Double vision: Simultaneous visualization of protein and RNA targets

ViewRNA Cell Plus Assay for antibody labeling and *in situ* hybridization in individual cells.

The complex interactions between transcription, translation, and posttranslational modifications are hidden from view in typical endpoint assays that measure either RNA or protein levels. While *in situ* hybridization (ISH) provides a method for examining the levels of specific RNA transcripts in individual cells, and immunocytochemistry (ICC) utilizes antibodies to visualize the localization of specific proteins (and protein modifications), the ability to simultaneously observe RNA and protein in a single cell has been thwarted by the incompatibility of ICC and ISH protocols and the inherent lack of sensitivity of traditional ISH methods for detecting low-abundance RNA species. Further complicating these analyses, commonly used ISH protocols have limited multiplexing capability, which further constrains its compatibility with other cell assays.

The Invitrogen™ ViewRNA™ Cell Plus Assay is a novel method that combines ViewRNA ISH technology—a proprietary fluorescent *in situ* hybridization (FISH) and sequential branched DNA (bDNA) amplification technique—with antibody-based protein detection to simultaneously visualize RNA and protein in individual cells (Figure 1). The ViewRNA Cell Plus Assay enables detection of up to three RNA targets (with single-molecule sensitivity thanks to the amplification protocol) in combination with immunophenotyping for cell-surface and intracellular proteins using both indirect and direct ICC, allowing an in-depth characterization of specific cell subpopulations.

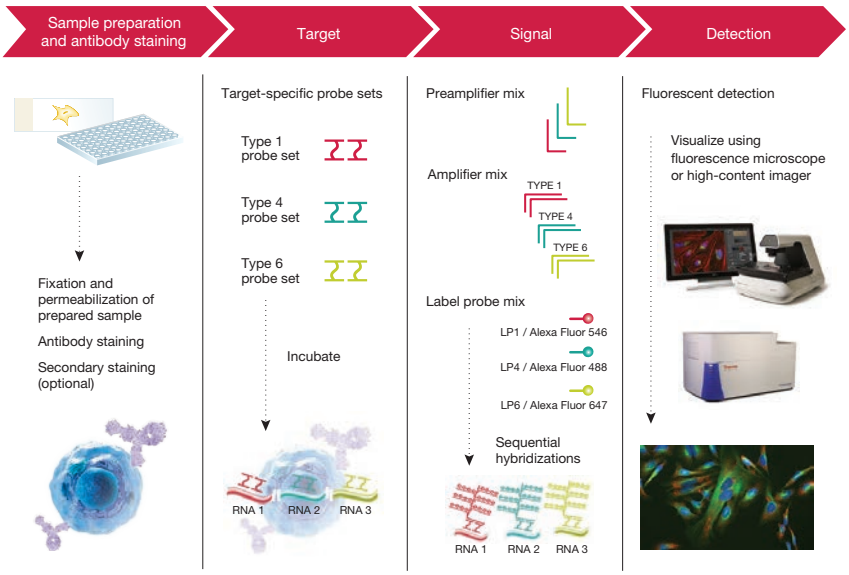

Figure 1. The ViewRNA Cell Plus Assay workflow. The workflow for the Invitrogen™ ViewRNA™ Cell Plus Assay Kit (Cat. No 88-19000-99) starts with fixation, permeabilization, and antibody labeling, followed by hybridization with RNA-specific target probes. This hybridization is then detected after branched DNA (bDNA) signal amplification using preamplifiers, amplifiers, and label probes. Labeled cells are analyzed on a fluorescence microscope or high-content imager.

A closer look at ViewRNA technology

Traditional FISH techniques that use large oligonucleotide sequences labeled with one to five fluorophores are generally limited by high background and low sensitivity due to non-specific binding and insufficient signal amplification. ViewRNA ISH assays incorporate a proprietary probe set design and bDNA signal amplification technology. A target-specific probe set of approximately 5 to 40 oligonucleotide pairs hybridizes to the target RNA of interest. An individual probe pair contains two oligonucleotides that are designed to bind adjacent to each other on the RNA transcript for bDNA signal amplification to take place. Signal amplification is achieved through a series of sequential hybridization steps with preamplifiers, amplifiers, and the fluorophore-conjugated label probes. The preamplifier molecules confer an additional level of specificity because they will hybridize to the RNA target only after both members of the oligonucleotide target probe set have bound to their target sequence. Multiple amplifier molecules subsequently hybridize to their respective preamplifier molecules. Finally, label probe oligonucleotides, which are conjugated to a fluorescent dye, hybridize to their corresponding amplifier molecules. 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.

As compared with traditional FISH, the ViewRNA ISH assays produce greater →

specificity, lower background, and higher signal-to-noise ratios. For example, to create images with similar discernible spots, traditional FISH techniques can require a 600-fold longer exposure and a 100-fold greater camera gain than ViewRNA ISH assays. Thus, under equivalent imaging conditions, the ViewRNA ISH assay is 100 times brighter than traditional FISH assays, with a two to three times higher signal-to-noise ratio [1].

