DETEK® Hrp

Horseradish Peroxidase Signal Generating System

Cat. No. 43820

For Research Use Only

INTRODUCTION

***DETEK*® Hrp Horseradish Peroxidase Signal Generating System** provides rapid and sensitive detection of biotinylated macromolecules. This Signal Generating System consists of the *DETEK*® Hrp Detection Reagent complex of streptavidin and biotin-labeled horseradish peroxidase, aminoethylcarbazole (AEC) chromogen and hydrogen peroxide substrate in reaction buffer. Detection of biotinylated macromolecules is based on the recognition of biotin by the biotin-binding protein, streptavidin, which is linked to the horseradish peroxidase enzyme. Bound horseradish peroxidase, in the presence of hydrogen peroxide, catalyzes the oxidation of AEC into a brick red precipitate. The *DETEK*® Hrp complex may be used for detection of any biotinylated product where precipitation at the site of the reaction is required, such as Southern, Northern and Western blots, dot blots and *in situ* hybridization.

REAGENTS PROVIDED

***DETEK*® Hrp Detection Reagent,** 1 ml
Concentrated complex of streptavidin and biotin-labeled horseradish peroxidase in phosphate buffered saline with stabilizer and preservative

Aminoethylcarbazole (AEC), 4 ml
40 mg/ml in dimethylformamide

8X Reaction Buffer/Substrate Reagent, 2 X 30 ml
Hydrogen peroxide in acetate buffer containing enhancers

STORAGE

1. Store the reagents at 2-8°C. The shelf-life of the AEC and the 8X Reaction Buffer/Substrate Reagent can be extended by storage at -20°C.
2. To increase the shelf-life of the Aminoethylcarbazole (AEC), remove the vial from the kit and store at -20°C.
3. The reagents are stable until the expiration date on the label when stored and used as directed.

WARNINGS

- **For RESEARCH use only**.
- Use a safety pipetting device for all pipeting. Never pipet by mouth.
- The ***DETEK*® Hrp Detection Reagent** contains thimerosal as preservative. Thimerosal contains mercury and is therefore poisonous. Avoid inhalation, ingestion and contact with skin. Wash thoroughly with soap and water if contact is made. Dispose of this solution in accordance with local regulations.
- **Aminoethylcarbazole (AEC)** is a potential carcinogen dissolved in dimethylformamide, a hazardous solvent. Avoid inhalation, ingestion and contact with skin. Wash thoroughly with soap and water if contact is made. Dispose of this solution in accordance with local regulations.

RECOMMENDED PROCEDURES

The ***DETEK*® Hrp Horseradish Peroxidase Signal Generating System** was specifically designed for detection of biotin-labeled molecules in a wide variety of formats. For optimal use in membrane hybridization applications and for in situ hybridization applications with biotin-modified probes, use the following procedures.

DETECTION OF BIOTIN-LABELED NUCLEIC ACID IN MEMBRANE HYBRIDIZATION APPLICATIONS

The ***DETEK*® Hrp Horseradish Peroxidase Signal Generating System** can be used for enzymatic detection of hybridized biotin-labeled nucleic acid following Southern, Northern and dot blot membrane hybridization procedures. The procedure described below details methods for use following membrane hybridization and stringency washing.

NOTE: All volumes are given for 10 cm X 10 cm blots.

1. **Blocking**

   Place the washed membrane in a sealable bag and incubate for 60 minutes with shaking at room temperature with a sufficient amount of blocking solution containing 0.1M Tris buffer, pH 7.5, 0.3M NaCl and 10% Liquid Blocking Reagent (Amersham Pharmacia RPN3601). Use enough blocking solution to completely suspend the membrane in the bag--*e.g.*, about 15 ml for a 10 cm X 10 cm blot.

2. **Incubation with Detection Reagent**
   As needed, prepare a sufficient amount of ready-to-use *DETEK*® Hrp Detection Reagent by diluting the concentrate 250- to 500-fold in blocking solution containing 0.1M Tris buffer, pH 7.5, 0.3M NaCl and 10% Liquid Blocking Reagent (Amersham Pharmacia RPN3601).

   Expel all of the blocking solution from the bag containing the membrane and replace with the solution of diluted detection reagent. Seal the bag and incubate with gentle shaking for 60 minutes at room temperature.

3. **Post-Detection Washing**

   Remove the membrane from the bag to a dish containing 100 ml of Detection Reagent Washing Solution (0.1M Tris Buffer, pH 7.5, 0.3M NaCl, 0.3% Tween 20) and incubate for 10 minutes with shaking. Repeat with 100 ml of fresh Detection Reagent Washing Solution twice for a total of three 10 minute washes.

4. **Pre-Development Washing**
   Wash the membrane twice for 5 minutes with shaking with 100 ml of 2X SSC (0.3M NaCl, 0.03M sodium citrate).

5. **Color Development**
   Prepare a sufficient amount of ready-to-use Color Reaction Mixture by diluting the **8X Reaction Buffer/Substrate Reagent** 8-fold (2.5 ml plus 17.5 ml of deionized or distilled water). Add 200 µl of **Aminoethylcarbazole (AEC)** to 20 ml of 1X Reaction Buffer/Substrate Reagent and mix thoroughly.

   Drain the membrane following the second Pre-Development Wash and place it in a clean dish or tray. Pour the Color Reaction Mixture onto the membrane and incubate for 30-60 minutes at room temperature without shaking, in the dark.

6. **Termination of Color Development and Preservation of the Membrane**
   Rinse the developed membrane 4 to 5 times with distilled or deionized water. Place the rinsed membrane on a piece of absorbent paper and allow the membrane to dry in the dark. Seal the dried membrane in a plastic bag and store in the dark. Color is retained on nylon membranes for years.

