

# Visualize single-molecule sensitivity

## A novel assay combining FISH and ICC

### Introduction

Immunocytochemistry (ICC) utilizes antibodies to visualize the localization of specific proteins in cells, while *in situ* hybridization (ISH) is a research tool for examining nucleic acid targets in cells. ISH enables researchers to determine the presence, localization, and/or changes in RNA levels. The ability to simultaneously visualize protein and RNA species in individual cells is necessary to address important biological questions. Additionally, studying both protein and RNA can be useful in cases where expression levels vary between mRNA and protein, as well as in studies that require the detection of posttranslational modifications. However, development of a combined technique has proved challenging due to the incompatibility of ICC and ISH protocols. Furthermore, traditional ISH methods have lacked sensitivity in detecting low-abundance RNA species and have limited multiplexing capability.

The Invitrogen™ ViewRNA™ Cell Plus Assay is a novel method that combines ICC with the ViewRNA technology, a proprietary fluorescence *in situ* hybridization (FISH) and sequential branched-DNA (bDNA) amplification technique, to visualize RNA with single-molecule sensitivity along with protein 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 immunocytochemistry, which allows for in-depth characterization of specific cell subpopulations.

The combined visualization of RNA and protein expression at the single-cell level is a valuable tool that has broad implications for advancing research across multiple fields of biology, such as:

- Analysis of sample heterogeneity
- Correlation between RNA and protein kinetics within the same cell
- Investigation of noncoding RNA expression, including microRNA (miRNA) and long noncoding RNA (lncRNA) in cellular subsets
- Analysis of mRNA expression levels when antibody is unavailable
- Tracking, visualization, and detection of viral RNA
- High-throughput compound screening

### Assay technology

FISH is a powerful technique that enables the specific detection of target RNA localization in fixed cells. Traditional FISH techniques using large oligonucleotide sequences labeled with 1–5 fluorophores are generally limited by high background and low sensitivity due to nonspecific binding and insufficient signal amplification.



## ViewRNA Cell Plus Assay principle

The assay workflow consists of several steps: 1) fixation and permeabilization, 2) antibody staining and optional secondary antibody signal amplification, 3) RNA target probe hybridization, 4) signal amplification using bDNA constructs, and 5) detection using a standard epifluorescence microscope or high-content imager. For simplicity, detection with only one of the 20–40 oligonucleotide target probe pairs per target RNA is depicted (Figure 2).

### Antibody staining, fixation, and permeabilization

Adherent cells or spun-down suspension cells are fixed and permeabilized prior to detection of surface or intracellular proteins. The cells are then stained with unconjugated and biotinylated antibodies followed by fluorescently labeled secondary reagents or fluorophore-conjugated antibodies to detect surface or intracellular

proteins, such as transcription factors, cytokines, or signaling molecules. After an additional fixation step, the cells are ready to proceed through the RNA hybridization and signal amplification steps.

### Target hybridization

A target-specific probe set contains 5–40 oligonucleotide pairs that hybridize to specific regions across the target RNA sequence. Subsequent signal amplification steps require that each oligonucleotide pair binds to the target RNA in adjacent positions. Three types of probe sets are currently available for RNA detection: type 1, which is labeled with Invitrogen™ Alexa Fluor™ 546 dye; type 4, which is labeled with Invitrogen™ Alexa Fluor™ 488 dye; and type 6, which is labeled with Invitrogen™ Alexa Fluor™ 647 dye (Figure 3). When detecting more than one RNA target in a single sample, each probe set must be a unique type to differentiate its signal from the others.

