Dako
In Situ Hybridization
Detection System
For Biotinylated Probes

Code K0601

Intended use
For In Vitro diagnostic use.

The In situ Hybridization Detection System (K0601) is intended for the detection of biotinylated DNA and RNA probes used with in situ hybridization procedures. The kit is optimized for use on routinely processed (formalin-fixed and paraffin-embedded) tissues, but may also be adapted for fresh-frozen tissues and cultured cells. Biotinylated probes must be supplied by the user.

Summary and explanation
In situ hybridization (ISH) procedures are used to localize specific nucleic acid (DNA or RNA) sequences within the cells of tissue specimens. In general, these methods involve mounting a specimen onto a microscope slide, hybridization of the target sequences with a labelled probe, and the specific detection of the probe molecules which form probe: target hybrids. ISH has been used to demonstrate a wide variety of specific nucleic acid sequences including viral DNA and RNA in infected tissues and cells, expressed mRNAs, and genomic sequences on chromosomes.

Recently, nonradioactive methods have been widely adopted as the methods of choice for probe labelling. The simplification of labelling methods and improvements in the detection sensitivity of non-radioactive procedures has made such methods attractive alternatives to traditional radioactive techniques of probe labelling. Among the nonradioactive nucleic acid labels which are available, biotin is the most widely used. Probes for ISH may be labelled either by chemical coupling of biotin to the probe or by the enzymatic incorporation of biotin-labelled nucleotides.

The highest sensitivities for the detection of biotinylated probes are achieved by the use of streptavidin, a tetrameric protein containing four extremely high-affinity (kd= 10-15 mol/L) binding sites for biotin. Unlike native avidin, streptavidin exhibits very low non-specific binding, due to its nearly neutral charge at physiological pH, and a lack of carbohydrate associated with the protein. The streptavidin used in this kit has been conjugated to the reporter enzyme alkaline phosphatase.

Principle of procedure
The ISH Detection System K0601 utilizes alkaline phosphatase (AP)-conjugated streptavidin to localize biotinylated probes. Following hybridization of the biotinylated probe to target sequences on the tissue a stringent wash solution containing a blocking agent is used to remove excess bound probe and to block non-specific binding sites on the tissue which may otherwise react with the detection reagents. The streptavidin-alkaline phosphatase conjugate is then allowed to react with the biotin groups on the hybridized probe molecules. Finally, the site of hybridization is visualized by the colorimetric reaction of the enzyme conjugate with its substrate, BCIP (5-bromo-4-chloro-3-indoly l phosphate), and the concomitant reduction of NBT (nitro blue tetrazolium). This reaction results in the deposition of an insoluble blue-purple product at the site of hybridization.

Reagents provided
Code K0601
The following materials, sufficient for processing at least 50 slides, are included in the ISH Detection System K0601.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x15 mL</td>
<td>Stringent Wash Concentrate</td>
</tr>
<tr>
<td>1x15 mL</td>
<td>Streptavidin, Alkaline Phosphatase Conjugated</td>
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</tbody>
</table>

Stringent wash concentrate (50x) containing 0.015 mol/L sodium azide.

Streptavidin-AP, in Tris-HCl buffer (1x) containing 0.015 mol/L sodium azide.
Materials required, but not supplied

- Biotinylated probe
- Pepsin-HCl digestion reagent (0.8% pepsin, 0.2N HCl) or Target Retrieval Solution, code S1700 for formalin-fixed tissue; ISH-qualified pepsin is available from Dako (code S3002)
- Tris-buffered saline solution (0.05 mol/L Tris-HCl, pH 7.6, 0.15 mol/L NaCl); ISH-qualified TBS is available in packets from Dako (code S3001)
- Alcohol solutions (95% and 100%)
- Deionized or distilled water
- Mounting medium, such as Glycergel Mounting Medium (code C0563) or Faramount (code S3025)
- Xylene or Histoclear
- Staining racks or Coplin jars
- Silanized or otherwise adhesively-treated microscope slides; ISH-qualified silanized slides are available from Dako (code S3003)
- Heating plate or oven
- Water bath
- Coverslips
- Absorbent wipes
- Gloves
- Humidified chamber for slide incubation
- Standard light microscope

Optional materials required, but not supplied

- Counterstain; Nuclear Fast Red is recommended, consult Reagent Preparation Section for formula

Precautions

**Product Specific**
1. For professional users.
2. Do not allow the tissue to dry out at any time during the detection procedure.
3. This product contains sodium azide (NaN₃), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, build-ups of NaN₃ may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.¹⁰
4. Do not expose the antibody conjugate or substrate chromogen reagents to strong light.