DETECTION OF BIOTIN-LABELED NUCLEIC ACID FOLLOWING *IN SITU* HYBRIDIZATION PROCEDURES

The ***DETEK®* Hrp Horseradish Peroxidase Signal Generating System** can be used for colorimetric detection of hybridized biotin-labeled nucleic acid following *in situ* hybridization procedures. The procedure described below details methods for use following hybridization and stringency washing.

1. **Incubation with Detection Reagent**
   As needed, prepare a sufficient amount of ready-to-use ***DETEK®* Hrp Detection Reagent** by diluting the concentrate 100- to 500-fold in:
   10mM phosphate buffer, pH 7.0 to 7.2
   0.15M NaCl
   0.025% Triton X-100
   0.3% gelatin
   Or, use **Dilution Buffer for Horseradish Peroxidase-Linked Detection Reagents** (Cat. No. 33804) to prepare ready-to-use *DETEK®* Hrp Reagent.
   Following stringency washing, soak the slide in phosphate buffered saline (ENZO Wash Buffer Salts, Cat. No. 33802). Remove the slide from the phosphate buffered saline soak and wipe around the specimen. Place 0.5-1 ml of diluted detection reagent on the slide and incubate the slide at 37°C for 30-60 minutes. Do not allow the slides to dry out during these procedures.

2. **Post-Detection Washing**
   Using a squeeze (wash) bottle filled with the phosphate buffered saline solution (ENZO Cat. No. 33802), rinse the slide with an even stream of wash buffer for 10 seconds. Soak the slides 2 to 3 times in phosphate buffered saline for 1-2 minutes to rinse off the Detection Reagent.

3. **Colorimetric Development**
   Prepare a sufficient amount of ready-to-use Color Reaction Mixture by diluting the **8X Reaction Buffer/Substrate Reagent** 8-fold (2.5 ml plus 17.5 ml of deionized or distilled H2O). Add 200 µl of **Aminoethylcarbazole (AEC)** to 20 ml of 1X Reaction Buffer/Substrate Reagent and mix thoroughly.
   Remove the slide from the last soak in wash buffer and carefully wipe around the specimen. Place 0.5-1.0 ml of Color Reaction Mixture on the slide and incubate for 15-30 minutes at 37°C.

4. **Termination of Color Development and Preservation of the Slides**
   Rinse the slide with phosphate buffered saline solution. Counterstain and mount the slide using an aqueous mounting medium.

DETECTION OF BIOTIN-LABELED PROTEINS IN IMMUNOBLOTTING PROCEDURES (WESTERN BLOTS)

The ***DETEK®* Hrp Horseradish Peroxidase Signal Generating System** can be used for colorimetric detection of biotin-labeled protein on immunoblots. SDS-polyacrylamide gel electrophoresis can be performed by standard techniques [Laemmli, U. K. (1970) Nature **227**: 680] followed by electrophoretic transfer to nitrocellulose or nylon membranes [Burnette, E. N. (1981) Anal. Biochem. **112**:195; Towbin, H. *et al.* (1979) Proc. Natl. Acad. Sci. USA **76**:4350]. For dot blots, the protein target can be applied directly onto the membrane. The procedure given here is for the detection steps following immobilization of the target protein on the membrane [Brower, M. *et al.* (1985) Anal. Biochem. **147**:382].

NOTE: All volumes are given for 10 cm X 10 cm blots.

1. **Pre-Blocking**
   After electroblotting (or dot blotting) of the target protein, place the membrane in a sealable bag and incubate overnight at 2-8°C (or 1 hour at room temperature) with a sufficient amount of Pre-Blocking Buffer [3% bovine serum albumin (BSA) in phosphate buffered saline]. Wash the membrane for 30 minutes at 37°C with 50 ml of PBS-Tween Buffer [0.3% Tween 20 in phosphate buffered saline].

2. **Incubation with Primary Antibody**
   As needed, prepare a sufficient amount of ready-to-use primary antibody in PBS-Tween Buffer. Incubate the membrane with diluted primary antibody for 60 minutes at 37°C with gentle shaking.
   After incubation with primary antibody, wash the membrane 3 times for 5 minutes each wash with 50 ml of PBS-Tween Buffer.

3. **Incubation with Biotin-Labeled Secondary Antibody**
   As needed, prepare a sufficient amount of ready-to-use biotin-labeled secondary antibody in PBS-Tween Buffer (the secondary antibody should be specific for the species in which the primary antibody was produced). Incubate the membrane with diluted biotin-labeled secondary antibody for 60 minutes at 37°C with gentle shaking.
   After incubation with secondary antibody, wash the membrane 3 times for 5 minutes each wash with 50 ml of PBS-Tween Buffer.

4. **Blocking**
   After washing off excess hapten-labeled secondary antibody, incubate the membrane with gentle shaking in Blocking Buffer [2%(w/v) BSA, 0.1% Tween 20 in phosphate buffered saline] for 30 minutes at room temperature.

5. **Incubation with Detection Reagent**
   As needed, prepare a sufficient amount of ready-to-use DETEK® Hrp Detection Reagent by diluting the concentrate 250- to 500-fold in phosphate buffered saline (ENZO Wash Buffer Salts, Cat. No. 33802).
   Place the membrane in a sealable bag, add the diluted Detection Reagent and incubate with gentle shaking for 60 minutes at room temperature.

6. **Post-Detection Washing**
   Remove the membrane from the bag to a dish containing 100 ml of PBS-Tween Buffer and incubate for 5 minutes with shaking. Repeat with 100 ml of fresh PBS-Tween Buffer twice for a total of four 5 minute washes.

7. **Color Development**
   Prepare a sufficient amount of ready-to-use Color Reaction Mixture by diluting the **8X Reaction Buffer/Substrate Reagent** 8-fold (2.5 ml plus 17.5 ml of deionized or distilled water). Add 200 µl of **Aminoethylcarbazole (AEC)** to 20 ml of 1X Reaction Buffer/ Substrate Reagent and mix thoroughly.
   Drain the membrane following the Post-Detcction Washes and place it in a clean dish or tray. Pour the Color Reaction Mixture onto the membrane and incubate for 30-60 minutes at room temperature without shaking, in the dark.

