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**DNA PROBE HYBRIDIZATION/DETECTION SYSTEM IN SITU KIT**  
**A Complete Hybridization & Immunodetection System**  
**Cat No. IH-60001 (IHD-0050): High Sensitivity 100 Slides**  
**Cat No. IH-60002 (IHD-0052): Ultra Sensitivity 100 Slides**

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**INSTRUCTION  
MANUAL**

ID-M10144  
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## I. INTRODUCTION

The DNA Probe Hybridization/Detection System - In Situ Kit is a complete hybridization and immunodetection system, incorporating the biotin-streptavidin amplification technology. Optimized for *in situ* hybridization, these user-friendly kits provide consistent results and maximum sensitivity to ensure economical and efficient use of the nucleic acid probes.

The DNA Probe Hybridization/Detection System is designed for the specific enzymatic detection of biotinylated DNA probes following hybridization to target DNA sequences

in tissue specimens. This kit can be used with a large variety of commercial or user labeled biotinylated probes. With the amplified system, stronger signal strength can be obtained with significantly smaller amounts of DNA. A high stringency hybridization solution is included for dilution of DNA probes.

For optimal results, please read and follow the instructions in this manual carefully. If you have any questions, contact Maxim Biotech Customer Service at (650) 871-1919.

## II. PRINCIPLE

In situ hybridization makes use of the high specificity of complementary nucleic acid binding to identify infectious agents in tissue sections<sup>1-5</sup>, to localize gene expression within individual cells<sup>6,7</sup>, or to detect specific DNA sequences in the genome of cells<sup>8,9</sup>. Briefly, the method involves deproteinization of fixed tissue sections mounted on slides, hybridization of the target nucleic acid sequence with a DNA or RNA probe, and detection of the hybridized probe to permit microscopic examination.

While early detection methods relied on the use of radioactive probes<sup>10,11</sup>, nonradioactive labeling methods have recently gained in popularity. The most widely used non-radioactive technique entails labeling the probe with biotin<sup>12,13</sup>. The hybridized probe is then detected by addition of enzyme-conjugated streptavidin followed by a suitable enzyme substrate, which produces a colored end product visible by light microscopy<sup>14</sup>. This conventional colorimetric reaction avoids the health hazards, disposal problems, and inherent instability of radiolabeled probes.

The DNA Probe hybridization/Detection System - In Situ Kit (High-Sensitivity and Ultra-Sensitivity) was developed at Maxim Biotech to provide the convenience of a colorimetric assay combined with sensitivity approaching that of radioactive methods.

For **High-Sensitivity Kit**: To detect a biotinylated probe hybridized to the target sequence, a streptavidin-AP

conjugate is applied. Upon addition of the single component BCIP/NBT solution (substrate), an intense blue signal appears at the specific site of the hybridized probe. This directly streptavidin-AP Conjugate linked to the biotinylated probe provides a rapid and highly sensitive detection method.

For **Ultra-Sensitivity Kit**: A higher sensitivity is obtained which is accomplished by amplification of the initial hybridization reaction using additional binding steps in which biotin is re-introduced into the system. These intermediate steps utilize the high affinity and high fidelity of antibody binding. To detect a biotinylated probe hybridized to the target sequence, an anti-biotin antibody (Linker 1) is first applied. This binds to the biotin on the hybridized probe. In order to re-introduce biotin into the system, an immunoglobulin (Linker 2) that is coupled with substantially higher numbers of biotin moieties than the probe is then added. Linker 2 recognizes and binds to the Linker 1 antibody. This additional layer initiates amplification of the signal.

To visualize the antibody/probe complex, streptavidin-AP conjugate is added next. Each biotin molecule on Linker 2, as well as any free biotin on the nucleic acid probe, is bound by a streptavidin moiety of the Conjugate. Each molecule of conjugate contains up to six alkaline phosphates molecules, providing further amplification of the reaction. Upon addition of the single component BCIP/NBT solution (substrate), an intense blue signal appears at the specific site of the hybridized probe.