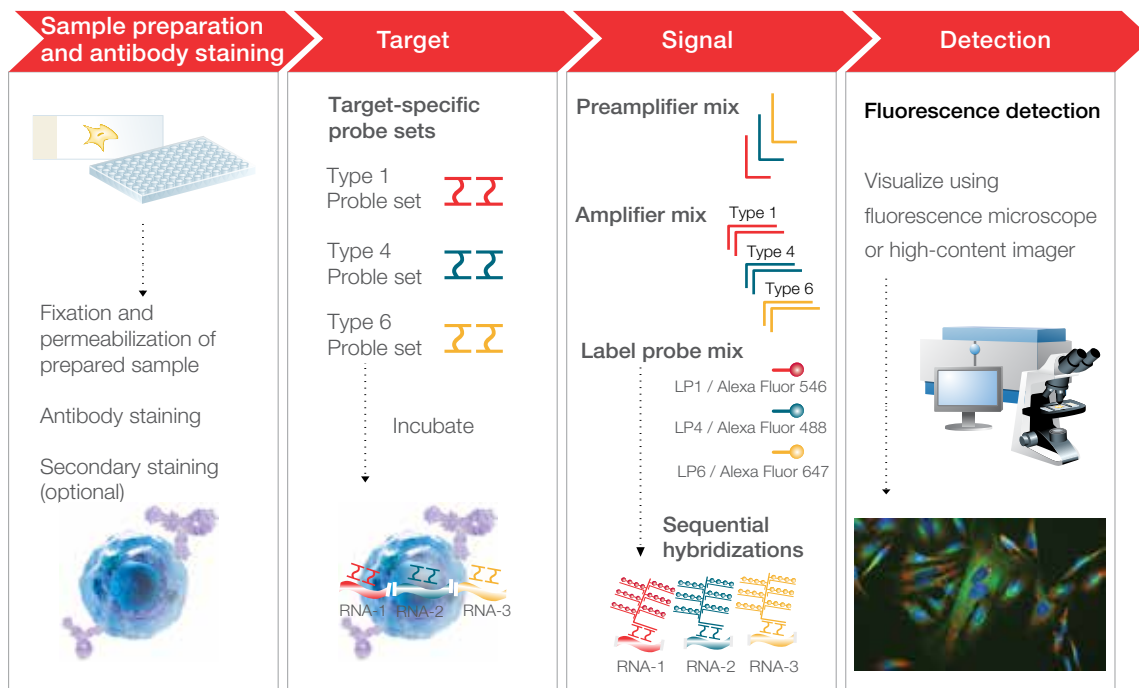


Figure 2. ViewRNA Cell Plus Assay workflow.



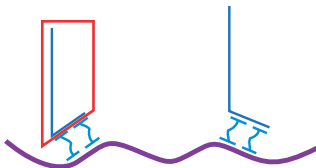
Probe set type	Fluorochrome label	Excitation wavelength (max)	Emission wavelength (max)	Laser excitation wavelength	Common filter set
Type 1	Alexa Fluor 546	556 nm	573 nm	488 or 532 nm	TRITC
Type 4	Alexa Fluor 488	490 nm	525 nm	488 nm	FITC
Type 6	Alexa Fluor 647	650 nm	665 nm	594 or 633 nm	Cy 5

Figure 3. Probe set hybridization. (Top) A schematic depicting how labeled probes hybridize to the target RNA is shown. (Bottom) Summary of probe sets currently available.

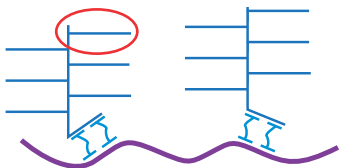
## Signal amplification

Signal amplification using bDNA technology is achieved through a series of sequential hybridization steps, which result in the formation of a tree-like structure. Pre-amplifier molecules hybridize to their respective pair of bound oligonucleotide probes to form the “trunk” of the tree. Multiple amplifier molecules hybridize to their respective pre-amplifier to create the “branches”. Finally, multiple label probes hybridize to the amplifiers to form the “leaves” of the tree (Figure 4). A fully assembled signal amplification tree contains 400 label probe-binding sites. If all target-specific oligonucleotides in a 20-oligonucleotide-pair probe set bind to the target RNA transcript, an 8,000-fold amplification can be achieved.

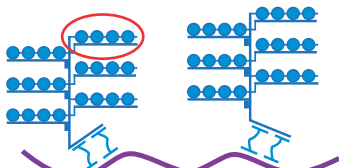
## Branched-DNA tree assembly



Step 1. Pre-amplifier (“trunk”) binds paired probe set



Step 2. Amplifier (“branches”) binds pre-amplifier



Step 3. Label probes (“leaves”) consisting of fluorochrome-conjugated oligos bind to multiple sites on amplifier

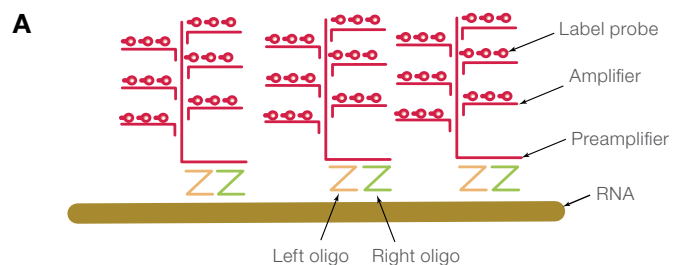
**Figure 4. Formation of bDNA.** A schematic depicting how signal amplification is achieved with bDNA technology.

## Fluorescence detection

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 imager equipped with the appropriate filter sets. (See Figure 3 for filter information.) Data shown within this paper was collected on a Zeiss Axio Vert microscope equipped with a mercury arc lamp, 20x (NA = 0.8), 40x (NA = 1.3), or 63x oil immersion (NA = 1.4), a MRm camera, and Zeiss AxioVision software. Further image analysis and quantitation was done using ImageJ freeware.

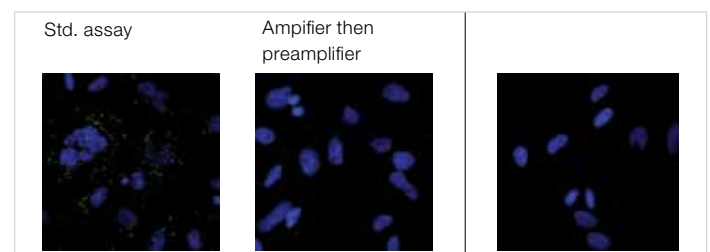
## Technology specificity

The bDNA technology achieves high target specificity with the use of oligonucleotide pairs in a design that results in signal amplification only when two adjacent target probe oligonucleotides (left oligonucleotide and right oligonucleotide) bind to the specific target. Following binding of the target probe pair, sequential binding of the pre-amplifier, amplifier, and label probe is necessary to generate the tree-like structure that produces an amplification of signal without background noise. To determine assay specificity, the ERBB2 type 1 probe set was used on HeLa cells with and without pre-amplifier, followed by amplifier and label probe. In this experiment, fluorescence signal was detected only when the ERBB2 probe set was combined with subsequent pre-amplifier, amplifier, and label probe incubation (Figure 5A). In contrast, when amplifier was added before the pre-amplifier, no signal was detected, indicating that amplifier binding is specific for pre-amplifier and does not bind nonspecifically to cells (Figure 5B). Moreover, no signal was detected with subsequent addition of pre-amplifier followed by label probe (due to the lack of amplifier), thus highlighting the specificity of the label probe for amplifier (Figure 5B). Omission of the ERBB2 probe set resulted in no detection of signal (data not shown). As expected, a negative control probe set for DapB, a bacterial gene in *Bacillus subtilis*, yielded no specific signal in HeLa cells using the standard protocol (Figure 5B, right).



