invitrogen

ViewRNA™ Tissue Fluorescence Assay

USER GUIDE

Catalog Numbers QVT0700, QVT4700, QVT0800, QVT4800, QVT0600C, QVT4600C, QVT0688B, QVT4688B, QVT0646B, QVT4646B, QVT0694B, QVT4694B, QVT0640B, and QVT4640B

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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

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Revision	Date	Description
A.0	15 April 2022	New document for ViewRNA™ Tissue Fluorescence Assay.

Contents

1 Product information

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

Product description

The Invitrogen™ ViewRNA™ Tissue Fluorescence Assay is designed for visualization of one to four target RNA molecules in formalin-fixed, paraffin-embedded (FFPE) or optimal cutting temperature (OCT) compound-embedded tissue sections that are prepared as described in this manual. The kit can also be used with paraformaldehyde-fixed cells.

In situ hybridization (ISH) techniques are used to visualize DNA or localize RNA molecules within cells, however, traditional methods are limited by low sensitivity and complicated probe synthesis. The ViewRNA™ Tissue Fluorescence Assay, which is based on highly specific branched DNA technology, provides robust *in situ* detection of up to four target mRNA molecules within tissue sections or cells with single-copy sensitivity.

Key features of the kit include the following:

- Suitable for formalin-fixed, paraffin-embedded (FFPE) tissue sections, OCT compound-embedded frozen tissue sections, and paraformaldehyde-fixed cells.
- Single RNA molecule sensitivity (one dot equals one RNA molecule).
- Detection of up to four target RNA molecules.
- Fluorescence detection with FITC, TRITC, Texas Red™, Deep Red/Cy™5, and/or NIR/Cy™7 channels.
- Compatible with traditional fluorescence imaging systems, including widefield and confocal systems.

Available kits and modules

Each ViewRNA™ Tissue Fluorescence Assay Core Kit is available in the sizes shown in the following table, based on the number of assays that can be performed with the kit.

Catalog numbers that appear as links open the web pages for those products.

Table 1 ViewRNA™ Tissue Fluorescence Assay Core Kit

Probe set	Cat. No.	Amount
ViewRNA™ Tissue Fluorescence 4-Plex Assay Core Kit (green, orange, deep red, near IR)		
• ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit • ViewRNA™ Tissue Alexa Fluor™ 488 (Type 4) Module • ViewRNA™ Tissue Alexa Fluor™ 546 (Type 1) Module • ViewRNA™ Tissue Alexa Fluor™ 750 (Type 10) Module	QVT0700	24 assays
	QVT4700	96 assays
ViewRNA™ Tissue Fluorescence 4-Plex Assay Core Kit (green, red, deep red, near IR)		
• ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit • ViewRNA™ Tissue Alexa Fluor™ 488 (Type 4) Module • ViewRNA™ Tissue Alexa Fluor™ 594 (Type 1) Module • ViewRNA™ Tissue Alexa Fluor™ 750 (Type 10) Module	QVT0800	24 assays
	QVT4800	96 assays
ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit		
ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor™ 647 (Type 6)	QVT0600C	24 assays
	QVT4600C	96 assays

Table 2 ViewRNA™ Tissue Alexa Fluor™ modules

Module[1]	Cat.	Amount
ViewRNA™ Tissue Alexa Fluor™ 488 (Type 4) Module	QVT0688B	24 assays
	QVT4688B	96 assays
ViewRNA™ Tissue Alexa Fluor™ 546 (Type 1) Module	QVT0646B	24 assays
	QVT4646B	96 assays
ViewRNA™ Tissue Alexa Fluor™ 594 (Type 1) Module	QVT0694B	24 assays
	QVT4694B	96 assays
ViewRNA™ Tissue Alexa Fluor™ 750 (Type 10) Module	QVT0640B	24 assays
	QVT4640B	96 assays

[1] Modules can be added to the ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor™ 647 (Type 6) for 2- to 4-plex detection.

Contents and storage

Each ViewRNA™ Tissue Fluorescence Assay Core Kit contains the following components, supplied in three boxes based on storage temperature.

- ViewRNA™ Tissue Fluorescence Assay Core Kit—Contains the reagents required for the detection of up to four mRNA targets.
- ViewRNA™ probe sets—Contain oligonucleotides specific to your RNA target of interest. Up to four different amplification systems (types) are used, consisting of pre-amplifier, amplifier, and label probe.

The ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor™ 647 (Type 6) includes the following components. Other kit configurations and individual reagents are also available (see "Available kits and modules" on page 6).

Table 3 ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor™ 647 (Type 6) (Cat. No. QVT0600C and QVT4600C)

Component	Description	Storage[1]
Box 1 of 3		
QVC Pre-Amplifier Mix	DNA in aqueous solution	–20°C
Amplifier Mix (Type 1, 4, 6, and 10) (25X)[2, 3]	DNA in aqueous solution	
Label Probe Mix (Type 6) (25X)[4]	DNA in aqueous solution	
100X DAPI	DAPI in aqueous solution	
Box 2 of 3		
100X Pretreatment Solution	Aqueous buffered solution	2–8°C
Protease QF	Enzyme in aqueous buffered solution	
Probe Set Diluent QT	Aqueous solution containing formamide and detergent	
Amplifier Diluent QF	Aqueous solution containing formamide and detergent	
Label Probe Diluent QF	Aqueous solution containing detergent	
Box 3 of 3		
100X Wash Buffer Component 1	Aqueous solution containing detergent	15–30°C
400X Wash Buffer Component 2	Aqueous solution	
Detergent Solution QC	0.1% Tween™ 20 in phosphate-buffered saline (PBS)	

[1] 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.