**General**
1. Read all instructions thoroughly before performing the assay.
2. Wear disposable gloves when handling any patient material and while performing the staining procedures.
3. As with any product derived from biological sources, proper handling procedures should be used.
4. Avoid microbial contamination of reagents or incorrect results may occur.
5. Incubation times or temperature other than those specified may give erroneous results; any such change must be validated by the user.
6. Do not use reagents beyond expiration date for prescribed storage method. If reagents are stored under any conditions other than those specified in the package insert, they must be validated by the user.
7. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
8. Unused solution should be disposed of according to local, State and Federal regulations.
9. Safety Data Sheet available for professional users on request.

**Danger**

**Stringent Wash Concentrate**: 1-5% Polyoxyethylene octyl phenyl ether; 1-5% Sodium chloride

- H318 Causes serious eye damage.
- P280 Wear eye or face protection.
- P264 Wash hands thoroughly after handling.
- P305 + P351 + P338 + P310 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.
Danger

**BCIP/NBT:** 1-5% Trometamol; 0.1-1% N.N-Dimethylformamide

**H360** May damage fertility or the unborn child.

**P201** Obtain special instructions before use.

**P202** Do not handle until all safety precautions have been read and understood.

**P281** Use personal protective equipment as required.

**P308 + P313** IF exposed or concerned: Get medical attention.

**P405** Store locked up.

**P501** Dispose of contents and container in accordance with all local, regional, national and international regulations.

Storage

Reagents of the ISH Detection System for Biotinylated Probes should be stored in the dark at 2–8°C.

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

Reagent preparation

**Tissue Pretreatment Reagents**

**Pepsin-HCl Digestion Reagent**
Pre-measured pepsin packets (code S3002) are available from Dako. Dissolve the entire contents of one pepsin packet in 250 mL of 0.2 N hydrochloric acid. Heat the digestion reagent to 37°C, using a water bath, just prior to use. Excess solution should be stored immediately at -20°C. Do not attempt to store digestion reagent once it has been heated.

**Target Retrieval Solution**
Ready-to-use solution (code S1700) is available from Dako. Fill a Coplin jar or other suitable container with Target Retrieval Solution and heat to 95°C in a water bath (do not boil).

**Tris-Buffered Saline (TBS)**
Dissolve the entire contents of one TBS powder packet (code S3001) in 1 liter of deionized water or distilled water. The diluted solution is stable for one month at 4°C.

**Stringent Wash Solution**
Remove the dropper tip from Bottle 2. Using the plastic pipette and calibrated test tube dispense 5 mL of the 50x Stringent Wash concentrate into 245 mL of distilled or deionized water. The stringent wash concentrate provided will make 1500 mL of diluted solution. If staining baths are used for bath runs during the stringent wash step, the diluted reagent will fill six 250 mL baths. Discard any unused, diluted solution.

**Nuclear Fast Red Counterstain (optional)**
Mix 0.1 g of Nuclear Fast Red with 100 mL of 5% aluminum sulfate solution. Heat the mixture to dissolve and filter through Whatman #1 filter paper. Add a grain of thymol for preservative.

Specimen preparation

**Tissue Processing**
In general, cross-linking fixatives are preferable over precipitating fixatives, due to the excellent retention of nucleic acids in cross-linked tissues. Over-fixation, however, can decrease the availability of nucleic acid for hybridization and cause higher background binding. Fixation times in buffered formalin should be 24 hours or less. It is recommended that fixation times be standardized to minimize tissue variability.

Cryostat sections from frozen tissues should be fixed in 100% acetone for 20 minutes and air dried. Cryostat sections generally do not require pretreatment prior to hybridization.

**Tissue Sectioning and Mounting**
Tissue sections should be three to five microns thick. The aggressive digestion and heating steps used in some ISH procedures require that tissue sections are well adhered to the glass slides. Slides which have been coated with organosilane (Silanized Slides, code S3003), are recommended. Paraffin sections which have been mounted on treated slides may be baked for 30 minutes at 55–60°C to ensure optimal adhesion of the sections to the slides (disregard for cryostat sections). A procedure for coating of normal slides with organosilane is given below.

**Coating of Glass Slides with Organosilane**
Immerse clean slides in 2% solution of 3-aminopropyl-triethoxysilane in acetone for two minutes. Rinse twice in distilled water and air dry. Store coated slides at room temperature in a dust-free container.

**Cell Preparations**
Cell preparations can be fixed just prior to hybridization, or fixed and stored desiccated at -70°C for several months.

