ViewRNA® Cell Plus Assay

Catalog Number: 88-19000

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

Product Information

REF **Contents:** ViewRNA® Cell Plus Assay
Catalog Number: 88-19000

Temperature Limitation: Storage conditions vary, see components list for details
LOT **Batch Code:** Refer to vial
Use By: Refer to vial
Contains sodium azide and <10% formaldehyde

Description

The ViewRNA® Cell Plus Assay is a novel assay that combines immunocytochemistry with ViewRNA® technology, a proprietary fluorescent in situ hybridization and sequential branched-DNA amplification technique, to visualize both RNA with single-molecule sensitivity and protein in individual cells. This assay enables simultaneous detection of up to three RNA targets in combination with immunophenotyping for cell surface or intracellular proteins using both indirect and direct immunocytochemistry to allow for detailed characterization of specific cell subpopulations.

This ViewRNA® Cell Plus Assay kit contains all the reagents needed to conduct the assay. Target Probe sets for genes of interest and antibodies are sold separately.

Components

ViewRNA® Cell Plus Fixation/Permeabilization Component A: cat. 00-19500-23, store at 2-8°C
ViewRNA® Cell Plus Fixation/Permeabilization Component B: cat. 00-19900-23, store at 2-8°C
ViewRNA® Cell Plus Blocking/Antibody Diluent: cat. 00-19600-54, store at 2-8°C
ViewRNA® Cell Plus Solution A Fixative: cat. 00-19950-101, store at 2-8°C
ViewRNA® Cell Plus Solution B Fixative: cat. 00-19700-12, store at 2-8°C
ViewRNA® Cell Plus Probe Set Diluent: cat. 19876, store at 2-8°C
ViewRNA® Cell Plus Amplifier Diluent: cat. 19877, store at 2-8°C
ViewRNA® Cell Plus Label Probe Diluent: cat. 19878, store at 2-8°C
ViewRNA® Cell Plus PBS (10X): cat. 19883, store at room temperature
ViewRNA® Cell Plus RNA Wash Buffer Component 1: cat. 19884, store at room temperature
ViewRNA® Cell Plus RNA Wash Buffer Component 2: cat. 19885, store at room temperature
ViewRNA® Cell Plus RNase Inhibitor (100X): cat. 00-19800-53, store at room temperature
ViewRNA® Cell Plus PreAmplifier Mix: cat. 19879, store at -20°C
ViewRNA® Cell Plus Amplifier Mix: cat. 19880, store at -20°C
ViewRNA® Cell Plus Label Probe Mix: cat. 19881, store at -20°C
ViewRNA® Cell Plus 100X DAPI: cat. 19882, store at -20°C

Applications Reported

This ViewRNA® Cell Plus Assay has been reported for use with immunocytochemistry and microscopy.

Applications Tested

This ViewRNA® Cell Plus Assay has been tested by fluorescence microscopy.

Related Products

00-19001 ViewRNA® Cell Plus Fixation/Permeabilization Buffer Set
00-4958 Fluoromount-G™
00-4959 Fluoromount-G™, with DAPI
44-0404 StainTray™
50-5699 Ki-67 Monoclonal Antibody (20Raj1), eFluor 660, eBioscience™ TDS DISABLED: ABMAINT SKU (20Raj1)
53-4502 alpha Tubulin Monoclonal Antibody (DM1A), Alexa Fluor 488, eBioscience™ TDS DISABLED: ABMAINT SKU (DM1A)
88-19002 ViewRNA® Cell Plus Cytospin Module Kit

Tel: 888.999.1371 or 858.642.2058 • Fax: 858.642.2046 • thermofisher.com/ebioscience • info@ebioscience.com

ViewRNA® Cell Plus Assay

Catalog Number: 88-19000

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

Tel: 888.999.1371 or 858.642.2058 • Fax: 858.642.2046 • thermofisher.com/ebioscience • info@ebioscience.com

invitrogen

View RNA™ Cell Plus Assay

Introduction

ViewRNA™ Cell Plus Assay is an *in situ* hybridization assay that combines the power of the ViewRNA™ branched DNA technology with antibody-mediated protein detection, using fluorescence microscopy to visualize both RNA and protein in individual cells. This assay enables simultaneous detection of up to three RNA targets or two RNA targets in combination with immunophenotyping for cell surface or intracellular proteins using both indirect and direct immunocytochemistry to allow further characterization of specific cell subpopulations.

In the ViewRNA Cell Plus assay, adherent cells or cytospun suspension cells are fixed and permeabilized prior to detection of surface or intracellular proteins. Unconjugated and biotinylated antibodies followed by fluorescent secondary reagents or fluorophore-conjugated antibodies can be used to detect surface or intracellular proteins of interest. Following protein detection, a proprietary fluorescent *in situ* hybridization (FISH) and branched DNA amplification technology is used to amplify the signal detection of an RNA transcript, rather than the target RNA itself. In the first hybridization step of the assay, a gene-specific oligonucleotide Target Probe Set that contains 20–40 probe pairs binds to the target RNA sequence. An individual probe pair is designed to bind adjacent to each other in order for signal amplification to occur. Signal amplification is then achieved through a series of sequential hybridization steps. The PreAmplifier molecules confer an additional level of specificity because they will hybridize to the Target Probes only when both halves of a respective probe pair have bound to their target sequence. Multiple Amplifier molecules subsequently hybridize to their respective PreAmplifier. Finally, Label Probe oligonucleotides conjugated to a fluorescent dye hybridize to their corresponding Amplifier molecules. A fully assembled signal amplification "tree" possesses 400 Label Probe binding sites. When all target-specific oligonucleotide probes in the Target Probe Set bind to the target RNA transcript, 8,000- to 16,000-fold amplification is achieved.

Thermo Fisher currently offers three different amplification structures that allow simultaneous measurement of up to three different RNA targets for multicolor microscopic analysis. Once the cells have been processed by ViewRNA Cell Plus Assay, the data can be collected and analyzed on an epifluorescent microscope or high-content imager equipped with the appropriate filter sets. The schematic on Image 1 illustrates the detection of two unique RNA targets and one protein target.

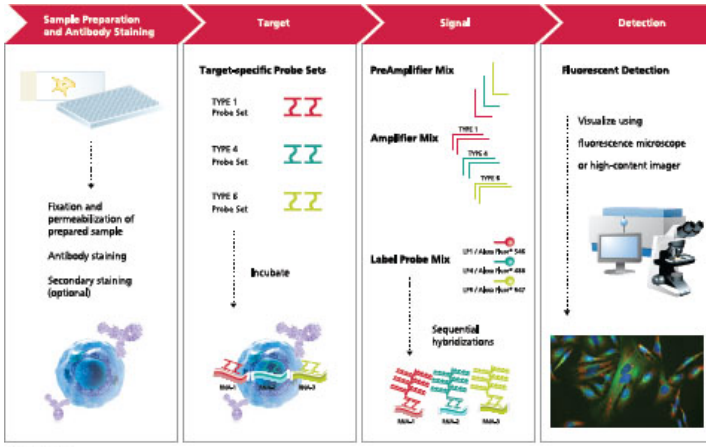


Image 1

Usage statement determined by Regulatory Affairs

ThermoFisher SCIENTIFIC

General notes

Assay specifications	
Sample type	Cultured cells, adherent or in suspension. See Appendix 5 for a complete list of validated cell types
Species	Mammalian
Plex level	Up to three RNA targets simultaneously; a combination of one to two antibodies and a single RNA; or one antibody and two RNA
Assay format	One 24-well plate with coverslips, twelve 8-well chamber slides, one-and-a-half 96-well plates, or eighty-four cytospin slides*
Protein/RNA detection	Fluorescent detection with FITC, Cy™3, Cy™5 filter sets
Antibody	Fluorescent dye-conjugated, purified, or biotinylated with fluorescent secondary detection**
RNA limit of detection	Single-molecule sensitivity

*Requires ViewRNA™ Cell Plus Cytospin Module Kit (cat. no. 88-19002-11).
**Validated antibody list can be found at www.Thermo Fisher.com.

Major instruments required	
Epifluorescent microscope for imaging	
Light source	100-watt mercury lamp or equivalent
Objective lens	20X (N.A. 0.8), 40X oil immersion (N.A. 1.3), or 63X oil immersion (N.A. 1.4)
Filter sets	• Excitation 358 nm/Emission 461 nm (DAPI) • Excitation 501 nm/Emission 523 nm (FITC) • Excitation 554 nm/Emission 576 nm (Cy™3) • Excitation 644 nm/Emission 669 nm (Cy™5)
Cooled-CCD camera	• 1.3 MP • 6.45 µm pixel size • >65% peak quantum efficiency (QE) • 6–10 e-read noise Common models include Exi™ Aqua (QImaging™), CoolSnap™ HQ² (Photometrics™), Flash 4.0 V2 (Hamamatsu)
Incubator	Incubator must be validated to maintain 40 ±1°C. We highly recommend validating the temperature of the incubator every three months using the QG ViewRNA™ Temperature Validation Kit (cat. no. QV0523).

Assay guidelines

1. Best results are obtained when starting with healthy cells. Always begin with cells that are in good physiological condition. Cells should be in active growth phase to preserve RNA integrity and minimize cell loss during processing.
2. This assay can utilize antibodies supplied by the customer if the antibodies have been tested for assay compatibility using ViewRNA™ Cell Plus Fixation/Permeabilization Buffer Set (cat. no. 00-19001) and found to stain at acceptable limits. A list of validated antibodies is available at **www.Thermo Fisher.com/viewrna-cell-plus-assay-validated-antibodies.**
3. This assay is highly temperature-dependent. Please ensure that the incubator holds temperature at 40 ±1°C. A significant reduction in signal will result from temperature deviations greater than 1°C. The incubator must be validated using QG ViewRNA™ Temperature Validation Kit (cat. no. QV0523) according to the instructions in Appendix 4 of this user manual.
4. This assay allows detection of up to three (3) RNA targets in a single sample:
   Type 1: Alexa Fluor™ 546. If visibility to naked eye is desired, use Type 1 or Type 4.
   Type 4: Alexa Fluor™ 488. If cells are known to exhibit high autofluorescence, use only for high-expressing genes.
   Type 6: Alexa Fluor™ 647
5. Protect samples from light after they have been stained with fluorophore-conjugated antibodies and labeled for RNA.
6. We recommend performing the assay in two days, as follows:

Day 1

- Fixation and permeabilization
- Antibody staining
- Target Probe hybridization

Day 2

- Signal amplification
- Sample mounting
- Microscopic analysis

Fluorochrome compatibility

1. Organic fluorochromes are compatible with this assay, such as DAPI, FITC, Alexa Fluor 488, eFluor™ 570, Alexa Fluor 546, eFluor™ 660, Alexa Fluor 647, and DRAQ5™.

Antibody staining compatibility

1. The fixation and permeabilization buffers in this kit are compatible with most Thermo Fisher™ antibodies to intracellular proteins when reported to work in immunocytochemistry, as well as to some surface proteins when reported to work with fixation and permeabilization. Antibodies that require methanol fixation conditions or those reported to only work on formalin-fixed paraffin-embedded (FFPE) sections are not compatible with this assay. Phosphorylation-specific antibodies from Thermo Fisher that are reported to work in immunocytochemistry are compatible. Please see the *Phospho Flow Cytometry Antibody Clone Buffer Selection Guide* or the technical data sheet of the individual antibody for more information.

   Note: It is highly recommended to empirically test the performance of the antibody using ViewRNA Cell Plus Fixation/Permeabilization Buffer Set (cat. no. 00-19001) before continuing on to the RNA hybridization portion of this assay.
2. Indirect detection of purified antibodies via a secondary antibody and/or tertiary reagent, as well as antibodies conjugated to biotin or fluorophores, have been used successfully in this assay. Please see the list of Thermo Fisher validated antibodies at **www.Thermo Fisher.com.**

Experimental design guidelines

1. To ensure proper assay performance, use Positive Control Probe Sets in every experiment. For example, beta-actin (ACTB) or GAPDH can be used in experiments with cell lines. See Appendix 5 for specific cell types and other recommended positive control genes.
2. Negative controls, such as samples with the target probe omitted or cells tested with a target probe that is not expressed in the cells of interest (e.g., DapB or FolA probes for bacterial mRNA), are highly recommended. Negative control samples comprised of or containing cells known to be negative for the gene of interest (e.g., unstimulated cells) are also recommended to confirm assay performance.
3. Please see Appendix 1 for examples of sample setup and experimental design.
4. To ensure that a sufficient amount of assay components is available, the recommended experimental setup should consist of three 8-well chamber slides at one time, 48 wells of a 96-well plate, 12 coverslips in a 24-well plate, or 24 cytospin slides.

ViewRNA Cell Plus Assay

Materials included

Store at 2–8°C

- ViewRNA™ Cell Plus Fixation/Permeabilization Component A (cat. no. 00-19500-23)
- ViewRNA™ Cell Plus Fixation/Permeabilization Component B (cat. no. 00-19900-23)[1]
- ViewRNA™ Cell Plus Blocking/Antibody Diluent (cat. no. 00-19600-54)
- ViewRNA™ Cell Plus Solution A Fixative (cat. no. 00-19950-101)[1]
- ViewRNA™ Cell Plus Solution B Fixative (cat. no. 00-19700-12)
- ViewRNA™ Cell Plus Probe Set Diluent (cat. no. 19876)[2]
- ViewRNA™ Cell Plus Amplifier Diluent (cat. no. 19877)[2]
- ViewRNA™ Cell Plus Label Probe Diluent (cat. no. 19878)

DANGER: [1]Contains formaldehyde, which is a poison and irritant. See Safety Data Sheet for complete information.