[2] For use with the ViewRNA™ Tissue Fluorescence 4-Plex Assay Core Kit (Cat. No. QVT0700, QVT4700, QVT0800, and QVT4800).

[3] ViewRNA™ target probe sets type 1, 4, and 10 are also available for separate purchase (see "Available kits and modules" on page 6).

[4] For use with the ViewRNA™ Tissue Fluorescence 1-Plex Assay Core Kit (Cat. No. QVT0600C and QVT4600C).

Table 4 ViewRNA™ Tissue Alexa Fluor™ modules

Module	Description	Storage[1]
ViewRNA™ Tissue Alexa Fluor™ 546 (Type 1) Module	Alexa Fluor™-conjugated DNA oligo in aqueous solution	–20°C
ViewRNA™ Tissue Alexa Fluor™ 594 (Type 1) Module		
ViewRNA™ Tissue Alexa Fluor™ 488 (Type 4) Module		
ViewRNA™ Tissue Alexa Fluor™ 750 (Type 10) Module		

[1] 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.

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.

Product	Cat. No.
Tissue-Tek Staining Dish (clear color), 3 dishes	NC9670850
Tissue-Tek Clearing Dish (green color), 2 dishes	NC9012500
TISSUE-TEK VERT 24 SLIDE RACK	NC9837976
Glass beaker, 1,000-mL	**MLS**
Fine Science Tools Dumont #3 Forceps, 12 cm	NC9341131
Pipettors—P20, P200, and P1000	**MLS**
Isolator, one of the following: • Hydrophobic barrier pen • Press-to-Seal™ Silicone Isolator, one well, 20 mm diameter, 0.5 mm deep	• NC9545623 • P18174
ProLong™ Glass Antifade Mountant	P36980
Fisherbrand™ Cover Glasses: Rectangles (24 × 55 mm)	12-544-18P
UltraPure™ DNase/RNase-Free Distilled Water, or equivalent double-distilled water (ddH_2O)	10977023
100% ethanol (200 proof)	**MLS**
PBS (10X), pH 7.4, RNase-free	AM9624
Xylene or National Diagnostics HISTO-CLEAR	**MLS**
37% formaldehyde	MFX04101 or equivalent

(continued)

Product	Cat. No.
Image-iT™ Fixative Solution (4% formaldehyde, methanol-free)	R37814
DAPI, FluoroPure™ grade (optional, for fluorescence detection)	D21490
ReadyProbes™ Tissue Autofluorescence Quenching Kit	R37630
Equipment	
Hybridization system: Dry incubator or oven capable of maintaining 40 ± 1°C	**MLS**
ViewRNA™ Temperature Validation Kit	QV0523
Water-proof remote probe thermometers, validated for 90–100°C	S66426
Slide-staining moisture chamber: Immuno Stain Moisture Chamber	NC0370987 or equivalent
Microscope and imaging equipment	AMF7000 or equivalent
Fume hood	**MLS**
Water bath capable of maintaining 40±1°C	**MLS**
Hotplate and hotplate stirrer	**MLS**
Table-top microtube centrifuge	**MLS**
Vortex mixer	**MLS**
Microplate shaker (optional, for washing steps)	**MLS**

2 Methods

Overview

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

- Part 1: Prepare samples, then hybridize the target probes (optional stopping point at end of procedure)
  - "Prepare the samples" on page 16
  - "Hybridize the target probe set (2.5 hours)" on page 21
- Part 2: Amplify and detect the signal (see "Amplify and detect the signal" on page 22)

Procedural guidelines

- Perform all procedural steps in a well-ventilated area at room temperature (RT) unless otherwise noted.
- Discard all reagents in accordance with local, state, and federal laws.
- 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 and temperatures, as variations can negatively affect the assay signal and background.
  - Double-check all reagent calculations, as correct reagent volumes and concentrations are critical.
- Do not mix and match kit components from different lots.
- 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 of 20 mm × 30 mm. Recommended tissue thickness is 5–10 µm.
- Determine the optimized conditions (heat pretreatment and protease digestion time) for your sample type before starting the procedure. If you do not know these optimized conditions, see Appendix B, "Sample pretreatment optimization procedures".
- Prepare all reagents and solutions with RNAase-free water, such as UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977023), unless otherwise indicated.

- 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.
- Calibrate the temperature of the hybridization system to 40°C using the ViewRNA™ Temperature Validation Kit.
- Ensure that the hybridization system is appropriately humidified.
- If you are 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 a slide-staining moisture chamber (Cat. No. NC0370987) to transfer slides to the incubator.
  - Incubate slides in the slide-staining moisture chamber with the lid closed to maintain moisture and prevent the slides from drying out.
  - 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.
- For best results, when using a dry oven that is set at 40 ± 1°C and slides that are in an enclosed plastic chamber (such as the Immuno Stain Moisture Chamber, Cat. No. NC0370987), we recommend adding extra water/tissue paper to the sides of the chamber. This system maintains moisture in the enclosed chamber and prevents the slides from drying out.
- (*Optional*) If you are using a microplate shaker for the wash steps, set the speed to 285 rpm. Place a slide rack in a clear staining dish containing the appropriate reagent and insert the slides into the rack. Manually lift the rack up and down 10 times. Put the lid on the staining dish and place it on a microplate shaker platform that is equipped with a non-skid pad. Shake for the recommended amount of time.
- Incorporate controls, both positive and negative, so that results are unambiguous and can be interpreted (see “Experiment design guidelines” on page 12).
- Use good washing techniques for all wash steps. Frequently, washing is performed too gently. Adequate washing is important for consistent low backgrounds.
- DO NOT let tissues dry out where indicated in the procedure.

