HISTAR Detection System

BIO-RAD

Reagents

Bio-Rad HISTAR Detection System (Catalog #STAR3000A) provides the following pre-titrated, pre-diluted reagents:

Component	Quantity
1 vial Serum Block, 5% normal serum in 0.01M Phosphate Buffered Saline (PBS) containing 1% sodium azide (NaN_3)	5 ml
1 vial Reagent Boost	5 ml
1 vial HRP Polymer	5 ml
1 vial Peroxide Blocking Reagent, 3% hydrogen peroxide (H_20_2)	5 ml
1 vial DAB Chromogen, 2.5% 3,3' diaminobenzidine in stabilizing buffer	2 ml
4 vials DAB Substrate Buffer, 0.1% H_20_2 in citric acid buffer	6 ml

Background

HISTAR Detection System - for use on human tissues only

The Bio-Rad HISTAR Detection System provides linking and labeling reagents intended to be used with species specific primary antibodies for visualizing cellular antigens in tissue specimens. Visualization is accomplished using an ultra-sensitive indirect labeling method that utilizes novel polymer labeling technology. Secondary antibodies are polymerized directly with horseradish peroxidase (HRP) into compact polymers bearing a high ratio of enzyme to antibody. This biotin-free system offers enhanced sensitivity and minimal background staining.

All labeling and blocking reagents are pre-diluted for immediate use, chromogen is provided in concentrated format. These products are suitable for use with the wide range of Bio-Rad primary antibody reagents.

This product is designed for the staining of human tissue sections only using mouse and rabbit primary antibodies — it has not been tested for use in other species.

Principle

Bio-Rad HISTAR Detection System reagents are designed specifically to enhance immunohistochemical tissue staining with a simple kit. Unlike ABC reagents that are mixed just prior to use and stable for a very short time, the kit is supplied in a chemically stable and ready-to-use format. This eliminates the need for multiple vials and extra steps in the procedure, minimizing hands-on time while maximizing convenience.

The kit can be used to demonstrate the presence of a variety of antigens using a two-step indirect labeling method. Tissue sections are deparaffinized, rehydrated, and separately incubated with two blocking reagents (Peroxide Blocking Reagent and Serum Block) to minimize non-specific background staining. The tissue is then sequentially incubated with primary antibody followed by two reagent preparations before addition of chromogen to visualize staining.

1. Primary antibody (user supplied mouse or rabbit monoclonal, or rabbit polyclonal) specific for the antigen of interest is added to the tissue sample.
2. Reagent boost prepares the system for HRP addition.
3. HRP polymer linking reagent conjugates HRP to the primary antibody.
4. DAB Chromogen and DAB Substrate Buffer mix are added to visualize the antigen detected by the primary antibody.

Warnings

1. For research use only, not for use in diagnostic procedures.
2. Sodium azide (NaN_3) is used in the reagents as a preservative and may react with lead or copper in drains to form explosive metal azides. Upon disposal, flush with a large volume of water to prevent azide build-up in drains.
3. Antibody components of this kit are optimized for sensitivity and specificity, interchanging vials with other detection systems is not recommended.
4. DAB Chromogen.
   DAB (3,3’ diaminobenzidine) in stabilizing buffer.
   DAB has been classified as a carcinogen and possible mutagen. It can cause irritation upon skin exposure. Avoid contact with unprotected skin surfaces when diluting with substrate buffer. If contacted, flush with water. Solutions containing DAB should be disposed of according to local regulations.
5. Do not use system components beyond expiration date.
6. Avoid dilution of system components unless called for in the procedure.

Materials and Equipment Required, but not Provided

Reagents

Primary antibody and control

1. Bio-Rad primary antibody or other antibody raised against a specific cellular antigen, diluted as per preparation of reagents below.
2. Appropriate control (for instance normal serum or negative control antibody of the same isotype and at the same concentration or dilution as the primary antibody) diluted as per preparation of reagents below.

Wash buffer

Phosphate Buffered Saline (PBS), p H7.4 (±0.2), or PBS prepared according to the following formula:

8.0 g sodium chloride (NaCl)

1.15 g sodium phosphate dibasic anhydrous (Na_2HPO_4)

0.2 g potassium chloride (KCl)

0.2 g potassium phosphate, monobasic anhydrous (KH_2PO_4)

Distilled water to 1 liter

Counterstain

1. Mayer's hematoxylin
2. Ammonia water, prepare as follows:

Add 1.4 ml of concentrated ammonium hydroxide to 250 ml of distilled water.