[2]Contains formamide, which is a teratogen, irritant, and possible carcinogen. See Safety Data Sheet for complete information.

Store at room temperature

- ViewRNA™ Cell Plus PBS (10X) (cat. no. 19883)
- ViewRNA™ Cell Plus RNA Wash Buffer Component 1 (cat. no. 19884)
- ViewRNA™ Cell Plus RNA Wash Buffer Component 2 (cat. no. 19885)
- ViewRNA™ Cell Plus RNase Inhibitor (100X) (cat. no. 00-19800-53)

Store -20°C

- ViewRNA™ Cell Plus PreAmplifier Mix (cat. no. 19879)
- ViewRNA™ Cell Plus Amplifier Mix (cat. no. 19880)
- ViewRNA™ Cell Plus Label Probe Mix (cat. no. 19881)
- ViewRNA™ Cell Plus 100X DAPI (cat. no. 19882)

Materials required, but not included

- Primary antibody: purified, biotinylated, or fluorophore-conjugated
- Secondary antibody and/or visualization reagent if not using a flurochrome-conjugated antibody: biotinylated and streptavidin-conjugated fluorophore (3-step protocol) or fluorophore-conjugated secondary antibody (2-step protocol)
- Isotype control antibody of the same species and isotype as the antibody of interest
- QG ViewRNA™ Probe Set(s) experimental and control
- 8-well chamber slide (Falcon, cat. no. 354108)
- Coverglasses, round 12 mm (Warner instruments, cat. no. CS-12R); 24-well plates (Corning Costar™, cat. no. 3526)
- Coverglasses for mounting (VWR, cat. nos. 48393059, 48393048)
- 96-well plates (Greiner Bio-One SensoplateTM Plus, cat. no. 655891)
- Parafilm™ wrap
- Clear nail polish (care should be taken to test for autofluorescence)
- Double-distilled water (ddH2O)
- Fluoromount-G™, with DAPI (cat. no. 00-4959-52), Fluoromount-G™ (cat. no. 00-4958-02), or ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, cat. no. P36931)
- Humidified incubation chamber or staining tray (cat. no. 44-0404-10)
- QG ViewRNA Temperature Validation Kit (cat. no. QVC0523)

Instruments and equipment

- Refer to Appendix 3 of this user manual for a guide on instrument setup.
- Dry incubator capable of maintaining 40 ±1°C (Refer to Appendix 4).

Optional accessories, not included

- ViewRNA Cell Plus Cytospin Module Kit (88-19002-11)
- ViewRNA Cell Plus Fixation/Permeabilization Buffer Set (00-19001)[1]

DANGER: [1]Contains formaldehyde, which is a poison and irritant. See Safety Data Sheet for complete information.

Experiment duration

Day 1 (~6–8 hours)

- Fixation and permeabilization of prepared sample
- Antibody staining
- Target Probe hybridization

Day 2 (4–5 hours)

- Signal amplification
- Sample mounting

Quick guide: ViewRNA Cell Plus Assay

Day 1

1. Fix and permeabilize with Fixation/Permeabilization Solution for 30 minutes at room temperature.
2. Wash three times in PBS with RNase Inhibitor.
3. Block with Blocking/Antibody Diluent with RNase Inhibitor for 20 minutes at room temperature.
4. Incubate with primary antibody for 1 hour at room temperature.
5. Wash three times in PBS with RNase Inhibitor.
6. (optional) If using an unconjugated primary antibody, stain with fluorophore-conjugated or biotin-conjugated secondary antibody for 1 hour at room temperature. Wash three times in PBS with RNase Inhibitor.
7. (optional) If using a biotinylated primary or secondary antibody, stain with streptavidin-conjugated fluorophore for 30 minutes at room temperature. Wash three times in PBS with RNase Inhibitor.
8. Fix with Fixation Solution for 1 hour at room temperature.
9. Wash three times in PBS with RNase Inhibitor.
10. Hybridize the Target Probe for 2 hours at 40°C.
11. Wash five times in Wash Buffer.
12. Store samples in Wash Buffer overnight.

Day 2

13. Hybridize the PreAmplifier for 1 hour at 40°C.
14. Wash five times in Wash Buffer.
15. Hybridize the Amplifier for 1 hour at 40°C.
16. Wash five times in Wash Buffer.
17. Hybridize the Label Probe for 1 hour at 40°C.
18. Wash five times with Wash Buffer.
19. Wash once in PBS.
20. Mount and coverslip.
21. Dry slides for 1–2 hours.
22. Visualize slides.

This protocol is for use with samples processed on coverslips in a 24-well plate

(To perform the protocol with 8-well chamber slides, 96-well plates, or cytospin slides, please refer to additional protocols in this user manual.)

This protocol has been validated with round 12-mm coverslips in a 24-well tissue culture plate. The reagents contained in this kit can be used for one plate.

Materials required for this protocol, but not included

- 24-well plate
- Round coverslips
- Poly-L-lysine (sterile) (optional)
- 70% and 100% ethanol
- Glass slides
- 1X PBS, pH 7.2 (sterile)
- Water (sterile, RNase-free)

Experimental procedure

Note: *Coverslips must be prepared using sterile culture conditions prior to plating cells. Buffer and solution preparations in this protocol are calculated to result in a slight excess to ensure that sufficient reagent is available for each sample. (For example, a preparation of 405 µL will be recommended when 400 µL will be used.) Coating of coverslips with poly-L-lysine substrate is optional and will depend on the cells used in the assay. The following coating protocol is a guideline only.—*

1. Dilute poly-L-lysine to 0.01% in sterile water or 1X PBS. Mix well and set aside.
2. Place one coverslip into each well of a sterile 24-well plate.
3. Sterilize the coverslips by adding 1 mL/well of 70% ethanol and soak for 15 minutes at room temperature.
4. Aspirate the 70% ethanol and rinse coverslips with 1 mL/well 100% ethanol.
5. Aspirate the 100% ethanol and allow coverslips to air dry completely for 15 minutes at room temperature.
6. Add 1mL/well poly-L-lysine and incubate for 15 minutes at room temperature.
7. Aspirate the poly-L-lysine and rinse the coverslips three times with 2 mL/well of 1X PBS.
8. Aspirate the PBS and plate cells. Cells should be plated at an appropriate density and allowed to attach to the well (e.g., 85,000 cells/well in a 24-well plate). Cells are usually plated one day prior to staining in order to achieve 60–80% confluency. For cells requiring extended treatment times prior to performing the assay, an optional stopping point is available after the fixation and permeabilization step to allow flexibility in the workflow.

Day 1: Fixation and permeabilization

1. **Prepare ViewRNA Cell Plus Fixation/Permeabilization Solution** by combining 1 part ViewRNA Cell Plus Fixation/Permeabilization Component A with 1 part ViewRNA Cell Plus Fixation/ Permeabilization Component B. Mix gently by inverting. This buffer will first be used in Step 3.

   Note: *Prepare 405 µL for each sample or well (or 9,720 µL for a 24-well plate). Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer.*

2. **Prepare 1X PBS with RNase Inhibitor** by diluting ViewRNA Cell Plus PBS (10X) to 1X with RNase-free ddH_2O. Then add ViewRNA Cell Plus RNase Inhibitor (100X) at a 1:100 dilution. Mix by inverting. If Day 1 of the protocol will be completed in its entirety, prepare 15.6 mL for each sample or well. This buffer will first be used in Step 5. Store at room temperature until ready for use.

   Note*:** *If stopping at the optional stopping point after Step 5, only prepare 3,360 µL for each sample or well for Day 1. You will need to freshly prepare additional* ***1X PBS with RNase Inhibitor *on Day 2.*

   Note: *Use of a dropper to add* ***1X PBS with RNase Inhibitor*** *to each well is a gentle method that is less likely to wash off cells. However, depending on the user, this method of washing may inadvertently consume more reagent and require the preparation of additional reagent.*

3. Remove 24-well plate from the tissue culture incubator, aspirate medium using a pipet or vacuum suction, and pipet 400 µL of **ViewRNA Cell Plus Fixation/Permeabilization Solution** into each well. Gently rock the 24-well plate to evenly distribute the solution over the cells. Incubate for 30 minutes at room temperature in a humidified staining tray.
4. **Prepare ViewRNA Cell Plus Blocking/Antibody Diluent Solution** by diluting ViewRNA Cell Plus RNase inhibitor (100X) to a 1:100 dilution with ViewRNA Cell Plus Blocking/Antibody Diluent. Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer. This buffer will first be used in Step 6. If Day 1 of the protocol will be completed in its entirety, prepare the following volumes:
   - If using fluorophore-conjugated primary antibodies, prepare 810 µL for each sample or well.
   - If using indirect detection of purified/unconjugated primary antibodies or biotinylated antibodies, prepare 1,620 µL for each sample or well.

   Note*:** *If the samples will be stored overnight for the optional stopping point after Step 5, prepare* ***ViewRNA Cell Plus Blocking/Antibody Diluent Solution *on the day that the samples will be run to ensure best performance.*

5. Aspirate ViewRNA Cell Plus Fixation/Permeabilization Solution, and wash the cells with **1X PBS with RNase Inhibitor** using a dropper or pipet to slowly and carefully add 800 µL/well. Then aspirate to remove the buffer. Repeat until the cells have been washed three times with 1X PBS with RNase Inhibitor.

   [OPTIONAL] Stopping point: You may stop the protocol at this point and continue later, if desired. Cover the cells with **1X PBS with RNase Inhibitor** (approximately 800 µL/well for a 24-well plate). To minimize evaporation, cover with a 24-well plate lid, seal with Parafilm to tightly cover the opening between the lid and plate, and then store in a humidified staining tray overnight at 2–8°C. Storing the samples longer than 24 hours is not recommended. If the protocol is halted at this step, prepare fresh **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** on Day 2.

   Note: *From this point on, it is critical that the cells are not allowed to dry out as this will cause increased levels of background staining and difficulty in interpreting staining results. It is best not to aspirate more than two wells at a time in order to reduce the possibility of the cells drying out.*

Day 1: Antibody staining

6. Overlay the cells with **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, which was prepared in Step 4 (use 400 µL for each sample or well). Gently rock the 24-well plate to evenly distribute the solution over the cells. Cover with the 24-well plate lid and incubate in a humidified staining tray for 20 minutes at room temperature.
7. Dilute primary or fluorophore-conjugated antibody(s) in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** according to the manufacturer's recommendation or as empirically determined, preparing 405 µL diluted antibody per sample or well. Aspirate **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution** and overlay 400 µL diluted primary antibody onto the cells. Gently rock the 24-well plate to evenly distribute the antibody solution over the cells. Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature. (The incubation time can be increased for proteins that are expressed at low levels.) Protect fluorophore-conjugated antibodies from light.
8. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using an unconjugated primary antibody that will be followed by a fluorophore-conjugated secondary (2-step protocol) or biotinylated secondary antibody and streptavidin tertiary reagent protocol, continue to Step 9 (3-step protocol).
    - If using a biotinylated primary antibody protocol, continue to Step 11 (2-step protocol).
    - If using fluorophore-conjugated antibody(s), continue to Step 13.
9. Dilute the fluorophore-conjugated or biotin-conjugated secondary antibody according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution**, preparing 405 µL per sample or well. Protect fluorophore-conjugated antibodies from light. Overlay 400 µL of the secondary antibody solution and gently rock the 24-well plate to evenly distribute the antibody solution over the cells. Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature.
10. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using a biotin-conjugated secondary antibody, continue to Step 11.
    - If using a fluorophore-conjugated secondary antibody, continue to Step 13.
11. Dilute streptavidin-conjugated fluorophore according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, and protect from light. Overlay the cells with 400 µL per well of the visualization reagent, cover with a 24-well plate lid, and incubate in a humidified staining tray for 30 minutes at room temperature.
12. Gently wash cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
13. Prepare **ViewRNA Cell Plus Fixation Solution** by combining 1 part **ViewRNA Cell Plus Solution** A Fixative with 7 parts **ViewRNA Cell Plus Solution B Fixative**. Prepare 405 µL for each sample or well. Aspirate the **1X PBS with RNase Inhibitor** and overlay the cells with 400 µL ViewRNA Cell Plus Fixation Solution for each sample. Gently rock the 24-well plate to evenly distribute the fixation solution over the cells. Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature.

    Note: *During this incubation, complete Steps 14–15.*
14. Thaw **QG ViewRNA Probe Set(s)** (Target Probes), including the Positive Control Target Probe Sets, at room temperature. Once thawed, maintain on ice until ready for use.
15. Pre-warm **ViewRNA Cell Plus Probe Set Diluent** to 40°C in a validated incubator (approximately 30 minutes).
16. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5. Cells should be maintained in the final **1X PBS with RNase Inhibitor** while preparing the Target Probe sets.

Day 1: Target probe hybridization

17. **Prepare ViewRNA Cell Plus Probe Solution** by diluting ViewRNA Probe Sets 1:100 in pre-warmed **ViewRNA Cell Plus Probe Set Diluent** and vortex briefly to mix. Prepare 405 µL for each sample or well.

    Note: *If adding more than one Target Probe per well, adjust the volume of* ***ViewRNA Cell Plus Probe Set Diluent*** *such that a final volume of 400 µL per sample or well is achieved.*
18. Aspirate the **1X PBS with RNase Inhibitor** and overlay the cells with **ViewRNA Cell Plus Probe Solution** (400 µL/well). Gently rock the 24-well plate to mix and distribute the diluted Target Probe(s). Cover with a 24-well plate lid and incubate in a humidified staining tray for 2 hours at 40 ±1°C in a validated incubator.

