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User Manual

ViewRNA™ ISH Tissue 2-Plex Assay

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When describing a procedure for publication using this product, please refer to it as the ViewRNA™ ISH Tissue 2-Plex Assay.

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Introduction

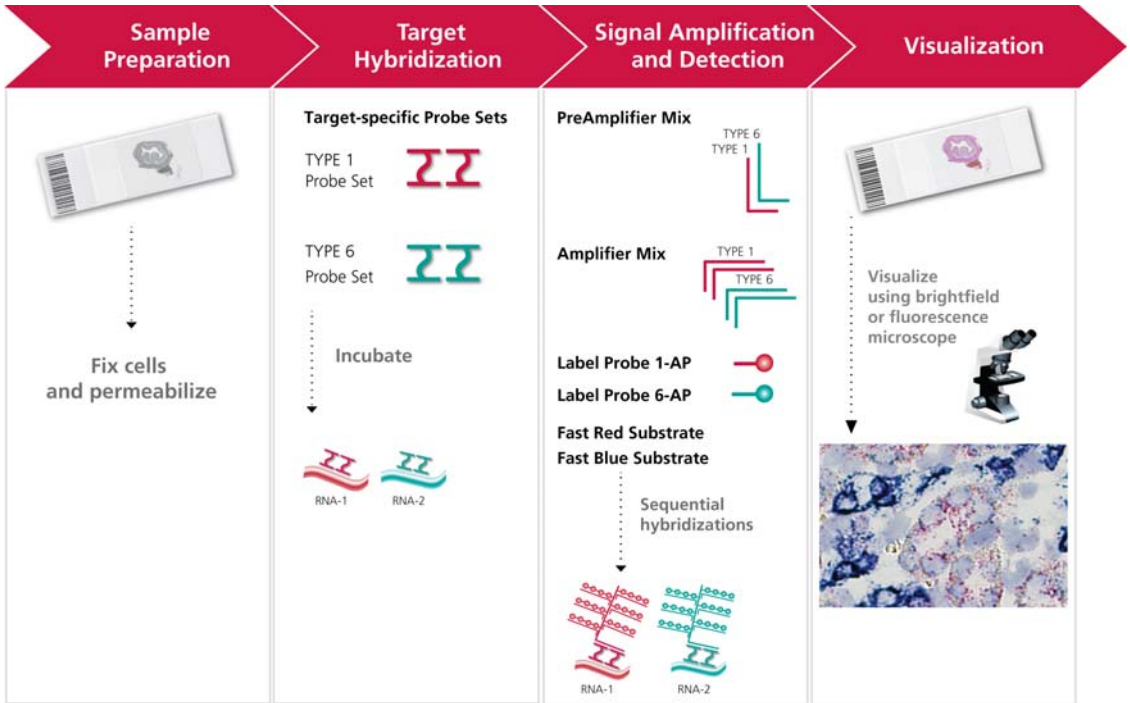
About This Manual

This manual provides complete instructions for performing the ViewRNA ISH Tissue 2-Plex Assay for visualization of one or two target RNAs in formalin-fixed paraffin-embedded (FFPE) samples prepared in accordance with the guidelines provided. Appendix E on page 41 provides a modified protocol for OCT-embedded frozen tissue sections.

Assay Overview

In situ hybridization (ISH) techniques are used to visualize DNA or localize RNAs within cells. However, *in situ* analysis of RNA, in particular, has always been limited by low sensitivity and complicated probe synthesis. The ViewRNA ISH Tissue 2-Plex Assay, based on highly specific, branched DNA signal amplification technology, provides robust simultaneous *in situ* detection of any two target mRNAs within FFPE tissue sections with single-copy sensitivity. Figure 1.1 shows an overview of the assay workflow.

Figure 1.1 ViewRNA ISH Tissue 2-Plex Assay Workflow



Sample Preparation. FFPE tissue sections are deparaffinized and pretreated to allow unmasking of RNA and probe accessibility.

Target Hybridization. Target specific probe pairs (indicated by ZZ and ZZ in Figure 1.1) hybridize to the target RNA. A typical mRNA probe set contains 20 oligonucleotide pairs. For simplicity, only one pair per mRNA target is shown in Figure 1.1. TYPE 1 and TYPE 6 probe sets are designed to generate red and blue signals, respectively.These separate yet compatible signal amplification systems provide the assay with multiplex capability.

Signal Amplification and Detection. Signal amplification is achieved via a series of sequential hybridization steps. PreAmplifiers hybridize to their respective pair of bound probe set oligonucleotides, then multiple amplifiers hybridize to their respective preamplifier. Next, TYPE-specific label probe oligonucleotides, conjugated to alkaline phosphatase, are sequentially hybridized to their corresponding amplifier molecules to provide up to 3,000-fold amplification per target RNA.

Visualization. Sequential hybridization of TYPE 6 label probe followed by addition of Fast Blue substrate and TYPE 1 label probe followed by addition of Fast Red substrate, produces blue and red precipitates (dots) respectively. The target mRNAs are visualized using a standard brightfield and/or fluorescent microscope.

Performance Highlights

Table 1.1 Performance Highlights

Specification	Description
Sample types	▪ Formalin-fixed paraffin-embedded (FFPE) tissue section or microarray ▪ OCT-embedded frozen tissue sections
Sensitivity	Single RNA molecule (one dot = one RNA molecule)
Plex	Detection of two target RNAs
Detection	Chromogenic and fluorescence
Nuclear stain	Hematoxylin and/or DAPI
Instrumentation	Brightfield and/or fluorescence microscope or scanner

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 QT, PreAmplifier Mix QT, and Amplifier Mix QT 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 and Equipment

The ViewRNA ISH Tissue 2-Plex Assay uses the following items, each sold separately and available in multiple sizes:

- ViewRNA ISH Tissue 2-Plex Assay Kit (see Table 1.2, QVT0012 – 24 assays, QVT0013 – 96 assays).
- ViewRNA TYPE 1 and TYPE 6 probe sets (see Table 1.3).

Table 1.4 and Table 1.5 list additional material and equipment requirements for the assay.

ViewRNA ISH Tissue 2-Plex Assay Kit

The kits are configured for processing a minimum of 6 assays (24 assay kit) or 12 assays (96 assay kit), respectively, per experiment.

Table 1.2 lists the components of the ViewRNA ISH Tissue 2-Plex Assay Kit and their recommended storage conditions. Refer to the component package insert for the quantity of individual components supplied. Kits are shipped in two parts, based on storage conditions, and have a shelf life of six months from the date of delivery when stored as recommended.

