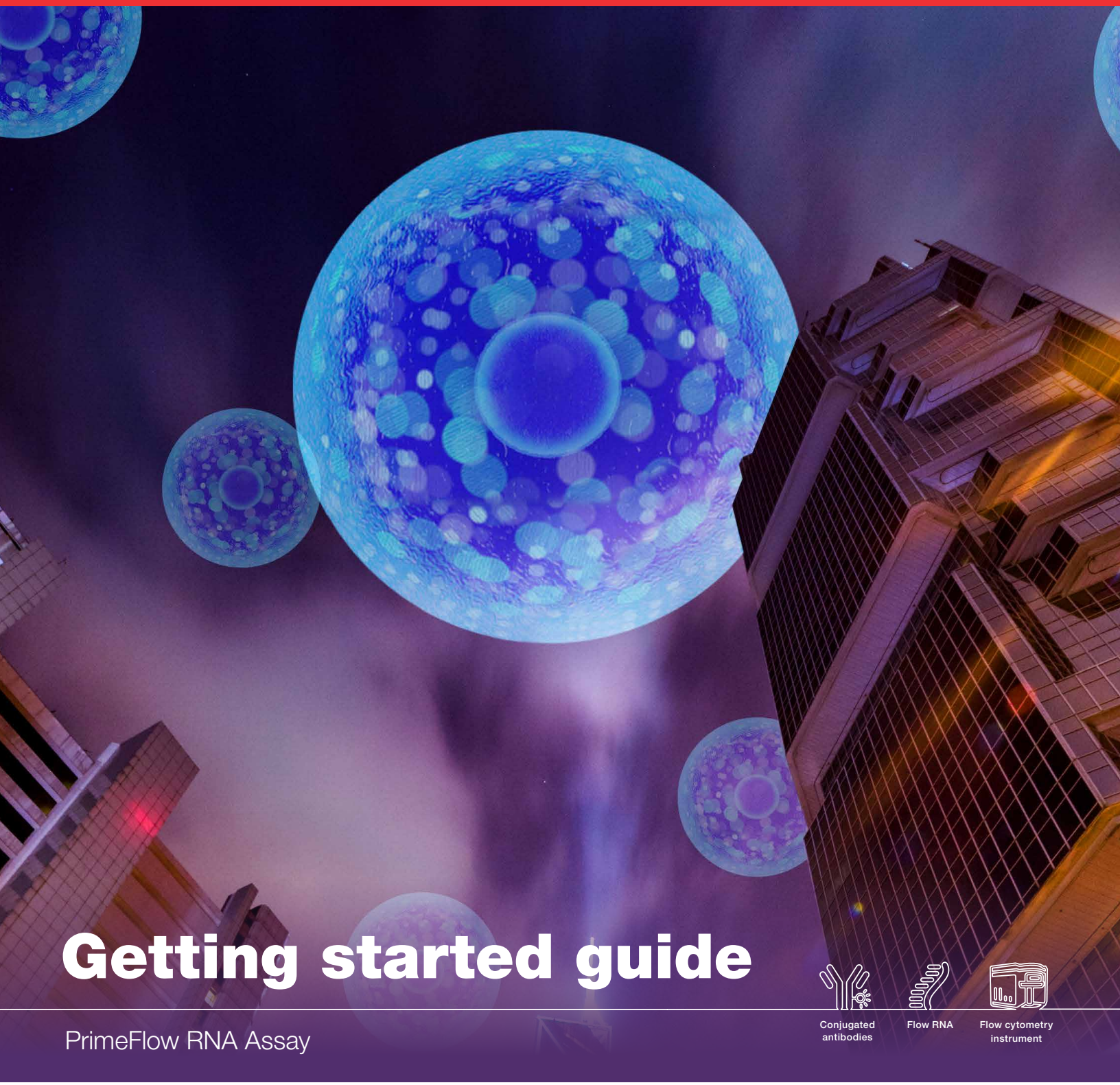
invitrogen



Getting started guide

PrimeFlow RNA Assay

ThermoFisher SCIENTIFIC

Contents

1. Introduction

What is the PrimeFlow RNA Assay?