    Note: *During this incubation, prepare* ***ViewRNA Cell Plus RNA Wash Buffer Solution****.*
19. **Prepare ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH_20, **ViewRNA Cell Plus RNA Wash Component 1**, and then **ViewRNA Cell Plus RNA Wash Component 2**. Preparing the solution in this order will minimize precipitation.

    Note:*Prepare 5.1 mL buffer for each sample. Prepare enough buffer in bulk to accommodate all samples and allow to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer. To prepare 5.1 mL, combine 5,059.2 µL of* ddH_2O*, 15.3 µL of* ***Wash Component 1****, and 25.5 µL of* ***Wash Component 2****, and then mix well. Watch for any visible precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature.*
20. Aspirate **ViewRNA Cell Plus Probe Solution** and gently wash the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** using a dropper or pipet to slowly and carefully add 800 µL/well. Then aspirate to remove buffer. Repeat these washes four more times. It is not necessary to incubate cells in the wash buffer for extended periods of time.

21. Cover the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** (approximately 800 µL/well). To minimize evaporation, cover with a 24-well plate lid, seal with Parafilm to tightly cover the opening between the lid and plate, and store in a humidified staining tray overnight. Do not store for longer than 24 hours at 2–8°C in the dark.

    Note: *This stopping point is recommended for ease of use and a more manageable workflow. However, if desired, Step 21 may be skipped.*

Day 2: Signal amplification

22. Pre-warm the samples to room temperature.
23. Pre-warm **ViewRNA Cell Plus Amplifier Diluent and ViewRNA Cell Plus Label Probe Diluent** to 40°C in a validated incubator (approximately 30 minutes).
24. Thaw **ViewRNA Cell Plus PreAmplifier Mix, ViewRNA Cell Plus Amplifier Mix**, and **ViewRNA Cell Plus Label Probe Mix** at room temperature. Vortex briefly to mix and place the tubes on ice until ready for use. Protect **ViewRNA Cell Plus Label Probe Mix** from light.
25. **Prepare ViewRNA Cell Plus PreAmplifier Solution** by diluting **ViewRNA Cell Plus PreAmplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent**. Vortex briefly to mix. Prepare 405 µL for each sample or well.
26. Aspirate **ViewRNA Cell Plus RNA Wash Buffer Solution** and overlay **ViewRNA Cell Plus PreAmplifier Solution** (400 µL/well). Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
27. As in Step 19, prepare **ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH_20, **ViewRNA Cell Plus Wash Component 1, ViewRNA Cell Plus Wash Component 2**. Preparing the solution in this order will minimize precipitation.

    If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and cool to room temperature. Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer.

    Note: *Prepare 14 mL buffer for each sample by combining 13,888 µL of ddH_2O, 42 µL of* ***Wash Component 1****, and 70 µL of* ***Wash Component 2****. Mix well.*
28. Aspirate **ViewRNA Cell Plus PreAmplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit in the final wash solution while preparing **ViewRNA Cell Plus Working Amplifier Mix Solution.**
29. **Prepare ViewRNA Cell Plus Working Amplifier Mix Solution** by diluting **ViewRNA Cell Plus AmplifierMix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent**. Vortex briefly to mix. Prepare 405 µL for each sample or well. Aspirate and overlay **ViewRNA Cell Plus Working Amplifier Solution** (400 µL/well). Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at 40°C ±1°C in a validated incubator.
30. Aspirate **ViewRNA Cell Plus Working Amplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow coverglasses to incubate with the final wash while preparing **ViewRNA Cell Plus Working Label Probe Mix Solution.**
31. **Prepare ViewRNA Cell Plus Working Label Probe Mix Solution** by diluting **ViewRNA Cell Plus Label Probe Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Label Probe Diluent**. Vortex briefly to mix and protect from light. Prepare 405 µL for each sample or well. Aspirate the Wash Buffer and overlay **ViewRNA Cell Plus Working Label Probe Mix Solution** (400 µL/well). Cover with a 24-well plate lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
32. Aspirate **ViewRNA Cell Plus Working Label Probe Mix Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit for 10 minutes in the final wash.
33. Prepare 1X PBS by diluting **ViewRNA Cell Plus PBS (10X)** with deionized water. Gently wash the cells once in 1X PBS. Mount the coverglasses one at a time while keeping them in PBS to ensure that they do not dry out.
34. To mount, label a glass slide with the appropriate description and place a drop of Fluoromount-G with DAPI (or other mounting medium) onto the slide.
35. Remove the coverslip from the 24-well plate using forceps and dab the edge to remove excess PBS.
36. Mount the coverslip with the cell side facing downwards onto the mounting medium.
37. Seal the edge of the coverslip with clear nail polish.
38. Allow slides to dry for 1–2 hours before visualizing. Protect from light.
39. Slides can be stored at 2–8°C and protected from light. For best results, the slides should be analyzed within 1–2 days.

This protocol is for use with 8-well chamber slides.

This protocol has been validated with 8-well chamber slides. The reagents contained in this kit can be used for 12 8-well chamber slides.

Materials required for assay on chamber slides, but not included

- Chamber slides
- Rectangular coverslips
- 1X PBS, pH 7.2 (sterile)
- Water (sterile, RNase-free)

Experimental procedure

Note: *Cells should be plated at an appropriate density and allowed to attach to the slide (e.g., 30,000 cells/well in an 8-well chamber slide). Cells are usually plated 1 day prior to staining in order to achieve 60–80% confluency. For cells requiring extended treatment times prior to performing the assay, an optional stopping point is available after the fixation and permeabilization step to allow flexibility in the workflow. Buffer and solution preparations in this protocol are calculated to result in a slight excess to ensure that sufficient reagent is available for each sample. (For example, a preparation of 405 µL is recommended when only 400 µL will be used.)*

Day 1: Fixation and permeabilization

1. **Prepare ViewRNA Cell Plus Fixation/Permeabilization Solution** by combining 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component A** with 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component B**. Mix gently by inverting. This buffer will first be used in Step 3.

   Note: *Prepare 105 µL for each sample or well (or 840 µL for an 8-well chamber slide). Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer.*

2. **Prepare 1X PBS with RNase Inhibitor** by diluting **ViewRNA Cell Plus PBS (10X)**to 1X with RNase-free ddH_2O. Then add **ViewRNA Cell Plus RNase Inhibitor (100X)** at a 1:100 dilution. Mix by inverting. If Day 1 of protocol will be completed in its entirety, prepare 3.6 mL for each sample or well. This buffer will first be used in Step 5. Store at room temperature until ready for use.

   Note: *If stopping at the optional stopping point after Step 5, only prepare 1,200 µL for each sample or well for Day 1. You will need to freshly prepare additional **1X PBS with RNase Inhibitor** on Day 2.*

   Note: *Use of a dropper to add **1X PBS with RNase Inhibitor** to each well is a gentle method that is less likely to wash off cells. However, depending on the user, this method of washing may inadvertently consume more reagent and require the preparation of additional reagent.*

3. Remove chamber slide with cells from the tissue culture incubator, aspirate medium using a pipet or vacuum suction, and pipet 100 µL of **ViewRNA Cell Plus Fixation/ Permeabilization Solution** into each well. Gently rock the chamber slide to evenly distribute the solution over the cells. Incubate for 30 minutes at room temperature in a humidified staining tray.

4. Prepare **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** by diluting **ViewRNA Cell Plus RNase inhibitor (100X)**to a 1:100 dilution with **ViewRNA Cell Plus Blocking/ Antibody Diluent**. Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer. This buffer will first be used in Step 6. If Day 1 of the protocol will be completed in its entirety, prepare the following volumes:
   - If using fluorophore-conjugated antibodies, prepare 210 µL for each sample or well.
   - If using indirect detection of purified/unconjugated primary antibodies or biotinylated antibodies, prepare 480 µL for each sample or well.

   Note: *If the samples will be stored overnight for the optional stopping point after Step 5, prepare ViewRNA Cell Plus **Blocking/Antibody Diluent Solution** on the day that the samples will be run to ensure best performance.*

5. Aspirate **ViewRNA Cell Plus Fixation/Permeabilization Solution**, and wash the cells with **1X PBS with RNase Inhibitor** using a dropper or pipet to slowly and carefully add 200 µL/well. Then aspirate to remove the buffer. Repeat until the cells have been washed three times with **1X PBS with RNase Inhibitor**.

   [OPTIONAL] Stopping point: You may stop the protocol at this point and continue later if desired. Cover the cells with **1X PBS with RNase Inhibitor** (approximately 400 µL/well). To minimize evaporation, use Parafilm to tightly cover the opening of the chamber slide before placing the lid onto the chamber slide. Store in a humidified staining tray overnight at 2–8°C. Storing the samples longer than 24 hours is not recommended. If the protocol is halted at this step, prepare fresh **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** on Day 2.

   Note: *From this point on, it is critical that the cells are not allowed to dry out as this will cause increased levels of background staining and difficulty in interpreting staining results. It is best not to aspirate more than two wells at a time in order to reduce the possibility of the cells drying out.*

6. Overlay the cells with **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, which was prepared in Step 4, using 100 µL for each sample or well. Gently rock the chamber slide to evenly distribute the solution over the cells. Cover with the chamber slide lid and incubate in a humidified staining tray for 20 minutes at room temperature.
7. Dilute primary or directly fluorophore-conjugated antibody(s) in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** according to the manufacturer's recommendation or as empirically determined, preparing 105 µL diluted antibody per sample or well. Aspirate **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** and overlay 100 µL diluted primary antibody onto the cells. Gently rock the chamber slide to evenly distribute the antibody solution over the cells. Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at room temperature. (The incubation time can be increased for proteins that are expressed at low levels.) Protect fluorophore-conjugated antibodies from light.
8. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using an unconjugated primary antibody that will be followed by a fluorophore-conjugated secondary antibody (2-step protocol) or biotinylated secondary antibody and streptavidin tertiary reagent, continue to Step 9 (3-step protocol).
    - If using a biotinylated primary antibody, continue to Step 11 (2-step protocol).
    - If using directly fluorophore-conjugated antibody(s), continue to Step 13.
9. Dilute the fluorophore-conjugated or biotin-conjugated secondary antibody according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution**, preparing 105 µL diluted antibody per sample or well. Protect fluorophore-conjugated antibodies from light. Overlay 100 µL of the secondary antibody solution and gently rock the chamber slide to evenly distribute the antibody solution over the cells. Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at room temperature.
10. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using a biotin-conjugated secondary antibody, continue to Step 11.
    - If using a fluorophore-conjugated secondary antibody, continue to Step 13.
11. Dilute streptavidin-conjugated fluorophore according to the manufacturer's recommendation or to an optimal dilution in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, and protect from light. Overlay the cells with 100 µL per well of the visualization reagent, cover with a chamber slide lid, and incubate in a humidified staining tray for 30 minutes at room temperature.
12. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
13. Prepare **ViewRNA Cell Plus Fixation Solution** by combining 1 part **ViewRNA Cell Plus Solution A Fixative** with 7 parts **ViewRNA Cell Plus Solution B Fixative**. Prepare 105 µL for each sample or well. Aspirate the **1X PBS with RNase Inhibitor** and overlay the cells with 100 µL of **ViewRNA Cell Plus Fixation Solution** for each sample. Gently rock the chamber slide to evenly distribute the fixation solution over the cells. Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at room temperature.

    Note: *During this incubation, complete Steps 14–15.*
14. Thaw **QG ViewRNA Probe Set(s)** (Target Probes), including the Positive Control Target Probe Sets, at room temperature. Once thawed, maintain on ice until ready for use.
15. Pre-warm **ViewRNA Cell Plus Probe Set Diluent** to 40°C in a validated incubator (approximately 30 minutes).
16. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5. Cells should be maintained in the final **1X PBS with RNase Inhibitor** while preparing the Target Probe Sets.

Day 1: Target probe hybridization

17. **Prepare ViewRNA Cell Plus Probe Solution** by diluting **ViewRNA Cell Plus Probe Sets** 1:100 in pre-warmed **ViewRNA Cell Plus Probe Set Diluent** and vortex briefly to mix. Prepare 105 µL for each sample or well.

    Note: *If adding more than one Target Probe per well, adjust the volume of **ViewRNA Cell Plus Probe Set Diluent** such that a final volume of 100 µL per sample or well is achieved.*

18. Aspirate the **1X PBS with RNase Inhibitor** and overlay the cells with **ViewRNA Cell Plus Probe Solution** (100 µL/well). Gently rock the chamber slide to mix and distribute the diluted Target Probe(s). Cover with a chamber slide lid and incubate in a humidified staining tray for 2 hours at 40 ±1°C in a validated incubator.

    Note: *During this incubation, prepare **ViewRNA Cell Plus RNA Wash Buffer Solution**.*

19. **Prepare ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH_20, **ViewRNA Cell Plus RNA Wash Component 1**, and then **ViewRNA Cell Plus RNA Wash Component 2**. Preparing the solution in this order will minimize precipitation.

    Note: *Prepare 1.6 mL buffer for each sample. Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer. To prepare 1.6 mL, add 1,587.2 µL of ddH_2O, 4.8 µL of **Wash Component 1**, and 8 µL of **Wash Component 2**, and then mix well. Watch for any visible precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature.*

20. Aspirate **ViewRNA Cell Plus Probe Solution** and gently wash the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** using a dropper or pipet to slowly and carefully add 200 µL/well. Aspirate to remove the buffer. Repeat these washes four more times. It is not necessary to incubate the cells in the wash buffer for extended periods of time.

21. Cover the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** (approximately 400 µL/well). To minimize evaporation, use Parafilm to tightly cover the opening of the chamber slide before placing the lid onto the chamber slide. Store in a humidified staining tray overnight. Do not store for longer than 24 hours at 2–8°C in the dark.