Mounting Media

Permanent Aqueous Mounting Medium (Bio-Rad #BUF058A/B/C)

Supplies

1. Wash bottle
2. Absorbent wipes
3. Diamond pencil
4. 60 minute timer
5. Coverslips
6. Staining tray
7. Light microscope

Preparation of Reagents: Primary Antibody and Negative Control

Many Bio-Rad primary antibodies are provided concentrated or pre-diluted and ready-to-use. If another antibody source is required, the user must determine the optimal dilution (titer) of the primary antibody in conjunction with the Bio-Rad HISTAR Detection System. This is done by preparing serial dilutions of the primary antibody using PBS with 0.1% sodium azide as a preservative. Carrier protein such as bovine serum albumin (BSA, 1% (w/v)) may also be added as a stabilizer for long term storage in diluted form.

Using serial dilutions of the primary antibody, stain serial sections of known positive tissues according to the staining procedure below. The dilution providing optimal staining intensity in contrast to minimal background should be selected.

Tissue Controls

Positive control

A positive control slide should be run each time specimens are stained to confirm that the staining system is working properly. The positive control slide is a tissue known to contain the antigen under study.

When running positive control tissues, the laboratory should be aware that the amount of antigen will vary within the tissue and some variations in staining may occur from slide to slide.

Negative control

A negative control slide should be run with each specimen being stained to check for nonspecific staining. The negative control slide is a duplicate of the tissue specimen slide which is sectioned and prepared at the same time.

However, on the negative control slide, the primary antibody is omitted and PBS, negative isotype control, normal rabbit serum, or pre-immune immunoglobulin of the corresponding isotype should be used instead. No specific staining should occur on the negative control tissue. Nonspecific background staining on the negative control will help the user differentiate positive staining from nonspecific background on the specimen.

Tissue Staining Procedure

Preparing tissue slides

Step	Procedure	Notes/Precautions
Paraffin-embedded tissue	▪ Cut paraffin sections 3-5 µm thick ▪ Adhere paraffin sections to slides with tissue adhesive or use slides pretreated with poly-L-lysine or silane ▪ Air dry sections for 2-16 hr. Preparation of slides up to this point may be done well in advance of staining	Tissues which are not properly adhered to slides may be lost during staining. Antigens can be destroyed by exposure to temperatures over 60^0C
Frozen tissue	▪ Cut frozen sections 6-10 µm thick ▪ Allow sections to dry out on slides under desiccant for 4 hr at room temperature (RT) ▪ Immediately before staining, fix sections in cold acetone for 10 min ▪ Flood or immerse slides in PBS for 10 min	
Prepare controls	▪ **Positive control:** a section from a known positive tissue should be cut and prepared in the same manner as the specimens ▪ **Negative control:** one additional section from each specimen block should be cut and prepared in the same manner as the specimens	Read section on **Tissue controls**
Deparaffanize and/or rehydrate slides	▪ Circle tissue sections with diamond pencil (to form a well for reagents on slide)	After rehydration do not allow slides to dry out at any time during staining procedure. If staining is not carried out immediately, flood slides with PBS buffer
Paraffin sections	BATH — TIME Xylene — 2 changes, 5 min each 100% alcohol — 2 changes, 3 min each 95% alcohol — 2 changes, 3 min each H_20 — 5 min H_20 — 2 changes, 2 min each PBS buffer — 5 min	
Frozen sections	Flood or immerse slides in PBS for 10 min	Do not allow sections to dry out during procedure

Antigen Retrieval

Antigen retrieval should be carried out on paraffin-embedded sections following rehydration according to protocols specific for the primary antibody used.

Staining

Step	Procedure
Vial 4: peroxide blocking reagent **For paraffin-embedded tissues only**	1. Wipe slides carefully around tissue sections. 2. Add 1-3 drops from vial 4 to cover section. 3. Incubate in a humidity chamber for 15 min. 4. Rinse three times (3x) with PBS.
Vial 1: Serum Block	1. Tap off excess buffer. Wipe slides carefully around tissue sections. 2. Add 1-3 drops from vial 1 to cover each section. 3. Incubate in a humidity chamber for 15 min. 4. Tap off excess buffer. 5. Do not rinse.
Primary antibody, negative control (user supplied)	1. Tap off excess serum and wipe carefully around tissue sections. 2. To each specimen and positive control, add 1-3 drops of primary antibody (user supplied) to cover each section. To the negative control add 1-3 drops of negative control (user supplied). 3. Incubate in humidity chamber for 30 min.
Vial 2. Reagent Boost (N.B shake vial prior to use)	1. Rinse 3x with PBS for 3 min. 2. Add 1-3 drops from vial 2 to cover each section. 3. Incubate in humidity chamber for 20 min. 4. Rinse 3x with PBS for 3 min.
Vial 3. HRP Polymer (N.B shake vial prior to use)	1. Tap off excess buffer. Wipe slides carefully around specimens. 2. Add 1-3 drops from vial 3 to cover each section. 3. Incubate in a humidity chamber for 30 min. During incubation, prepare the substrate solution per the instructions for vials A and B. 4. Rinse 3x with PBS for 3 min.
Prepare substrate solution Vial A: DAB Chromogen Vial B: DAB Substrate Buffer **Note: DAB Substrate is stable for up to 6 hr**	DAB Substrate Buffer preparation. 50 test systems: Add 0.2 ml DAB Chromogen from vial A to 5 ml DAB Substrate Buffer from vial B. Mix gently.
Substrate solution	1. Tap off excess buffer. Wipe slides carefully around tissue sections. 2. Add 1-3 drops of substrate solution to cover each section. 3. Incubate in a humidty chamber for 3-5 min. 4. Rinse well with distilled H_20 then flood with distilled H_20 and incubate for 1 min. 5. Discard unused portions of substrate solution (see warnings).
Counterstain	1. Flood slides (or immerse) with Mayer's hematoxylin and incubate for 5 min. 2. Rinse slides with tap water (run gently to avoid washing off specimens). 3. Flood with ammonia water and incubate for 10 sec to develop counter-stain. 4. Rinse gently with running tap water.
Mounting	Add 1 drop of permanent Aqueous Mounting Medium (#BUF058A/B/C).