The Invitrogen™ PrimeFlow™ RNA Assay employs fluorescence *in situ* hybridization (FISH) with branched-DNA (bDNA) signal amplification for the simultaneous detection of up to four RNA targets. This assay can also be used in combination with immunolabeling of both cell-surface and intracellular proteins using fluorophore-conjugated antibodies and detection by flow cytometry. The PrimeFlow RNA Assay can detect messenger RNA (mRNA), long noncoding RNA (lncRNA), and microRNA (miRNA).

What is bDNA signal amplification?

bDNA signal amplification is achieved through sequential hybridization steps with preamplifiers, amplifiers, and fluorophore-conjugated label probes (Figure 1). A fully assembled signal amplification "tree" has 400 label-probe binding sites. When all target-specific oligonucleotides in the probe set bind to the target RNA transcript, 8,000- to 16,000-fold amplification can be achieved.

The purpose of this guide is to provide all the necessary information to help you get started with the PrimeFlow RNA Assay and walk you through the design and workflow of an experiment using the PrimeFlow RNA Assay.

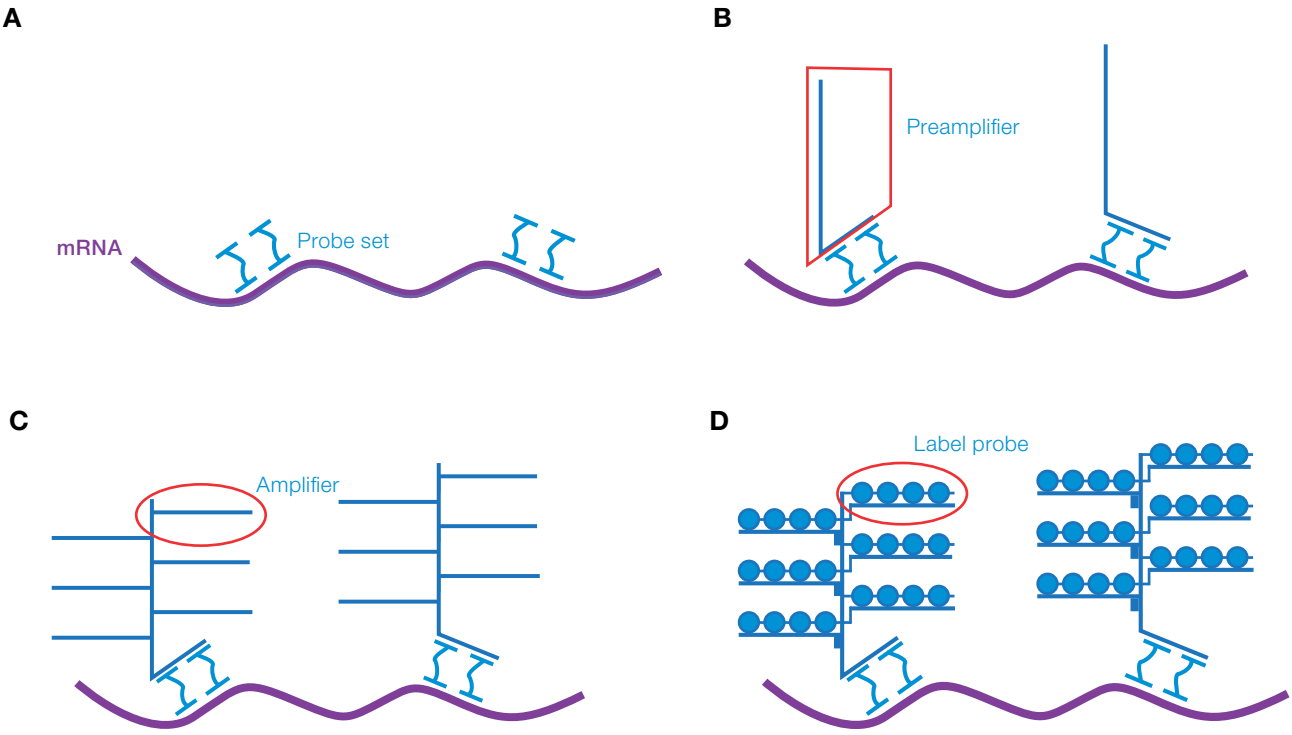


Figure 1. Signal amplification by sequential hybridization of oligonucleotides. (A) Gene-specific probe sets are hybridized to target RNA transcripts. **(B)** Preamplifier ("trunk") binds to a probe set. **(C)** Amplifiers ("branches") bind to multiple sites on the preamplifier. **(D)** Fluorophore-conjugated label probes ("leaves") bind to multiple sites on the amplifiers.

2. Workflow summary

PrimeFlow RNA Assay workflow summary

In the PrimeFlow RNA Assay workflow, cells are first labeled with cell-surface antibodies, fixed and permeabilized, and then labeled with intracellular antibodies. Next, these cells are sequentially hybridized with probes specific to the RNA targets, and hybridized targets are detected after bDNA signal amplification.

