DAKO Working Procedure

DAKO LSAB®+ Kit, Peroxidase Universal
Code No.: K 0690
Lot No.: ____

Applications

Instructions

These instructions apply to the **Universal Large Volume DAKO LSAB®+ Kit, Peroxidase** (L.V. DAKO LSAB®+ Kit, HRP) for use with primary antibodies from **RABBIT, MOUSE and GOAT** supplied by the user.

Intended Use

FOR LABORATORY USE.

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 ethanol, B-5, Bouins, and neutral buffered formalin may be used.

Summary and Explanation

The purpose of immunohistochemical (IHC) staining techniques is to allow for thevisualization of tissue (cell) antigens. Originally this was accomplished by the direct technique using enzymes conjugated directly to an antibody with known antigenic specificity. Although this technique lacked the sensitivity of later methods, it allowed the direct visualization of tissue antigens using a standard light microscope.1

The sensitivity of IHC techniques was significantly improved with the development of an indirect method. In this two-step method several enzyme-labelled secondary antibodies reacted with the antigen-bound primary antibody. Subsequently the peroxidase-antiperoxidase (PAP) methodology was introduced which used a three-step method consisting of the sequential application of primary antibody, link antibody and a PAP complex. This method rendered greater sensitivity than traditional two-step indirect techniques.2 Finally, the strong affinity of avidin for biotin was exploited in the three-step avidin-biotin complex (ABC) method developed by Hsu et al.3 This method provided a further increase in sensitivity over other existing methods.

The L.V. DAKO LSAB®+ Kit, HRP utilizes a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules.4

The L.V. DAKO LSAB®+ Kit, HRP is a sensitive and versatile IHC staining procedure which permits the simultaneous processing of numerous specimens in just over one hour. When compared to the ABC method, labelled avidin-biotin methods have been shown to provide an increase in sensitivity of up to eight times.5-8 The L.V. DAKO LSAB®+ Kit, HRP is an extremely sensitive LSAB method, and, as a result, optimal dilutions of primary antibody are up to 20 times higher than those used for the more traditional PAP technique, and several fold greater than those used in traditional ABC or LSAB methods. This kit offers an enhanced signal generating system for the detection of antigens present in low concentrations, or for increased staining intensity in compensation for low titer primary antibodies. Primary antibodies produced in rabbit, mouse or goat are labelled by the biotinylated link antibody provided in this kit. 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 LSAB method. Endogenous peroxidase activitycan be quenched by first incubating the specimens for five minutes in 3% hydrogen peroxide (supplied by user). The specimens are then incubated with an appropriately

characterized and diluted rabbit, mouse or goat primary antibody, followed by sequential incubations with biotinylated link antibody and peroxidase-labelled streptavidin. Staining is completed after incubation with substrate-chromogen solution (supplied by user). For optimal sensitivity DAKO® Large Volume DAB+ (Code No. K3467) is recommended for substrate-chromogen.

Reagents

The following materials are included in this kit:

Manual Staining Reagents

Quantity	Description
1x110 mL	Link : Biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate buffered saline (PBS), containing carrier protein and 15mM sodium azide.
1x110 mL	Streptavidin Peroxidase: Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and anti-microbial agents.

DAKO Autostainer Reagents*

10x11 mL	Link : Biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins in phosphate buffered saline (PBS), containing carrier protein and 15mM sodium azide.
10x11 mL	Streptavidin Peroxidase: Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and anti-microbial agents.

*DAKO Autostainer is available only in North America, South America, Australia and New Zealand.

MATERIALS REQUIRED BUT NOT SUPPLIED

Primary antibody and negative control reagent

Positive and negative control specimens

Xylene, toluene or xylene substitutes

Absolute ethanol

95% ethanol

3% hydrogen peroxide

Distilled water

Wash bottles

Wash solution, not containing sodium azide

Timer (2-30 minutes)

Absorbent wipes

Substrate-chromogen reagents, for optimal sensitivity DAKO Large Volume DAB+ (Code No. K3468) is recommended due to its increased sensitivity;

alternatively, DAKO¨ DAB Chromogen tablets (Code No. S3000), Large Volume DAKO¨ AEC Substrate System, Ready-to-use (Code No. K3464) or Large Volume DAKO¨ AEC Substrate System (Code No. K0696) can be used.

