For chromogenic in situ detection of DNA in cells and tissues

DNADetector™

Chromogenic *In situ* Hybridization Kit

HRP Detection System

Catalog Number
60-03-00

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INTENDED USE

KPL's DNADetector™ Chromogenic *in situ* Hybridization Kit is designed for non-radioactive *in situ* detection of DNA in fixed cells and tissue sections using biotin-labeled DNA probes, enzyme-streptavidin conjugate and chromogenic substrate. Applications include detection and localization of viral infection in cells and tissues and detection of integration of exogenous DNA into the genome of cells.

INTRODUCTION

In situ hybridization was first described for the localization of nucleic acids in tissues, chromosomes and nuclei by Pardue and Gall[1]. The method comprises three basic steps: fixation of a specimen (cells or tissue) on a microscope slide, hybridization of labeled probe to homologous fragments of genomic DNA and enzymatic detection of the tagged probe:target hybrids.

The DNADetector Kit utilizes a rapid and easy to use biotin-streptavidin system for highly sensitive, non-radioactive, chromogenic detection of DNA *in situ*. A biotin-labeled nucleic acid probe is hybridized to target DNA on a microscope slide containing fixed cells or tissue. The biotin is recognized and binds with high affinity to streptavidin labeled with horseradish peroxidase (HRP). The enzymatically tagged hybridization event is detected using a chromogenic substrate specific for HRP, which deposits a blue precipitate at the site on the specimen where hybridization has occurred. Finally, the slide is counterstained with a red dye to provide maximum contrast to the blue signal.

Due to the diversity of specific cells, tissues and probe hybridization conditions, it may be necessary to optimize tissue fixation and deproteinization, slide preparation and hybridization conditions for your system. Specific recommendations are discussed in the appropriate section below.

MATERIALS AND EQUIPMENT

NOTE: Kit components require multiple storage temperatures. Please note the appropriate storage conditions for each component and place the components at the recommended temperature immediately upon receipt of the kit. The control slide provided in the kit may be stored at 2 - 8°C for up to 2 weeks, for long term stability it should be stored at -20°C.

The DNADetector™ Chromogenic *in situ* Hybridization Kit is packaged in two separate boxes in order to store and ship the components at the appropriate temperatures.

60-03-00-A contains:

Kit Component	Catalog Number	Volume	Storage Temperature
Hybridization Buffer (2X)	50-86-01	1 x 1.0 mL	2 - 8°C
Formamide	50-86-02	1 x 1.0 mL	2 - 8°C
DEPC-Treated H_2O	50-86-03	1 x 1.0 mL	2 - 8°C
Control Probe Hybridization Cocktail	60-00-08	1 x 50 µL	2 - 8°C
Streptavidin-HRP Conjugate (amber vial)	474-3002	1 x 0.1 mL	2 - 8°C
Conjugate Diluent Buffer	50-82-03	1 x 5.0 mL	2 - 8°C
Biotin Wash Solution Concentrate (10X)	50-63-09	3 x 200 mL	2 - 8°C
Control Slide	60-00-01	1 slide	-20°C

60-03-00-B contains:

Kit Component	Catalog Number	Volume	Storage Temperature
20X SSC	50-86-04	1 x 500 mL	Room Temperature
TrueBlue™ Peroxidase Substrate	71-00-68	1 x 50 mL	Room Temperature
Orcein (nuclear counterstain)	71-01-00	1 x 25 mL	Room Temperature
Eosin Y (10X) (cytoplasmic counterstain)	71-02-00	1 x 10 mL	Room Temperature

The TrueBlue Substrate may appear light blue to light yellow/orange after extended storage. Product stability and performance are not affected by variations in solution color.

Sufficient reagents are provided for the hybridization and detection of 50 samples. Reagents are stable for a minimum of one year when stored as directed.

REQUIRED SUPPLIES AND EQUIPMENT NOT INCLUDED

- Glass Coplin jar or equivalent, for high temperature incubations (above 37°C)
- Plastic or glass Coplin jars or equivalent, for room temperature incubations
- Forceps
- Gloves
- Humidified chamber (see Preparation of Reagents, page 9)
- Water baths at 37°C and 60°C
- Incubator or heat block at 95°C
- Biotin-labeled nucleic acid probe
- Micropipettors and sterile tips
- Autoclaved 1.5 mL microfuge tubes
- Autoclaved or sterile-filtered (0.2 µ) molecular biology grade H_2O
- Treated microscope slides
- Rubber cement or HybCovers and HybHolder
- Organic mounting media, such as Permount (Fisher Scientific)
- Glass coverslips and parafilm
- Microscope

Additional Supplies Needed for Tissue

- Xylene
- Reagent grade ethanol solutions (100%, 80%, 70% and 50%)
- PBS

WARNINGS AND PRECAUTIONS

1. Read ALL instructions thoroughly before using the kit.
2. Wear gloves when handling kit components and slides. Some components of the kit are sensitive to DNA degrading enzymes such as DNases.
3. Some kit components contain hazardous materials (formamide, acetic acid or microcide). Handle these reagents using accepted laboratory safety procedures. Dispose of hazardous chemicals according to local, state and federal regulations.
4. The streptavidin conjugate, substrate and counterstains are light sensitive. Keep caps closed and avoid prolonged exposure to light.
5. For slide washes at temperatures above 37°C, the use of glass Coplin jars is recommended to achieve proper temperature equilibration of slides.

