CISH Poly HRP Detection Kit for DIG Labeled Probe

Cat No.	Quantity
52-0025RUO	4 mL Ready-To-Use

Intended Use

For Research Use Only.

This kit is intended for use with digoxigenin (DIG) labeled probe supplied by Genemed for qualitative detection of targeted nucleic acid using Chromogenic *In Situ* hybridization (CISH) methodology by light microscopy on routine formalin-fixed, paraffin-embedded (FFPE) tissue section.

Interpretation of any positive or negative staining shall be supported by implementation of a proper control.

Summary And Explanation

Chromogenic *In Situ* Hybridization (CISH) can detect change in chromosome copy number and gene amplification using conventional peroxidase reactions under the light microscopy on formalin-fixed, paraffin-embedded (FFPE) tissues. Labeled nucleic acid probe hybridize (bind), *in situ*, to specific complementary nucleic acid in the sample. Tissue morphology and probe hybridization result can be viewed simultaneously.

This kit is a non-biotin system and utilizes Mouse anti-DIG to locate where the DIG labeled probe which binds to the target nucleic acid. Poly HRP (horseradish peroxidase) for mouse conjugate is then applied and binds to the Mouse anti-DIG. This complex is observed through the use of a substrate-chromogen solution (DAB), which when added, results in a colored precipitate at the probe location. The staining result is easily observable by light microscopy. As the DAB signal is permanent, results may be stored for a long period, creating a permanent test record. With the CISH immunodetection methodology, analysis of results is both fast and easy. The most important advantage of CISH is that detection of genetic aberrations as well as verification of histopathology can be done simultaneously.

Reagents Supplied

Reagent A: One bottle of ready-to-use **Casein Blocking Solution**, 4 mL.
Reagent B: One bottle of ready-to-use in **Mouse anti-Digoxigenin** antibody, 4 mL.
Reagent C: One bottle of ready-to-use in **Poly HRP Conjugate for Mouse**, 4 mL.
Reagent D1: One bottle of 2X **DAB Chromogen Solution**, 4 mL.
Reagent D2: One bottle of 2X **DAB Buffer Solution**, 4 mL.

Storage

Store at 2-8°C. Do not freeze.
All performance claims are void after the kit expiration date.

Materials Required But Not Supplied

Digoxigenin (DIG) labeled probe (Genemed offers CISH Probes that are Ready-To-Use and optimized with CISH Poly HRP Detection Kit for DIG Labeled Probe)
Super Frost Plus slides
Positive and Negative Control Specimens
Microscope Slides, Positively Charged
Xylene
70%, 85%, 95%, and 100% Ethanol (EtOH)
Ethanol
Coverslips
Rubber cement (optional)
Wash Buffer - 1X PBS/Tween 20 (0.05%)
Hematoxylin (Cat No. 10-0027, 10-0049)
ISH Tissue Pretreatment kit (Genemed Cat No. 10-0173RUO)
SSC Buffer (20X) (Genemed Cat No. 10-0029RUO)
Reagent Water

Genemed Biotechnologies, Inc
458 Carlton Ct.
South San Francisco, CA 94080, U.S.A.
Tel: 650-952-0110
Fax: 650-952-1060

Equipment

Timer
Pipette (p20, p1000)
Pipette tips
Slide rack
Hot plate, aluminum foil, and 1 liter beaker
Slide warmer
37°C Incubator
Heating block with digital temperature display and humidity slide box OR
PCR thermal cycler with a slide block OR
Hybridizer
Water bath (capable of maintaining 75-80°C temperature range)
Coplin jars and staining jars
Light Microscopy

Procedural Notes

The reagents for this kit have been designed to be Ready-To-Use (except DAB Chromogen and Buffer are 2X). Any dilutions or alterations of the kit reagents may give erroneous staining results.

The directions accompanying this kit provide step by step instructions for optimal staining. Any change in procedure or incubation times may give erroneous staining results.

All reagents should be allowed to equilibrate at room temperature readily before usage. All incubations should be performed at room temperature in a humid environment.

Do not allow the tissue section to dry out at any point in the staining procedure. The reagents are for single use.

