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ViewRNA™ Tissue Assay

USER GUIDE

Single and duplex *in situ* hybridization detection of mRNA in tissue

for use with:
ViewRNA™ Tissue Core Kit
ViewRNA™ Tissue Assay Fast Red Module
ViewRNA™ Tissue Assay Fast Blue Module
ViewRNA™ Tissue Assay DAB Module

Catalog Numbers QVT0400, QVT0410, QVT0420, and QVT0430
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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.

Revision history: MAN0018633 H.0 (English)

Revision	Date	Description
H.0	2 August 2023	The ViewRNA™ Tissue Core Kit and ViewRNA™ Tissue Assay Fast Blue Module formats were changed and the ViewRNA™ Tissue Assay Fast Red Module and ViewRNA™ Tissue Assay DAB Module were added. Associated procedures were updated.
G.0	9 June 2023	In step 1 of "Perform counterstain with DAPI" corrected volume from 6 µL to 60 µL.
F.0	16 August 2022	• In the kit, Fast Red tablets were replaced with ViewRNA™ Fast Red Substrates 1, 2, and 3. Associated procedures were updated: – "Apply Fast Red staining solution (75 minutes)" – "(Optional) Perform counterstain (25 minutes)" – "Mount slides for imaging" – "Mount slides with Dako ultramount mount medium" was removed. – "Mount slides with ADVANTAGE mounting medium was removed. • A workflow was added (page 12), and additional minor edits throughout clarified the 1-plex and 2-plex workflows. • The following sections were moved to a new Appendix "Guidelines for experimental design": – "Probe set considerations" "Assigning colors to target mRNA in 1- vs 2-plex assays" Guidelines for microscopy and imaging" • Thermo Fisher Scientific or Fisher Scientific options for some of the required materials were added. • Updated to the latest document template, with associated changes to safety, legal, format, and style.
E.0	20 December 2021	• The Wash 2 amount was updated from 10 mL to 20 mL in "Wash slides (8 minutes)". • Appendix D: Using frozen tissues with ViewRNA™ Tissue Assay was removed. • The document was updated to the current document template, with associated updates to trademarks, logos, licensing, and warranty.
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.

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

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1 Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The ViewRNA™ Tissue Assay is used for visualization of one or two target RNAs in tissue samples prepared in accordance with the guidelines provided in this manual.

In situ hybridization 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™ Tissue Assay, based on highly specific branched DNA signal amplification technology, provides robust *in situ* detection of one or two target mRNAs within tissue sections with single-copy sensitivity.

Key features:

- Suitable for formalin-fixed paraffin-embedded (FFPE) or cryo tissue sections.
- Single RNA molecule sensitivity (one dot equals one RNA molecule).
- Detection of two target RNAs.
- Chromogenic and fluorescent detection.
- Compatible with some colorimetric counterstains and DAPI.
- Compatible with brightfield and fluorescence microscopes or scanners.

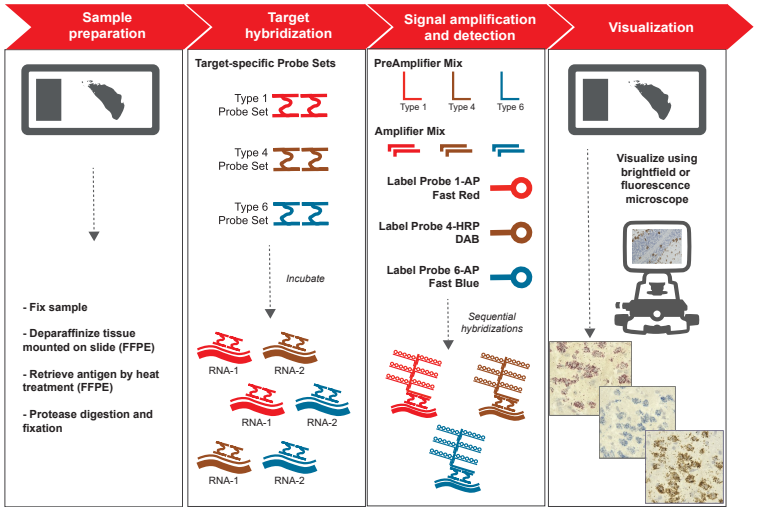

Figure 1 ViewRNA™ Tissue Assay.
The ViewRNA™ Tissue Assay, based on branched-DNA signal amplification technology, can be used to measure two targets simultaneously and has the sensitivity and robustness to measure single mRNA molecules in single cells.

Contents and storage

The ViewRNA™ Tissue Assay consists of a core kit and three modules, each sold separately:

- ViewRNA™ Tissue Core Kit—Contains the reagents required for *in situ* hybridization to RNA targets. Does not contain the specific target probes (ordered separately, see "ViewRNA™ Probe Sets" on page 6) or the detection reagents to visualize the targets using brightfield microscopy of 1 or 2 mRNA targets using the individual or multiple ViewRNA™ color modules.
- ViewRNA™ Tissue Assay Fast Red Module—Contains the reagents for the detection of an RNA target using the Fast Red substrate.
- ViewRNA™ Tissue Assay Fast Blue Module—Contains the reagents for the detection of an RNA target using the Fast Blue substrate.
- ViewRNA™ Tissue Assay DAB Module—Contains the reagents for the detection of an RNA target using the DAB substrate.

ViewRNA™ Probe Sets

To order TYPE 1, TYPE 4, and TYPE 6 probe sets, go to **thermofisher.com/viewrnatissue**.

ViewRNA™ Tissue Core Kit

Each ViewRNA™ Tissue Core Kit is supplied in two boxes based on storage temperature. Reagents have a minimum shelf life of 6 months from date of receipt when stored as recommended. See kit labels for expiration dates and the package insert for component quantities. Kits are configured for processing a minimum of 24 assays per experiment.

