Agilent FISH General Purpose Reagents

FISH Hybridization Buffer
FISH FFPE Hybridization Buffer
IQFISH Fast Hybridization Buffer
FISH Wash Buffer 1
FISH Wash Buffer 2
FISH Mounting Buffer
FISH Mounting Buffer with DAPI
ISH Pepsin Kit

Protocol

Version F0, September 2015

For Laboratory Use.

Agilent Technologies

Notices

Manual Part Number

G9400-90000

Edition

Version F0, September 2015

Printed in USA

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CAUTION

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In this Guide...

This document describes how to use the Agilent FISH General Purpose Reagents to perform fluorescence *in situ* hybridization (FISH) on a spread of chromosomes or with tissues that have been preserved with FFPE.

If you have comments about this protocol, send an email to techservices@agilent.com.

Contents

1 Before You Begin

This chapter provides important information on getting started with a FISH experiment.

FISH overview

Agilent's FISH general purpose reagents are designed for use in fluorescence *in situ* hybridization (FISH) protocols.

For the FISH General Purpose Reagents Protocols provided in Chapter 2 and Chapter 3, the starting material is a spread of interphase or metaphase chromosomes fixed to a glass microscope slide. Prepare the slide using a technique that is compatible with FISH. Store the slides at –20°C until ready to use.

If your starting material is a formalin-fixed paraffin-embedded (FFPE) tissue sample, follow the protocol provided in Chapter 4, "FISH Protocol on FFPE Samples". This protocol uses either the Agilent IQFISH Fast Hybridization Buffer (Agilent p/n G9415A or G9416A) or the Agilent FISH FFPE Hybridization Buffer (Agilent p/n G9410A), in addition to the necessary histology reagents.

Storage conditions for the FISH general purpose reagents

Store the following reagents at –20°C.

- FISH Hybridization Buffer
- FISH FFPE Hybridization Buffer
- IQFISH Fast Hybridization Buffer
- FISH Mounting Buffer (with DAPI)
- FISH Mounting Buffer (without DAPI)

Store the following reagents at room temperature.

- FISH Wash Buffer 1
- FISH Wash Buffer 2

Catalog information for the FISH general purpose reagents

Table 1 contains a list of the Agilent part numbers for the FISH general purpose reagents.

Table 1 Agilent catalog information

Part Number	Description
G9400A	FISH Hybridization Buffer, 100 µl
G9410A	FISH FFPE Hybridization Buffer, 100 µl
G9415A	IQFISH Fast Hybridization Buffer, 200 µl
G9416A	IQFISH Fast Hybridization Buffer, 6 x 200 µl
G9401A	FISH Wash Buffer 1, 500 ml
G9402A	FISH Wash Buffer 2, 500 ml
G9403A	FISH Mounting Buffer,100 µl
G9404A	FISH Mounting Buffer, with DAPI, 100 µl
G9405A	FISH Hybridization Buffer and Mounting Buffer with DAPI, 100 µl each
G9406A	FISH Hybridization Buffer and Mounting Buffer with DAPI, 300 µl each
G9407A	FISH Hybridization Buffer and Mounting Buffer, 100 µl each
G9408A	FISH Hybridization Buffer and Mounting Buffer, 300 µl each
G9411A	ISH Pepsin Kit

2
FISH Protocol with Overnight Hybridization on non-FFPE Samples

This chapter provides instructions on how to perform a FISH experiment using a protocol that includes an overnight hybridization.

CAUTION If your sample slides contain formalin-fixed paraffin-embedded (FFPE) tissues, do not use this protocol. To perform FISH with FFPE samples, follow the protocol provided in Chapter 4, "FISH Protocol on FFPE Samples".

Required Reagents and Equipment

Table 2 contains a list of reagents and equipment that are required for the protocol in this chapter.

If your starting material is an FFPE sample, use the protocol provided in the next chapter, Chapter 4.

Table 2 Required Reagents and Equipment

Description
Glass microscope slide of fixed chromosomes
Agilent FISH Hybridization Buffer*
Agilent FISH Wash Buffer 1*
Agilent FISH Wash Buffer 2*
Agilent FISH Mounting Buffer*, with or without DAPI
Fluorescently labeled FISH probe(s)
Cover slips
Rubber cement
Hybridization chamber e.g. Dako Hybridizer, part number S245030
Dako Humidity Strips, part number S245230-2
Coplin jars
Ethanol, 200-proof
Nuclease-free dH_20
Formamide and 20× SSC only needed if using option 2 for denaturation/hybridization (see "Option 2: Denature the probe and chromosomal DNA separately" on page 14)
Epifluorescence microscope with appropriate filter cubes

* See Table 1 for catalog information.

Preparing the Probes

Prepare the probe hybridization mixtures

1 In a 1.5-ml microcentrifuge tube, combine the components in Table 3.

The volumes listed are for 1 slide; scale up the volumes as needed.

NOTE Fluorescently labeled probes are light-sensitive. To limit photo-bleaching, keep the probes and any mixture containing the probes in the dark as much as possible.