6. Proper temperatures of heat blocks, water baths and slide warmers are very important. It is necessary to achieve a temperature of at least 95°C for the specified time in order to denature the probe and target DNA.
7. Keep all reagents at the designated temperatures stated on the labels to maintain and prolong shelf life.
8. Do not allow slides to dry out during the hybridization and detection procedures.

EXPERIMENTAL DESIGN

1. **Slide Preparation**

   In order to ensure that specimens will remain attached to the slide during the *in situ* hybridization protocol, it is recommended that you pre-treat or "sub" your slides with an adhesive agent before mounting specimens. Reagents such as poly-L-lysine[12], 3-aminopropyltriethoxysilane (AES) and gelatin[13] have all been successfully used as subbing agents. The outline for AES treatment is as follows:

   a. Incubate clean, dry glass slides in 2% AES (Aldrich) in acetone for 2 minutes.
   b. Rinse slides 2 times in dH_2O and air dry.
   c. Store slides at room temperature in a closed, dust-free container.
   d. Handle slides only by the corners.

2. **Cell Preparation**

   Cells can be applied to the slide using different techniques depending on the amount of sample available, the cell type being used, and the applicability of your system to different slide growth chambers and the availability of Cytospin centrifuges.

   Use of chamber slides for growth of cells will allow the optimization of growth conditions to obtain a well spread and dense monolayer of cells. This may facilitate the ability to detect multiple *in situ* hybridization signals in a sample. Once optimal growth results are obtained, the medium may be removed and the cells then fixed on the slide.

   Cells from cytological samples or cell cultures can be smeared on the slide to form a thin layer and then fixed on the slide. When only a small amount of cells are available it may be advantageous to concentrate the cells in a defined area of the slide using a Cytospin centrifuge. Cells are then fixed on the slide.

3. **Slide Fixation**

   Optimal fixation conditions are critical for successful *in situ* hybridization. The reagents used for fixation, as well as the length of time a specimen is fixed, must be determined empirically for each sample

type, as these parameters will influence morphological integrity and hybridization signal. There are two types of fixatives: precipitating fixatives and cross-linking fixatives. Precipitating fixatives, like ethanol or methanol/acetone, do not require further treatment with proteolytic enzymes. However, cells fixed by these methods are susceptible to loss of target nucleic acids. Cross-linking fixatives like formaldehyde or glutaraldehyde prevent loss of nucleic acids from their *in situ* origin, but subsequent proteolytic digestion is required in order to make the target nucleic acids accessible to probe. Further information on fixation may be found in references 9 - 11.

4. **Paraffin-Embedded Tissue Sample Preparation**

Paraffin-embedded, formalin/paraformaldehyde fixed tissue sections of 4 - 6 micron thickness may be mounted on slides using the following method:

a. Float paraffin-embedded tissue sections in clean, distilled water.
b. Dip a silanized slide under the tissue section and scoop the section onto the slide by lifting the slide and attached tissue section out of the water.
c. Gently remove any bubbles that may have become trapped under the section with a soft brush.
d. Allow the sections to dry on the slides.
e. Bake the slides for 1 - 2 hours at 90°C or at 65°C overnight.
f. Store slides at room temperature in a closed container.

5. **Deparaffinization and Rehydration of Cell or Tissue Sections**

Paraffin-embedded, mounted specimens must be deparaffinized, then pretreated with agents such as proteases, acids or detergents[2] to loosen the cross-links formed by fixatives and allow efficient access of the probe to the target.

Incubate the slides sequentially in the series of solutions specified below. All incubations should be performed in Coplin jars. Use fresh reagents for each batch of slides (8 - 10 slides). Gently agitate the jars every few minutes during each incubation.

Number	Reagent
1	Xylene
2	Xylene
3	95% Ethanol
4	95% Ethanol
5	80% Ethanol
6	70% Ethanol
7	50% Ethanol
8	molecular biology grade water

After the slides have been rehydrated, continue with protease digestion.