Table 1 ViewRNA™ Tissue Core Kit [Cat. No. QVT0400 (24 assays), includes Cat. No. QVT0400A and Cat. No. QVT0400B]

Product	Component	Description	Storage
ViewRNA™ Tissue Core Reagents (Cat. No. QVT0400A)	100X Pretreatment Solution	Aqueous buffered solution	2–8°C
	Probe Set Diluent	Aqueous solution containing formamide, detergent, and blocker	
	PreAmplifier Mix	DNA in aqueous solution containing formamide and detergent	
	Amplifier Mix	DNA in aqueous solution containing formamide and detergent	
	Label Probe Diluent	Aqueous solution containing detergent	
	Protease Solution	Enzyme in aqueous buffered solution	2–8°C Do not freeze

Table 1 ViewRNA Tissue Core Kit [Cat. No. QVT0400 (24 assays), includes Cat. No. QVT0400A and Cat. No. QVT0400B] *(continued)*

Product	Component	Description	Storage
ViewRNA™ Tissue Wash Buffers (Cat. No. QVT0400B)	Wash Buffer Component 1	Aqueous buffered solution containing detergent	15–30°C
	Wash Buffer Component 2	Aqueous buffered solution	

ViewRNA™ Tissue Assay Fast Red Module

Reagents have a minimum shelf life of 6 months from date of receipt when stored as recommended. See kit labels for expiration dates and the Package Insert for component quantities.

Table 2 ViewRNA™ Tissue Assay Fast Red Module [Cat. No. QVT0410 (24 assays)]

Component	Description	Storage
Fast Red Reagent 1	Red precipitating substrate component 1 for the detection of alkaline phosphatase activity	2–8°C
Fast Red Reagent 2	Red precipitating substrate component 2 for the detection of alkaline phosphatase activity	
Fast Red Reagent 3 - lyo	Red precipitating substrate component 3 for the detection of alkaline phosphatase activity (lyophilized)	
Fast Red Reaction Buffer	Buffer required for preparation of Red Substrate	
AP (Alkaline Phosphatase) Enhancer Solution	Aqueous buffered solution to enhance the fluorescence signal from Fast Red Substrate development	
Label Probe Type 1 - AP	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2–8°C Do not freeze

ViewRNA™ Tissue Assay Fast Blue Module

Reagents have a minimum shelf life of 6 months from date of receipt when stored as recommended. See kit labels for expiration dates and the Package Insert for component quantities.

Table 3 ViewRNA™ Tissue Assay Fast Blue Module [Cat. No. QVT0420 (24 assays)]

Component	Description	Storage
Fast Blue Reagent 1	Blue precipitating substrate component 1 for the detection of alkaline phosphatase activity	2–8°C
Fast Blue Reagent 2	Blue precipitating substrate component 2 for the detection of alkaline phosphatase activity	
Fast Blue Reagent 3	Blue precipitating substrate component 3 for the detection of alkaline phosphatase activity	
Fast Blue Reaction Buffer	Buffer required for preparation of Blue Substrate	
AP Stop Solution	Aqueous buffered solution intended for the inactivation of residual Label Probe Type 6 - AP activity after the Fast Blue Substrate development	
Label Probe Type 6 - AP	Alkaline phosphatase-conjugated oligonucleotide in aqueous buffered saline	2–8°C Do not freeze

ViewRNA™ Tissue Assay DAB Module

Reagents have a minimum shelf life of 6 months from date of receipt when stored as recommended. See kit labels for expiration dates and the Package Insert for component quantities.

Table 4 ViewRNA™ Tissue Assay DAB Module [Cat. No. QVT0430 (24 assays)]

Component	Description	Storage
DAB Solution	DAB precipitating substrate component for the detection of horseradish peroxidase activity	2–8°C
DAB Reaction Buffer	Buffer required for preparation of DAB Substrate	
Peroxidase Quencher	Aqueous solution intended for the inactivation of endogenous peroxidase present in tissue	
Label Probe Type 4 - HRP	Horseradish peroxidase-conjugated oligonucleotide in aqueous buffered saline	2–8°C Do not freeze

ViewRNA™ Tissue Assay Products

The ViewRNA™ Tissue Core Kit and ViewRNA™ Modules can be purchased separately to create a ViewRNA™ assay for detection of up to two RNA targets. ViewRNA™ 1-Plex and 2-Plex Kits containing the ViewRNA™ Tissue Core Kit and different combinations of the ViewRNA™ Modules are also available.

Product	Contents	Cat. No.	Unit size
ViewRNA™ Tissue Core Kit	• ViewRNA™ Tissue Core Reagents (Cat. No. QVT0400A) • ViewRNA™ Tissue Wash Buffers (Cat. No. QVT0400B)	QVT0400	24 assays
ViewRNA™ Tissue Assay Fast Red Module	ViewRNA™ Tissue Assay Fast Red Module	QVT0410	24 assays
ViewRNA™ Tissue Assay Fast Blue Module	ViewRNA™ Tissue Assay Fast Blue Module	QVT0420	24 assays
ViewRNA™ Tissue Assay DAB Module	ViewRNA™ Tissue Assay DAB Module	QVT0430	24 assays
ViewRNA™ Fast Red 1-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay Fast Red Module (Cat. No. QVT0410)	QVT0410C	24 assays
		QVT4410C	96 assays
ViewRNA™ Fast Blue 1-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay Fast Blue Module (Cat. No. QVT0420)	QVT0420C	24 assays
		QVT4420C	96 assays
ViewRNA™ DAB 1-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay DAB Module (Cat. No. QVT0430)	QVT0430C	24 assays
		QVT4430C	96 assays
ViewRNA™ Red-Blue 2-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay Fast Red Module (Cat. No. QVT0410) • ViewRNA™ Tissue Assay Fast Blue Module (Cat. No. QVT0420)	QVT0412C	24 assays
		QVT4412C	96 assays
ViewRNA™ Red-Brown 2-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay Fast Red Module (Cat. No. QVT0410) • ViewRNA™ Tissue Assay DAB Module (Cat. No. QVT0430)	QVT0422C	24 assays
		QVT4422C	96 assays
ViewRNA™ Blue-Brown 2-Plex Kit	• ViewRNA™ Tissue Core Kit (Cat. No. QVT0400) • ViewRNA™ Tissue Assay Fast Blue Module (Cat. No. QVT0420) • ViewRNA™ Tissue Assay DAB Module (Cat. No. QVT0430)	QVT0433C	24 assays
		QVT4433C	96 assays

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier. Catalog numbers that appear as links open the web pages for those products.