Table 3 Probe Hybridization Mixture

Component	Volume per reaction
FISH Hybridization Buffer	7 µl
Labeled FISH probe(s)	X µl (typically 1 µl/probe; up to 3 probes/mixture)
dH_20	X µl (enough to bring final volume to 10 µl)

2 Flick the tube several times, or vortex the tube for several seconds, to thoroughly mix the contents, then briefly spin it in a microcentrifuge.

Proceed directly to "Denaturing and Hybridizing the Probe and Chromosomal DNA". **Do not put the probe hybridization mixture on ice.**

Denaturing and Hybridizing the Probe and Chromosomal DNA

CAUTION If your sample slides contain formalin-fixed paraffin-embedded (FFPE) tissues, do not use this protocol. To perform FISH with FFPE samples, follow the protocol provided in Chapter 4, "FISH Protocol on FFPE Samples".

Before you can hybridize the probes to the chromosomes on the slide, both the probes and the chromosomal DNA must be denatured to create single-stranded DNA. Choose 1 of the 2 denaturation protocols that are provided here:

Option 1: Co-denature the probe and chromosomal DNA: In this protocol, you first prepare the slide for denaturation, then you apply the probe mixture to the slides and incubate the slides. You then incubate the slides at 78°C to denature both the probe and the chromosomal DNA. Once you complete the protocol, proceed to "Wash the slides" on page 16.

Option 2: Denature the probe and chromosomal DNA separately: In this protocol, you heat the slide and the probe mixture to the denaturing temperature of 73°C before you apply the probe to the slide. For some cell types, you may attain better results with this option. Once you complete the protocol, proceed to "Wash the slides" on page 16.

Option 1: Co-denature the probe and chromosomal DNA

Prepare the slides and reagents

1 Remove the slides from the –20°C freezer and incubate them at room temperature for 10–30 minutes.

2 Prepare 3 Coplin jars of the following solutions.

- 70% ethanol
- 85% ethanol
- 100% ethanol

If your slides have a label glued to one edge, fill the Coplin jars with enough solution to cover the spread of chromosomes without wetting the label.

3 Put up to 5 slides in the jar of 70% ethanol and incubate for 1 minute at room temperature.

4 Transfer the slides to the jar of 85% ethanol and incubate for 1 minute at room temperature.

5 Transfer the slides to the jar of 100% ethanol and incubate for 1 minute at room temperature. Then, remove the slides and put them on a clean benchtop to allow them to air dry at room temperature.

Denature the probe and chromosomal DNA together

1 Once the slides are dry, add the probe hybridization mixture:

   a Pipet 5 µl of probe hybridization mixture directly onto one of the prepared slides so that the area of the slide that contains the chromosomes is covered in liquid. Immediately cover with a cover slip (18×18 mm).

   If using larger cover slips, you can increase the volume of the probe hybridization mixture up to 10 µl.

   b Gently press down or lightly tap on the cover slip to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.

   Avoid moving the cover slip around on the slide.

   c Seal the edges of the cover slip with rubber cement.

   Make sure the rubber cement is applied to all edges of the slide to avoid evaporation of the probe hybridization mixture.

   d Repeat step a through step c for all slides.

2 Incubate the slides at 78°C for 5 minutes to denature the DNA.

   Perform this incubation on a heat block or in a Dako Hybridizer or other hybridization chamber.

   You may need to adjust the incubation time and temperature at this step to achieve optimal results for your specific cell type.

Hybridize the probes to the chromosomes

- Transfer the slides to 37°C and incubate overnight.

  Perform this incubation in a Dako Hybridizer or other hybridization chamber with a dark, humid environment.

  At the end of the incubation, proceed directly to "Wash the slides".

Option 2: Denature the probe and chromosomal DNA separately

Prepare the slides and reagents

1 Remove the slides from the –20°C freezer and incubate them at room temperature for 10–30 minutes.

2 Prepare 100 ml of Denaturation Solution by combining:
70 ml formamide
10 ml 20× SSC
20 ml dH_20

3 Fill a Coplin jar with the Denaturation Solution, and then warm the solution to 73°C using the following procedure:

a Put the Coplin jar in a programmable, room temperature water bath.

b Adjust the water bath setting to 73°C.

c Incubate the jar in this water bath for 30 minutes.

NOTE Putting the Coplin jar directly into a 73°C water bath may cause the jar to crack.

4 Prepare 3 additional Coplin jars of the following solutions.

- 70% ethanol
- 85% ethanol
- 100% ethanol

If your slides have a label glued to one edge, fill the Coplin jars with enough solution to cover the spread of chromosomes without wetting the label.

Denature the chromosomal DNA

1 Put up to 5 slides in the jar of prewarmed Denaturation Solution and incubate at 73°C for 5 minutes.

You may need to adjust the time and temperature of the denaturation to achieve optimal results for your specific cell type.

2 Transfer the slides to the jar of 70% ethanol and incubate for 1 minute at room temperature.

3 Transfer the slides to the jar of 85% ethanol and incubate for 1 minute at room temperature.

4 Transfer the slides to the jar of 100% ethanol and incubate for 1 minute at room temperature. Then, remove the slides and put them on a clean benchtop to allow them to air dry at room temperature.

Denature the probe DNA

- While the slides are drying, incubate the probe hybridization mixture at 73°C for 5 minutes to denature the probe DNA.

