Agilent Dako

CE

General Instructions
For In Situ Hybridization

Purpose
These general instructions apply to in situ hybridization (ISH) reagents from Agilent. Always consult the specific Instructions for Use for your product for specific instructions on the use of the product.

The information in this General Instructions for in situ hybridization are guidelines only. A procedure is provided in the specific Instructions for Use for each in situ hybridization probe. Optimal procedures must be determined and verified by the user.

NOTE: Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Principle of Procedure
Agilent Dako ISH probes may be used together with a variety of ISH reagents for demonstration of specific genetic events in tissue samples. In general, ISH staining techniques allow the genetic events to be observed in a morphological context.[1] The basic principle in manual ISH is that after deparaffinization and rehydration, specimens are heated in Pre-Treatment Solution followed by proteolytic digestion, e.g. using Pepsin. The probe mix is then applied to the specimens, which are placed on a heating block or hybridization chamber for denaturation of DNA or RNA and hybridization of the probe. After a stringent wash, the specimens are mounted with Fluorescence Mounting Medium containing DAPI and coverslipped for subsequent analysis using a fluorescence microscope. Some Agilent Dako ISH products are specifically designed for automated use on the Dako Omnis instrument. The basic principle is the same, however all steps, except mounting and coverslipping, are performed onboard Dako Omnis.

The following fluorescence in situ hybridization (FISH) probe types are available from Agilent:

Break-apart probe
Break apart probes consist of two child probes, designed to be on opposite sides of the translocation breakpoint for a given gene, each labeled in a different color. These probes generate signals in normal cells that are closely matched in size and colocalized (two fusion signals). Following a translocation, the signals are “broken apart” and no longer colocalize, resulting in: one red/orange, one green, one fusion.

	Normal cells: Signals are closely matched in size and colocalized (two fusion signals)
	Translocation: The signals are “broken apart” and no longer colocalized (for example: one red/orange, one green, one fusion)

Gene amplification
In normal diploid cells in metaphase or interphase, a gene amplification probe will generate two signals (red/orange) corresponding to the gene of interest and two signals (green) corresponding to the centromere region of the chromosome where the gene is. In cells with a gain or amplification of the gene or chromosome, the number of signals will be greater.

	Normal cells: Two signals (orange) corresponding to the gene of interest and two signals (green) corresponding to the centromere region of the chromosome where the gene is (for example: two red/orange, two green)
	Amplification: The number of signals is greater (for example: six red/orange, two green)

Rearrangements/dual fusion probe
Dual fusion probes consist of two child probes, each designed to target a given gene and each labeled in a different color. These probes generate signals in normal cells that are closely matched in size and are separated by more than one signal distance (for example: two red/orange signals, two green signals). Following a translocation, the signals are colocalized, producing fusion signals (for example: one red/orange, one green, two fusion).

	Normal cells: Signals are closely matched in size and separated by more than one signal distance (for example: two red/orange, two green)

Agilent

Rearrangement: Signals are colocalized, producing fusion signals (for example: one red/orange, one green, two fusion)

The following chromogenic in situ hybridization (CISH) probe types are available from Agilent:

Detection of mRNA/RNA
Oligonucleotide-based probes that bind to mRNA or RNA are conjugated with fluorescein isothiocyanate (FITC) to enable detection using the Anti-FITC-AP CISH Accessory Kit (Dako Omnis) (Code K5899). When used together with this kit, staining is identified by bright field microscopy as dark blue/purple staining at the site at the sites where the probe is bound.

Detection of PNA Probes
PNA probe is conjugated with FITC to enable detection using Dako PNA ISH Detection Kit (Code K5201). When used together with this kit, staining is identified by bright field microscopy as dark blue/black color at the site of hybridization.

Materials Required, but Not Supplied
See individual Instructions for Use for specific recommendation of procedure. Not all the below listed materials may be required.