### III. SUPPLIED MATERIALS AND REAGENTS

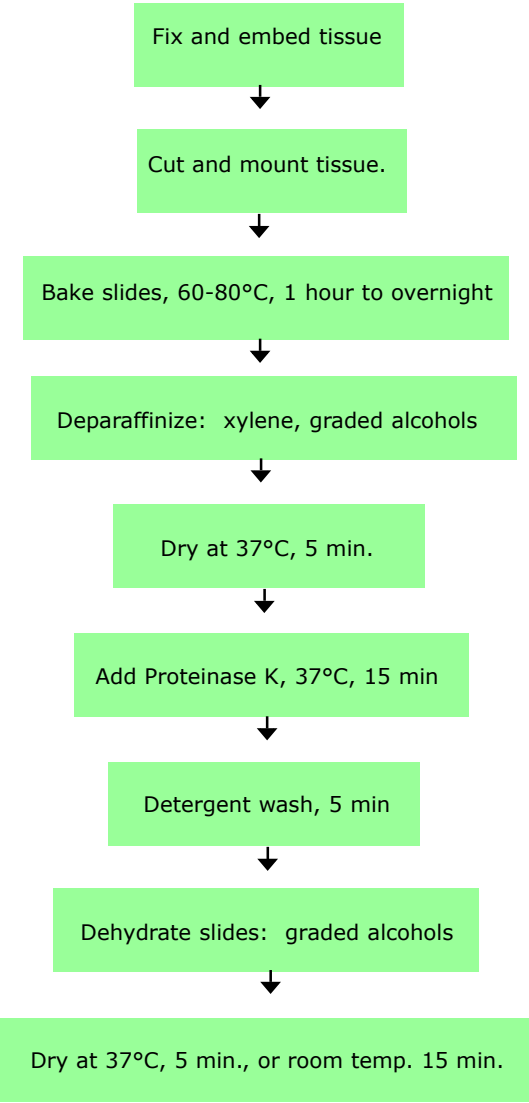
Kit Component	Container Color	Amount No.	Volume	IH-60001	IH-60002
Biotinylated Housekeeping (GAPDH) Gene Probe	blue	1 vial	1 ml	+	+
Proteinase K	brown	1 vial	4 mg	+	+
DNase and RNase free Dilution Buffer		1 vial	2 ml	+	+
Hybridization Solution	white	1 vial	6 ml	+	+
Protein Block (20X)		1 bottle	50 ml	+	+
Rnase A (15 ug/ml)	Purple	1 vial	6 ml	+	+
Mouse Anti-Biotin Linker 1	green	1 vial	6 ml	-	+
Biotin-Goat Anti-Mouse Ig Linker 2	yellow	1 vial	6 ml	-	+
Streptavidin-AP Conjugate	red	1 vial	6 ml	+	+
Substrate	brown	1 vial	6 ml	+	+
Detergent Wash Buffer (20 X)		1 bottle	100 ml	+	+
Instruction Manual				+	+

