CE

Dako
EnVision®+ Dual Link System-HRP (DAB+)

Code K4065

Intended use

For In Vitro diagnostic use.

These instructions apply to the Dako EnVision+ Dual Link System-HRP (DAB+).

This kit is intended for use with primary antibodies from **mouse** and **rabbit** supplied by the user for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues or cell preparations. Tissues processed in a variety of fixatives including ethanol, B-5, Bouin's, zinc formalin, and neutral buffered formalin may be used.

Summary and explanation

The EnVision+ Dual Link System-HRP is a two-step IHC staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequently, nonspecific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. All reagents in the EnVision+ Dual Link System-HRP with substrate (excluding the Liquid DAB+ Substrate-Chromogen) are ready-to-use. This system is an extremely sensitive method and, as a result, optimal dilutions of the primary antibody are up to 20 times higher than those used for the traditional PAP technique and several-fold greater than those used for the traditional ABC or LSAB methods. This protocol offers an enhanced signal generating system for the detection of antigens present in low concentrations or for low titer primary antibodies. Primary antibodies produced in mouse or rabbit react well with the labelled polymer. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper controls.

Principles of procedure

Any endogenous peroxidase activity is quenched by incubating the specimen for 5–10 minutes with Dual Endogenous Enzyme Block. The specimen is then incubated with an appropriately characterized and diluted mouse or rabbit primary antibody, followed by incubation with the labelled polymer using a recommended 30 minute incubation for each. *It should be noted that for antibodies requiring enzyme digestion or target retrieval, it may be necessary to increase incubation times of the primary antibody and labelled polymer by 5–10 minutes.* Staining is completed by a 5–10 minute incubation with 3,3'-diaminobenzidine (DAB+) substrate-chromogen which results in a brown-colored precipitate at the antigen site. (DAB is a potential carcinogen; see Precautions Section.)

Reagents provided

Code K4065
The following materials, sufficient for 150 tissue sections based upon 100 µL per section, are included in this kit:

Quantity	*Description*
1x15 mL	**Dual Endogenous Enzyme Block** DUAL ENDOGENOUS ENZYME BLOCK 0.3% hydrogen peroxide containing sodium azide and levamisole.
1x15 mL	**Labelled Polymer** LABELLED POLYMER-HRP Peroxidase labelled polymer conjugated to goat anti-mouse and goat anti-rabbit immunoglobulins in Tris-HCl buffer containing stabilizing protein and an antimicrobial agent.
1x18 mL	**Substrate Buffer** DAB+ SUBSTRATE BUFFER Substrate buffer solution, pH 7.5, containing hydrogen peroxide and a preservative.
1x1 mL	**DAB+ Chromogen** DAB+ CHROMOGEN 3,3'-diaminobenzidine chromogen solution.

Materials required, but not supplied

Deionized or Distilled water
Ethanol, absolute and 95%
Xylene, toluene or xylene substitutes
Primary antibodies and negative control reagent
Slides, poly-L-lysine coated or Silanized Slides (code S3003) (see Adherence of Paraffin-Embedded Tissue)
Control tissue, positive and negative
Ammonium hydroxide, 15 mol/L diluted to 0.037 mol/L
Counterstain; aqueous based, such as Mayer's Hematoxylin or Lillie's Modified Mayer's Hematoxylin (code S3002 for automated use; code S3001 for manual use) (see Reagent Preparation Section)
Faramount, Aqueous Mounting Medium, Ready-to-use (code S3025) is recommended for aqueous mounting; or nonaqueous permanent mounting medium, Ultramount (code S1964) or Permanent Mounting Medium (Code S3026)
Coverslips
Light microscope (20x–800x)
Absorbent wipes
Staining jars or baths
Timer (capable of 3–40 minute intervals)
Wash bottles
Wash Buffer Solution, such as Automation Buffer (code S3006), TBS (code S3001) or PBS (code S3024)

Precautions

1. For professional users.
2. 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.
3. Do not substitute reagents from other lot numbers or from kits of other manufacturers.
4. Enzymes and substrate-chromogens may be affected adversely if exposed to excessive light levels. Do not store kit components or perform staining in strong light, such as direct sunlight.
5. Incubation times or temperatures other than those specified may give erroneous results; any such changes must be validated by the user.[1]
6. As with any product derived from biological sources, proper handling procedures should be used.
7. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
8. Unused solution should be disposed of according to local, State and Federal regulations.
9. Safety Data Sheet available for professional users on request.