**B** ERBB2 type1

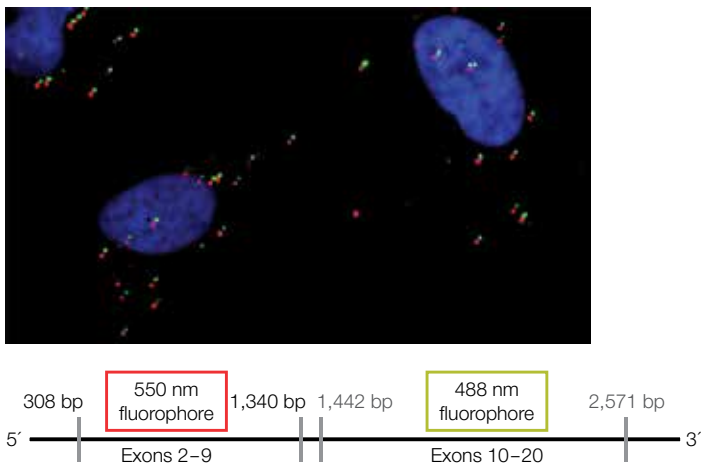
DapB type1



**Figure 5. The bDNA technology used in the ViewRNA Cell Plus Assay is specific.** (A) Diagram of the design principles underlying bDNA technology that result in target specificity. (B) HeLa cells were incubated with either the type 1 ERBB2 probe set using standard assay conditions (left) or the type 1 ERBB2 probe set with amplifier added before incubation with pre-amplifier (center). (Right) HeLa cells were incubated with the type 1 DapB negative control probe set using standard assay conditions.

## Technology sensitivity

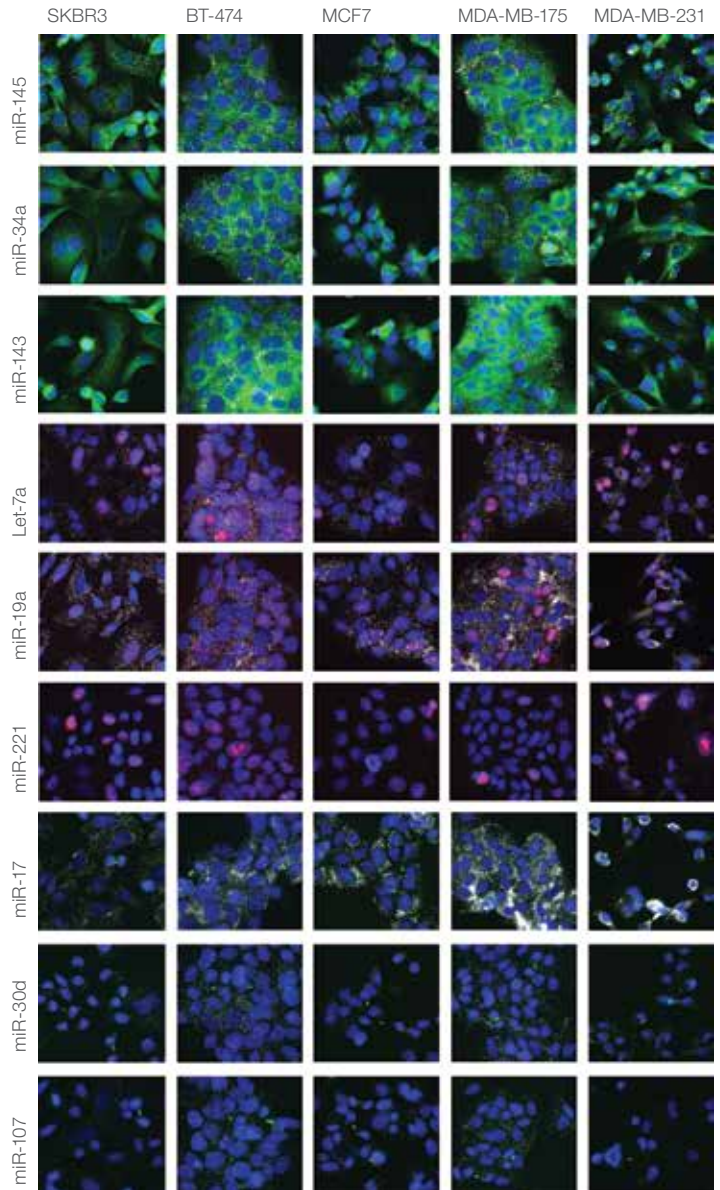
To demonstrate single-molecule sensitivity in which one dot is equivalent to one target molecule, the ViewRNA assay was performed with two target probe sets that were designed to recognize different regions of the ERBB2 (Her2) mRNA. One probe set targeted the region between exons 2 through 9 (Alexa Fluor 546 dye, red dots) and the other probe set targeted the region between exons 10 and 20 (Alexa Fluor 488 dye, green dots). Although resolving these two signals was not possible on a single target, images were captured slightly offset to enable visualization of both signals. If one dot is equivalent to the detection of one target, one would expect to see pairs of red and green dots as is shown in Figure 6.