8. **Termination of Color Development and Preservation of the Membrane**
   Rinse the developed membrane 4 to 5 times with distilled or deionized water. Place the rinsed membrane on a piece of absorbent paper and allow the membrane to dry in the dark. Seal the dried membrane in a plastic bag and store in the dark. Color is retained on nylon membranes for years.

For Technical Assistance call ENZO:
Toll free from the U.S. and Canada: 1-800-221-7705
All others: 631-694-7070
Fax: 631-694-7501

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USER'S GUIDE
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In Situ HYBRIDIZATION AND DETECTION PROCEDURES

for Hapten-Modified DNA and Oligonucleotide Probes

DNA and oligonucleotide probes labeled with hapten-modified nucleotides are particularly suitable for *in situ* hybridization and detection applications. Using ENZO's versatile modular labeling systems, different hapten labels can be used to produce modified nucleic acid probes for a variety of hybridization studies. To enhance the utility of ENZO's labeling and detection systems, a summary of acceptable methods for *in situ* hybridization and detection with hapten-modified DNA and oligonucleotide probes is contained in this User's Guide.

For *in situ* hybridization studies of high copy number sequences and in applications such as chromosome painting, the fluorescent deoxynucleotides--Fluorescein-12-dUTP, Rhodamine-5-dUTP and Coumarin-6-dUTP--are extremely useful labels. Because **the probe is the signal**, the hybridization test is simply a matter of hybridization and stringency washing. Methods for use of such probes are included in this User's Guide.

ENZO offers the highest quality reagents for the *in situ* detection of probes modified with Biotin, Digoxigenin or Fluorescein hapten labels so that each of these haptens can provide access to single copy sequences in various *in situ* hybridization and detection procedures. To assist the researcher with *in situ* hybridization analyses, this User's Guide presents a compendium of methods for these studies. The methods described here can be carried out with a combination of ready to use ENZO reagents or the researcher can use this guide as a starting point for designing and preparing unique reagents to meet specific needs.

PREPARATION AND PRETREATMENT OF TISSUE SECTIONS AND FIXED CELLS

SLIDE PREPARATION

Tissue Specimens

Tissue Fixation

Acceptable methods for fixation of tissue include preservation in 10% buffered formalin followed either by paraffin embedding or freezing for later sectioning. Formalin fixation should not be allowed to proceed for longer than 8-24 hours. If necessary, formalin-fixed tissue may be stored in 70% alcohol at 2-8°C prior to embedding or freezing.

Tissue Sectioning

Sections, either paraffin-embedded or frozen or formalin-fixed frozen sections of 4-6 microns in thickness should be applied to specimen slides that have been pre-treated to enhance adherence of the tissue during the hybridization and detection procedures (*e.g.*, ENZO Slide Packs, Cat. Nos. 31802/20, 31802/100, 31803/20 or 31803/100).

Slide Fixation

Following application of the sections to the slides, the slides should be baked to fix the tissue to the slides. Bake tissue-mounted slides vertically for 2-18 hours at 60-80°C. Fixed slides can be stored at room temperature.

Deparaffinization

Paraffin-embedded tissue sections MUST be deparaffinized before proceeding to pretreatment and *in situ* hybridization and detection procedures.

Bake the slides at 60-80°C for 5-15 minutes to melt residual paraffin. Soak the slides sequentially in a series of Coplin jars containing the following solutions, for the times indicated:

Soak Number	*Reagent*	*Duration of Soak*
1	Xylenes	10 minutes
2	Xylenes	2 minutes
3	100% Alcohol	1 minute
4	100% Alcohol	1 minute
5	90% Alcohol	1 minute
6	70% Alcohol	1 minute
7	50% Alcohol	1 minute
8	Deionized water	1 minute

Use fresh reagents in each jar for each batch of slides and discard in appropriate waste containers when finished.

Caution: Xylenes saturate rapidly with paraffin. Use fresh xylenes for each batch of 5-8 slides.

After the final soak (number 8, deionized water), allow slides to dry completely by incubation for 5-10 minutes at 37°C.

Proceed to Slide Pretreatment Procedures.

Cellular Specimens

Adherent Cell Lines

For surface-dependent adherent cell lines, cell suspensions in growth medium can be placed on glass slides for a sufficient time (4 to 24 hours) to allow the cells to attach to the surface. Slides containing attached cells should be rinsed by dipping in phosphate buffered saline (ENZO Cat. No. 33802). The cells should then be fixed by allowing the slides to stand in acetone for 5 minutes. If RNA is the intended target of the hybridization study, the cells should be fixed with 4% paraformaldehyde.

Exfoliated Cells

For epithelial cells and cells of epithelial lineage, such as exfoliated cells from oral or cervical scrapings, collect cells into phosphate buffered saline and concentrate by centrifugation. Following resuspension in a minimal amount of saline, the cells can be allowed to dry onto slides. Once dried, the cells should be fixed by allowing the slides to stand in a Coplin jar of cold (0°C) anhydrous alcohol for 5 minutes. Again, if RNA is the target of the hybridization study, the cells should be fixed with 4% paraformaldehyde.

Lymphoid Cells

For non-adherent cells, lymphoma cell lines and blood cells in general, cells should be collected by centrifugation and rinsed with phosphate buffered saline. Remove excess saline from the cell pellet and fix the cells with an appropriate fixative such as alcohol, acetone, or paraformaldehyde. The suspension of fixed cells can then be spread onto glass slides. After drying, permanently fix the slides by allowing the slides to stand for 5 minutes in a Coplin jar of fixative. Alternatively, cell pellets can be fixed with formalin or paraformaldehyde and then embedded in paraffin and sectioned as for tissue.