The PrimeFlow RNA Assay currently offers four unique amplifications of bDNA structures that allow simultaneous measurement of up to four different RNA targets for multicolor flow cytometry analysis.

Protocol flowchart

Day 1

Sample preparation

Antibody fixation and permeabilization

Harvest cells

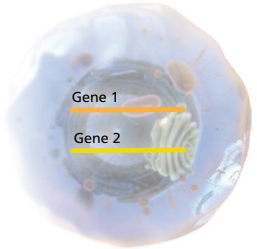
Prepare single-color compensation controls*

Stain cells with an eBioscience Fixable Viability Dye

Stain cells with antibodies to cell-surface antigens

Fix and permeabilize cells in the presence of RNase inhibitors

Stain cells with antibodies to intracellular antigens (optional)



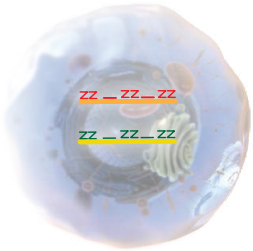
Target hybridization

Target probe hybridization

Add target probes to cell suspension

Incubate at 40°C for 2 hr

ZZ Gene-specific
ZZ label extenders (LE)



* If using compensation beads provided in the kit, the preparation should be done on day 2.

General precautions on experiments

- Prepare buffers (PrimeFlow RNA Fixation Buffers 1 and 2, and RNA Permeabilization Buffer with RNase Inhibitors) each time as necessary for sample preparation. Do not prepare buffer in advance to cover multiple experiments for different days.
- Control the incubator temperature in target hybridization steps (40 ± 1°C) accurately.
- When diluting and adding antibodies, probes, and labeling reagents in the sample preparation, target hybridization, and signal amplification steps, place the tip directly onto the liquid surface to avoid making bubbles in the liquid.
- During permeabilization of cells, take precautions to avoid precipitation after adding PrimeFlow Permeabilization Buffer with RNase Inhibitors to samples by following these steps:
  - Centrifuge > discard supernatant > suspend carefully in the residual 100 µL volume (using markings on the tube as a guide and checking to make sure the solution becomes cloudy as uniformly as possible).
- After the target probe hybridization step, be sure to use the specialized tube attached to the kit.