Table 1.2 ViewRNA ISH Tissue 2-Plex Assay Kit Components and Storage Conditions

Component	Description	Storage
100X Pretreatment Solution	Aqueous buffered solution	2-8 °C
Protease QF[a]	Enzyme in aqueous buffered solution	2-8 °C
Probe Set Diluent QT	Aqueous solution containing formamide, detergent, and blocker	2-8 °C
Label Probe Diluent QF	Aqueous solution containing detergent	2-8 °C
PreAmplifier Mix QT	DNA in aqueous solution containing formamide and detergent	2-8 °C
Amplifier Mix QT	DNA in aqueous solution containing formamide and detergent	2-8 °C
Label Probe 6-AP[a]	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered solution	2-8 °C
Blue Buffer	Buffer required for preparation for Blue Substrate	2-8 °C
Blue Reagent 1	Blue precipitating substrate component 1 for the detection of alkaline phosphatase activity	2-8 °C
Blue Reagent 2	Blue precipitating substrate component 2 for the detection of alkaline phosphatase activity	2-8 °C
Blue Reagent 3	Blue precipitating substrate component 3 for the detection of alkaline phosphatase activity	2-8 °C
AP Enhancer Solution	Aqueous buffered solution	2-8 °C
Fast Red Tablets	Red precipitating substrate for the detection of alkaline phosphatase activity	2-8 °C
Naphthol Buffer	Buffer required for preparation of Red Substrate	2-8 °C
Label Probe 1-AP[a]	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2-8 °C
AP Stop QT	Aqueous buffered solution intended for the inactivation of residual LP6-AP activity after the Fast Blue substrate development	15-30 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15-30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15-30 °C

[a] *IMPORTANT! Do not freeze.*

ViewRNA Probe Sets

In addition to the ViewRNA ISH Tissue 2-Plex Assay Kit, ViewRNA TYPE 1 and TYPE 6 probe sets specific to your targets of interest must be purchased separately. Probe sets are available in multiple sizes and should be stored at –20 °C. Refer to the package insert for quantities provided and design specificities.

Table 1.3 ViewRNA Probe Set and Storage Conditions

Component	Description	Storage
ViewRNA TYPE 1 Probe Set	RNA-specific oligonucleotides to your RNA target of interest. TYPE 1 probe sets are compatible with the TYPE 1 Signal Amplification system which includes PreAmplifier Mix QT, Amplifier Mix QT, Label Probe 1-AP, and Fast Red Substrate. Refer to the package insert for design specificities.	–20 °C
ViewRNA TYPE 6 Probe Set	RNA-specific oligonucleotides to your target of interest. TYPE 6 probe sets are compatible with the TYPE 6 Signal Amplification system which includes PreAmplifier Mix QT, Amplifier Mix QT, Label Probe 6-AP, and Fast Blue Substrate.	–20 °C

Additional Required Materials and Equipment

Table 1.4 and Table 1.5 list other materials and equipment that are required to perform the ViewRNA ISH Tissue 2-Plex Assay. Do not substitute materials or suppliers.

Table 1.4 Required Materials

Item	Source	Part Number
Tissue Tek Staining Dish (clear color), 3	Affymetrix American Master Tech Scientific	QVC0502 LWS20WH
Tissue Tek Clearing Agent Dish (green color), 2	American Master Tech Scientific	LWS20GR
Tissue Tek Vertical 24 Slide Rack, 1	Affymetrix American Master Tech Scientific	QVC0503 LWSRA24
1000 mL glass beaker	Major laboratory supplier	
Forceps	Major laboratory supplier	
Pipettes – P20, P200, P1000	Major laboratory supplier	
Hydrophobic Barrier Pen	Affymetrix Vector Laboratories	QVC0500 H4000
Mounting media ▪ UltraMount Permanent Mounting Medium ▪ HistoMount Mounting Solution (used only in conjunction with UltraMount) ▪ ADVANTAGE Mounting Medium	 DAKO Life Technologies Innovex	 S1964 00-8030 NB300
Rectangular cover glass, 24 mm x 55 mm	VWR Affymetrix	48382-138 QVC0501
Aluminum foil	Major laboratory supplier	
Double-distilled water (ddH_20)	Major laboratory supplier	
100% ethanol (200 proof)	VWR VWR	89015-512 89125-188

Table 1.4 Required Materials (Continued)

Item	Source	Part Number
10X PBS, pH 7.2-7.4	Calbiochem/EMD or equivalent	6504
Gill's Hematoxylin I	American Master Tech Scientific	HXGHE1LT
xylene or Histo-Clear	National Diagnostics or equivalent	HS-200
37% formaldehyde	EMD or equivalent	FX0410-1
27-30% ammonium hydroxide	VWR or equivalent	JT9726-5
DAPI (optional, for fluorescence detection)	Life Technologies or equivalent	D3571

Table 1.5 Required Equipment

Item	Source	Part Number
Either of the following hybridization systems: ▪ ThermoBrite System (110/120 V) 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 VWR or Major laboratory supplier	07J91-010 (110V), 07J68-001 100493380
ViewRNA Temperature Validation Kit	Affymetrix	QV0523
Water-proof remote probe thermometers, validated for 90-100 °C	VWR	46610-024
Fume hood	Major laboratory supplier	
Isotemp hot plates	Fisher Scientific	11-300-49SHP (120V) 11-302-49SHP (230V)
Table-top microtube centrifuge	Major laboratory supplier	
Water bath capable of maintaining 40 ± 1 °C	Major laboratory supplier	
Vortexer	Major laboratory supplier	
Dry incubator or oven capable of maintaining 60 °C for baking slides	Affymetrix or equivalent	QS0704 (120V) or QS0714 (220V)
Microplate shaker (optional, for washing steps)	VWR or equivalent	12620-926
Microscope and imaging equipment	See Table 1.7 on page 6.	

Microscopy and Imaging Equipment Guidelines

The stains used to label RNA in the ViewRNA ISH Tissue 2-Plex Assay can be visualized using brightfield or fluorescence microscopy.

