Dako
LSAB®2 System-HRP
for use on Rat Specimens

Code K0609

Intended use

For research use only. Not for use in diagnostic procedures.

These instructions apply to the LSAB 2 System-HRP for use on **RAT SPECIMENS**, with primary antibodies from **Rabbit and Mouse** supplied by the user.

This kit, consisting of labelled streptavidin biotin (LSAB) reagents, is intended for the qualitative demonstration of antigens in paraffin-embedded tissues, cryostat tissues and cell preparations. Tissues processed in a variety of fixatives, including neutral buffered formalin, ethanol, B-5, and Bouin's, may be used.

Summary and explanation

The LSAB 2 System-HRP for use on Rat Specimens utilizes a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules.[1,2] Primary antibodies produced in either rabbit or mouse are labelled by the biotinylated link antibody provided in this kit, without cross-reactivity with the rat specimen. The color reaction is developed using a substrate-chromogen solution, resulting in a colored precipitate at the antigen site.

Principles of procedure

The technique used in this kit is based on the labelled streptavidin-biotin (LSAB) method. Endogenous peroxidase activity can be quenched by first incubating the specimen for 5 minutes in 3% hydrogen peroxide (user supplied). The specimen is then incubated with an appropriately characterized and diluted rabbit or mouse primary antibody, followed by sequential 10-minute incubations with biotinylated link antibody and peroxidase-labelled streptavidin. Staining is completed after incubation with substrate-chromogen solution (user supplied).

Reagents provided

The following materials, sufficient for at least 150 tissue sections based on 100 µL per section, are included in this kit:

Quantity	*Description*
1x15 mL	**LINK** **BIOTINYLATED LINK** Biotinylated anti-rabbit and anti-mouse immunoglobulins (absorbed to abolish crossreactivity with serum proteins of several species including rat, human and bovine) in PBS containing carrier protein and 0.015 mol/L sodium azide.
1x15 mL	**STREPTAVIDIN PEROXIDASE** **STREPTAVIDIN-HRP** Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and an antimicrobial agent.

Materials required, but not supplied

Primary antibody and negative control reagent
Control tissue, positive and negative
Xylene, toluene or xylene substitutes
Ethanol, absolute and 95%
Endogenous blocking reagent such as Peroxidase Block (code S2001), Dual Endogenous Enzyme Block (code S2003), or 3% hydrogen peroxide
Distilled or deionized water
Wash bottles
Wash solution **(not containing sodium azide)**
Timer
Absorbent wipes
Substrate-chromogen reagents, such as AEC Substrate System (code K3464) or Liquid DAB Chromogen (code K3466)
Staining jars
Counterstain, aqueous based, such as Mayer's Hematoxylin or Lillie's Modified Mayer's Hematoxylin (code S3301 for automated use; code S3302 for manual use)
0.037 mol/L ammonium hydroxide
Mounting medium, such as Faramount, Aqueous Mounting Medium, Ready-to-use (code S3025) or Glycergel ® Mounting Medium (code C0563)
Coverslips
Light microscope (20-800x)

Precautions

1. The device is not intended for clinical use including diagnosis, prognosis, and monitoring of a disease state, and it must not be used in conjunction with patient records or treatment.
2. For professional users.
3. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, NaN_3 may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
4. As with any product derived from biological sources, proper handling procedures should be used.[3]
5. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
6. Unused solution should be disposed of according to local, State and Federal regulations.

Storage

Store at 2–8°C. Do not freeze.

Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user.[4] There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the product is suspected, contact Dako Technical Support.

Reagent preparation

Wash Solution
0.05 mol/L Tris-HCl, pH 7.6, containing 0.015 mol/L NaCl and 0.05% Tween 20, without sodium azide (Automation Buffer, code S3006), PBS, 0.05 mol/L Tris-HCl buffer or Tris Buffered Saline (TBS, code S3001), pH 7.2-7.6, **not containing sodium azide** are suitable wash solutions. Distilled water may be used for rinsing the hydrogen peroxide, substrate-chromogen solution, and counterstain. Unused wash solution may be stored at 2–8°C. Discard if solution becomes cloudy.

Primary Antibody and Negative Control Reagent
This kit is designed for the use of mouse or rabbit primary antibodies on rat specimens. Information on cross-reactivity to rat proteins is available for many Dako concentrated antibodies, however, optimal dilutions must be determined experimentally by the user. Dilutions should be prepared in an appropriate buffer containing a carrier protein such as 1% bovine serum albumin (or use Antibody Diluent, code S0809). For most antibodies an incubation time of 10 minutes is recommended.