**Figure 6. Validation of single-molecule detection.** The ViewRNA assay was performed with two target probe sets that recognize different regions of the ERBB2 (Her2) mRNA as shown. Red: Type 1 probe set that binds a region between exons 2 and 9. Green: Type 4 probe set that binds a region between exons 10 and 20. Blue: DAPI-stained nuclei.

## Orthogonal verification

The ViewRNA Cell Plus Assay was also verified by examining a variety of microRNAs with known relative levels of expression as determined by microarray analysis and real-time quantitative PCR of both tissue and breast cancer cell lines [2–4]. The expression of these selected microRNAs was examined in multiple luminal or basal breast cancer cell lines. As shown in Figure 7, miR-107 showed the highest copy number per cell in BT-474 and MDA-MB-175 cells (>20 copies) compared to two other luminal breast cancer cell lines, SKBR3 and MCF7, and the more basal cell line MDA-MB-231. This data is consistent with the microRNA array results published by Riaz et al. [4]. Moreover, investigation of miR-221, reported to exhibit very low expression based on microarray analysis of luminal cell types, was present in fewer than 5 copies/cell in the luminal cell lines BT-474 and MDA-MB-175 when examined by the ViewRNA Cell Plus Assay.



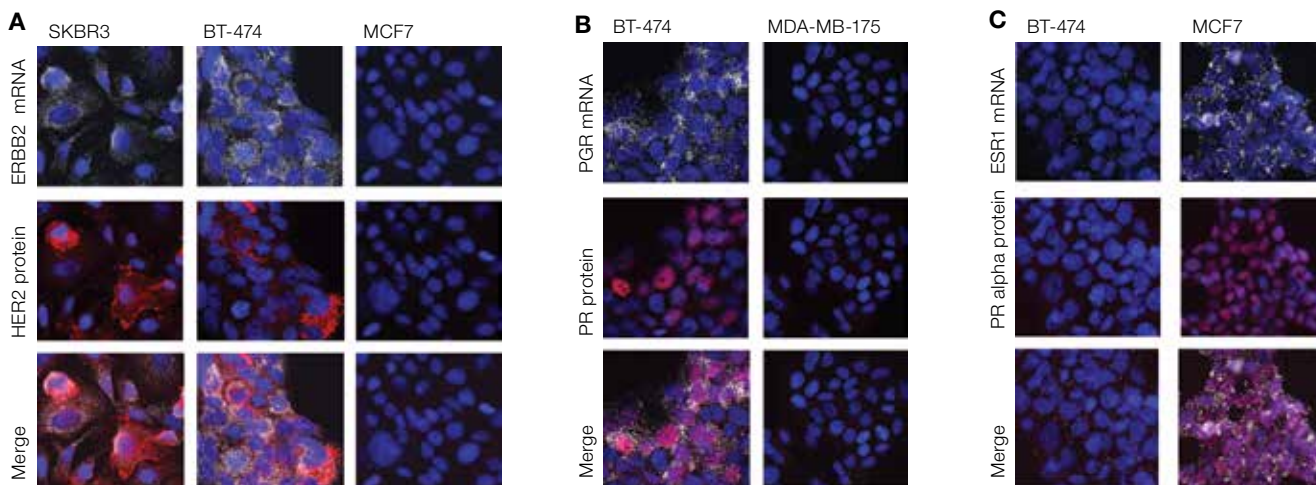
**Figure 7. The ViewRNA Cell Plus Assay can detect differences in microRNA expression profiles in human breast cancer cells.** Expression of microRNA along with tubulin, Ki-67, and survivin protein is similar in ductal versus adenocarcinoma breast cancer cell lines. The high expression of Let-7a, miR-17, and miR-19a observed with the ViewRNA Cell Plus Assay is supported by published data obtained by microarray analysis and real-time quantitative PCR. (Top three rows) miR-145, miR-34a, and miR-143 are expressed at similar levels between the different cell lines examined. Green: alpha-tubulin Alexa Fluor 488 dye. White: Type 1 probe sets specific to miR-145, miR-34a, or miR-143. Blue: DAPI-stained nuclei. (Middle three rows) Let-7a and miR-19a are highly expressed across the different breast cancer cell lines. miR-221 is expressed at very low levels, with MDA-MB-231 cells expressing slightly higher levels than the other cell lines. Red: Invitrogen™ eBioscience™ Ki-67 eFluor™ 660 dye. White: Type 1 Let-7a, miR-19a, or miR-221. Blue: DAPI-stained nuclei. (Bottom three rows) miR-17 is highly expressed across the different breast cancer cell lines, with SKBR3 cells exhibiting slightly lower expression than the others. Green: Survivin conjugated with Alexa Fluor 488 dye. White: type 1 miR-17, miR-30d, or miR-107. Blue: DAPI-stained nuclei.