The ViewRNA Cell Plus Assay workflow

The ViewRNA Cell Plus Assay workflow consists of four steps: 1) fixation, permeabilization, and antibody staining (with optional secondary antibody signal amplification), 2) RNA target probe hybridization, 3) signal amplification using bDNA constructs, and 4) detection using a standard epifluorescence microscope or high-content imaging system. Figure 1 depicts hybridization with three different target probe sets for multiplex detection of three target RNAs.

In step 1, adherent cells or centrifuged suspension cells are fixed and permeabilized prior to detection of surface or intracellular proteins. The cells are then stained with unconjugated, biotinylated, or fluorescent primary antibodies, followed by fluorescent secondary reagents if needed. In our development of the ViewRNA Cell Plus Assay, we assessed a broad panel of ICC-compatible antibodies specific for structural proteins, transcription factors, organelles, and surface markers. Our results show that signal intensity and resolution produced by the ViewRNA Cell Plus Assay is comparable to that produced by standard ICC protocols. Antibody compatibility with this assay should be verified using the Invitrogen™ ViewRNA™ Cell Plus Fixation/Permeabilization Buffer Set, and antibodies should be titrated for optimal performance, as in all ICC experiments. After an additional fixation step, the cells are ready to proceed through the RNA hybridization and signal amplification steps.

The RNA hybridization step (step 2) and subsequent bDNA amplification (step 3) require an RNA-specific target probe set containing 5 to 40 oligonucleotide pairs that hybridize to adjacent regions in the target RNA. Three types of target probe sets are currently available for RNA detection, namely Type 1, which is labeled with Invitrogen™ Alexa Fluor™ 546 dye (Ex/Em = 556/573 nm), Type 4, which is labeled with Invitrogen™ Alexa Fluor™ 488 dye (Ex/Em = 495/519 nm), and Type 6, which is labeled with Invitrogen™ Alexa Fluor™ 647 dye (Ex/Em = 650/668 nm). When detecting more than one RNA target in a single sample, each target probe set must be a unique type to differentiate its signal from the others. Once the cells have been processed by the ViewRNA Cell Plus Assay, the data can be collected and analyzed on an epifluorescence microscope or high-content imaging system equipped with the appropriate filter sets (step 4).

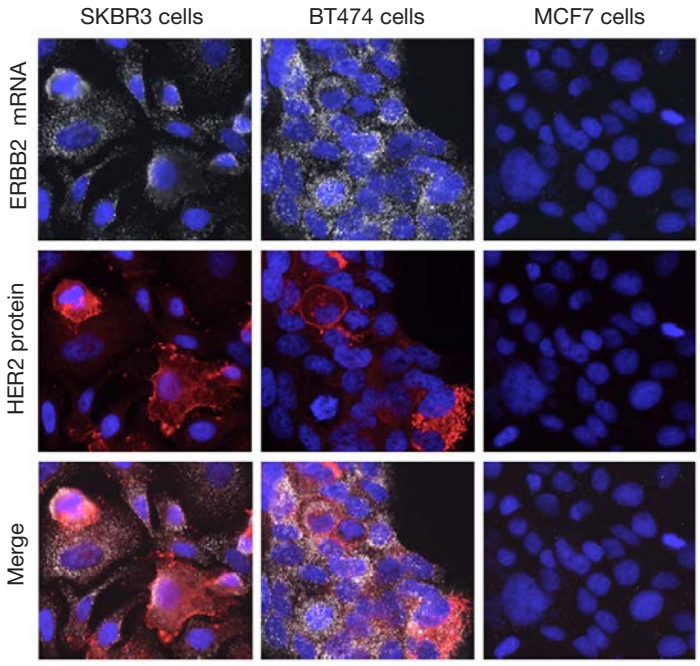

Figure 2. Use of the ViewRNA Cell Plus Assay to examine ERBB2 mRNA and HER2 protein expression levels simultaneously in three different human breast cancer cell lines. Using the Invitrogen™ ViewRNA™ Cell Plus Assay Kit (Cat. No. 88-19000-99), we labeled the HER2 protein with an Invitrogen™ eBioscience™ eFluor™ 570 anti–human ErbB2/HER2 antibody (Cat. No. 41-9757-82), and ERBB2 mRNA expression was assessed with a Type 6 (Alexa Fluor 647) label probe specific for ERBB2 mRNA. We observed ERBB2 mRNA (white) localized to the cytoplasm while HER2 protein (red) is found predominantly in the membrane in both SKBR3 and BT474 cells. MCF7 cells, which do not show ERBB2 gene amplification, were negative for ERBB2 mRNA and HER2 protein expression. Nuclei were stained with DAPI nucleic acid stain (blue).