Item	Source
Instruments and equipment	
One 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) and 07J68-001 (ThermoBrite™ system) • Thermo Fisher systems: QS0720 (0.7 cu. ft.) QS0704 (1.4 cu. ft.) QS0700 (3 cu. ft.) QS0701 (4.9 cu. ft.) • MLS
Dry incubator or oven capable of maintaining 60°C, for baking slides	• Thermo Fisher systems: – QS0720 (0.7 cu. ft.) – QS0704 (1.4 cu. ft.) – QS0700 (3 cu. ft.) – QS0701 (4.9 cu. ft.) • MLS
Electron Microscopy Sciences Immuno Stain Moisture Chamber Black, or equivalent (for staining)	NC0370987
ViewRNA™ Temperature Validation Kit	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
Vortex mixer	MLS
Microplate shaker (optional, for washing steps)	MLS
Microscope and imaging equipment	See “Guidelines for microscopy and imaging” on page 42
Tissue Tek Staining Dish (clear color), 3	StatLab LWS20WH

(continued)

Item	Source
Tissue Tek Clearing Agent Dish (green color), 2	StatLab LWS20GR
StatLab TISSUE-TEK VERT 24 SLIDE RACK	Fisher Scientific™ NC9837976
1000-mL glass beaker	MLS
Fine Science Tools Dumont #3 Forceps, 12cm	Fisher Scientific™ NC9341131
Pipettes – P20, P200, P1000	MLS
ReadyProbes™ Hydrophobic Barrier Pap Pen	R3777
Consumables	
Fisherbrand™ Cover Glasses: Rectangles (24 × 55 mm)	Fisher Scientific™ 12-544-18P
Aluminum foil	MLS
Mounting media, one of the following	
Histomount Mounting Solution	008030
ProLong™ Glass Antifade Mountant with NucBlue™ Stain (Hoechst 33342)	P36981
SlowFade™ Glass Antifade Mountant	S36917
Epredia™ Cytoseal™ 60 Mountant	8310-4
Reagents	
Double-distilled water (ddH_20)	MLS
100% ethanol (200 proof)	MLS
PBS (10X), pH 7.4, RNase-free	AM9624
Histo-Clear or xylene	50-329-51 (National Diagnostics HS-200) or equivalent
37% formaldehyde	410731000, EMD Millipore™ FX0410-1, or equivalent

Table 5 (*Optional*) Materials for Gill's counterstaining (for bright field microscopy)

Item	Source
Gill's Hematoxylin No. 1	50-261-10 or equivalent
27-30% ammonium hydroxide	VWR™ JT-9726-5 or equivalent

Table 6 (*Optional*) Materials for DAPI counterstaining (for fluorescence detection)

Item	Source
DAPI, FluoroPure™ grade (optional, for fluorescence detection)	D21490
NucBlue™ Fixed Cell ReadyProbes™ Reagent	R37606

Workflow

The ViewRNA™ Tissue Assay can be run as a 1-plex assay in a single long day or broken up over two days for added flexibility. Two days is a better workflow for a 2-plex assay. The procedure includes two parts:

- Part 1—"Prepare sample and hybridize target probe" on page 17 (optional stopping point at the end of Part 1 procedure)
- Part 2—"Amplify and detect signal" on page 23 (performed on day 2 if using the optional Part 1 stopping point)

IMPORTANT! If performing multiplex staining, the optimal order is the DAB Module (Type 4 - HRP) followed by the Fast Blue Module (Type 6 - AP) followed by the Fast Red Module (Type 1 - AP).

① Part 1: Prepare the sample and hybridize the target probe

2 hr:55m – 4 h:10 m

Prepare tissue sections

1. Bake slides (FFPE only) and prepare reagents (65 minutes; page 18)
   - "Prepare solutions and equipment for target probe hybridization" on page 17
   - For 1-day protocol: "Prepare the reagents for amplification and detection (30–60 minutes)" on page 19
2. Deparaffinize the tissue sections (FFPE only, 30 minutes; page 19)
3. Draw a hydrophobic barrier (10–20 minutes; page 20)
4. Perform heat pretreatment (FFPE only, 10–25 minutes; page 21)
5. Perform protease digestion and fixation (30–50 minutes; page 22)

2 h:18 m

Perform target probe set hybridization, then wash slides

1. Perform target probe set hybridization (2 hours and 10 minutes; page 22)
2. Wash slides (8 minutes; page 23)

- (*Optional*) Store slides
- Proceed to "Part 2: Amplify and detect signal" ② on page 13

② Part 2: Amplify and detect signal

1 h:6 m – 2 h:11 m

Perform preamplifier then amplifier hybridizations

1. For 2-day protocol: "Prepare the reagents for amplification and detection (30–60 minutes)" on page 19
2. For 2-day protocol: wash stored slides (5 minutes; page 24)
3. Perform preamplifier hybridization (38 minutes; page 24)
4. Perform amplifier hybridization (28 minutes; page 24)

Proceed to amplify and detect signal

- For 1-plex assays, proceed to:
  - "DAB Module: Perform Label Probe Type 4 - HRP hybridization, then stain" ③ on page 14,
  - "Fast Blue Module: Perform Label Probe Type 6 - AP hybridization, then stain" ④ on page 14, OR
  - "Fast Red Module: Perform Label Probe Type 1 - AP hybridization, then stain" ⑤ on page 15
- For 2-plex assays with:
  - DAB/Fast Blue, proceed to ③ on page 14 followed by ④ on page 14
  - DAB/Fast Red, proceed to ③ on page 14 followed by ⑤ on page 15
  - Fast Blue/Fast Red, proceed to ④ on page 14 followed by ⑤ on page 15

③ DAB Module: Perform Label Probe Type 4 - HRP hybridization, then stain

42 – 52 m

Perform Label Probe Type 4 - HRP hybridization

1. Hybridize with Label Probe Type 4 - HRP (32 minutes; page 25)
2. Stain with DAB (10–20 minutes; page 25)

Proceed to the appropriate section, according to the assay type

- For 2-plex assays, proceed to:
  - "Fast Blue Module: Perform Label Probe Type 6 - AP hybridization, then stain" ④ on page 14, OR
  - "Fast Red Module: Perform Label Probe Type 1 - AP hybridization, then stain" ⑤ on page 15
- For 1-plex assays, proceed to "Counterstain and mount slides for imaging" ⑥ on page 15