Table 1.6 Stains for ViewRNA ISH Tissue 2-Plex Assay

Detect:	Staining Reagent	Stain Color	
		Brightfield View	**Fluorescence View**
RNA 1 using TYPE 1 probe	Fast Red	Red	Red
RNA 2 using TYPE 6 probe	Fast Blue	Aqua blue	Far red
Nucleus	Hematoxylin/DAPI	Light purple-blue	Blue

Table 1.7 ViewRNA ISH Tissue 2-Plex Assay Imaging Options

Viewing and Digital Capturing Options	Microscope Type	Recommended Microscope/ System	Required Optics	Recommended Filters
Brightfield viewing	Standard brightfield microscope	▪ Leica DM series ▪ Nikon E series ▪ Olympus BX series ▪ Zeiss Axio Lab/Scope/Imager ▪ Or equivalent	Requires 20 and 40X objectives	Requires neutral density filters and/or color filters for white balancing
Fluorescence viewing and image capture	Microscope with camera and fluorescence options Verify that the camera does not have infrared blocking filter	▪ Leica DMA series ▪ Nikon E series ▪ Olympus BX series ▪ Zeiss Axio Lab/Scope/Imager ▪ Or equivalent	▪ Requires 20 and 40X objectives ▪ Numerical aperture (NA) $\geq$ 0.5	For Fast Red Substrate, use Cy3/TRITC filter set: Excitation: 530 ±20 nm Emission: 590 ±20 nm Dichroic: 562 nm For Fast Blue Substrate, use custom filter set[a]: Excitation: 630 ± 20 nm Emission: 775 ± 25 nm Dichroic: 750 nm For DAPI filter set: Excitation: 387/11 nm Emission: 447/60 nm
Automated image capture in brightfield and/or fluorescence mode	Digital pathology scanner system	▪ Aperio ScanScope AT/XT/CS, use FL version for fluorescence ▪ Leica SCN400-F ▪ Olympus Nanozoomer RS ▪ Or equivalent	Recommend scanning at 40X when expression is low	Compatible to above

[a]*Recommended vendor: Semrock Cy7-B/Alexa 750 filter modified with excitation filter FF02-628/40-25.*

Assay Guidelines

Tissue Preparation Guidelines

This section provides critical guidelines for preparation of FFPE tissue blocks, FFPE tissue slides, and tissue microarray (TMA) slides for use with the ViewRNA ISH Tissue 2-Plex Assay. Samples prepared outside of these guidelines may not produce the best results.

FFPE/TMA Tissue Block Preparation

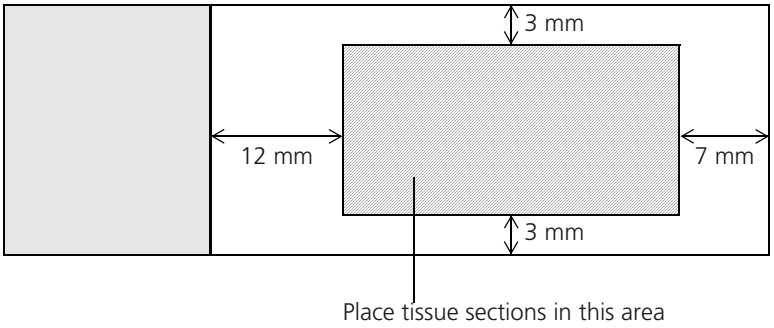
- Immediately place freshly dissected tissues in ≥ 20 volumes of fresh 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) at room temperature (RT) for 16-24 hr. Trim larger specimens to ≤ 3 mm thickness to ensure faster diffusion of the fixative into the tissue.
- Rinse, dehydrate, and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

FFPE/TMA Tissue Slide Preparation

- Section FFPE tissue to a thickness of 5 ± 1 µm.
- If working with TMAs, core size should be ≥ 1.0 mm diameter.
- Maximum tissue area is 20 mm x 30 mm and should fit within the hydrophobic barrier.
- Mount sections as shown in Figure 2.1 on page 8 onto one of the following types of positively-charged glass slides:
  - Leica Non Clipped X-tra® Slides, 1 mm white (P/N 3800200 in U.S., Canada, and Asia Pacific regions or P/N 3800210 in Europe).
  - Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, P/N 12-550-15). Avoid other colored labels as they tend to give high background.
- Air dry freshly-mounted sections at RT overnight or at 37 °C for 5 hr.
- Bake slides at 60 °C for 1 hr to immobilize tissue sections.
- Storage:
  - Short-term – Store sections in a slide box at RT for up to 2 weeks.
  - Long-term – Store sections in a slide box at –20 °C for up to 1 year (avoid freeze/thaw).
- Slides can be shipped at the temperature at which they were originally stored.

NOTE: See ***Guidelines for Working with Tissue Microarrays*** **on page 11 for more information .**

Figure 2.1 Correct Tissue Section Placement on Glass Side



Experiment Design Guidelines

Assay Controls

We recommend running one positive and one negative control slide, based on your sample type, in every ViewRNA ISH Tissue 2-Plex Assay. 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.

Replicates

We recommend running all assays in duplicate.

Sample Pretreatment Optimization

The pretreatment of tissue sections is critical for the success of all *in situ* assays. Pretreatment for the ViewRNA Tissue 2-Plex Assay 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 *Appendix A, Sample Pretreatment Optimization Procedures* on page 31). 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* on page 35 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 31, 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 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 on page 36).

Assessment of Endogenous Alkaline Phosphatase

The ViewRNA ISH Tissue 2-Plex 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. While the problem is more prevalent in fresh frozen tissues, it has also been observed in some FFPE samples.

To empirically determine the level of endogenous AP activity in your tissue type, perform the pretreatment protocol as instructed for fresh frozen or 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 RT for 15 min just before the probe hybridization but after the sample has undergone protease treatment, 10% NBF fixation and 2 washes in 1X PBS.

Probe Set Considerations

Probe sets of the same TYPE can be combined to create a target panel ("pan") 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 on 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 mRNAs in 1- vs. 2-Plex Assay

The ViewRNA ISH Tissue 2-Plex Assay has multiplexing capability, allowing *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. See Appendix C, *Modified Protocols for 1-Plex Assay* on page 35 for instructions on how to shorten the length of the assay when developing Fast Red or Fast Blue as a single-plex.

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 Stop QT 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. Please refer to Table 1.7 on page 6 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 co-localized, 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 co-localization 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.

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 2-Plex 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 if there is one optimization condition that will be suitable for all of the sample types.

ViewRNA ISH Tissue 2-Plex Assay Procedure

About the ViewRNA ISH Tissue 2-Plex Assay Procedure

ViewRNA ISH Tissue 2-Plex 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 – Sample Preparation and Target Probe Set Hybridization (optional stopping point)
- Part 2 – Signal Amplification and Detection

Important Procedural Notes and 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 *Appendix A, Sample Pretreatment Optimization Procedures* on page 31.
- 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.