Day 2

Signal amplification | **Detection**

Signal amplification

Add PrimeFlow RNA PreAmp mix to cell suspension

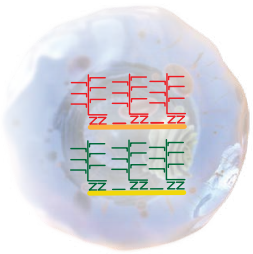
Incubate at 40°C for 1.5 hr

Add PrimeFlow RNA Amp mix to cell suspension

Incubate at 40°C for 1.5 hr

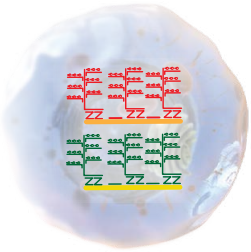
Add label probes to cell suspension

Incubate at 40°C for 1 hr

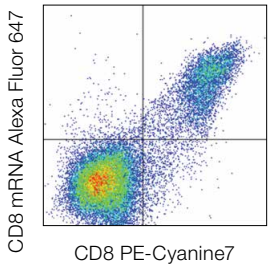


Detection and analysis

Perform Attune NxT Flow Cytometer setup, compensation, and analysis



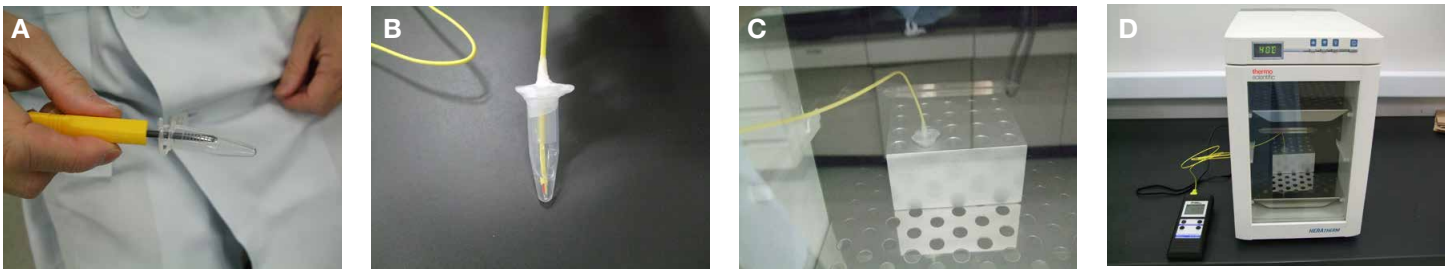
Process cells using a flow cytometer



3. Things to consider

Precisely control the temperature of incubator

The assay is highly dependent on temperature. Ensure that the incubator holds the temperature at 40 ± 1°C. A significant reduction in signal will result from temperature deviations greater than 1°C. To ensure the correct temperature control in samples, follow the steps for setting up the Invitrogen™ ViewRNA™ Temperature Validation Kit (Figure 2).



Make a hole through the cap of a 1.5 mL tube.

Place electric sensor from the ViewRNA Temperature Validation Kit through the tube.

Put a tube with a code to the metal heat block in an incubator.

Overview of setup.

Figure 2. Steps for setting up the ViewRNA Temperature Validation Kit.

Select compatible dyes for cell-surface and/or intracellular labeling of proteins

Compatible dyes

- Organic fluorescent dyes (Invitrogen™ FITC, eBioscience™ eFluor™ 450, eFluor™ 506, eFluor™ 660, Alexa Fluor™ 700, Brilliant™ Violet, Super Bright dyes, etc.)
- Most fluorescent proteins (Invitrogen™ PE, PE-eFluor™ 610, PE-Cyanine5, PE-Cyanine5.5, PE-Cyanine7, APC, APC-eFluor™ 780, etc.)

Incompatible dyes

- Invitrogen™ eBioscience™ PerCP-Cyanine 5.5, PerCP-eFluor™ 710
- Invitrogen™ Qdot™ nanocrystal, eBioscience™ eVolve dye–conjugated antibodies

Make sure you have the right buffer for your target

If your target is microRNA, Invitrogen™ PrimeFlow™ microRNA Pretreatment Buffer (Cat. No. 88-18006) is recommended. This reagent helps ensure that you get improved signal and better sensitivity for miRNA.

Make sure you have V-bottom plates

When the assay is processed in a 96-well plate, V-bottom plates are recommended; do not use flat-bottom plates. A modified protocol for the use of polystyrene 96-well plates is available in Appendix 7 of the PrimeFlow RNA Assay User Manual.

Make sure you use a swinging-bucket centrifuge

To maximize cell recovery, use a swinging-bucket centrifuge. Using fixed-angle centrifuge will result in significant cell loss.

Determine the best probe set for your target

- Four types of probe sets are currently available for RNA detection
- Select different types of probe sets depending on the expression level of RNA (Table 1)
- For multiplex analysis with immunolabeling of both cell-surface and intracellular proteins, use fluorophore-conjugated antibodies

Table 1. Probe sets for RNA detection.