Guidelines for assigning fluorescence channels (types) to probe sets

The ViewRNA™ Tissue Assay allows *in situ* detection of up to four mRNA targets simultaneously when using all four fluorescence channels. Before staining the tissue, view the tissue under a microscope and assign the most non-abundant target to the fluorescence channel with the least background. Order the probe sets with this selection in mind.

Probe set type	Fluorophore	Fluorescence channel	Ex/Em maxima (nm)	Considerations
6	Alexa Fluor™ 647	Deep Red/Cy™5	650/665	Most sensitive with the least tissue autofluorescence expected. Assign to non-abundant target.
1	Alexa Fluor™ 594[1]	Texas Red™	590/617	Excellent sensitivity with the least tissue autofluorescence expected. Assign to non-abundant target.
	Alexa Fluor™ 546[1]	TRITC/RFP	556/573	Good sensitivity with some tissue autofluorescence expected. Assign to non-abundant target.
4	Alexa Fluor™ 488	FITC/GFP	496/519	Okay sensitivity with the most tissue autofluorescence expected. Assign to negative or positive control, or high-abundance target.
10	Alexa Fluor™ 750	NIR/Cy™7	749/775	Okay sensitivity with the least tissue autofluorescence expected. Assign to negative or positive control, or high-abundance target.

[1] Alexa Fluor™594 or Alexa Fluor™ 546 can be used, depending on the microscope setting.

Experiment design guidelines

Assay controls

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

Negative control

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

The negative control can be one of the following:

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

Table 5 Example: Negative control target probes

Probe No.	Type	Target	Species	Detection
VF6-10407-VC	6	dapB	Bacteria	Alexa Fluor™ 647
VF1-11712-VC	1			Alexa Fluor™ 546 or Alexa Fluor™ 594
VF4-10408-VC	4			Alexa Fluor™ 488
VF10-10409-VC	10			Alexa Fluor™ 750

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, then dilute the panel of probe sets 1:40 to create a working probe set solution for use as a positive control.

Table 6 Example: Positive control target probes

Probe No.	Type	Target	Species	Detection
VA6-10337-VC	6	GAPDH	Human	Alexa Fluor™ 647
VA1-10119-VC	1			Alexa Fluor™ 546 or Alexa Fluor™ 594
VA4-10641-VC	4			Alexa Fluor™ 488
VA10-10458-VC	10			Alexa Fluor™ 750

Table 6 Example: Positive control target probes *(continued)*

Probe No.	Type	Target	Species	Detection
VA6-10506-VC	6	ACTB	Human	Alexa Fluor™ 647
VA1-10351-VC	1			Alexa Fluor™ 546 or Alexa Fluor™ 594
VA4-10293-VC	4			Alexa Fluor™ 488
VA10-10504-VC	10			Alexa Fluor™ 750

Replicates

We recommend running all assays in duplicate.

Probe set considerations

The typical design for a ViewRNA™ probe set consists of 40 unlabeled oligos, or 20 pairs of oligos per RNA target, and spans approximately 1,000 bases of the target transcript to achieve maximal sensitivity. Each oligo is ~20 bp long. 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 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.

Before you begin

Prepare buffers and reagents

Prepare sufficient volume of the following reagents for the size of your washing/incubator chambers and the number of replicates.

- Prepare 1X PBS (RNase-free)—Dilute PBS (10X), pH 7.4, RNase-free (Cat. No. AM9624) 1:10 with ddH_2O.
- Prepare 1 L of Wash Buffer—Combine the following reagents in the order indicated to avoid precipitation.

Note: The volume of Wash Buffer can be adjusted depending on experimental requirements.

Component	Volume
ddH_2O	900 mL
100X Wash Buffer Component 1	10 mL
400X Wash Buffer Component 2	2.5 mL
ddH_2O	To 1 L

- Prepare 100 mL of storage buffer by combining the following reagents.

Note: The volume of storage buffer can be adjusted depending on experimental requirements.

Component	Volume
400X Wash Buffer Component 2	30 mL
ddH_2O	70 mL

- Thaw the following reagents at room temperature.
  - Probe set{s)
  - QVC Pre-Amplifier Mix
  - Amplifier Mix
  - Label Probe Mix

  After the probe set(s) have thawed, keep on ice until use.

- Pre-warm the following reagents to 40 ± 1°C.
  - Amplifier Diluent QF
  - Label Probe Diluent QF
  - 1X PBS (RNase-free)
  - Probe Set Diluent QT

Prepare equipment

- Ensure that the hybridization system is set to 40 ± 1°C and that it is appropriately humidified.
- Prepare the slide-staining moisture chamber (Cat. No. NC0370987 or equivalent)—Add moist tissue to the sides of the chamber to prevent slides from drying out.

  Use this chamber to transfer slides to the incubator and during incubation steps.