Pretreatment:
Dako Pre-Treatment Solution (20x) (Code GM301 and included in Code K5799)
Dako Pepsin Diluent (10x) (Included in Code K5799)
Pepsin (Code S3002/GM302 and included in Code K5799)

Hybridization:
FISH probe(s)

Detection:
Anti-FITC-AP CISH Accessory Kit (Dako Omnis) (Code K5899)
PNA ISH Detection Kit (Code K5201)

Stringent wash:
Stringent Wash Buffer (20x) (Code GM303 and included in code K5799)

Mounting:
Dako Fluorescence Mounting Medium (Code GM304, S3023, and included in Code K5799)
Mounting medium, Dako Faramount Mounting Medium (Code S3025) or Dako Glycergel Mounting Medium (Code C0563)

Equipment and accessories:
Absorbent wipes
Adjustable pipettes
Calibrated partial immersion thermometer (range 37–100 °C)
Clearify (GC810)
Coverslips (18 x 18 mm or 22 x 22 mm and 24 x 50 mm or 24 x 60 mm)
Dako Coverslip Sealant (Included in Code K5799)
Dako Omnis (Code GI100)
Dako Omnis Mixing Device (Code GC116)
Dako Omnis ISH Lid (Code GC102)
Fluorescence microscope with appropriate filter cubes
Forceps
Graded ethanol (70%, 85%, 95% and/or 100%)
Heating block for digestion (37 ±2 °C)
Heating block or hybridization oven for denaturation (66 ±2 °C or 82 ±2 °C)
Humid hybridization chamber for hybridization (45 ±2 °C)
ISH Cleaning Solution (Code GC207)
ISH Ethanol Solution, 96% (Dako Omnis) (Code GM300)
Light microscope (4–40X objective magnification)
Microcentrifuge
Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar)
Microscope slides, Dako Silanized Slides (Code S3003) or Superfrost Plus slides
Microwave oven
Positive Control RNA CISH (Dako Omnis) (Code G111702-2)
Staining jars or baths
Timer (capable of 0–60 minute intervals)
Vortex mixer
Wash Buffer (20x) (Code GC807)
Water bath with lid (capable of maintaining 37 ±2 °C, 55 ±2 °C, 63 ±2 °C and from 95 °C to 99 °C)
Water (distilled water or de-ionized water)
Xylene

Storage
Proper storage and handling of reagents and samples are essential for the performance. Do not use the product after the expiration date printed on the outside of the reagent package. Unless otherwise specified in the product's IFU, the expiry date on the label is valid for unopened as well as opened (in-use) vials when handled according to instructions. If products are stored under any conditions other than

those specified, they must be validated by the user. Return vials to specified storage conditions directly after use.

There are no obvious visual signs to indicate incorrect product storage or handling of products during the products shelf life. If a problem is suspected with the product during the shelf life that cannot be explained by incorrect product storage or handling, or other variations in laboratory procedures, contact Agilent Pathology Support. Refer to the Troubleshooting and Quality Control sections for more information.

ISH stained slides should be stored according to recommendations in the specific Instructions for use. In general, store FISH-stained slides in the dark at –18 to 8 °C as some fading of stained slides may occur if slides are exposed to light or high temperatures. In general, store CISH-stained slides in the dark at room temperature as some fading of stained slides may occur if slides are exposed to high temperature.

Specimen Preparation
These are guidelines only. Optimal procedures must be determined and verified by the user.

Specimens must be handled to preserve the tissue for ISH staining. Standard methods of tissue processing should be used for all specimens. Prior to ISH staining, tissues must be fixed and processed. Fixation prevents autolysis and necrosis of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing includes dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues. The most common fixatives for ISH tissue preparations are discussed below.

For specific information regarding tissue fixation and processing, see references 2 and 3. Consult local Health and Safety regulations.

Paraffin-embedded tissue
General comments
The most common tissue fixative is 10% (v/v) neutral phosphate-buffered formalin, commonly referred to in the EU as 4% w/v buffered formalin. Agilent Dako Reagents for ISH have been successfully validated with paraffin-embedded tissues fixed in 10% formalin. This is referred to as formalin-fixed, paraffin-embedded (FFPE) tissue sections.

It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Specimens should be blocked into a thickness of 3 or 4 mm, fixed in formalin and dehydrated and cleared in a series of ethanol and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. Tissue specimens should be cut into sections of 4–5 µm. After sectioning, tissues should be mounted on recommended microscope glass slides. See references 2–4, the probe(s) Instructions for Use(s) and the protocol(s) supplied with the fixing reagent(s) for additional information regarding tissue fixation.