Essential Keys for a Successful Assay

- Prepare samples following *Tissue Preparation Guidelines* on page 7.
- 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. Click  for a helpful video.
- 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. Click  for a helpful video.
- Incorporate controls, both positive and negative, so that results are unambiguous and can be interpreted. See *Experiment Design Guidelines* on page 8.

Part 1: Sample Preparation and Target Probe Hybridization

Table 3.1 ViewRNA ISH Tissue 2-Plex Assay – Sample Preparation and Target Probe Hybridization

Step	Action
1 Bake Slides **65 min**	**A.** Set the dry oven or hybridization system to 60 ± 1 °C. **B.** Label the slides with a pencil. **C.** Bake the slides following the instructions below: ▪ Dry oven – Insert slides into the slide rack and bake slides for 60 min. ▪ ThermoBrite System – Keep the lid open and bake slides for 60 min. Make sure that the temperature of the ThermoBrite System is validated with the lid 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 – Work 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.** Prepare 200 mL Storage Buffer (for optional stopping point) – 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 – Work 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 400 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.** Prewarm 40 mL 1X PBS and Probe Set Diluent QT to 40 ± 1 °C. **K.** Optional – If performing both parts of the assay in 1 day: ▪ Prewarm PreAmplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF to 40 °C . ▪ Briefly spin down the Label Probe 1-AP, Label probe 6-AP, and Blue reagents, then place on ice until use. ▪ Bring Fast Red Tablets, Napthol Buffer, Blue Buffer, and AP Enhancer Solution to RT.

Table 3.1 ViewRNA ISH Tissue 2-Plex Assay – Sample Preparation and Target Probe Hybridization (Continued)

Step		Action
2	**Prepare Buffers, Reagents, and Equipment While Slides Bake (continued)**	**L.** Optional – If using a microplate shaker for the washes (optional), follow the steps below. Click  for a helpful video. **1.** Set the speed to 550 rpm. **2.** Place a slide rack in a clear staining dish containing the appropriate reagent and insert the slides into the rack. **3.** 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 min**	**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 RT for 5 min. 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 RT for 5 min with frequent agitation. **E.** Repeat Step D above. **F.** Remove the slide rack from the xylene and wash the slides twice, each time with 200 mL of 100% ethanol for 5 min with frequent agitation. **G.** Remove the slides from the rack and place them face up on a paper towel to air dry for 5 min at RT. **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 min 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 min at RT. **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 min at RT. **F.** Remove the slide rack from the Histo-Clear and wash the slides twice, each time with 200 mL of 100% ethanol for 5 min with frequent agitation. **G.** Remove the slides from the rack and place them face up on a paper towel to air dry at RT for 5 min.
4	**Draw Hydrophobic Barrier** **40 min**	**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: **1.** Place the slide over the template image, making sure that the tissue sections fall inside the blue rectangle. **2.** Lightly trace the thick blue rectangle 2-4 times with the hydrophobic barrier pen to ensure a solid seal. **3.** Allow for barrier to dry at RT for 20-30 min. Begin the next step while the barrier is drying. 

Table 3.1 ViewRNA ISH Tissue 2-Plex Assay – Sample Preparation and Target Probe Hybridization (Continued)

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>5</td><td>Heat Pretreatment
10 - 25 min, depending on optimized time</td><td>
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. Click  for a helpful video.
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* on page 31.
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 min 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 RT overnight.
</td></tr>
<tr><td>6</td><td>Protease Digestion and Fixation
30 - 50 min, depending on optimized time</td><td>
A. Prepare the working protease solution using the table below as a guide. Dilute the Protease QF 1:100 in prewarmed 1X PBS and briefly vortex to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.

<table>
<tr><th colspan="2">Working Protease Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Protease QF</td><td>4 µL</td></tr>
<tr><td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
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 the *Sample Pretreatment Optimization Procedures* on page 31.
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 min at RT under a fume hood.
I. Wash the slides twice, each time with 200 mL of fresh 1X PBS for 1 min with frequent agitation.
</td></tr>
</table>

Table 3.1 ViewRNA ISH Tissue 2-Plex Assay – Sample Preparation and Target Probe Hybridization (Continued)

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>7</td><td>Target Probe Set Hybridization
2 hr and 10 min</td><td>
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 QT 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 QT to the "negative control" or probe negative control slide.
<table>
<tr><th colspan="2">Working Probe Set Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Probe Set Diluent QT (prewarmed to 40 °C)</td><td>380 µL</td></tr>
<tr><td>ViewRNA TYPE 1 Probe Set</td><td>10 µL</td></tr>
<tr><td>ViewRNA TYPE 6 Probe Set</td><td>10 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
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 prewarmed Probe Set Diluent QT 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.
</td></tr>
<tr><td>8</td><td>Wash Slides
8 min</td><td>
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 RT for 2 min with constant and vigorous agitation. Click  for a helpful video.
D. If you plan to perform the assay over the course of two days, go to Step 9 Optional Stop Point. Otherwise, proceed to Step 12 PreAmplifier Hybridization on page 18 to complete the entire assay in one day.
</td></tr>
<tr><td>9</td><td>Optional Stop Point
1 minute</td><td>
A. Store slides in a clear staining dish containing 200 mL of Storage Buffer at RT for up to 24 hr. 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 RT for use in Part 2: Signal Amplification and Detection on page 18.
D. If using a ThermoBrite System, rewet the ThermoBrite Humidity Strips in ddH_2O.
E. Proceed to Step 10 Prepare Additional Buffers and Reagents on page 18 when you are ready to continue the assay.
</td></tr>
</table>

Part 2: Signal Amplification and Detection

Table 3.2 ViewRNA ISH Tissue 2-Plex Assay – Signal Amplification and Detection

Step		Action
10	**Prepare Additional Buffers and Reagents** **5 min**	**A.** Pour Gill's Hematoxylin into a clear staining dish and store at RT protected from light until use. **B.** Prewarm PreAmplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF buffers to 40 °C. **C.** Briefly spin down the Label Probe 1-AP , Label probe 6-AP, and Blue reagents. Place them on ice. **D.** Bring Fast Red Tablets, Naphthol Buffer, AP Enhancer Solution, and Blue Buffer to RT.
11	**Wash Slides** **5 min**	**A.** Remove the slides from Storage Buffer. **B.** Wash the slides 2 times, each time with 200 mL of fresh Wash Buffer at RT for 2 min with constant and vigorous agitation.
12	**PreAmplifier Hybridization** **35 min**	**A.** Swirl the PreAmplifier Mix QT 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 PreAmplifier Mix QT to each tissue section. **C.** Transfer slides to the hybridization system and incubate at 40 °C for 25 min.
13	**Wash Slides** **8 min**	**A.** Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer. **B.** After incubation, decant the PreAmplifier Mix QT 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 RT for 2 min with constant and vigorous agitation.
14	**Amplifier Hybridization** **20 min**	**A.** Swirl the Amplifier Mix QT 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 QT to each tissue section. **C.** Transfer slides to the hybridization system and incubate at 40 °C for 15 min.
15	**Wash Slides** **8 min**	**A.** Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer. **B.** After incubation, decant the Amplifier Mix QT 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 RT for 2 min with constant and vigorous agitation.