6. **Proteinase K Digestion of Cells and Tissue Sections Fixed With Cross-linking**

Fixatives
Optimal removal of proteins from specimens that have been fixed using cross-linking fixation methods is necessary to expose target nucleic acids for hybridization. The degree of deproteinization necessary must be determined empirically as it depends on the type of tissue used, the method of fixation, the thickness of the tissue section and whether the nucleic acid target is nuclear or cytoplasmic. Overdigestion may result in release of the target DNA from the tissue or loss of tissue morphology, while underdigestion prevents access of the probe to the target and will result in a weak hybridization signal. It is recommended that you titrate the amount of protease used and perform a time course to determine the optimal conditions for your samples. Proteases used in *in situ* hybridization include Proteinase K, pronase, trypsin and 0.2N HCl. Proteinase K is the universal deproteinization agent. Prepare Proteinase K and digest specimen following manufacturer's recommendations.

7. **Probe Preparation**
Use of this kit requires a biotin-labeled nucleic acid probe. A variety of methods are available for the biotinylation of nucleic acids for *in situ* hybridization. Riboprobes may be generated from RNA with the addition of a biotinylated ribonucleotide triphosphate during *in vitro* transcription[2]. Fragments of DNA greater than 100 nucleotides in length may be labeled enzymatically either by nick translation[3 – 4], random priming[5] or PCR biotinylation[6 – 7]. Oligonucleotides can be labeled during their synthesis by covalent attachment of a biotin or after synthesis by enzymatic end-labeling. DNA may also be labeled using a photoactivated biotin analog[8].

It is important that the probe generated is between 20 - 30 bases if it is an oligo and 50 - 1000 base pairs in length if it is a genomic probe. The probe needs to be labeled in such a way that it has the optimal number of biotin groups to allow for greatest signal, yet not too many biotins that they interfere with hybridization or with the binding of streptavidin to the biotin. If high background is encountered, it may be due to the presence of the labeling components. Purification of the biotinylated probe over a gel filtration column specifically designed for biotinylated DNA (such as SELECT-B, G-50 column from 5 Prime → 3 Prime, Inc.) is recommended.

8. **Hybridization :**

Due to the unique melting temperatures and hybridization properties of different probes and target sequences, it will be necessary to optimize hybridization conditions for each probe and target specimen you use. The use of formamide lowers the melting temperature of the nucleic acid:nucleic acid hybrids, so hybridizations may be performed at lower temperatures. When the formamide concentration is increased and the hybridization temperature remains stable, the stringency of hybridization is increased. Formamide is supplied separately from the rest of the hybridization cocktail to allow for the optimization of hybridization conditions.

In addition to modifying formamide concentration, it may also be necessary to optimize the temperature at which you perform the hybridization reactions. Too low or non-stringent hybridization temperatures may result in high background due to non-specific hybridization or hybridization to shorter than desired stretches of homology.

Hybridization temperatures which are too high may result in loss of the ability of the probe to hybridize to the target at all. In addition, prolonged exposure of cells and tissue specimens to temperatures above 50°C may result in morphological alterations and structural abnormalities. Also, keep in mind that detection of single-copy genes is more difficult than detecting multi-copy genes. The hybridization conditions and probe concentration may need to be optimized depending upon the abundance of your target gene.

RESULTS

Areas of positive activity should appear light blue to purple, with little to no staining in areas where antigen is not present. For long-term preservation of results, store slides in the dark.

- ♦ Always include positive and negative controls.
- ♦ To decrease background staining, include detergents (NP-40 or Triton X) in the antibody diluent and wash, or use hypertonic wash buffer[4].
- ♦ If ANY of the following results are seen, antibody concentration (primary, secondary or HRP-Streptavidin) MUST be reduced:
  - * Color fades or floats off section during wash or dehydration
  - * Overall high background.
  - * Excessively dark or clumped staining
  - * Particles of dye scattered over the section

SOLUTION PREPARATION

0.1M Tris-HCl:

a. Dissolve 121 g Tris in 500 mL reagent quality water.
b. Adjust pH to 7.6 with 2M HCl (approximately 300 mL).
c. QS to 1 Liter with reagent quality water to obtain 1M stock solution.
d. Dilute 1 part stock solution from Step c. with 9 parts reagent quality water and mix well.

0.01M Phosphate Buffered Saline (PBS):

a. Dissolve 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 500 mL reagent quality water.
b. Adjust pH to 7.4 with 2M HCl.
c. QS to 1 Liter with reagent quality water.

PREPARATION OF REAGENTS

1. **2X SSC:** Dilute 20X SSC 1/10 in autoclaved or sterile-filtered water. 2X SSC is stable at room temperature for 1 year.