Danger

Dual Endogenous Enzyme Block: <0.1% 3(2H)-Isothiazolone, 5-chloro-2-methyl-, mixt. with 2-methyl-3(2H)-isothiazolone

H314	Causes severe skin burns and eye damage.
H317	May cause an allergic skin reaction.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P261	Avoid breathing vapor.
P264	Wash hands thoroughly after handling.
P272	Contaminated work clothing should not be allowed out of the workplace.
P304 + P340 + P310	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. Immediately call a POISON CENTER or physician.
P301 + P310 + P330 + P331	IF SWALLOWED: Immediately call a POISON CENTER or physician. Rinse mouth. Do NOT induce vomiting.
P303 + P361 + P353 + P363 + P310	IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water or shower. Wash contaminated clothing before reuse. Immediately call a POISON CENTER or physician.
P302 + P352	IF ON SKIN: Wash with plenty of water/...
P333 + P313	If skin irritation or rash occurs: Get medical attention.
P305 + P351 + P338 + P310	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

Danger

DAB+ Chromogen: 1–5% biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride

H350	May cause cancer.
H341	Suspected of causing genetic defects.
P201	Obtain special instructions before use.
P202	Do not handle until all safety precautions have been read and understood.
P280	Wear protective gloves. Wear eye or face protection. Wear protective clothing.
P308 + P313	IF exposed or concerned: Get medical attention.
P405	Store locked up.
P501	Dispose of contents and container in accordance with all local, regional, national and international regulations.

As a main rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper work procedure, the dangerous properties of the product and the necessary safety instructions.

Storage

Reagents of the EnVision+ Dual Link System-HRP are to be stored at 2–8°C. Do not freeze. Do not use after expiration printed on reagent vials and kit label. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user.[1]

Alteration in the appearance of any reagent, such as precipitation, may indicate instability or deterioration. In such cases, the reagent(s) is (are) not to be used.

There are no obvious signs to indicate instability of these products. Therefore, positive and negative controls should be tested simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variation in laboratory procedures and a problem with the kit is suspected, contact Dako Technical Support.

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. These are guidelines only. Optimal procedures must be determined and verified by the user. For specific information regarding tissue fixation and processing, refer to Dako's *General Instructions for Immunohistochemical Staining.*

Reagent preparation

Wash Buffer Solution
Investigation has shown that 0.05 mol/L Tris-HCl, pH 7.6, containing 0.15 mol/L NaCl and 0.05% Tween 20, without sodium azide, significantly aids in minimizing background staining with this system. A recommended wash buffer is Automation Buffer (code S3006). Wash buffers containing sodium azide inactivate horseradish peroxidase and result in negative staining. Store unused buffer at 2□8°C. Discard buffer if cloudy in appearance.

MANUAL STAINING
Tissue sections should be washed in up to three fresh baths of Automation Buffer (code S3006), TBS (code S3001) or PBS (code S3024) for 3□5 minutes between each step of the EnVision+ Dual Link System staining protocol.

Primary Antibody
Ready-to-use antibodies for the EnVision+ Dual Link System are available from Dako. Concentrated antibodies are also available from Dako; however, optimal dilutions must be determined experimentally by the user. Due to the high sensitivity of the EnVision+ Dual Link System, primary antibody dilutions may range from 5- to 20-fold greater than those used in traditional IHC methods. If concentrated antibodies are to be used, dilutions should be prepared using Antibody Diluent (code S0809). For most primary antibodies used with this kit, an incubation time of 30 minutes is sufficient.