Hybridize the probes to the chromosomes

1 Add the probe hybridization mixture to the slides:

   a Pipet 5 µl of probe hybridization mixture directly onto one of the prepared slides so that the area of the slide that contains the chromosomes is covered in liquid. Immediately cover with a cover slip (18×18 mm).

   If using larger cover slips, you can increase the volume of the probe hybridization mixture up to 10 µl.

   a Gently press down or lightly tap on the cover slip to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.

   Avoid moving the cover slip around on the slide.

   b Seal the edges of the cover slip with rubber cement.

   Make sure the rubber cement is applied to all edges of the slide to avoid evaporation of the probe hybridization mixture.

   c Repeat step a through step b for all slides.

2 Transfer the slides to 37°C and incubate overnight.

   Perform this incubation in a Dako Hybridizer or other hybridization chamber with a dark, humid environment.

   At the end of the incubation, proceed directly to “Wash the slides”.

Washing and Viewing Slides

Wash the slides

1 Fill a Coplin jar with FISH Wash Buffer 1, and warm the buffer to 73°C using the following procedure.

   a Put the jar in a programmable, room temperature water bath.

   b Adjust the water bath setting to 73°C.

   c Incubate the jar in this water bath for 30 minutes.

NOTE Putting the Coplin jar directly into a 73°C water bath may cause the jar to crack.

2 Fill a second Coplin jar with FISH Wash Buffer 2. Leave this jar at room temperature.

3 Carefully remove the cover slip from one of the slides and then immediate put the slide in the jar of pre-warmed Wash Buffer 1, moving the slide up and down in the jar 2–3 times to agitate it. Repeat this step for up to 5 slides.

4 Allow the slides to sit in the jar of Wash Buffer 1 for 2 minutes at 73°C.

5 Transfer the slides to the Coplin jar of Wash Buffer 2, moving each slide up and down in the jar 2–3 times as you transfer it.

6 Allow the slides to sit in the jar of Wash Buffer 2 for 1 minute at room temperature.

7 Remove the slides from the jar and carefully remove any remaining rubber cement. Allow the slides to air dry in the dark at room temperature.

8 Repeat step 4 through step 7 for all slides, working in batches of no more than 5 slides at a time.

9 Continue to air dry the slides until they are all completely dry.

Once the slides are dry, proceed to "View the results", or store the slides at –20°C in the dark to be viewed later. Allow stored slides to come to room temperature before viewing.

View the results

1. Pipet 10 µl of the appropriate FISH Mounting Buffer (with or without DAPI) onto the slide so that the area of the slide that contains the chromosomes is covered. Immediately add a cover slip.
2. Gently press down or lightly tap on the cover slip to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.
3. View the slide under a fluorescence microscope.

Store slides at –20°C in the dark.

3
FISH Protocol with IQ Fast Hybridization on non-FFPE Samples

This chapter provides instructions on how to perform a FISH experiment using a protocol that includes a 90-minute hybridization.

CAUTION If your sample slides contain formalin-fixed paraffin-embedded (FFPE) tissues, do not use this protocol. To perform FISH with FFPE samples, follow the protocol provided in Chapter 4, "FISH Protocol on FFPE Samples".

Required Reagents and Equipment

Table 4 contains a list of reagents and equipment that are required for the protocol in this chapter.

If your starting material is an FFPE sample, use the protocol provided in the next chapter, Chapter 4.

Table 4 Required Reagents and Equipment

Description
Glass microscope slide of fixed chromosomes
Reagents from the Dako Cytology FISH Accessory Kit (Dako part number K5477) Dako Wash Buffer (20×) Dako Stringent Wash Buffer (20×) Dako Coverslip Sealant Dako Fluorescence Mounting Medium
1100-watt microwave oven
Agilent IQFISH Fast Hybridization Buffer*
Fluorescently labeled FISH probe(s)
Cover slips
Hybridization chamber e.g. Dako Hybridizer, part number S245030
Dako Humidity Strips, part number S245230-2
Coplin jars
Ethanol, 200-proof
Nuclease-free dH_20
2× SSC
Epifluorescence microscope with appropriate filter cubes
Agilent FISH Mounting Buffer with DAPI* (if using a DAPI/FITC/Cy3 triple filter for visualization)

* See Table 1 for catalog information.

Preparing the Slides and Probes

Prepare the reagents

1 Prepare 50 ml of the following solutions in separate Coplin jars.
- 70% ethanol
- 85% ethanol
- 100% ethanol

2 Prepare 50 ml of 2× SSC in a heat-resistant Coplin jar.

NOTE In place of 2× SSC, a MES-based pretreatment solution can also be used.

3 Prepare 500 ml of 1× Dako Wash Buffer by combining:
25 ml 20× Dako Wash Buffer
475 ml H_20

The volumes indicated in these instructions are appropriate for processing slides in Coplin jars. If you are processing your slides in an alternative container, you can scale the volumes as needed. For example, for a slide staining dish, prepare 200 ml of each of the ethanol solutions, 200 ml of 2× SSC, and 2 L of 1× Dako Wash Buffer.

