. The HRP enzymes of the streptavidin complex then catalyze the substrate/chromogen reaction to form a colored reaction product (brown to black when using DAB as the chromogen) at the antigen site. A biological stain is then used to visualize the whole tissue section.

Principle of Procedure

The **IHC Select® Immunoperoxidase Secondary Detection System** uses biotin-avidin-HRP complexed antibodies to detect rabbit or mouse IgG in the Primary Antibody. The primary antibody binds to specific antigens present in the specimen. Any excess antibody is removed by washing. Next, the Secondary Antibodies Reagent (conjugated to biotin) is added and will react with the primary antibody present on the tissue. Unbound antibody is removed by washing. The sample is then incubated with the Streptavidin-HRP solution, which binds to the biotin-labeled secondary antibody present on the tissue. Unbound enzyme is removed by washing. The chromogenic development reagent, 3,3' diaminobenzidine (DAB Substrate), is then added and reacts with the HRP attached to the HRP-streptavidin-biotin-antibody complex. The HRP activity on the chromogenic substrate results in the deposit of brown to black insoluble precipitate at those antigenic sites containing the specific epitopes recognized by the primary antibody. Tissues processed in a variety of fixatives including neutral buffered formalin, B-5, and Bouin's may be used.

Materials Provided

The following materials are included in the kit:

Cat. No. DAB150	Cat. No. DAB500	Description
20783 15 mL	20773 50 mL	Blocking Reagent: normal goat serum in phosphate buffered saline (PBS) containing carrier protein
20782 15 mL	20775 50 mL	***Secondary Antibodies: biotinylated goat anti-mouse IgG and/or goat anti-rabbit IgG (depending on kit) in PBS, containing carrier protein
20777 15 mL	20774 50 mL	Streptavidin HRP: Diluted in Tris Buffered Saline
71895 2 mL	71897 4 mL	DAB Chromogen A: 3,3' Diaminobenzidine diluted in TBS
71896 2 x 15 mL	71898 2 x 50 mL	DAB Chromogen B: hydrogen peroxide diluted in TBS, containing Tween® 20
20780 15 mL	20785 50 mL	Hematoxylin: Mayer's hematoxylin counter stain
20776 275 mL	20784 2 x 500 mL	20X Rinse Buffer: TBS

Materials Required But Not Provided

- Primary rabbit or mouse IgG antibodies (such as **MILLIPORE IHC Select® Prediluted Primary Antibodies**)
- Positive and negative tissue control slides
- Positively charged microscope slides
- Glass cover slips (24 x 40mm, No. 1)
- Distilled or deionized water
- Tween® 20
- Permanent mounting medium, Eukitt® American Histology or equivalent
- 3% laboratory grade hydrogen peroxide
- 10.0% neutral buffered formalin[15]
- Xylene, ACS grade
- Ethanol, absolute (100%)
- Latex, rubber or 100% nitrile gloves (xylene resistant)
- Timer
- Fume hood
- Light microscope equipped with Plan Achromat or equivalent lenses
- 60°C drying oven

Stability and Storage

When stored at 2° to 8°C, the **IHC Select® Immunoperoxidase Secondary Detection System** is stable up to the expiration date printed on the kit label. Do not freeze or expose to elevated temperatures. Discard any remaining reagents after the expiration date. Do not store kit components or perform staining in strong light, such as direct sunlight.

Precautions

- Pooling, substitution or alteration of any reagent may cause erroneous results.
- Do not mix reagents from different lots.
- Do not allow the slides to dry at any time during the staining procedure.
- Handle all specimens and materials coming in contact with them as potentially infectious materials. Disinfect with 0.05% sodium hypochlorite.
- Do not mouth pipette reagents. If ingested, seek professional assistance or contact a Poison Control Center immediately.
- Diaminobenzidine (DAB) may be carcinogenic. This solution may cause irritation upon skin contact. Avoid inhalation, ingestion, or skin contact and wear gloves when handling DAB. If skin contact occurs, flush affected area with water. Spills may be 'neutralized' with bleach[25,26]. Dispose of according to local regulations.
- Inhalation or ingestion of xylene or the highly allergenic fixative formaldehyde is harmful. These materials should be handled with gloves. Prepare in a fume hood. If skin or eye contact occurs, wash thoroughly with copious amounts of water.
- Wear disposable gloves when handling any human material and while performing the staining procedure.