See individual probe Instructions for Use for specific recommendation of fixative. Alternative fixatives and use on frozen tissue are only suitable for specific products and should always be determined and verified by the user.

Tissue fixation in formaldehyde-based solution (neutral buffered formalin)
Most formaldehyde-based fixatives contain 10% formalin, a neutral salt to maintain tonicity, and a buffered system to maintain pH. These fixatives are well tolerated by tissues and exhibit good histological penetration. However, shrinkage or distortions may occur in poorly fixed and embedded tissue specimens. Fix small blocks of tissue (10.0 x 10.0 x 3.0 mm) in 5–10 mL of neutral buffered formalin per block for 24 hours (12–72 hours). Prior to in situ hybridization, dehydrate, clear, and embed tissue sections as described below in *Processing and paraffin-embedding*.

Processing and paraffin-embedding
After fixation, processing may be completed using an automatic tissue processor. Tissues are dehydrated using graded ethanols, cleared with xylene or xylene substitute, and infiltrated with paraffin wax. The tissue is subsequently embedded with paraffin wax in molds or cassettes, which facilitate tissue sectioning. To minimize denaturing of target specific sites, do not expose tissues to temperatures that exceed 60 °C during processing. Tissue blocks may be stored or sectioned on completion of embedding. It is recommended that tissue sections mounted on slides should be stained within 6 months of sectioning when held in the dark at 2–8 °C (preferred), or at room temperature up to 25 °C.

Adherence of paraffin-embedded tissue sections to microscope slides
Collect sectioned tissues (4–5 µm) from paraffin-embedded blocks on clean glass slides. Dry the tissue sections at 58 ±2 °C for a maximum of one hour. For increased adhesion of tissue sections during staining procedures, use of Dako Silanized Slides (Code S3003) or Superfrost Plus slides is suggested, see individual Instructions for Use for specific recommendation of slide type. When using charged or silanized slides specifically omit any adhesives in the mounting water bath, such as gelatin, glue and/or commercially produced protein-containing products. Coated slides are strongly recommended for staining procedures requiring proteolytic digestion or heat-induced target retrieval.

Deparaffinization and rehydration
Prior to manual staining, tissue slides must be deparaffinized to remove embedding media and rehydrated. Avoid incomplete removal of paraffin. Residual embedding media will result in increased nonspecific or reduced staining.

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 2 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 2 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in diluted Wash Buffer. Commence staining procedure as outlined in Instructions for use.

Xylene and ethanol solutions should be changed after a maximum of 200 slides. Xylene substitutes may be used. Consult the Instructions for Use of the used reagent.

Pretreatment and Proteolytic Digestion
Fixation in formalin is suitable because the induced protein-protein and protein-nucleic acid cross-links preserve the tissue efficiently while retaining morphology. However, the macromolecular network introduced by formalin significantly reduces the access of ISH probes to target DNA and RNA. The nucleus may be made accessible with pretreatment at high temperature and proteolytic digestion using pepsin prior to in situ hybridization. The optimal pepsin incubation time depends in the fixation history of the tissue and should be determined by the user. For specific recommendations for pretreatment, see the Instructions for Use provided with each probe.

General pretreatment guidelines when using Agilent Dako probes

Probe	Pretreatment Solution	Proteolytic digestion
Probes for use on Dako Omnis	Pretreatment solution onboard Dako Omnis (automated process)	Pepsin onboard Dako Omnis (automated process)
Probes for manual use	Pretreatment solution held at 98 °C using either a water bath or a microwave oven.	Droplets of concentrated pepsin directly on the tissue (at room temperature or at 37 °C on at heating plate) or in a pepsin bath at 37 °C using diluted pepsin.

Always consult Instructions for Use for optimal pretreatment method for the individual probes.

Use the timing recommended in the specified Instructions for Use of pepsin. Overdigestion may result in nonspecific staining and/or unacceptable morphology. Continue with the staining procedure according to the recommended instructions.