Probe type/fluorescent label	Laser	Channel	Expression level of detected gene	Sensitivity of the probe
Type 1/Alexa Fluor 647	633 (red)	APC, Alexa Fluor 647, eFluor 660	Low	
Type 10/Alexa Fluor 568	561 (yellow)	PE-Texas Red, PE-eFluor 610, Alexa Fluor 568	Low	
Type 4/Alexa Fluor 488	488 (blue)	FITC, Alexa Fluor 488	Medium to high	
Type 6/Alexa Fluor 750	633 (red)	APC-Cy7, APC-eFluor 780, Alexa Fluor 750	Medium to high	

Set controls to obtain clear results

The following controls are recommended to obtain clear results. Figure 3 demonstrates an example of control and sample placement.

- Positive-control probe sets (RPL13A for human, ACTB for mouse, etc.)
- Negative-control probe sets (samples with the target-specific probe omitted, or samples labeled with a probe against a target not expressed in the cells of interest)
- Single-color compensation samples
- Fluorescence minus one (FMO) controls

Sample #	FVD eFluor 450	Protein			RNA			
		CD3-SB600	CD8-PE	CD14-PE-Cy7	Tbet-Alexa Fluor 647	CD8-Alexa Fluor 488	ACTB-Alexa Fluor 750	
1								← Positive control
2								Single-color compensation sample
3								
4								
5								
6								
7								
8								
9								FMO controls
10								
11								
12								
13								
14								
15								
16								

Figure 3. Example of controls that are required for an experiment having a viability marker along with detection of three proteins and three RNA targets.

4. Checklist—what you'll need

Reagents

- ☐ PrimeFlow RNA Assay Kit
- ☐ PrimeFlow target probe set (Find targets at **thermofisher.com/primeflow**)
- ☐ Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer

Optional

- ☐ For protein detection: fluorescently labeled antibodies (Find at **thermofisher.com/antibody**)
- ☐ For viability check: viability marker (fixable viability dyes, Invitrogen™ LIVE/DEAD™ fixable dyes, etc.)
- ☐ For microRNA detection: Invitrogen™ PrimeFlow™ microRNA Pretreatment Buffer

Controls

- ☐ Positive-control probe sets (RPL13A for human, ACTB for mouse, etc.)
- ☐ Negative-control probe sets (samples with the target-specific probe omitted, or samples labeled with a probe against a target not expressed in the cells of interest)
- ☐ Single-color compensation samples
- ☐ Fluorescence minus one (FMO) controls

Instruments

- ☐ Flow cytometer:
  - Three lasers: blue (488 nm), yellow-green (561 nm), and red (633 nm or similar)
  - Detection optics optimized for FITC, PE-eFluor 610 (PE-Texas Red), APC, and APC-eFluor 780 (APC-Cyanine7)
- ☐ Incubator:
  - Capable of maintaining temperature at 40 ± 1°C
- ☐ Metal heat block for 1.5 mL microcentrifuge tube, placed inside the validated incubator
- ☐ ViewRNA Temperature Validation Kit (Cat. No. QV0523)
- ☐ Swinging-bucket centrifuge with adaptors for 15 mL conical tubes and 1.5 mL microcentrifuge tubes
- ☐ Aspirator system for washing—aspiration rate adjusted to 0.5 mL/sec; can use in-house vacuum line or vacuum pump

