

RISH™ AP Detection Kit

(Chromogenic Detection of Digoxigenin Labeled Probes)

Control Number: 901-RI0213-082417

Catalog Number: RI0213 KG

Description: 6ml (Approximately 40 tests)

Intended Use:

For In Vitro Diagnostic Use

Summary & Explanation:

The Biocare RISH™ AP Detection Kit is to be used for the revealing of RISH probes with the red end-point. Under optimal conditions, RISH probes specifically hybridize to cognate mRNAs or DNA in formalin-fixed paraffin-embedded (FFPE) tissues. The Biocare RISH AP Detection Kit provides reagents and materials for the preparation, pretreatment, hybridization and detection of digoxigenin labeled probes.

Species Reactivity: Digoxigenin labeled probes on human tissues

Cellular Localization: Cytoplasmic or nuclear dependant upon probe

Known Applications:

in situ hybridization on formalin-fixed paraffin-embedded (FFPE) tissues

Supplied As:

1. RISHzyme™ (RI0200G) 6ml
2. RISHzyme™ Buffer (RI0201G) 6ml
3. RISH™ Secondary Reagent (RI0203G) 6ml
4. RISH™ AP Tertiary Reagent (RI0212G) 6 ml
5. Warp Red™ Chromogen (WR806CHE) 0.7ml
6. Warp Red™ Substrate Buffer (WR806BFH) 25ml
7. Mixing Vial (RMVL103)

Materials and Reagents Needed But Not Provided:

Decloaking Chamber™ (pressure cooker)*

RISH Retrieval Solution

RISH Probe

IQ Kinetic Slide Stainer* or other hybridization oven

IQ Aqua Sponge*

Microscope slides, positively charged

Desert Chamber* (Drying oven)

Positive and negative tissue controls

Xylene (Could be substituted with xylene substitute*)

Ethanol or reagent alcohol

Deionized or distilled water

TBS wash buffer (TWB945)*

Hematoxylin*

Bluing reagent*

Mounting medium*

Peroxidase*

HybriSlip™ (or equivalent)*

Thermal Test Strips*

* Biocare Medical Products: Refer to a Biocare Medical catalog for further information regarding catalog numbers and ordering information. Certain reagents listed above are based on specific application and detection system used.

Storage and Stability:

Store kit at 2°C to 8°C. Do not use after expiration date printed on vials. If reagents are stored under conditions other than those specified in the package insert, they must be verified by the user. Diluted reagents should be used promptly; any remaining reagent should be stored at 2°C to 8°C.

Protocol Recommendations:

1. Deparaffinization

- a. Deparaffinize slides as per standard procedures.
- b. Perform 5 minute hydrogen peroxide block.
- c. Wash with distilled water, and place onto IQ Stainer at room temperature (RT).

2. Protein Digestion/ Retrieval

The following recommendations should be used as a starting point for tissues fixed 24 hours or longer. Tissues fixed less than 24 hours may require a further dilution of RISHzyme to buffer and/ or a heat pretreatment at lower temperature.

- a. Prepare digestion reagent (1:2) by combining 1 part enzyme to 1 part buffer. If tissues appear over-digested, consider: 1:3, 1:4 or 1:5 digestion reagent to buffer. (Working solution is stable for 7 days at 4°C).
- b. Place 200 µl onto tissue sample for 1 minute at RT.
- c. Wash twice with distilled water, 2 minutes each wash.
- d. Retrieve section with RISH Retrieval using Biocare's Decloaking Chamber*, followed by a wash in distilled water.
 - i. Suggested parameters: 90°C for 15 minutes, followed by 10 minute cool down in RISH Retrieval. If tissues appear over-digested, retrieve sections at 80°C or lower for 15 minutes followed by 10 minute cool down in RISH Retrieval.
 - ii. Wash in distilled water
 - iii. See technical notes if using a water bath or hotplate

3. Probe Hybridization

- a. Use Kimwipe to wipe off excess water around tissue section.
- b. Apply 20 µl of RISH probe onto tissue section and cover slip with 22×22mm cover slip.
- c. Place slides onto a preheated IQ Kinetic Slide Stainer** or humidity chamber at 37°C (DNA targeting probes) for 60 minutes or 55°C (mRNA targeting probes) for 30-60 minutes.

Note: **RISH DNA Targeting Probes (CMV, etc)** require denaturation at 95°C for 5 minutes prior to the 60 minute hybridization at 37°C.

4. Post-Hybridization Washing

- a. Remove slides from incubation and put directly into TBS at RT. Briefly agitate until cover slip comes off.
- b. Wash 5 minutes in TBS wash buffer at 55°C. Then, place slides in TBS wash buffer at RT for 5 minutes. Slight agitation in buffers and stringency wash is recommended.