Preparation Of Slides before staining

Paraffin-Embedded Tissue Sections**:**

1. Tissues fixed in neutral buffered formalin for 12-24 hours prior to paraffin embedding are suitable for use. Tissue sections (4-5 µm thick) must be mounted on Superfrost Plus microscope slides.
2. Air dry slides, or dry at 37°C, and then bake 2-4 hours at 60°C.
3. Routine de-paraffinization and rehydration of tissue section.

Immerse in Xylene	2 times, 5 min. each
Soak in 100% EtOH	2 times, 3 min. each
Wash in dH_2O	3 times, 2 min. each

   (If next step cannot proceed immediately, air dry slides after soaking in 100% EtOH two times instead of washing slides in dH_2O three times.)
4. Heat Pretreatment (**The most critical step for successful performance**. Refer also to Genemed Cat. No. 10-0173RUO).

   The slides/ specimens must be boiled or heated ≥98°C for 15 min. in Tris-EDTA Buffer. We recommend that the hotplate be used for this step. (For protocols using a pressure cooker with a pressure and temperature gauge or microwave oven with temperature gauge, please contact Genemed Technical Support.)

   i. Place slides on slides rack.
   ii. Heat the Tris-EDTA Buffer in a beaker on a hotplate until it is steadily boiling, and at ≥ 98°C. To prevent buffer from evaporating, the beaker should be covered with either a glass cover or aluminum foil.
   iii. Place slides in the boiling solution, cover the beaker, and boil for 15 min.
   iv. Transfer slides immediately to dH_2O at RT (15-30°C) and wash three times, 2 min. each.
5. Enzyme Pretreatment (**A critical step for successful performance.** Refer also to Genemed Cat. No. 10-0173RUO).

   i. Equilibrate the Enzyme Pretreatment Reagent (Reagent B) to RT (15-30°C).
   ii. Add enough Reagent B to cover the tissue section and incubate for 10 min. at RT.
   iii. Wash in dH_2O three times, 2 min. each.

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6. Dehydration in graded ethanol series

70% EtOH	5 min.
85% EtOH	5 min.
95% EtOH	5 min.
100% EtOH	5 min.
100% EtOH	5 min.

7. Air dry slides for at least 20 minutes.
8. Label slides with pencil.
9. Denaturation and Hybridization

   (Use either a PCR machine with slide block or heating block with digital temperature display and humidity slide chamber with 37°C incubator)

   i. Add 15 µL of DIG labeled probe to the center of the 22 x 22 mm coverslip. (Depending on tissue size, more or less probe may be required)
   ii. Place coverslip, probe side down, on the appropriate area of the tissue sample on slide.
   iii. Seal coverslip to prevent evaporation during incubation.
   Use a 5 mL syringe containing rubber cement and topped with an 18G ½" needle, carefully apply a thin layer of rubber cement to the edges of the coverslip, slightly overlapping onto the slide. Allow rubber cement to dry (~10 min.) to prevent coverslip from sliding off slide.
   iv. Denature probe by incubating slides at 92°C for 5 min. (To do this, place the slides in the slide block of a PCR machine set to at 92°C, or on a 92°C heating block with digital temperature display).
   v. Place slides in the slide block of a PCR machine set at 37°C and incubate for >10 hours (overnight), or place slides in a humidity chamber set at 37°C for >10 hours (overnight).

10. Stringent wash

    i. Prepare two Coplin jars containing 0.5X SSC buffer, one at room temperature (RT), the other heated to 75°C. Prepare 0.5X SSC, dilute 1 part of Genemed SSC Buffer 20X Cat. No. 10-0029 with 39 parts of reagent water.
    ii. After hybridization, carefully peel off rubber cement. Remove coverslip. Do not let the tissue section dry.
    iii. Rinse slides briefly in the jar containing RT SSC, then immerse slides for 5 min. in the jar containing SSC at 75°C for 5 min..
    iv. Wash slides in dH_2O for 3 times, 2 min. each.

Control Slides

Three types of control slides are necessary for proper interpretation.

Positive Tissue Control – A tissue containing the desired gene or chromosome copy number changes.

Negative Tissue – A tissue that contains normal copy number of the targeted gene or chromosome.