Pre-treat the slides

1 Add the slides to a heat-resistant Coplin jar containing 50 ml of 2× SSC. Loosely cover the jar with a lid.

NOTE Make sure that steam can escape from the jar either by making holes in the jar lid prior to use or by leaving the lid slightly ajar during microwave use.

2 Transfer the jar to a 1100-watt microwave oven and heat at 100% power until the solution just begins to boil (approximately 1–3 minutes).
Proceed immediately to step 3 when the solution begins to boil.

3 Maintain the solution at just below boiling temperature in the microwave oven by heating at 10–20% power for an additional 10 minutes. Monitor the solution during the 10-minute incubation step. If boiling is observed, reduce the power level.

   A steam-sensing microwave oven may also be used. In this case, heat the sample using the steam sensing function for 10 minutes.

4 Remove the jar from the microwave oven and carefully remove the lid (do not remove the slides). Allow the slides to cool in the solution for 15 minutes at room temperature.

5 Transfer the slides to a jar of 1× Dako Wash Buffer and incubate for 3 minutes at room temperature. After 3 minutes, replace with fresh 1× Dako Wash Buffer and incubate for an additional 3 minutes at room temperature.

6 Transfer the slides to a jar of 70% ethanol and incubate for 1–2 minutes at room temperature.

7 Transfer the slides to a jar of 85% ethanol and incubate for 1–2 minutes at room temperature.

8 Transfer the slides to a bath of 100% ethanol and incubate for 1–2 minutes at room temperature.

9 Remove the slides and allow them to air dry completely at room temperature. While the slides are drying, prepare the FISH probes as described in the following section.

Prepare the probe hybridization mixtures

1 Thaw the vial of IQFISH Fast Hybridization Buffer at room temperature. Mix the buffer solution thoroughly by vortexing for 15 seconds maximum speed. Briefly spin the vial to collect the liquid.

NOTE The hybridization buffer may separate into two layers when frozen and thawed. Make sure that the buffer has been mixed well and is a homogeneous suspension before use.

2 If frozen, thaw probes at room temperature. Mix by vortexing then briefly spin the vial to collect the liquid.

3 Combine the components in Table 5 in a 1.5-ml microcentrifuge tube.

The volumes listed are for 1 slide; scale up the volumes as needed.

NOTE Fluorescently labeled probes are light-sensitive. To limit photo-bleaching, keep the probes and any mixture containing the probes in the dark as much as possible.

Table 5 Probe Hybridization Mixture

Component	Volume per reaction
IQFISH Fast Hybridization Buffer	9 µl
Labeled FISH probe(s)	X µl (typically 1 µl/probe; up to 3 probes/mixture)*

* If labeled FISH probes are supplied pre-mixed (e.g. orange-red probe and green probe in single tube), add 1 µl of the pre-mixed probe. If labeled probes are supplied in separate tubes, add 1 µl of each probe.

4 Flick the tube several times, or vortex the tube for several seconds, to thoroughly mix the contents, then briefly spin it in a microcentrifuge.

5 Proceed directly to "Denaturing and Hybridizing the Probe and Chromosomal DNA". **Do not put the probe hybridization mixture on ice.**

Denaturing and Hybridizing the Probe and Chromosomal DNA

Denature the probe and chromosomal DNA

1. Pipet 5–10 µl of probe hybridization mixture directly onto one of the prepared slides so that the area of the slide that contains the chromosomes is covered in liquid. Immediately cover with a cover slip (18×18 mm or 22×22 mm).
2. Gently press down or lightly tap on the cover slip to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.

   Avoid moving the cover slip around on the slide.
3. Seal the edges of the cover slip with Dako Coverslip Sealant.

   Make sure the Coverslip Sealant is applied to all edges of the slide to avoid evaporation of the probe hybridization mixture.
4. Repeat step 1 through step 3 for all slides.
5. Incubate the slides at 66°C for 10 minutes to denature the DNA.

   Perform this incubation in a Dako Hybridizer or other hybridization chamber.

   You may need to adjust the incubation time and temperature at this step to achieve optimal results for your specific cell type.

Hybridize the probes to the chromosomes

- Transfer the slides to 45°C and incubate for 90 minutes.

  Perform this incubation in a Dako Hybridizer or other hybridization chamber with a dark, humid environment.

  At the end of the incubation, proceed directly to “Washing and Viewing Slides”.

NOTE

You can perform the FISH protocol with IQ Fast Hybridization on non-FFPE samples without pre-treating the slides in 2× SSC if you make the following protocol modifications:

- Omit step step 1 through step 5 on page 21 and proceed directly to the ethanol dehydration step (step 6 on page 21).
- In step 5 on page 23, denature the probe and chromosomal DNA at 90°C for 5 minutes (instead of 66°C for 10 minutes).
- Immediately following the 10-minute wash in Dako Stringent Wash buffer at 63°C (step 6 on page 25), perform an additional wash in dH_20 at 37°C for 1 minute before proceeding to step 7.

Note that, even with the above modifications, omitting the pre-treatment in 2× SSC may negatively impact probe signal morphology.