Laboratories at High Elevations
At certain higher elevations (above 1372 m (4500 feet)), boiling of the pretreatment solution may occur prior to achieving the desired optimal temperature. In such situations, a recommended alternative procedure is to heat the slides at the maximum achievable temperature and to extend the incubation time of the slides in the target retrieval solution until the desired staining intensity is achieved.[5] An additional possible solution is to use a closed pressure system such as a pressure cooker to achieve 121 ℃. Drying of tissue sections at elevated temperatures must only be performed in a calibrated oven with uniform heat distribution. Each laboratory must determine the best method and target retrieval time for their particular circumstances.

Consult references 2 and 3 for further details on specimen preparation.

Staining Procedure
When using probes on Dako Omnis, use the recommended reagents and the validated protocol for optimal staining performance. When using probes and options for manual use, refer to specific Instructions for Uses for probes and to specific Accessory Kits Instructions for recommended procedures.

NOTE: Any modification of the recommendations outlined in the Instructions for Use must be validated by the user.

Quality Control
Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. For use of controls, refer to specific Instructions for Uses for probes and accessory reagents.

Assay Verification
Prior to initial use of a probe or kit containing ISH reagents in a diagnostic procedure, the user should verify the assay by testing it on a series of lab-supplied tissues with known ISH performance characteristics representing known positive and negative tissues. The quality control guidelines of the College of American Pathologists (CAP) Accreditation Program Molecular Pathology Checklist[6] contain additional information. Tissues listed in the product specific Instructions for Use, Performance Characteristics Section are suitable for assay verification.

Staining Evaluation
The specific staining pattern is described in the individual Instructions for Use. For evaluation, objectives of 4–40X magnification are appropriate for CISH stained slides on a bright field microscope and objectives of 10–100X magnification are appropriate for FISH-stained slides on fluorescence microscope equipped with appropriate filter cubes. The entire tissue section should be considered, avoiding edge effects, necrotic areas, areas with ambiguous borders and areas with other obvious artifacts. Pay attention to areas with heterogenic distribution of signals and follow guidelines in the specific IFU.

Interfering Substances
Residual embedding media resulting from incomplete removal of paraffin may lead to excessive background staining and/or increased nonspecific staining. Tissue marking dyes (especially lime and orange/yellow-colored inks) may show orange or green autofluorescence, interfering with interpretation of the FISH staining.[7] Fluorescence detection requires the use of specialized low autofluorescence immersion oil to avoid autofluorescence that will interfere with image quality.[8]

General Limitations
1. ISH is a multistep diagnostic process that requires specialized training in the selection, fixation and processing of tissue; selection of reagents; preparation of the ISH slide; and interpretation of the staining results.
2. ISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may affect probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Ready-to-use probes are prediluted and optimized for use with specific staining systems. When used in conjunction with other than the specified reagents and protocols these are no longer ready-to-use and must be re-optimized and validated according to the clinical laboratory's ISH validation protocol.
4. Unless specifically claimed in the instructions, the performance characteristics of probes used for ISH have not been determined for other laboratory techniques.
5. Improper storage and use of reagents may lead to erroneous results
6. The user should always ensure adherence to the maintenance schedule for the automated staining instrument. Lack of adherence to the maintenance schedule may give erroneous results.

Troubleshooting
Refer to the Troubleshooting section in specific Instructions for Use or see table below for common problems and solutions.

Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Below is a list of common problems and solutions when using ISH probes with recommended products and validated protocols.