④ Fast Blue Module: Perform Label Probe Type 6 - AP hybridization, then stain

1 h:19 m

Perform Label Probe Type 6 - AP hybridization

1. Hybridize with Label Probe Type 6 - AP (32 minutes; page 26)
2. Stain with Fast Blue (47 minutes; page 27)

35 m

Proceed to the appropriate section, according to the assay type

- For 2-plex assays with Fast Blue/Fast Red
  a. Quench Label Probe Type 6 - AP (35 minutes; page 27)
  b. Proceed to "Fast Red Module: Perform Label Probe Type 1 - AP hybridization, then stain" ⑤ on page 15
- For 1-plex assays or 2-plex assays with DAB/Fast Blue, proceed to "Counterstain and mount slides for imaging" ⑥ on page 15

⑤ Fast Red Module: Perform Label Probe Type 1 - AP hybridization, then stain

1 h:47 m

Perform Label Probe Type 1 - AP hybridization

1. Hybridize with Label Probe Type 1 - AP (32 minutes; page 28)
2. Stain with Fast Red (75 minutes; page 28)
3. Proceed to "Counterstain and mount slides for imaging" ⑥ on page 15

⑥ Counterstain and mount slides for imaging

25 m

(*Optional*) Counterstain

- Use colorimetric counterstains for brightfield microscopy (page 29)
- Use DAPI for fluorescent imaging (page 29)

Mount slides for imaging (page 30)

2 Methods

Before you begin

- Read and familiarize yourself with the guidelines described in Appendix B, “Guidelines for experimental design”.
  - “Procedural guidelines” on page 16
  - “Controls and replicates” on page 39
  - “Probe set considerations” on page 40
  - “Assigning colors to target mRNA in 1- vs. 2-plex assays” on page 40
  - “Guidelines for microscopy and imaging” on page 42
- If needed, consider optimizing sample pretreatment (see Appendix C, “Sample pretreatment optimization procedures”).
- **IMPORTANT!** If performing multiplex staining, the optimal order is the DAB Module (Type 4 - HRP) followed by the Fast Blue Module (Type 6 - AP) followed by the Fast Red Module (Type 1 - AP).

Procedural 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 of kits.
- 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, see “About pretreatment optimization” on page 44.
- Throughout the procedure, dedicate the Tissue Tek staining dishes as follows:
  - Clear staining dish for formaldehyde.
  - 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.
  - Care should be taken to not allow PBS to contaminate the alkaline phosphatase colorimetric reaction because it can decrease sensitivity of detection.

- 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.
- 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 “Controls and replicates” on page 39).

Prepare sample and hybridize target probe

Before you begin

Prepare solutions and equipment for target probe hybridization

Note: For FFPE tissue sections, these steps can be performed while the slides bake (see “Bake slides (65 minutes) [FFPE only]” on page 18).

Verify that the hybridization system is set to 40±1°C and that it is appropriately humidified.

- Prepare 3 L of 1X PBS: add 300 mL of 10X PBS and 2.7 L of ddH_2O to a 3-L capacity container.
- Prepare 200 mL of 10% NBF (4% formaldehyde in PBS), working in fume hood: add 178 mL of 1X PBS + 22 mL of 37% formaldehyde to a 200-mL capacity container and mix well.

- Prepare 4 L of Wash Buffer:
  a. Add the components in the order listed to a 4-L capacity container, then mix well:

Reagent	Volume
ddH_2O	3 L
Wash Buffer Component 1	36 mL
Wash Buffer Component 2	20 mL

  b. Adjust the total volume to 4 L with ddH_2O.

- Prepare 500 mL of 1X Pretreatment Solution: add 5 mL of 100X Pretreatment Solution and 495 mL of ddH_2O to a 1-L glass beaker.

- Ensure the availability of:
  - 600 mL of 100% ethanol
  - 1.4 L of ddH_2O
  - 600 mL of xylene or 600 mL of Histo-Clear

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

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

- If using the optional stopping point, prepare 200 mL of Storage Buffer: add 60 mL of Wash Buffer Component 2 and 140 mL of ddH_2O to a 200-mL capacity container.

- (*Optional*) If using a microplate shaker for the washes, set the speed to 285 rpm.

  To wash slides on the microplate shaker: 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.

Bake slides (65 minutes) [FFPE only]

1. Set the dry oven or hybridization system to 60±1°C.
2. Label the slides with a pencil.
3. 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.

Prepare the reagents for amplification and detection (30–60 minutes)

- If performing both parts of the assay in 1 day, prepare these reagents for amplification and detection on day 1.
- Otherwise, prepare them before washing the stored slides (2-day protocol).
- Pre-warm the following reagents to 40°C.
  - PreAmplifier Mix
  - Amplifier Mix
  - Label Probe Diluent
- Briefly centrifuge the following reagents, then place on ice until use.

For DAB Module	For Fast Red Module	For Fast Blue Module
Label Probe Type 4 - HRP	Label Probe Type 1 - AP	Label Probe Type 6 - AP

- Bring the following reagents to room temperature.

For DAB Module	For Fast Red Module	For Fast Blue Module
Peroxidase Quencher	Fast Red Reaction Buffer	Fast Blue Reaction Buffer
DAB Solution	Fast Red Reagent 1	Fast Blue Reagent 1
DAB Reaction Buffer	Fast Red Reagent 2	Fast Blue Reagent 2
	Fast Red Reagent 3	Fast Blue Reagent 3
	AP Enhancer Solution	AP Stop Solution

- (*Optional*) If you plan to perform a counterstain, ensure the availability of counterstain reagents.
  - For bright field microscopy:
    - For colorimetric counterstains, follow the protocol of the product.
  - For fluorescence detection: Prepare 200 mL of 3 µg/mL DAPI in 1X PBS. Store in the dark at 4°C until use.

Deparaffinize the tissue sections (30 minutes) [FFPE only]

Deparaffinize with xylene

Work in a fume hood

1. Pour 200 mL of xylene into a green clearing agent dish.
2. Transfer the rack of baked slides to the green clearing dish containing the xylene.
3. Incubate the slides at room temperature for 5 minutes. Agitate frequently by moving the rack up and down.
4. Discard the used xylene and refill with another 200 mL of fresh xylene. Incubate slides at room temperature for 5 minutes with frequent agitation.

5. Repeat step 4.

6. 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.

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

Deparaffinize with Histo-Clear

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

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

3. Bake the slide for 3 minute to melt the paraffin.

4. 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.

5. 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.

6. 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.