Specimen Collection

Specimens should be from fresh autopsy or biopsy specimens, and fixed as soon as possible. Routinely fixed, paraffin-embedded specimens are recommended. It is recommended that proper in-house quality control and assay verification measures be taken to determine suitability of specimen types for immunohistochemical methodologies[5,16, 18].

Specimen Preparation

Specimens fixed in 10.0% neutral buffered formalin for 6-24 hours at room temperature (18º to 25ºC) are optimal[27]. Fixation should not exceed 24 hours. Fixatives other than neutral buffered formalin, such as B5 (Formal sublimate), zinc formalin or Bouin's may be used. It is recommended that the user verify optimal conditions[11,15,18]. Following are some factors to be considered when evaluating the suitability of the users' tissue processing procedures:

Mercuric-Chloride Fixation (B5 and Zenker's)

Mercuric chloride fixatives, such as B5 and Zenker's frequently include a neutral salt to maintain tonicity and may be mixed with other fixatives. Mercuric chloride fixatives are poor histological penetrators and are not well tolerated by tissue specimens. Consequently, small tissue blocks (less than 3 mm thick) and short fixation periods (2-4 hours) are recommended.

After fixation, the tissue block(s) should be rinsed well with water and placed in 70% ethanol for wet storage or until tissue processing and paraffin embedding can be completed. Prior to immunostaining, clear tissue sections of mercury deposits using a 0.5% iodine solution in 0.5% ethanol for 5 minutes, followed by 5% sodium thiosulfate solution for 5 minutes. Alternatively, the iodine may be combined with the xylene in the first deparaffinization step.

Note: *Exercise the necessary precautions when handling and disposing of reagents containing mercury compounds.*

Formaldehyde-Based Fixation (Neutral Buffered Formalin and Bouin's) Most formaldehyde-based fixatives contain 10% formalin, a neutral salt to maintain tonicity, and a buffering system to maintain pH. These fixatives are well tolerated by tissues, exhibit good histological penetration and are well suited for labeled streptavidin-biotin immunostains. Specimens should be fixed from 6-12 hours depending on tissue thickness.

Bouin's solution is an alternative formaldehyde-based fixative which contains picric acid and is suitable for use on all tissues except kidney. Excessively fixed tissues become brittle and adversely affect the appearance and quantity of lipids. After fixation, remove yellow color by treating with a saturated solution of lithium carbonate in 70% ethanol for 2 minutes. Follow fixation with dehydration, clearing, infiltration and embedding.

Osseous Tissues[16,17]

Osseous tissues should be decalcified in acid prior to tissue processing to facilitate tissue cutting and prevent damage to microtome blades.

Note: *Decalcification of tissues may impact staining intensity. Therefore, exercise caution when using this specimen type in immunohistochemical staining.*

Optimal Specimen Handling and Storage

Prior to formalin-fixation and paraffin embedding, specimens should be handled carefully and promptly to avoid compromising specimen integrity. Once the tissues are fixed and paraffin-embedded, they are stable for several years when stored in the dark at room temperature (18º to 25ºC).

Tissue Sectioning, Handling and Placement on Microscope Slides.

1. Section the tissues approximately four microns thick for optimum resolution with staining.
2. Place the sections into a container with deionized or distilled water preheated to 40º to 44ºC to relax the sections from compression due to sectioning. Do not add gelatin or polylysine, or any other agent to the water.
3. Place the sections on the positively charged microscope slides as flat and wrinkle free as possible to optimize stain contact with the tissues.
4. Place the tissue on the slide with the painted portion side up.

   Note: *After placing the tissue sections on the slides, store slides at 18 - 25°C in the dark up to one month. Prior to use, the tissue sections should be dried (with a conventional or microwave oven) onto the slides.*
5. Dry the tissue sections on the slides by heating in an oven at 60°C for a minimum of 60 minutes to ensure that any moisture trapped under the tissue section is completely eliminated by melting of the paraffin and evaporation of the water droplets. This eliminates the "water spotting" phenomenon when reading the slides.

6. Deparaffinize tissues by dipping into the specified containers containing approximately 200ml each of xylene. Use a separate container of xylene for each of the steps and washes.