Deposit cells onto silanized slides by smearing or cytocentrifugation and allow to dry.
Fix the cells by immersing slides in 100% acetone for 20 minutes at room temperature.

Air dry the slides at room temperature for 10–20 minutes. The slides are now ready for hybridization or storage at -70°C.

**Procedure**

**Tissue Pretreatment**

Tissues fixed in cross-linking fixatives such as formalin require pretreatment to provide the hybridization probe access to target nucleic acid sequences. This can be accomplished using either proteolytic digestion (i.e. Pepsin, code S3002), or by heating in combination with Target Retrieval Solution (code S1700).

The optimal digestion time, whether by pepsin-HCl digestion or target retrieval, must be determined by the user, and will vary according to the tissue type and extent of fixation. This can be done by starting with a three to five minute digestion and increasing the time as necessary. Too much digestion will destroy the morphology of the tissue; too little will result in low hybridization signals.

An alternative to enzymatic digestion is to heat the sections at 95°C in Target Retrieval Solution. This method preserves tissue morphology better than digestion, but may result in higher overall background staining. Heat pretreatment works best on tissues which are improperly fixed and therefore prone to disintegrate when digested.

**Hybridization and Stringency Temperatures**

Hybridization and stringency temperatures for Biotinylated probes are specified in the instructions supplied with each probe. The temperatures specified for both hybridization and stringent wash have been optimized for each probe under the conditions used for this procedure.

Both temperatures are determined by the melting temperature ($T_M$) of the hybridized probe. As a general rule, the optimal hybridization temperature is 25°C below the $T_M$ and the stringent wash temperature is between 10–20°C below the calculated $T_M$. $T_M$ can be calculated according to the following formula for DNA:DNA hybrids longer than 20 bases:

$$T_M = 81.5 + 16.6 \left( \log M \right) + 0.41 \times \left( \%G + C \right) - 0.61 \times \left( \% \text{formamide} \right) - \frac{500}{N}$$

where:
- $M$ = Na⁺ in moles/liter
- $N$ = shortest chain in the hybrid

The $T_M$ for DNA:RNA hybrids is 10–15°C higher. The $T_M$ for RNA:RNA hybrids is 20–25°C higher. The $T_M$ for oligonucleotides between 14–20 base pairs may be calculated according to the formula:

$$T_M = 2 \times \text{number of AT pairs} + 4 \times \text{number of GC pairs}$$

Do not allow the tissue sections to dry out once the hybridization procedure has begun. Sections that are allowed to dry will exhibit high levels of background staining.

The color of the positive signals produced by the BCIP/NBT will have a purple hue, and background may be more prominent when tissues are mounted with an aqueous mounting medium. Mounting with a permanent medium will result in bluer reaction signals and very low background levels, but some signal may be lost during the tissue dehydration and clearing steps.

**A note on RNAse**

RNAse contamination is a legitimate concern when probing for RNA targets. Bare hands, old laboratory solutions and glassware, and biological specimens are all sources of contamination. RNAse-free conditions should be employed for all steps prior to and during hybridization. This includes wearing gloves to handle all specimens, reagents, slides, etc. Any solutions and containers which will be used prior to or during hybridization should be made RNAse-free. DEPC-treatment of solutions is very effective at destroying RNAse activity (see Molecular Cloning: A Laboratory Manual, by Sambrook, Fitsch, and Maniatis for directions on how to use DEPC). Containers may be treated with 70% EtOH for five minutes, rinsed with DEPC or otherwise RNAse-free water, and finally treated with 3% H₂O₂ for 10 minutes to inactivate RNAeses. In practice, we have found that water taken directly from the deionizing or distilling apparatus, fresh bottles of chemicals, and disposable plastic lab ware are usually free of RNAse and do not require treatment.

**ISH Procedure**

The biotinylated detection system is compatible with either double or single-stranded probes and may be used for DNA or mRNA targets. The procedures for the various probe/target combinations are essentially the same except for hybridization times and temperatures, stringent wash temperatures and the use of a denaturing step for double-stranded DNA probes. The user should refer to the instructions provided with the probe for specific information regarding denaturing of the probe and stringency conditions.

Prior to beginning the procedure, read all instructions thoroughly with particular attention to incubation times and temperatures. The times and temperatures specified are critical for successfully using this procedure. Any change in the procedure may result in decreased performance of the assay.
Prepare and prewarm the tissue pretreatment solution. If proteolytic digestion will be used, prepare the pepsin-HCl reagent and warm to 37°C. If heat pretreatment will be used, preheat Target Retrieval Solution to 95°C.

Dispense distilled or deionized water into several staining baths or Coplin jars.

Prepare TBS and dispense into four baths of 250 mL each.