Proceed to Slide Pretreatment Procedures.

SLIDE PRETREATMENT PROCEDURES

Proteinase Pretreatment of Tissues and Cells

Introduction

Formalin-fixed tissue and cells fixed with crosslinking fixatives need to be made permeable to the reagents used for *in situ* hybridization and detection. Thus, both formalin-fixed tissue and paraformaldehyde-fixed cells should be proteinase treated prior to hybridization and detection.

Use ENZO *PathoGene*® Tissue Preparation Kit, Cat. No. 32800, for preparation and proteinase pretreatment of formalin-fixed tissue specimens. Alternatively, follow these procedures for preparation and use of Proteinase K for pretreatment of formalin-fixed tissue or formaldehyde-fixed cells.

Stock Solution

Prepare a stock solution of Proteinase K (at a concentration of 2.5 mg/ml) by dissolving 5 mg of good quality Proteinase K (*e.g.*, ENZO Cat. No. 33801) in 2 ml of phosphate (or Tris) buffered saline. Store convenient portions of the solution at ≤-20°C. Freeze all tubes that are not required for immediate use. The separate frozen tubes of Proteinase K Stock Solution are to be used for later batches of slides. Once frozen, the enzyme is stable for at least one year. Once defrosted, **do not refreeze**.

Working Solutions

As needed, use the vials of Proteinase K Stock Solution to prepare working strength solutions of Proteinase K. Initial experiments with formalin-fixed tissue should be performed with 10-fold dilutions of the 2.5 mg/ml Stock Solution. More dilute Working Solutions may be required to preserve morphology. Once an appropriate dilution of the proteinase has been determined for the types of tissue being analyzed, routine experiments can be performed at the pre-determined concentration. Do not store diluted Proteinase K solutions, the working solutions should be used immediately and unused material should be discarded.

Paraformaldehyde-fixed cells will require more dilute preparations of Proteinase K than formalin-fixed tissue. Initial experiments might be performed with 20- to 50-fold dilutions of the 2.5 mg/ml Stock Solution.

Pretreatment Procedure

Following drying of deparaffinized embedded tissue specimens (see above) and following drying of paraformaldehyde fixed cellular slides, add **0.35 ml to 0.5 ml of freshly prepared Proteinase K Working Solution**. Incubate the slides at **37°C for 15 minutes**.

Tap off Proteinase K and soak slides at room temperature for 1 minute in a Coplin jar filled with buffered saline wash buffer. Gently agitate the Coplin jar while soaking the slides.

NOTE: Shorter or longer incubation times can be used. However, to optimize signal response, titration of the Proteinase K is necessary for each new tissue type.

Tap off wash buffer and proceed to Quench Treatment and/or Dehydration Procedures.

Pre-Incubation Treatment of Cell Slides

Introduction

Cell slides that have been fixed with alcohol or acetone DO NOT require proteinase pretreatment. In fact, such procedures would completely destroy the cells. However pre-incubation can help to prepare the slides for the *in situ* hybridization and detection procedure. Slides that have been pre-incubated yield more detailed results than slides that have not been pre-incubated.

Pre-Incubation Procedure

After fixation and drying of the slides, add **0.35 ml to 0.5 ml of buffered saline to each specimen slide**. **Incubate on a slide warmer for 15 minutes at 37°C**. Use ENZO Wash Buffer Salts, Cat. No. 33802, when using a peroxidase-based detection reagent or ENZO *SignaSure*® Wash Buffer, Cat. No. 33803, when using an alkaline phosphatase-based detection reagent.

Tap off buffer and proceed to Quench Treatment and/or Dehydration Procedure.

Quench Treatment

Introduction

When using peroxidase-based detection reagents it is often necessary to quench the activity of endogenous peroxidase enzymes. This can be carried out using the Quench Reagent and methods provided in the ENZO *PathoGene*® Tissue Preparation Kit, Cat. No. 32800.

Alternatively, follow these procedures.

Procedure

Apply 0.5 ml of phosphate buffered saline containing 3% H_2O_2 to each specimen slide. Incubate at 37°C for 10-15 minutes.

Rinse off quench reagent using a stream of wash buffer (phosphate buffered saline) from a squeeze (wash) bottle.

Proceed to the Dehydration Procedure.

Dehydration Procedure

Introduction

Following proteinase pretreatment, pre-incubation, and/or Quench Treatment, **all slides must be dehydrated prior to beginning the *in situ* hybridization and detection procedures**.

Procedure

Dehydrate slides by incubations at room temperature in the following series of reagents for one minute in each:

Soak Number	*Reagent*	*Duration of Soak*
1	Deionized water	1 minute
2	50% Alcohol	1 minute
3	70% Alcohol	1 minute
4	100% Alcohol	1 minute

Dry the slides at 37°C for 5-10 minutes or at room temperature. The slides can be stored desiccated at 4°C, for one week or more before beginning the *in situ* hybridization and detection procedures. Once the *in situ* hybridization procedure has been started, however, the slides must be processed through the entire hybridization and detection procedure without interruption.

IN SITU HYBRIDIZATION PROCEDURES

PROBE PREPARATION

Nick Translated Probes

To prepare probe for *in situ* hybridization, add concentrated DNA probe to give a final concentration of 0.2 to 5 μg/ml in a medium containing 1X to 5X SSC or SSPE, 10% to 50% formamide, and 1% to 10% dextran sulfate. Alternatively, use ENZO *In Situ* Hybridization Buffer, Cat. No. 33808, for preparation of ready to use probe reagent for *in situ* hybridization studies.