Problem	Probable Cause	Suggested Action
1. No signals or weak signals	1a. Wrong storage conditions used for reagents.	1a. Check that reagents have been stored correctly according to recommended storage conditions.
	1b. Reagent is used past its expiration date.	1b. Ensure reagent is not used past its expiration date.
	1c. Reagent is used past its onboard stability.	1c. Ensure reagent is not used past its onboard stability.
	1d. Inappropriate fixation method used.	1d. Ensure that patient tissue is not fixed for too short or too long a time period, and that the correct fixative was used.
	1e. Microscope not functioning properly	1e. Check the microscope and ensure that the used filters are suitable for use with the probe mix fluorochromes
	1f. Damaged ISH lids.	1f. Check integrity of ISH lids.
	1g. Correct diluent is not used to dilute concentrated reagents.	1g. Ensure that correct solution is used to dilute concentrated reagents.
	1h. Incorrect pretreatment method is used.	1h. Ensure that correct pre-treatment specified in Instructions for Use is used.
	1i. Faded signals	1i. Avoid long microscopic examination and minimize exposure to strong light sources.
	1j. Reagents have been exposed to high temperatures during transport or storage.	1j. Check storage conditions. Ensure that dry ice was present when the consignment was received.
	1k. Stringent wash conditions incorrect.	1k. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash.
	1l. Pre-treatment conditions incorrect.	1l. Ensure that the recommended pre-treatment temperature and time are used.
	1m. Evaporation of probe during hybridization using manual method.	1m. Ensure sufficient humidity in the hybridization chamber
2. Areas without signal	2a. Air bubbles caught during placement of cover slip after probe application	2a. Avoid air bubbles. If observed, gently tap them away using forceps.
	2b. Air bubbles caught during mounting	2b. Avoid air bubbles. If observed, gently tap them away using forceps.
	2c. Probe volume too small.	2c. Ensure that the probe volume is large enough to cover the area under the coverslip.
	2d. Probe not fully covering staining area.	2d. Ensure correct placement probe on tissue.
3. Excessive background staining	3a. Inappropriate tissue fixation.	3a. Ensure that recommended fixative is used.
	3b. Prolonged exposure of hybridized section to strong light	3b. Avoid long microscopic examination and minimize exposure to strong light.
	3c. Paraffin incompletely removed.	3c. Ensure that paraffin is removed completely from sections.
	3d. Probe volume too small.	3d. Ensure that the probe volume is large enough to cover the area under the coverslip.
	3e. Nonspecific binding of reagents to tissue.	3e. Ensure that correct fixation method of the specimen is used and avoid large areas of necrosis.
	3e. Correct diluent or distilled/de-ionized water is not used to dilute concentrated reagents.	3e. Ensure that correct solution is used to dilute concentrated reagents.
	3f. Incorrect pretreatment method is used.	3f. Ensure that correct pre-treatment specified in Instructions for Use is used.
4. Poor tissue morphology	4a. Incorrect Pepsin treatment	4a. Change to another of the digestion methods. Ensure that the pepsin is stored at the correct temperature.
	4b. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.	4b. Attempt a staining protocol with shorter pepsin incubation time. Ensure that the section thickness is 4-6 µm.
	4c. Necrosis or ethanol fixation of tissue	4c. Ensure proper fixation of tissue prior to processing and paraffin-embedding.
	4d. Incorrect pretreatment conditions may result in unclear or cloudy appearance.	4d. Ensure that the recommended pre-treatment temperature and time are used.
5. High level of auto fluorescence on slide including areas without FFPE tissue	5a. Use of expired or non-recommended glass slides	5a. Choose glass slides as specified. Ensure that the glass slides have not passed expiry date.
6. Tissue detaches from slides	6a. Use of incorrect glass slides.	6a. Choose glass slides as specified.

References

1. Mollerup J and Nielsen KB; In Situ Hybridization. Chapter 5, in 1st Edition of Companion and Complementary Diagnostics – From Biomarker Discovery to Clinical Implementation, page 93-109. Edited by Jan Trøst Jørgensen, Academic Press (2019).
2. Taylor C and Rudbeck L. Education Guide: Immunohistochemical Staining Methods. Sixth Edition. 2013. Available at www.agilent.com.
3. Kiernan JA; Histological and Histochemical Methods: Theory and Practice. New York: Pergamon Press 1981.
4. Sheehan DC and Hrapchak BB; Theory and Practice of Histotechnology. St. Louis: C.V. Mosby Co. 1980.
5. Shi SR, Key ME, Kalra KL; Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1991; 39:741-48.
6. Molecular Pathology Checklist, CAP Accreditation Program, 2017.
7. Evin Gulbahce H. Interference of tissue-marking dyes with fluorescence in situ hybridization assays. Arch Pathol Lab Med 2019; 143:1299.
8. Alamri A Nam JY, Blancato JK. Fluorescence in situ hybridization of cells, chromosomes, and formalin-fixed paraffin-embedded tissues, Methods Mol Biol. 2017; 1606: 265–279.

Agilent Technologies Singapore (International) Pte Ltd.
No. 1 Yishun Avenue 7
Singapore, 768923
Tel. +44 161 492 7050
www.agilent.com

Revision [01]
Date of issue: 2022.03

Changes since last revision	
Revision [01]	Creation