Negative Control Reagent
Ideally, a negative control reagent contains an antibody which exhibits no specific reactivity with human tissues or normal/non-immune serum in the same matrix/solution as the diluted primary antibody. The negative control reagent should be the same subclass and animal species as the primary antibody, diluted to the same immunoglobulin or protein concentration as the diluted primary antibody using the same diluent. The incubation period for the negative control reagent should correspond to the primary antibody. For convenience, a universal negative control reagent for mouse primary antibodies and universal negative control reagent for rabbit primary antibodies are available from Dako, supplied as ready-to-use products. For specific information regarding negative control reagent preparation, refer to Dako's *General Instructions for Immunohistochemical Staining.*

Substrate-Chromogen Solution
The following protocol for the preparation of 1 mL of the DAB+ substrate-chromogen solution is sufficient for up to 10 tissue sections or up to 5 cell smears.

STEP 1 Depending on the number of slides to be stained, transfer enough 1 mL aliquots of Substrate Buffer into an appropriately sized container.
STEP 2 For each 1 mL of buffer, add one drop (20 µL) of Liquid DAB+ Chromogen. Mix immediately.

The prepared DAB+ substrate-chromogen solution is stable approximately 5 days when stored at 2□8°C. This solution should be mixed thoroughly prior to use. Any precipitate developing in the solution does not affect staining quality. Used in its entirety, the 18 mL of Substrate Buffer provided will provide adequate volume for 150 tests. An excess volume may remain.

Mounting Media
Faramount, Aqueous Mounting Medium, Ready-to-use (code S3025) is recommended for aqueous mounting. For permanent mounting, use Ultramount (code S1964) or Permanent Mounting Medium (code S3026).

Staining procedure

Procedural Notes

The user should read these instructions carefully and become familiar with the kit contents prior to use. See the Precautions Section.

The Dual Endogenous Enzyme Blocking Solution and EnVision+ Dual Link System polymer should be equilibrated to room temperature prior to immunostaining. Likewise, all incubations are optimized for performance at room temperature. 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.

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 EnVision+ Dual Link System-HRP can be further increased by lengthening the incubation times of Steps 2 and 3 for 5□10 minutes.

MANUAL STAINING PROTOCOL

STEP 1 ENDOGENOUS ENZYME BLOCK
Tap off excess buffer. Using a lint-free tissue (such as Kimwipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagent within the prescribed area.
Apply enough Dual Endogenous Enzyme Block to cover specimen.
Incubate 5□10 (±1) minutes.
Rinse gently with distilled water or buffer solution from a wash bottle (do not focus flow directly on tissue) and place in a fresh buffer bath.

STEP 2 PRIMARY ANTIBODY OR NEGATIVE CONTROL REAGENT
Tap off excess buffer and wipe slides as before.
Apply enough optimally diluted primary antibody or negative control reagent to cover specimen.
Incubate 30 (±1) minutes.
Rinse gently with buffer solution from a wash bottle (do not focus flow directly on tissue) and place in a fresh buffer bath.
If the staining procedure must be interrupted, slides may be kept in a buffer bath following incubation of the primary antibody (Step 2) for up to one hour at room temperature without affecting the staining performance.

STEP 3 LABELLED POLYMER-HRP
Tap off excess buffer and wipe slides as before.
Apply enough Labelled Polymer to cover specimen.
Incubate 30 (±1) minutes.
Rinse slides as in Step 2 and place in buffer bath for 5 (±1) minutes.

STEP 4 SUBSTRATE-CHROMOGEN
Wipe slides as before.
Apply enough substrate-chromogen solution to cover specimen (see Reagent Preparation Section).
Incubate 5□10 (±1) minutes.
Rinse gently with distilled water from a wash bottle (do not focus flow directly on tissue). Collect substrate-chromogen waste in a hazardous materials container for proper disposal.