### IV. NEEDED MATERIALS AND REAGENTS

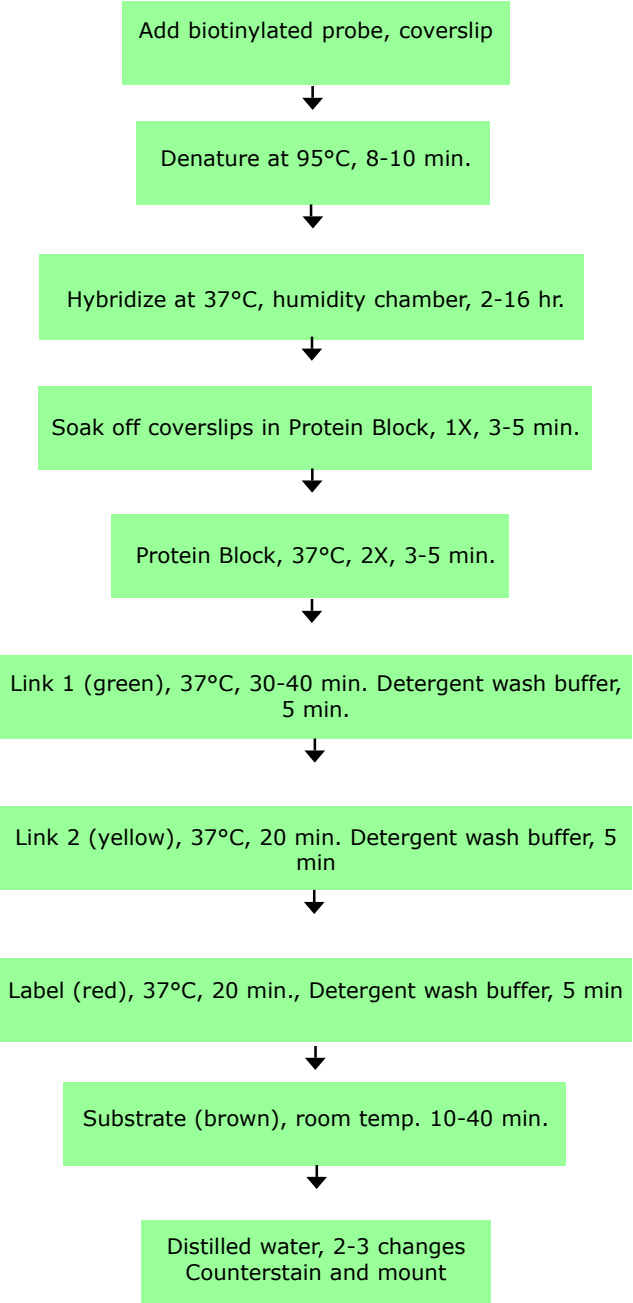
- Adhesive-coated microscope slides
- Oven(s) or incubator(s), 37°C - 45°C
- Forceps
- Coplin jars or staining dishes slide staining racks
- Timer
- Xylene
- Ethanol
- PBS
- Pipettes
- Your biotinylated DNA probes
- Test and positive control positive control tissue/cells (See Maxim catalog - positive control slide section)
- Distilled or deionized H<sub>2</sub>O
- Light microscope
- Coverslips
- Heating block or oven 95°C
- Humidity chamber
- Absorbent wipes
- Counterstain, nuclear fast red recommended
- Mounting medium

# V. SUMMARY OF HYBRIZATION/DETECTION PROCEDURE

**Figure 1**  
**Sample Preparation**



**Figure 2**  
**Hybridization and Detection**



## VI. PROCEDURE

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### A. PREPARATION OF REAGENTS:

#### 1. Proteinase K

- 10X concentration Proteinase K solution

To reconstitute the Proteinase K, add the entire contents (2 ml) of the DNase, RNase free dilution buffer (may precipitate during storage, warm it up before use) vial to the vial containing the lyophilized Proteinase K powder (4 mg), and mix gently. Any portion of the resulting 10X concentrated Proteinase K solution that is not to be used immediately should be divided into small aliquots and stored frozen at -20°C. Each 0.1 ml of 10X concentrated solution yields 1 ml of ready-to-use 1X Proteinase K solution sufficient for use on 5-10 slides.

- 1X Proteinase K solution

Remove aliquot(s) of 10X concentration Proteinase K solution from the freezer and allow to thaw. Dilute this solution 10 fold with deionized or distilled water. (Add 1 part of 10X concentration solution to 9 parts of water.) The resulting 1X Proteinase K solution is ready for immediate use. *NOTE:* The ready-to-use 1X Proteinase K solution is stable at room temperature for only one hour.

#### 2. Protein Block Buffer:

Dilute 50 ml of 20X concentrated Protein Block buffer into 1000 ml distilled water. The resulting 1X Protein Block buffer is ready to use and may be stored at 4°C.