Warm a heating block or oven to 90°C if the probe requires denaturing.

Warm a humid chamber to the appropriate hybridization temperature.

**STEP 1 DEPARAFFINIZATION**

**OPTIONAL:** Slides with paraffin sections may be warmed for 30 minutes in a 60°C oven just prior to deparaffinization to soften the paraffin, which may help in its removal.

Immerse sections in two changes of xylene or Histoclear for five minutes each.

Immerse sections in two changes of 100% alcohol, followed by three changes of 95% alcohol, for one minute each. This removes residual xylene and rehydrates the tissue.

Rinse sections in several changes of water to remove residual alcohol.

**STEP 2 TISSUE PRETREATMENT**

The conditions listed below are optimized for paraffin-embedded tissues that have been formalin-fixed according to recommendations listed in Reagent Preparation Section. Over-fixed tissues may require longer digestion times in order to achieve adequate staining. Either of the following methods may be used:

**Pepsin Digestion**

Immerse sections in prewarmed pepsin-HCl for 3–10 minutes at 37°C (digestion conditions may need to be tailored to the particular tissue used).

Wash sections in several changes of distilled water to remove residual pepsin.

**Heat Pretreatment**

Immerse sections in prewarmed Target Retrieval Solution for 20–40 minutes at 95°C.

Cell preparations and cryostat sections which have been acetone-fixed do not require pretreatment. If the slides have been stored at -70°C, allow them to come to room temperature before proceeding with hybridization.

**STEP 3 HYBRIDIZATION**

Remove slides from water and carefully wipe excess water from around each tissue section to avoid excessive dilution of the probe. When hybridizing with probes for mRNA it especially important to work with small numbers of slides at a time to avoid drying of the tissue sections, allowing the sections to dry at this step may result in nonspecific binding of the probe to nuclei.

Apply one drop (approximately 15 µL) of probe in hybridization solution to the specimen. Place a coverslip over the specimen and hybridization solution, taking care not to introduce bubbles under the coverslip.

For double-stranded DNA probes/targets, lay the slides on a flat heating block which has been warmed to 90°C. Orient the slides with the coverslips facing up and the tissue sections directly over the heating surface. Heat the sections for five minutes at 90°C to denature the probe and target DNA. A 90°C oven may also be used for this step. However, to ensure efficient heat transfer to the slides, place them on a metal tray or shelf. Transfer the slides to a humid chamber which has been prewarmed to the appropriate hybridization temperature. The required hybridization period will depend on the probe used. Refer to the instructions supplied with the probe for specific temperature and incubation time.

While the slides are hybridizing, prepare the stringent wash solution and prewarm it to the specified temperature in a water bath.

**STEP 4 STRINGENT WASH**

Remove the slides from the humid chamber and immerse them in a TBS bath for 5–10 minutes. Soak the slides in the TBS solution until their coverslips slide off (do not put the slides in a staining rack for this step; it will make the coverslips hard to remove).

Immerse the slides in a fresh TBS bath for two minutes. A staining rack may be used for this step.

Immerse the slides in the warmed stringent wash solution and incubate at the appropriate temperature (see probe instructions) for 30 minutes.

Immerse the slides in a fresh TBS bath for five minutes.

Remove the slides from the TBS and carefully wipe the excess fluid from around the sections.

**STEP 5 DETECTION**
To prevent drying of slides, perform detection steps in humid chamber. Place slides on a level surface and apply enough streptavidin-AP reagent to each section to completely cover the tissue. Incubate for 20 minutes at room temperature. Pour off the streptavidin-AP reagent from each slide and immerse the slides in a fresh TBS bath for five minutes.

Remove the slides from the TBS and carefully wipe the excess fluid from around the sections. Place the slides on a level surface and apply enough BCIP/NBT substrate solution to each section to completely cover the tissue. Incubate the slides at room temperature. The time required for substrate development will vary with target concentration. High copy targets are usually visualized in 60 minutes or less; low target concentrations may necessitate the use of longer substrate incubations. If long incubations are used, it is recommended that the reaction be monitored each hour to avoid overstaining.

Pour off the substrate solution from each slide and immerse the slides in a distilled water bath for five minutes.

STEP 6 COUNTERSTAINING AND COVERSLIP MOUNTING

If desired, tissues may be counterstained by immersing slides in Nuclear Fast Red (see Reagent Preparation Section) for one to five minutes, followed by a rinse in distilled water for one minute. For mounting with a permanent non-aqueous medium, immerse the slides for one minute each in 95% then 100% alcohol, followed by one minute in Histoclear or xylene.