2. **Hybridization Cocktail:** The amount of hybridization cocktail necessary for each reaction depends on the size of the specimen. A volume of 10 - 50 µL is usually sufficient to cover most samples.
   In order to allow optimization of hybridization conditions, hybridization cocktail is supplied in three components: 100% Formamide (amber vial), 2X Hybridization Buffer (yellow cap) and DEPC Water (white cap). The stringency of hybridization can be optimized by modifying the final concentration of formamide between 0 - 50% in the hybridization cocktail.
   50% formamide is most commonly used for hybridization cocktail. To prepare a 50% formamide hybridization cocktail: mix equal volumes 2X Hybridization Buffer and Formamide (i.e. 50 µL 2X Hybridization Buffer + 50 µL Formamide = 100 µL of hybridization cocktail). If a lower formamide concentration is desired, decrease the amount of formamide and adjust the final volume with DEPC water (i.e. to prepare a 20% formamide hybridization cocktail: mix 50 µL 2X Hybridization Buffer + 20 µL Formamide + 30 µL DEPC Water = 100 µL of hybridization cocktail). If formamide is not needed in the hybridization cocktail, mix equal volumes of 2X Hybridization Buffer and DEPC Water (i.e. 50 µL 2X Hybridization Buffer + 50 µL DEPC Water = 100 µL of hybridization cocktail).

3. **Probe Hybridization Cocktail:** It is recommended that you use biotinylated probe at a concentration of 1.0 ng/µL. Depending upon the size of your specimen, you may need 10 - 50 µL of hybridization cocktail. The probe should take no more than 5% of the final volume of the cocktail.

If your probe is too dilute, place 20 ng of probe in a microfuge tube and dry the probe down using a speed vac or lyophilizer. Add 10 µL of 2X hybridization buffer to the microfuge tube containing the dry DNA. Mix the solution gently to resuspend the DNA, then add the desired amount of formamide.

Note: Detecting single-copy genes is more difficult than detecting multi-copy genes. The hybridization conditions and probe concentration may need to be optimized depending on the abundance of your target gene and the specific activity of your biotinylated probe.

4. **Humidified Chamber:** It is important to perform some incubations in a humidified chamber to prevent evaporation of reagents and dehydration of the specimen. A humidified chamber can be constructed using a small box with a lid. Line the bottom of the box with a layer of paper towels soaked with water. To construct an elevated area to place slides, place two pipettes or rods in parallel on the towels. Close the lid on the chamber and incubate at the appropriate temperature in an incubator to equilibrate. Place the slides inside the box across the pipettes. Alternatively, if you are using a covered-water bath for incubations, a tray filled with a foam block can be used. Soak the foam block with water and place the slides on the foam. Place the lid over the water bath.

CONTROL SLIDE HYBRIDIZATION AND DETECTION PROTOCOL

A 2-well Control Slide and Control Hybridization Cocktail are included to facilitate familiarization with the methodology utilized and to serve as a test system for the type of results you should expect to see. It is recommended that you perform this control assay before attempting use of the kit for your test specimens.

Control Slide Hybridization

1. Remove Control Slide from envelope. Save the drying paper provided with the slide for later use.
2. Pre-warm a humidified chamber or water bath to 37°C. Pre-warm a water bath to 60°C.
3. Prepare 50 µL hybridization cocktail as follows:

2X Hybridization Buffer (yellow cap)	25 µL
Formamide (amber vial)	25 µL
Total Volume	50 µL

4. PreHybridization: Apply 20 µL hybridization cocktail to both the (+) and (-) wells. Save the remaining 10 µL hybridization cocktail for the "no probe" control well (-).
5. Incubate for 30 minutes in a 37°C waterbath on top of a foam float or in a humidified chamber.
6. Pre-warm an incubator or oven to 95°C.

7. Shake off the hybridization cocktail from the slide and blot dry using the drying paper provided.
   Note: Be careful not to touch the inner surface of the well with the paper.
   Note: The Control Probe Hybridization Cocktail vial (blue cap) contains a small volume of solution. Before opening, spin the vial briefly in a microcentrifuge to concentrate the solution to the bottom of the tube.
8. Hybridization: Add 10 µL of the Control Probe Hybridization Cocktail (blue cap) to the (+) well. Add the remaining 10 µL of the prepared hybridization cocktail (from step 2) to the (-) well. Cover the slide with a gasketed HybCover and place in a HybHolder. Alternatively, the slide may be covered with a coverslip.
9. Place the slide at 95°C and incubate for 2 minutes to denature the target and probe DNA.
10. Transfer the slide to a 37°C humidified chamber and incubate for 1 hour.
11. Immediately after starting hybridization, make enough 2X SSC for two incubations in Coplin jars. Pre-warm 2X SSC to 60°C in one glass Coplin jar in a water bath. Place the other aliquot of 2X SSC in a Coplin jar at room temperature.
12. Following hybridization, remove the cover from the slide and place the slide in the Coplin jar containing 2X SSC at 60°C for 5 minutes.
13. Transfer the slide to the Coplin jar containing 2X SSC at room temperature and incubate for 5 minutes.