    Note: *This stopping point is recommended for ease of use and a more manageable workflow. However, if desired, Step 21 may be skipped.*

Day 2: Signal amplification

22. Pre-warm the samples to room temperature.
23. Pre-warm **ViewRNA Cell Plus Amplifier Diluent** and **ViewRNA Cell Plus Label Probe Diluent** to 40°C in a validated incubator (approximately 30 minutes).
24. Thaw **ViewRNA Cell Plus PreAmplifier Mix**, **ViewRNA Cell Plus Amplifier Mix**, and **ViewRNA Cell Plus Label Probe Mix** at room temperature. Vortex briefly to mix and place the tubes on ice until ready for use. Protect **ViewRNA Cell Plus Label Probe Mix** from light.
25. **Prepare ViewRNA Cell Plus PreAmplifier Solution** by diluting **ViewRNA Cell Plus PreAmplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent** and vortex briefly to mix. Prepare 105 µL for each sample or well.
26. Aspirate **ViewRNA Cell Plus RNA Wash Buffer Solution** and overlay **ViewRNA Cell Plus PreAmplifier Solution** (100 µL/well). Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
27. As in Step 19, prepare **ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH_20, **ViewRNA Cell Plus Wash Component 1**, and then **ViewRNA Cell Plus Wash Component 2**. Preparing the solution in this order will minimize precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature. Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer.

    Note: *Prepare 3.6 mL buffer for each sample by combining 3,571.2 µL of ddH_2O, 10.8 µL of **Wash Component 1**, and 18 µL of **Wash Component 2**. Mix well.*

28. Aspirate **ViewRNA Cell Plus PreAmplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit in the final wash solution while preparing **ViewRNA Cell Plus Working Amplifier Mix Solution**.
29. **Prepare ViewRNA Cell Plus Working Amplifier Mix Solution** by diluting **ViewRNA Cell Plus Amplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent**. Vortex briefly to mix. Prepare 105 µL for each sample or well. Aspirate and overlay **ViewRNA Cell Plus Working Amplifier Solution** (100 µL/well). Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at 40°C ±1°C in a validated incubator.
30. Aspirate the **ViewRNA Cell Plus Working Amplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit in the final wash while preparing **ViewRNA Cell Plus Working Label Probe Mix Solution**.
31. **Prepare ViewRNA Cell Plus Working Label Probe Mix Solution** by diluting **ViewRNA Cell Plus Label Probe Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Label Probe Diluent**. Vortex briefly to mix and protect from light. Prepare 105 µL for each sample or well. Aspirate the Wash Buffer and overlay **ViewRNA Cell Plus Working Label Probe Mix Solution** (100 µL/well). Cover with a chamber slide lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
32. Aspirate **ViewRNA Cell Plus Working Label Probe Mix Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to incubate for 10 minutes in the final wash.

33. Prepare 1X PBS by diluting **ViewRNA Cell Plus PBS (10X)** with deionized water. Gently wash the cells once in 1X PBS.
34. Carefully remove the chamber from the slide while keeping cells wet with 1X PBS.
35. Mount and coverslip using Fluoromount-G with DAPI. Seal the edge of the coverglass with clear nail polish.
36. Allow the slides to dry for 1–2 hours before visualizing. Protect from light.
37. Slides can be stored at 2–8°C and protected from light. For best results, the slides should be analyzed within 1–2 days.

This protocol is for use with 96-well plates.

This protocol has been validated on glass bottom 96-well plates. The reagents contained in this kit can be used for one-and-a-half 96-well plates.

Materials required, but not included

- 96-well glass bottom imaging plate (Greiner Bio-One Sensoplate Plus, cat no. 655891 or equivalent low autofluorescence plate). It is recommended that plates be evaluated for autofluorescence prior to use in performing this assay.

Experimental procedure

Note: *Cells should be plated at an appropriate density and allowed to attach to the well (e.g., 15,000 cells/well in a 96-well plate). Cells are usually plated one day prior to staining in order to achieve 60–80% confluency. For cells requiring extended treatment times prior to performing the assay, an optional stopping point is available after the fixation and permeabilization step to allow flexibility in the workflow. Buffer and solution preparations in this protocol are calculated to result in a slight excess to ensure that sufficient reagent is available for each sample. (For example, a preparation of 405 µL is recommended when only 400 µL will be used.)*

Day 1: Fixation and permeabilization

1. **Prepare ViewRNA Cell Plus Fixation/Permeabilization Solution** by combining 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component A** with 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component B**. Mix gently by inverting. This buffer will first be used in Step 3.

   Note: *Prepare 55 µL for each sample or well (or 5,280 µL for a 96-well plate). Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer.*

2. **Prepare 1X PBS with RNase Inhibitor** by diluting **ViewRNA Cell Plus PBS (10X)**to 1X with RNase-free ddH_2O. Then add **ViewRNA Cell Plus RNase Inhibitor (100X)** at a 1:100 dilution. Mix by inverting. If Day 1 of protocol will be completed in its entirety, prepare 1.6 mL for each sample or well. This buffer will first be used in Step 5. Store at room temperature until ready for use.

   Note: *If stopping at the optional stopping point after Step 5, only prepare 420 µL for each sample or well for Day 1. You will need to freshly prepare additional **1X PBS with RNase Inhibitor** on Day 2.*

   Note: *Use of a dropper to add **1X PBS with RNase Inhibitor** to each well is a gentle method that is less likely to wash off cells. However, depending on the user, this method of washing may inadvertently consume more reagent and require the preparation of additional reagent.*

3. Remove the 96-well plate from the tissue culture incubator, aspirate medium using a pipet or vacuum suction, and pipet 50 µL of **ViewRNA Cell Plus Fixation/Permeabilization Solution** into each well. Gently rock the plate to evenly distribute the solution over the cells. Incubate for 30 minutes at room temperature in a humidified staining tray.
4. **Prepare ViewRNA Cell Plus Blocking/Antibody Diluent Solution** by diluting **ViewRNA Cell Plus RNase inhibitor (100X)** to a 1:100 dilution with **ViewRNA Cell Plus Blocking/ Antibody Diluent**. Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer. This buffer will first be used in Step 6. If Day 1 of the protocol will be completed in its entirety, prepare the following volumes:
   - If using fluorophore-conjugated antibodies, prepare 110 µL for each sample or well.
   - If using indirect detection of purified/unconjugated primary antibodies or biotinylated antibodies, prepare 220 µL for each sample or well.

   Note: *If the samples will be stored overnight for the optional stopping point after Step 5, prepare **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** on the day that the samples will be run to ensure best performance.*

5. Aspirate **ViewRNA Cell Plus Fixation/Permeabilization Solution**, and wash the cells with **1X PBS with RNase Inhibitor** using a dropper or pipet to slowly and carefully add 100µL/well. Then aspirate to remove the buffer. Repeat until the cells have been washed three times with **1X PBS with RNase Inhibitor**.

   [OPTIONAL] Stopping point: You may stop the protocol at this point and continue later if desired. Cover the cells with **1X PBS with RNase Inhibitor** (approximately 100 µL/well). To minimize evaporation, cover with a 96-well plate lid, use Parafilm to tightly cover the opening between the lid and plate, and store in a humidified staining tray overnight at 2–8°C. Storing samples for longer than 24 hours is not recommended. If the protocol is halted at this step, prepare fresh **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** on Day 2.

   Note: *From this point on, it is critical that the cells are not allowed to dry out as this will cause increased levels of background staining and difficulty in interpreting staining results. It is best not to aspirate more than two wells at a time in order to reduce the possibility of the cells drying out.*

Day 1: Antibody staining

6. Overlay the cells with **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, which was prepared in Step 4 (use 50 µL for each sample or well). Gently rock the plate to evenly distribute the solution over the cells. Cover with a 96-well plate lid and incubate in a humidified staining tray for 20 minutes at room temperature.
7. Dilute primary or fluorophore-conjugated antibody(s) in **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution** according to the manufacturer's recommendation or as empirically determined, preparing 55 µL diluted antibody per sample or well. Aspirate **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** and overlay 50 µL diluted primary antibody onto the cells. Gently rock the 96-well plate to evenly distribute the antibody solution over the cells. Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature. (The incubation time can be increased for proteins that are expressed at low levels.) Protect fluorophore-conjugated antibodies from light.
8. Gently wash the cells three times in 1X PBS with RNase Inhibitor as described in Step 5.
    - If using an unconjugated primary antibody that will be followed by a fluorophore-conjugated secondary antibody (2-step protocol) or biotinylated secondary antibody and streptavidin tertiary reagent protocol, continue to Step 9 (3-step protocol).
    - If using a biotinylated primary antibody protocol, continue to Step 11 (2-step protocol).
    - If using directly fluorophore-conjugated antibody(s), continue to Step 13.
9. Dilute the fluorophore-conjugated or biotin-conjugated secondary antibody according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution**, preparing 55 µL diluted antibody per sample or well. Protect fluorophore-conjugated antibodies from light. Overlay 50 µL of the secondary antibody solution and gently rock the 96-well plate to evenly distribute antibody solution over the cells. Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature.
10. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using a biotin-conjugated secondary antibody, continue to Step 11.
    - If using a fluorophore-conjugated secondary antibody, continue to Step 13.
11. Dilute streptavidin-conjugated fluorophore according to the manufacturer's recommended or to an optimized dilution in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, and protect from light. Overlay the cells with 50 µL per well of the visualization reagent, cover with a 96-well plate lid, and incubate in a humidified staining tray for 30 minutes at room temperature.
12. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
13. Prepare **ViewRNA Cell Plus Fixation Solution** by combining 1 part **ViewRNA Cell Plus Solution A Fixative** with 7 parts **ViewRNA Cell Plus Solution B Fixative**. Prepare 55 µL for each sample or well. Aspirate the 1X PBS with RNase Inhibitor and overlay the cells with 50 µL of **ViewRNA Cell Plus Fixation Solution** for each sample. Gently rock the 96-well plate to evenly distribute the fixation solution over the cells. Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at room temperature.

    Note: *During this incubation, complete Steps 14–15.*
14. Thaw **QG ViewRNA Cell Plus Probe Set(s)** (Target Probes), including the Positive Control Target Probe Sets, at room temperature. Once thawed, maintain on ice until ready for use.
15. Pre-warm **ViewRNA Cell Plus Probe Set Diluent** to 40°C in a validated incubator (approximately 30 minutes).
16. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5. Cells should be maintained in the final 1X PBS with RNase Inhibitor while preparing the Target Probe Sets.

Day 1: Target probe hybridization

17. **Prepare ViewRNA Cell Plus Probe Solution** by diluting ViewRNA Probe Sets 1:100 in pre-warmed **ViewRNA Cell Plus Probe Set Diluent** and vortex briefly to mix. Prepare 55 µL for each sample or well.

    Note: *If adding more than one Target Probe per well, adjust the volume of* ***ViewRNA Cell Plus Probe Set Diluent*** *such that a final volume of 50 µL per sample or well is achieved.*
18. Aspirate **1X PBS with RNase Inhibitor** and overlay cells with **ViewRNA Cell Plus Probe Solution** (50 µL/well). Gently rock the 96-well plate to mix and distribute the diluted Target Probe(s). Cover with a 96-well plate lid and incubate in a humidified staining tray for 2 hours at 40 ±1°C in a validated incubator.

    Note: *During this incubation, prepare* **ViewRNA Cell Plus RNA Wash Buffer Solution**.
19. **Prepare ViewRNA Cell Plus RNA Wash Buffer Solution** by adding the components in the following order: ddH_20, **ViewRNA Cell Plus RNA Wash Component 1**, and then **ViewRNA Cell Plus RNA Wash Component 2**. Preparing the solution in this order will minimize precipitation.

    Note: *Prepare 800 µL buffer for each sample. Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer. To prepare 800 µL, add 793.5 µL of* ddH_2O*, 2.4 µL of* ***Wash Component 1****, and 4 µL of* ***Wash Component 2****, and then mix well. Watch for any visible precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature.*

20. Aspirate **ViewRNA Cell Plus Probe Solution** and gently wash the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** using a dropper or pipet to slowly and carefully add 100 µL/well. Aspirate to remove the buffer. Repeat these washes four more times. It is not necessary to incubate cells in the wash buffer for extended periods of time.
21. Cover the cells with **ViewRNA Cell Plus RNA Wash Buffer Solution** (approximately 100 µL/well). To minimize evaporation, cover with a 96-well plate lid, use Parafilm to seal the opening between the lid and plate, and store in a humidified staining tray overnight. Do not store for longer than 24 hours at 2–8°C in the dark.

    Note: *This stopping point is recommended for ease-of- use and a more manageable workflow. However, if desired, Step 21 may be skipped.*

Day 2: Signal amplification

22. Pre-warm the samples to room temperature.
23. Pre-warm **ViewRNA Cell Plus Amplifier Diluent** and **ViewRNA Cell Plus Label Probe Diluent** to 40°C in a validated incubator (will require approximately 30 minutes).
24. Thaw **ViewRNA Cell Plus PreAmplifier Mix, ViewRNA Cell Plus Amplifier Mix**, and **ViewRNA Cell Plus Label Probe Mix** at room temperature. Vortex briefly to mix and place the tubes on ice until ready for use. Protect **ViewRNA Cell Plus Label Probe Mix** from light.
25. Prepare **ViewRNA Cell Plus PreAmplifier Solution** by diluting **ViewRNA Cell Plus PreAmplifier Mix** 1:25 in pre-warmed **ViewRNA Cell Plus Amplifier Diluent** and vortex briefly to mix. Prepare 55 µL for each sample or well.
26. Aspirate **ViewRNA Cell Plus RNA Wash Buffer Solution** and overlay **ViewRNA Cell Plus PreAmplifier Solution** (50 µL/well). Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
27. As in Step 19, prepare **ViewRNA Cell Plus RNA Wash Buffer Solution** by adding the components in the following order: ddH_20, **ViewRNA Cell Plus Wash Component 1**, and then **ViewRNA Cell Plus Wash Component 2**. Preparing the solution in this order will minimize precipitation. If precipitation is observed, warm solution to 37°C, gently mix to dissolve, cool to room temperature. Prepare buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose of any unused buffer.