Staining jars

Counterstain

37mM ammonium hydroxide

Mounting media, such as DAKO¨ Faramount (Code No. S3025) or DAKO Glycergel¨ (Code No. C0563)

Coverslips

Standard light microscope (20-800x)

Precautions

A. Product Specific

1. FOR LABORATORY USE.
2. Sodium azide which is used as a preservative is toxic if ingested. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.9,10
3. Do not use reagents beyond the expiration date.
4. Do not substitute reagents from other lot numbers or from kits of other manufacturers.
5. Do not store kit components or perform staining in strong light, such as direct sunlight.

B. General

1. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions.11
2. Inhalation or ingestion of the highly allergenic fixative formaldehyde is harmful. Prepare in hood. If swallowed, induce vomiting. If skin or eye contact occurs, wash thoroughly with water.
3. Organic reagents are flammable. Do not use near open flame.
4. Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.
5. Avoid microbial contamination of reagents as incorrect results may occur.
6. Avoid splashing of reagents or generation of aerosols.

Reagent Preparation

A. Wash Solution

PBS, 0.05M Tris-HCl buffer or Tris Buffered Saline (DAKO¨ PBS, Code No. S3024, DAKO¨ TBS, Code No. S3001), pH 7.2-7.6, *not containing sodium azide* are suitable wash solutions. Distilled water may be used for rinsing off the hydrogen peroxide (Section 9.B. Step 1), substrate-chromogen solution, and counterstain. Unused wash solution may be stored at 2-8¡C. Discard if solution becomes cloudy.

B. Primary Antibody and Negative Control Reagent

DAKO LSAB¨ Ready-to-use Primary Antibodies and Negative Controls (N-series) are *not* recommended for use with this kit. However, DAKO offers concentrated monoclonal and polyclonal antibodies suitable for use in IHC. Due to the high sensitivity of the L.V. DAKO LSAB®+ Kit, HRP, primary

antibody dilutions may range from five- to twenty-fold greater than those used in traditional IHC methods. Optimal dilutions must be determined experimentally by the user. Dilutions should be prepared using 0.05M Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin or DAKO¨ Antibody Diluent (Code No. S0809). For primary antibodies an incubation time of 30 minutes is recommended.

C. Substrate-Chromogen Solution

DAKO¨ Large Volume DAB+ (Code No. K3468) is recommended for use with the L.V. DAKO LSAB¨+ Kit, HRP due to its increased sensitivity. Alternatively, the DAKO¨ DAB Chromogen tablets (Code No. S3000), the Large Volume DAKO¨ AEC Substrate System, Ready-to-use (Code No. K3464) or the Large Volume DAKO¨ AEC Substrate System (Code No. K0696) can be used. Please follow the instructions provided with each substrate system for substrate-chromogen preparation.

D. Counterstain

DAB chromogen yields an alcohol insoluble end-product and can be used with alcohol-based hematoxylin. The colored end-product of the AEC substrate-chromogen reaction is alcohol soluble and should only be used with aqueous-based counterstains such as

Mayers hematoxylin. When using hematoxylin, counterstaining is completed by immersing tissue sections into 37mM ammonia water. Ammonia water is prepared by mixing 2.5 mL 15M (concentrated) ammonium hydroxide with 1 liter water. Unused ammonia water may be stored at room temperature in a tightly capped bottle.

E. Mounting Media

DAKO¨ Faramount, Aqueous Mounting Medium, Ready-to-use (Code No. S3025) or DAKO Glycergel¨ Mounting Medium (Code No. C0563) is recommended for aqueous mounting. Liquify DAKO Glycergel¨ by warming to approximately 40 ± 5¡C prior to use. A nonaqueous mounting medium can be used with DAB chromogen.

Storage and Handling

Reagents of the L.V. DAKO LSAB®+ Kit, HRP are to be stored at 2-8¡C. Do not freeze.

Specimen Collection and Preparation

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.*12

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.13 It is thus important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Where possible, the use of thinner specimens coupled with shorter fixation times is recommended. Prolonged exposure to fixatives may result in the

masking of antigens and hence contribute to reduced staining. Zenkers fluid, B-5, and Bouins have often been recommended as milder fixatives for paraffin-embedded specimens.14,15

A. Paraffin-Embedded Tissue

Tissue Fixation in Formaldehyde-Based Solutions

For optimal fixation in neutral buffered formalin, tissue should be cut in blocks of approximately 1.0ÊxÊ1.0ÊxÊ0.5 cm, and immediately placed in 5-10 mL formalin per block. The tissue should not remain in formalin for more than 24 hours or more than three hours in B-5.

