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ViewRNA™ ISH Tissue Assay

Catalog Numbers 19931, 19932, 19942, 19943

Publication Number MAN0018633
Revision D.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0018633

Revision	Date	Description
D.0	12 October 2020	Removed use of frozen tissue from the manual.
C.0	20 February 2020	Updated manufacturing address.
B.0	13 January 2020	Protocol update because one of the kit components has been replaced.
A.0	14 April 2019	New user guide.

Contents

Product information

Product description

The ViewRNA™ ISH Tissue Assay is used for visualization of one or two target RNAs in formalin-fixed paraffin-embedded (FFPE) samples prepared in accordance with the guidelines provided in this manual. **ViewRNA™ ISH Tissue Assay is not recommended for OCT embedded frozen tissue sections.**

Assay overview

In situ hybridization (ISH) techniques are used to visualize DNA or localize RNAs within cells. Whereas *in situ* analysis of RNA has traditionally been limited by low sensitivity and complicated probe synthesis, the ViewRNA™ ISH Tissue Assay, based on highly specific branched DNA signal amplification technology, provides robust *in situ* detection of one or two target mRNAs within FFPE tissue sections with single-copy sensitivity.

Features

- Suitable for formalin-fixed paraffin-embedded (FFPE) tissue section.
- Single RNA molecule sensitivity (one dot = one RNA molecule).
- Detection of two target RNAs.
- Chromogenic and fluorescent detection.
- Compatible with hematoxylin and DAPI.
- Compatible with brightfield and fluorescence microscopes or scanners.

Kit contents and storage

The ViewRNA™ ISH Tissue Assay consists of three modules, each sold separately:

- **ViewRNA™ ISH Tissue Assay, Core Kit**: contains the reagents required for the detection of a single mRNA target using the Fast Red substrate.
- **ViewRNA™ ISH Tissue Assay, Blue Module (for 2-plex assays only)**: contains reagents for the detection of a second RNA target using the Fast Blue Substrate. Both the Blue Module and the Core Kit are required to perform a ViewRNA™ ISH Tissue 2-plex assay.
- **ViewRNA™ TYPE1 and TYPE6 Probe Sets**: contains oligonucleotides specific to your RNA target of interest. Two different amplification system

"TYPEs" are used (consisting of Pre-amplifier, Amplifier, Label Probe, and Substrate). TYPE 1 probe sets and amplification systems are compatible with Fast Red, and TYPE 6 systems with Fast Blue.

Types of ViewRNA™ ISH Tissue Assay Kits

Each ViewRNA™ Tissue Assay Core Kit or Blue Module comes in two sizes, based on the number of assays that can be performed with it.

Kit	Cat. No.	Amount
ViewRNA™ Tissue Assay Core Kit	19931	24 assays
	19942	96 assays
ViewRNA™ Tissue Assay Blue Module	19932	24 assays
	19943	96 assays

ViewRNA™ ISH Tissue Assay, Core Kit

Each ViewRNA™ Tissue Assay Core Kit contains the following components, supplied in two boxes based on storage temperature. Please refer to kit labels for expiration dates and to the Package Insert for component quantities. Kits are configured for processing a minimum of 6 assays per experiment.

For 19931; For 19942 (96 assays per kit) multiply the quantity of components by 4.

Part number*	Component	Quantity/ 24 assay kit	Description	Storage
17428 / QVT0500	100X Pretreatment Solution	21 mL	Aqueous buffered solution	2-8°C box 19939
18844 / QVT0510	Probe Set Diluent	12 mL	Aqueous solution containing formamide, detergent, and blocker	
18978 / QVT0513	AP (Alkaline Phosphatase) Label Probe Diluent	24 mL	Aqueous solution containing detergent	
18874	Pre-Amplifier Mix	10 mL	DNA in aqueous solution containing formamide and detergent	
18875	Amplifier Mix	10 mL	DNA in aqueous solution containing formamide and detergent	
17430 / QVT0508	AP (Alkaline Phosphatase) Enhancer Solution	10 mL	Aqueous buffered solution	
19941	ViewRNA™ Fast Red Reaction Buffer	20 mL	Buffer required for reaction of ViewRNA™ Fast Red substrate with Alkaline Phosphatase (AP)	
19944	ViewRNA™ Fast Red Substrate 1	320 µL	Aqueous solution of ViewRNA™ Fast Red precipitating substrate for the detection of alkaline phosphatase activity	
19945	ViewRNA™ Fast Red Substrate 2	320 µL		
19946	ViewRNA™ Fast Red Substrate 3	320 µL		
17420 / QVT0512	100X Protease Solution	180 µL	Enzyme in aqueous buffered solution	2-8°C Do Not Freeze box 19939
18994	AP (Alkaline Phosphatase) Label Probe Type 1	45 µL	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	
18871	100X Wash Buffer Component 1	144 mL	Aqueous buffered solution containing detergent	15-30°C box 19940
18872	400X Wash Buffer Component 2	280 mL	Aqueous buffered solution	

*Component part numbers are not catalog numbers but can be requested as custom part numbers, with a quote, if needed.

Separate catalog reagents can be bought separately according to the following table.

Part number	Component	Quantity/ 24 assay kit	Description	Storage
QVT0500	100X Pretreatment Solution	21 mL	Aqueous buffered solution	2-8°C
QVT0510	Probe Set Diluent	12 mL	Aqueous solution containing formamide, detergent, and blocker	
QVT0513	AP (Alkaline Phosphatase) Label Probe Diluent	24 mL	Aqueous solution containing detergent	
QVT0508	AP (Alkaline Phosphatase) Enhancer Solution	10 mL	Aqueous buffered solution	
QVT0517	AP (Alkaline Phosphatase) Reaction stop solution	10 mL	Aqueous buffered solution designed for the inactivation of residual Label Probe 6-AP activity after the Fast Blue Substrate development	
QVT0512	100X Protease Solution	180 µL	Enzyme in aqueous buffered solution	2-8°C Do Not Freeze

ViewRNA™ ISH Tissue Assay, Blue Module

Each ViewRNA™ Tissue Assay Blue Module contains the following components. Refer to kit labels for expiration dates and to the Package Insert for component quantities.

For 19932: For 19943 (96 assays per kit) multiply the quantity of components by 4				
Part Number*	**Component**	**Quantity/ 24 assay kit**	**Description**	**Storage**
18847	ViewRNA™ Fast Blue Reaction Buffer	20 mL	Buffer required for reaction of ViewRNA™ Fast Red substrate with Alkaline Phosphatase (AP)	2-8°C
18980	ViewRNA™ Fast Blue Substrate 1	420 µL	Aqueous solution of ViewRNA™ Fast Red precipitating substrate for the detection of alkaline phosphatase activity	
18981	ViewRNA™ Fast Blue Substrate 2	420 µL		
18982	ViewRNA™ Fast Blue Substrate 3	420 µL		
10748 / QVT0517	AP (Alkaline Phosphatase) Reaction stop solution	10 mL	Aqueous buffered solution designed for the inactivation of residual Label Probe 6-AP activity after the Fast Blue Substrate development	
18861	AP (Alkaline Phosphatase) Label Probe for Type 6	45 µL	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2-8°C Do not freeze

* Component part numbers are not catalog numbers but can be requested as custom part numbers, with a quote, if needed.

Safety warnings and precautions

- Formaldehyde is a poison and an irritant. Avoid contact with skin and mucous membranes. Use in a fume hood.
- Ammonium hydroxide is highly volatile. Use in a fume hood.
- Xylene is both flammable and an irritant. Avoid inhalation and contact with skin. Use in a fume hood.
- Probe Set Diluent, Pre-amplifier Mix, and Amplifier Mix contain formamide, a teratogen, irritant and possible carcinogen. Avoid contact with mucous membranes.
- DAPI is a possible mutagen. Avoid contact with skin and mucous membranes.
- Perform all procedural steps in a well-ventilated area at room temperature (RT) unless otherwise noted.
- Discard all reagents in accordance with local, state, and federal laws.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**www.fisherscientific.com**) or other major laboratory supplier.

Materials	**Source**
Tissue Tek Staining Dish (clear color)	Thermo Fisher Scientific, QVC0502 American Master Tech Scientific, LWS20WH
Tissue Tek Clearing Agent Dish (green color)	American Master Tech Scientific, LWS20GR
Tissue Tek Vertical 24 Slide Rack	Thermo Fisher Scientific, QVC0503
1000 mL glass beaker	MLS
Forceps	Thermo Fisher Scientific, QVC0501
Pipettes – P20, P200, P1000	MLS
Hydrophobic Barrier Pen	Thermo Fisher Scientific, QVC0500 Vector Laboratories, H4000
Mounting media • Dako UltraMount Permanent Mounting Medium • Slowfade Glass Antifade non-curing mountant • Prolong Glass Antifade Curing mountant • Cytoseal 60	 • Agilent, S196430-2 • Thermo Fisher Scientific, S36916 • P36981 • Thermo Fisher Scientific, 8310-4
Rectangular cover glass, 24 mm x 55 mm	Thermo Fisher Scientific, QVC0506
Aluminum foil	MLS
Double-distilled water (dd$H_2$0)	MLS
100% ethanol (200 proof)	MLS
10X PBS, pH 7.2-7.4 RNase-Free	Thermo Fisher Scientific, AM9625
Gill's Hematoxylin I	American Master Tech Scientific, HXGHE1LT
xylene or Histo-Clear	National Diagnostics, HS-200 or equivalent
37% formaldehyde	EMD Millipore, FX0410-1 or equivalent
27-30% ammonium hydroxide	VWR, JT-9726-5 or equivalent
100X DAPI (optional, for fluorescence detection)	Thermo Fisher Scientific, QVC0515
Equipment	**Source**
Either of the following hybridization systems: • ThermoBrite System and ThermoBrite Humidity Strips • Tissue culture incubator with >85% humidity and 0% CO_2 and 3 aluminum slide racks for transferring slides to incubator during hybridization	 • Abbott, 07J91-010 (110V) & 07J68-001 • MLS
ViewRNA™ Temperature Validation Kit	Thermo Fisher Scientific, QV0523
Water-proof remote probe thermometers, validated for 90–100°C	VWR, 46610-024
Fume hood	MLS
Fisherbrand™ Isotemp™ Advanced Stirring Hotplate or equivalent	Fisher Scientific SP88857290
Table-top microtube centrifuge	MLS
Water bath capable of maintaining 40±1°C	MLS