In contrast, we observed higher expression of miR-221 in the basal cell line MDA-MB-231 cells (>20 copies/cell), which is consistent with microRNA array analysis. We also found that, in accordance with results reported in Fix et al. [3], steady-state levels of Let-7a, miR-107, and miR-17 in MCF-7 cells were higher than those of miR-34a, miR-30d, and miR-221. Progesterone receptor-positive (PR+) breast cancer cell lines have been reported to exhibit a 1.8-fold increase in miR-30d expression compared to PR-negative breast cancer cell lines [2]. Using the ViewRNA Cell Plus Assay, we also observed that miR-30d expression is 1.5-fold higher in BT-474 cells, which are PR+, than in MCF-7 cells, which are PR- (data not shown). Overall, the ViewRNA Cell Plus Assay produced results that were consistent with previously published microarray and real-time quantitative PCR data. However, one major advantage of this assay is the increased visibility of cell-to-cell differences in relative expression levels, a facet that is not easily captured by non-single cell methods.

### Correlation between protein and mRNA

The central dogma of molecular biology states that DNA gives rise to RNA, which in turn gives rise to protein. 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 specificity of the ViewRNA Plus Cell Assay. To show the relationship

between transcription and translation, ERBB2/HER2, PR, and estrogen receptor alpha (ER) mRNA and protein were visualized in several breast cancer cell lines of known HER2, PR, and ER status. The ViewRNA Cell Plus Assay was used to visualize HER2 protein using a fluorophore-conjugated antibody to HER2 (Invitrogen™ eBioscience™ Anti-Human ErbB2/HER2 eFluor™ 570 conjugate), while ERBB2 mRNA expression was assessed with a type 6 probe set. SKBR3 and BT-474 cells are characterized as HER2+, while MCF-7 cells are HER2-. The data in Figure 8A demonstrate that ERBB2 mRNA is present in all cells within the SKBR3 and BT-474 cultures. However, expression of HER2 protein is heterogeneous within the cell populations and localizes primarily to the cell membrane with some protein detected in the cytoplasm. Interestingly, our data show that the level of HER2 protein varies from cell to cell. While the differences may reflect biological heterogeneity within the cultured cells, they may also be assay-dependent. While HER2 protein was detectable in many of the cells, some which possessed only a minimal level of ERBB2 mRNA did not produce a detectable HER2 signal. This result may have arisen due to several reasons, namely limitations in the detection of HER2 protein by the antibody, cell-to-cell differences in translation of ERBB2 mRNA, or differences in degradation of HER2 protein. Interestingly, despite its extremely low expression levels, ERBB2 mRNA is still detectable in MDA-MB-175 cells, which have been documented as HER2-.

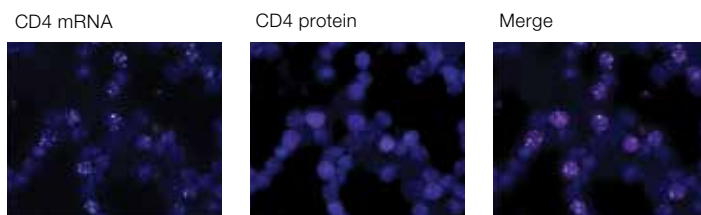


**Figure 8. The ViewRNA Cell Plus Assay can be used to examine mRNA and protein expression levels simultaneously.** (A) Expression of ERBB2 mRNA and HER2 protein expression in human breast cancer cells. Our data demonstrate that ERBB2 mRNA (white) is localized to the cytoplasm while HER2 protein (red) is found predominantly in the membrane. MCF7 cells were negative for ERBB2 mRNA and HER2 protein. Nuclei were stained with DAPI (blue). (B) PR mRNA and protein expression in human breast cancer cells. PR mRNA (white) was detected using a type 1 probe set and was found to be localized in the cytoplasm. PR protein was detected using Invitrogen™ eBioscience™ Anti-PR eFluor™ 660 antibody (red) and was observed in the nucleus. BT-474 cells express PR while MDA-MB-175 cells do not. Nuclei were stained with DAPI (blue). (C) ER alpha mRNA and protein expression in human breast cancer cells. ER mRNA (white) was detected using a type 1 probe set and was found to be localized to the cytoplasm. ER protein, as detected by Invitrogen™ anti-estrogen receptor-purified antibody followed by anti-mouse IgG1 eFluor 660 antibody (red), was observed in the nucleus. BT-474 cells express very low levels of ER mRNA and ER protein was not detected. On the other hand, MCF-7 cells express both ER mRNA and protein. Nuclei were stained with DAPI (blue).

However, ERBB2 protein is not detectable in these cells (data not shown), thereby highlighting differences in capability between mRNA and protein detection. Under optimal assay conditions, mRNA signal amplification can be 8,000-fold higher than a protein signal, which possesses much more limited amplification (<5-fold). This limitation may contribute to the differences observed in the ability to detect mRNA versus protein levels. As expected, ERBB2 mRNA and HER2 protein were not detected in MCF-7 and MDA-MB-231 cells (data not shown).