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

Draw hydrophobic barrier (15–20 minutes)

1. Dab the hydrophobic barrier pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.

2. To create a hydrophobic barrier:
   a. Place the slide over the template image, making sure that the tissue sections fall inside the blue rectangle.

   b. Lightly trace the thick blue rectangle 2 to 4 times with the hydrophobic barrier pen to ensure a solid seal.

   c. Allow the barrier to dry at room temperature for 15–20 minutes.

d. If using the DAB Module, apply sufficient Peroxidase Quencher to cover the tissue while the barrier is drying (without wetting the hydrophobic barrier) to quench endogenous peroxidase activity. Incubate for 15–20 minutes at room termperature, then rinse the tissue in ddH_2O before proceeding to “Perform heat pretreatment (10–25 minutes, depending on optimized time) [FFPE only]” on page 21.

While the barrier is drying, preheat 1X Pretreatment Solution as described in “Perform heat pretreatment (10–25 minutes, depending on optimized time) [FFPE only]” on page 21.

Perform heat pretreatment (10–25 minutes, depending on optimized time) [FFPE only]

For information on optimization of the heat pretreatment, see Appendix C, “Sample pretreatment optimization procedures”.

1. 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.
2. Load the slides into the vertical slide rack.
3. 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 “About pretreatment optimization” on page 44.
4. 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.
5. Repeat the wash one more time with 200 mL of fresh ddH_2O.
6. 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.

Perform protease digestion and fixation (30–50 minutes, depending on optimized time)

1. Prepare the working protease solution by diluting the Protease Solution 1:100 in prewarmed 1X PBS (for example, 4 µL of Protease Solution added to 396 µL of 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.

2. 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.

3. Place the slides face up on a flat, elevated platform (for example, Eppendorf Tubes™ 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.

4. Transfer the slides to the hybridization system and incubate at 40°C for the optimal time as determined in "About pretreatment optimization" on page 44.

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

6. 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.

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

8. 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.

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

Perform target probe set hybridization (2 hours and 10 minutes)

1. 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. See "Negative control" on page 39.

Reagent	1-plex (400 µL total volume)	2-plex (400 µL total volume)
Probe Set Diluent (prewarmed to 40°C)	390 µL	380 µL
ViewRNA™ TYPE 1/4/6 probe set (not provided)	10 µL	10 µL
ViewRNA™ TYPE 1/4/6 probe set (not provided)	—	10 µL

2. 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.

3. 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.

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

Wash slides (8 minutes)

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

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

3. 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.

- If you plan to perform the assay over the course of two days, proceed to "(Optional stopping point) Store slides".
- Otherwise, proceed to "Amplify and detect signal" on page 23 to complete the entire assay in one day.

(*Optional stopping point*) Store slides

1. 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.

2. Discard 1X Pretreatment Solution, 10% NBF, remaining protease and probe set working solutions.

3. Store the remaining 1X PBS and Wash Buffer at room temperature for use in "Amplify and detect signal".

4. If using a ThermoBrite™ System, rewet the ThermoBrite™ Humidity Strips in ddH_2O.

5. Proceed to "Amplify and detect signal" on page 23 when you are ready to continue the assay.

Amplify and detect signal

Ensure that the reagents for amplification and detection have been prepared, as described in "Prepare the reagents for amplification and detection (30–60 minutes)" on page 19.

If slides have been stored (2-day protocol only)—wash stored slides (5 minutes)

Note: Before washing the stored slides, ensure that the reagents for amplification and detection have been prepared, as described in "Prepare the reagents for amplification and detection (30–60 minutes)" on page 19.

1. Remove the slides from Storage Buffer. Transfer the slide rack to a clear staining dish containing Wash Buffer, and wash for 2 minutes with frequent agitation.
2. Decant Wash Buffer, refill with 200 mL of fresh Wash Buffer, and wash for 2 minutes with frequent agitation. Repeat this step once more for a total of 3 washes.
3. Proceed to "Perform preamplifier hybridization (38 minutes)" on page 24.

Perform preamplifier hybridization (38 minutes)

1. Swirl the PreAmplifier Mix bottle briefly to mix the solution.
2. 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, then immediately add 400 µL of PreAmplifier Mix to each tissue section.
3. Transfer slides to the hybridization system and incubate at 40°C for 25 min.
4. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
5. After incubation, decant the PreAmplifier Mix from the slides and insert them into the slide rack.
6. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 2 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.
7. Proceed to "Perform amplifier hybridization (28 minutes)".

Perform amplifier hybridization (28 minutes)

1. Swirl the Amplifier Mix bottle briefly to mix the solution.
2. 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.
3. Transfer slides to the hybridization system and incubate at 40°C for 15 min.
4. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

5. After incubation, decant the Amplifier Mix from the slides and insert them into the slide rack.

6. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 2 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.

For DAB Module: Perform Label Probe Type 4 - HRP hybridization (32 minutes)

1. Briefly centrifuge Label Probe Type 4 - HRP before using.

2. Prepare the Working Label Probe Type 4 - HRP Solution using the following table as a guide. Dilute Label Probe Type 4 - HRP 1:1000 in prewarmed 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 Type 4 - HRP Solution	Volume (400 µL total volume per slide)
Label Probe Diluent (pre-warmed to 40°C)	399.6 µL
Label Probe Type 4 - HRP	0.4 µL

3. 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 Type 4 - HRP Solution to each tissue section.

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

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

6. After incubation, decant the Working Label Probe Type 4 - HRP Solution from the slides and insert them into the slide rack.

7. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.

8. Proceed to "Apply DAB staining solution (10–20 minutes)" on page 25.

Apply DAB staining solution (10–20 minutes)

1. Prepare 500 µL of DAB staining solution per slide (each slide requires 400 µL). For 12 slides prepare 5 mL of staining solution, adjusting the volume for more or fewer slides.
   a. Add 500 µL of DAB Buffer and 15 µL of DAB Solution to a 1.5-mL microcentrifuge tube and vortex.

      Note: For 12 slides, prepare the DAB staining solution in a 15-mL conical tube and vortex.

   b. Protect from light until use by wrapping the tube in aluminum foil.

2. 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 DAB staining solution.

3. Incubate at room temperature for 5–15 minutes.

   Note: Monitoring the DAB color development by brightfield microscopy can help optimize development of the signal.

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

5. After incubation, decant the DAB staining solution from the slides and insert them into the slide rack.

6. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.