Equipment	Source
Vortexer	MLS
Dry incubator or oven capable of maintaining 60°C for baking slides with ability to control (+/1°C), temperature at 40°C	Thermo Fisher Scientific: • QS0720 (0.7 ft^3) • QS0704 (1.4 ft^3) • QS0700 (3 ft^3) • QS0701 (4.9 ft^3)
Microplate shaker (optional, for washing steps)	Thermo Fisher Scientific, 88880023 or 88880024
Microscope and imaging equipment	(See "Guidelines for microscopy and imaging", page 15)

Before you begin

Probe set considerations

To order custom probes or to search through 6000+ designed ready to order probes, visit: **thermofisher.com/order/custom-oligo/branchedna**

Probe sets of the same TYPE can be combined to create a target panel or cocktail. For example, identifying epithelial cells could be easily accomplished by pooling different cytokeratin probe sets of the same type, such as TYPE 1, KRT5, KRT7, KRT8, KRT10, KRT19, KRT19 and KRT20, into a single assay. However, we do not recommend combining more than 10 targets for any one signal amplification system, be it TYPE 1 or TYPE 6.

How the probe sets are diluted to generate a panel depends on the application. For example, if the goal is to identify all of the epithelial cells or to assess RNA integrity, then each probe set can be diluted 1:40. However, when using a panel of housekeeping gene probe sets for optimizing pretreatment conditions, the probe sets (e.g., ACTB, GAPD and PPIB) should be pooled at equal volumes to form the panel, and then diluted 1:40 to create the working probe set solution. This ensures that the panel expression is sufficiently high but not saturated so that the differences in signal between pretreatment conditions can be distinguished.

The typical design for a ViewRNA™ Probe Set consists of 40 unlabeled oligos, or 20 pairs of oligos per RNA target, and spans approximately 1000 bases of the target transcript to achieve maximal sensitivity. The binding of these oligo pairs side-by-side to the target sequence serves as a base upon which the signal amplification is built, and is the core of the assay's sensitivity and specificity. Using multiple pairs of oligos in a single probe set ensures that there are many opportunities for the probe to bind to the target's unmasked/accessible regions so as to achieve the maximal signal amplification possible for that particular RNA target molecule. When working with smaller targets or applications such as splice variants or RNA fusions, the available number of oligo pairs in the probe set is naturally reduced, and this will directly impact the sensitivity of the assay. That is, the probes will have fewer opportunities to find the unmasked areas of the target in order to generate signal at that location. In these cases, increasing the probe set concentration used in the assay from 1:40 to 1:30 or 1:20 might increase the sensitivity. However, note that there is always a general trade-off between sensitivity and specificity.

Assigning colors to target mRNA in 1 vs. 2-plex assays

The ViewRNA™ ISH Tissue Assay allows in situ detection of up to two mRNA targets simultaneously, using the ViewRNA™ TYPE 1 and/or TYPE 6 probe sets. The standard workflow of the assay is designed to automatically assign Fast Red signal to TYPE 1 and Fast Blue signal to TYPE 6 probe sets. While both the Fast Red and Fast Blue signals that form are easily visible under brightfield, the red dots generally have a much higher contrast than the blue dots, especially in the presence of hematoxylin. Thus, when the detection of only one target (1-plex assay) is desired, we recommend using either TYPE 1 or TYPE 6 probe set and developing the signal as Fast Red. The Core Kit (SKU 19931) is configured for use with TYPE 1 probe sets.

When performing a 2-plex assay, we recommend assigning the TYPE 1 probe set (Fast Red) to the more important target of the two. Reserve the TYPE 6 probe set (Fast Blue) for the less critical target, such as a housekeeping gene. Due to the nature of the chromogenic assay and the sequential development of Fast Blue before Fast Red signals, large quantities of blue precipitate that are deposited, particularly when a TYPE 6 target is expressed homogeneously at high level, have the potential to partially block subsequent hybridization of the TYPE 1 Label Probe and consequently the development of the Fast Red signal. For this reason, the target assigned to Fast Blue should preferably have lower expression than the one assigned to Fast Red to ensure against potential interference with Fast Red signal development downstream.

If only medium and high expressing housekeeping targets are available in a particular tissue type and the critical target of interest has low to medium expression, a 2-plex assay can still be performed by assigning Fast Red to the housekeeping target and Fast Blue to the second target. Brightfield detection of the Fast Blue signal for a medium expressing transcript could still be easily done, while fluorescent detection would provide a more sensitive alternative for detecting a low expressing target tagged with Fast Blue.

Fluorescent mode guidelines

The advantage of using alkaline phosphatase-conjugated label probe for the enzymatic signal amplification is the availability of substrates with dual property, such as Fast Red and Fast Blue, which allows for both chromogenic and fluorescent detection of the targets. However, for a 2-plex assay in which both Label Probe 1 and Label Probe 6 are conjugated to the same alkaline phosphatase, the enzymes conjugates are unable to differentiate between Fast Red and Fast Blue if both substrates are added simultaneously. As a result, the enzymatic signal amplification has to be performed sequentially in order to direct substrate/color specificity to each target. Additionally, complete inactivation of the first alkaline phosphatase-conjugated label probe (LP6-AP) is necessary, especially when employing fluorescence mode for the detection of the targets. Otherwise, the residual LP6-AP activity can also convert Fast Red substrate in subsequent step into a red signal even at locations where TYPE 1 target is not present, giving a false impression that the Fast Blue and Fast Red signals are co-localized. For this reason, it is absolutely necessary to quench any residual LP6-AP activity with the ViewRNA™ AP Reaction Stop Solution prior to proceeding with the second label probe hybridization and development of the Fast Red color as this will ensure specific signals in fluorescent mode and brighter aqua blue dots in chromogenic mode.

Fast Red has a very broad emission spectrum and its bright signal that can bleed into adjacent Cy5 channel if one uses the standard Cy3/Cy5 filter sets for imaging. For this reason, it is critical that the recommended filter set for Fast Blue detection be used to avoid spectral bleed through of the Fast Red signal into the Fast Blue channel and interfering with Fast Blue detection. See "Guidelines for microscopy and imaging", page 15 for exact filter set specifications.

Limitations of chromogenic in situ assay in co-localization studies

When employing the ViewRNA™ ISH Tissue 2-Plex Assay for co-localization studies, it is crucial to understand the assay's strengths and limitations. By definition, a requisite for in situ detection is target accessibility. While the assay, with its branched DNA technology, has the capability to detect RNA molecules down to single-copy sensitivity and the probe sets are designed to maximize the binding opportunities to all accessible regions of the targets, the overall detection for any given target is only as good as the unmasking of the target site is able to provide. This essentially means that in situ assays in general are only capable of relative and not absolute detection. That is, not every single molecule of a given target can be detected. So in practice, even if two RNA targets are theoretically expected to be colocalized, only a subset these two transcripts will be detected as being so due to lack of complete target accessibility.

Another factor that can limit the use of this assay for co-localization studies is the nature of chromogenic assay and the sequential development of Fast Blue then Fast Red signals. In chromogenic assay, the enzyme converts the substrate into color precipitates and deposits them at the site where the RNA molecule is localized. Because the Fast Blue and Fast Red substrates are sequentially developed in the ViewRNA™ ISH Tissue 2-Plex Assay, the Fast Blue precipitates that are formed first and deposited have the potential to partially block subsequent hybridization of the TYPE 1 Label Probe, by masking its binding sites on a nearby/co-localized target and consequently affecting the development of the Fast Red signal. This is yet another form of accessibility issue that needs to be considered when performing colocalization studies and analyzing the data obtained from such studies. Consequently, even when two targets are co-localized, only a subpopulation of the two is actually observed as such because of target accessibility, be it at the probe hybridization step due to incomplete unmasking or at the label probe hybridization step due to masking of the binding site by the deposition of the Fast Blue precipitates.

Experiment design guidelines

Assay controls

We recommend running one positive and one negative control slide in each assay, based on your sample type. This will allow you to qualify and interpret your results.

Negative control

This slide undergoes the entire assay procedure and assesses the assay background from different levels.

The negative control can be one of the following:

- Omit the target probe set. A no probe negative control.
- Use a probe set designed to the sense strand of the target – A more target-specific negative control used to subtract assay background when assessing results.
- Use a probe set for a target not present in your tissue sample – A more general negative control used to subtract assay background when assessing results, for example, the bacterial gene dapB.

Positive control

This slide undergoes the entire assay procedure using a probe set against an ubiquitous or tissue-specific target that has consistent, medium-high to high, but not saturating, expression level. A positive control ensures that the assay procedure has been successfully run. Examples of positive control targets include:

- Housekeeping Genes: ACTB, GAPD, or UBC.
- Housekeeping Gene Panel: A panel of several housekeeping genes can be pooled and used as a positive control whenever the expression level of any one given housekeeping gene is unknown in the tissue of interest. For example, pool ACTB, GAPD and PPIB probe sets at equal volumes to form a panel, and then dilute the panel of probe sets 1:40 to create a working probe set solution for use as a positive control. Order the positive and negative control probe sets at **thermofisher.com/us/en/home/life-science/cell-analysis/cellular-imaging/in-situ-hybridization-ish/viewrna-ish-assays/viewrna-cell-plus-assay.html**
- The recommended probe sets for human and mouse are below. ViewRNA probes sets are very specific for each species, and probe set for one species usually does not work for other species.