The data shown in Figure 8B demonstrate that although PR mRNA and protein expression in BT-474 cells is heterogeneous throughout the culture, there is good correlation between mRNA and protein levels within individual cells. Cells expressing high levels of PR mRNA also exhibit strong protein staining within the nucleus. Nevertheless, a subpopulation of BT-474 cells was negative for PR mRNA and protein. Negative controls for both mRNA (no probe and nonspecific probe) and protein (isotype-matched antibody) were run in parallel with and without detection of any signal (data not shown). As expected, MDA-MB-231 cells were negative for PR mRNA and protein (data not shown).

Data produced by the ViewRNA Cell Plus Assay demonstrate that ER mRNA and protein expression is correlative in all MCF-7 cells even though the levels of expression vary from cell to cell (Figure 8B). BT-474 cells have been reported to express ER protein [4], and its mRNA was detected but at very low levels (<10 copies/cell). Interestingly, even the use of a purified antibody in combination with a secondary antibody to detect ER protein, a method that should provide signal amplification, did not result in a positive signal. This result emphasizes the increased sensitivity in mRNA detection within this assay, which is due to the signal amplification achieved by bDNA technology, compared to protein signal amplification.

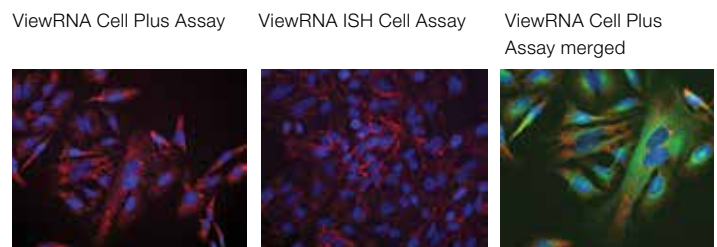


**Figure 9. Examination of CD4 mRNA and protein expression in C57BL/6 mouse splenocytes using the ViewRNA Cell Plus Assay.** CD4 mRNA was detected using a type 1 probe set and was found localized to the cytoplasm (white). CD4 protein was detected on the cell surface using Invitrogen™ anti-CD4 biotin followed by Invitrogen™ eBioscience™ streptavidin eFluor™ 660 conjugate (red). Nuclei were stained with DAPI (blue).

Figure 9 demonstrates the power of the ViewRNA Cell Plus Assay to assess a heterogeneous primary cell population. As expected, examination of spun-down C57BL/6 mouse splenocytes demonstrated that a subpopulation of cells expresses CD4 mRNA with good correlation between CD4 mRNA and protein expression. Since heterogeneity may have important implications on biological processes following treatments or within experimental models, assessing the heterogeneity of a cell culture or endogenous model system is important. The ability to examine this attribute constitutes one of the strengths of this single-cell assay.

### Comparison of the ViewRNA Cell Plus Assay mRNA detection to the ViewRNA ISH Cell Assay

To compare the sensitivity of mRNA detection in the ViewRNA Cell Plus Assay versus the ViewRNA ISH Cell Assay, HeLa cells from the same culture were plated on duplicate plates and then beta-actin and PPIB mRNA were detected using either type 1 or type 6 probe sets, respectively. Images were acquired using similar exposure times for the type 6 PPIB mRNA signal (i.e., 2,100 ms and 2,380 ms). An exposure time of 268 ms was used to detect the type 1 beta-actin mRNA signal, while 569 ms was used for the ViewRNA ISH Cell Assay. In this experiment, beta actin mRNA and PPIB were detected with greater sensitivity using the ViewRNA Cell Plus Assay as evidenced by the shorter exposure time needed for detecting beta actin mRNA and the improved signal for PPIB mRNA (Figure 10). Distribution of the mRNAs in each assay was equivalent.



**Figure 10. Comparison of beta actin mRNA and PPIB mRNA detection between the ViewRNA Cell Plus Assay and the ViewRNA ISH Cell Assay.** Merged image of ViewRNA Cell Plus Assay (far right) shows detection of beta actin and PPIB mRNA along with alpha-tubulin protein, which was detected using Anti-Tubulin Alexa Fluor 488 (green) antibody. Beta-actin mRNA (red) and PPIB mRNA (white). Nuclei are stained with DAPI (blue).

## Comparison of ViewRNA Cell Plus Assay protein staining to immunocytochemistry (ICC) antibody staining

To date, we have evaluated the performance of more than 100 ICC-compatible antibodies (comprised of a mix of unconjugated or purified, biotinylated, and fluorophore-conjugated formats) for use in the ViewRNA Cell Plus Assay. To accomplish this, antibody performance was tested either in the context of the full assay (antibody staining and RNA detection) or by using the Invitrogen™ ViewRNA™ Cell Plus Fixation/Permeabilization Buffer Set (Cat. No. 00-19001) according to the antibody staining portion of the protocol. We found that 95% of the antibodies tested that perform optimally with formaldehyde-fixed cells also perform well with the ViewRNA Cell Plus Assay. Antibodies that require methanol fixation were generally found to be unsuitable for this assay.