7. Proceed to the appropriate section, according to the assay type:

   - For 2-plex assays, proceed to "For Fast Blue Module: Perform Label Probe Type 6 - AP hybridization (32 minutes)" on page 26 or "For Fast Red Module: Perform Label Probe Type 1 - AP hybridization (32 minutes)" on page 28.
   - For 1-plex assays, proceed to "(Optional) Perform counterstain (25 minutes)" on page 29 or "Mount slides for imaging" on page 30.

For Fast Blue Module: Perform Label Probe Type 6 - AP hybridization (32 minutes)

1. Briefly centrifuge Label Probe Type 6 - AP before using.

2. Prepare the Working Label Probe Type 6 - AP Solution using the following table as a guide. Dilute Label Probe Type 6 - AP 1:1000 in prewarmed 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 Type 6 - AP Solution	Volume (400 µL total volume per slide)
Label Probe Diluent (pre-warmed to 40°C)	399.6 µL
Label Probe Type 6 - AP	0.4 µL

3. 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 Type 6 - AP Solution to each tissue section.

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

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

6. After incubation, decant the Working Label Probe Type 6 - AP Solution from the slides and insert them into the slide rack.

7. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.

8. Proceed to "Apply Fast Blue staining solution (47 minutes)".

Apply Fast Blue staining solution (47 minutes)

1. Prepare 400 µL of Fast Blue staining solution for each slide. For 12 slides prepare 5 mL of staining solution, adjusting the volume for more or fewer slides.
   a. Add 5 mL of Fast Blue Reaction Buffer and 105 µL of Fast Blue Reagent 1 to a 15-mL conical tube and vortex.
   b. Add 105 µL of Fast Blue Reagent 2 and vortex.
   c. Add 105 µL Fast Blue Reagent 3 and briefly vortex.
   d. Protect from light until use by wrapping the tube in aluminum foil.
2. 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 staining solution.
3. Transfer slides to a moisture chamber, then incubate in the dark at room temperature for 30 minutes.
4. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.
5. After incubation, decant the Fast Blue staining solution from the slides and insert them into the slide rack.
6. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.
7. Proceed to the appropriate section, according to the assay type:
   - For 2-plex assays with Fast Blue/Fast Red, proceed to “Quench Label Probe Type 6 - AP (35 minutes)” on page 27.
   - For 1-plex assays or 2-plex assays with DAB/Fast Blue, proceed to “(Optional) Perform counterstain (25 minutes)” on page 29 or “Mount slides for imaging” on page 30.

Quench Label Probe Type 6 - AP (35 minutes)

1. 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.
2. Place the slides face up on a flat, elevated platform, then immediately add 400 µL of AP Stop Solution. Transfer to the moisture chamber, then incubate in the dark at room temperature for 30 minutes.
3. Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.
4. After incubation, decant the AP Stop Solution from the slides and insert them into the slide rack.
5. Wash the slides twice, each time in 200 mL of fresh 1X PBS at room temperature for 1 minute with frequent agitation.

6. 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 minute.

7. Proceed to "For Fast Red Module: Perform Label Probe Type 1 - AP hybridization (32 minutes)".

For Fast Red Module: Perform Label Probe Type 1 - AP hybridization (32 minutes)

1. Briefly centrifuge Label Probe Type 1 - AP before using.

2. Prepare the Working Label Probe Type 1 - AP Solution using the following table as a guide. Dilute Label Probe Type 1 - AP 1:1000 in prewarmed 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 Type 1 - AP Solution	Volume (400 µL total volume per slide)
Label Probe Diluent (pre-warmed to 40°C)	399.6 µL
Label Probe Type 1 - AP	0.4 µL

3. 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 Type 1 - AP Solution to each tissue section.

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

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

6. After incubation, decant the Working Label Probe Type 1 - AP Solution from the slides and insert them into the slide rack.

7. Wash the slides with 200 mL of fresh Wash Buffer at room temperature for 3 minutes with constant and vigorous agitation. Repeat this step two more times for a total of 3 washes.

8. Proceed to "Apply Fast Red staining solution (75 minutes)".

Apply Fast Red staining solution (75 minutes)

1. 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.

2. 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 staining solution.

3. Prepare 400 µL of Fast Red staining solution for each slide. For 12 slides prepare 5 mL of staining solution, adjusting the volume for more or fewer slides.
   a. Add 5 mL of Fast Red Reaction Buffer and 80 µL of Fast Red Reagent 1 to a 15-mL conical tube and vortex.

   b. Add 80 µL of Fast Red Reagent 2 and vortex.

c. Add 80 µL of Fast Red Reagent 3 and briefly vortex.

d. Protect from light until use by wrapping the tube in aluminum foil.

4. 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 staining solution onto each tissue section.

5. Transfer slides to the moisture chamber, then incubate in the dark at room temperature for 60 minutes.

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

7. After incubation, decant the Fast Red staining solution from the slides and insert them into the slide rack.

8. Rinse off the excess Fast Red staining solution from the slides by moving the slide rack up and down for 1 minute.

9. Proceed to "(Optional) Perform counterstain (25 minutes)" on page 29 or "Mount slides for imaging" on page 30.

(*Optional*) Perform counterstain (25 minutes)

Perform counterstain

1. For colorimetric counterstains, follow the protocol of the product.

2. Proceed to "Mount slides for imaging" on page 30.

Perform counterstain with DAPI

Counterstain with DAPI for fluorescent imaging.

1. Prepare 1X DAPI counterstain in one of the following ways:
   - Add 2 drops of NucBlue™ Fixed Cell ReadyProbes™ Reagent per mL of 1X PBS.
   - Prepare a 3 µg/mL solution of DAPI, FluoroPure™ grade by adding 60 µL of a 10 mg/mL solution of DAPI, FluoroPure™ grade (1/3333) to 200 mL of 1X PBS.

2. Move the slide rack to a clear staining dish containing 200 mL of 1X DAPI counterstain, or place 400 µL of DAPI counterstain on each tissue section. Incubate the slides for 1–5 minutes, then rinse them in 200 mL of fresh ddH_2O by moving the slide rack up and down for 1 minute.

3. 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.

4. Place the slides face up on a paper towel, then air dry in the dark.

5. Ensure slide sections are completely dry before mounting (~20 minutes).

6. Proceed to "Mount slides for imaging".

Mount slides for imaging

The ViewRNA™ Tissue Assay with with DAB, Fast Blue, and Fast Red substrates is compatible with most aqueous mountants. For fluorescent imaging, the mountants containing antifades are highly recommended.