    Note: *Prepare 1.8 mL buffer for each sample by combining 1,785.6 µL of ddH_2O, 5.4 µL of* ***Wash Component 1****, and 9 µL of* ***Wash Component 2****. Mix well.*

28. Aspirate **ViewRNA Cell Plus PreAmplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit in the final wash solution while preparing **ViewRNA Cell Plus Working Amplifier Mix Solution.**
29. **Prepare ViewRNA Cell Plus Working Amplifier Mix Solution** by diluting **ViewRNA Cell Plus Amplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent**. Vortex briefly to mix. Prepare 55 µL for each sample or well. Aspirate and overlay **ViewRNA Cell Plus Working Amplifier Solution** (50 µL/well). Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at 40°C ±1°C in a validated incubator.
30. Aspirate **ViewRNA Cell Plus Working Amplifier Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow cells to sit in the final wash while preparing **ViewRNA Cell Plus Working Label Probe Mix Solution**.
31. Prepare **ViewRNA Cell Plus Working Label Probe Mix Solution** by diluting **ViewRNA Cell Plus Label Probe Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Label Probe Diluent**. Vortex briefly to mix and protect from light. Prepare 55 µL for each sample or well. Aspirate the Wash Buffer and overlay **ViewRNA Cell Plus Working Label Probe Mix Solution** (50 µL/well). Cover with a 96-well plate lid and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
32. Aspirate **ViewRNA Cell Plus Working Label Probe Mix Solution** and gently wash the cells five times with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow the cells to sit for 10 minutes in the final wash.
33. Prepare 1X PBS by diluting **ViewRNA Cell Plus PBS (10X)** with deionized water. Dilute DAPI to a 1:100 dilution in 1X PBS and vortex to mix. Add 50 µL/well and incubate for 5 minutes at room temperature.
34. Gently wash the cells once in 1X PBS (100 µL/well).
35. Add 1X PBS to a volume of 150 µL/well and image immediately.

This protocol is for use with cytospin samples.

This protocol has been validated on cytospun suspension cells on glass slides. This platform type requires **ViewRNA Cell Plus Assay**, as well as additional reagents contained in **ViewRNA Cell Plus Cytospin Module Kit** to perform the assay on 84 samples. Typically, slides are grouped and washed in coplin jars in groups of six. It is recommended that 24 samples or slides should be run at one time.

Materials required, but not included

- ViewRNA Cell Plus Cytospin Module Kit (cat. no. 88-19002-11)
- Glass slides
- Cytospin chambers/cuvetters
- Cytospin blotting paper
- Coplin jars
- Orbital shaker
- Cytospin centrifuge

Experimental procedure

***Note**: Cells must be cytospun onto a glass slide prior to starting **ViewRNA Cell Plus Assay**. Additional **ViewRNA Cell Plus PBS (10X)** (cat. no. QVC0508), **ViewRNA Cell Plus Wash Buffer** (cat. no. QG0507), and **ViewRNA Cell Plus RNase Inhibitor** are required for this procedure due to the larger volumes required. These additional reagents are provided in **ViewRNA Cell Plus Cytospin Module Kit**. Prepare a labeled glass slide, chamber/ cuvette, and blotting paper. Add up to 200 µL of suspended cells to the chamber/cuvette. Spin at 100 rpm for 2 minutes. Carefully remove the slide and allow to air dry. Proceed with ViewRNA Cell Plus Assay. Buffer and solution preparations in this protocol are calculated to result in a slight excess to ensure that sufficient reagent is available for each sample. (For example, a preparation of 405 µL is recommended when only 400 µL will be used.)*

Day 1: Fixation and permeabilization

1. **Prepare ViewRNA Cell Plus Fixation/Permeabilization Solution by** combining 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component A** with 1 part **ViewRNA Cell Plus Fixation/Permeabilization Component B**. Mix gently by inverting. This buffer will first be used in Step 3.

   ***Note:** Prepare 105 µL for each sample or slide. Prepare enough buffer in bulk to accommodate all samples and allow buffer to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer.*

2. **Prepare 1X PBS with RNase Inhibitor** by diluting **ViewRNA Cell Plus PBS (10X)** to 1X with RNase-free ddH_2O. Then add **ViewRNA Cell Plus RNase Inhibitor (100X)** at a 1:100 dilution. Mix by inverting. If Day 1 of protocol will be completed in its entirety, prepare 2,880 mL for 24 samples or slides (four coplin jars). This buffer will first be used in Step 5. Store at room temperature until ready for use.

   ***Note:** If stopping at the optional stopping point after Step 5, only prepare 768 mL for four coplin jars (assuming six slides/jar) for Day 1. You will need to freshly prepare additional 1X PBS with RNase Inhibitor on Day 2.*

3. Pipet 100 µL of **ViewRNA Cell Plus Fixation/Permeabilization Solution** per slide. To minimize evaporation of reagents and to help spread the solution evenly over the cells, use forceps to gently overlay the cells with Parafilm that is cut to fit over the cells on the slide. Incubate the slides for 30 minutes at room temperature in a humidified staining tray.
4. **Prepare ViewRNA Cell Plus Blocking/Antibody Diluent Solution** by diluting **ViewRNA Cell Plus RNase inhibitor (100X)** to a 1:100 dilution with **ViewRNA Cell Plus Blocking/ Antibody Diluent**. Prepare enough buffer in bulk to accommodate all samples and allow to equilibrate to room temperature. This buffer should be prepared fresh. Dispose any unused buffer. This buffer will first be used in Step 6. If Day 1 of protocol will be completed in its entirety, prepare the following volumes:
   - If using fluorophore-conjugated antibodies, prepare 210 µL for each sample or slide.
   - If using indirect detection of purified/unconjugated primary antibodies or biotinylated antibodies, prepare 480 µL for each sample or slide.

   ***Note:** If the samples will be stored overnight for the optional stopping point after Step 5, prepare ViewRNA Cell Plus Blocking/Antibody Diluent Solution on the day that the samples will be run to ensure best performance.*
5. Gently remove the Parafilm and wash the slides in coplin jars containing 45 mL/jar of **1X PBS with RNase Inhibitor** on an orbital shaker. Repeat until the slides have been washed three times with 1X PBS with RNase Inhibitor.

   [OPTIONAL] Stopping point: You may stop the protocol at this point and continue later if desired. Store the slides in a coplin jar containing **1X PBS with RNase Inhibitor** (approximately 45 mL/jar). To minimize evaporation, use a cap to tightly cover the coplin jar, and store it overnight at 2–8°C. Storing samples longer than 24 hours is not recommended. If the protocol is halted at this step, prepare fresh **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** on Day 2.

   ***Note:** From this point on, it is critical that the cells are not allowed to dry out as this will cause increased levels of background staining and difficulty in interpreting staining results. It is recommended to leave the slides in the wash buffer until ready to add antibody.*

Day 1: Antibody staining

6. Overlay the cells with **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, which was prepared in Step 4, using 100 µL for each slide. To minimize evaporation of reagents and to help spread the solution evenly over the cells, use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 20 minutes at room temperature.
7. Dilute primary or fluorophore-conjugated antibody(s) in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** according to the manufacturer's recommendation or as empirically determined, preparing 105 µL diluted antibody per sample or slide. Tap off **ViewRNA Cell Plus Blocking/Antibody Diluent Solution** onto a paper towel and overlay 100 µL of diluted primary antibody onto the cells. To minimize evaporation of reagents and to help spread the solution evenly over the cells, use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 1 hour at room temperature. (The incubation time can be increased for proteins that are expressed at low levels.) Protect fluorophore-conjugated antibodies from light.
8. Gently wash the slides three times in **1X PBS with RNase Inhibitor** as described in Step 5.

- If using an unconjugated primary antibody that will be followed by a fluorophore- conjugated secondary antibody protocol (2-step protocol) or biotinylated secondary antibody and streptavidin tertiary reagent protocol, continue to Step 9 (3-step protocol).
- If using a biotinylated primary antibody protocol, continue to Step 11 (2-step protocol).
- If using directly fluorophore-conjugated antibody(s), continue to Step 13.

9. Dilute the fluorophore-conjugated or biotin-conjugated secondary antibody according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/ Antibody Diluent Solution** (prepare 105 µL per sample or slide). Protect fluorophore- conjugated antibodies from light. Overlay 100 µL of the secondary antibody solution. To minimize evaporation of reagents and to help spread the solution evenly over the cells, use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 1 hour at room temperature.
10. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
    - If using a biotin-conjugated secondary antibody, continue to Step 11.
    - If using a fluorophore-conjugated secondary antibody, continue to Step 13.
11. Dilute streptavidin-conjugated fluorophore according to the manufacturer's recommendation or to an optimized dilution in **ViewRNA Cell Plus Blocking/Antibody Diluent Solution**, and protect from light. Overlay the cells with 100 µL per slide of the visualization reagent.Gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 30 minutes at room temperature while protected from light.
12. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5.
13. Prepare **ViewRNA Cell Plus Fixation Solution** by combining 1 part **ViewRNA Cell Plus Solution A Fixative** with 7 parts **ViewRNA Cell Plus Solution B Fixative**. Prepare 105 µL for each sample or slide. Tap off the **1X PBS with RNase Inhibitor** onto a paper towel and overlay the cells with 100 µL of **ViewRNA Cell Plus Fixation Solution** for each sample. Gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 1 hour at room temperature.

    Note: *During this incubation, complete Steps 14–15.*
14. Thaw the **QG ViewRNA Cell Plus Probe Set(s)** (Target Probes), including the Positive Control Target Probe Sets, at room temperature. Once thawed, maintain on ice until ready for use.
15. Pre-warm **ViewRNA Cell Plus Probe Set Diluent** to 40°C in a validated incubator.
16. Gently wash the cells three times in **1X PBS with RNase Inhibitor** as described in Step 5. Cells should be maintained in the final 1X PBS with RNase Inhibitor while preparing the Target Probe Sets.

Day 1: Target probe hybridization

17. **Prepare ViewRNA Cell Plus Probe Solution** by diluting **ViewRNA Cell Plus Probe Sets** to a 1:100 dilution in pre-warmed **ViewRNA Cell Plus Probe Set Diluent** and vortex briefly to mix. Prepare 105 µL for each sample or slide.

    Note: *If adding more than one Target Probe per well, adjust the volume of* ***ViewRNA Cell Plus Probe Set Diluent*** *accordingly such that a final volume of 100 µL per sample/slide is achieved.*
18. Remove slides from the copin jars and dab edges to remove excess buffer. Overlay cells with **ViewRNA Cell Plus Probe Solution** (100 µL/slide). To minimize evaporation of reagents and to help spread the solution evenly over the cells, use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide. Incubate the slides in a humidified staining tray for 2 hours at 40 ±1°C in a validated incubator.

    Note: *During this incubation, prepare ViewRNA Cell Plus RNA Wash Buffer Solution.*
19. **Prepare ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH20, **ViewRNA Cell Plus RNA Wash Component 1**, and then **ViewRNA Cell Plus RNA Wash Component 2**. Preparing the solution in this order will minimize precipitation.

    Note: *Prepare 1,080 mL buffer for four coplin jars (assuming six slides/jar). Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer. To prepare 1,080 mL, add 1,071.4 mL of ddH_2O, 3.2 mL of* ***Wash Component 1****, and 5.4 mL of* ***Wash Component 2****, and then mix well. Watch for any visible precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature.*
20. Gently remove Parafilm and tap off excess Probe solution on a paper towel. Wash the slides in the coplin jars with 45 mL/jar of **ViewRNA Cell Plus RNA Wash Buffer Solution** on an orbital shaker. Repeat these washes four more times. It is not necessary to incubate the cells in the wash buffer for extended periods of time.
21. Store the slides in the coplin jars with **ViewRNA Cell Plus RNA Wash Buffer Solution** (approximately 45 mL/jar). To minimize evaporation, use a cap to tightly cover the coplin jar and store overnight. Do not store for longer than 24 hours at 2–8°C in the dark.

    Note: *This stopping point is recommended for ease of use and a more manageable workflow. However, if desired, Step 21 may be skipped.*

Day 2: Signal amplification

22. Pre-warm the samples to room temperature.
23. Pre-warm **ViewRNA Cell Plus Amplifier Diluent** and **ViewRNA Cell Plus Label Probe Diluent** to 40°C in a validated incubator (approximately 30 minutes).