Oligonucleotide Probes

To prepare probe for *in situ* hybridization, add concentrated oligonucleotide probe to give a final concentration of 50 to 100 ng/ml in a medium containing 1X to 5X SSC or SSPE, 10% to 50% formamide, and 1% to 10% dextran sulfate. Alternatively, use ENZO *In Situ* Hybridization Buffer, Cat. No. 33808, for preparation of ready to use oligonucleotide probe reagents for *in situ* hybridization studies.

HYBRIDIZATION AND POST HYBRIDIZATION WASHING

Hybridization

To hybridize to target DNA in fixed, proteinase pretreated tissues and in fixed cells, place a drop of ready to use probe reagent on the specimen and cover with a coverslip, being careful not to trap bubbles under the coverslip. Heat the slide on a heating block set to 95°C ± 3°C for 8-10 minutes for tissue specimens and 4-5 minutes for cellular specimens. Remove slide to a 37°C slide warmer and incubate for 30-60 minutes (up to 24 hours, depending upon the probe concentration and the target nucleic acid level).

Note that extended hybridizations are best carried out in an humidified chamber.

To hybridize to target RNA in fixed, pretreated tissues and cells, pre-hybridize the slide with hybridization buffer for 10-20 minutes at 37°C. Rinse the slide in buffered saline and then add probe reagent. When using double stranded (nick translated) DNA probe reagents, denature the probe reagent (probe in hybridization buffer) by incubation at 90-95°C prior to addition to the slide. When using single stranded (oligonucleotide) probe reagents, pre-warm the probe reagent to 37°C prior to addition to the slide. Place a drop of probe reagent onto the specimen and cover with a coverslip. Place the slide on a 37°C slide warmer and incubate for 30-60 minutes (up to 24 hours, depending upon the probe concentration and the target nucleic acid level).

Note that extended hybridizations are best carried out in an humidified chamber.

Post Hybridization Washing

After hybridization, remove the coverslip from the slide and soak the slide in a phosphate or Tris buffered saline solution (*e.g.*, ENZO Wash Buffer Salts, Cat. No. 33802, or ENZO *SignaSure*® Wash Buffer, Cat. No. 33803, respectively) for 5 minutes at room temperature.

Remove the slide from the buffer and carefully wipe around the specimen. Pipet 0.5 ml to 1 ml of a post hybridization wash solution onto the slide and incubate the slide at 37°C for 10-20 minutes.

Nick Translated Probes

Use either ENZO Cat. No. 33809, *In Situ* Hybridization Wash Reagent, or a solution containing 50% deionized formamide in 0.2X to 1X SSC. Incubate at 37°C for 10-20 minutes.

When hybridizing to an RNA target, post hybridization washing can be carried out at an elevated (50-60°C) temperature.

Oligonucleotide Probes

Use 37°C pre-warmed 1X SSC for 10 minutes at 37°C, followed by 37°C pre-warmed 0.2X SSC for 10 minutes at 37°C for post hybridization washes.

Following post hybridization incubations, soak the slide twice for 2 minutes in buffered saline.

The slides are now ready for *In Situ* Detection Procedures.

IN SITU DETECTION PROCEDURES

FLUORESCENT PROBES

Fluorescein-labeled Probes

Counterstain cells with an Evans Blue counterstain, 0.05 mg/ml in buffered saline, for 2-5 minutes at room temperature. Rinse with a stream of buffered saline from a squeeze (wash) bottle. Tap off excess buffer. Mount with alkaline glycerol (50% glycerol in 0.05M Tris buffer, pH 9.5).

To counterstain DNA or chromosomes, use 0.5 μg/ml propidium iodide or 75 ng/ml 4',6-diamino-2-phenylindole (DAPI). Slides can be mounted in a solution containing 9 parts glycerol, 2.3% 1,4-diaza-bicyclo-[2,2,2]-octane and 1 part 0.2M Tris HCl, pH 8.0.

View results with a microscope equipped for epifluorescence with filters for excitation at 494 nm.

Rhodamine-labeled Probes

To counterstain DNA or chromosomes, use 75 ng/ml 4',6-diamino-2-phenylindole (DAPI). Slides can be mounted in a solution containing 9 parts glycerol, 2.3% 1,4-diaza-bicyclo-[2,2,2]-octane and 1 part 0.2M Tris HCl, pH 8.0.

View results with a microscope equipped for epifluorescence with filters for excitation at 596 nm.

Coumarin-labeled Probes

Mount slides in an alkaline glycerol solution and view with a microscope equipped for epifluorescence with filters for excitation at 399 nm.

HAPTEN-MODIFIED PROBES

Standard Detection Procedures

Dilution of Detection Reagent

Dilute the appropriate Detection Reagent as follows:

Horseradish Peroxidase detections using *DETEK*® Hrp, *DigDETEK*® Hrp or *FluorDETEK*® Hrp Signal Generating System:

Dilute 100- to 500-fold in:

- 10mM phosphate buffer, pH 7.0-7.2
- 0.15M NaCl
- 0.025% Triton X-100
- 0.3% gelatin

Or, use ENZO Dilution Buffer for Horseradish Peroxidase-linked Detection Reagents (Cat. No. 33804).

When using oligonucleotide probes, add ENZO Liquid Blocking Reagent (Cat. No. 45704) to 5% (v/v) to the above solution.

Alkaline Phosphatase detections using *DETEK*® Alk, *DigDETEK*® AP or *FluorDETEK*® AP Signal Generating System:

Dilute 50- to 200-fold in:

- 0.1M Tris Buffer, pH 7.5
- 0.1M NaCl
- 0.1mM $ZnCl_2$
- 0.03% BSA

Or, use ENZO Dilution Buffer for Alkaline Phosphatase-linked Detection Reagents (Cat. No. 33805).

When using oligonucleotide probes, add ENZO Liquid Blocking Reagent (Cat. No. 45704) to 5% (v/v) to the above solution.