  Positive and Negative control probes

	Assay ID #	Gene Symbol	Species*	Probe Type	Detection Label
Positive Control	VA1-10119-VT	GAPDH	Human	1	Fast Red
	VA6-10337-VT	GAPDH	Human	6	Fast Blue
	VB1-10150-VT	GAPDH	Mouse	1	Fast Red
	VB6-10574-VT	GAPDH	Mouse	6	Fast Blue
	VA1-10351-VT	ACTB	Human	1	Fast Red
	VA6-10506-VT	ACTB	Human	6	Fast Blue
	VB1-10350-VT	ACTB	Mouse	1	Fast Red
	VB6-12823-VT	ACTB	Mouse	6	Fast Blue
	VA1-10148-VT	Ppib	Human	1	Fast Red

	VA6-10509-VT	Ppib	Human	6	Fast Blue
	VB1-10064-VT	Ppib	Mouse	1	Fast Red
	VB6-11226-VT	Ppib	Mouse	6	Fast Blue
Negative Control	VF1-11712-VC	dapB	Bacteria	1	Fast Red
	VF6-10407-VC	dapB	Bacteria	6	Fast Blue

Replicates We recommend running all assays in duplicate.

Guidelines for microscopy and imaging

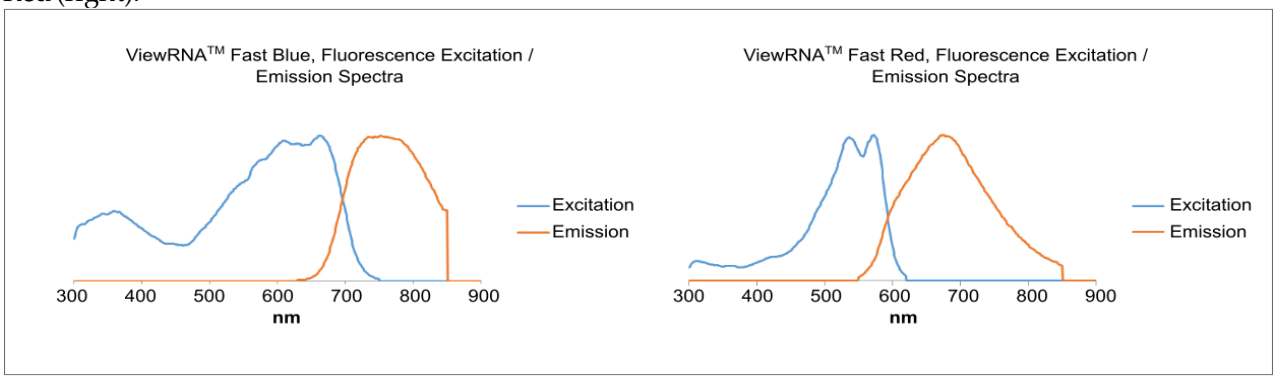
Imaging and Analysis:

- ViewRNA™ ISH Tissue Assay can be imaged with most brightfield, or fluorescent microscope, or slide scanner, such as EVOS cell imaging system, or CellInsight™ CX7 high content platform
- Use 20X to 40X objectives. 40X Objective is recommended for low abundant targets.
- For choosing the right filter sets for fluorescent imaging, see Figure 1. For traditional microscope filter set, see the following table.

To Detect	Staining reagents	Bright field staining color	Fluorescence traditional filter sets*
mRNA labeled with Type 1 probe set	ViewRNA™ Fast Red	Red	RFP/TRITC, or Texas-Red
mRNA labeled with Type 6 probe set	ViewRNA™ Fast Blue	Aqua Blue	Cy5.5, or Cy7
Optional nuclear counter stain	Hematoxylin	Light Purple, light blue	N/A
	DAPI	N/A	DAPI

*For optimized filter/laser settings, see Figure 1.

Figure 1: Fluorescence excitation/Emission spectra of ViewRNA™ Fast Blue (left), and ViewRNA™ Fast Red (right).



ViewRNA™ ISH Tissue assay guidelines

- The procedure assumes running a maximum of 12 slides at a time and that the size of the section does not exceed the maximum coverage area recommended.
- Do not mix and match kit components from different lots.
- Before beginning the procedure, know the optimized conditions (heat treatment time and protease digestion time) for your sample type. If you do not know these optimized conditions, refer to "Sample pretreatment optimization procedures" on page 33.
- Throughout the procedure, dedicate the Tissue Tek staining dishes as follows:
    - Clear staining dish for formaldehyde.
    - Green staining dish for Gill's hematoxylin.
    - Green staining dish for xylene/Histo-Clear.
    - The remaining two clear staining dishes can be used interchangeably for 1X PBS, 100% ethanol, Wash Buffer, ddH_2O, Storage Buffer, and DAPI. Rinse staining dishes between steps with ddH_2O.
- If using a humidified tissue culture incubator (without CO_2) as the hybridization system:
    - Verify that the water jacket or bottom tray is filled with water.
    - Use an aluminum slide rack to transfer slides to the incubator.
    - Do not leave the incubator door open longer than necessary when transferring slides, particularly during the protease optimization procedure. This will help maintain the required temperature.
- Typical processing times included in the assay procedure assume that the preparations for the following step are being done during the incubation periods.
- Prepare samples following the "Tissue preparation guidelines", page 43.
- Organize the preparation of the assay before you start:
    - Verify that all materials and equipment are available.
    - Be mindful of the incubation times/temperatures, as variations can negatively affect assay signal and background.
    - Double-check all reagent calculations, as correct reagent volumes and concentrations are critical.
- Employ good washing techniques. Frequently, washing is performed too gently. Adequate washing is important for consistent low backgrounds.
- Calibrate temperatures for hybridization system (to 40°C) and dry oven (to 60°C) using the ViewRNA™ Temperature Validation Kit.
- Ensure that hybridization system is appropriately humidified.
- DO NOT let tissues dry out where indicated in the procedure.
- Incorporate controls, both positive and negative, so that results are unambiguous and can be interpreted (see "Experiment design guidelines", page 13).

Experimental procedure

The ViewRNA ISH™ Tissue Assay can be run in a single long day or broken up over two days for added flexibility. The procedure includes two parts:

- Part 1: "Prepare sample and hybridize target probe" (optional stopping point at end of procedure)
- Part 2: "Amplify and Detect Signal"

Prepare sample and hybridize target probe

1. Bake slides (65 minutes)
   a. Set the dry oven or hybridization system to 60±1°C.
   b. Label the slides with a pencil
   c. Bake the slides. With a dry oven, insert slides into the slide rack and bake for 60 minutes. With a ThermoBrite System, keep the lid open and bake for 60 minutes. If necessary, adjust the ThermoBrite System so that it is at the correct temperature while the lid is open.
2. Prepare buffers, reagents, and equipment while slides bake
   a. Verify that the hybridization system is set to 40± 1°C and that it is appropriately humidified.
   b. Prepare 3 L 1X PBS: add 300 mL 10X PBS and 2.7 L ddH_2O to a 3 L capacity container.
   c. Prepare 200 mL 10% NBF (4% formaldehyde in PBS), working in fume hood: add 178 mL 1X PBS + 22 mL 37% formaldehyde to a 200 mL capacity container and mix well.
   d. Prepare 4 L Wash Buffer: add the components below in the order listed to a 4 L capacity container and mix well:
      - 3 L ddH_2O
      - 36 mL Wash Comp 1
      - 10 mL Wash Comp 2

      Adjust the total volume to 4 L with ddH_2O.
   e. Prepare 500 mL 1X Pretreatment Solution: add 5 mL 100X Pretreatment Solution and 495 mL ddH_2O to a 1 L glass beaker.
   f. If using the optional stopping point, prepare 200 mL Storage Buffer: add 60 mL Wash Comp 2 and 140 mL ddH_2O to a 200 mL capacity container.
   g. Prepare 1 L of 0.01% ammonium hydroxide, working in a fume hood: add 0.33 mL 30% ammonium hydroxide and 999.67 mL ddH_2O in a 1 L capacity container.
   h. Ensure the availability of:
      - 600 mL 100% ethanol
      - 1.4 L ddH_2O
      - 600 mL xylene or 600 mL Histo-Clear
      - 200 mL Gill's Hematoxylin I
      - 200 mL of 3 µg/mL DAPI in 1X PBS (optional, for fluorescence detection). Store in the dark at 4 °C until use.

i. Thaw probe set(s). Mix, briefly centrifuge to collect contents, and place on ice until use.

j. Pre-warm 40 mL 1X PBS and Probe Set Diluent to 40±1°C.

k. If performing both parts of the assay in 1 day:

- Pre-warm Pre-amplifier Mix, Amplifier Mix, and Label Probe Diluent to 40°C .
- Briefly spin down the Label Probe 1-AP, Label probe 6-AP, and Blue reagents (for 2-plex), then place on ice until use.
- Bring Fast Red Substrate, Red Reaction Buffer, Blue Buffer (for 2-plex), and AP Enhancer Solution to room temperature.

l. Optional: if using a microplate shaker for the washes, set the speed to 285 rpm. Place a slide rack in a clear staining dish containing the appropriate reagent and insert the slides into the rack. Manually lift the rack up and down 10 times. Put the lid on the staining dish and place it on a microplate shaker platform that is equipped with a non-skid pad. Shake for the recommended amount of time.

3. Deparaffinization (30 minutes)

If using xylene (work in a fume hood):

a. Pour 200 mL of xylene into a green clearing agent dish.

b. Transfer the rack of baked slides to the green clearing dish containing the xylene.

c. Incubate the slides at room temperature for 5 minutes. Agitate frequently by moving the rack up and down.

d. Discard the used xylene and refill with another 200 mL of fresh xylene. Incubate slides at room temperature for 5 minutes with frequent agitation.

e. Repeat Step 3d above.

f. Remove the slide rack from the xylene and wash the slides twice, each time with 200 mL of 100% ethanol for 5 minutes with frequent agitation.

g. Remove the slides from the rack and place them face up on a paper towel to air dry for 5 minutes at room temperature.

If using Histo-Clear:

a. Pour 200 mL of Histo-Clear into a green clearing dish and insert an empty slide rack.

b. Set the dry oven or hybridization system to 80±1°C.

c. Bake the slide for 3 minutes to melt the paraffin.