Washing and Viewing Slides

Wash the slides

1 Prepare 100 ml of diluted Dako Stringent Wash Buffer by combining:
5 ml of 20× Dako Stringent Wash Buffer
95 ml of dH_20

Divide the diluted Dako Stringent Wash Buffer between two heat-resistant Coplin jars.

2 Leave one of the jars containing diluted Dako Stringent Wash Buffer at room temperature. Preheat the other jar to 63°C using the following procedure.

a Put the jar into a programmable, room temperature, circulating water bath. Cover the jar with a lid to help stabilize the temperature and avoid evaporation.

b Adjust the water bath setting to 63°C.

c Monitor the temperature inside the jar using a calibrated thermometer to ensure that it reaches 63°C.

NOTE Putting the Coplin jar directly into a 63°C water bath may cause the jar to crack.

3 Remove the slides from the Dako Hybridizer or hybridization chamber.

4 Working with one slide a time, gently remove the Coverslip Sealant with forceps and then remove the cover slip. As you finish with each slide, put the slide into the room temperature jar containing the diluted Dako Stringent Wash Buffer.

5 After you have removed the cover slips from all the slides, transfer the slides from the room temperature jar of diluted Stringent Wash buffer to the 63°C jar of diluted Dako Stringent Wash Buffer in the water bath. Agitate each slide for 10 seconds by vigorously moving it up and down within the jar. Close the jar lid and then the water bath lid.

6 Incubate the slides at 63°C for exactly 10 minutes.

7 Remove the slides and transfer to the jar of 1× Dako Wash Buffer. Incubate the slides at for 1 minute at room temperature.

8 Transfer the slides to a second jar containing 1× Dako Wash Buffer. Incubate the slides for an additional 3 minutes at room temperature.

Dehydrate the samples

1 Put the slides in a jar of 70% ethanol and incubate for 1 minute at room temperature.

2 Transfer the slides to a jar of 85% ethanol and incubate for 1 minute at room temperature.

3 Transfer the slides to a jar of 100% ethanol and incubate for 1 minute at room temperature.

4 Remove the slides and allow them to air dry completely at room temperature, in the dark.

View the results

1 Pipet 10 µl of Dako Fluorescence Mounting Medium onto the slide so that the sample is covered. Immediately add a cover slip.

NOTE If using a DAPI/FITC/Cy3 triple filter for visualization, use Agilent FISH Mounting Buffer with DAPI (part number 9404A) in place of the Dako Fluorescence Mounting Medium to minimize photo-bleaching.

2 Gently tap on the cover slip with forceps to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.

3 View the slide under a fluorescence microscope.

Store slides at –20°C in the dark.

4
FISH Protocol on FFPE Samples

This chapter provides instructions on how to perform a FISH experiment using FFPE samples.

Required Reagents and Equipment

Table 6 contains a list of reagents and equipment that are required for the FISH protocol for FFPE samples.

Table 6 Required reagents and equipment

Description
Glass microscope slide of FFPE tissue slice
Reagents from the Dako Histology Accessory Kit, Dako part number K5799 Dako Pre-Treatment Solution (20×) Dako Wash Buffer (20×) Dako Pepsin Diluent (10×) Dako Pepsin Solution Dako Stringent Wash Buffer (20×) Dako Coverslip Sealant Dako Fluorescence Mounting Medium
Agilent ISH Pepsin Kit, Agilent part number G9411A (if additional Pepsin is needed [a])
Xylene or xylene substitute (e.g. CitriSolv)
Circulating water bath with programmable heater
Lintless tissues, e.g. KimWipes
Agilent FISH FFPE Hybridization Buffer [b] OR Agilent IQFISH Fast Hybridization Buffer [b]
Fluorescently labeled FISH probe(s)
Cover slips
Hybridization chamber e.g. Dako Hybridizer, part number S245030
Dako Humidity Strips, part number S245230-2
Coplin jars
Ethanol, 200-proof
Nuclease-free dH_20

Table 6 Required reagents and equipment

Description
Epifluorescence microscope with appropriate filter cubes
Agilent FISH Mounting Buffer with DAPI [b] (if using a DAPI/FITC/Cy3 triple filter for visualization[c])

[a] Pepsin is provided in the Dako Histology Accessory Kit (Dako p/n K5799). The Agilent ISH Pepsin Kit, (Agilent p/n G9411A), provides additional pepsin, if needed for the protocol.

[b] See Table 1 for catalog information.

[c] Single and double filters are preferable to triple filters as they provide better signal intensity and photo-stability.

Preparing the Slides for FISH

Prepare the Dako histology reagents

1 Prepare 50 ml of diluted Dako Pre-Treatment Solution in a heat-resistant Coplin jar by combining:
2.5 ml of 20× Dako Pre-Treatment Solution
47.5 ml of dH_20

The diluted Dako Pre-Treatment Solution must be heated to 98°C for use in the protocol below. Heating to 98°C may be done either by using a water bath, or by using a microwave oven. If using a water bath to heat the solution, do the preheating steps described on page 31 during the slide preparation steps below (under "De-paraffinize the samples"). If using a microwave oven, leave the Dako Pre-Treatment Solution at room temperature until it is used on page 32.