Optional

- ☐ For 96-well plate assay: V-bottom shape 96-well plates

5. Frequently asked questions (FAQs)

Q: Which species are compatible with the PrimeFlow RNA Assay?	**A:** We have tested the PrimeFlow RNA Assay on mouse and human cells. The assay is expected to work on other mammalian species and has been reported to work in some nonmammalian species. However, this should be determined empirically.
Q: When using the PrimeFlow RNA Assay kit with the PrimeFlow microRNA Pretreatment Buffer, can we combine miRNA and mRNA staining?	**A:** Yes, it is possible to perform any combination of miRNA and mRNA up to a total of four targets.
Q: Can you detect rare populations in a heterogeneous mix of cells using the PrimeFlow RNA Assay?	**A:** This assay can be used to detect cell populations that represent greater than 1% of the total cells.
Q: What is the minimum length of targeted sequence needed to design the probe sets for use with the PrimeFlow RNA Assay?	**A:** For optimal sensitivity, a minimum of 1 kb is recommended to design target probe sets with sufficient sensitivity for medium- and high-expressing genes. For low-expressing genes, a minimum of 2 kb of sequence is recommended.
Q: When using the PrimeFlow RNA Assay, what is the sensitivity (limit of detection) for RNA staining?	**A:** Under fully optimized conditions, we estimate that 10–20 copies can be detected per cell for Type 1 or Type 10; and about 30 copies per cell for Type 4 or Type 6. The actual sensitivity may vary depending on the specific target.
Q: Can you design custom probes?	**A:** By request, PrimeFlow probe sets can be designed and synthesized at no additional cost. Please provide the following information when ordering: accession number (including version or GI number) or RNA sequence for the target of interest, species, gene name or symbol, PrimeFlow probe set type, and any special design requirements. Please contact **flowsupport@thermofisher.com** for more information.
Q: What can I use the PrimeFlow RNA Assay for?	**A:** It can be used for the following key application areas: • Probing mRNA when an antibody to the protein target is unavailable • Analyzing mRNA expression at the single-cell level • Comparing RNA and protein kinetics in the same cell • Detecting miRNA • Detecting viral RNA in infected cells • Verifying single-cell RNA sequencing results
Q: Is the PrimeFlow RNA Assay compatible with live- and dead-cell determination?	**A:** Yes.
Q: Is the PrimeFlow RNA Assay compatible with extracellular and intracellular staining?	**A:** Yes.

invitrogen

6. Ordering information

Product	Quantity	Cat. No.
PrimeFlow probe sets	Go to step 4 under the ordering information at **thermofisher.com/primeflow**	
PrimeFlow RNA Assay Kit* • PrimeFlow RNA Tubes • PrimeFlow RNA Fixation Buffer 1A • PrimeFlow RNA Fixation Buffer 1B • PrimeFlow RNA Permeabilization Buffer (10X) • PrimeFlow RNA Fixation Buffer 2 (8X) • PrimeFlow RNA Wash Buffer • PrimeFlow RNA Target Probe Diluent • PrimeFlow RNA PreAmp Mix • PrimeFlow RNA Amp Mix • PrimeFlow RNA Label Probe Diluent • PrimeFlow RNA Storage Buffer • PrimeFlow RNase Inhibitors (100X) • PrimeFlow Compensation Kit • IC Fixation Buffer	40 tests 100 tests	88-18005-204 88-18005-210
ViewRNA Temperature Validation Kit	1	QV0523
eBioscience Flow Cytometry Staining Buffer	200 mL	00-4222-57
Optional: Is your target microRNA? This buffer helps ensure you get improved signal and better sensitivity for miRNA.		
PrimeFlow microRNA Pretreatment Buffer	100 tests	88-18006
Optional: Will the assay be processed in a 96-well plate?		
PrimeFlow 96-well plate	10 packets	44-17005-46

* The PrimeFlow RNA Assay Kit provides a complete buffer system, compensation kit, and reagents for detecting up to four RNA transcripts in mammalian cells optionally labeled with antibodies that recognize cell-surface or intracellular proteins.

7. Additional resources to help you get started

Resource

- Use our Custom Branched DNA Probe Set Tool at **thermofisher.com/custom-bDNA**
- Find fluorescently labeled antibodies for protein detection at **thermofisher.com/antibody**
- Learn more about the Invitrogen™ Attune™ NxT Flow Cytometer at **thermofisher.com/attune**
- See publications citing the use of the PrimeFlow RNA Assay at **thermofisher.com/primeflowpublications**
- View webinars about the PrimeFlow RNA Assay at **thermofisher.com/primeflow**

Find out more at **thermofisher.com/primeflow**

ThermoFisher SCIENTIFIC