In this evaluation, we have assessed a broad panel of antibodies with specificities against structural proteins, transcription factors, organelles, and surface markers. Our results show that signal intensity and resolution of detail is maintained with the ViewRNA Cell Plus Assay compared to our standard immunocytochemistry protocol. For example, Figure 11 shows the results of detecting golgin-97, COX IV, and Ki-67 in HeLa cells using our standard immunocytochemistry protocol and the ViewRNA Cell Plus Assay. The signal intensity and localization of golgin-97, which is typically found on the trans face of the Golgi apparatus, was maintained as expected with the ViewRNA Cell Plus Assay protocol. COX IV, an enzyme that plays a role in the mitochondrial respiratory chain, was also

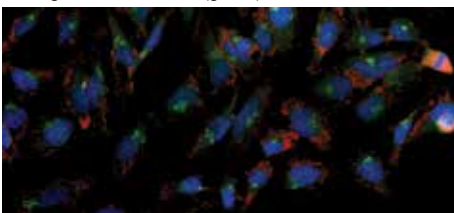
visualized easily with the expected staining pattern using the ViewRNA Cell Plus Assay protocol, but with a slightly lower intensity compared to the immunocytochemistry protocol. Finally, detection of Ki-67, which is expressed within the nuclei of dividing cells, was equivalent in intensity and localization with both protocols. Based on our studies, we conclude that antibodies that are suitable for ICC or IHC (i.e., optimized for formaldehyde fixation) are likely to be compatible with the ViewRNA Cell Plus Assay. Nevertheless, we recommend using the ViewRNA Cell Plus Fixation/Permeabilization Buffer Set to establish acceptable performance criteria prior to running the complete ViewRNA Cell Plus Assay.

## ViewRNA Cell Plus Assay precision: day-to-day variability and operator variability Intra-assay variability (slide format)

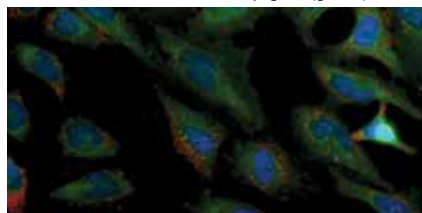
To assess intra-assay variability using primary cells, splenocytes from a single C57BL/6 mouse were isolated and cytopun onto slides prior to undergoing ViewRNA Cell Plus Assay using the mouse beta-2 microglobulin (β2m) type 4 probe set and the mouse CD8a type 1 probe set. Mouse beta-2 microglobulin mRNA is expected to be expressed equivalently in all cells, while CD8a mRNA is expressed by a subpopulation of the splenocytes. This experiment was performed in triplicate (i.e., three separate slides) and the number of dots per cell was counted. A total of 15 cells was counted per sample. Our data demonstrate that this assay produces good intra-assay performance with coefficient of variation (CV) values for all targets below 13% (Figure 12).

### ViewRNA Cell Plus Assay

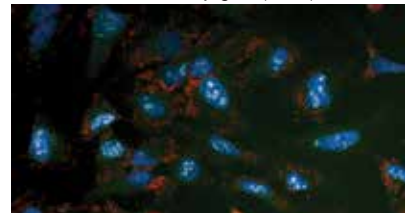
Golgin-97 eFluor 660 (green)



COX IV Alexa Fluor 488 conjugate (green)

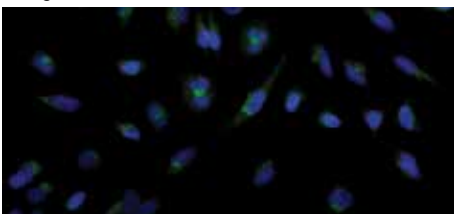


Ki-67 eFluor 660 conjugate (white)