2 Prepare 500 ml of diluted Dako Wash Buffer by combining:
25 ml of 20× Dako Wash Buffer
475 ml of dH_20

NOTE Volumes may be scaled for slide processing in alternative containers. For example, if using a staining dish, prepare 200 ml of 1× Dako Pre-Treatment solution and 2L of 1× Dako Wash Buffer.

De-paraffinize the samples

NOTE Sample maturation as described in step 1 below is optional. If performed, it may reduce background noise for some samples.

1 **Optional:** Incubate the slides with the FFPE samples at 60°C for 1 hour to mature the samples.

Perform this incubation in a Dako Hybridizer or other hybridization chamber or heating block.

2 Transfer the slides to a jar of xylene and incubate for 5 minutes. Replace with fresh xylene and incubate the slides for another 5 minutes.

NOTE Always tap off excess liquid from the slides when transferring them between solutions.

3 Transfer the slides to a jar of 100% ethanol. Incubate the slides for 2 minutes at room temperature. Replace with fresh 100% ethanol and incubate the slides for another 2 minutes.

4 Transfer the slides to a jar of 70% ethanol. Incubate the slides for 2 minutes at room temperature. Replace with fresh 70% ethanol and incubate the slides for another 2 minutes.

5 Transfer the slides to a jar of diluted Dako Wash Buffer. Incubate the slides for 2–5 minutes at room temperature.

Pre-treat the samples with Dako Pre-Treatment Solution

In this step, slides are incubated in Dako Pre-Treatment Solution held at 98°C, then cooled to room temperature. Heating of the pre-treatment solution may be done either using a water bath (follow the steps directly below) or using a microwave oven (follow the steps on page 32). At the end of the pre-treatment, proceed to "Digest proteins using Pepsin" on page 33.

Option 1: Pre-treat using water bath-heated Dako Pre-Treatment Solution

1 Preheat the diluted Dako Pre-Treatment Solution to 98°C using the following procedure. This pre-heating step should be started during sample maturation (step 1 on page 30).

   a Put the Coplin jar containing the diluted Dako Pre-Treatment Solution into a programmable, room temperature, circulating water bath.

   b Adjust the water bath setting to 98°C.

   c Monitor the temperature inside the jar using a calibrated thermometer to ensure that the temperature reaches a minimum of 95°C without reaching the boiling point of 100°C.

NOTE Putting the Coplin jar directly into a 98°C water bath may cause the jar to crack.

2 Transfer the de-paraffinized sample slides to the preheated jar of diluted Dako Pre-Treatment Solution in the 98°C water bath. Leaving the jar in the 98°C water bath, incubate the slides for 10 minutes.

3 Remove the jar containing the slides from the water bath and carefully remove the lid from the jar (do not remove the slides). Allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

4 Transfer the slides to a jar of diluted Dako Wash Buffer. Incubate the slides for 3 minutes at room temperature. Replace with fresh diluted Dako Wash Buffer and incubate the slides for another 3 minutes.

5 Remove the slides from the jar and tap off the excess liquid.

Option 2: Pre-treat using microwave oven-heated Dako Pre-Treatment Solution

NOTE

When using the microwave heating method, make sure that steam can escape from the Coplin jar either by making holes in the jar lid prior to use or by leaving the lid ajar during microwave use.

1 Add the de-paraffinized sample slides to the Coplin jar containing 50 ml of diluted Dako Pre-Treatment Solution at room temperature.

2 Place the jar in a 1100-watt microwave oven and heat at 100% power until the solution just begins to boil (approximately 1 to 3 minutes). Proceed immediately to step 3 when the solution begins boiling.

3 Maintain the solution at just below boiling temperature in the microwave oven by heating at 10-20% power for an additional 10 minutes.

   Monitor the solution during the 10-minute incubation step. If boiling is observed, reduce the power level.

4 Carefully remove the lid from the jar (do not remove the slides). Allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

5 Transfer the slides to a jar of diluted Dako Wash Buffer. Incubate the slides for 3 minutes at room temperature. Replace with fresh diluted Dako Wash Buffer and incubate the slides for another 3 minutes.

6 Remove the slides from the jar and tap off the excess liquid.

Digest proteins using Pepsin

In this step, the proteins in the sample are digested with Pepsin. The digest may be done either with droplets of Pepsin (follow the steps in Option 1 directly below) or in a Pepsin bath (follow the steps in Option 2). At the end of the digest, proceed to "Dehydrate the samples" on page 34.

Option 1: Digest proteins using Pepsin droplets

1. Using a KimWipe or other lintless tissue, carefully dry the area of the slide around the sample without disturbing the sample.
2. Using the Pepsin from the Dako Histology Accessory Kit or the Agilent ISH Pepsin Kit, add 5 drops of cold (2–8°C) Pepsin to each slide, ensuring that the sample is covered. Add more cold Pepsin as necessary to cover the sample.
3. Put the slides, with the Pepsin on top, on a hot plate or in a Dako Hybridizer set to 37°C. Incubate the slides at 37°C for 3–18 minutes.

NOTE

The optimal incubation time for step 3 may vary between sample slides due to differences in the way the tissue samples were fixed and embedded. As a starting point, Agilent recommends a 6-minute incubation.