When using an aqueous mounting medium, such as Glycergel Mounting Medium (code C0563), slides should be mounted directly without dehydration.

Quality control

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls. Tissues with known presence or absence of the gene sequence should be used as controls.

Positive controls

Controls should be fresh specimens which are fixed and processed in the same manner as the patient sample(s). Positive control tissues are indicative of correctly prepared tissue and proper staining techniques. One positive control tissue for each set of test conditions should be included in each staining run. If positive control tissues fail to demonstrate positive staining, results with the test specimens should be considered invalid. A positive control probe is needed for determining the suitability of tissue fixation for ISH and the correct performance of the detection reagents. For example, positive control (Human DNA) Biotinylated DNA Probe (code X1414) may be used to demonstrate genomic DNA in nuclei.

Negative controls

One negative control tissue, fixed and processed in a manner identical to the patient sample(s) should be stained with each probe used in each staining run to verify specificity. The tissue should not exhibit specific staining and serves as an indicator of non-specific staining. Negative control tissues should be used as an interpretive aid to distinguish specific staining from non-specific staining results. If staining occurs in the negative control tissue, results with the patient specimen should be considered invalid.

To evaluate specificity of the hybridization reaction, a non-hybridizing labelled probe may be used as a negative control e.g. Negative Control (Plasmid) DNA Biotinylated Probe (code X1415). To evaluate non-specific staining by the detection reagents, one can carry out the hybridization procedure with just the hybridization buffer (code S3305). The presence of background using this control is indicative of non-specific binding of the detection reagents. This problem may occur if the sections are allowed to dry out during the detection procedure.

Staining interpretation

Positive signals, corresponding to areas of hybridization, will appear as blue or blue-purple regions within individual cells of the tissue. Overdevelopment of the substrate-chromogen may result in black signals.

For hybridization involving DNA targets, counterstaining with nuclear counterstains may make the interpretation of positive signals difficult if too much counterstain is used. The use of Hematoxylin is not recommended, since the blue color of the positive reaction is easily obscured by the blue counterstain. A light counterstain of the tissue section using Nuclear Fast Red is recommended if additional morphological detail is desired.

Limitations

Negative results may be caused by a variety of procedural factors, including improper tissue fixation, mishandling of reagents, or performing incubations at improper temperatures. Target sequences which exist only at very low copy number may go undetected. Consult the Troubleshooting Chart for solutions to some commonly encountered problems.

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
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<tbody>
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(127948-001)
<table>
<thead>
<tr>
<th>Problem</th>
<th>Causes</th>
</tr>
</thead>
</table>
| 1. Overall bluish background | 1a. Tissue was allowed to dry out during staining.  
1b. Insufficient washing times  
1c. Tissue section folded during mounting of slide.  
1d. Insufficient stringency.  
1e. Endogenous biotin. |
| | 1a. Keep wet at all times.  
1b. Check times.  
1c. Re-mount sections.  
1d. Raise stringent wash temperature.  
1e. Block using Biotin Blocking System (code X0590) |
| 2. No signal observed with positive control probe | 2a. Target DNA was not denatured.  
2b. Tissue DNA has been over-fixed.  
2c. Tissue has been over-digested  
2d. Stringent wash temperature too high  
2e. Stained tissue was kept too long in clearing/dehydration solutions.  
2f. Inactive staining reactions.  
2g. Target RNA is degraded. |
| | 2a. Check temperature of heating block.  
2b. Use only formalin-fixed tissues; restrict fixation times to less than 24 hours.  
2c. Check temperature, time, and reagent concentrations of digestion reaction.  
2d. Check incubation temperature.  
2e. Follow recommended times to avoid solubilization of stain.  
2f. Check expiration date of kit.  
2g. Use RNase-free conditions prior to hybridization; fix tissues immediately following excision. |
| 3. Tissue sections lost from slide during procedure | 3a. Treated slides were not used.  
3b. Tissue was over-digested  
3c. Tissue was over-heated during denaturation step. |
| | 3a. Use pre-treated slides or treat slides with organosilane.  
3b. Check temperature, time, and reagent concentrations of digestion reaction.  
3c. Check heating block temperature. |
| 4. Poor tissue morphology | 4a. Tissue was over-digested.  
4b. Tissue was over-heated during denaturation step. |
| | 4a. Check temperature, time, and reagent concentrations of digestion reaction.  
4b. Check heating block temperature. |

Note: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call Dako Technical Support for further assistance.

References
<table>
<thead>
<tr>
<th>REF</th>
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<th>Temperature limitation</th>
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<td>Consult instructions for use</td>
<td>Authorized representative in the European Community</td>
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