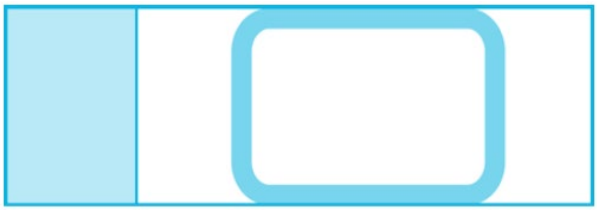
d. Immediately insert the warm slides into the Histo-Clear and agitate frequently by moving the rack up and down for 5 minutes at room temperature.

e. Discard the used Histo-Clear and refill the dish with another 200 mL of fresh Histo-Clear. Agitate frequently by moving the rack up and down for another 5 minutes at room temperature. Repeat this step once more for a total of 3 washes in Histo-Clear.

f. Remove the slide rack from the Histo-Clear and wash the slides twice, each time with 200 mL of 100% ethanol for 5 minutes with frequent agitation.

g. Remove the slides from the rack and place them face up on a paper towel to air dry at room temperature for 5 minutes.

4. Draw hydrophobic barrier (40 minutes)
    a. Dab the hydrophobic barrier pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.
    b. To create a hydrophobic barrier:
        - Place the slide over the template image, making sure that the tissue sections fall inside the blue rectangle.
        - Lightly trace the thick blue rectangle 2-4 times with the hydrophobic barrier pen to ensure a solid seal.
        - Allow for barrier to dry at room temperature for 20-30 minutes. Begin the next step while the barrier is drying.



5. Heat pretreatment (10-25 minutes, depending on optimized time)
    a. Tightly cover the beaker containing the 500 mL of 1X Pretreatment Solution with aluminum foil, place it on a hot plate, and heat the solution to a temperature of 90-95°C. Use a waterproof probe thermometer to measure and maintain the temperature of the solution at 90-95°C during the pretreatment period.
    b. Load the slides into the vertical slide rack.
    c. Using a pair of forceps, submerge the slide rack into the heated 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate at 90-95°C for the optimal time as determined in "Sample pretreatment optimization procedures", page 33.
    d. After pretreatment, remove the slide rack with forceps, submerge it into a clear staining dish containing 200 mL of ddH_2O, and wash for 1 minute with frequent agitation.
    e. Repeat the wash one more time with 200 mL of fresh ddH_2O.
    f. Transfer the slide rack to a clear staining dish containing 1X PBS.

    IMPORTANT: Do not let the tissue sections dry out from this point forward. After heat pretreatment, sections can be stored covered in 1X PBS at room temperature overnight.
6. Protease digestion and fixation (30-50 minutes, depending on optimized time)
    a. Prepare the working protease solution by diluting the Protease 1:100 in prewarmed 1X PBS (e.g. 4µL Protease added to 396 µL 1X PBS prewarmed to 40°C) and briefly vortex to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.
    b. Remove each slide and flick it to remove excess 1X PBS. Without completely drying out the sections, tap the slides on the edge and then wipe the backside on a laboratory wipe.
    c. Place the slides face up on a flat, elevated platform (e.g., Eppendorf tube rack for easier handling) and immediately add 400 µL of the working protease solution onto the tissue section. Make sure that the tissue section is covered with working protease solution. It may be necessary to spread the solution with a pipette tip.

d. Transfer the slides to the hybridization system and incubate at 40 °C for the optimal time as determined in "Sample pretreatment optimization procedures", page 33.

e. Pour 200 mL of 1X PBS into a clear staining dish and insert an empty slide rack into the dish.

f. After the incubation, decant the working protease solution from the slides, insert the slides into the rack and wash gently but thoroughly by moving the rack up and down for 1 min.

g. Repeat the wash one more time with another 200 mL of fresh 1X PBS.

h. Transfer the slide rack to a clear staining dish containing 200 mL of 10% NBF and fix for 5 minutes at room temperature under a fume hood.

i. Wash the slides twice, each time with 200 mL of fresh 1X PBS for 1 minute with frequent agitation.

7. Target probe set hybridization (2 hours and 10 minutes)

a. Prepare the working probe set solution using the table below as a guide. Dilute the ViewRNA™ Probe Set 1:40 in prewarmed Probe Set Diluent and briefly vortex to mix. Scale reagents according to the number of assays to be run and include one slide volume overage.

Note: Add only 400 µL of Probe Set Diluent to the "negative control" or probe negative control slide.

1-plex (400 µL total volume)	**Volume**
Probe Set Diluent (prewarmed to 40°C)	390 µL
ViewRNA™ TYPE 1	10 µL
2-plex (400 µL total volume)	**Volume**
Probe Set Diluent (prewarmed to 40°C)	380 µL
ViewRNA™ TYPE 1 Probe Set	10 µL
ViewRNA™ TYPE 6 Probe Set	10 µL

b. Remove each slide and flick it to remove excess 1X PBS. Without completely drying out the sections, tap the slides on the edge and then wipe the backside on a laboratory wipe

c. Place the slides face up on a flat, elevated platform and immediately add 400 µL of pre-warmed Probe Set Diluent to the negative probe control and 400 µL of working probe set solution to each test sample.

d. Transfer the slides to the hybridization system and incubate at 40°C for 2 hr.

8. Wash slides (8 minutes)

a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

b. After incubation, decant the working probe set solution from the slides and insert them into the slide rack.

c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 2 minutes with constant and vigorous agitation.

d. If you plan to perform the assay over the course of two days, proceed to Step 9. Otherwise, proceed to "Amplify and detect signal" to complete the entire assay in one day.

9. Optional stopping point (1 minute)
    a. Store slides in a clear staining dish containing 200 mL of Storage Buffer at room temperature for up to 24 hours. Cover the dish with a lid or sealing film to prevent evaporation.
    b. Discard 1X Pretreatment Solution, 10% NBF, remaining protease and probe set working solutions.
    c. Store the remaining 1X PBS and Wash Buffer at room temperature for use in "Amplify and detect signal".
    d. If using a ThermoBrite System, rewet the ThermoBrite Humidity Strips in ddH_2O.
    e. Proceed to "Amplify and detect signal" when you are ready to continue the assay.

Amplify and detect signal

1. Prepare additional buffers and reagents (5 minutes)
    a. Pour Gill's Hematoxylin into a clear staining dish and store at room temperature protected from light until use.
    b. Pre-warm Pre-amplifier Mix, Amplifier Mix, and Label Probe Diluent buffers to 40°C.
    c. Briefly spin down the Label Probe 1-AP, Label probe 6-AP (for 2-plex), and Blue reagents (for 2-plex). Place them on ice.
    d. Bring Fast Red Substrate, Red Reaction Buffer, AP Enhancer Solution, and Blue Buffer (for 2-plex) to room temperature.
2. Wash slides (5 minutes)
    a. Remove the slides from Storage Buffer. Transfer slide rack to a clear staining dish containing Wash buffer, and wash for 2 minutes with frequent agitation.
    b. Decant Wash Buffer, refill with 200 mL fresh Wash Buffer, and wash for 2 minutes with frequent agitation. Repeat this step once more for a total of 3 washes.
3. Pre-amplifier hybridization (35 minutes)
    a. Swirl the Pre-amplifier Mix bottle briefly to mix the solution.
    b. Remove each slide and flick it to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of Pre-amplifier Mix to each tissue section.
    c. Transfer slides to the hybridization system and incubate at 40°C for 25 min.
4. Wash slides (8 minutes)
    a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
    b. After incubation, decant the Pre-amplifier Mix from the slides and insert them into the slide rack.
    c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 2 minutes with constant and vigorous agitation.

5. Amplifier hybridization (20 minutes)
    a. Swirl the Amplifier Mix bottle briefly to mix the solution.
    b. Remove each slide and flick it to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of Amplifier Mix to each tissue section.
    c. Transfer slides to the hybridization system and incubate at 40°C for 15 min.
6. Wash slides (8 minutes)
    a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
    b. After incubation, decant the Amplifier Mix from the slides and insert them into the slide rack.
    c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 2 minutes with constant and vigorous agitation.

IMPORTANT: For 1-plex assays, proceed directly to step 12: Label Probe 1-AP Hybridization, and omit steps 7–11. For 2-plex assays, continue with step 7.

7. Label Probe 6-AP hybridization (20 minutes)
    a. Briefly spin down Label Probe 6-AP before using.
    b. Prepare the Working Label Probe 6-AP Solution using the table below as a guide. Dilute Label Probe 6-AP 1:1000 in pre-warmed Label Probe Diluent and briefly vortex to mix. Scale reagents according to the number of assays to be run and include overage of one slide's volume.

Working Label Probe 6-AP Solution per slide (400 µL total volume)	Volume
Label Probe Diluent (pre-warmed to 40°C)	399.6 µL
Label Probe 6-AP	0.4 µL

    c. Remove each slide and flick to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of Working Label Probe 6-AP Solution to each tissue section.
    d. Transfer the slides to the hybridization system and incubate at 40°C for 15 min.
8. Wash slides (12 minutes)
    a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
    b. After incubation, decant the Working Label Probe 6-AP Solution from the slides and insert them into the slide rack.
    c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation.
9. Apply Fast Blue Substrate (35 minutes)
    a. Prepare the Fast Blue Substrate: Add 5 mL of Blue Buffer and 105 µL of Blue Reagent 1 to a 15 mL conical tube and vortex. Add 105 µL of Blue Reagent 2 and vortex. Add 105 µL Blue Reagent 3 and briefly vortex. Protect from light by wrapping in aluminum foil until use.

b. Remove each slide and flick it to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of Fast Blue Substrate.

c. Incubate in the dark at room temperature for 30 minutes.

10. Wash slides (12 minutes)

a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

b. After incubation, decant the Fast Blue Substrate from the slides and insert them into the slide rack.

c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation.