4. Remove the slides from the Dako Hybridizer or hot plate and tap off the excess Pepsin.
5. Transfer the slides to a jar of diluted Dako Wash Buffer. Incubate the slides for 3 minutes at room temperature. Replace with fresh diluted Dako Wash Buffer and incubate the slides for another 3 minutes.

Option 2: Digest proteins in a Pepsin bath

1. In a heat-resistant Coplin jar, prepare a diluted Pepsin solution using either the Pepsin and Pepsin Diluent from the Dako Histology Accessory Kit or the Pepsin and Pepsin Diluent from the Agilent ISH Pepsin Kit. Dilute the Pepsin and Pepsin Diluent 1:10 with nuclease-free H_20.

   For example, for 60 ml of 1× Pepsin solution, combine:
   6 ml of Pepsin
   6 ml of 10× Pepsin Diluent
   48 ml of nuclease-free H_20

2 Put the Coplin jar containing the Pepsin solution into a 37°C water bath.

3 Once the Pepsin solution is heated to 37°C, add the slides to the jar. Incubate the slides at 37°C for 30 minutes.

NOTE The optimal incubation time for step 3 may vary between sample slides due to differences in the way the tissue samples were fixed and embedded. As a starting point, Agilent recommends a 30-minute incubation.

4 Transfer the slides to a jar of diluted Dako Wash Buffer. Incubate the slides for 3 minutes at room temperature. Replace with fresh diluted Dako Wash Buffer and incubate the slides for another 3 minutes.

Dehydrate the samples

1 Put the slides in a jar of 70% ethanol and incubate for 2 minutes at room temperature.

2 Transfer the slides to a jar of 85% ethanol and incubate for 2 minutes at room temperature.

3 Transfer the slides to a bath of 100% ethanol and incubate for 2 minutes at room temperature.

4 Remove the slides and allow them to air dry completely at room temperature. While the slides are drying, prepare the FISH probes as described in the following section.

Hybridizing to FISH Probes

Two separate hybridization protocols (listed below) are provided in this section. Make sure that you are following the probe preparation and hybridization steps appropriate for your Hybridization Buffer.

- Hybridize probes using **IQFISH Fast Hybridization Buffer** (page 35, below)
- Hybridize probes using **FISH FFPE Hybridization Buffer** (page 37)

Option 1: Hybridize probes using IQFISH Fast Hybridization Buffer (Fast Hybridization Protocol)

Prepare the probe hybridization mixture

1 Thaw the vial of IQFISH Fast Hybridization Buffer at room temperature. Mix the buffer solution thoroughly by vortexing for 15 seconds or by repeated pipetting. Briefly spin the vial to collect the liquid.

NOTE The hybridization buffer may separate into two layers when frozen and thawed. Make sure that the buffer has been mixed well and is a homogeneous suspension before use.

2 If frozen, thaw probes at room temperature. Mix by vortexing then briefly spin the vial to collect the liquid.

3 In a 1.5-ml microcentrifuge tube, combine the components in Table 7.

The volumes listed are for 1 slide; scale up the volumes as needed.

NOTE Fluorescently labeled probes are light-sensitive. To limit photo-bleaching, keep the probes and any mixture containing the probes in the dark as much as possible.

Table 7 Probe Hybridization Mixture for IQFISH Fast Hybridization Buffer

Component	Volume per reaction
Agilent IQFISH Fast Hybridization Buffer	9 µl
Labeled FISH probe(s)	X µl (typically 1 µl/probe; up to 3 probes/mixture)

* If labeled FISH probes are supplied pre-mixed (e.g. orange-red probe and green probe in single tube), add 1 µl of the pre-mixed probe. If labeled probes are supplied in separate tubes, add 1 µl of each probe.

4 Flick the tube several times, or vortex the tube for several seconds, to thoroughly mix the contents, then briefly spin it in a microcentrifuge.

At the end of this step, proceed directly to "Denature the probe and chromosomal DNA in IQFISH Fast Hybridization Buffer". **Do not put the probe hybridization mixture on ice.**

Denature the probe and chromosomal DNA in IQFISH Fast Hybridization Buffer

1 Pipet 10 µl of the probe hybridization mixture directly onto a prepared slide so that the sample is covered in liquid. Immediately cover with a glass cover slip (18×18 mm or 22×22 mm).

If you see air bubbles between the slide and cover slip, gently tap the cover slip with forceps until the air bubbles are gone.

2 Seal the edges of the cover slip with Dako Coverslip Sealant.

Make sure that the Coverslip Sealant seals all edges of the cover slip to avoid evaporation of the probe hybridization mixture.

3 Put the slides in a Dako Hybridizer or other hybridization chamber set to 80°C (see note below). Incubate the slides for 10 minutes to denature the probes and chromosomal DNA.

NOTE In most assays, higher signals are observed when 80°C is used for denaturation. If the background is unacceptably high using 80°C, the denaturation temperature can be lowered to 66°C.

Hybridize the probes to the chromosomes in IQFISH Fast Hybridization Buffer

1 Adjust the temperature on the Dako Hybridizer or hybridization chamber to 45°C. Hybridize the probes to the chromosomes by incubating the slides at 45°C for an additional 60 to 120 minutes.