Please refer to the manufacturers fixation protocol supplied with the fixing reagents for proper procedures. Processing may be completed using an automatic tissue processor, graded alcohols, xylene or xylene substitute, and paraffin wax. Temperatures greater than 60¡C MUST be avoided. Rapid high temperature processing destroys antigenicity.

Tissues fixed in mercuric chloride-containing fixatives (e.g. Zenkers, B-5, etc.) should be de-Zenkerized prior to application of the primary antibody.

Formaldehyde is known to induce steric changes in the antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. Enzymatic digestion of tissue sections prior to incubation with primary antibody may recover some of these sites. Deparaffinized and rehydrated tissue sections can be digested for six minutes at room temperature in a solution containing 0.025% protease Type XXIV and 0.025% CaCl2, in Tris-HCl buffer, pH 7.2-7.6. The use of coated slides is recommended for greater adherence of tissue sections to the glass slides. Rinse thoroughly with distilled water and continue with the staining procedure as outlined in Section 9.B. Step 1. Other proteolytic enzymes, such as proteinase K (DAKO¨ Proteinase K, Code No. S3004 or DAKO¨ Proteinase K, Ready-to-use, Code No. S3020), pepsin (DAKO¨ Pepsin, Code No. S3002), or trypsin can also be used.

Tissue Fixation in Ethanol

The following procedure for fixation in ethanol is recommended: immerse the tissue blocks in absolute ethanol for 48 hours at room temperature, followed by two 1-hour baths in fresh xylene and two 1-hour baths in liquid paraffin. The tissue may then be embedded.

Adherence of Tissue Sections to Slides

Poly-L-lysine coated or DAKO¨ Silanized Slides (Code No. S3003) are recommended for proper adherence of tissue sections during staining procedures. The use of coated slides is especially recommended when enzymatic predigestion is performed. The tissue sections should be dried onto the slides at temperatures not to exceed 37¡C.

Deparaffinization and Rehydration

The embedding medium must be completely removed from the specimen. Any residual medium can cause an increase in background and obscure specific staining. Xylene and alcohol solutions should be changed after every 40 slides. Toluene or xylene substitute such as Histoclear may be used in place of xylene.

1. Place slides in xylene bath and incubate for five minutes. Repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for three minutes. Repeat once.

3. Tap off excess liquid and place slides in 95% ethanol for three minutes. Repeat once.

4. Tap off excess liquid and place slides in distilled or deionized water for 30 seconds. Unless proteolytic predigestion is required, commence staining procedure as outlined in Section 9.B. Step 1.

B. Frozen Tissue

Cryostat sections (five to eight microns) should be cut from snap-frozen tissue blocks (approximately 1.0 x 1.0 x 0.5 cm) and air-dried for 2-24 hours. Dried sections are ready for immediate processing or may be wrapped air-tight and stored frozen at -20¡C or lower.

If stored frozen, sections should be brought to room temperature before unwrapping. Tissue sections may be fixed in acetone for 10 minutes. Alternatively, fixation may be performed after sectioning and air-drying, prior to frozen storage. Allow sections to air-dry after fixation. Submerge slides in buffer bath for five minutes, then commence staining procedure as outlined in Section 9.B., Step 2.

Staining Procedure

A. Procedural Notes

Before using this kit for the first time the user should read the instructions carefully and become familiar with the contents. the colors of solutions are keyed to the condensed instructions on the inside cover of the kit box. see Section 5 for precaution information.

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

All kit reagents should be equilibrated to room tempera-ture prior to performing the procedure; likewise, all incubations should be performed at room temperature.

Do not allow tissue sections to dry during the staining procedure. Provided that the workplace is not exposed to drafts, the slides need not be covered during the recommended incubation times. However, if incubations are prolonged, cover or place slides in a humid environment.

If the staining protocol must be interrupted, slides may be kept in the buffer bath which follows the link incubation (Step 3) for up to one hour without affecting staining performance.

B. Staining Protocol

STEP 1 HYDROGEN PEROXIDE

Tap off excess water and carefully wipe around specimen.

Apply enough user supplied 3% hydrogen peroxide to cover specimen.

Incubate five minutes.

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 user prepared primary antibody or negative control reagent to

cover specimen.

Incubate 30 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 YELLOW drops of Link to cover specimen.

Incubate 15 minutes. For further enhancement of sensitivity incubate for 30 minutes.

Rinse slide as in Step 2.