11. Quench Label Probe 6-AP (35 minutes)

a. Remove each slide and flick it to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of AP Reaction Stop Solution. Incubate in the dark at room temperature for 30 minutes.

b. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.

c. After incubation, decant the AP Reaction Stop Solution from the slides and insert them into the slide rack.

d. Wash the slides twice, each time in 200 mL of fresh 1X PBS at room temperature for 1 minute with frequent agitation.

e. Replace the 1X PBS with 200 mL of fresh Wash Buffer and rinse any residual PBS from the slides by moving the slide rack up and down for 1 min.

12. Label Probe 1-AP hybridization (20 minutes)

a. Briefly spin down Label Probe 1-AP before using.

b. Prepare the Working Label Probe 1-AP Solution using the table below as a guide. Dilute Label Probe 1-AP 1:1000 in pre-warmed Label Probe Diluent and briefly vortex to mix. Scale reagents according to the number of assays to be run and include overage of one slide's volume.

Working Label Probe 6-AP Solution per slide (400 µL total volume)	Volume
Label Probe Diluent (pre-warmed to 40°C)	399.6 µL
Label Probe 1-AP	0.4 µL

c. Remove each slide and flick to remove the Wash Buffer. Without completely drying out the sections, tap the slide on its edge and then wipe the backside on a laboratory wipe. Place the slides face up on a flat, elevated platform and immediately add 400 µL of Working Label Probe 1-AP Solution to each tissue section.

d. Transfer the slides to the hybridization system and incubate at 40°C for 15 minutes.

13. Wash slides (12 minutes)
    a. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
    b. After incubation, decant the Working Label Probe 1-AP Solution from the slides and insert them into the slide rack.
    c. Wash the slides 3 times, each time with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation.
14. Apply Fast Red Substrate (75 minutes)
    a. Remove each slide and remove the Wash Buffer by flicking. Tap the slide on its edge and then wipe the backside on a laboratory wipe without letting the sections dry out. Place slides face up on a flat, elevated surface.
    b. Immediately add 400 µL of the AP Enhancer Solution to each tissue section and incubate at room temperature for 5 minutes while preparing the Fast Red Substrate.
    c. Prepare the 400 µL ViewRNA™ Fast Red staining solution for each slide: For 12 slides make 5 mL staining solution, but adjust the volume for more or less slides. Add 5 mL of ViewRNA™ Fast Red Reaction Buffer and add 80 µL of ViewRNA™ Fast Red substrate 1 to a 15 mL conical tube and vortex. Add 80 µL of ViewRNA™ Fast Red substrate 2 and vortex. Add 80 µL ViewRNA™ Fast Red substrate 3 and briefly vortex. Protect from light by wrapping in aluminum foil until use.
    d. Decant the AP Enhancer Solution and flick the slide twice to completely remove any excess AP Enhancer Solution. Tap the slide on its edge then wipe the backside on a laboratory wipe. Immediately add 400 µL of Fast Red Substrate onto each tissue section.
    e. Transfer the slides to the hybridization system and incubate at room temperature for 60 minutes.
    f. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
    g. After incubation, decant the ViewRNA™ Fast Red Staining solution from the slides and insert them into the slide rack.
    h. Rinse off the excess ViewRNA™ Fast Red Staining solution from the slides by moving the slide rack up and down for 1 minute.
15. Optional Counterstain (25 minutes)

    Counter staining with Gill's hematoxylin for bright field microscopy:
    a. Transfer the slide rack to the clear staining dish containing the 200 mL of Gill's hematoxylin and stain at room temperature.
    b. Wash the slides 3 times, each time with 200 mL of fresh ddH_2O for 1 minute by moving the slide rack up and down.
    c. Pour off the ddH_2O, refill with 200 mL of 0.01% ammonium hydroxide and incubate the slides for 10 seconds. Unused 0.01% ammonium hydroxide can be stored at room temperature for up to 1 month.
    d. Wash the slides once more in 200 mL of fresh ddH_2O by moving the rack up and down for 1 minute.

    Counter staining with DAPI for fluorescent imaging.
    a. Prepare 200 mL of 1X DAPI staining solution by adding 2 drops of NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606) per mL of

1X PBS or 3 µg/mL solution in PBS of DAPI, FluoroPure™ grade (Cat. No. D21490).

b. Move the slide rack into a clear staining dish containing 200 mL 1X DAPI Staining solution. Incubate the slides for 1-5 min, then rinse them in 200 mL of fresh ddH_2O by moving the slide rack up and down for 1 minute.

c. Remove the slides from the slide rack and flick to remove the excess ddH_2O. Tap the slide on its edge then wipe the backside on a laboratory wipe. Place them face up onto a paper towel to air dry in the dark.

16. Mounting for imaging

ViewRNA™ ISH Tissue Assay is compatible with most aqueous and non-aqueous Mountants. For Fluorescent imaging, the Mountants containing Antifades are highly recommended. When mounted with a curing, hardening mountant, slides can be stored for years at 2-8°C.

Mounting with Aqueous mountant:

a. Follow the manufacturer's instruction for mounting. ViewRNA™ ISH Tissue Assay has been tested with ProLong™ Glass Antifade Mountant (Cat. No. P36980), SlowFade Diamond Antifade Mountant (Cat. No. S36963), and DAKO Ultramount mounting medium (Agilent Cat. No. S1964).

Mounting with non-Aqueous mountant:

a. For dehydration, we recommend 2 washes for 1 minute each with 95% ethanol, then another 2 washes for 1 minute each with 100% ethanol.

b. For optional clearing, we recommend 3 washes of 1 minute each with Richard-Allan Scientific™ Signature Series Clear-Rite™ 3 (Cat. No. 6901TS).

Note: Avoid using Xylene for clearing or dehydration steps. Xylene will dissolve ViewRNA™ Fast Blue or ViewRNA™ Fast Blue precipitates.

Mount with a non-aqueous mountant:

a. We tested VectaMount™ (Vector Laboratories Cat. No. H-5000) successfully.

Troubleshooting

Observation	Probable Cause	Recommended Action
Weak or No Signals	Incorrect pretreatment conditions	Repeat pretreatment assay optimization procedure to determine optimal heat treatment time and protease digestion time that will strike a balance between morphology and signal. • Under-pretreatment yields good morphology but poor signal due to insufficient unmasking of target. • Over-pretreatment yields poor morphology and loss of signal due to over digestion.
	Sample preparation	Immediately place freshly dissected tissues in ≥20 volumes of fresh 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) at room temperature for 16–24 hours.
	Tissue over-fixed after protease digestion	Make sure the tissue sections are not fixed for more than 5 minutes in 10% NBF after protease digestion.
	RNA in tissue is degraded	Verify tissue fixation: • Immediately place freshly dissected tissues in ≥20 volumes of fresh 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) for 16–24 hours at room temperature. • If fixation cannot be performed immediately, be sure that the tissue is placed on dry ice or in liquid nitrogen to prevent RNA degradation. • Use positive control probe set(s) such as one for a housekeeping gene or a housekeeping gene panel (ACTB, GAPD and UBC) to assess RNA integrity.
	Reagents applied in wrong sequence	Apply target probe sets, Pre-amplifier Mix, Amplifier Mix, Label Probe-AP and substrates in the correct order.
	Gene of interest not expressed	• Verify expression using other tissue lysate methods such as QuantiGene™ Singleplex or QuantiGene Plex Assays.

		• Run the same probe set on known samples that have been confirmed to express the target of interest.
	Incorrect storage condition	Store the components at the storage condition as written on the component label or kit boxes.

Observation	Probable Cause	Recommended Action
Weak or No Signals	Hybridization temperature not optimal	Calibrate the hybridization system at 40°C using a ViewRNA™ Temperature Validation Kit (QV0523).
	Mounting solution contained alcohol	Use the recommended mounting media to mount your tissue (see Step 25: Mount and Image). Avoid any mounting solution containing alcohol.
	Tissue dries up during hybridization steps	Recommendations for hybridization systems: • Ensure the hybridization system is appropriately humidified and that door/lid is closed during hybridization steps. • Make sure the hybridization system is placed on a level bench. • Calibrate the hybridization system to 40°C using the ViewRNA™ Temperature Validation Kit (QV0523). Prevent sections from drying out: • Prepare enough reagents and use the recommended volumes for each step of the assay. • Ensure that you have a solid seal when drawing your hydrophobic barriers. • Add all working reagents onto the slides before moving them to the 40°C hybridization system.
	Tissue dries up during processing	Keep tissue sections moist starting from the heat pretreatment step: • Add respective reagents immediately after decanting solution from the slides. • Keep tissue exposure to air as short as possible before adding hybridization reagents. • Add all working reagents onto the slides before moving them to the 40°C hybridization system.
	Fast Red and Fast Blue Substrate solutions not freshly prepared	Prepare Fast Red and Fast Blue Substrate solutions immediately before use.
	Small targets, splice variants or RNA fusions	Doing one or both of the following may increase sensitivity, but it should be noted that there is always a general trade-off between specificity and sensitivity:

		• Increase probe set concentration by diluting target probe set 1:30 instead of 1:40 and hybridize for 2 hr. • Decrease hybridization temperature from 40°C to 38°C. • Increase Fast Red incubation time to 45 min.

Observation	Probable Cause	Recommended Action
Weak or no signals	Probe set hybridization temperature, time, and/or concentration not optimal	Decrease hybridization temperature from 40°C to 38°C and increase the probe set concentration by diluting the target probe set 1:30 instead of 1:40. Hybridize for 2 hr.
	Label Probe-AP concentration too low	• Verify that the correct concentrations were used. • Increase the recommended concentration for Label Probe-AP. If this is necessary, it may result in higher background.
	Dark hematoxylin stain reduces visibility of the blue dots	• Tissues with lower cell density require longer hematoxylin incubation than tissues with higher cell density. It may be helpful to titrate incubation times. • Increase the lamp brightness during viewing. • View under a 40X objective. • Image using fluorescent mode.
High background	Tissue dries up during processing	Prevent tissue sections from drying out after the pretreatment step: • Ensue that you have a solid seal when drawing your hydrophobic barrier. • Prepare enough reagents and use the recommended volume for each step of the assay. • Add respective reagents immediately after decanting solution from the slides. • Keep tissue exposure to air as short as possible before adding hybridization reagents. • Make sure that the hybridization system is appropriately humidified. • Make sure the hybridization system is set at 40°C and that the lid/door is closed during hybridization steps. • Process as few or as many slides at a time as you are comfortable doing.
	Incomplete removal of paraffin	• Use fresh xylene or Histo-Clear solution. • Immediately submerge the warm slides into the Histo-Clear solution after baking.
	Insufficient washing	• Move the slide rack up and down with constant and vigorous agitation. Click for a helpful video. • Increase wash incubation time by 1 minute per wash.