#### 3. Detergent Wash Buffer

Dilute 50 ml of 20X concentrated Detergent wash buffer into 1000 ml distilled water. The resulting 1X Detergent wash buffer is ready to use and may be stored at room temperature. The concentrated solution supplied is sufficient to prepare 1 liters of 1X Detergent wash buffer. The 20X Detergent wash buffer may be precipitated during low temperature storage. However, it will not affect its performance. It may be stored at room temperature after diluting to 1X.

### B. SPECIMEN COLLECTION

This kit is designed for use with routine formalin-fixed, paraffin-embedded tissue sections and cold acetone-fixed tissue culture cell slides. For best results, an overnight grown tissue cultured cells should be fixed in 100% cold acetone for 10 minutes and specimens should be fixed in 10% neutral buffered formalin for 5 to 20 hours. Longer fixation may require prolonged incubation with Proteinase K. (See Tissue Preparation, section VI.C.6.) Over-fixation may result in weak staining of positive tissue. Tissue processing conditions should be standardized in order to obtain consistent, reliable results. Use of a positive control DNA probe which is provided in the kit against human genomic DNA is recommended to assess tissue processing. It will show dark blue positive signals on all human cells' origin on the nucleus. (See Quality Control, section VII.)

### C. TISSUE PREPARATION

1. Cut serial tissue sections 4-6 micron thick and float them in a protein-free water bath.
2. Carefully position the sections on slides that have been treated with a suitable slide adhesive: poly-L-lysine or silane.
3. Bake the slides in a vertical position at 60-80°C for 1 hour to overnight. Longer baking yields better adhesion of tissues to the slides.

## VI. PROCEDURE Continued

4. Deparaffinize the tissue sections by standard methods. As a suggested procedure, immerse the slides sequentially in the following solutions at room temperature for the indicated times.

Reagent	Incubation time (minutes)
Xylene	5
100% Ethanol	2
95% Ethanol	1
70% Ethanol	1
Deionized water	1

5. After the final rinse, allow the slide to dry completely by incubating them at 37°C for 5 minutes.
6. To each tissue section, apply 2-3 drops of freshly diluted 1X Proteinase K solution. (See section VI.A.1.) Be sure to cover and surround each section completely in order to achieve effective deproteinization. Incubate slides at 37°C for 10-15 minutes. *NOTE:* A 15 minutes digestion is satisfactory in Positive Control Slide and most cases. Variations in tissue processing may require that the incubation time be increased or decreased for optimal deproteinization and preservation of tissue morphology.
7. Dehydrate slides by immersing them sequentially in the following solutions at room temperature for the indicated times:

Reagent	Incubation time (minutes)
Distilled water	1
70% Ethanol	1
95% Ethanol	1
100% Ethanol	1

8. Dry the slides by incubating them at 37°C for 5 minutes. Figure 1 Summarizes the procedure for tissue preparation. The slides are now ready for hybridization and detection.

If necessary, slides may be stored dry for up to one month after tissue preparation. If slides have come into contact with any moisture, repeat the dehydration process (step 7) and dry the slides (step 8) before beginning the hybridization and detection procedure.

### D. HYBRIDIZATION AND DETECTION

*NOTE:* The tissue sections should not be allowed to dry out at any time during the dehydration and staining procedures. Protein block and Detergent wash buffer need to be prewarmed at 37°C before use.

All reagents used during hybridization and detection should be warmed to room temperature before use.

#### I. DNA in situ Hybridization:

1. Place 1 drop of the working DNA probe/hybridization solution on the tissue section. Place a coverslip over each slide. Be careful to avoid trapping any air bubbles.
2. Place the slides with coverslips in an oven or heating block at 95°C for 8-10 minutes to denature the DNA.
3. Place the slides in a humidity chamber and incubate at 37°C for 1-2 hours, or longer if desired, to allow hybridization of the probe with the target nucleic acid.