5. Detection of Probe

- a. Remove slides from TBS and use a Kimwipe to wipe around the edges of tissue. Apply PAP Pen, if necessary.
- b. Place slides onto RT IQ Stainer or slide rack.
- c. Decant TBS, and put 4 drops of RISH Secondary Reagent (3) onto tissue sample, and incubate for 15 minutes.
- d. Wash with TBS twice, 2 minutes each.
- e. Decant TBS. Add 4 drops RISH AP Tertiary Reagent (4) onto tissue sample and incubate for 15 minutes.
- f. Wash with TBS twice, 2 minutes each.
- g. Decant TBS. Apply 4 drops of prepared Warp Red (5-6) to tissue samples and incubate for 5 to 10 minutes (mix 1 drop chromogen to 2.5 ml of buffer).
- h. Wash with distilled water and examine slides on microscope prior to counterstaining.

6. Counterstaining

- a. Briefly soak slides in CAT Hematoxylin for 5-6 seconds. Immediately rinse with distilled water. Excessive counterstaining will obscure specific signal. Reduce time in hematoxylin if too dark.
- b. Soak slides in Tacha's Bluing Solution for 5-6 seconds, and rinse with distilled water.

7. Cover Slipping

- a. Dehydrate through graded alcohols and finish in xylene.
- b. Apply 1 drop of mounting media to an appropriate cover slip and mount.
- c. Allow to dry.

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Technical Notes:

The Biocare RISH AP Detection Kit has been developed to detect digoxigenin labeled RISH probes by *in situ* hybridization. Routinely processed (FFPE) tissues in which the presence of cognate nucleic acid target is anticipated can be used.

Biocare's IQ Kinetic Slide Stainer was used for hybridization and post-hybridization detection steps of RISH probes. Detection steps can also be programmed on an automated staining system (see below).

Commercially available hybridization chambers can be used, but measures should be taken to ensure that chamber is hermetically sealed during hybridization. Both incubator and humidity chamber must be at 55 °C (mRNA targeting probes) or at 37°C (DNA targeting probes) when hybridizing probe.

*If a Decloaking Chamber or pressure cooker is not available, consider using a water bath or hot plate for retrieval. Place RISH Retrieval (1X) in glass (Pyrex) container and heat solution until the appropriate temperature is achieved (90°C). Heat slides in this solution for 15 minutes. Remove slides after incubation, and immediately wash in distilled water. Proceed with probe hybridization.

**The IQ Stainer can be used as an incubation and humidity chamber by using the IQ Aqua Sponge. Saturate IQ Aqua Sponge with distilled water, and place on hot bar set to 55°C (mRNA targeting probes) or 37°C (DNA targeting probes) for hybridization. Use the clear plastic hood to contain heat and moisture.

If using the IQ1000 (single hot bar) place slides onto rack and denature on hot bar at 95°C for 5 minutes. After denaturation, remove rack and place on bench. Turn off hot bar and unplug unit. Cool hot bar (3-5 minutes) with running tap water until bar approximates 35-40°C. Re-set hot bar to hybridization temperature (37°C). Place water-saturated IQ Aqua sponge and a thermometer onto hot bar before hybridization. Check the temperature on the hot bar. It should not be higher than 40°C. Place rack with slides onto sponge, cover unit and incubate for 1 hour.

If probe appears cloudy, briefly vortex and heat to hybridization temperature (37°C or 55°C) before application.

The use of probe in amounts less than recommended may lead to inconsistent results.

Detection of RISH probes may be performed on Biocare's IntelliPATH™ Automated Stainer with IntelliPATH™ Warp Red™ Chromogen Kit (IPK5024). Contact Biocare's Technical Support Staff for protocol recommendations.

Bone Marrow Biopsies:

For optimal results, bone marrow biopsies should be fixed for 24 hours in 10% neutral buffered formalin (NBF) or zinc formalin prior to decalcification. Biocare recommends for preservation of RNA integrity that bone marrows are decalcified in a formic acid or 10% EDTA based solution^{1,2}. However, there are many methods of fixation and decalcification used in the clinical laboratory. The table below represents parameters and tissues that have tested positive for kappa / lambda mRNA RISH probes in our laboratory. All decalcification methods, including those mentioned, should be empirically determined for optimal pretreatment parameters. Biocare's protocol recommendations for the ratio of enzyme to buffer (1:2) should be used as a starting point for tissues fixed 24 hours or longer. Tissues fixed less than 24 hours may require a dilution of RISHzyme to buffer of 1:4 and a heat pretreatment of 80°C for 15 minutes to prevent digestion artifacts.