	Hybridization temperature not optimal	Calibrate the hybridization system at 40°C using the ViewRNA™ Temperature Validation Kit (QV0523).
	Concentration of hybridization reagents too high	Double check the dilution calculation for all working solutions.

Observation	Probable Cause	Recommended Action
High background	Suboptimal pretreatment conditions	Perform the pretreatment optimization procedure to determine the optimal heat treatment and protease digestion time.
	Concentration of hybridization reagents too high	Double check the dilution calculation for all working solutions.
	Label Probe-AP concentration too high	• Verify that the correct concentrations were used. • Decrease the recommended concentration for Label Probe-AP.
Diffused signals	Tissue dries up during processing	Prevent tissue sections from drying out after the pretreatment step: • Ensure that you have a solid seal when drawing your hydrophobic barrier. • Prepare enough reagents and use the recommended volume for each step of the assay. • Add respective reagents immediately after decanting solution from the slides. • Limit tissue exposure to air before adding hybridization reagents. • Make sure that the hybridization system is appropriately humidified. • Make sure the hybridization system is set at 40°C and that the lid/door is closed during hybridization steps. • Process as few or as many slides at a time as you are comfortable doing.
	Incomplete removal of AP Enhancer	Ensure that excess AP Enhancer is removed by decanting the AP Enhancer and flicking the slides twice prior to adding Fast Red Substrate.
	Insufficient washing	Make sure tissues are washed twice in 1X PBS after protease digestion and twice again after subsequent fixing in 10% NBF.
	Fast Red Substrate and Fast Blue Substrate solutions not freshly prepared	Prepare Fast Red and Fast Blue Substrate solutions immediately before use.

	Slides are not dried before mounting	Ensure that the sections are completely dry (~20 min) before mounting.
	Mounting solution contained alcohol	Use the recommended mounting media to mount your tissue (see "Amplify and detect signal" step 16, page 25). Avoid any mounting medium containing alcohol or any cover slipping method requiring alcohol dehydration.

Observation	Probable Cause	Recommended Action
Endogenous alkaline phosphatase activity	Endogenous alkaline phosphatase activity	Verify alkaline phosphatase activity by incubating protease-treated sample with Fast Red Substrate or Fast Blue Substrate. If endogenous AP activity is present, diffused signals (which can be weak or strong) will appear. Inactivate endogenous AP with 0.2 M HCl at room temperature for 10 minutes before the protease step. Wash samples twice with 1X PBS before proceeding to protease digestion.
Tissue detachment from slide	Improper tissue preparation	Make sure that the tissue is prepared as recommended in the "Tissue preparation guidelines", page 43, including fixation time and reagent, thickness of sections, brand of positively charged glass slide, and baking of the sections at 60 °C for 1 hour before storing at –20 °C.
	Insufficient baking of slides	Verify that the 60 minutes at 60 °C baking step was performed prior to storage of slides at –20 °C and again just before the deparaffinization step to ensure adhesion of tissue to slide.
	Incorrect pretreatment conditions	Perform full pretreatment optimization procedure to determine optimal heat treatment and protease digestion time.
	Temperature of heat pretreatment condition too high	Make sure the temperature is within the tolerance range of 90-95°C. For fatty soft tissue such as breast, adjust to 90°C.
	Proteinase treatment is too long or at too high a concentration.	Reduce proteinase concentration and/or incubation time.
Poor cell morphology	Incorrect pretreatment conditions	Perform full pretreatment optimization procedure to determine optimal heat treatment and protease digestion time. See "Sample pretreatment optimization procedures", page 33.
	Tissue sample not fixed properly	Make sure that freshly dissected tissues are fixed in 10% NBF or 4% PFA for 16-24 hr.
	Section thickness is variable or not optimal	Make sure microtome is calibrated and tissue is sectioned at 5 ± 1 µm.

Pink non-specific background where paraffin was	Incomplete removal of paraffin	• Use fresh Histo-Clear or xylene for the indicated amount of time during the dewaxing step. • Use 3 changes of Histo-Clear instead of 2 changes.
	Polymerization of poor quality paraffin	• Melt paraffin at 80°C for 3 minutes and remove paraffin using 3 changes of fresh Histo-Clear. • Do not bake the slides at a temperature higher than 60°C.
Observation	**Probable Cause**	**Recommended Action**
High non-specific binding on glass slide	Incompatible glass slide	• Use the recommended glass slides: • Leica Non-Clipped X-tra® Slide, 1 mm White Cat. No. 3800200 or 3800210 • Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, Cat. No. 12-550-15); avoid other colored labels as they tend to give high background. • Test each new batch of slides by running the entire assay, including probe set on empty slides with hydrophobic barriers (without fixed tissues) to determine if the slides are suitable for the assay.
	Insufficient washing	• Move the slide rack up and down with constant and vigorous agitation. • Increase wash incubation time by 1 minute per wash.
	Concentration of hybridization reagents was too high	Confirm that the dilution calculations are correct for all working solutions.
Hydrophobic barrier falls off	Incompatible glass slide	• Use the recommended glass slides: o Leica Non-Clipped X-tra® Slide, 1 mm White Cat. No. 3800200 or 3800210 o Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, Cat. No. 12-550-15); avoid other colored labels as they tend to give high background. • Test each new batch of slides by drawing a hydrophobic barrier onto an empty slide (without fixed tissue), allow it to dry for 20-30 min, boil in pretreatment solution for 40 minutes to determine if the hydrophobic barrier is intact and the slides are suitable for the assay.
	Incorrect hydrophobic pen	Use the recommended Hydrophobic Barrier Pen (QVC0500 or Vector Laboratories H4000).
	Hydrophobic barrier was not completely dried	Be sure that the hydrophobic barrier is completely dry before proceeding to the next step. This can be 20-30 minutes or longer depending on how heavily the barrier is created.

TYPE 1 target signals observed in the channel for TYPE 6 target	Spectral bleed through of Fast Red signal	Check to make sure that the filter set for Fast Blue is as recommended.
	Incorrect filter set for Fast Blue signal	Use the correct filter set. See "Guidelines for microscopy and imaging", page 15 for recommended filter set specifications for Fast Blue.

Observation	Probable Cause	Recommended Action
Fast Red signal for TYPE 1 target is weak or different in 2-plex vs. 1-plex	Cross-inhibition of LP1-AP by Fast Blue precipitate	Assign lower expressing target to TYPE 6 (Fast Blue) and higher expressing target to TYPE 1 (Fast Red).
	Co-localization of TYPE 1 and TYPE 6 targets	• Perform a 1-plex assay for each target. • Assign lower expressing target to TYPE 6 (Fast Blue) and higher expressing target to TYPE 1 (Fast Red). • If co-localization study is desired, try reducing development time for Fast Blue from 30 minutes to 10-15 minutes.
Co-localized Fast Blue and Fast Red signals when using only TYPE 6 probe set in a 2-plex assay	Residual LP6-AP activity	• Do not omit Step 20: Quench Label Probe 6-AP. • Be sure to quench LP6-AP activity with AP Reaction Stop Solution for the entire 30 minutes.

Sample pretreatment optimization procedures

About pretreatment optimization

Critical to any *in situ* assay is the balance between the adhesion of the tissue to the glass surface, crosslinking of the target molecules to the cellular structures by chemical fixatives and the subsequent unmasking of the RNA targets by heat treatment and protease digestion for the probes to hybridize. For the ViewRNA™ ISH Tissue Assay, this balance between signal strength and tissue morphology is largely sample dependent (tissue types as well as the modes of fixation and sample preparation) and can be achieved by optimizing the pretreatment conditions to empirically determine the optimal time for heat treatment and protease digestion.

When optimizing the pretreatment conditions for your tissue type, choose a target that is known to be expressed in the tissue of interest with medium to medium-high levels of expression. This will avoid possible signal saturation that may be associated with extremely high expressing targets and allow for detectable changes in the signals to be assessed as a function of the different pretreatment conditions. In general, a housekeeping gene with medium-high expression, such as GAPD or ACTB, can be used for this purpose. Once the optimal pretreatment conditions are determined, they can generally be used for most targets within the particular tissue. If the transcript is expressed at an extremely low level, the optimal pretreatment condition may need to be one that favors signal over morphology.

Sample pretreatment optimization setup

Ten FFPE tissue sections from the same block are treated with different pretreatment conditions prior to target probe hybridization step. Slide 7 serves as a "no probe control", while the remaining 9 slides are processed with the control target probe set.

Table B.1, page 37 provides sample pretreatment conditions for some common tissues. If samples are limited, see Table B.2, page 38.

Table A.1: Pretreatment Optimization Setup

Protease Incubation Time (min)	Heat Pretreatment Time (min)			
	0	5	10	20
0	Slide 1 Morphology reference			
10		Slide 2	Slide 5	Slide 9
20		Slide 3	Slide 6 Slide 7 No Probe Control	Slide 10
40		Slide 4	Slide 8	

Before starting the pretreatment optimization protocol, read "About the ViewRNA™ ISH Tissue assay guidelines", page 16.

The pretreatment optimization procedure for the ViewRNA™ ISH Tissue Assay is divided into two parts that can be performed in a single day or over two days:

- Part 1: Sample preparation and target probe set hybridization (optional stopping point).
- Part 2: Signal Amplification and Detection.