Prepare the samples

Prepare FFPE tissue

Before you begin

Prepare the following solutions.

- 1X Pretreatment Solution (500 mL)—Dilute the 100X Pretreatment Solution 1:100 with ddH_2O.
- Deparaffinization solutions—Prepare 70%, 85%, and 90% ethanol in ddH_2O.

Deparaffinize tissue sections with xylene (~35 minutes)

Work in a fume hood.

Perform the following washes (in the order indicated) at room temperature with frequent agitation.

Note: HISTO-CLEAR can also be used to deparaffinize samples. Follow the manufacturer's instructions.

Step	Wash solution	Time
1	Xylene	1 × 10 minutes or 3 × 5 minutes
2	Xylene:100% ethanol (1:1)	2 × 5 minutes
3	100% ethanol	2 × 5 minutes
4	95% ethanol	1 × 5 minutes
5	85% ethanol	1 × 5 minutes
6	70% ethanol	1 × 5 minutes
7	1X PBS	3 × 5 minutes

Perform heat pretreatment (~45 minutes)

1. Tightly cover the beaker containing the 500 mL of 1X Pretreatment Solution with aluminum foil, place it on a hot plate, then heat the solution to 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, then incubate at 90–95°C for the appropriate amount of time as determined in Appendix B, "Sample pretreatment optimization procedures".

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

5. Repeat the wash with 200 mL of fresh ddH_2O.

6. Transfer the slide rack to a clear staining dish containing 1X PBS, then proceed to apply a hydrophobic barrier (see "Apply a hydrophobic or silicone barrier to the sample (~30 minutes)" on page 18) .

IMPORTANT! Do not let the tissue sections dry out from this point forward.

STOPPING POINT After heat pretreatment, the samples can be stored overnight in 1X PBS at room temperature.

Prepare OCT compound-embedded tissue (16–19 hours)

Prepare a 4% paraformaldehyde (methanol free) or formaldehyde solution in 1X PBS, then chill on ice before use.

1. Day 1—Add the frozen tissues to the pre-chilled 4% paraformaldehyde (methanol free) or formaldehyde solution, then fix overnight (16-19 hours) at 4°C.

2. Day 2—Wash the slides with 1X PBS for 1 minute.

3. Proceed to apply a hydrophobic barrier (see "Apply a hydrophobic or silicone barrier to the sample (~30 minutes)" on page 18) .

IMPORTANT! Do not let the tissue sections dry out from this point forward.

STOPPING POINT After fixation, the samples can be stored overnight in 1X PBS at room temperature.

Prepare adherent cells grown on a coverslip in a 24-well plate

Follow this procedure to prepare adherent cells grown on a coverslip in a 24-well cell-culture plate. For optimal adhesion, we recommend growing cells on poly-L-lysine coated coverslips.

1. Prepare a 4% paraformaldehyde (methanol free) solution in 1X PBS.

2. Wash the cells with 1X PBS, then incubate with 4% paraformaldehyde (methanol free) solution for 30 minutes at room temperature.

3. Wash the cells with 1X PBS.

4. Incubate the cells with Detergent Solution QC for 5 minutes at room temperature.

5. Wash the cells two times with 1X PBS.

6. Proceed to perform the protease digestion (see “Perform protease digestion (~30 minutes)” on page 19).

Apply a hydrophobic or silicone barrier to the sample (~30 minutes)

1. To keep the reagents in contact with the sample, use one of the following methods.

Barrier method	Action
Hydrophobic barrier pen	1. Dab the hydrophobic barrier pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution. 2. Place the slide over the template image, making sure that the tissue sections fall inside the blue rectangle. 3. Lightly trace the thick blue rectangle 2 to 4 times with the hydrophobic barrier pen. 4. Allow the barrier to dry at room temperature for 10-20 minutes, then retrace to ensure a complete seal. 
(Recommended) Press-to-Seal™ Silicone Isolator, one well, 20 mm diameter, 0.5 mm deep	Position a silicone isolator on the surface of the slide, then press to seal. For adhesive silicone isolators, peel off the liner using forceps, then place the gasket, adhesive side down, on the slide. Press to seal.

2. Proceed to perform the protease digestion (next section).

Perform protease digestion (~30 minutes)

Ensure that 1X PBS is pre-warmed to 40°C before starting this procedure.

1. Prepare the working protease solution—Dilute Protease QF 1:100 in prewarmed 1X PBS (e.g. 4µL Protease QF added to 396 µL 1X PBS prewarmed to 40°C), then briefly vortex to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.
2. Remove each slide from the 1X PBS, then flick to remove excess buffer. Do not over-dry.
3. Place the slides face-up on a flat, elevated platform (e.g., Eppendorf Tubes™ rack for easier handling), then immediately add 400 µL of the working protease solution to 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. Place the slides in a slide-staining moisture chamber, then incubate in a hybridization system set at 40 ± 1°C for the appropriate amount of time according to the tissue type.

   IMPORTANT! For best results, add extra water and tissue paper to the sides of the moisture chamber to prevent the slides from drying out.

Tissue	Time
Breast	15 minutes
Lung	20 minutes
Pancreas	10 minutes
Brain	10 minutes

[1] For a complete list of tissues and optimal protease digestion times, see Appendix B, “Sample pretreatment optimization procedures”.