    - xylene 4 x 5 minutes
    - 100% ethanol 2 x 1 minute
    - 70% ethanol/water 2 x 1 minute
    - 30% ethanol/water 2 x 1 minute
    - Distilled or Deionized water 2 x 1 minute

    Note: *Tissue sections should be used the same day they are deparaffinized.*

Tissue Pretreatment:

1. Use one of the recognized retrieval methods i.e., steam, microwave, pressure cooker or hotplate to recover tissue antigenicity[24].

    Note*: The method of Microwave retrieval is patented. It is up to the user to obtain the appropriate licenses.*

2. Blot dry the area around the tissue and then use a PAP Pen® to create a hydrophobic barrier to contain the Staining Reagents.

3. Draw 2 parallel lines with a PAP Pen®, across the narrow width of each slide one line on each side of the tissue.

4. Allow the PAP Pen® barrier to dry for at least 2 minutes at room temperature but ensure that the tissue remains hydrated.

    Note*: Leave space between the tissue and the PAP Pen® barrier as the PAP Pen® solution may adversely affect staining of the tissue, causing false negative results.*

5. Using a Superfrost Pen, mark on the top of each slide which antibody will be used to stain the slide.

6. Apply two or more drops of the 3% hydrogen peroxide to completely cover the specimen.

7. Incubate **10 minutes** in a hydrated incubation enclosure.

8. Follow Rinse Procedure as described in next section

    Note: *Do not allow tissues to dry out during the staining procedure.*

Preparation of Reagents

IHC Rinse Buffer

Total Volume 1X Rinse Buffer desired	Volume 20X Rinse Buffer	Volume Distilled Water	Tween® 20 (recommended)
50 mL	2.5 mL	47.5 mL	50 µL
100 mL	5.0 mL	95.0 mL	100 µL
150 mL	7.5 mL	142.5 mL	150 µL
200 mL	10.0 mL	190.0 mL	200 µL
250 mL	12.5 mL	237.5 mL	250 µL

1. Follow above chart to make up desired volume of 1X Rinse Buffer**.**
2. Using a sterile pipette, remove a desired volume of 20X Rinse Buffer and pipette it into a 250 ml rinse buffer bottle.
3. Add the desired volume of deionized or distilled water to make the final desired volume of 1X Rinse Buffer. If Tween® 20 is used (recommended but not required) add it at this time to a final concentration of 0.1%, and mix thoroughly. Use this 1X Rinse Buffer in the Staining Procedure.

Chromogen Reagent

1. Determine the volume of Chromogen Reagent required for the total number of slides according to the table below.
2. Prepare the Chromogen Reagent by adding appropriate volumes of DAB Chromogen A and DAB Chromogen B in a 1:25 ratio using a separate mixing bottle. Use table below as a guide.

   Note: *Use separate pipette tips for DAB Chromogen A and DAB Chromogen B reagents. Contamination will occur if separate pipette tips are not used*.

# of tests	1	5	10	15	20
Drops of A (µL of A)	1 (25µL)	5 (125µL)	10 (250µL)	15 (375µL)	20 (500 µL)
mL of B	0.625mLs	3.125mLs	6.250mLs	9.375mLs	12.5mLs

Drops of Chromogen A should be approximately 25µL measured with a micropipettor or using Samco #292 Transfer Pipets.

Rinse procedure

1. Pour off solution and gently tap the edge of each slide onto a paper towel to remove excess solution from each slide.
2. Holding slide horizontally, gently add Rinse Buffer to the slide dropwise to flood the tissue specimen.
3. Pour off the Rinse Buffer, and repeat steps 2 and 3 for a total of 5 times.
4. Apply 4 drops of Rinse Buffer to the tissue and incubate for a minimum of 2 minutes.
5. Pour off Rinse Buffer, and gently tap the edge of each slide remove excess solution from each slide.
6. Follow Steps 2 and 3 for a total of 5 times and remove excess Rinse Buffer.

Staining Procedure

Blocking Reagent

1. Apply two drops of the Blocking Reagent (blue colored reagent) to the specimen.

   Note: *Larger specimens may require more than two drops – cover specimen completely*

2. Incubate **5 minutes** in an enclosed container.
3. While holding the slide at a 45^{o} angle, gently rinse the specimen with 1X Rinse Buffer for a minimum of 15 seconds. Tap the end of the slide onto a paper towel to remove excess Rinse Buffer.