We do not recommend stopping the procedure at any point in the assay unless specifically indicated.

Sample preparation and target probe hybridization

1. Bake slides
   a. See "Prepare sample and hybridize target probe" steps 1 to 4, page 17.
2. Heat pretreatment (10-25 minutes)
   a. Tightly cover the beaker containing the 500 mL of 1X Pretreatment Solution with aluminum foil, place it on a hot plate and heat the solution to a temperature of 90-95°C. Use a waterproof probe thermometer to measure and maintain the temperature of the solution at 90-95°C during the pretreatment period.
   b. Set slide 1 aside on the lab bench.
   c. Load slides 9 and 10 into the vertical slide rack.
   d. Using a pair of forceps, submerge the slide rack into the heated 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate at 90-95°C for 10 min.
   e. At the end of the 10 min, add slides 5, 6, 7 and 8 to the rack in the 90-95°C 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate for 5 min.
   f. At the end of the 5 min, add slides 2, 3, 4 into the rack in the 90-95°C 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate for 5 min.

g. After the pretreatment, remove the slide rack with forceps, submerge it into a clear staining dish containing 200 mL of ddH_2O and wash for 1 minute with frequent agitation.

h. Repeat the wash one more time with another 200 mL of fresh ddH_2O.

i. Transfer the slide rack to a clear staining dish containing 1X PBS.

j. **IMPORTANT**: From this point forward, do not let the tissue sections dry out. Tissue sections that have been heat treated can be stored covered in 1X PBS at room temperature for up to one week. Continue with step 3 below when ready.

3. Protease digestion and fixation (30-50 minutes)

a. Prepare the Working Protease Solution using the table below as a guide. Dilute the Protease 1:100 in pre-warmed 1X PBS and briefly vortex to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.

Reagent	Volume
Protease	4 µL
1X PBS (pre-warmed to 40°C)	396 µL

b. Leave slide 1 on the lab bench as it is excluded from this step.

c. Begin by removing slides 4 and 8 and flicking each to remove excess 1X PBS. Tap the slide on its edge then wipe the backside on a laboratory wipe. Leave remaining slides in 1X PBS.

d. Place slides 4 and 8 face up on a flat, elevated platform (e.g., an Eppendorf tube rack for ease of handling) and immediately add 400 µL of the working protease solution onto the tissue section. It may be necessary to spread the solution with a pipette tip.

e. Transfer the slides to the hybridization system and incubate at 40°C for 20 min.

f. After 19 min, remove slides 3, 6, 7 and 10 from the clear staining dish and flick off excess 1X PBS. Without completely drying out the sections, tap the slides on their edges and then wipe the backsides on a laboratory wipe.

g. Place slides 3, 6, 7, and 10 face up on a flat, elevated platform and immediately add 400 µL of the working protease solution onto the tissue section.

h. Transfer the slides to the hybridization system and incubate at 40°C for 10 min.

i. After 9 min, remove slides 2, 5 and 9 from the clear staining dish and flick off excess 1X PBS. Without completely drying out the sections, tap the slides on their edges and then wipe the backsides on a laboratory wipe.

j. Place slides 2, 5, and 9 face up on a flat, elevated platform and immediately add 400 µL of the working protease solution onto the tissue section.

k. Transfer the slides to the hybridization system and incubate at 40°C for 10 min.

l. Pour 200 mL of 1X PBS into a clear staining dish and insert an empty slide rack into it.

m. At the end of 10 minutes (40 minutes total of incubation time), decant the working protease solution from the slides, insert the slides into the rack and wash gently but thoroughly by moving the rack up and down for 1 minute.
n. Repeat the wash one more time with another 200 mL of fresh 1X PBS before adding slide 1 to the rack.
o. Transfer the slide rack, containing all 10 slides, to a clear staining dish containing 200 mL of 10% NBF and fix at room temperature for 5 minutes under a fume hood.
p. Wash the slides twice, each time with 200 mL of fresh 1X PBS for 1 minute with frequent agitation.
q. Proceed to "Prepare sample and hybridize target probe" step 7, page 20 to continue the assay.

Sample pretreatment lookup table

Table B.1 shows a list tissues that were prepared according to the guidelines outlined in this manual (see "Tissue preparation guidelines", page 43) and optimized using the recommended pretreatment assay optimization procedure. This table provides a reference or a starting point to minimize the number of test conditions if you do not have sufficient slides to perform the full recommended pretreatment optimization procedure.

The conditions listed here are specific to tissues prepared in 10% NBF and may not be applicable to tissue prepared in 4% PFA. If you chose to use any of the pretreatment conditions listed in the lookup table, include a "negative control" slide to assess whether the assay background is clean and cellular morphology is well-defined.

Table B.1: Sample Pretreatment Optimization Lookup Table

Species	Tissue	Optimal Conditions (minutes)		Range of Tolerance (Heat pretreatment, Protease)
		Heat pretreatment at 90-95°C	Protease at 40°C	
Human	Brain	20	10	(10, 10) (10, 20)
	Breast	20	15	(25, 15) (30, 20) (25, 20)
	Colon	5	20	(5, 10)
	Kidney	20	10	
	Liver	20	20	(10, 20)
	Lung	10	20	
	Lymph Node	10	20	
	Nasal polyp	5	5	
	Osteoarthritic tissue	20	20	
	Pancreas	10	10	(10, 20) (5, 10)
	Prostate	10	20	(5, 10) (20, 10) (10, 10)
	Salivary gland	10	10	(5, 10)
	Skin	5	10	
	Tonsil	10	20	
	Thyroid	10	20	

Species	Tissue	Optimal Conditions (minutes)		Range of Tolerance (Heat pretreatment, Protease)
		Heat pretreatment at 90-95°C	Protease at 40°C	
Rat	Kidney	10	20	(10, 10) (20, 20)
	Liver	10	20	
	Spleen	20	10	
	Thyroid	10	20	
Mouse	Bone	20	20	
	Brain	10	10	
	Heart	10	40	(20, 20)
	Kidney	20	20	(10, 20)
	Liver	20	20	(5, 40) (10, 20)
	Lung	10	20	
	Retina	10	10	
Salmon	Heart	10	10	
	Muscle	10	20	
Monkey	Mucosal rectum	10	20	

If your tissue type is not listed in Table B.1, and you have only limited slides available for the pretreatment optimization, Table B.2 provides the recommended heat treatment and protease incubation times that will likely give the best chance of achieving an acceptable pretreatment conditions for your ViewRNA™ ISH Tissue Assay.

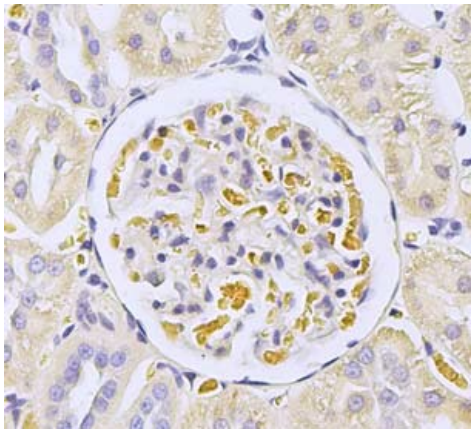
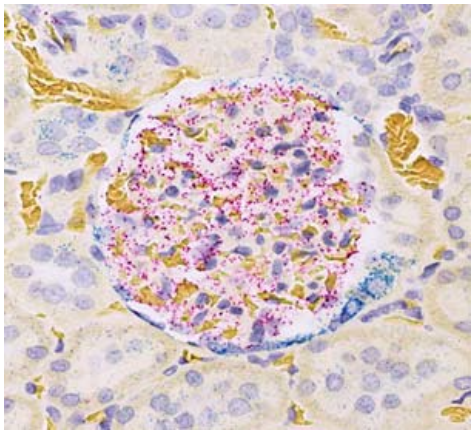
Table B.2: Heat Treatment and Protease Incubation Times for Limited Optimization

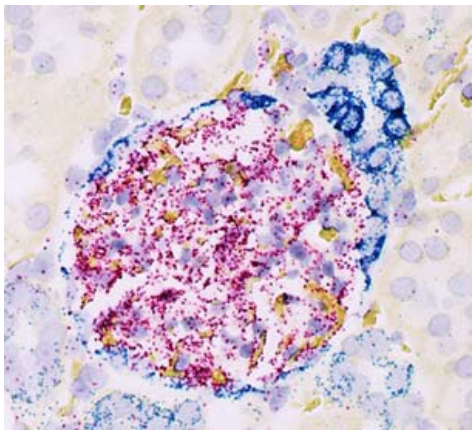
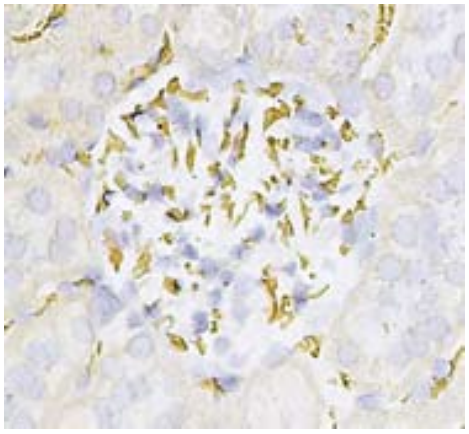
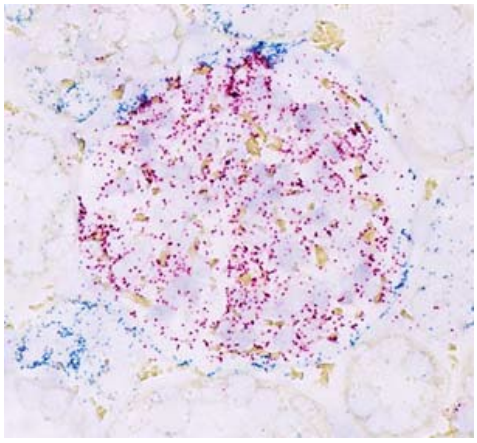
Number of Available Slides	Heat Pretreatment Time (min)	Protease Time (min)
3	5	10
	10	10
	10	20
5	5	10
	5	20
	10	10
	10	20
	20	10
7	5	10
	5	20
	10	10
	10	2
	20	10
	20	20
	0	0

Assessing pretreatment conditions

This section provides sample images obtained from the ViewRNA ISH™ Tissue Assay, performed on rat kidney tissue, to illustrate the effects of optimal and suboptimal pretreatment conditions on Arbp signal strength versus morphology and to demonstrate how data gathered from the in situ assay can be analyzed to determine target expression.