STEP 4 STREPTAVIDIN PEROXIDASE

Wipe slide as before.

Apply enough RED drops of Streptavidin to cover specimen.

Incubate 15 minutes. For further enhancement of sensitivity incubate for 30 minutes.

Rinse slide as before.

STEP 5 SUBSTRATE-CHROMOGEN SOLUTION

Wipe slide as before.

Apply enough of the user prepared substrate-chromogen solution to cover specimen.

Incubate as recommended in instructions provided with substrate-chromogen.

Rinse *gently* with distilled water from wash bottle.

STEP 6 COUNTERSTAIN (optional)

Cover specimens with hematoxylin or place slides in a bath of hematoxylin (see SectionÊ6.D.). 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 ammonia water (see Section 6.D.).

Place in distilled or deionized water for two minutes.

STEP 7 MOUNTING

Specimen may be mounted and coverslipped with an aqueous-based mounting medium such as DAKO" Faramount (Code No. S3025) or DAKO Glycergel" (Code No. C0563). A nonaqueous mounting medium may 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

A. 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.

B. 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.

C. Negative Control Reagent

A negative control reagent, allowing the recognition of nonspecific staining should be used with each specimen. This will allow improved interpretation of specific staining at the antigen site. Alternatively, an antibody not specific for any specimen antigen, diluted in Tris-HCl buffer, pH 7.2-7.6, containing 1% bovine serum albumin or DAKO® Antibody Diluent (Code No. S0809) may be used.

For other suggested procedures to be used in quality control assessment, please see references 16 and 17.

Interpretation of Staining

Examine the positive control specimen for the presence of a colored end-product at the site of the target antigen. DAB chromogen yields a characteristic brown end-product whereas AEC chromogen yields a red 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 low 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.

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 LSAB®+ 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 may be DAKO

Protein Block Serum-Free (Code No. X0909), or a blocking solution

can be made from normal swine serum such as DAKO" Serum Normal, Swine (Code No. X0901) diluted to 20% in 0.05M 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).18,19 EABA can be suppressed by sequential 10-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 Section 9.B. StepÊ1.

Endogenous peroxidase or pseudoperoxidase activity can be found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase as well as in eosinophils.20,21 In formalin-fixed tissue this activity can be inhibited by incubating the tissue in 3% hydrogen peroxide for five minutes prior to the application of primary antibody. Blood and bone marrow smears can be treated with DAKO" Peroxidase Blocking Reagent (Code No. 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.

Necrotic or degenerated cells will offer stain nonspecifically, therefore examine only intact cells for staining interpretations.13

Excessive or incomplete counterstaining may compromise proper interpretation of results.

Troubleshooting

PROBLEM	PROBABLE CAUSE	SUGGESTED ACTION
1. No staining of any slides	1. a. Reagents not used in proper order	1. a. Review application of reagents
	b. Sodium azide in buffer bath	b. Use fresh azide-free buffer
	c. Substrate-chromogen reagent mixed incorrectly	c. Make a fresh substrate-chromogen using the instructions included with the product
2. Weak staining of all slides	2. a. Sections retain too much solution after wash bath	2. a. Gently tap off excess solution before wiping around section
	b. Substrate-chromogen mixture too old	b. Prepare fresh substrate-chromogen solution
	c. Slides not incubated long enough with antibodies or substrate mixture	c. Review recommended incubation times
3. Excessive background staining in all slides	3. a. Specimens contain high endogenous peroxidase activity	3. a. Incubate slides with fresh hydrogen peroxide (see Section 12)
	b. Paraffin incompletely removed	b. Use fresh xylene or toluene baths. If several slides are stained simultaneously, the second xylene bath should contain fresh xylene
	c. Slides not properly rinsed	c. Use fresh solutions in buffer baths and wash bottles
	d. Faster than normal substrate reaction due to e.g. excessive room temperature	d. Use shorter incubation time with substrate-chromogen solution
	e. Sections dried during staining procedure	e. Use humidity chamber. Wipe only three to four slides at a time before applying reagent
	f. Nonspecific binding of reagents to tissue section	f. Apply a blocking solution containing an irrelevant protein (see Section 12)
	g. Primary antibody too concentrated	g. Use higher dilution of the primary antibody

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 the DAKO Technical Services Department for further assistance at 800/424-0021.

Additional information on staining techniques and specimen preparation can also be found in the Handbook-Immunochemical Staining Methods,22 available from DAKO Corporation, Atlas of Immunohistology,23 and Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis.24

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