STEP 5 HEMATOXYLIN COUNTERSTAIN (optional)
Immerse slides in a bath of aqueous hematoxylin. Length of incubation depends on the strength of Hematoxylin used.
Rinse gently in a distilled water bath.
Dip slides 10 times into a bath of 0.037 mol/L ammonia or similar bluing agent.
Rinse slides in a bath of distilled or deionized water for 2□5 minutes.
Proceed to mounting.

Mounting

Specimens may be mounted and coverslipped with an aqueous-based mounting medium such as Faramount, Aqueous Mounting Medium, Ready-to-use (code S3025). For permanent mounting, use Ultramount (code S1964) or Permanent Mounting Medium (code S3026).
Note: Slides may be read when convenient. However, some fading may occur if slides are exposed to strong light over a period of one week. To minimize fading, store slides in the dark at room temperature (20□25°C).

Quality control

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls. Consult the quality control guidelines of the American Pathologists (CAP) Certification Program for Immunohistochemistry and references 3 through 5 for additional information. Refer to the specification sheet of each primary antibody used for details regarding sensitivity and immunoreactivity.

Refer to Dako's *General Instructions for Immunohistochemical Staining* for further information on positive and negative controls.

Staining interpretation

Refer to Dako's *General Instructions for Immunohistochemical Staining* for interpretation guidelines.

General limitations

Refer to Dako's *General Instructions for Immunohistochemical Staining* for general limitations.

Product specific limitations

Use of old or unbuffered fixatives, or exposure of tissues to excessive heat (greater than 60°C) during processing may result in decreased staining sensitivity.

Endogenous peroxidase or pseudoperoxidase activity can be found in hemoproteins such as hemoglobin, myoglobin, cytochrome, and catalase as well as in eosinophils.[2,3] This activity can be inhibited by incubating specimens with Dual Endogenous Enzyme Block of the EnVision+ Dual Link System-HRP for five to ten minutes prior to the application of the primary antibody. Blood and bone marrow smears and frozen tissues can also be treated with this reagent. Alternately, a solution of methanol-hydrogen peroxide can be used. Some antigens may become denatured with this procedure.

Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit nonspecific staining with horseradish peroxidase.[4]

Normal/non-immune 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.

The reagents supplied in this kit have been optimally diluted. Further dilution may result in loss of antigen detection.

Troubleshooting MANUAL

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 antibodies or substrate.	2b. Review recommended incubation times.
3. Excessive background in all slides.	3a. Specimens contain high peroxidase activity.	3a. Use longer incubation time of Dual Endogenous Enzyme Block.
	3b. Paraffin incompletely removed.	3b. Use fresh xylene or toluene baths. If several slides are stained simultaneously, the second xylene bath should contain fresh xylene.
	3c. Slides not properly rinsed.	3c. Use fresh solutions in buffer baths and wash bottles. Use Automation Buffer as wash buffer (see Reagent Preparation Section).
	3d. Faster than normal substrate reaction due to e.g. excessive room temperature.	3d. Use shorter incubation time with substrate-chromogen solution.
	3e. Sections dried during staining procedure.	3e. Use humidity 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).
	3g. Antibody too concentrated.	3g. 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 Dako Technical Support for further assistance.

Additional information on staining techniques and specimen preparation can be found in the *Handbook – Immunochemical Staining Methods*[5] (available from Dako), *Atlas of Immunohistology*[6] and *Immunoperoxidase Techniques, A Practical Approach to Tumor Diagnosis.*[7]

References

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6. Tubbs RR, et al. Atlas of Immunohistology. Chicago: Amer Soc Clin Pathol Press 1986
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Explanation of symbols

REF	Catalogue number		Temperature limitation	IVD	In vitro diagnostic medical device
	Manufacturer	LOT	Batch code		Contains sufficient for <n> tests
	Use by		Consult instructions for use	EC REP	Authorized representative in the European Community

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