## VI. PROCEDURE Continued

4. Soak the slides in Protein Block Buffer at 37°C until the coverslips fall off. Be careful not to tear the tissue. Allow slides to remain in the buffer for 3 minutes after the coverslips are removed.
5. Wash the slide again with Protein Block (prewarmed) at 37°C for 3 min., 2X.
6. Carefully wipe excess buffer from around the tissue section. Place 1-2 drops of Linker 1 (green) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 40 minutes. (Skip For High-Sensitive Kit)
7. Tap off excess reagent. Rinse slides in Detergent wash buffer for 5 minutes. (Skip For High-Sensitive Kit)
8. Carefully wipe off excess buffer from around the tissue section. Put 1-2 drops of Linker 2 (yellow) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 20 minutes.
9. Tap of excess reagent. Rinse slides in Detergent wash buffer for 5 minutes. (Skip For High-Sensitive Kit)
10. Carefully wipe off excess buffer from around the tissue section. Place 1-2 drops of Conjugate (red) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 20 minutes.
11. Tap of excess reagent. Rinse slides in Detergent wash buffer for 5 minutes.
12. Carefully wipe excess buffer from around the tissue section. Place 1-2 drops of substrate (brown) on tissue section. Incubate at room temperature for about 10 minutes, or until color development is complete.
13. *NOTE:* Color development can be monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Color begins to appear after 3-5 minutes, usually reaching sufficient development after 10 minutes. A 20-40 minute incubation may be necessary for weekly stained slides.
14. Tap off excess reagent, Rinse slides in 2-3 changes of distilled water.

Slides may be counterstained using nuclear fast red, eosin, or any other contrasting stain as desired. Nuclear Fast Red is recommended to study morphology.

### **II. RNA in situ Hybridization:**

1. Place 1 drop of the working DNA probe/hybridization solution on the tissue section. Place a coverslip over each slide. Be careful to avoid trapping any air bubbles.
2. Place the slides with coverslips in an oven or heating block at **70°C** for 8-10 minutes to denature the Secondary structure of RNA.
3. Place the slides in a humidity chamber and incubate at 37°C for 3-4 hours, or longer if desired, to allow hybridization of the probe with the target nucleic acid.
4. Soak the slides in 1X Detergent wash at 37°C until the coverslips fall off. Be careful not to tear the tissue.
5. *Place 1-2 drops of RNase A (15 ug/ml) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 30 minutes.*
6. Wash the slide with Protein Block (prewarmed) at 37°C for 3 min., 3X.
7. Carefully wipe excess buffer from around the tissue section. Place 1-2 drops of Linker 1 (green) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 40 minutes. (Skip For High-Sensitive Kit)

## VI. PROCEDURE Continued

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8. Tap off excess reagent. Rinse slides in Detergent wash buffer for 5 minutes. (Skip For High-Sensitive Kit)
9. Carefully wipe off excess buffer from around the tissue section. Put 1-2 drops of Linker 2 (yellow) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 20 minutes.
10. Tap off excess reagent. Rinse slides in Detergent wash buffer for 5 minutes. (Skip For High-Sensitive Kit)
11. Carefully wipe off excess buffer from around the tissue section. Place 1-2 drops of Conjugate (red) on tissue section. Place slides in humidity chamber. Incubate at 37°C for 20 minutes.
12. Tap off excess reagent. Rinse slides in Detergent wash buffer for 5 minutes.
13. Carefully wipe excess buffer from around the tissue section. Place 1-2 drops of substrate (brown) on tissue section. Incubate at room temperature for about 10 minutes, or until color development is complete.
14. *NOTE:* Color development can be monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Color begins to appear after 3-5 minutes, usually reaching sufficient development after 10 minutes. A 20-40 minute incubation may be necessary for weakly stained slides.
15. Tap off excess reagent, Rinse slides in 2-3 changes of distilled water.
16. Slides may be counterstained using nuclear fast red, eosin, or any other contrasting stain as desired. Nuclear Fast Red is recommended to study morphology.

Sections can be mounted with a permanent mounting medium (e.g. Permount from Fisher Scientific). Prior to application of a permanent mounting medium, the sections should be dehydrated by sequentially dipping the slides in graded alcohols, once in 95%, then twice in 100% ethanol, and then in xylene.