5. Remove the protease solution, then thoroughly wash the slides in 1X PBS for 1 minute, shaking up and down.
6. Repeat the wash three more times with fresh 1X PBS.
7. Proceed to fix the slides (see “Fix the slides” on page 20).

(Optional) Prepare samples for microRNA detection

Follow this procedure to prepare samples for microRNA detection. Any mRNA type/channel can be replaced with a microRNA probe set.

1. Prepare 1X PrimeFlow™ microRNA Pretreatment Buffer.

   Note: Prepare fresh 1X PrimeFlow™ microRNA Pretreatment Buffer for each experiment.

   a. Combine 100 µL of PrimeFlow™ microRNA Pretreatment Concentrate (4X) with 300 µL of PrimeFlow™ microRNA Pretreatment Diluent per sample.
   For 5 samples, combine 0.5 mL of PrimeFlow™ microRNA Pretreatment Concentrate (4X) with 1.5 mL of PrimeFlow™ microRNA Pretreatment Diluent.

   b. Invert gently to mix — do not vortex. Protect from light, then store at room temperature until use.

2. Add 400 µL of 1X PrimeFlow™ microRNA Pretreatment Buffer to each sample.

3. Incubate for 15 minutes in the dark at room temperature.
4. Wash the slides 3 times with 1X PBS, 1 minute each time, with frequent agitation.
5. Proceed to fix the slides (see “Fix the slides” on page 20).

Discard any unused 1X PrimeFlow™ microRNA Pretreatment Buffer.

Fix the slides

1. Prepare a 4% paraformaldehyde (methanol free) or formaldehyde solution in 1X PBS.
2. Transfer the slides to the fixative solution, then incubate for 5 minutes at room temperature.
3. Wash the slides 3 times with 1X PBS, 1 minute each time, with frequent agitation.
4. Proceed to hybridize the target probe set (see “Hybridize the target probe set (2.5 hours)” on page 21).

Hybridize the target probe set (2.5 hours)

Ensure that Probe Set Diluent QT is pre-warmed to 40°C before starting this procedure.

1. Prepare working probe set solution—Dilute the probe set in pre-warmed Probe Set Diluent QT as indicated, then briefly vortex to mix. Prepare sufficient volume to add 400 µL per sample.

Experimental requirements	Recommended probe set dilution
Multiplex detection	1:40[1]
Low-abundance targets	1:30 or 1:20
High-abundance targets	≥1:40

[1] For component volumes, see Table 7.

Table 7 Example: Component volumes for multiplex detection

Reagent	1-Plex (400 µL/slide total volume)	2-Plex (400 µL/slide total volume)	3-Plex (400 µL/slide total volume)	4-Plex (400 µL/slide total volume)
Probe Set Diluent QT (pre-warmed to 40°C)	390 µL	380 µL	370 µL	360 µL
ViewRNA™ type 6 target probe set	10 µL	10 µL	10 µL	10 µL
ViewRNA™ type 1 target probe set	—	10 µL	10 µL	10 µL
ViewRNA™ type 4 target probe set	—	—	10 µL	10 µL
ViewRNA™ type 10 target probe set	—	—	—	10 µL

2. Remove each slide from the 1X PBS, then flick to remove excess buffer. Do not over-dry.

3. Place the slides face-up on a flat, elevated platform, then immediately add 400 µL of pre-warmed Probe Set Diluent QT to the no-probe negative control and 400 µL of the working probe set solution to each test sample.

4. Place the slides in a slide-staining moisture chamber, then incubate in the hybridization system set at 40 ± 1°C for 2 hours.

   IMPORTANT! For best results, add extra water and tissue paper to the sides of the moisture chamber to prevent the slides from drying out.

5. Wash the slides three times with fresh Wash Buffer, 2 minutes each time, with frequent agitation.

Amplify and detect the signal

Perform hybridization (1 hour)

Ensure that Amplifier Diluent QF is pre-warmed to 40°C before starting this procedure.

1. If stored, transfer the slides to Wash Buffer, then wash for 2 minutes with frequent agitation.
2. Remove the Wash Buffer, then wash two more times with fresh Wash Buffer for a total of three washes.
3. Prepare working PreAmplifier Mix solution—Briefly swirl the bottle of QVC PreAmplifier Mix to mix the solution, then dilute 1:25 in pre-warmed Amplifier Diluent QF. Prepare sufficient volume to add 400 µL per sample.
4. Remove each slide from the Wash Buffer, then flick to remove excess buffer. Do not over-dry.
5. Place the slides face-up on a flat, elevated platform, then immediately add 400 µL of the pre-warmed working PreAmplifier Mix solution to each sample.
6. Place the slides in a slide-staining moisture chamber, then incubate in a hybridization system set at 40 ± 1°C for 30 minutes.

   IMPORTANT! For best results, add extra water and tissue paper to the sides of the moisture chamber to prevent the slides from drying out.
7. Wash the slides three times with fresh Wash Buffer, 2 minutes each time, with frequent agitation.
8. Prepare working Amplifier Mix solution—Briefly swirl the bottle of Amplifier Mix to mix the solution, then dilute 1:25 in pre-warmed Amplifier Diluent QF. Prepare sufficient volume to add 400 µL per sample.
9. Remove each slide from the Wash Buffer, then flick to remove excess buffer. Do not over-dry.
10. Place the slides face-up on a flat, elevated platform, then immediately add 400 µL of the pre-warmed working Amplifier Mix solution to each sample.
11. Return the slides to the slide-staining moisture chamber, then incubate in a hybridization system set at 40 ± 1°C for 30 minutes.