24. Thaw **ViewRNA Cell Plus PreAmplifier Mix, ViewRNA Cell Plus Amplifier Mix**, and **ViewRNA Cell Plus Label Probe Mix** at room temperature. Vortex briefly to mix and place the tubes on ice until ready for use. Protect **ViewRNA Cell Plus Label Probe Mix** from light.
25. **Prepare ViewRNA Cell Plus PreAmplifier Solution** by diluting **ViewRNA Cell Plus PreAmplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent** and vortex briefly to mix. Prepare 105 µL for each sample or slide.
26. Remove slides from coplin jars, and dab edges to remove excess buffer. Overlay the cells with **ViewRNA Cell Plus PreAmplifier Solution** (100 µL/slide). Use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide, and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
27. As in Step 19, prepare **ViewRNA Cell Plus RNA Wash Buffer Solution** by combining the components in the following order: ddH_20, **ViewRNA Cell Plus Wash Component 1**, and then **ViewRNA Cell Plus Wash Component 2**. Preparing the solution in this order will minimize precipitation. If precipitation is observed, warm the solution to 37°C, gently mix to dissolve, and then cool to room temperature. Prepare enough buffer in bulk to accommodate all samples. This buffer should be prepared fresh. Dispose any unused buffer.

    Note: *Prepare 2,880mL buffer for four coplin jars (assuming six slides/jar) by combining 2,857 mL of ddH2O, 8.6 mL of Wash Component 1, and 14.4 mL of Wash Component 2. Mix well.*
28. Gently remove the Parafilm and wash slides in coplin jars five times with 48 mL/jar of **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow slides to incubate in the final wash solution while preparing **ViewRNA Cell Plus Working Amplifier Mix Solution**.
29. **Prepare ViewRNA Cell Plus Working Amplifier Mix Solution** by diluting **ViewRNA Cell Plus Amplifier Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Amplifier Diluent**. Vortex briefly to mix. Prepare 105 µL for each sample or slide. Remove the slides from the coplin jars and dab edges to remove excess buffer. Overlay slides with **ViewRNA Cell Plus Working Amplifier Solution** (100 µL/slide). Use forceps to gently overlay the cells with Parafilm that is cut to fit over the cells on the slide. Incubate in a humidified staining tray for 1 hour at 40°C ±1°C in a validated incubator.
30. Gently remove Parafilm and wash the slides in coplin jars with 48 mL/jar of **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow slides to sit in final wash while preparing **ViewRNA Cell Plus Working Label Probe Mix Solution**.
31. Prepare **ViewRNA Cell Plus Working Label Probe Mix Solution** by diluting **ViewRNA Cell Plus Label Probe Mix** to a 1:25 dilution in pre-warmed **ViewRNA Cell Plus Label Probe Diluent**. Vortex briefly to mix and protect from light. Prepare 105 µL for each sample or slide. Remove slides from coplin jars and dab edges to remove excess buffer. Overlay **ViewRNA Cell Plus Working Label Probe Mix Solution** (100 µL/slide). Use forceps to gently overlay the cells with Parafilm cut to fit over the cells on the slide, and incubate in a humidified staining tray for 1 hour at 40 ±1°C in a validated incubator.
32. Gently remove Parafilm and wash the slides with **ViewRNA Cell Plus RNA Wash Buffer Solution** as described in Step 20. Allow slides to incubate for 10 minutes in the final wash.
33. Prepare 1X PBS by diluting **ViewRNA Cell Plus PBS (10X)** with deionized water. Gently wash the slides one time in coplin jars with 1X PBS (approximately 45 mL/jar).
34. To eliminate drying out, remove slides from coplin jars, one at a time, and dab the edge to remove excess buffer.
35. Mount and coverslip using Fluoromount-G with DAPI. Seal the edge of the coverglass with clear nail polish.
36. Allow slides to dry for 1–2 hours before visualizing. Protect from light.
37. Slides can be stored at 2–8°C and protected from light. For best results, the slides should be analyzed within 1–2 days.

Appendix

Appendix 1: Experimental design, imaging, and analysis

Note: *Before starting the ViewRNA Cell Plus assay, please ensure that:*

1. The tissue culture slide, culture dish, or multi-well plate to be used does not exhibit autofluorescent properties. Please see suggested culture slides, culture dishes, and multi-well plates within the specific protocols. If you are not using one of the recommended platform products, it is suggested that you perform the experiment described in the "Assessing non-assay-related background".
2. The cells to be used for the assay are healthy and in good morphological and physiological condition.
3. The incubator has been calibrated and can hold a temperature of 40 ±1°C. Temperature can be measured and validated using ViewRNA Temperature Validation Kit (cat. no. QV0523).

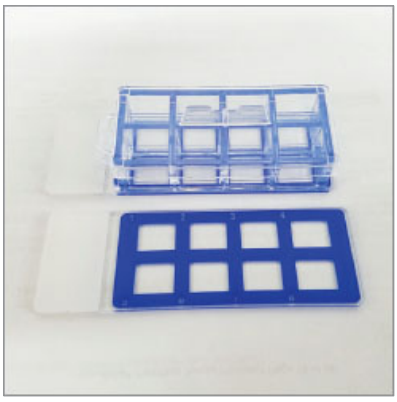


Figure 1

Table 1. Sample experimental setup. The following experimental setup is an example of how one could assess ERBB2 (HER2/neu) mRNA and Ki-67 protein expression in HeLa cells using Falcon™ 8-Well Culture Slides (Figure 2). As a positive control for RNA expression and to visualize the cell cytoplasm, a Probe Set specific for the housekeeping gene GAPDH is included.

Well #	Well description	Type 1	Type 4	Type 6	Antibody 1	Antibody 2
1	No probe control	-	-	-	-	-
2	Non-specific Target Probe Set/isotype control	dapB (*E.coli* gene)	dapB (*E.coli* gene)	-	Mouse IgG1 eFluor™ 660	-
3	Experimental well	ERBB2	GAPDH	-	Anti-Human Ki-67 eFluor™ 660	-

Well #1 No probe control: This sample will not be incubated with any Target Probe Sets or antibodies. However, this well will be incubated with all of the same solutions as your experimental well (e.g., ViewRNA Cell Plus Blocking/Antibody Diluent with RNase Inhibitor during the antibody incubation step and ViewRNA Cell Plus Probe Set Diluent during the Probe Set incubation step). Additionally, Well #1 will be incubated with ViewRNA Cell Plus PreAmplifier, Amplifier, and Label Probe Mixes. If a secondary antibody and tertiary detection reagent will be used for antibody detection in the experimental well, it would also be included in this well as a control and measure of non-specific signal.

Well #2 Non-specific Target Probe Set/isotype control: This sample will be incubated with a control probe set that is the same type as the experimental Probe Set. We recommend the dapB Probe Set, which hybridizes with *E. coli* dapB mRNA. Alternatively, one can use a scrambled or sense-strand Probe Set. Well #2 should also contain an isotype control antibody at the same concentration and of the same species and isotype as your experimental antibody.

Well #3 Experimental well: This sample will be incubated with Anti-Human Ki-67 eFluor 660, ERBB2 Type 1, and GAPDH Type 4 Probe Sets.

After performing the ViewRNA Cell Plus assay and mounting the slide, the samples can be imaged (see below for imaging strategy and analysis).

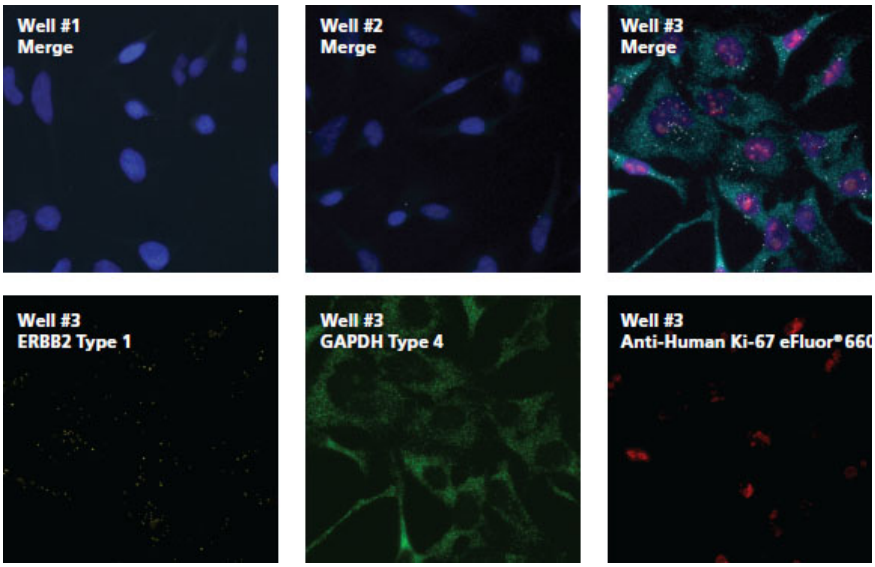


Figure 2. Expected results from the experimental setup shown in Table 1. Top row: Well #1: HeLa cells, no probe control. Well #2: Staining of HeLa cells with Mouse IgG1 K Isotype Control eFluor™ 660 (cat. no. 50-4714), dapB Type 1 and dapB Type 4 Target Probe Sets. Well #3: Staining of HeLa cells with Anti-Human Ki-67 eFluor 600 (cat. no. 50-5699, red), ERBB2 Type 1 (white), and GAPDH Type 4 (green) Target Probes. Nuclei were counterstained with DAPI. Colocalization of the Ki-67 signal with DAPI appears as pink. **Bottom row:** Individual/single-channel fluorescent images for Well #3.

Imaging strategy

Filter sets needed for imaging this experiment

- Excitation 358 nm / Emission 461 nm (for the detection of DAPI)
- Excitation 501 nm / Emission 523 nm (FITC; for the detection of GAPDH Type 4)
- Excitation 554 nm / Emission 576 nm (Cyanine3; for the detection of ERBB2 Type 1)
- Excitation 644 nm / Emission 669 nm (Cyanine5; for the detection of Anti-Human Ki-67 eFluor 660)

Non-optimal filters may limit the excitation or detection of the fluorophore used in the experiment. Not every fluorophore is compatible with microscopy. Please make sure to use fluorophores that can be adequately excited by the light source on your microscope and detected with the filter sets listed above.

Objective lens

For imaging larger cells such as fibroblasts or epithelial cells, a 20X objective can be sufficient. If desired, a 40X oil immersion (N.A. 1.3) lens can also be used. For smaller cells, such as human peripheral blood cells or mouse splenocytes, we recommend first locating, focusing, and observing your sample using a 20X objective. However, a 40X or 100X oil immersion (N.A. 1.3) lens is recommended for imaging. For the above experiment with HeLa cells (a human epithelial cell line), all images were taken using a 20X objective.

Imaging

ViewRNA Cell Plus Assay offers single-copy RNA sensitivity combined with protein detection in individual cells in a multiplex assay format. When RNA is expressed at low levels, distinct dots dispersed throughout the cytoplasm can be observed. By contrast, when a target RNA is highly expressed, such as the case with a housekeeping transcript, strong and uniform fluorescent signal is present throughout the cytoplasm due to the difficulty of distinguishing individual dots when so many are present. For target RNA expressed at low levels, longer exposure times may be required to detect the signal, which can lead to increased background signal that appears as tiny grains. This type of grainy background signal should not be confused with true RNA signal, which appears as distinct, uniform dots.

When imaging your samples, it is important to consider the order in which to image the different signals to limit photobleaching and to obtain the sharpest image possible. In the experimental setup outlined above, ERBB2 has a relatively low expression level in HeLa cells (less than 60 dots per cell). However, Type 1 is the brightest of the three Target Probe Set types. Hence, we recommend that you image your Type 1 Probe Set signal first. In the above example, after focusing on and capturing the image for ERBB2, the filter was changed to capture the Type 4 image. Once again, you should focus and capture your image. Typically, it is not necessary to adjust the focus of your camera if it has not moved since capturing the Type 1 RNA image. In the event that the focal plane has drifted, refocus the camera prior to obtaining the next image. In the above example, GAPDH is highly expressed and relatively bright. However, if you observe a dim signal for your RNA targets, we suggest capturing the image as immediately as possible after capturing the Type 1 signal, which may require minimizing the time it takes to focus on the RNA signal (dots). After imaging the Type 4 signal in the above example, the filter was switched in order to image the Anti-Human Ki-67 eFluor 660 signal. We suggest that you image the antibody signal last as it is typically more robust than the RNA signal. If you capture an antibody signal before the RNA signal, the focus will need to be re-adjusted. Finally, the filter was changed to image DAPI in the example above. After capturing the images of your experimental sample(s), proceed to image the negative controls using the same exposure times for each filter set as you did for the experimental sample.

Interpretation and analysis of results

ViewRNA Cell Plus Assay offers protein and single-copy RNA sensitivity at the single-cell level. Antibodies to a variety of proteins (e.g., cell structure, phosphorylated proteins, transcription factors) can be used to visualize protein in the context of RNA with each dot representing one target transcript. The amount of protein and the level of target gene expression can be quantified by measuring the number of dots per cell.

In the experimental images shown in Figure 2, target transcripts with different expression levels in untreated HeLa cells were detected using ViewRNA Cell Plus Assay. ERBB2, represented by the white signal, has a relatively low expression level in HeLa cells (less than 60 dots per cell). GAPDH has an immense number of transcripts, resulting in homogeneous fluorescent signal throughout the cytoplasm (represented by the green signal).

For targets such as ERBB2, quantification of gene expression levels can be performed manually by counting the average number of dots per cell. However, for very high-expressing targets such as GAPDH, quantification should be performed by measuring the total signal intensity level within the entire cell. Highly expressed housekeeping genes such as ACTB or GAPDH are useful in defining cell boundaries

for software analysis and for counting dots representing other genes. It is important to note that even in established cell lines, the cell population is heterogeneous due to clonal variations. Hence, the expression levels of a given target within a cell population are expected to be heterogeneous. As such, it is necessary to measure a representative number of cells in the population and calculate the average. Avoid cells with nuclei that exhibit abnormal morphology (fractured, pinched, or split). Instead, include only those cells with normal-appearing nuclei, as indicated by round or oval DAPI counterstaining (blue in the Figure 2). Image analysis software, including CyteSeer (Vala Sciences), MetaMorph™ (Molecular Devices), and ImageJ (Freeware from NIH), should facilitate quantitation when a large number of samples are handled. Contact vendors for specific details on how to quantify the number of dots and the signal intensity at the single-cell level.