Table 3.2 ViewRNA ISH Tissue 2-Plex Assay – Signal Amplification and Detection (Continued)

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>16</td><td>Label Probe 6-AP Hybridization
20 min</td><td>
A. Briefly vortex and 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 prewarmed Label Probe Diluent QF and briefly vortex to mix. Scale reagents according to the number of assays to be run and include one slide volume overage.
<table>
<tr><th colspan="2">Working Label Probe 6-AP Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>399.6 µL</td></tr>
<tr><td>Label Probe-6AP</td><td>0.4 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
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.
</td></tr>
<tr><td>17</td><td>Wash Slides
12 min</td><td>
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 RT for 3 min with constant and vigorous agitation.
</td></tr>
<tr><td>18</td><td>Apply Fast Blue Substrate
35 min</td><td>
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 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. Transfer the slides to the hybridization system and incubate in the dark at RT for 30 min.
</td></tr>
<tr><td>19</td><td>Wash Slides
12 min</td><td>
A. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
B. After incubation, decant the working 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 RT for 3 min with constant and vigorous agitation.
</td></tr>
<tr><td>20</td><td>Quench Label Probe 6-AP
35 min</td><td>
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 Stop QT. Incubate in the dark at RT for 30 min.
B. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
C. After incubation, decant the AP Stop Buffer 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 RT for 1 min 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.
</td></tr>
</table>

Table 3.2 ViewRNA ISH Tissue 2-Plex Assay – Signal Amplification and Detection (Continued)

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>21</td><td>Label Probe 1-AP Hybridization
20 min</td><td>
A. Briefly vortex and 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 prewarmed Label Probe Diluent QF and briefly vortex to mix. Scale reagents according to the number of assays to be run and include one slide volume overage.
<table>
<tr><th colspan="2">Working Label Probe 1-AP Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>399.6 µL</td></tr>
<tr><td>Label Probe 1-AP</td><td>0.4 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
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-AP solution to each tissue section.
D. Transfer the slides to the hybridization system and incubate at 40 °C for 15 min.
</td></tr>
<tr><td>22</td><td>Wash Slides
12 min</td><td>
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 RT for 3 min with constant and vigorous agitation.
</td></tr>
<tr><td>23</td><td>Apply Fast Red Substrate
45 min</td><td>
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 slides face up on a flat, elevated platform.
B. Immediately add 400 µL of the AP Enhancer Solution to each tissue section and incubate at RT for 5 min while preparing the Fast Red Substrate.
C. Prepare the Fast Red Substrate – Add 5 ml of Naphthol Buffer and one Fast Red Tablet to a 15 ml conical tube. Vortex at high speed to completely dissolve the tablet. Protect from light until use by wrapping the tube in aluminum foil.
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 40 °C for 30 min.
F. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
G. After incubation, decant the Fast Red Substrate solution from the slides and insert them into the slide rack.
H. Rinse off the excess Fast Red Substrate from the slides by moving the slide rack up and down for 1 min.
</td></tr>
</table>

Table 3.2 ViewRNA ISH Tissue 2-Plex Assay – Signal Amplification and Detection (Continued)

Step	Action
24 Counterstain **25 min**	**A.** Transfer the slide rack to the clear staining dish containing the 200 mL of Gill's hematoxylin and stain for 5-10 sec at RT. **B.** Wash the slides 3 times, each time with 200 mL of fresh ddH_2O for 1 min 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 RT 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 min. **E.** Optional – If you plan to view slides using a fluorescent microscope, move the slide rack into a clear staining dish containing 200 mL DAPI (3 µg/mL). Stain the slides for 1 min, then rinse them in 200 mL of fresh ddH_2O by moving the slide rack up and down for 1 min. **F.** 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. **G.** Ensure that slide sections are completely dry before mounting (~20 min).
25 Mount and Image **40 min**	**If using DAKO Ultramount mounting medium:** ▪ **For no coverslipping (20X viewing or imaging):** **A.** Place the slide flat on a counter top with specimen facing up. **B.** Dab the first 2-3 drops of Ultramount onto a paper towel to remove bubbles. **C.** Apply a sufficient amount of Ultramount to completely cover the specimen with a thin layer (3-4 drops) of mounting medium. **D.** Place slides horizontally in a 70 °C oven/incubator to dry the mounting medium. Allow 10-30 min for the mounting medium to harden completely. The drying time depends on the amount of mounting medium applied. **E.** Image or store slides at RT. ▪ **For post mounting with coverslip (crisper 20X or 40X viewing or imaging):** **A.** Work in a fume hood and follow the no coverslipping procedure. **B.** Make sure that the Ultramount is completely hardened. **C.** Allow the slides to come to RT. **D.** Apply HistoMount directly on top of the dried Ultramount. **E.** Place coverslip on top and allow to air dry at RT for 15 min. **F.** Image or store slides at RT. **If using ADVANTAGE mounting medium:** **A.** Place a 24 mm x 55 mm cover glass horizontally onto a clean, flat surface. **B.** Dab the first 2-3 drops of mounting media onto a paper towel to remove bubbles. **C.** Add 2 drops of the ADVANTAGE medium directly onto the middle of the cover glass. **D.** Use a pipette tip to draw out any air bubbles in the droplets. **E.** Invert the specimen slide and slowly place it onto the mounting medium at an angle. Make sure that the tissue comes into contact with the mounting medium first before completely letting go of the glass slide to overlap with the cover glass. **F.** After mounting, flip the slide over and place it on its edge on a laboratory wipe to soak up and remove excess mounting medium. Allow the slide to dry at RT in the dark for 15 min. Do not bake the slides to speed up the drying process. **G.** To prevent bubble formation, seal all 4 edges of the cover glass with a flat black-colored nail polish (iridescent or colored nail polish can autofluoresce and interfere with fluorescent imaging). **H.** Image the results using a brightfield and/or fluorescence microscope. Store slides at RT.