    IMPORTANT! For best results, add extra water and tissue paper to the sides of the moisture chamber to prevent the slides from drying out.
12. Wash the slides three times with fresh Wash Buffer, 2 minutes each time, with frequent agitation.

Perform label probe hybridization (1-plex assay only) (1 hour)

Ensure that Label Probe Diluent QF is pre-warmed to 40°C before starting this procedure.

1. Prepare working Label Probe Mix solution—Briefly swirl the bottle of Label Probe Mix to mix the solution, then dilute 1:25 in pre-warmed Label Probe Diluent QF. Prepare sufficient volume to add 400 µL per sample.

   Note: For multiplex experiments, mix each label probe at 1:25 concentration in Label Probe Diluent QF.

Table 8 Example: Component volumes for multiplex detection

Reagent	1-Plex (400 µL/slide total volume)	2-Plex (400 µL/slide total volume)	3-Plex (400 µL/slide total volume)	4-Plex (400 µL/slide total volume)
Label Probe Diluent QF (pre-warmed to 40°C)	384 µL	368 µL	352 µL	336 µL
Label Probe Mix (Type 6) (25X)	16 µL	16 µL	16 µL	16 µL
ViewRNA™ Tissue Alexa Fluor™ 546 (Type 1) Module or ViewRNA™ Tissue Alexa Fluor™ 594 (Type 1) Module	—	16 µL	16 µL	16 µL
ViewRNA™ Tissue Alexa Fluor™ 488 (Type 4) Module	—	—	16 µL	16 µL
ViewRNA™ Tissue Alexa Fluor™ 750 (Type 10) Module	—	—	—	16 µL

2. Vortex briefly to mix. Protect from light.
3. Remove each slide from the Wash Buffer, then flick to remove excess buffer. Do not over-dry.
4. Place the slides face-up on a flat, elevated platform, then immediately add 400 µL of the pre-warmed working Label Probe Mix solution to each sample.
5. Place the slides in a slide-staining moisture chamber, then incubate in a hybridization system set at 40 ± 1°C for 30 minutes.

   IMPORTANT! For best results, add extra water and tissue paper to the sides of the moisture chamber to prevent the slides from drying out.

6. Remove the Label Probe Mix solution, then wash the slides two times with fresh Wash Buffer, 2 minutes each time, with frequent agitation.

7. Perform a final wash with Wash Buffer for 10 minutes with frequent agitation. Do not wash the slides in Wash Buffer for longer than 30 minutes.

8. Proceed to the next step according to your experimental requirements.
   - To reduce tissue autofluorescence—See "(Optional) Reduce tissue autofluorescence" on page 24.
   - To perform a counterstain—See "Perform counterstain" on page 24.

(Optional) Reduce tissue autofluorescence

1. Remove each slide from the Wash Buffer, then flick to remove excess buffer. Do not over-dry.

2. Wash the slides three times with fresh 1X PBS, 3 minutes each time, at room temperature with frequent agitation.

3. Prepare the ReadyProbes™ Tissue Autofluorescence Quenching Kit—Combine equal volumes of Components A, B, and C.

4. Add 400 µL of the prepared ReadyProbes™ solution to each sample, then incubate for 5 minutes at room temperature.

5. Wash the slides three times with fresh 1X PBS, 3 minutes each time, at room temperature with frequent agitation.

6. Proceed to perform the counterstain (see "Perform counterstain" on page 24).

Perform counterstain

1. Prepare working DAPI solution—Dilute 100X DAPI 1:100 in 1X PBS, then briefly vortex to mix. Protect from light. Prepare sufficient volume to add 400 µL per sample.

2. Aspirate the Wash Buffer, then add 400 µL of working DAPI solution to each sample.

3. Incubate for 10 minutes at room temperature.

4. Remove the working DAPI solution, then wash slides with 1X PBS.

5. Repeat the wash with fresh 1X PBS.

Mount, then image the slides

1. Mount samples on glass slides using ProLong™ Glass Antifade Mountant, then allow to cure overnight.

2. Image the samples using any fluorescence microscope, including widefield, confocal, slide-scanner, or high-content analyzer. Ensure that the appropriate filter settings are selected for each channel according to the following table.

Note: To detect single RNA puncta, we highly recommend using a numerical aperture (NA) objective, such as Olympus™ 40X Objective, X-Apo, 0.95NA/0.18WD, correction collar (Cat. No. AMEP4907), with the EVOS™ M7000 Imaging System (Cat. No. AMF7000).

Probe set type	Fluorophore	Fluorescence channel	Ex/Em maxima (nm)
6	Alexa Fluor™ 647	Deep Red/Cy™5	650/665
1	Alexa Fluor™ 594[1]	Texas Red™	590/617
	Alexa Fluor™ 546[1]	TRITC/RFP	556/573
4	Alexa Fluor™ 488	FITC/GFP	496/519
10	Alexa Fluor™ 750	NIR/Cy™7	749/775
—	DAPI	UV/Violet	358/461

[1] Alexa Fluor™594 or Alexa Fluor™ 546 can be used, depending on the microscope setting.