Control Slide Detection

1. Dilute the HRP-Streptavidin (amber vial, 474-3002) 1/100 in Conjugate Diluent Buffer (i.e. 1.0 µL HRP-SA + 100 µL conjugate diluent Buffer). Prepare enough to add 20 - 50 µL per sample.
2. Shake off the 2X SSC from the slide, and blot the slide with drying paper. Do not allow the specimens to dry.
3. Immediately add 20 - 50 µL of the diluted HRP-Streptavidin to the sample. Place a HybCover, coverslip or piece of parafilm over the specimen to prevent evaporation. Place slide in a 37°C humidified chamber for 20 minutes.
4. Prepare 1X Biotin Wash Solution (dilute Biotin Wash Solution Concentrate 1/10 in autoclaved or sterile-filtered water, i.e. 10 mL Biotin Wash Solution Concentrate + 90 mL H_2O). Dispense to 3 Coplin jars at room temperature.
5. Wash slides 5 minutes in each successive Coplin jar. Shake off Biotin Wash Solution from the slide and blot dry.
6. Apply TrueBlue™ to the slide, making sure to cover the edges of the specimen.
7. Observe the development of the color under the microscope to determine the optimal development time. Development should be optimal within 2 - 3 minutes at room temperature.

8. When the signal has reached the desired intensity, stop color development by soaking the slide in a Coplin jar containing picopure water for 5 - 10 seconds.
9. Air dry the slide for at least 5 minutes.
10. Counterstain the sample as described below, using Orcein to stain nuclei or Eosin Y to stain cytoplasm.

COUNTERSTAIN

Nuclear Counterstain:

1. Apply Orcein to the slide, making sure to cover the edges of the specimen.
2. Incubate for 3 - 5 minutes at room temperature.
3. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
4. Air dry slide for 30 minutes.
5. Dehydrate and mount slide as described below.

Cytoplasmic Counterstain:

1. Prepare enough 1X Eosin Y to cover the specimen (approximately 0.5 mL per slide). Dilute 10X Eosin Y Concentrate 1/10 in autoclaved picopure water (i.e. 50 µL Eosin Y Concentrate + 450 µL H_2O). Mix well and store at room temperature.
2. Apply 1X Eosin Y to the slide, making sure to cover the edges of the specimen.
3. Incubate for 1 - 2 minutes at room temperature.
4. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
5. Air dry slide for 30 minutes.
6. Dehydrate and mount slide as described below.

Dehydrate And Mount Slide:

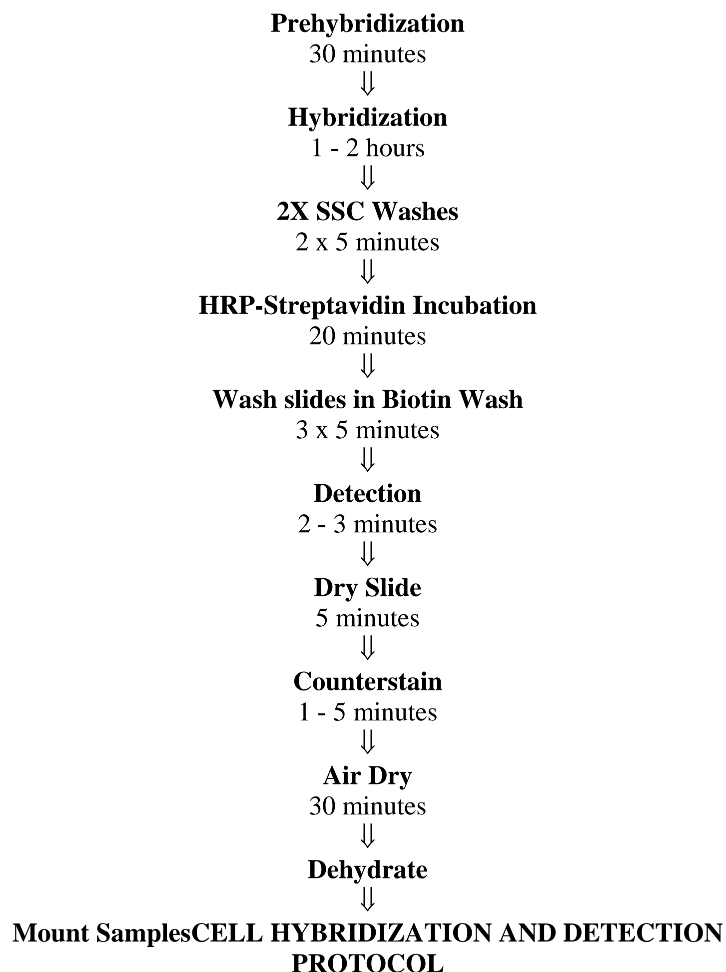
1. Dip slide 5 times in a Coplin jar containing 95% EtOH. Transfer to a second Coplin jar containing 95% EtOH and dip 5 more times. Let the ethanol evaporate.
2. Dip slide in 2 changes of xylene. Blot the excess xylene from the slide.
3. Mount with an organic mounting media, such as Permount and coverslip.
4. Let the mounted slide dry overnight.
5. View the slide under a microscope.