## VII. QUALITY CONTROL

Each in situ hybridization assay should include control slides to confirm that

1. the detection system is working properly
2. the positive or negative staining is specific
3. the correct procedure has been followed.

**Positive control tissue:** The positive tissue slide should be prepared from tissue or cells that are known to contain the target nucleic acid. (See Maxim Catalog - Immunohistochemistry: Positive Control Tissue Section)

**Positive control probe:** The positive probe slide should be prepared from a section of the test tissue that is processed in a manner identical to the test section but is hybridized with a probe that is known to be complementary to a sequence in the test tissue (e.g. human genomic DNA probe). Ideally, the control probe should be similar in length and GC content to the test probe. (See Maxim Catalog - Molecular Biology: Stock-Oligo Section, Biotinylated Oligonucleotide Probes are available for human, bacteria, parasites and viruses)

**Negative control tissue:** The negative probe slide should be prepared from a section of the test tissue that is processed in a manner identical to the test section but is hybridized with a heterologous probe that is not complementary to any sequence in the test tissue (e.g. plant genomic DNA probe). Ideally, control probe should be similar in length and GC content to the test probe.

## VIII. TROUBLESHOOTING

Proper use of this hybridization/detection system will result in an intense blue-black stain at the specific site of the hybridized probe in positive test tissue and positive controls. If staining is absent from any positive control slides, or present in any negative control slides, the test should be considered invalid. If deviation from the expected results occurs, please consult the following trouble shooting guide for assistance. The interpretation of any test result is solely the responsibility of the user.

Observation:	Possible Cause:
A. Sections fall off slides:	<ol style="list-style-type: none"><li>1. Sections not floated in protein-free water bath. Gelatin interfering with adhesion.</li><li>2. Adhesive absent or insufficient.</li><li>3. Insufficient baking of tissue to slides.</li><li>4. Over-digestion with Proteinase K. Try decreasing digestion time or lowering concentration of enzyme.</li><li>5. Denaturing temperature too high or time of denaturing too long.</li></ol>
B. High background staining:	<ol style="list-style-type: none"><li>1. Probe was too concentrated, causing spillover of signal.</li><li>2. Slides dried out during incubations.</li><li>3. Washing steps omitted or too short.</li><li>4. Detergent wash buffer not used after Conjugate incubation.</li></ol>
C. Weak Staining:	<ol style="list-style-type: none"><li>1. Reagents not warmed to room temperature, or room temperature too low. Try longer incubations.</li><li>2. Probe or target DNA not sufficiently denature. Check heating block temperature. Or try increasing time of denaturation.</li><li>3. Hybridization incomplete. Try increasing time of hybridization.</li></ol>

Correct treatment of tissues prior to and during fixation, embedding, and sectioning is important for obtaining optimal results. Inconsistent results may be due to variations in tissue processing, as well as inherent variations in tissue. The results from in situ hybridization must be correlated with other laboratory findings. If you have questions regarding either the use of the reagents in this kit or the results obtained, contact Maxim Customer Service.

## IX. PRECAUTIONS AND STORAGE

### Precautions

The Linker 1, Linker 2, and Conjugate reagents in this kit contain sodium azide. The National Institute of Occupational Safety and Health (NIOSH) has issued a bulletin citing the potential explosion hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide is added at minimal concentration, it is recommended that a copious amount of water be used to flush the drain pipeline after disposal of these reagents in the plumbing system.

The Hybridization Solution reagent in this kit contains formamide. Formamide is classified as a teratogen and pregnant workers should keep exposure to a minimum. Avoid inhalation, ingestion, and contact with unprotected skin. If skin contact occurs, wash thoroughly with soap and water.

### Storage

The reagents in this kit are to be stored at 2-8°C. Reconstituted Proteinase K (10X concentrated solution) should be stored frozen in small aliquots at -20°C.

### Expiration

When stored under the recommended conditions, the performance of the reagents in this kit is guaranteed for 12 months.

## X. REFERENCES

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