Fluorescence Detection of Biotin-labeled Probes using *DETEK*® FS, Fluorescent Streptavidin Detection Reagent:
For use of *DETEK*® FS (Cat. No. 43821), reconstitute one vial of the lyophilized reagent in 1 ml of deionized or distilled water. Store small portions (*e.g.*, 100 µl) of the reconstituted reagent frozen at -20°C until required for use. Protecting the reagent from light, dilute 100 µl of reconstituted reagent with 5 ml of:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

Or, use ENZO Dilution Buffer for Fluorescence-linked Streptavidin Detection Reagents (Cat. No. 33806).

When using oligonucleotide probes, add ENZO Liquid Blocking Reagent (Cat. No. 45704) to 5% (v/v) to the above solution.

These solutions can also be used for Streptavidin conjugated with other fluorescent dyes such as Rhodamine, Texas Red®, etc.

Fluorescence Detection using Fluorescent Hapten-Specific Antibodies:
For use of various fluorescence-linked hapten specific antibodies, dilute the antibody as recommended by the manufacturer in:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

Or, use ENZO Dilution Buffer for Antibody Detection Reagents (Cat. No. 33807).

When using oligonucleotide probes, add ENZO Liquid Blocking Reagent (Cat. No. 45704) to 5% (v/v) to the above solution.

Incubation with Detection Reagent

Tap off excess buffer from the last saline rinse after the Post Hybridization Wash, above. Carefully wipe around the specimen and place 0.5 ml to 1 ml of diluted Detection Reagent on the slide. Incubate the slide at 37°C for 30-60 minutes.

Post-Detection Washing

Horseradish Peroxidase and Alkaline Phosphatase Detections: Using a squeeze (wash) bottle filled with the appropriate buffered saline solution (phosphate buffered saline for horseradish peroxidase detections and Tris buffered saline for alkaline phosphatase detections), rinse the slide with an even stream of wash buffer for 10 seconds. Soak the slide 2 to 3 times in buffered saline for 1-2 minutes to rinse off the Detection Reagent.

Fluorescence-linked Detection Reagents: Using a squeeze (wash) bottle filled with phosphate buffered saline containing 0.1% Triton X-100, rinse the slide with an even stream of buffer for 10 seconds. Soak the slide 2 to 3 times in phosphate buffered saline containing 0.1% Triton X-100 for 1-2 minutes to rinse off the Detection Reagent.
Slides processed in this manner are now ready for counterstaining and viewing by epifluorescence. For counterstaining, see section on use of fluorescent probes.

Colorimetric Development

Horseradish Peroxidase Detections: Place 0.5 ml to 1 ml of 0.01M sodium acetate buffer pH 5.0, 0.04M NaCl, containing 0.025% H_2O_2, and 0.2 mg/ml aminoethylcarbazole (AEC) on the slide and incubate for 20-30 minutes at 37°C (for improved results, use ENZO Cat. No. 43825 for AEC/H_2O_2 color reactions, or use Cat. No. 43826 for Diaminobenzidine(DAB)/H_2O_2 color reactions).

Alkaline Phosphatase Detections: Place 0.5 ml to 1 ml of 0.1M Tris Buffer, pH 9.5, 0.1M NaCl, 0.01M $MgCl_2$, containing 0.165 mg/ml NBT and 0.33 mg/ml BCIP, on the slide and incubate for 20-40 minutes at 37°C, protected from direct light (for consistently high quality results, use ENZO Cat. No. 43827 for NBT/BCIP color reactions, or use Cat. No. 43828 for INT/BCIP color reactions).

Termination of Color Development and Preservation of the Slides

Soak the slides in buffered saline for 1-2 minutes to rinse off the reaction mixtures. Counterstain and mount the slides as desired.

Double Antibody Enhanced Detection Procedures

Blocking Non-Specific Binding

To prevent non-specific binding of antibody detection reagents to hybridized fixed cells and tissues it is often necessary to block the tissue or cell preparation following hybridization and post hybridization washing. After the last saline soak following the Post Hybridization Wash, tap off excess wash buffer and wipe around the specimen. Pipet 0.5 ml to 1 ml of blocking solution onto the slide and incubate for 10 to 15 minutes at 37°C. A typical blocking solution consists of non-immune serum or a solution consisting of:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

Alternatively, use ENZO Dilution Buffer for Antibody Detection Reagents (Cat. No. 33807) to block non-specific binding of antibody reagents.

Tap off Blocking Buffer and soak the slide twice for 2 minutes in fresh phosphate buffered saline.

Dilute the appropriate Detection Reagent as follows:

Preparation of Ready to Use Antibody Detection Reagents

Until stability data have been established for each particular antibody preparation, prepare ready to use reagent only as needed. A good buffer for use with antibodies consists of:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

Alternatively use ENZO Dilution Buffer for Antibody Detection Reagents (Cat. No. 33807) for dilution of unmodified or fluorescent antibodies, but NOT for dilution of enzyme-linked antibodies.

For use of specific antibody combinations and for Double Antibody Enhancement Procedures follow the outline below:

DETEK® 1-f

For use of *DETEK*® 1-f, (ENZO Cat. No. 43818), dilute Rabbit anti-Biotin 100- to 500-fold and dilute Fluorescein-conjugated Goat anti-Rabbit IgG 200-fold. Fluorescein-conjugated Goat anti-Rabbit IgG should be diluted only as needed and should be protected from light.

Double Antibody Enhancement

For double antibody enhancement procedures using Rabbit anti-Biotin (ENZO Cat. No. 43861), dilute the concentrated Primary antibody 100- to 500-fold in:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

Alternatively, use ENZO Dilution Buffer for Antibody Detection Reagents (Cat. No. 33807), in which the diluted antibody is stable for up to six months when stored at 2-8°C in this buffer. Warm to room temperature prior to use.