CONTROL SLIDE EXPECTED RESULTS

There should be no significant background staining. The infected cells in the (+) well should be dark blue to purple. If Orcein counterstain was used, nuclear material not bound by probe should appear light to dark pink. If Eosin Y counterstain was used, the cytoplasm should be stained light to dark red. The (-) well should appear pink to red due to the absence of probe in the hybridization cocktail.

CELL HYBRIDIZATION AND DETECTION FLOW CHART

Total time: 3 - 4 hours



CELL HYBRIDIZATION AND DETECTION PROTOCOL

Cell Hybridization

1. Prepare cells as described in Cell Preparation (page 5).
2. Pre-warm a humidified chamber or water bath to 37°C. Pre-warm a water bath to 60°C.
3. Prepare hybridization cocktail (see Preparation of Reagents, page 9). Make enough for both prehybridization and hybridization. Allow about 10 - 50 µL per specimen, per treatment.
4. Prehybridization: Apply 10 - 50 µL hybridization cocktail to each sample. Save the remaining hybridization cocktail for addition of the biotin-labeled probe (step 9).

5. Incubate for 30 minutes in a 37°C waterbath on top of a foam float or in a humidified chamber.
6. Shake off hybridization cocktail from the slide and blot the slide with drying paper.
7. Note: Be careful not to touch the specimen with the paper.
8. Pre-warm an incubator or oven to 95°C.
9. Hybridization: Add biotinylated probe to the remaining hybridization cocktail to a concentration of 1.0 ng/µL (see Preparation of Reagents, page 9). Apply 10 - 50 µL hybridization cocktail containing probe to the specimen.
10. Cover the slide with a gasketed HybCover and put the slide in a HybHolder. Alternatively, the slide may be covered with a coverslip.
11. Place the slide at 95°C and incubate for 2 minutes to denature the target and probe DNA.
12. Transfer the slide to a 37°C humidified chamber and incubate for 1 - 2 hours.
13. Immediately after starting hybridization, make enough 2X SSC for two incubations in Coplin jars. Pre-warm 2X SSC to 60°C in one glass Coplin jar in a water bath. Place the remaining 2X SSC in a Coplin jar at room temperature.
14. Following hybridization, remove the cover from the slide and place the slide in the Coplin jar containing pre-warmed 2X SSC at 60°C for 5 minutes.
15. Transfer the slide to the Coplin jar containing 2X SSC at room temperature and incubate for 5 minutes.

Cell Detection

1. Dilute the HRP-Streptavidin (amber vial, 474-3002) 1/100 in Conjugate Diluent Buffer (i.e. 1.0 µL HRP-SA + 100 µL Conjugate Diluent Buffer). Prepare enough to add 20 - 50 µL per sample.
2. Shake off the 2X SSC from the slide and blot the slide with drying paper. Do not allow the specimens to dry.
   Note: Be careful not to touch the specimen with the paper.
3. Immediately add 20 - 50 µL of the diluted HRP-Streptavidin to the samples. Place a HybCover, coverslip or piece of parafilm over the specimen to prevent evaporation. Place slide in a 37°C humidified chamber for 20 minutes.
4. Prepare 1X Biotin Wash Solution (dilute Biotin Wash Solution Concentrate 1/10 in autoclaved or sterile-filtered water, i.e. 10 mL Biotin Wash Solution Concentrate + 90 mL H_2O). Dispense to 3 Coplin jars at room temperature.
5. Wash slides 5 minutes in each successive Coplin jar. Shake off Biotin Wash Solution from the slide and blot dry.
6. Apply TrueBlue to the slide, making sure to cover the edges of the specimen.

7. Observe the development of color under the microscope to determine the optimal development time. Development should be optimal within 2 - 3 minutes at room temperature.
8. When the signal has reached the desired intensity, stop color development by soaking the slide in a Coplin jar containing picopure water for 5 - 10 seconds.
9. Air dry the slide for at least 5 minutes.
10. Counterstain the sample as described below, using Orcein to stain nuclei or Eosin Y to stain cytoplasm.

COUNTERSTAIN

Nuclear Counterstain:

1. Apply Orcein to the slide, making sure to cover the edges of the specimen.
2. Incubate for 3 - 5 minutes at room temperature.
3. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
4. Air dry slide for 30 minutes.
5. Dehydrate and mount slide as described below.