Primary Antibody OR negative control reagent

1. Apply two drops of the **Primary Antibody OR** a **negative control reagent** to the specimen.

   Note: *If not using* ***IHC Select™ Prediluted Primary Antibodies,*** *optimal antibody titer must be determined prior to running assay.*

2. Incubate **10 minutes** in an enclosed container.
3. Follow Rinse Procedure (outlined above)

Secondary Antibodies

1. Apply two drops of the Secondary Antibodies (yellow colored reagent) to the specimen.
2. Incubate **10 minutes** in an enclosed container.
3. Follow Rinse Procedure (outlined above)

Streptavidin HRP

1. Apply two drops of the Streptavidin HRP (pink colored reagent) to the specimen.
2. Incubate **10 minutes** in an enclosed container.
3. Follow Rinse Procedure (outlined above)

Chromogen Reagent

1. Apply sufficient volume of the freshly prepared Chromogen Reagent (light to dark brown colored reagent) to completely cover the specimen.
2. Incubate **10 minutes** in an enclosed container.
3. Follow Rinse Procedure (outlined above)

Hematoxylin Counter Stain

1. Apply two drops of the Hematoxylin Counter Stain solution (bluish red color) to the specimen.
2. Incubate **1 minute** in an enclosed container.
3. Follow Rinse Procedure (outlined above)
4. Place the slides directly into a container filled with deionized water. Hold here until the next step.

Mounting Media and Coverslipping

1. Remove slides from the water, and apply 3 drops of an aqueous-based mounting media to the tissue and apply a coverslip.

   Or

   Dehydrate through a graded series of alcohols, immerse in Xylenes, apply 3 drops Xylene based mounting media (e.g., Permount) and apply a coverslip.

Quality Control

Laboratory differences in tissue processing and technical procedures may significantly affect results, necessitating regular performance of in-house controls, in addition to the following procedures. Consult the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry and references 5, 18 and 19 for additional information. Refer to the package insert of each primary antibody used for details regarding sensitivity and immunoreactivity.

Positive Control Specimens: Controls should be fresh surgical, biopsy or autopsy specimens fixed, processed and embedded in the same manner as the patient sample(s). Positive tissue controls should include those weakly positive with the primary antibody in order to detect changes in reagent sensitivity. It is recommended that one positive tissue control be included for each set of test conditions in each staining run.

If the positive tissue controls fail to demonstrate the appropriate staining reaction, results with the test specimens should be considered invalid.

Negative Tissue Control: Use Negative Tissue Control, which has been fixed and processed identical to that of the patient sample(s) with each staining run. This tissue should show absence of specific staining, and provide an indication of non-specific background staining. It should also be used as an aid in the Interpretation of Results. The variety of different cell types present in most tissue sections offers internal negative control sites, but the user should verify this.

If specific staining occurs in the Negative Tissue Control, results with the patient specimens should be considered invalid.

Assay Verification: Each lot of primary antibody should be tested to verify antibody's specificity by testing on a series of known positive and negative tissues with known immunohistochemical characteristics. Refer to the quality control procedure outlined above and to the quality control guidelines of the College of American Pathologists (CAP) Certification Program for Immunohistochemistry. Also, references 5, 18, 19, and 20 may be reviewed for additional information.

The Purpose of the Daily Quality Control

Control Tissue:	**Specific 1° Antibody**	**Nonspecific 1° Antibody***
Positive Control: Tissues or cells containing target antigen to be detected (could be located in patient tissue). *The ideal control is weakly positive staining tissue to be most sensitive to antibody degradation.*	Controls all steps of the analysis. Validates reagent and procedures used for staining.	Detection of non-specific background staining.
Negative Control: Tissues or cells expected to be negative (could be located in patient tissue or positive control tissue).	Detection of unintended antibody cross-reactivity to cells / cellular components.	Detection of non-specific background staining.
Patient Tissue:	Detection of specific staining	Detection of non-specific background staining.

***** Same source and type as the specific antibody but not directed against the same target antigen, to detect non-specific antibody binding, e.g., binding of Fc portion of antibody by the tissue. Examples of expected staining can be seen in reference 12.

Interpretation of Results

The positive and negative tissue controls should be examined first to ascertain that all reagents are functioning properly. The presence of a brownish reaction product at the site of the target antigen is indicative of positive reactivity. Counterstain will be pale to dark blue coloration of the cell nuclei. If the positive or negative tissue controls fail to demonstrate appropriate staining, any results with the test specimens should be considered invalid.