Table C.1: Assessing Pretreatment Conditions: Synpo and SPP1 Expression in Rat Kidney FFPE Tissue

Heat Pretreatment Time (min)	Protease Digestion Time (min)	Brightfield Image	Results Interpretation
0	0		**Untreated Morphology Reference Slide; +Probes (Synpo and SPP1)** • Good morphology • Intact cellular structure • Good hematoxylin counterstaining of nuclei • Little or no signal (dots) observed
5	10		**Insufficient Pretreatment or Over Fixation of Tissue; +Probes (Synpo and SPP1)** • Good morphology • Intact cellular structure • Strong hematoxylin counterstaining of nuclei • Weak, diffused and non-ubiquitous signal • Few number of dots

Heat Pretreatment Time (min)	Protease Digestion Time (min)	Brightfield Image	Results Interpretation
10	20		**Optimal Pretreatment and Sample Preparations; +Probes (Synpo and SPP1)** • Good morphology • Cellular structures and boundaries are retained and still identifiable • Good hematoxylin counterstaining of nuclei • Strong, punctated and ubiquitous signals in (+) probe sample and clean background in (-) probe sample
10	20		**Optimal Pretreatment and Sample Preparations; No Probes** • Clean background • Acceptable morphology and cellular architecture • Good hematoxylin counterstaining of nuclei
10	40		**Over Pretreatment or Under Fixation; + Probes (Synpo and SPP1)** • Poor morphology • Loss of cellular structure and boundaries due to excessive heat treatment and protease digestion • Poor hematoxylin counterstaining of nuclei • Weak signal and fewer number of dots

Analyzing target expression

Each observable punctated dot represents a single RNA molecule within the cell that the ViewRNA™ ISH Tissue Assay is able to detect, assuming the RNA target is intact and properly unmasked for the probe to access. These dots are typically uniform in size. However, smaller than average size dots can also be present, and this usually indicates that the transcript is not properly unmasked, or that the RNA target is not intact, resulting in the binding of only one or a few pairs of oligonucleotides from the probe set.

Conversely, a larger than average size dot can occur when multiple targets are found clustered in the same physical area. Naturally, with everything being equal, an RNA target with a low expression will yield fewer numbers of dots than one with a high expression.

In quantifying the results to assess the RNA target expression, it is important to consider the pattern and number of dots observed in the "Negative Control", such as bacterial dapB or sense strand of the target, in order to confidently differentiate between low expressing targets and non-specific background dots. The ViewRNA™ ISH Tissue Assay typically has an average background of < 1 dot/10 cells. Consequently, as long as your target is consistently showing an expression level above the "Negative Control" threshold, even if the RNA target expression is extremely low (e.g., 1 dot/every 2 cells), you can trust that the detection is real.

Tissue preparation guidelines

This section provides guidelines for preparation of FFPE tissue blocks, FFPE tissue slides, and tissue microarray (TMA) slides. Samples prepared outside of these guidelines may produce inadequate results.

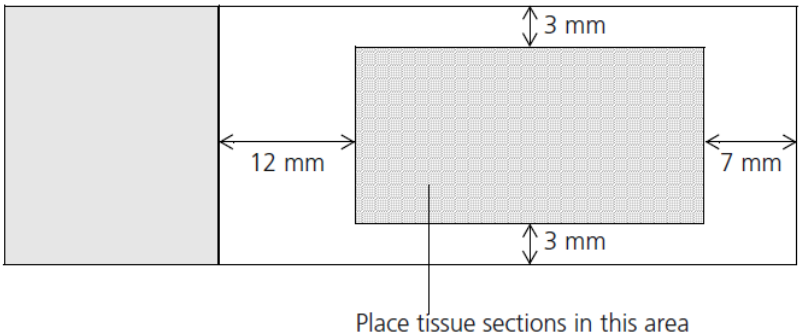
Prepare FFPE/TMA tissue block

1. Immediately place freshly dissected tissues in ≥20 volumes of fresh 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) at room temperature for 16–24 hours. Trim larger specimens to ≤3 mm thickness to ensure faster diffusion of the fixative into the tissue.
2. Rinse, dehydrate, and embed in a paraffin block.
3. Store FFPE tissue blocks at room temperature.

Prepare FFPE/TMA tissue slide

1. Section FFPE tissue to a thickness of 5 ± 1 µm.
2. If working with TMAs, core size should be ≥1.0 mm diameter.
3. Maximum tissue area is 20 mm × 30 mm and should fit within the hydrophobic barrier.
4. Mount sections as shown in Figure 2.1 onto one of the following types of positively-charged glass slides:
   - Leica Non-Clipped X-tra™ Slides, 1 mm white (Cat. No. 3800200 in U.S., Canada, and Asia Pacific regions or Cat. No. 3800210 in Europe).
   - Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, Cat. No. 12-550-15). Avoid other colored labels as they tend to give high background.
5. Air dry freshly-mounted sections at room temperature overnight or at 37°C for 5 hours.
6. Bake slides at 60°C for 1 hours to immobilize tissue sections.
7. Storage
8. Slides can be shipped at the temperature at which they were originally stored.

Figure 2.1 Correct Tissue Section Placement on Glass Side



Sample pretreatment optimization

The pretreatment of tissue sections is critical for the success of all in situ assays, and consists of heat treatment and protease digestion. These pretreatment steps help to unmask the RNA targets, allowing for better probe accessibility and thereby increasing assay signal. However, excessive pretreatment can have a negative effect on tissue morphology. Thus, we recommend optimizing the pretreatment conditions for each new tissue type (see "Sample pretreatment optimization procedures", page 33). Once the optimal pretreatment conditions are determined, they can generally be used for most targets within the particular tissue. In instances when the transcript is particularly rare or expressed at an extremely low level, the optimal pretreatment condition may need to be one that favors signal over morphology.

Refer to the "Sample pretreatment lookup table", page 37 for heat treatment and protease conditions that we have found to be optimal for a number of tissues prepared according to the recommended guidelines in this manual using 10% NBF. This table serves as a reference or starting point only and may not be applicable to tissues prepared using 4% PFA. If you do not obtain the desired results, we recommend performing either the full or limited "Sample pretreatment optimization procedures" on page 33, depending on availability of your samples.

When optimizing pretreatment conditions for TMAs, it is important to understand that it is impossible to identify one condition that is ideal for every tissue type on the array. The optimal pretreatment conditions in such a case would be one that maximizes the number of cores with assay signal and minimizes the number of cores lost due to excessive heat treatment and protease digestion. Due to their high cost and limited quantity, TMAs would greatly benefit from the limited pretreatment optimization procedure, since only as few as three slides might be necessary (see Table B.2, page 37).

Guidelines for working with tissue microarrays

Process TMA slides using the same assay procedures but with the following two modifications:

- Increase the initial baking step time from 60 to 90 min. This additional baking time will increase the tissue attachment to the slide, reducing the risk of small (> 1 mm) core sections falling off during assay procedure.
- Increase the volume/slide of the protease working solution to prevent tissues at the edge of the TMA from drying out.

When designing TMAs to be used in the ViewRNA™ ISH Tissue Assay, it is important to understand that only one optimized condition can be used when running the assay. Therefore, if you want multiple tissue types within the same TMA block, we recommend running an optimization procedure on each individual FFPE tissue type to identify the most favorable pretreatment boiling and protease condition. Based on the optimal condition of the tissue morphology, signal strength, and residual cores, you can judge whether there may be one optimization condition that will be suitable for all of the sample types.

Assessment of endogenous alkaline phosphatase

The ViewRNA™ ISH Tissue Assay uses alkaline phosphatase to convert a chromogenic substrate into a colored signal. For this reason it is important to assess the level of endogenous alkaline phosphatase (AP) activity in your tissue of interest prior to performing the assay.

Certain types of tissue (such as stomach, intestine, placenta and mouse embryo) are known to possess high levels of endogenous AP activity that can interfere with the assay.

To empirically determine the level of endogenous AP activity in your tissue type, perform the pretreatment protocol as instructed for fresh FFPE tissue. After the protease treatment and fixation in 10% NBF, wash the samples in 1X TBS (Sigma, T5912-1L) and incubate the sections with either Fast Blue Substrate or Fast Red Substrate.

If present, endogenous AP can be inactivated with 0.2 M HCl / 300 mM NaCl at room temperature for 15 minutes just before the probe hybridization but after the sample has undergone protease treatment, 10% NBF fixation and 2 washes in 1X PBS.

Safety

Chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

WARNING! CHEMICAL HAZARD. All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

General safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "Safety Data Sheets (SDS)")

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications.

ATTENTION! BIOHAZARD. Les échantillons biologiques tels que les tissus, les fluides corporels et le sang des humains et d'autres animaux ont la possibilité de transmettre des maladies infectieuses. Suivre tous les règlements municipaux, provinciaux/provincial et / ou nationales en vigueur. Porter des lunettes de protection approprié, des vêtements et des gants.

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories*
  (stock no. 017-040-00547-4;
  www.cdc.gov/OD/ohs/biosfty/bmbl4/bmbl4toc.htm)
- Occupational Safety and Health Standards, Bloodborne Pathogens
  (29 CFR§1910.1030;
  www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at:
  www.cdc.gov

In the EU:

- Check your local guidelines and legislation on biohazard and biosafety precaution, and the best practices published in the World Health Organisation (WHO) Laboratory Biosafety Manual, third edition
  www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Documentation and support

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- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

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