Troubleshooting

Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. Visit our website at www.affymetrix.com/panomics for an updated list of FAQs and product support literature.

Table 4.1 Technical Support Contact Information

Location	Affymetrix
North America	Tel: 1.877.726.6642 option 1, then option 3 E-mail: pqbhelp@affymetrix.com
Europe	Tel: +43 1 7964040-120 E-mail: tech@ebioscience.com
Asia	Tel: +81 3 6430 430 E-mail: techsupport_asia@affymetrix.com

Weak or No Signals

Table 4.2 Troubleshooting Weak or No Signal

Probable Cause	Recommended Action
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 RT for 16-24 hours.
Tissue over-fixed after protease digestion	Make sure the tissue sections are not fixed for more than 5 min 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 RT. ▪ 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, PreAmplifier Mix QT, Amplifier Mix QT, Label Probe-AP and substrates in the correct order.
Gene of interest not expressed	▪ Verify expression using other tissue lysate methods such as QuantiGene, QuantiGene Plex assay, or Affymetrix array. ▪ Run the same probe set on known samples that have been validated 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.
Hybridization temperature not optimal	Calibrate the hybridization system at 40°C using a ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523).
Mounting solution contained alcohol	Use the recommended mounting media to mount your tissue (see *Step 25 Mount and Image* on page 21). 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 (Affymetrix 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.

Table 4.2 Troubleshooting Weak or No Signal (Continued)

Probable Cause	Recommended Action
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 to 38 °C. ▪ Increase Fast Red incubation time to 45 min.
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

Table 4.3 Troubleshooting High Background

Probable Cause	Recommended Action
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 min per wash.
Hybridization temperature not optimal	Calibrate the hybridization system at 40 °C using the ViewRNA Temperature Validation Kit (Affymetrix P/N QV0523).
Concentration of hybridization reagents too high	Double check the dilution calculation for all working solutions.
Suboptimal pretreatment conditions	Perform the pretreatment optimization procedure to determine the optimal heat treatment and protease digestion time.
Label Probe-AP concentration too high	▪ Verify that the correct concentrations were used. ▪ Decrease the recommended concentration for Label Probe-AP.

Diffused Signals

Table 4.4 Troubleshooting Diffused Signals

Probable Cause	Recommended Action
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 *Step 25 Mount and Image* on page 21). Avoid any mounting medium containing alcohol or any cover slipping method requiring alcohol dehydration.

Endogenous Alkaline Phosphatase Activity

Table 4.5 Troubleshooting Endogenous Alkaline Phosphatase Activity

Probable Cause	Recommended Action
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 RT for 10 min before the protease step. Wash samples twice with 1X PBS before proceeding to protease digestion.

Tissue Detachment From Slide

Table 4.6 Troubleshooting Tissue Detachment From Slide

Probable Cause	Recommended Action
Improper tissue preparation	Make sure that the tissue preparation is as recommended in *Tissue Preparation Guidelines* on page 7, including fixation time and reagent, thickness of sections, brand of positively charged glass slide, and baking of the sections at 60 °C for 1 hr before storing at –20 °C.
Insufficient baking of slides	Verify that the 60 min 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 of a concentration.	Reduce proteinase concentration and/or incubation time.

Poor Cell Morphology

Table 4.7 Troubleshooting Poor Cell Morphology

Probable Cause	Recommended Action
Incorrect pretreatment conditions	Perform full pretreatment optimization procedure to determine optimal heat treatment and protease digestion time. See Appendix A on page 31.
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.

High Non-Specific Binding on Glass Slide

Table 4.8 Troubleshooting Non-specific Binding on Glass Slide

Probable Cause	Recommended Action
Incompatible glass slide	▪ Use the recommended glass slides: ▫ Leica Non-Clipped X-tra® Slide, 1 mm White P/N 3800200 or 3800210 ▫ Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, P/N12-550-15); avoid other colored labels as they tend to give high background. ▪ Prevalidate 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. Click  for a helpful video. ▪ Increase wash incubation time by 1 min per wash.
Concentration of hybridization reagents was too high	Confirm that the dilution calculations are correct for all working solutions.

Pink Non-Specific Background Where Paraffin Was

Table 4.9 Troubleshooting Pink Non-Specific Background Where Paraffin Was

Probable Cause	Recommended Action
Incomplete removal of paraffin	▪ Be sure to 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 the paraffin at 80 °C for 3 min and remove paraffin using 3 changes of fresh Histo-Clear. ▪ Do not bake the slides at a temperature higher than 60 °C.

Hydrophobic Barrier Falls Off

Table 4.10 Troubleshooting the Hydrophobic Barrier

Probable Cause	Recommended Action
Incompatible glass slide	▪ Use the recommended glass slides: ▫ Leica Non-Clipped X-tra® Slide, 1 mm White P/N 3800200 or 3800210 ▫ Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific, P/N12-550-15); avoid other colored labels as they tend to give high background. ▪ Prevalidate 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 min 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 (Affymetrix 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 min or longer depending on how heavily the barrier is created.

Fast Red Signal for TYPE 1 Target is Weak or Different in 2-Plex Versus 1-Plex

Table 4.11 Troubleshooting Weak or Different Fast Red Signal for TYPE 1 Target in 2-Plex Versus 1-Plex

Probable Cause	Recommended Action
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 min to 10-15 min.

TYPE 1 Target Signals Observed in the Channel for TYPE 6 Target

Table 4.12 Troubleshooting TYPE 1 Target Signals Observed in the Channel for TYPE 6 Target

Probable Cause	Recommended Action
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 *Microscopy and Imaging Equipment Guidelines* on page 6 for recommended filter set specifications for Fast Blue.

Co-localized Fast Blue and Fast Red Signals When Using Only TYPE 6 Probe Set

Table 4.13 Troubleshooting Co-localized Fast Blue and Fast Red Signals When Using Only TYPE 6 in a 2-Plex Assay

Probable Cause	Recommended Action
Residual LP6-AP activity	▪ Do not omit Step 20 on page 19 – *Quench Label Probe 6-AP*. ▪ Be sure to quench LP6-AP activity with AP Stop QT for the entire 30 min.