Detect the primary Rabbit anti-Biotin antibody with a good quality biotin-conjugated anti-Rabbit IgG secondary antibody. Dilute the biotin-conjugated secondary antibody in:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

according to manufacturer's recommendations. Alternatively, use ENZO Dilution Buffer for Antibody Detection Reagents (Cat. No. 33807).

Other hapten-specific primary antibodies (*e.g.*, anti-Digoxigenin or anti-Fluorescein antibodies), coupled with a biotin-conjugated secondary antibody can be used in similar double antibody enhancement procedures. Such procedures can be used to convert detection of any hapten into a biotin-detection procedure.

NOTE: For double antibody enhancement procedures using other hapten-specific antibodies and hapten-modified secondary antibodies, follow the manufacturer's recommendations for dilution of the antibodies using:

10mM phosphate buffer, pH 7.0 to 7.2
0.15M NaCl
0.3% gelatin
0.05% sodium azide

as dilution buffer (or, use ENZO Cat. No. 33807).

Application of Antibody Reagents

Tap off excess buffer from the last saline rinse following blocking of the slides, above. Carefully wipe around the specimen and place 0.5 ml to 1 ml of diluted primary antibody detection reagent on the slide. Incubate the slide at 37°C for 20 minutes.

Soak slide twice for 2 minutes in fresh phosphate buffered saline.

Tap off excess buffer from the last saline rinse, above. Carefully wipe around the specimen and place 0.5 ml to 1 ml of diluted secondary antibody detection reagent on the slide and incubate the slide at 37°C for 20 minutes.

Soak slide twice for 2 minutes in phosphate buffered saline.

Detection of Antibody Enhanced Slides

Following application and washing of secondary antibody, the ultimate detection procedure will, of course, depend upon the choice of detection reagent. While it is difficult to cover every potential situation in this protocol, several recommendations follow:

Fluorescent Secondary Antibody (e.g., fluorescein, rhodamine, Texas Red®, etc., conjugated secondary antibodies) as in DETEK® 1-f:

Following application and washing of the fluorescent secondary antibody, place 0.5 ml to 1 ml of an appropriate counterstain on the slide (*e.g.*, Evans Blue for Fluorescein-labeled secondary antibody) and incubate for 1-2 minutes at room temperature.

Using a squeeze (wash) bottle filled with phosphate buffered saline, rinse the slide with an even stream of buffer. (If excess background fluorescence is evident, use 0.1% Triton X-100 in the washing steps following application of the fluorescent antibody.)

Tap off excess buffer and mount in alkaline glycerol. View results using appropriate filters for excitation at 494 nm.

Enzyme-modified Secondary Antibody (e.g., horseradish peroxidase- or alkaline phosphatase -conjugated secondary antibody):

Continue to use phosphate buffered saline (ENZO Cat. No. 33802, ENZO Wash Buffer Salts) when using horseradish peroxidase. Switch to Tris buffered saline, (ENZO Cat. No. 33803, *SignaSure*® Wash Buffer) when using alkaline phosphatase for colorimetric development. For colorimetric development, follow the instructions above, or, for horseradish peroxidase development of the slides with Aminoethylcarbazole or Diaminobenzidine, use ENZO Cat. No. 43825 or 43826, respectively. For alkaline phosphatase development of the slides with NBT/BCIP or INT/BCIP use ENZO Cat. No. 43827 or 43828, respectively.

Hapten-modified Secondary Antibody in Double Antibody Enhancement Procedures (e.g., biotin-, digoxigenin- or fluorescein-conjugated secondary antibody):

Use ENZO Signal Generating Systems, *DETEK*® Hrp or *DETEK*® Alk (ENZO Cat. Nos. 43820 or 43822, respectively) for Biotin detection; *DigDETEK*® Hrp or *DigDETEK*® AP (ENZO Cat. Nos. 43830 or 43832, respectively) for Digoxigenin detection; or *FluorDETEK*® Hrp or *FluorDETEK*® AP (ENZO Cat. Nos. 43840 or 43842, respectively) for Fluorescein detection. Follow the instructions given above (Standard Detection Procedures) for use of these reagents in double antibody enhanced detections.

NOTE: Continue to use phosphate buffered saline (ENZO Cat. No. 33802, ENZO Wash Buffer Salts) when using horseradish peroxidase. Be sure to switch to Tris buffered saline, (ENZO Cat. No. 33803, *SignaSure*® Wash Buffer) when using alkaline phosphatase for ultimate colorimetric development. Follow instructions included with these *DETEK*® reagents for colorimetric development of the slides.

For Technical Assistance call ENZO:
Toll free from the U.S. and Canada: 1-800-221-7705
All others: 631-694-7070
Fax: 631-694-7501

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HYBRIDIZATION AND DETECTION PROCEDURES FOR NICK TRANSLATED DNA PROBES

In Situ Hybridization Applications

Nick translated DNA probes are extremely well suited for *in situ* hybridization applications. For ease of use, ENZO offers a complete line of ready-to-use *Simply Sensitive*™ *In Situ* Detection Systems for use with Biotin-labeled probes. The Systems provide reagents for detection using either horseradish peroxidase development (colorimetric with either Aminoethylcarbazole and H_2O_2, Cat. No. 32830, or Diaminobenzidine and H_2O_2, Cat. No. 32840) for high-target-level applications or alkaline phosphatase development (colorimetric with Nitroblue tetrazolium, NBT, and 5-Bromo-4-chloro-3-indolyl phosphate, BCIP, Cat. No. 32870, or Iodonitrotetrazolium violet, INT, and BCIP, Cat. No. 32860) for low-target-level applications. The systems also provide for fluorescence detection of biotin-labeled probes with fluorescein-conjugated streptavidin, Cat. No. 32850. Each system provides sufficient reagents for the detection of 20 slides. In addition, for extra high sensitivity, each of the *in situ* detection systems is available in a ready-to-use *UltraSensitive*™ *Enhanced In Situ* format for the development of up to 30 slides.