Assessing non-assay-related background

Background signal can come from a variety of sources, including: (1) autofluorescence from the cell type tested, (2) the materials used to coat the cover slips, and (3) the glass slides. To assess and identify whether any of these reagents or materials contribute to background signal, we recommend performing the following experiment to assess the sample background prior to performing any assays.

Experimental design to test for background signal

1. Grow cells overnight in your culture slide, culture dish, or multi-well plates on a coverglass either uncoated or coated with the appropriate matrix protein.
2. After the overnight incubation, fix the cells with ViewRNA Cell Plus Fixation/Permeabilization solution for 30 minutes at room temperature.
3. Wash once with PBS and mount using Fluoromount-G with DAPI.
4. Visualize the samples using an epifluorescent microscope with the filter sets intended for future experiments.

Expected results

The background fluorescence of the glass surface should be minimal with strong, nuclear DAPI staining. No smears, bright spots, or debris should be observed in the 488 nm, 550 nm, or 650 nm channels. If any of the materials used results in such a high level of autofluorescence that it would hinder the detection of your specific signals, we recommend checking the reagents used and verifying that they have been validated for use with ViewRNA Cell Plus Assay.

Some cell lines will exhibit high autofluorescence, particularly in the 488 nm channel. This is a normal biological property of the cells. If this occurs, you can use the 488 nm dye for detecting a highly expressed gene (e.g., housekeeping gene) whereas an RNA with lower expression can be detected using the 550 nm or 650 nm Label Probes (Type 1 or 6, respectively). If the type of cell line being used is not critical, perform the assay using an alternate cell line.

Appendix 2: Examples of expected results

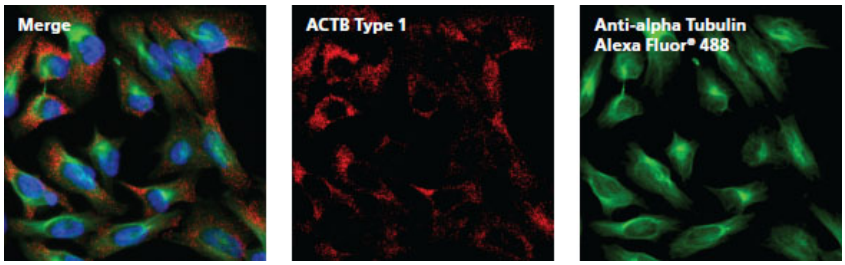


Figure 3. Staining of HeLa cells with ACTB (beta-Actin) Type 1 Target Probe (red) and Anti-alpha Tubulin Alexa Fluor™488 (cat.no. 53-4502, green). Nuclei were counterstained with DAPI (blue).

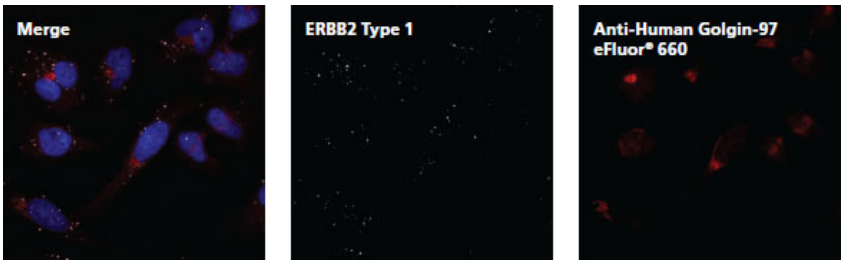


Figure 4. Staining of HeLa cells with ERBB2 (Her2/neu) Type 1 Target Probe (white) and Anti-Human Golgin-97 eFluor™ 660 (cat. no. 50-9767, red). Nuclei were counterstained with DAPI (blue).

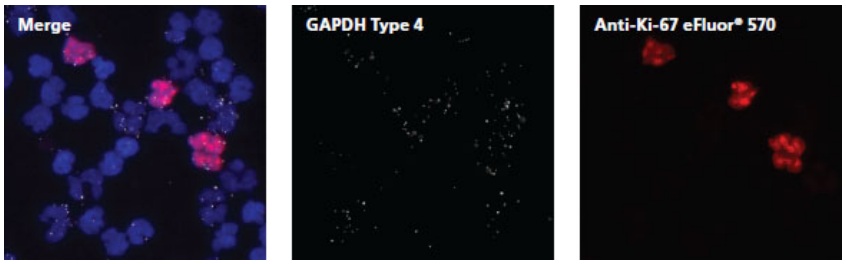


Figure 5. Staining of normal human peripheral blood cells with GAPDH Type 4 Target Probe (white) and Anti-Human Ki-67 eFluorTM 570 (cat. no. 41-5699, red). Nuclei were counterstained with DAPI (blue).

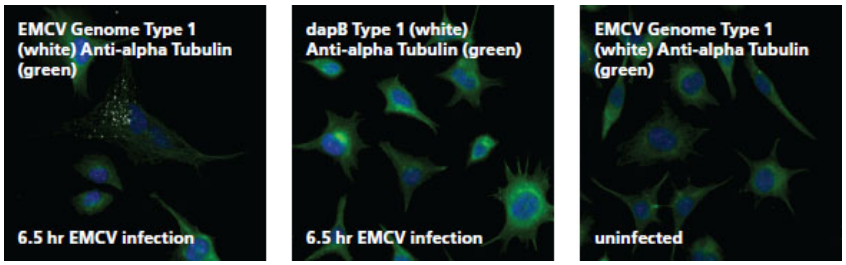


Figure 6. L929 mouse fibroblast cells infected with encephalomyocarditis virus (EMCV) for 6.5 hours were stained with EMCV genome Type 1 Target Probe (left) or E. coli-specific dapB Type 1 Target Probe (center) and Anti-alpha Tubulin Alexa FluorTM 488 (cat. no. 53-4502). Uninfected L929 cells are shown on the right. Nuclei were counterstained with DAPI (blue).

Appendix 3: Instrument and equipment setup guide

This guide illustrates the setup of typical equipment and their specifications for ViewRNA Cell Plus Assay. Consult your equipment manufacturers to ensure that the equipment meets the appropriate specifications. The critical equipment required for this assay includes: incubator, humidity chamber, fluorescence microscope, aspiration system for washing, and temperature validation kit.

Equipment	Specifications
1.Incubator with humidified chamber and temperature probe inside 	1. Validated to maintain 40 ±1°C **Example shown:** Incubator (cat. no. QS0704 or QS0712) with StainTray™ device (cat. no. 44-0404) shown on bottom shelf of incubator
2. Fluorescence microscope equipped with monochrome camera 	1. Mercury arc lamp or LED illumination source 2. Detection optics optimized for DAPI, FITC/Alexa Fluor™ 488, TRITC, Alexa Fluor™ 647, Alexa Fluor™ 546 **Example shown:** Axiovert with MRm camera (Zeiss) A list of filters, optics, and camera specifications
3. Aspiration system for washing 	1. Aspiration rate adjusted to 0.5 mL/sec. Can use in-house vacuum line or vacuum pump **Examples shown:** Vacuum bottle (Argos Technologies, model EV432) Aspirator (Argos Technologies, model EV514)
4. QG ViewRNA™ Temperature Validation Kit 	1. NIST traceable thermometer with temperature probe **Example shown:** Thermo Fisher (cat. no. QV0523)
5. Humidity chamber 	1. Humidified tray in which to place chamber slides, 24-well plates with coverglasses, 96 well plates, cytospin slides **Example shown:** StainTray™ device (cat. no. 44-0404)
6. Optional cytospin 	1. Cytospin in which to attach suspension cells to slide **Example shown:** Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific cat. no. A78300003)

Appendix 4: Temperature validation procedure for incubator

Temperature control is critical for the success of ViewRNA Cell Plus Assay. Improper hybridization temperature will result in high background and/or weak signal. The incubator should be validated before use by following these instructions. This temperature validation procedure is appropriate for the chamber slide, glass slide, and plate-based protocols in this user manual.

Materials required

- Incubator capable of maintaining temperature at 40 ±1°C (cat. no. QS0704 or QS0712)
- QG ViewRNA™ Temperature Validation Kit (cat. no. QV0523)

Calibration Procedure

Step I: Prepare the incubator.

1. Turn on the incubator.
2. Set the temperature to 40°C.
3. Place the staining tray into the incubator near the center of the middle shelf of the incubator.
4. Allow the incubator and staining tray to equilibrate overnight.

Step II: Assemble the temperature validation unit.

1. Insert the battery to activate the digital thermometer.
2. Add water to bottom of humidified chamber or staining tray.
3. Insert the Type-K beaded probe into the digital thermometer, and place the other end of the probe in the humidified chamber, resting on the supports where the chamber slides would be placed (not in the water).
4. Turn on the digital thermometer.

Step III: Measure and adjust the temperature of the incubator.

1. Close the door, making sure that there is sufficient slack in the wiring.
2. Wait 15 minutes for the temperature to equilibrate.
3. Record the temperature. If necessary, adjust the temperature settings so that the digital thermometer reads 40°C. After adjustment, allow the incubator and staining tray to equilibrate. Then recheck the temperature.
4. Repeat step 3 to adjust temperature until the incubator is 40 ±1°C.

Note: We recommend calibrating the incubator at least once a month to ensure accuracy

Step IV: Assess incubator temperature uniformity.

1. Repeat Step III to measure the temperature at multiple positions within the incubator to determine temperature uniformity.

   Note: The temperature for all positions should be 40 ±1°C.

Step V: Assess temperature ramp-up time.

1. Remove the probe from the pre-warmed staining tray from Step III, and allow it to return to room temperature.
2. Open the incubator door for 1 minute and then place the probe back into the staining tray. Close the door. Measure the time needed for the temperature to return to 40°C and monitor the temperature profile during recovery.
3. Repeat Steps V1–2 two more times.

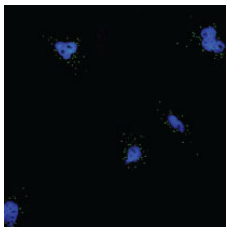
   Note: Do not use the incubator for the assay if it takes more than 10 minutes to return to 40°C or if it overshoots by more than 2°C during recovery.

Appendix 5: Validated cells and recommended positive control genes

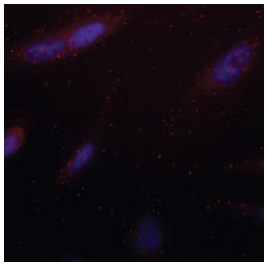
Cell type	Recommended positive control RNA
HeLa	GAPDH, ACTB
BT474	GAPDH, ACTB
C2C12	GAPDH, ACTB
C6	GAPDH, ACTB
F9	GAPDH, ACTB
L929	GAPDH, ACTB
MCF7	GAPDH, ACTB
MDA-MB-231	GAPDH, ACTB
MDA-MB-175	GAPDH, ACTB
PC12	GAPDH, ACTB
THP1	GAPDH, ACTB
SKBR3	GAPDH, ACTB
U937	GAPDH, ACTB
Human peripheral blood mononuclear cells (PBMC)	ACTB
Mouse bone marrow-derived macrophage	GAPDH, ACTB
Mouse splenocytes	GAPDH, ACTB

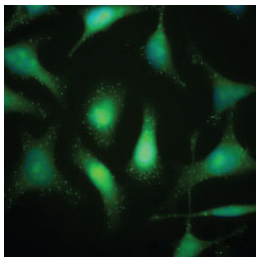
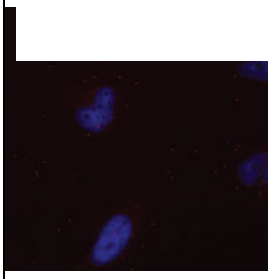
Appendix 6: Troubleshooting

Poor cell retention

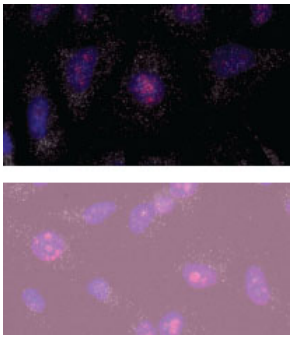
Problem	Possible reasons	Solutions
 Fewer cells than expected	Poor cell adhesion to the glass surface	1. Coat the coverslip surface with extracellular matrices, such as collagen, poly-L-lysine, or poly-D-lysine, to facilitate adhesion. 2. Use the protocol for suspension cells if cell adhesion is extremely poor.
	Cell detachment during aspiration and dispensing of the solutions	1. Avoid aspirating and dispensing solutions directly on top of the cells. 2. Dispense and aspirate reagents at a slower rate from the side of the well.
	Cell density not optimal	1. Cells that are too confluent may be prone to peeling and sloughing off the glass surface, especially during wash steps. 2. For most cells, optimal confluency is between 60% and 85%. Nevertheless, a titration experiment may be required to obtain the optimal density for cell retention and desired staining.