Substrate-Chromogen Solution
The AEC Substrate System (code K3464) can be used with the LSAB2 System-HRP for use on Rat Specimens. Alternatively, the Liquid DAB Chromogen (code K3466) can be used. Please follow the instructions provided with each substrate system for substrate-chromogen preparation.

Counterstain
The colored end-product of the AEC substrate-chromogen reaction is alcohol soluble and should only be used with aqueous-based counterstains such as Mayer's hematoxylin. DAB chromogen yields an alcohol insoluble end-product and can be used with alcohol-based hematoxylin. When using hematoxylin, counterstaining is completed by immersing tissue sections into 0.037 mol/L ammonia water. Ammonia water is prepared by mixing 2.5 ml of 15 mol/L (concentrated) ammonium hydroxide with 1 liter water. Unused ammonia water may be stored at room temperature in a tightly capped bottle.

Mounting Media
Faramount, Aqueous Mounting Medium, RTU (code S3025) or Glycergel Mounting Medium (code C0563) are recommended for aqueous mounting. Liquefy Glycergel by warming to approximately 40°C immediately before use. A non-aqueous mounting medium can be used with DAB chromogen.

Specimen preparation

Prior to IHC staining, tissues must be fixed and processed. Fixation prevents autolysis and putrefaction of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing includes dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues. Optimal procedures must be determined and verified by the user.

Specimens processed in a variety of fixatives may be used. The choice of fixative and method is best made by the user within the context of their own laboratory and institutional constraints. For recommended methods and techniques of specimen fixation, please refer to *Histological and Histochemical Methods: Theory and Practice*.[5]

Paraffin-Embedded Tissue
Fixation and Processing
Survival of tissue antigens for immunological staining may depend on the type and concentration of fixative, on fixation time, and on the size of the tissue specimen to be fixed.[6] It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Where possible, use of thinner specimens coupled with shorter fixation times is recommended. Prolonged exposure to fixatives may result in the masking of antigens and contribute to reduced staining. Zenker's fluid, B-5, and Bouin's have often been recommended as milder fixatives for paraffin-embedded specimens.[7,8]

After fixation, tissues are dehydrated using graded alcohols, cleared with xylene or xylene substitute, and infiltrated with paraffin wax. The tissue is subsequently embedded with paraffin wax in molds or cassettes

which facilitate tissue sectioning. To minimize denaturing of antigens, do not expose tissues to temperatures in excess of 60°C during processing.

Tissue blocks may be stored or sectioned on completion of embedding. Properly fixed and paraffin-embedded tissues will keep indefinitely if stored in a cool place.

Mounting and Deparaffinization of Tissue Sections
Collect sectioned (4 µm) tissues cut from paraffin-embedded blocks on clean glass slides. Dehydrate in an oven for one to two hours at 60°C or less or allow to air dry for 15 to 24 hours. For increased adhesion of tissue sections during immunostaining procedures, use of poly-L-lysine coated slides, charged slides or Silanized Slides (code S3003) is suggested. Coated slides are strongly recommended for staining procedures requiring proteolytic digestion or heat-induced target retrieval. Slides with paraffin-embedded tissue sections can be kept indefinitely if stored in a cool place.

Prior to staining, tissue sections must be deparaffinized to remove embedding media, rehydrated, and placed in bath of wash buffer. Avoid incomplete removal of paraffin. Residual embedding media will result in increased nonspecific staining.

Proteolytic Digestion and Target Retrieval (Heat-Induced Epitope Retrieval)
Formaldehyde is known to induce conformational changes in antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. However, these sites may be revealed with proteolytic digestion or target retrieval of tissue sections prior to immunostaining. Proteinase K (codes S3004 or S3020), Proteolytic Enzyme, RTU (code S3007), Target Retrieval Solution (codes S1699, S1700), or Target Retrieval Solution pH 9.0 (codes S2367, S2368) are recommended.

Cryostat Tissue
Frozen sections should be cut from snap-frozen tissue blocks and air-dried for 2–24 hours. Dried sections can be fixed in room temperature acetone for 10 minutes or in buffered formylacetone for 30 seconds. Allow sections to air-dry until completely dehydrated. Proceed with immunostaining or wrap slides in aluminum foil and store at -20°C or lower for up to three to six months. Equilibrate wrapped, frozen sections to room temperature prior to use. Prior to staining place slides in bath of wash buffer.

Cell Smears
Smears may be air-dried for 2–24 hours and processed for immediate staining or wrapped in aluminum foil and stored at -20°C or lower for up to three to six months. Air-dried or thawed smears may be fixed for 90 seconds in acetone-methanol (1:1). Fixation in acetone-methanol-formalin (10:10:1) is also acceptable. Prior to staining place slides in bath of wash buffer.