A Troubleshooting

Observation	Possible cause	Recommended action
Weak or no signal	The pretreatment conditions were incorrect.	Repeat the pretreatment optimization procedure to determine the optimal heat treatment and protease digestion time that will provide 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 was not optimal.	Immediately place freshly dissected tissues in a minimum of 20 volumes of fresh 4% paraformaldehyde (PFA) at room temperature for 16–24 hours.
	Tissues were over-fixed after the protease digestion.	Make sure the tissue sections are not fixed beyond the time indicated in the procedure.
	RNA in the tissue was degraded.	Verify tissue fixation: • Immediately place freshly dissected tissues in a minimum of 20 volumes of fresh 4% paraformaldehyde (PFA) for 16–24 hours at room temperature. • If fixation cannot be performed immediately, ensure 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 were applied in the wrong sequence.	Apply target probe sets, QVC PreAmplifier Mix, Amplifier Mix QT, and Label Probe Mix in the correct order.
	Gene of interest was 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.

Observation	Possible cause	Recommended action
Weak or no signal *(continued)*	Storage conditions were incorrect.	Store the components as indicated on the component label or kit boxes.
	Hybridization temperature was not optimal.	Calibrate the hybridization system at 40°C using the ViewRNA™ Temperature Validation Kit (Cat. No. QV0523).
	Tissue dried out during hybridization steps.	Do not let the sample dry out during hybridization. Use a humidified enclosed chamber during hybridization.
	Probe set hybridization temperature, time, and/or concentration were 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.
High background	Paraffin removal was incomplete.	Use fresh xylene or HISTO-CLEAR solution.
		Immediately submerge the warm slides into the HISTO-CLEAR solution after baking.
	Slides were not washed sufficiently.	Move the slide rack up and down with constant and vigorous agitation.
		Increase wash incubation time by 1 minute per wash.
	Hybridization temperature was not optimal.	Calibrate the hybridization system at 40°C using the ViewRNA™ Temperature Validation Kit (Cat. No. QV0523).
	Tissue dried out during processing/hybridization.	Do not let the sample dry out during hybridization. Use a humidified enclosed chamber during hybridization.
	Concentration of hybridization reagents was too high.	Double-check the dilution calculation for all working solutions.
	The pretreatment conditions were suboptimal.	Perform the pretreatment optimization procedure to determine the optimal heat treatment and protease digestion time.
	Autofluorescence was observed in the tissue.	Some tissues can have high levels of autofluorescence. To minimize autofluorescence, we recommend the following: • Use Alexa Fluor™ 647 and Alexa Fluor™ 594 channels if autofluorescence is observed in FITC and/or orange channels. • Use ReadyProbes™ Tissue Autofluorescence Quenching Kit (Cat. No. R37630) before mounting the slides.

B

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 Fluorescence 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.

Pretreatment optimization procedure

Before beginning the procedure, determine the optimized conditions (heat pretreatment time and protease digestion time) for your sample type. The optimal time for both heat pretreatment and protease digestion is the time in which a maximum signal is obtained with minimal background, while also retaining tissue morphology and adherence to the slide.

To determine the optimized conditions for your sample type, we recommend testing ten FFPE tissue sections from the same block with different pretreatment conditions before the target probe hybridization step (see Table 9). If needed, fewer slides can be used if tissue samples are limited.

Table 9 Pretreatment optimization setup

Protease digestion time at 40°C	Heat pretreatment time at 90-95°C			
	0 minutes	5 minutes	10 minutes	20 minutes
0 minutes	Slide 1 Morphology reference			
10 minutes		Slide 2	Slide 5	Slide 9
20 minutes		Slide 3	Slide 6 Slide 7 (no-probe control)[1]	Slide 10
40 minutes		Slide 4	Slide 8	

[1] Slide 7 serves as a no-probe control. The remaining 9 slides are processed with the control target probe set.

Pretreatment conditions for common tissue types

The following table lists common tissue types that were prepared according to the guidelines outlined in this manual and optimized using the recommended pretreatment optimization procedure.The table should be used as 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.

Table 10 Pretreatment conditions for common tissue types

Species	Tissue	Optimal conditions	
		Heat pretreatment time at 90-95°C	Protease digestion time at 40°C
Human	Brain	20 minutes	10 minutes
	Breast	20 minutes	15 minutes
	Colon	5 minutes	20 minutes
	Kidney	20 minutes	10 minutes
	Liver	20 minutes	20 minutes

Table 10 Pretreatment conditions for common tissue types *(continued)*

Species	Tissue	Optimal conditions	
		Heat pretreatment time at 90-95°C	Protease digestion time at 40°C
Human	Lung	10 minutes	20 minutes
	Lymph node	10 minutes	20 minutes
	Nasal polyp	5 minutes	5 minutes
	Osteoarthritic tissue	20 minutes	20 minutes
	Pancreas	10 minutes	10 minutes
	Prostate	10 minutes	20 minutes
	Salivary gland	10 minutes	10 minutes
	Skin	5 minutes	10 minutes
	Tonsil	10 minutes	20 minutes
	Thyroid	10 minutes	20 minutes
Rat	Kidney	10 minutes	20 minutes
	Liver	10 minutes	20 minutes
	Spleen	20 minutes	10 minutes
	Thyroid	10 minutes	20 minutes
Mouse	Bone	20 minutes	20 minutes
	Brain	10 minutes	10 minutes
	Heart	10 minutes	40 minutes
	Kidney	20 minutes	20 minutes
	Liver	20 minutes	20 minutes
	Lung	10 minutes	20 minutes
	Retina	10 minutes	10 minutes
Salmon	Heart	10 minutes	10 minutes
	Muscle	10 minutes	20 minutes
Monkey	Rectum	10 minutes	20 minutes

C Application of dual multiplexed RNA ISH-immunohistochemistry (IHC)

Dual multiplexed RNA ISH-IHC procedure

The following example procedure describes dual mRNA and protein detection in cryopreserved mouse brain using the ViewRNA™ Tissue Fluorescence Assay and anti-NeuN primary antibody (see “Dual multiplexed RNA ISH-IHC application example” on page 32). Optimize the procedure in this section for your application.