Performance Considerations

1. Peroxide blocking reagent, vial 4, can be used with HRP systems to reduce nonspecific staining which can occur due to endogenous peroxidase present in the specimen tissue sections reacting with the substrate. This blocking treatment should be used only on paraffin-embedded tissues. Hydrogen peroxide may remove frozen sections from the slide.
2. Serum Block, vial 1, can be used to reduce background staining which may result from nonspecific adsorption of antibodies to certain types of proteins usually associated with collagen. The incubation period for normal serum may be extended to reduce background staining.
3. The DAB Substrate solution must be prepared when indicated and used within 6 hr.

Troubleshooting

Problem: no staining or weak staining

Possible causes:

1. Improper fixation; antigens are sensitive to fixation. Timing is important for preservation of immunoreactivity. If tissues are over-fixed, the antigen binding sites can be masked or blocked. If the fixative is too harsh, the antigens may be destroyed and staining will not be possible. Tissue type and condition make a difference. Certain antigens are unstable and unless fresh tissue is used, little if any staining will be possible. Tissue should be fresh biopsy or surgical tissue, autopsy tissue may be unsatisfactory due to proteolysis. Correct preparation of tissue is critical for the preservation of immunoreactivity.
2. Results may differ in frozen and paraffin sections due, in part, to antigen solubility and fixation procedures.
3. No antigen in specimen. If the positive control used is a specimen known to possess the antigen, it should stain and confirm that the staining system is working. Be aware that variations in antigenicity can occur within the tissue block of the positive control.
4. DAB Substrate solution is improperly prepared, or it is used more than 6 hr after preparation.
5. Primary antibody not used at the proper dilution.
6. Primary antibody not added to specimens, or reagents used in incorrect order.
7. Tissues allowed to dry out on the slide. Once sections have been rehydrated, they must be kept moist.
8. Sodium azide in buffer baths. This inactivates peroxidase, preventing the stain from developing.
9. Tissue is incompletely deparaffanized.
10. Over-fixation of specimen resulting in masking/blocking of antigen sites inhibiting primary antibody binding. Use a Bio-Rad Antigen Retrieval Buffer (#HIS003B, #BUF025A/B/C, #BUF026A or #BUF027A) to pre-treat specimens after deparaffinization/rehydration and prior to step 1 of the staining procedure.

Problem: excessive background staining

Possible causes:

1. Large amounts of free antigen present in specimen due to autolysis or tissue necrosis.
2. Large amounts of free antigen normally found in certain tissues, such as hCG in placental tissue and in trophoblastic cells.
3. Failure to use Serum Block, vial 1, to block nonspecific adsorption of antibodies. If the reagent was used, the incubation period may be extended. See **performance considerations on page 7.**
4. Heavy counterstain interfering with interpretation.
5. Specimen drying out during staining procedure.
6. Wash steps between reagents not performed as vigorously as needed.

Problem: artifactual staining

Possible causes:

1. Poor specificity of the primary antibody or use of the primary antibody at incorrect dilution.
2. Non-uniform cutting and mounting of tissue resulting in reagent entrapment and artifactual staining.
3. Endogenous peroxidase staining in certain tissues. Interpret with caution using the negative control slide as a reference. Hydrogen peroxide should be used as a blocking agent with HRP systems for paraffin-embedded tissues. See **performance considerations on page 7**.
4. Loose tissue fragments entrapping staining reagents. Interpret staining only in the plane of the section.

Limitations

1. Quality of staining is dependent on the specificity and affinity of the user supplied primary antibody, and of immunoreactivity of the specimens. Immunoreactivity may be dependent upon fixation.
2. Due to inherent variations in specimens, inconsistent staining should not be considered unusual. It is important, therefore, to correlate staining results with other laboratory findings and controls. Evaluation and interpretation of staining achieved is solely the responsibility of the user.

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