Cytoplasmic Counterstain:

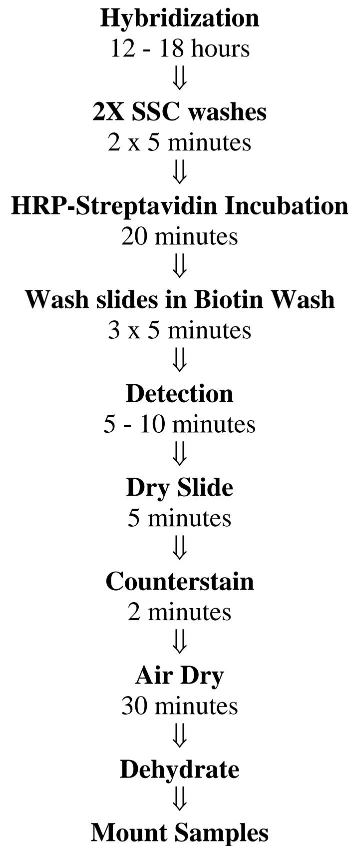
1. Prepare enough 1X Eosin Y to cover the specimen (approximately 0.5 mL per slide). Dilute 10X Eosin Y Concentrate 1/10 in autoclaved picopure water (i.e. 50 µL Eosin Y Concentrate + 450 µL H_2O). Mix well and store at room temperature.
2. Apply 1X Eosin Y to the slide, making sure to cover the edges of the specimen.
3. Incubate for 1 - 2 minutes at room temperature.
4. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
5. Air dry slide for 30 minutes.
6. Dehydrate and mount slide as described below.

Dehydrate And Mount Slide:

1. Dip slide 5 times in a Coplin jar containing 95% EtOH. Transfer to a second Coplin jar containing 95% EtOH and dip 5 more times. Let the ethanol evaporate.
2. Dip slide in 2 changes of xylene. Blot the excess xylene from the slide.
3. Mount with an organic mounting media, such as Permount and coverslip.
4. Let the mounted slide dry overnight.
5. View the slide under a microscope.

TISSUE HYBRIDIZATION AND DETECTION FLOW CHART

Total time: 14 - 20 hours (includes 12 - 18 hour hybridization)



TISSUE HYBRIDIZATION AND DETECTION PROTOCOL

Tissue Hybridization:

1. Prepare tissue sections as described in Tissue Preparation (page 6).
2. Pre-warm a heat block or oven to 95°C. Pre-warm a humidified chamber or water bath to 37°C.
3. Prepare sufficient hybridization cocktail for each sample (see Preparation of Reagents, page 9). Allow about 10 - 50 µL per specimen, per treatment.
4. Hybridization: Add biotinylated probe to the hybridization cocktail (see Preparation of Reagents, page 9). Apply 10 - 50 µL hybridization cocktail containing biotinylated probe to the specimen. No prehybridization is required.
5. Cover the sample with a gasketed HybCover and put the slide in a HybHolder. Alternatively, cover the slide with a coverslip and seal the slide around the edges with rubber cement.

6. Place the slide at 95°C and incubate for 10 minutes to denature the probe and target DNA simultaneously.
7. Immediately transfer the slide to a 37°C humidified chamber and incubate for 12 - 18 hours.
8. The next day: Pre-warm a water bath to 60°C. Before proceeding to post-hybridization washes, make enough 2X SSC for two incubations in Coplin jars. Pre-warm 2X SSC to 60°C in one glass Coplin jar in a water bath. Place the remaining 2X SSC in a Coplin jar at room temperature.
9. Remove the HybCover or sealed coverslip from the slide and place the slide in the pre-warmed glass Coplin jar containing 2X SSC at 60°C for 5 minutes.
10. Transfer the slide to the Coplin jar containing 2X SSC at room temperature and incubate for 5 minutes.

Tissue Detection:

1. Dilute the HRP-Streptavidin (amber vial, 474-3002) 1/100 in Conjugate Diluent Buffer (i.e. 1.0 µL HRP-SA + 100 µL Conjugate Diluent Buffer). Prepare enough to add approximately 100 µL per sample.
2. Shake off the 2X SSC from the slide and blot the slide with drying paper. Do not allow the specimens to dry.
3. Note: Be careful not to touch the specimen with the paper.
4. Immediately add 100 µL of the diluted HRP-Streptavidin to the samples. Place a HybCover, coverslip or piece of parafilm over the specimen to prevent evaporation. Place slide in a 37°C humidified chamber for 20 minutes.
5. Prepare 1X Biotin Wash Solution (dilute Biotin Wash Solution Concentrate 1/10 in autoclaved or sterile-filtered water, i.e. 10 mL Biotin Wash Solution Concentrate + 90 mL H_2O). Dispense to 3 Coplin jars at room temperature.
6. Wash slides 5 minutes in each successive Coplin jar. Shake off Biotin Wash Solution and blot dry.
7. Apply TrueBlue™ to the slide, making sure to cover the edges of the specimen.
8. Observe the development of the color under the microscope to determine the optimal development time. Development should be optimal within 5 - 10 minutes at room temperature.
9. When the signal has reached the desired intensity, stop color development by soaking the slide in a Coplin jar containing picopure water for 5 - 10 seconds.
10. Air dry the slide for at least 5 minutes.
11. Counterstain the sample as described below, using Orcein to stain nuclei or Eosin Y to stain cytoplasm.