In addition to aqueous mountants, ViewRNA™ Tissue Assay DAB Module staining is compatible with xylene/ethanol dehydration and organic mountants. Avoid mountants containing alcohol when staining with the ViewRNA™ Tissue Assay Fast Blue Module and ViewRNA™ Tissue Assay Fast Red Module.

Follow the manufacturer's instruction for mounting.

The ViewRNA™ Tissue Assay has been tested with ProLong™ Glass Antifade Mountant with NucBlue™ Stain (Hoechst 33342) and SlowFade™ Glass Antifade Mountant.

When mounted with a curing, hardening mountant, slides can be stored for years at 2–8°C.

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Troubleshooting

Observation	Possible cause	Recommended action
Weak or no signal	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, PreAmplifier Mix, Amplifier Mix, Label Probe-AP, Label Probe-HRP, and substrates in the correct order.

Observation	Possible cause	Recommended action
Weak or no signal *(continued)*	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.
	Hybridization temperature not optimal	Calibrate the hybridization system at 40°C using the ViewRNA™ Temperature Validation Kit (Cat. No. QV0523).
	Mounting solution contained alcohol	Use the recommended mounting media to mount the tissue (see "Mount slides for imaging" on page 30). When staining with the Fast Blue and Fast Red Substrate solutions, avoid any mounting solution containing alcohol and use aqueous-based mountants. DAB Substrate staining is compatible with both aqueous- and alcohol-based mountants.
	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 (Cat. No. 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.

Observation	Possible cause	Recommended action
Weak or no signal *(continued)*	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.
	DAB, Fast Red, and Fast Blue Substrate solutions not freshly prepared	Prepare DAB, Fast Red, and Fast Blue Substrate solutions immediately before use.
	Fast Red incubation time not optimal	Increase Fast Red incubation time.
	Label Probe-4 HRP treated slide washed in other buffers before DAB staining	Refrain from rinsing slide in PBS or ddH2O prior to DAB labeling.
	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 hours. • Decrease hybridization temperature from 40°C to 38°C. • Increase Fast Red incubation time to 45 minutes.
	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 hrs.
	Label Probe concentration too low	Verify that the correct concentrations were used.
		Increase the recommended concentration for Label Probe. If this is necessary, it may result in higher background.

Observation	Possible cause	Recommended action
Weak or no signal *(continued)*	Dark hematoxylin stain reduces visibility of the blue dots	Decrease or omit the hematoxylin staining. 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	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.
		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 (Cat. No. QV0523).
	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. • Keep tissue exposure to air as short as possible before adding hybridization reagents. • Ensure that the hybridization system is appropriately humidified. • Ensure that 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.
	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.

Observation	Possible cause	Recommended action
High background *(continued)*	Label Probe-AP or Label Probe-HRP concentration too high or incubation time too long	Verify that the correct concentrations were used.
		Decrease the recommended concentration for Label Probe.
		Decrease the incubation time–especially for DAB color development which is often very rapid.
	Fast Red incubation time not optimal	Decrease Fast Red incubation time.
Diffused signals	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.
	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.
	DAB, Fast Red, and Fast Blue Substrate solutions not freshly prepared	Prepare DAB, Fast Red, and Fast Blue Substrate solutions immediately before use.
	Fast Red incubation time not optimal	Decrease Fast Red incubation time.
	Slides are not dried before mounting	Ensure that the sections are completely dry (~20 minutes) before mounting.

Observation	Possible cause	Recommended action
Diffused signals *(continued)*	Mounting solution contained alcohol	Use the recommended mounting media to mount the tissue (see "Mount slides for imaging" on page 30). When staining with the Fast Blue and Fast Red Substrate solutions, avoid any mounting medium containing alcohol or any cover slipping method requiring alcohol dehydration. DAB Substrate staining is compatible with xylene/ethanol dehydration and organic mountants.
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.
Endogenous peroxidase activity	Endogenous peroxidase activity	Verify peroxidase activity by incubating protease-treated sample with DAB Substrate. If endogenous HRP activity is present, diffused signals (which can be weak or strong) will appear especially in red blood cells (RBCs). Inactivate endogenous peroxidase with the Peroxidase Quencher (see substep 2d on page 21) or with 1–3% H_2O_2 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, 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.

Observation	Possible cause	Recommended action
Tissue detachment from slide *(continued)*	Protease treatment is too long or at too high a concentration.	Reduce protease 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 setup" on page 45.
	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.
High non-specific binding on glass slide	Incompatible glass slide	Use the recommended glass slides: • Leica Non-Clipped X-tra™ Slide, 1 mm White (Fisher Scientific™, Cat. No. 3800200 or 3800210) • Fisherbrand™ Superfrost™ PLUS™ Microscope Slides (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.

Observation	Possible cause	Recommended action
Hydrophobic barrier falls off	Incompatible glass slide	Use the recommended glass slides: • Leica Non-Clipped X-tra™ Slide, 1 mm White (Fisher Scientific™, Cat. No. 3800200 or 3800210) • Fisherbrand™ Superfrost™ PLUS™ Microscope Slides (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 minutes, 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 ReadyProbes™ Hydrophobic Barrier Pap Pen (Cat. No. R3777).
	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. For recommended filter set specifications for Fast Blue, see “Guidelines for microscopy and imaging” on page 42.
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	Follow the procedure described in “Quench Label Probe Type 6 - AP (35 minutes)” on page 27.
		Be sure to quench LP6-AP activity with AP Stop Solution for the entire 30 minutes.

B Guidelines for experimental design

Controls and replicates

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.

Replicates

We recommend running all assays in duplicate.

Probe set considerations

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™ 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 or TYPE 4 probe set and developing the signal with DAB. The ViewRNA™ Fast Red 1-Plex Kit (Cat. No. QVT0410C) is configured for use with TYPE 1 probe sets, while the ViewRNA™ DAB 1-Plex Kit (Cat. No. QVT0430C) is configured for use with TYPE 4 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 the 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 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” on page 42, for exact filter set specifications.

Limitations of chromogenic *in situ* assay in co-localization studies

When employing the ViewRNA™ 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™ 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.