- **Blocking solution—**1X PBS, 10% goat serum, 0.1% Tween™ and 0.1% Triton-X™ detergents
- **Primary antibody—**2.5-hour incubation at room temperature with rabbit anti-NeuN primary antibody (1:200 dilution; Cat. No. PA578639)
- **Wash solution—**1X PBA, 0.1% Tween™ detergent
- **Secondary antibody—**1-hour incubation at room temperature with goat anti-rabbit Alexa Fluor™ Plus 488 secondary antibody (1:500 dilution; Cat. No. A32731TR)

Determine the optimized conditions (heat pretreatment and protease digestion time) for your sample type before starting this procedure. If you do not know these optimized conditions, see Appendix B, “Sample pretreatment optimization procedures”.

1. Follow the protocol, starting with sample preparation (page 16) through label probe hybridization (page 23).
2. Incubate with primary antibody according to the manufacturer's recommendation.
3. Wash three times with 1X PBS, plus 0.1% Tween™ detergent.
4. Incubate with secondary antibody according to the manufacturer's recommendation.
5. Wash three times with 1X PBS, plus 0.1% Tween™ detergent.
6. Proceed to counterstain the slides with DAPI solution (see “Perform counterstain” on page 24).

Dual multiplexed RNA ISH-IHC application example

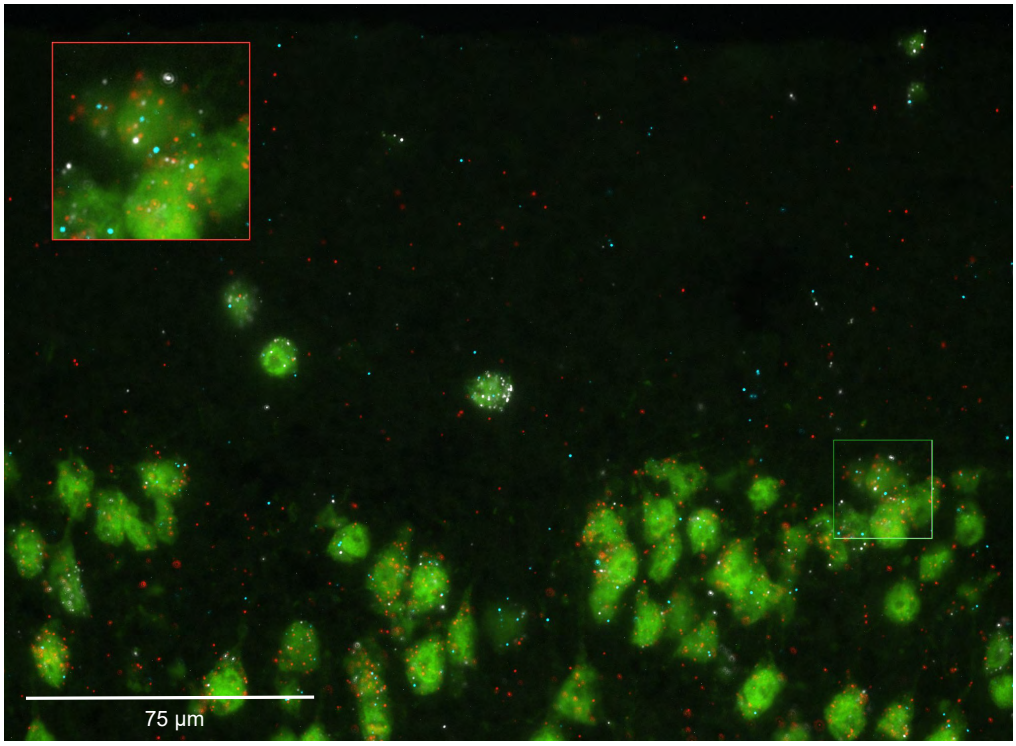

Figure 1 Dual multiplexed RNA ISH-IHC in mouse brain tissue using the ViewRNA™ Tissue Fluorescence Assay

Synaptopodin (Synpo; red), Cannabinoid Receptor 1 (Cnr1; white), and RNA polymerase II subunit A (Polr2a; blue) mRNA were detected in cryopreserved mouse brain tissue probed with ViewRNA™ probe sets. The tissue was then co-stained with NeuN primary antibody (Cat. No. PA578639) and Alexa Fluor™ Plus 488 secondary antibody (Cat. No. A32731TR). The probe sets were detected using the ViewRNA™ Tissue Fluorescence Assay with Alexa Fluor™ 594 (Type 1) for Synpo (red), Alexa Fluor™ 647 (Type 6) for Cnr1 (white), and Alexa Fluor™ 750 (Type 10) for Polr2a (blue). NeuN staining is shown in green. The fluorescent image was taken using the EVOS™ M7000 Imaging System and post-processed for visualization.

D

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
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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