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, cross-linking 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 2-Plex 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 set of 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 on page 35 provides sample pretreatment conditions for some common tissues. If samples are limited, see Table B.2 on page 36.

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, please read the sections on *Important Procedural Notes and Guidelines* on page 13 and *Essential Keys for a Successful Assay* on page 13

The pretreatment optimization procedure for the ViewRNA ISH 2-Plex Tissue 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

Table A.2 Sample Pretreatment Optimization Procedure – Sample Preparation and Target Probe Set Hybridization

Step		Action
1	**Bake Slides**	See Step 1 to Step 4 starting on page 14.
2	**Heat Pretreatment 10-25 min**	**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. Click  for a helpful video. **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 min 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. **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 RT for up to one week. Continue with Step 3 on page 33.

Table A.2 Sample Pretreatment Optimization Procedure – Sample Preparation and Target Probe Set Hybridization (Continued)

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3 Protease Digestion and Fixation
30-50 min</td><td>
A. Prepare the working protease solution using the table below as a guide. Dilute the Protease QF 1:100 in prewarmed 1X PBS and briefly vortex to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.
<table>
<tr><th colspan="2">Working Protease Solution per Slide</th></tr>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Protease QF</td><td>4 µL</td></tr>
<tr><td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr>
<tr><td>Total volume</td><td>400 µL</td></tr>
</table>
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 min (40 min 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 min.
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 RT for 5 min under a fume hood.
P. Wash the slides twice, each time with 200 mL of fresh 1X PBS for 1 min with frequent agitation.
Q. Proceed to *Step 7 Target Probe Set Hybridization* on page 17 to continue the assay procedure.
</td></tr>
</table>

Sample Pretreatment Lookup Table

Table B.1 shows a list tissues that were prepared according to the guidelines outlined in this manual (*Tissue Preparation Guidelines* on page 7) 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.

Please note that the conditions listed here are specific to tissues prepared using 10% NBF and may not be applicable to tissue prepared using 4% PFA. If you chose to use any of the pretreatment conditions listed in the lookup table, it is important to 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

Tissue Information		Optimal Conditions (Min)		Range of Tolerance (Min)
Species	**Type**	**Heat treatment at 90-95 °C**	**Protease at 40 °C**	**(Heat treatment, Protease)**
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	
Rat	Kidney	10	20	(10,10) (20,20)
	Liver	10	20	
	Spleen	20	10	
	Thyroid	10	20	

Table B.1 Sample Pretreatment Optimization Lookup Table (Continued)

Tissue Information		Optimal Conditions (Min)		Range of Tolerance (Min)
Species	**Type**	**Heat treatment at 90-95 °C**	**Protease at 40 °C**	**(Heat treatment, Protease)**
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 2-Plex Assay.

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

Number of Available Slides	Heat Treatment 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

Evaluating Results

Assessing Pretreatment Conditions

This section provides sample images obtained from the ViewRNA ISH Tissue 2-Plex 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

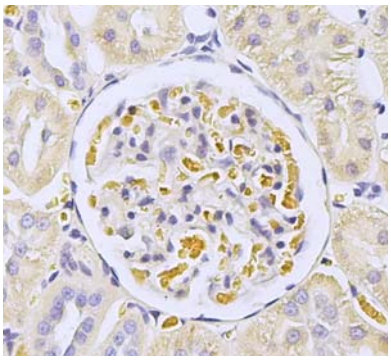
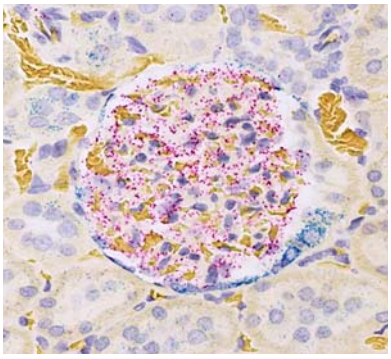
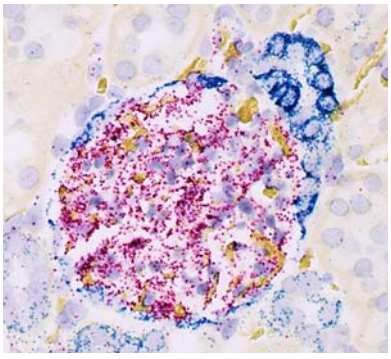
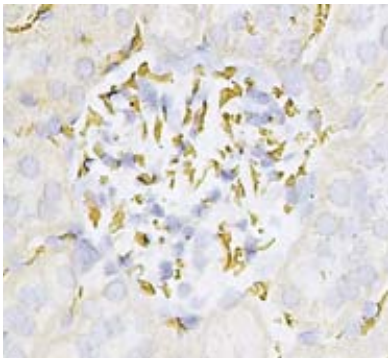
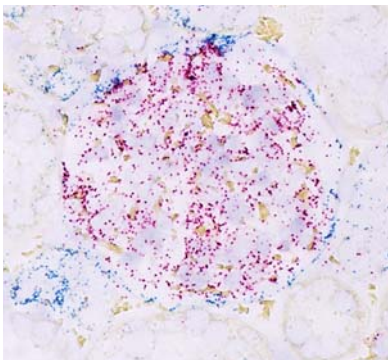
Heat Pretreatment Time (Min)	Protease Digestion Time (Min)	Brightfield Image	Results Interpretation
0	0		**Untreated Morphology Reference Slide + Probes (Synpo and SPP1)** ▪ Good morphology ▪ Intact cellular structure ▪ Good hematoxylin counterstaining of nuclei ▪ Little or no signal (dots) observed
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
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

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

Heat Pretreatment Time (Min)	Protease Digestion Time (Min)	Brightfield Image	Results Interpretation
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 2-Plex 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 2-Plex 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 reliably real.

Modified Protocols for a 1-Plex Assay

This appendix provides modified and shortened assay procedures for performing a 1-plex assay using the ViewRNA ISH Tissue 2-Plex Assay Kit. Whether your preference for target detection is Fast Red or Fast Blue, both TYPE 1 and TYPE 6 probe sets can be used.