Nonspecific staining, if present, will be of a diffuse appearance. Sporadic light staining of connective tissue may also be observed in sections from excessively formalin fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells often stain nonspecifically.

Test Tissue: Patient or test specimens stained with the primary antibody should be examined last. Positive staining intensity should be assessed within the context of any nonspecific background staining of the negative reagent control. The absence of a specific positive staining reaction can be interpreted as no antigen detected. If necessary, use a panel of antibodies to identify false-negative reactions.

Limitations

1. Results should be interpreted by qualified personnel under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
2. Tissue staining is dependent upon the proper handling and processing of tissues prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Shrinkage or distortions may occur in poorly fixed and embedded tissue specimens.
4. Excessive or incomplete counterstaining may compromise proper interpretation of results.
5. The **IHC Select® Immunoperoxidase Secondary Detection System** is intended for use with rabbit or mouse IgG primary antibody only.
6. If the **IHC Select® Prediluted Primary Antibodies** are not used with the detection kit, the end user must first determine the optimal titer.
7. The reagents supplied in this kit have been optimally diluted for use following the provided instructions for IHC on prepared tissue sections and should not be diluted.
8. Unexpected positive staining in tumors may be from expression of an antigen not usually expressed in morphologically similar normal cells, or from persistence or acquisition of an antigen in a neoplasm that develops morphologic and immunohistochemical features associated with another cell lineage (divergent differentiation).

9. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues[19].

10. Normal/nonimmune sera from the same animal source as the secondary antisera used in blocking steps may cause false-negative or false-positive results due to auto-antibodies or natural antibodies.

11. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products.

12. Non-specific reactions due to pseudoperoxidase activity (erythrocytes), and endogenous peroxidase activity (cytochrome C), or endogenous biotin (liver, breast, brain, kidney) depending on the type of immunostain used[13] may result in false-positive results.

13. Use of unbuffered fixatives, or exposure of tissues to excessive heat (greater than 60ºC) during processing may result in decreased staining intensity.

14. False negative results may be seen in improperly fixed tissues (e.g., overfixed).

15. False positive results may be seen due to non-specific binding to Fc receptors. In some cases the application of an alternative blocking reagent may be useful (see Quality Control, Negative Control Reagent section above). False positive results may also be seen in necrotic tissues.

16. The biotinylated secondary antibodies have some limited cross reactivities (the antibodies directed against mouse primary antibodies have limited reactivities to rat primary antibodies).

17. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues[19].

18. Endogenous avidin-binding activity (EABA) has been noted in frozen sections of liver (entire hepatic nodule) and kidney (tubular epithelium), as well as in frozen and formalin-fixed lymphoid tissues (paracortical histiocytes)[21,22]. When the potential presence of EABA is of concern to interpretation, it may be suppressed by sequential 20-minute incubations, first with 0.1% avidin and then with 0.01% biotin in 0.05M Tris-HCl buffer, pH 7.2-7.6 prior to initiating the staining run.

19. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase[23].

Troubleshooting

PROBLEM	PROBABLE CAUSE	SUGGESTED ACTION
Tissue sections wash off slides.	Additives in water bath during transfer of tissue sections to slides.	Remove all additives from water bath.
No staining of any slides	Reagents not used in proper order. DAB mixed incorrectly.	Refer to Staining Procedure proper use.
Controls acceptable but test specimens not stained.	Tissues not properly prepared.	Follow procedure for proper specimen preparation.
Weak staining of all slides.	DAB Solutions old or prepared incorrectly.	Prepare fresh solutions according to Staining Procedure.
Specific staining too dark.	DAB Solutions not properly made.	Prepare fresh solutions according to Staining Procedure.
Excessive background all slides.	Specimens not properly prepared, paraffin not adequately removed. Slides not properly rinsed.	Follow procedure for proper specimen preparation.
No background, or low background on control slides, but high background on test slides.	Specimens contain nonspecific background. Specimens not properly prepared.	Follow recommended specimen preparation procedure

Additional information on staining techniques and specimen preparation can also be found in the "Atlas of Immunohistology"[20] and "Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis."[7]

Contact MILLIPORE Technical Service for additional assistance at (800) 437-7500

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