Staining Protocol

Reagent A, B, and C should be equilibrated to room temperature prior to use. Each incubation should be performed at RT unless otherwise indicated. Throughout the entire procedure, unless otherwise indicated, it is important that the tissue section does not dehydrate.

1.	Immerse slides in 3% H_2O_2 in water	10 min.
2.	Wash in 1X PBS/Tween 20 (0.05%)	3 times, 2 min. each
3.	Add Casein Blocking Solution (Reagent A) to cover the section	10 min.
4.	Blot off blocking reagent. DO NOT RINSE.	
5.	Add Mouse anti-DIG antibody (Reagent B) to cover the section	**15 min.** For Centromere probe **30 min.** For Gene specific probe
6.	Wash in 1X PBS/Tween 20 (0.05%)	3 times, 2 min. each
7.	Add Poly HRP Conjugate for Mouse (Reagent C) to cover the section	**15 min.** For Centromere probe **30 min.** For Gene specific probe
8.	Wash in 1X PBS/Tween 20 (0.05%)	3 times, 2 min. each
9.	During the wash, Prepare Ready-To-Use DAB substrate solution. Mix equal volume (1:1 ratio) of DAB Chromogen Solution (D1) to DAB Buffer Solution (D2).	

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	10. Add Substrate-Chromogen Solution (DAB), 2-3 drops/slide	**10 min.** For Centromere probe **30 min.** For Gene specific probe
	11. Place slides in slide rack.	
	12. Wash with running tap water.	2 min.

Counterstaining and Coverslipping

1.	Counterstain tissue with hematoxylin.	1-2 min.
2.	Wash with running tap water.	2 min.
3.	Dehydrate in graded EtOH series. (70%, 85%, 95%, 100%, 100%)	2 min. each grade
4.	Immerse in Xylene.	2 times, 2 min. each
5.	Coverslip.	

Bright field Microscopy

Examine hybridization results and tissue morphology simultaneously using a bright field microscope and a 40x dry objective.

Interpretation Of Staining Results

Step 1: Review Positive and Negative Controls. Do not proceed to next step if the staining intensity or background staining does not meet requirements.

Step 2: Score the tested specimens.

Troubleshooting

1. Throughout the entire procedure, unless otherwise indicated, it is important that the tissue section does not dehydrate.
2. Pretreatment (The critical step for successful performance):

   Refer also to Genemed ISH Tissue Pretreatment Kit, Cat. No. 10-0173RUO. Heat Pretreatment and Enzyme Pretreatment is the most critical step for successful ISH performance. The FFPE tissue sections must be boiled or heated above 98°C for 15 minutes in Tris-EDTA Buffer. Different enzyme incubation time (3-15 minutes) may be required, depending on tissue type and fixation method. For most tissues using standard fixation procedure, 5-10 minutes enzyme pretreatment at 37°C or 10-15 minutes at room temperature will produce the best ISH results. Be sure to pre-warm the Enzyme Pretreatment Reagent to room temperature prior to adding to the tissue section.

   Excessive epitope retrieval of FFPE tissues could result in damage of tissue morphology or tissue sections becoming detached from the slide. Inadequate epitope retrieval of FFPE tissue could result in weaker staining. Enzyme pretreatment of the tissue section should be evaluated first before signal enumeration and scoring. If nuclei are not counterstained and there is an absent or very weak ISH signal, this may be due to nuclear loss as the result of excessive enzyme pretreatment. If nuclei counterstain is strong but ISH signal is absent in the nuclei, this may be due to under pretreatment of enzyme.
3. Probe denaturation at a temperature lower than recommended by the protocol may result in a weak or absent CISH signal.
4. Hybridization performed for shorter time periods, or stringent washes performed at higher temperatures, than recommended by the protocol may produce a decrease in or complete loss of the CISH signal. Stringent washes performed at too low a temperature may result in high background.
5. Genemed's DIG labeled probe, ISH Tissue Pretreatment Kit, and reagents provided with the kit are necessary for the CISH assay. Using other reagents may result in high background, decrease or loss of CISH signal.

Symbols

Catalog No.

Batch No.


Temperature Range


Use By

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