Staining procedure

Procedural Notes
The user should read these instructions carefully and become familiar with the product content prior to use.

The reagents and instructions supplied have been designed for optimal performance. Further dilution of the product reagents or alteration of incubation times or temperatures may give erroneous results.

All reagents should be equilibrated to room temperature prior to immunostaining. Likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Dried tissue sections may display increased nonspecific staining. Cover slides exposed to drafts. If prolonged incubations are used, place tissues in a humid environment.

The sensitivity of the LSAB 2 System-HRP for use on Rat Specimens can be further increased by lengthening the incubation times of Steps 2, 3 and 4 to 30 minutes each.

Staining Protocol

STEP 1: HYDROGEN PEROXIDE
Tap off excess water and carefully wipe around specimen.
Apply enough endogenous enzyme blocking reagent to cover specimen.
Incubate 5–10 minutes, depending on concentration of endogenous enzyme in the tissue tested.
Rinse gently with distilled water or wash solution from a wash bottle and place in fresh buffer bath.

STEP 2: PRIMARY ANTIBODY AND NEGATIVE CONTROL REAGENT
Tap off excess buffer and wipe slide as before.
Apply enough primary antibody or negative control reagent to cover specimen.
Incubate 10 (±1) minutes unless otherwise specified.
Rinse gently with wash solution from a wash bottle and place in buffer bath.

STEP 3: LINK
Immediately tap off excess buffer and wipe slide as before.
Apply enough link to cover specimen.

Incubate 10 (±1) minutes.
Rinse slide as in Step 2.

STEP 4: STREPTAVIDIN PEROXIDASE
Wipe slide as before.
Apply enough Streptavidin to cover specimen.
Incubate 10 (±1) minutes.
Rinse slide as before.

STEP 5: SUBSTRATE-CHROMOGEN SOLUTION
Wipe slide as before.
Apply enough of the prepared substrate-chromogen solution to cover specimen.
Refer to instructions provided with substrate-chromogen for recommended incubation times.
Rinse gently with distilled water from wash bottle.

STEP 6: COUNTERSTAIN (optional)
Cover specimens with hematoxylin or place slides in a bath of hematoxylin. Incubate for two to five minutes, depending on the strength of the hematoxylin used.
Rinse gently with distilled water from a wash bottle.
Dip 10 times into a wash bath filled with 0.037 mol/L ammonia (optional)
Place in distilled or deionized water for 2 minutes.

STEP 7: MOUNTING
Specimens may be mounted and coverslipped with an aqueous-based mounting medium such as Dako Faramount, Aqueous Mounting Medium, RTU (code S3025) or Glycergel® Mounting Medium (code C0563). A non-aqueous, permanent mounting medium can be used with DAB chromogen.

NOTE: The AEC reaction product is soluble in organic solvents and therefore not compatible with toluene- or xylene-based, permanent mounting media.

Quality control

Positive Control Specimen
In order to ascertain that all kit reagents are functioning properly, a positive control specimen should accompany each staining run. It should be noted that the known positive control specimen should *only* be utilized for monitoring the accurate performance of the kit reagents. If positive control specimens fail to demonstrate positive staining, labelling of test specimens should be considered invalid.

Negative Control Specimen
A negative control specimen stained with primary antibody should be used with each staining run to verify the specificity of the primary antibody. If staining occurs in the negative control specimen, results with the test specimen should be considered invalid.

Negative Control Reagent
A negative control reagent, allowing the recognition of nonspecific staining should be used with each specimen. This will improve interpretation of specific staining at the antigen site. Alternatively, an irrelevant antibody not specific for any specimen antigen, diluted in Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin (or use Antibody Diluent, code S0809).

For other suggested procedures to be used in quality control assessment, please consult references 9 and 10.

Staining interpretation

Examine the positive control specimen for the presence of a colored end-product at the site of the target antigen. AEC chromogen yields a characteristic red end-product whereas DAB chromogen yields a brown end-product. The presence of these colors can be interpreted as a positive staining result, indicating proper performance of kit reagents. The absence of specific staining in the negative control specimen confirms the specificity of the primary antibody.

Examination for any nonspecific staining present on the negative control reagent slide is recommended next. Nonspecific staining, if present, is of rather diffuse appearance and is frequently observed in connective tissue.

Test specimens stained with primary antibody should then be examined. Positive staining intensity should be assessed within the context of any background staining of the negative control reagent. The presence of a colored end-product can be interpreted as a positive staining result. The absence of a staining reaction can be interpreted as a negative staining result.