COUNTERSTAIN

Nuclear Counterstain:

1. Apply Orcein to the slide, making sure to cover the edges of the specimen.
2. Incubate for 2 minutes at room temperature.
3. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
4. Air dry slide for 30 minutes.
5. Dehydrate and mount slide as described below.

Cytoplasmic Counterstain:

1. Prepare enough 1X Eosin Y to cover the specimen (approximately 0.5 mL per slide). Dilute 10X Eosin Y Concentrate 1/10 in autoclaved picopure water (i.e. 50 µL Eosin Y Concentrate + 450 µL H_2O). Mix well and store at room temperature.
2. Incubate for 3 - 5 minutes at room temperature.
3. Rinse 5 - 10 seconds in a Coplin jar containing picopure water.
4. Air dry slide for 30 minutes.
5. Dehydrate and mount slide as described below.

Dehydrate And Mount Slide:

1. Dip slide 5 times in a Coplin jar containing 95% EtOH. Transfer to a second Coplin jar containing 95% EtOH and dip 5 more times. Let the ethanol evaporate.
2. Dip slide in 2 changes of xylene. Blot the excess xylene from the slide.
3. Mount with an organic mounting media, such as Permount and coverslip.
4. Let the mounted slide dry overnight.
5. View the slide under a microscope

TROUBLESHOOTING

Problem	Possible Cause	Corrective Measure
No signal.	Target and Probe were not denatured.	• Check temperature of heating apparatus. • Increase denaturation time or temperature.
	Tissue or cells were over-fixed.	• Decrease length of fixation step. • Use another fixation methodology.

No signal.	Tissue was underdigested.	• Increase temperature, time or concentration of protease digestion reaction.
	Tissue was overdigested.	• Decrease temperature, time or concentration of protease digestion reaction.
	Poor hybridization reaction.	• Increase denaturation time or temperature. • Increase probe concentration. • Increase hybridization time. • Decrease hybridization temperature. • Modify concentration of formamide in hybridization cocktail.
	Post-hybridization wash too stringent.	• Decrease temperature or time of post-hybridization wash.
	Labeling of probe with biotin was insufficient.	• Repeat labeling reaction with fresh reagents.
Target specimen lost from slide.	Slide was pretreated improperly.	• Treat slide with AES. • Try another method of slide pretreatment.
	Tissue was overdigested.	• Decrease temperature, time or concentration of protease digestion reaction.

Problem	Possible Cause	Corrective Measure
Poor morphology.	Tissue was overdigested.	• Decrease temperature, time or concentration of protease digestion reaction.
	Tissue was overheated during denaturation step.	• Check the temperature of the heating apparatus. • Decrease the time or temperature of denaturation.
High background.	Endogenous peroxidase activity in tissue.	• Use Universal Block before incubation with streptavidin conjugate.
	Probe hybridizes to non-homologous sequences.	• Test probe in Southern Blot of the target genomic DNA. • Modify size of probe. • Use a different probe. • Increase stringency of post-hybridization washes. • Reduce probe concentration. • Increase hybridization temperature or formamide concentration.
	Tissue was allowed to dry during detection.	• Keep tissue wet during detection step.
	Endogenous biotin present in tissue.	• Include control sample with no probe in hybridization cocktail. • Block specimen with free streptavidin and then saturate streptavidin with biotin.

For additional support, contact KPL Technical Services at 800-638-3167 or 301-948-7755 or by e-mail at reagents at kpl.com.

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RELATED PRODUCTS

Product	Size	Catalog Number
Universal Block	100 mL	71-00-61
Detector™ Random Primer DNA Biotinylation Kit	30 reactions	60-01-00
Detector PCR DNA Biotinylation Kit	30 reactions	60-01-01
DNADetector™ Fluorescent in situ Hybridization Kit, CY™3/DAPI	30 reactions	60-05-00

REORDERING INFORMATION

The following components of the DNADetector Chromogenic *in situ* Hybridization Kit, HRP System are available for purchase separately. Please reorder using the following information:

Product	Size	Catalog Number
HRP-Streptavidin	1.0 mL	474-3000
TrueBlue Peroxidase Substrate	50 mL	71-00-64
Orcein	25 mL	71-01-01
20X SSC	1000 mL	50-86-05
Biotin Wash Solution (10X)	2 x 100 mL	50-63-06

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