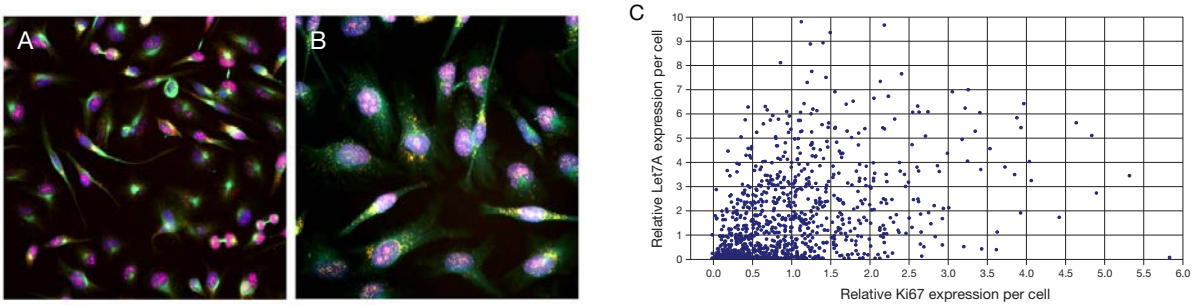

Figure 3. Use of the ViewRNA Cell Plus Assay to examine Let7A mRNA and Ki67 protein expression levels simultaneously in NIH/3T3 cells. After labeling NIH/3T3 cells with Invitrogen™ eBioscience™ eFluor™ 660 anti-Ki67 antibody to visualize Ki67 protein expression (purple), cells were hybridized with a Type 6 (Alexa Fluor 647) label probe specific for Let7A mRNA expression (yellow to red) using the Invitrogen™ ViewRNA™ Cell Plus Assay Kit (Cat. No. 88-19000-99). The cells were counterstained for tubulin (green, labeled with an anti–α-tubulin primary antibody and an Invitrogen™ Alexa Fluor™ 488 secondary antibody) and nuclei (blue, detected with DAPI nucleic acid stain). The cells were imaged using either **(A)** the Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective or **(B)** the Thermo Scientific™ CellInsight™ CX7 High-Content Analysis Platform with a 40x objective. Ki67 expression (purple) is limited to the nucleus, whereas Let7A expression (yellow to red) is primarily seen in the cytoplasm. **(C)** Using Thermo Scientific™ HCS Studio™ Cell Analysis Software available with the CellInsight CX7 instrument, the relative expression of Let7A/cell was plotted vs. the relative expression of Ki67/cell (cell number = 994). This scatter plot indicates a possible relationship between expression of Let7A microRNA and the protein Ki67.

ViewRNA Cell Plus Assay in action

Although studies have shown that the correlation between levels of RNA and protein products varies widely, a general relationship between transcription level and protein presence can be used to assess the accuracy and specificity of the ViewRNA Plus Cell Assay. To show the relationship between transcription and translation, ERBB2 RNA and HER2 protein expression were visualized in several breast cancer cell lines of known HER2 status (Figure 2). SKBR3 and BT474 cells are characterized as $HER2^+$, while MCF7 cells are $HER2^-$. The data in Figure 2 demonstrate that ERBB2 mRNA is present in all cells within the SKBR3 and BT474 cultures; however, expression of HER2 protein is heterogeneous within the cell population and localizes primarily to the cell membrane, with some protein detected in the cytoplasm.

Figure 3 shows the use of the ViewRNA Cell Plus Assay for the detection of both Let7A microRNA, which is known to play a role in cell proliferation, and the protein Ki67, a proliferation marker. The expression levels of specific microRNAs, which are noncoding RNA, are particularly difficult to measure, and the bDNA amplification method used in the ViewRNA Cell Plus Assay is one of the few methods sensitive enough to detect microRNA. Cells labeled with the ViewRNA Cell Plus Assay Kit can be imaged using an epifluorescence microscope such as the Invitrogen™ EVOS™ FL Auto 2 Imaging System, or with a high-content analysis instrument such as the Thermo Scientific™ CellInsight™ CX7 High-Content Analysis Platform, which facilitates the analysis of the relative expression and colocalization of microRNA and protein at the single-cell level.

Learn more about the ViewRNA Cell Plus Assay Kit

The ViewRNA Cell Plus Assay combines highly sensitive ISH for visualizing RNA at single-molecule sensitivity with ICC for protein detection in individual cells. This assay enables simultaneous detection of up to three RNA targets in combination with immunophenotyping for cell-surface and intracellular proteins using both indirect and direct ICC. The combined visualization of RNA and protein expression at the single-cell level is a valuable tool for correlating RNA and protein levels in single cells, analyzing sample heterogeneity, tracking viral RNA and protein, and a variety of other areas of cell biology research.

The ViewRNA Cell Plus Assay Kit contains the key reagents needed to conduct the assay; target-specific probe sets for genes of interest and antibodies for protein detection are sold separately. For more information, including recent publications and customer webinars as well as available probe sets and ordering guidelines, visit **thermofisher.com/viewrnacellplusbp76**. ■

Reference

1. Battich N, Stoeger T, Pelkmans L (2013) *Nat Methods* 10:1127–1133.

Product	Quantity	Cat. No.
ViewRNA™ Cell Plus Assay Kit	1 kit	88-19000-99
ViewRNA™ Cell Plus Cytospin Module Kit	1 kit	88-19002-11
ViewRNA™ Cell Plus Fixation/Permeabilization Buffer Set	1 kit	00-19001