Check for completion of decalcification by in-house methods and process tissues into paraffin according to standard procedures.

Fixation	Decalcification	Digestion of RISHzyme: buffer	Bone marrow positive by RISH
NBF	10% HNO ₃	1:2-1:4	Plasma cell myeloma, Thoracic myeloma
NBF+zinc	*FA / Form	1:2-1:4	Bone marrow clinically undefined
NBF	RDO	1:2-1:4	Bone marrow clinically undefined
NBF+zinc	RDO	1:2-1:4	Bone tumor plasma cell myeloma

*FA / Form = formic acid / formaldehyde (Formical-4)

Precautions

1. Some components in this kit are classified as hazardous. Consult the MSDS for each component in order to determine appropriate handling procedures. Visit the Biocare Medical website at <http://biocare.net/support/msds/> for the current MSDS.
2. Specimens, before and after fixation, and all materials exposed to them should be handled as if capable of transmitting infection and disposed of with proper precautions. Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents or specimens come in contact with sensitive areas, wash with copious amounts of water.
3. Microbial contamination of reagents may result in an increase in nonspecific staining.
4. Incubation times or temperatures other than those specified may give erroneous results. The user must validate any such change.
5. Do not use reagent after the expiration date printed on the vial.

Follow the DNA probe specific protocol recommendations according to the RISH AP Detection Kit data sheet. If atypical results occur, contact Biocare's Technical Support at 1-800-542-2002.

Troubleshooting guide:

No Staining

1. Critical reagent (such as probe) omitted
2. Incorrect hybridization temperature (check temperature)
3. Staining steps performed incorrectly or in the wrong order
4. Low or compromised target RNA / DNA
5. Detection reagent incubations too short
6. Improperly mixed substrate and/or chromogen solution(s)

Weak Staining

1. Tissue is either over-fixed or under-fixed
2. Probe incubation time too short
3. Low expression of RNA, contamination of tissues with RNases or RNA degradation (mRNA targeting probes)
4. Compromised genomic or target DNA
5. Over-development of substrate
6. Omission of critical reagent
7. Incorrect procedure in reagent preparation
8. Improper procedure in steps
9. Incorrect hybridization temperature (check temperature) used

Non-specific or High Background Staining

1. Tissue is either over-fixed or under-fixed
2. Substrate is overly developed
3. Tissue was inadequately rinsed
4. Deparaffinization incomplete
5. Tissue damaged or necrotic
6. Sections dried during hybridization
7. Too high HIER temperature

Tissues Falling off Slide

1. Slides were not positively charged
2. A slide adhesive was used in water bath
3. Tissue was not dried properly
4. Tissue contained too much fat
5. Tissue may be over digested

Specific Staining too Dark

1. Incubation of probe, secondary or tertiary too long

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Limitations:

The optimum parameters and protocols for a specific application can vary. These include, but are not limited to: fixation, enzymatic digestion, incubation times, tissue section thickness and detection kit used. Due to the superior sensitivity of these unique reagents, the recommended hybridization and incubation times listed are not applicable to other detection systems, as results may vary. The data sheet recommendations and protocols are based on exclusive use of Biocare products. Ultimately, it is the responsibility of the investigator to determine optimal conditions. The clinical interpretation of any positive or negative staining should be evaluated within the context of clinical presentation, morphology and other histopathological criteria by a qualified pathologist. The clinical interpretation of any positive or negative staining should be complemented by morphological studies using proper positive and negative internal and external controls as well as other diagnostic tests.

Quality Control:

Refer to CLSI Quality Standards for Design and Implementation of Immunohistochemistry Assays; Approved Guideline-Second edition (I/LA28-A2). CLSI Wayne, PA, USA (www.clsi.org). 2011

References:

1. Beck RC, *et al.* Automated colorimetric in situ hybridization (CISH) detection of immunoglobulin (Ig) light chain mRNA expression in plasma cell (PC) dyscrasias and non-Hodgkin lymphoma. *Diagn Mol Pathol.* 2003 Mar; 12(1):14-20.
2. Shibata Y, *et al.* Assessment of decalcifying protocols for the detection of specific RNA by non-radioactive in situ hybridization in calcified tissues. *Histochem Cell Biol.* 2000 Mar; 113(3):153-9.
3. Iwasaki Y, *et al.* Differential expression of IFN-gamma, IL-4, IL-10, and IL-1 beta mRNAs in decalcified tissue sections of mouse lipopolysaccharide-induced periodontitis mandibles assessed by in situ hybridization. *Histochem Cell Biol.* 1998 Apr; 109(4):339-47.