Table D.1 Modified 1-Plex Protocol for Fast Red Detection Using TYPE 1 or TYPE 6 Probe Sets

Probe Set Designation	Modified Protocol
TYPE 1	**A.** Perform the assay as directed through *Step 15 Wash Slides* on page 18. **B.** Omit *Step 16 Label Probe 6-AP Hybridization* to *Step 20 Quench Label Probe 6-AP* on page 19. **C.** Continue with *Step 21 Label Probe 1-AP Hybridization* to *Step 25 Mount and Image* on page 21.
TYPE 6	**A.** Perform the assay as directed through *Step 17 Wash Slides* on page 19. **B.** Omit *Step 18 Apply Fast Blue Substrate* to *Step 22 Wash Slides* on page 20. **C.** Continue with *Step 23 Apply Fast Red Substrate* to *Step 25 Mount and Image* on page 21.

Table D.2 Modified 1-Plex Protocol for Fast Blue Detection Using TYPE 1 or TYPE 6 Probe Sets

Probe Set Designation	Modified Protocol
TYPE 1	**A.** Perform the assay as directed through *Step 15 Wash Slides* on page 18. **B.** Replace Label Probe 6-AP with Label Probe 1-AP in *Step 16 Label Probe 6-AP Hybridization* on page 19. **C.** Continue with *Step 17 Wash Slides* to *Step 19 Wash Slides* on page 19. **D.** Omit *Step 20 Quench Label Probe 6-AP* to *Step 23 Apply Fast Red Substrate* on page 20. **E.** Continue with *Step 24 Counterstain* to *Step 25 Mount and Image* on page 21.
TYPE 6	**A.** Perform the assay as directed through *Step 19 Wash Slides* on page 19. **B.** Omit *Step 20 Quench Label Probe 6-AP* to *Step 23 Apply Fast Red Substrate* on page 20. **C.** Continue with *Step 24 Counterstain* to *Step 25 Mount and Image* on page 21.

Using Frozen Tissues with ViewRNA ISH Tissue 2-Plex Assay

About This Appendix

This appendix provides procedural modification for running the ViewRNA ISH Tissue 2-Plex Assay on fresh frozen or OCT-embedded frozen tissue sections. This modified assay protocol has been tested on the following OCT-embedded frozen tissue samples:

- Bovine – Ovary
- Human – Colon, skin, testis
- Mouse – Brain, duodenum, eye, liver, lung, pancreas, skin, spinal cord
- Rat – Brain, spinal cord

Important Procedural Notes

- This protocol requires overnight fixation in chilled 4% NBF prior to starting Day 1 of the assay. See *Step 2 Fix Tissue Overnight* on page 42.
- Samples should be freshly sectioned at 12 ± 1 µm and mounted onto one of the following positively charged glass slides.
  - Leica Non-Clipped X-tra® Slides, 1 mm white (P/N 3800200 in US, Canada, and Asia Pacific regions or P/N 3800210 in Europe).
  - Fisherbrand™ Superfrost™ Plus Slides, white label (Fisher Scientific Cat# 12-550-15); avoid other colored labels as they tend to give high background.
- Prepared frozen tissue slides should be used immediately in the assay or can be stored at –80 °C for up to 6 months.
- Perform optimization for Protease only (see Table A.1 on page 31 for recommended times). No heat treatment step required.

Modifications to Part 1: Sample Preparation and Target Probe Hybridization

The following procedural steps replace Step 1 to Step 5 in *Part 1: Sample Preparation and Target Probe Hybridization* on page 14 .

Table E.1 ViewRNA ISH Tissue 2-Plex Assay – Sample Preparation and Target Probe Hybridization for Frozen Tissues

Step		Action
1	**Prepare and Chill 10% NBF**	Add 178 mL 1X PBS and 22 mL 37% formaldehyde to a 200 mL capacity container. Mix well and chill on ice for 1 hr.
2	**Fix Tissue Overnight**	Pour chilled 10% NBF into a clear staining dish and insert an empty slide rack into the solution. Insert frozen tissue slides into the slide rack and incubate at 4 °C for 16-18 hr.
3	**Prepare Buffers, Reagents, and Equipment**	**A.** Verify that the hybridization system is set to 40 ± 1 °C and appropriately humidified. **B.** Prepare 2 L 1X PBS – Add 200 mL 10X PBS and 1.8 L ddH_2O to a 2 L capacity container. **C.** Prepare 200 mL of 50% ethanol – Add 100 mL 100% ethanol and 100 mL ddH_2O to a 200 mL capacity container. **D.** Prepare 200 mL of 70% ethanol – Add 60 mL 100% ethanol and 140 mL ddH20 to a 200 mL capacity container. **E.** 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. **F.** Prepare 200 mL Storage Buffer (for optional stopping point) – Add 60 mL Wash Comp 2 and 140 mL ddH_2O to a 200 mL capacity container. **G.** Ensure availability of: ▪ 1000 mL ddH_2O ▪ 200 mL Gill's Hematoxylin I ▪ 200 mL of 3 µg/mL DAPI in 1X PBS (optional for fluorescent detection), store in the dark at 4 °C until use. **H.** Thaw probe set(s). Mix, briefly centrifuge to collect content and place on ice until use. **I.** Prewarm 10 mL of 1X PBS and Probe Set Diluent QF to 40 °C. **J.** Optional for 1-day assay: ▪ Prewarm PreAmplifier Mix QT, Amplifier Mix QT, and Label Probe Diluent QF to 40 °C . ▪ Briefly spin down the Label Probe 1-AP, Label Probe 6-AP, and Blue Reagents. Place on ice. ▪ Bring Fast Red Tablets, Napthol Buffer, Blue Buffer, and AP Enhancer Solution to RT. ▪ Prepare 1 L of 0.01% ammonium hydroxide – Work in a fume hood. Add 0.33 mL 30% ammonium hydroxide and 999.67 mL ddH2O in a 1 L capacity container. **K.** Optional – If using a microplate shaker for the washes, set the speed to 550 rpm. Click  for a video with detailed instructions.
4	**Wash Slides**	Remove slide rack from the 10% NBF and wash the slides twice, each time with 200 mL of 1X PBS for 1 min with frequent agitation.
5	**Tissue Dehydration**	**A.** Dehydrate the tissue by sequentially soaking the rack of slides in 50%, 70% and then 100% ethanol in a clear staining dish, each time at RT for 10 min without agitation. **B.** Remove the slide rack from the 100% ethanol and drain the excess on a paper towel. **C.** Transfer the entire rack of slides to a 60 °C dry incubator/oven and bake the slides for 60 min. **Note:** Following the baking step, continue with the assay within 1 hr, beginning with *Step 4 Draw Hydrophobic Barrier* on page 15 and skipping to *Step 6 Protease Digestion and Fixation* on page 16. The heat treatment step is NOT REQUIRED for frozen tissues.