2 Proceed to "Washing and Viewing Slides" on page 39.

Option 2: Hybridize probes using FISH FFPE Hybridization Buffer (Overnight Hybridization Protocol)

Prepare the probe hybridization mixtures

1 In a 1.5-ml microcentrifuge tube, combine the components in Table 8.

The volumes listed are for 1 slide; scale up the volumes as needed.

NOTE Fluorescently labeled probes are light-sensitive. To limit photo-bleaching, keep the probes and any mixture containing the probes in the dark as much as possible.

Table 8 Probe Hybridization Mixture for FISH FFPE Hybridization Buffer

Component	Volume per reaction
Agilent FISH FFPE Hybridization Buffer	7 µl
Labeled FISH probe(s)	X µl (typically 1 µl/probe; up to 3 probes/mixture)
dH_20	X µl (enough to bring final volume to 10 µl)

2 Flick the tube several times, or vortex the tube for several seconds, to thoroughly mix the contents, then briefly spin it in a microcentrifuge.

At the end of this step, proceed directly to "Denature the probe and chromosomal DNA in FISH FFPE Hybridization Buffer". **Do not put the probe hybridization mixture on ice.**

Denature the probe and chromosomal DNA in FISH FFPE Hybridization Buffer

1. Pipet 10 µl of the probe hybridization mixture directly onto a prepared slide so that the sample is covered in liquid. Immediately cover with a glass cover slip (18×18 mm or 22×22 mm).

   If you see air bubbles between the slide and cover slip, gently tap the cover slip with forceps until the air bubbles are gone.

2. Seal the edges of the cover slip with Dako Coverslip Sealant.

   Make sure that the Coverslip Sealant seals all edges of the cover slip to avoid evaporation of the probe hybridization mixture.

3. Put the slides in a Dako Hybridizer or other hybridization chamber set to 90°C. Incubate the slides for 5 minutes at 90°C to denature the probes and chromosomal DNA.

Hybridize the probes to the chromosomes overnight in FISH FFPE Hybridization Buffer

1. Adjust the temperature on the Dako Hybridizer or hybridization chamber to 37°C. Incubate the slides at 37°C overnight (14–20 hours) to hybridize the probes to the chromosomes.
2. Proceed to "Washing and Viewing Slides" on page 39.

Washing and Viewing Slides

Wash the slides

1 Prepare 100 ml of diluted Dako Stringent Wash Buffer by combining:
5 ml of 20× Dako Stringent Wash Buffer
95 ml of dH_20

Divide the diluted Dako Stringent Wash Buffer between two heat-resistant Coplin jars.

2 Leave one of the jars containing diluted Dako Stringent Wash Buffer at room temperature. Preheat the other jar to 65°C using the following procedure.

a Put the jar into a programmable, room temperature, circulating water bath. Cover the jar with a lid to help stabilize the temperature and avoid evaporation.

b Adjust the water bath setting to 65°C.

c Monitor the temperature inside the jar using a calibrated thermometer to ensure that it reaches 65°C.

NOTE Putting the Coplin jar directly into a 65°C water bath may cause the jar to crack.

3 Remove the slides from the Dako Hybridizer or hybridization chamber.

4 Working with one slide a time, gently remove the Coverslip Sealant with forceps and then remove the cover slip. As you finish with each slide, put the slide into the room temperature jar containing the diluted Dako Stringent Wash Buffer.

5 After you have removed the cover slips from all the slides, transfer the slides from the room temperature jar of diluted Stringent Wash buffer to the 65°C jar of diluted Stringent Wash Buffer in the water bath. Close the jar lid and then the water bath lid. Incubate the slides at 65°C for exactly 10 minutes.

6 Remove the slides and transfer to a room temperature jar of diluted Dako Wash Buffer (not the Stringent Wash Buffer). Incubate the slides for 3 minutes. Replace with fresh diluted Dako Wash Buffer and incubate the slides for another 3 minutes.

Dehydrate the samples

1 Put the slides in a jar of 70% ethanol and incubate for 2 minutes at room temperature.
2 Transfer the slides to a jar of 85% ethanol and incubate for 2 minutes at room temperature.
3 Transfer the slides to a jar of 100% ethanol and incubate for 2 minutes at room temperature.
4 Remove the slides and allow them to air dry completely at room temperature, in the dark.

View the results

1 Pipet 10 µl of Dako Fluorescence Mounting Medium onto the slide so that the sample is covered. Immediately add a cover slip.

NOTE If using a DAPI/FITC/Cy3 triple filter for visualization, use Agilent FISH Mounting Buffer with DAPI (part number 9404A) in place of the Dako Fluorescence Mounting Medium to minimize photo-bleaching.

2 Gently tap on the cover slip with forceps to spread the liquid evenly under the cover slip and, if possible, to remove bubbles.
3 View the slide under a fluorescence microscope.

Store slides at –20°C in the dark.

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In This Book

This document describes how to use the Agilent FISH General Purpose Reagents to perform fluorescence in situ hybridization (FISH) on a spread of chromosomes or with tissues that have been preserved with FFPE.

Version F0, September 2015

G9400-90000

Agilent Technologies