For economy and specific developmental studies, use *DETEK*® Hrp or *DETEK*® Alk Signal Generating Systems (Cat. Nos. 43820 or 43822) for colorimetric *in situ* detection of biotin-labeled probes. Use *DigDETEK*® Hrp or *DigDETEK*® AP Signal Generating Systems (Cat. Nos. 43830 and 43832) for colorimetric *in situ* detection of probes labeled with digoxigenin. Use *FluorDETEK*® Hrp or *FluorDETEK*® AP Signal Generating Systems (Cat. Nos. 43840 or 43842) for colorimetric *in situ* detection of probes labeled with fluorescein.

For Biotin-, Digoxigenin- and Fluorescein-labeled Nick Translated probes, most *in situ* hybridizations and detections can be carried out as outlined here.

In Situ Hybridization with Nick Translated Probes:

1. **Preparation of Slides**

   Choose a reliable method for preservation and fixation of the cells and/or tissue to be analyzed. Use ENZO *PathoGene*® Tissue Preparation Kit, Cat. No. 32800, for preparation and proteinase pre-treatment of formalin-fixed, paraffin-embedded tissue specimens. If using a horseradish peroxidase detection system, inactivate endogenous peroxidases by pre-treatment with a Quench Reagent such as provided in the ENZO *PathoGene*® Tissue Preparation Kit.

2. **Denaturation of Probe and Target DNA and Hybridization**

   A typical hybridization buffer for *in situ* hybridization of nick translated probes consists of 50% deionized formamide, 2X SSC or SSPE, 5% Dextran Sulfate plus 0.5-5 μg/ml labeled DNA probe (or, use ENZO *In Situ* Hybridization Buffer, Cat. No. 33808).

   To hybridize to target DNA in fixed, pre-treated tissues and cells, place a drop of probe hybridization mix on the specimen and cover with a coverslip. Heat the slides on a heat block set to 95°C for 8-10 minutes for tissue specimens and 4-5 minutes for cellular specimens.

   Remove the slides to a 37°C slide warmer and incubate for 60 minutes up to 24 hours (depending upon the probe concentration). (Note: Extended hybridization times may require use of an humidified chamber.)

3. **Post Hybridization Washing**

   After hybridization, remove the coverslip from the slide and soak the slide in a buffered saline solution (phosphate buffered saline for horseradish peroxidase developments, ENZO Cat. No. 33802; Tris buffered saline for alkaline phosphatase developments, ENZO Cat. No. 33803) for 5 minutes at room temperature.

   Remove the slide from the buffer and wipe around the specimen. Place 0.5 ml to 1 ml of a Post Hybridization Wash Solution (typically, 0.2X to 1X SSC, 50% deionized formamide) (use ENZO *In Situ* Hybridization Wash Reagent, Cat. No. 33809) on the slide and incubate the slide at 37°C for 10 to 20 minutes.

   Soak the slides 2 to 3 times in buffered saline for 1 to 2 minutes to rinse off the Post Hybridization Wash Solution.

   The slides are now ready for the Detection Procedure.

Detection of Nick Translated Probes following *in situ* Hybridizations:

1. **Incubation with Detection Reagent**

   Dilute the appropriate Detection Reagent as follows (use ENZO *In Situ* Dilution Buffers Cat. Nos. 33804 or 33805):

   Horseradish Peroxidase detections using *DETEK*® Hrp, *DigDETEK*® Hrp or *FluorDETEK*® Hrp Signal Generating System**:** Dilute 100- to 500-fold in:

   10mM phosphate buffer, pH 7.0 to 7.2
   0.15M NaCl
   0.025% Triton X-100
   0.3% gelatin

   Alkaline Phosphatase detections using *DETEK*® Alk, *DigDETEK*® AP or *FluorDETEK*® AP Signal Generating System: Dilute 100- to 500-fold in:

   0.1M Tris Buffer, pH 7.5
   0.1M NaCl
   0.1mM $ZnCl_2$
   0.03% BSA

   Remove the slide from buffer and wipe around the specimen. Place 0.5 ml to 1 ml of diluted detection reagent on the slide and incubate the slide at 37°C for 30 to 60 minutes

2. **Post-Detection Washing**

   Using a squeeze (wash) bottle filled with the appropriate buffered saline solution (phosphate buffered saline for horseradish peroxidase detections and Tris-buffered saline for alkaline phosphatase detections), rinse the slide with an even stream of wash buffer for 10 seconds. Soak the slides 2 to 3 times in buffered saline for 1 to 2 minutes to rinse off the Detection Reagent.

3. **Colorimetric Development**

   Horseradish Peroxidase Detections: Place 0.5 ml to 1 ml of 0.01M sodium acetate buffer, pH 5.0, 0.04M NaCl, containing 0.025% H_2O_2, and 0.2 mg/ml Aminoethylcarbazole (for enhanced results, use ENZO Cat. No. 43825 for AEC/H_2O_2 color reactions, or use Cat. No. 43826 for Diaminobenzidine/H_2O_2 color reactions) on the slide and incubate for 20 to 30 minutes at 37°C.

   Alkaline Phosphatase Detections: Place 0.5 ml to 1 ml of 0.1M Tris Buffer, pH 9.5, 0.1M NaCl, 0.01M $MgCl_2$, containing 0.165 mg/ml NBT and 0.33 mg/ml BCIP, on the slide and incubate for 20 to 40 minutes at 37°C, protected from direct bright light (use ENZO Cat. No. 43827 for NBT/BCIP color reactions, or use Cat. No. 43828 for INT/BCIP color reactions).

4. **Termination of Color Development and Preservation of the Slides**

   Soak the slides in buffered saline for 1 to 2 minutes to rinse off the reaction mixtures. Counterstain and mount the slides as desired.

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