Guidelines for microscopy and imaging

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

To detect…	Staining reagent	Stain color	
		Brightfield view	Fluorescent view
RNA using TYPE 1 probe	Fast Red	Red	Red (RFP channel)
RNA using TYPE 6 probe	Fast Blue	Fast Blue	Far red (Cy5.5™/Cy7™ channel)
RNA using TYPE 4 probe	DAB	Brown	—
Nucleus	Hematoxylin/DAPI	Light blue/gray	Blue

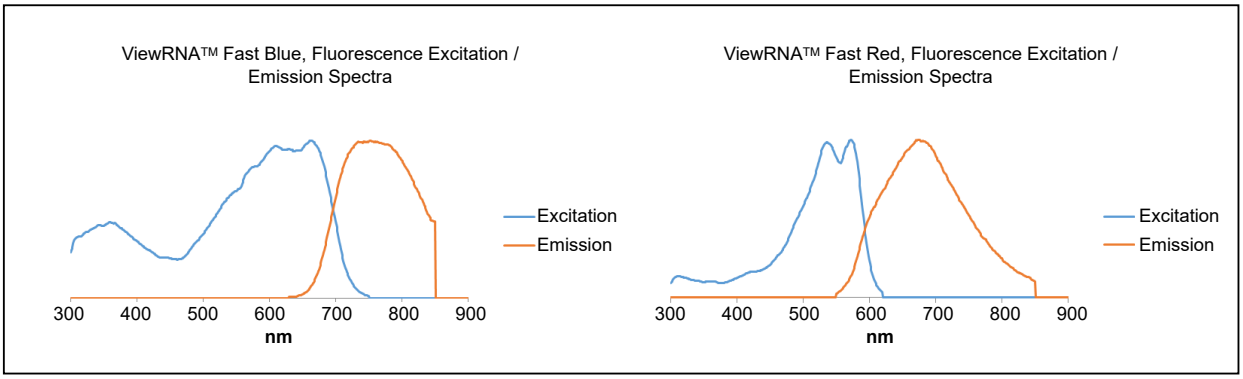


Figure 2 Fluorescence excitation/Emission spectra of ViewRNA™ Fast Blue (left), and ViewRNA™ Fast Red (right).

Viewing and digital capture options

	Brightfield viewing	Fluorescence viewing and image capture	Automated image capture (brightfield or fluorescence)
Microscope Type	Standard brightfield microscope	Microscope with camera and fluorescence options. Verify that the camera does not have infrared blocking filter.	Digital pathology scanner system
Recommended Microscope	Leica DM series Nikon™ E series Olympus™ BX series Zeiss™ Axio Lab/Scope/Imager (or equivalent)		Aperio ScanScope AT/XT/CS, use FL version for fluorescence Leica SCN400-F Olympus™ Nanozoomer RS
Required Optics	Requires 20X and 40X objectives	Requires 20 and 40X objectives Numerical aperture (NA) >0.5	Recommend scanning at 40X when expression is low
Recommended Filters	Requires neutral density filters and/or color filters for white balancing	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[1]: • Excitation: 630±20 nm • Emission: 775±25 nm • Dichroic: 750 nm For DAPI filter set: • Excitation: 387/11 nm • Emission: 447/60 nm	

[1] Recommended vendor: Semrock Cy7™-B/Alexa 750 filter modified with excitation filter FF02-28/40-25

C 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™ 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 8 on page 48 provides sample pretreatment conditions for some common tissues. If samples are limited, see Table 9 on page 49.

Table 7 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, see "Procedural guidelines" on page 16.

The pretreatment optimization procedure for the ViewRNA™ 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 the slides (see "Bake slides (65 minutes) [FFPE only]" on page 18).
2. Perform 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 minutes.

e. At the end of the 10 minutes, 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 minutes.

f. At the end of the 5 minutes, 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 minutes.

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.

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 when ready.

3. Perform protease digestion and fixation (30–50 minutes).

a. Prepare the Working Protease Solution using the following table 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 (for example, an Eppendorf Tubes™ 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 minutes.

f. After 19 minutes, 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 minutes.

i. After 9 minutes, 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 minutes.

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.

Proceed to “Perform target probe set hybridization (2 hours and 10 minutes)” on page 22, to continue the assay.

Sample pretreatment lookup

Table 8 shows a list of tissues that were prepared according to the guidelines outlined in this manual 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 8 Sample Pretreatment Optimization Lookup

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	—

Table 8 Sample Pretreatment Optimization Lookup *(continued)*

Species	Tissue	Optimal Conditions (minutes)		Range of Tolerance (Heat pretreatment, Protease)
		Heat pretreatment at 90-95°C	Protease at 40°C	
Human	Thyroid	10	20	—
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 8, and you have only limited slides available for the pretreatment optimization, Table 9 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™ Tissue Assay.

Table 9 Heat Treatment and Protease Incubation Times for Limited Optimization

Number of Available Slides	Heat Pretreatment Time (minutes)	Protease Time (minutes)
3	5	10
	10	10
	10	20
5	5	10
	5	20

Table 9 Heat Treatment and Protease Incubation Times for Limited Optimization *(continued)*

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

D

Evaluation of results

Assessment of pretreatment conditions

This section provides sample images obtained from the ViewRNA™ 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 10 Assessment of Pretreatment Conditions: Synpo and SPP1 Expression in Rat Kidney FFPE Tissue

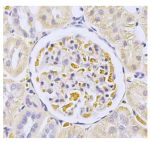
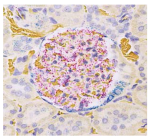
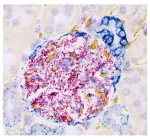
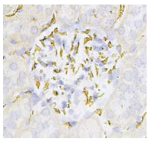
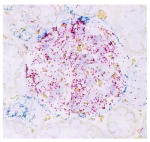
Heat Pretreatment Time (min)	Protease Digestion Time (min)	Brightfield Image	Results Interpretation
0	0		**Untreated Morphology Reference Slide; +Probes (Synpo and SPP1)** • Good morphology • Intact cellular structure • Good hematoxylin counterstaining of nuclei • Little or no signal (dots) observed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

Analysis of target expression

Each observable punctated dot represents a single RNA molecule within the cell that the ViewRNA™ 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. (See “Negative control” on page 39.) The ViewRNA™ 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 (for example, 1 dot/every 2 cells), you can trust that the detection is real. (See “Negative control” on page 39.)

E

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- 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. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
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