Use only intact cells for interpretation since necrotic or degenerated cells often stain nonspecifically. Precipitates may form if, for example, specimens are allowed to dry during the staining procedure. This may be apparent at the edge of the specimen. Use of 40x magnification for scanning will minimize this potential misinterpretation.

Depending on the length of the incubation time in hematoxylin, counterstaining will result in pale to dark blue coloration of cell nuclei.

Product specific limitations

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, or sectioning may produce artifacts or false- negative results.

Results will not be optimal if old or unbuffered fixatives are used, or if excessive heat is used during embedding or during attachment of sections to slides.

False-positive results may be seen due to nonspecific binding of proteins. Although Dako LSAB2 Kits do not require the use of a separate blocking reagent, in some cases the application of a blocking reagent containing an irrelevant protein, prior to incubation with the primary antibody may be useful for reducing background. A recommended blocking reagent is Protein Block Serum-Free (code X0909), or a blocking solution can be made from normal swine serum such as Serum Normal, Swine (code X0901) diluted to 20% in 0.05 mol/L Tris-HCl buffer, pH 7.2–7.6.

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 tissue (paracortical histiocytes).[11,12] EABA can be suppressed by sequential 20-minute incubations, first with 0.1% avidin and then with 0.01% biotin in 0.05 mol/L Tris-HCl buffer, pH 7.2–7.6, prior to peroxidase quenching or use Biotin Blocking System (code X0590), prior to Staining Protocol, Step 1 in Staining Procedure.

Endogenous peroxidase or pseudoperoxidase activity can be found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase as well as in eosinophils.[13,14] In formalin-fixed tissue this activity can be inhibited by incubating the tissue in 3% hydrogen peroxide for 5 minutes prior to the application of primary antibody. Blood and bone marrow smears can be treated with Peroxidase Blocking Reagent (code S2001), however, this procedure does not abolish the reddish-brown pigment of hemoproteins. A solution of methanol-hydrogen peroxide can also be used, however, some antigens may become denatured with this procedure.

Excessive or incomplete counterstaining may compromise proper interpretation of results.

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Aldehyde-containing fixatives, such as formalin and glutaraldehyde frequently result in increased background staining. Improper fixation, freezing, thawing, washing, drying, heating or sectioning may produce artifacts, antibody trapping or false negative results. False positive staining may also be caused by cross-reactivity of other IHC staining reagents to e.g. endogenous alkaline phosphatase, endogenous peroxidase, pseudoperoxidase, endogenous avidin-binding activity or by nonspecific reaction with necrotic or degenerated cells.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[15]

Troubleshooting

Problem	*Probable Cause*	*Suggested Action*
1. No staining of any slides.	1a. Reagents not used in proper order.	1a. Review application of reagents.
	1b. Sodium azide in buffer bath.	1b. Use fresh, azide-free buffer.
2. Weak staining of all slides.	2a. Sections retain too much solution after wash bath.	2a. Gently tap off excess solution before wiping around section.
	2b. Slides not incubated long enough with reagent(s).	2b. Review recommended incubation times.
	2c. Primary antibody too dilute.	2c. Use higher concentration of the primary antibody.
3. Excessive background staining in all slides.	3a. Specimens contain high endogenous peroxidase activity.	3a. Use longer incubation time of Peroxidase Block.
	3b. Paraffin incompletely removed.	3b. Use fresh xylene or toluene baths. If several slides are deparaffinized simultaneously, use a second xylene bath.
	3c. Slides not properly rinsed.	3c. Use fresh solutions in buffer baths and wash bottles.
	3d. Faster than normal substrate-chromogen reaction due to excessive room temperature.	3d. Use shorter incubation time with substrate-chromogen solution.
	3e. Sections dried during staining procedure.	3e. Use humid chamber. Wipe only three to four slides at a time before applying reagent.
	3f. Nonspecific binding of reagents to tissue section.	3f. Apply a blocking solution containing an irrelevant protein (see Staining Interpretation section).

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako Technical Support for further assistance.

Additional information on staining techniques and specimen preparation can be found in the *Handbook Immunochemical Staining Methods*[16] (available from Dako), *Atlas of Immunohistology*[17] and *Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis.*[18]

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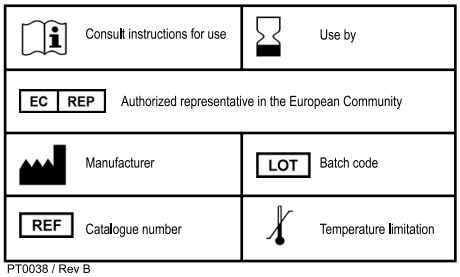

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