Non-specific signal

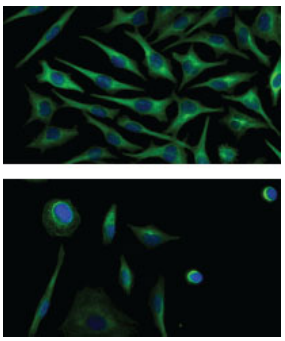
Problem	Possible reasons	Solutions
Non-specific signal adjacent to cell  Dots visible outside cells (not debris)	Variability in slide lots; expired culture slides	Use non-expired lots of chamber slides, coverslips, and/or 96-well plates. If the problem persists, try an alternate lot or a different manufacturer. Please see the suggested platforms and suppliers.
	Insufficient washing	Increase the number of washes or wash volume.
	Non-specific binding of Probe Sets, PreAmplifier, Amplifier, or Label Probes to glass surface	Verify non-specific binding by performing the assay without cells in the presence and absence of coating materials. Use the recommended vendors for plates, chamber slides, and coverglasses.
	Staining with DAPI results in signal outside the nucleus due to the presence of mycoplasma	If working with a cell line, it may be contaminated with bacteria or mycoplasma which will bind DAPI. Use sterile technique for cell culture. Discard or treat mycoplasma-contaminated cultures.
	Grainy staining or clumps of fluorescent material present due to antibody solution	Ensure that the optimal antibody concentration is used and that the antibody is free of any precipitation.
	Samples dried during assay	Ensure that sufficient solution covers the entire sample at all times during the assay.

Problem	Possible reasons	Solutions
Non-specific signal within cells from Antibody or RNA staining  No probe negative control with high background inside the cells	Insufficient washing after fixation and permeabilization	Increase the number of washes or wash volume. Ensure that the wash buffer is adequately spread over the entire sample.rease the number of washes or wash volume. Ensure that the wash buffer is adequately spread over the entire sample.
	Autofluorescence	Some cell lines may exhibit high autofluorescence, particularly around 488 nm. This is a normal biological property of the cell. If this occurs, use the 488 nm channel for detecting a highly expressed gene or protein.
	Primary and/or secondary antibody concentration too high	1. Titrate both the primary and secondary antibodies to determine the optimal concentration. 2. Different cell types may require different concentrations of antibodies
	Non-specific secondary antibody	1. Some secondary antibodies may cross-react with other species, thereby recognizing the primary antibody while also binding non-specifically to the cells in the experiment. 2. To determine the amount of nonspecific binding, include a control sample with only the secondary antibody. 3. Use species-specific (cross-absorbed) and isotype-specific secondary antibodies.
	Fc portion of secondary antibody binds non-specifically to Fc receptors on the membranes of macrophages, monocytes, granulocytes, and a subset of lymphocytes	1. Use secondary antibodies that are F(ab')2 fragments instead of intact antibodies. 2. Use directly-conjugated antibodies and eliminate the use of secondary antibodies in the experiment.
	Non-optimal antibody incubation time	Extended incubation of some antibodies can lead to increased background. Optimize both the antibody concentration and time of incubation.
	Insufficient washing after hybridization	Increase the number of washes or wash volume.
	Samples were allowed to dry	Ensure that sufficient solution covers the entire sample at all times during the assay.
No probe negative control with high background within and outside of the cells	Insufficient washing	1. Ensure the wash buffer is used at room temperature. 2. Follow instructions for wash steps in the protocol. 3. Ensure uniformity in cell resuspension by gentle vortexing.
	Excessive Target Probe used	Dilute Target Probe to a 1:100 dilution.

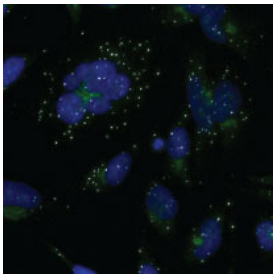
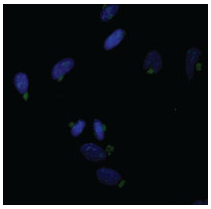
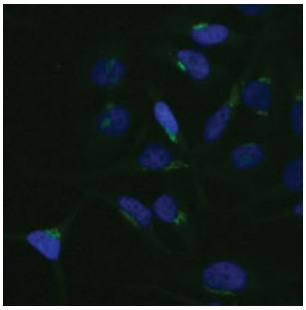
Non-specific autofluorescence

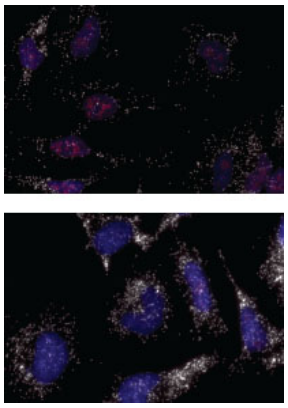
Problem	Possible reasons	Solutions
Autofluorescence  HeLa cells plated on a Fisher Scientific Falcon™ 8-well culture slide (top) or an unsuitable culture slide with autofluorescent properties (bottom) were stained with Anti-Human Ki-67 eFluor™ 660 (cat. no. 50-5699) (pink, nuclear staining) and Let-7a Type 1 Target Probe (white dots). Nuclei were counterstained with DAPI.	Background from slide	1. Under a microscope, ensure that the glass slide being used does not exhibit intrinsic autofluorescent properties. 2. Please see recommended chamber slides.
	Incorrect filters used for visualization of signal	Check filter specifications and replace filters used in the imaging system if necessary.
	Autofluorescence from the nail polish used in the chamber slide, glass coverslip format, or coating material	1. Using a microscope, check nail polish on a glass slide for autofluorescent properties. 2. Ensure the coating material used does not exhibit autofluorescent properties by comparing coated and non-coated glass surfaces with a microscope.

Poor cell morphology

Problem	Possible reasons	Solutions
 Healthy (top) and dying (bottom) L929 mouse fibroblasts stained with Antialpha Tubulin AlexaFluor™ 488 (cat. no. 53-4502). Nuclei were counterstained with DAPI.	Dead or dying cells	1. Characterize cells prior to performing ViewRNA Plus Cell Plus Assay by staining representative cells with a viability dye. Use cultures with greater than 80% viability. 2. Examine culture morphology to identify dead or dying cells. Only use cultures with healthy cells.
	Bubbles in the mounting medium	1. Excessive bubbles in the medium can obscure the cells. Ensure that bubbles are removed during the mounting process. 2. Remove bubbles in the mounting medium prior to attaching the coverslip by gently brushing the bubbles to the edge of the well or coverslip.
	Cell density not optimal	1. Cells that are too confluent can result in poor imaging and signal segregation. 2. For most cells, confluency between 60% and 85% is optimal. However,a careful titration may be needed to identify the optimal density for viability and staining.

Weak or no RNA signal or antibody staining

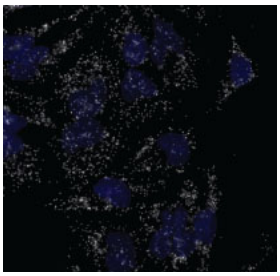
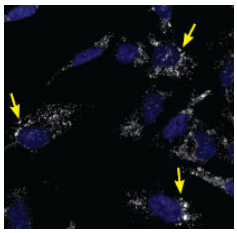
Problem	Possible reasons	Solutions
Weak or no RNA signal  Positive control staining of HeLa cells with Anti-Human Golgin-97 eFluor™ 660 (cat. no. 50-9767) (green) and ERBB2 (HER2/ neu) Type 1 Target Probe (white dots).  Example of applying PreAmplifier Mix and Amplifier Mix in the reverse order, resulting in no RNA signal.  Example of using too high of a temperature for RNA Detection Steps. Probe set, PreAmplifier, Amplifier, and Label Probe incubations were performed in an incubator at 43°C, which resulted in no RNA signal. Nuclei were counterstained with DAPI	Incorrect use of PreAmplifier and/or Amplifier	Ensure that the Probe Set, PreAmplifier Mix, Amplifier Mix, and Label Probe Mix are added in the correct order and to the appropriate samples.
	Inaccurate hybridization temperature	1. Hybridization reactions must be performed at 40 ±1 °C. 2. Minimize traffic in and out of the incubator. The temperature tolerance for this assay is 1-2°C. 3.Ensure that the incubator has been stable at 40°C for at least 12 hours before starting the assay as the temperature may drift during initial incubator setup. 4. Temperature fluctuations will occur when opening the incubator. Minimize time when the incubator is open. 5. Measure the temperature using QG ViewRNA Temperature Validation Kit (cat. no. QV0523).
	Target is not expressed in the cells being assayed	Use a positive control probe set such as a housekeeping gene to validate the assay procedure.
	Target is expressed at very low levels	1. Consider a new high-sensitivity Probe Set that would increase the number of probes. 2. Use a Type 1 probe, which provides a very bright and easy to detect signal.
	Mounting medium is obscuring signal	Unvalidated or non-recommended mounting media can result in reduced signal and increased background, making detection of signal (dots) difficult. For best results, use a recommended mounting medium.

Problem	Possible reasons	Solutions
Weak or no RNA signal (continued)	Samples left too long in ViewRNA™ Cell Plus RNA Wash Buffer solution	Follow protocol for appropriate wash times.
	Photobleaching of fluorescent signals	1. Keep Label Probe Mix in the dark during experiment. 2. After adding Label Probe Mix Solution, protect samples from light. 3. Minimize exposure of samples to fluorescent light when viewing at the microscope.
	Reagents did not come into contact with the cells when using the 24-well plate coverslip protocol	1. Ensure that the cell-seeded cover slips are facing up during the assay. 2. To confirm which side of the coverslip contains the cells, hold the coverslip up to the light and scrape a small surface area with fine tipped forceps.
	Inappropriate microscope setup or operation	Ensure that the microscope used for analysis is in good working order and that your light source, objectives, filters, and exposure times for image acquisition are selected properly (see Major Instrument Requirements).
	RNase Inhibitor not used	Ensure that the 1X PBS with RNase Inhibitor solution in Step 5 contains RNase Inhibitor at a final dilution of 1:100.
Weak or no antibody signal  HeLa cells stained with 10 µg/mL (top; optimal concentration) or 2 µg/mL (bottom, non-optimal concentration) Anti-Human Ki-67 eFluor™ 660 (cat. no. 50-5699) (pink, nuclear staining) and Let-7a Type 1 Target Probe (white dots). Nuclei were counterstained with DAPI.	Non-optimal antibody concentration	Titrate both the primary and secondary antibodies in order to determine the optimal concentration. Different cell types may require different concentrations of antibodies
	Secondary antibody does not recognize the primary antibody.	1. The secondary antibody must be directed against the species in which the primary antibody was raised (e.g., if the primary antibody is raised in mouse, use an anti-mouse secondary antibody). 2. For primary antibodies that are of IgM subtype, it may be preferential to use an isotype-specific secondary antibody (e.g., anti-mouse IgM FITC).
	The secondary antibody or fluorophore-conjugated tertiary reagent was not stored in the dark.	Avoid exposing fluorophore-conjugated antibodies or tertiary reagents to light.
	Primary or secondary antibody not stable.	1. If improperly stored, antibodies can degrade and lose reactivity. Replace the antibody with a new lot. 2. Confirm storage conditions to avoid loss of antibody performance.

Weak or no RNA signal or antibody staining

Problem	Possible reasons	Solutions
Weak or no antibody signal (continued)	Non-optimal antibody incubation time	Antigens that are expressed at lower levels may require longer primary antibody incubation times. Note that this may lead to increased background.
	Antibody does not recognize antigen following fixation and permeabilization	1. Antibodies that require methanol fixation are not compatible with this assay. To test for compatibility, perform the antibody staining portion of the assay using ViewRNA Cell Plus Fixation/ Permeabilization Buffer Set (cat. no. 00-19001, not provided). A list of validated antibodies is available at www.Thermo Fisher.com/viewrna-cell-plus-assay-validated-antibodies. 2. Phosphorylation-specific antibodies from Thermo Fisher that are reported to work in immunocytochemistry are compatible. Please refer to the specific antibody technical data sheet for details. 3. Not all immunocytochemistry (ICC) antibodies will work with the required fixation/permeabilization conditions for this assay. One should perform the ICC portion of this assay for antibody compatibility before proceeding to the RNA detection step to ascertain performance of the specific antibody.
	Mounting medium decreases signal	Use recommended mounting media listed.
	Protein not expressed on cells of interest	1. Confirm that the protein of interest is expressed in the cell type used in the assay. 2. Include a positive control cell line when possible.
	Protein of interest is not abundantly expressed or unable to discriminate weak staining from background staining	1. Compare experimental staining to an isotype control or biological negative control to determine any background present. 2. Consider using a secondary antibody and tertiary detection reagent to amplify the signal. 3. Use a positive control, such as cells known to express the protein of interest.
	Inappropriate microscope setup or operation	Ensure that the microscope is in good working order and that the light source, objectives, filters, and exposure times for image acquisition are selected properly.
	Photobleaching of fluorescent signals	Minimize exposure of samples to fluorescent light when viewing on the microscope. See the "Imaging and analysis" section in Appendix 1.

Variable staining within a sample

Problem	Possible reasons	Solutions
Variable RNA signal or antibody staining  Sample of HeLa cells stained with Let-7a Type 1 Target Probe (white dots) thatdid not dry out.  Sample was allowed to dry for 5 minutes before fixation/ permeabilization, which resulted in unexpected aggregation of the RNA signal (yellow arrows). Nuclei were counterstained with DAPI.	Samples were allowed to dry	Ensure adequate coverage of solution over the entire sample at all times during the assay.
	Insufficient mixing of reagents	1. Pre-warm all hybridization buffers to dissolve any precipitation prior to use. 2. Briefly vortex all working hybridization solutions before use.
	Uneven distribution of antibody	1. Antibody concentrated in one location can cause increased background in that area and decreased signal in other areas of the sample. 2. Mix antibodies thoroughly prior to overlaying onto the cells. Tip slide with chambers to allow even distribution of antibody over the entire area.

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **thermofisher.com/support**.

Corporate entity: Life Technologies | Carlsbad, CA 92008 USA | Toll Free in USA 1.800.955.6288