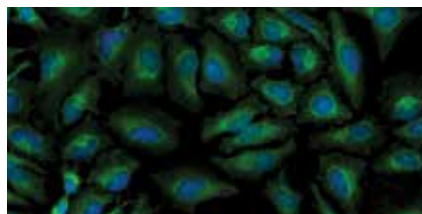


### Immunocytochemistry

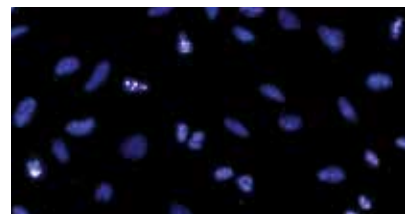
Golgin-97



COX IV



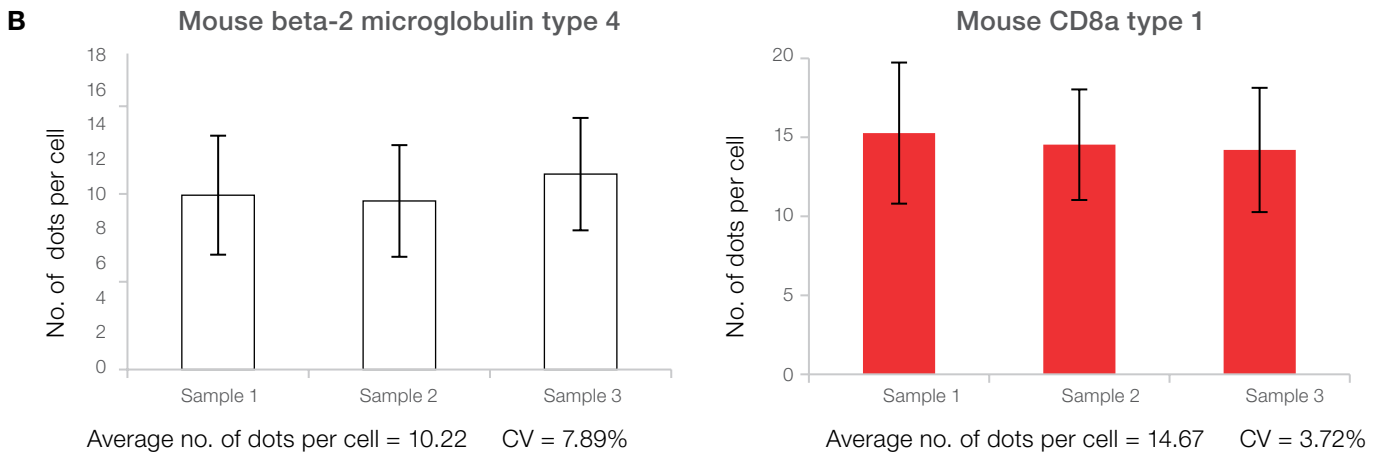
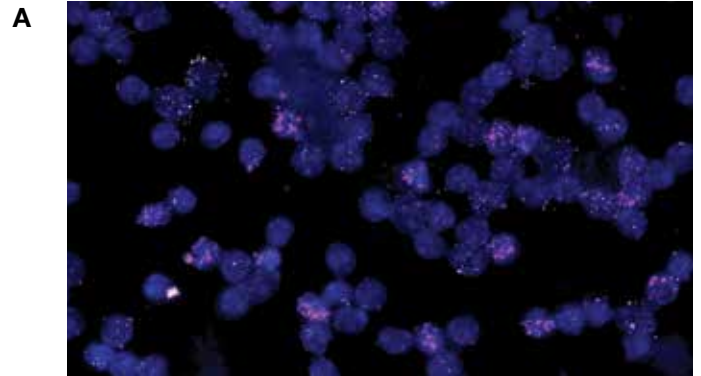
Ki-67



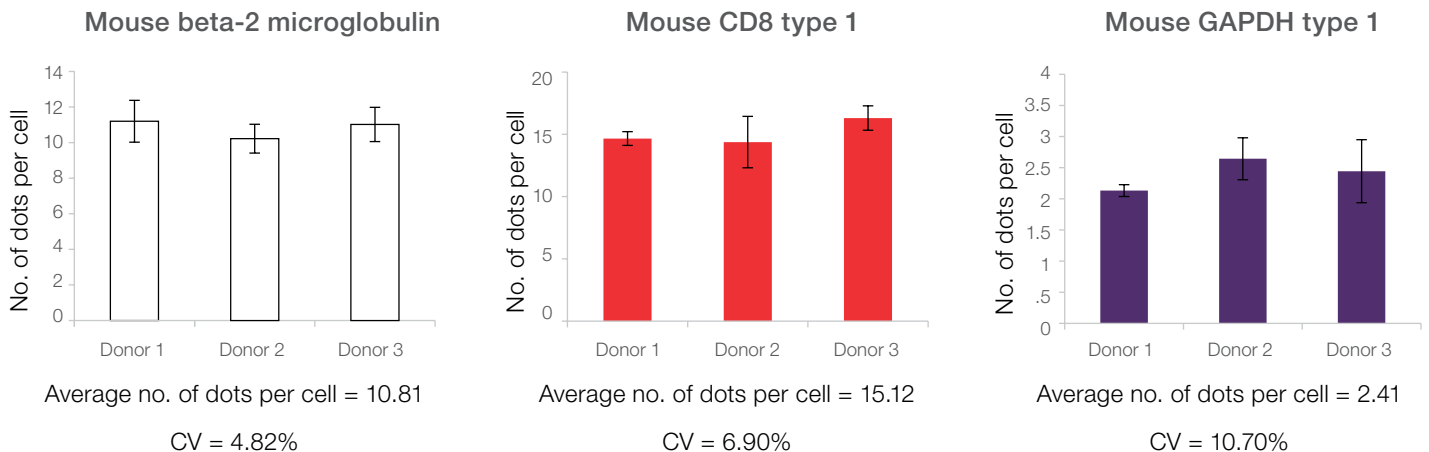
**Figure 11. Detection of golgin-97, COX IV, and Ki-67 protein by antibody staining using the ViewRNA Cell Plus Assay and traditional immunocytochemistry staining protocols. Nuclei are stained with DAPI (blue).**



In addition to evaluating intra-assay performance, donor-to-donor variability was analyzed using the same target probe sets as above. Splenocytes were isolated from three separate C57BL/6 donors and cytopspun onto slides prior to undergoing the ViewRNA Cell Plus Assay using the mouse beta-2 microglobulin type 4 probe set, CD8a type 1 probe set, and GAPDH type 6 probe set. The number of dots per cell was counted in 45 cells from each donor. As shown in Figure 13, the CV values for all targets were below 11%, indicating both robust intra-assay performance and minimal donor variation across the three mRNA targets tested.



**Figure 12. Intra-assay reproducibility of beta-2 microglobulin and CD8a mRNA detection in C57BL/6 splenocytes using slides in the ViewRNA Cell Plus Assay.** (A) Photomicrograph of mouse beta-2 microglobulin mRNA (white) and mouse CD8a mRNA (pink). Nuclei are stained with DAPI (blue). (B) The mouse beta-2 microglobulin and mouse CD8a mRNA signals were quantified by counting the number of dots/cell in 15 cells per sample. The error bars represent the standard deviation.

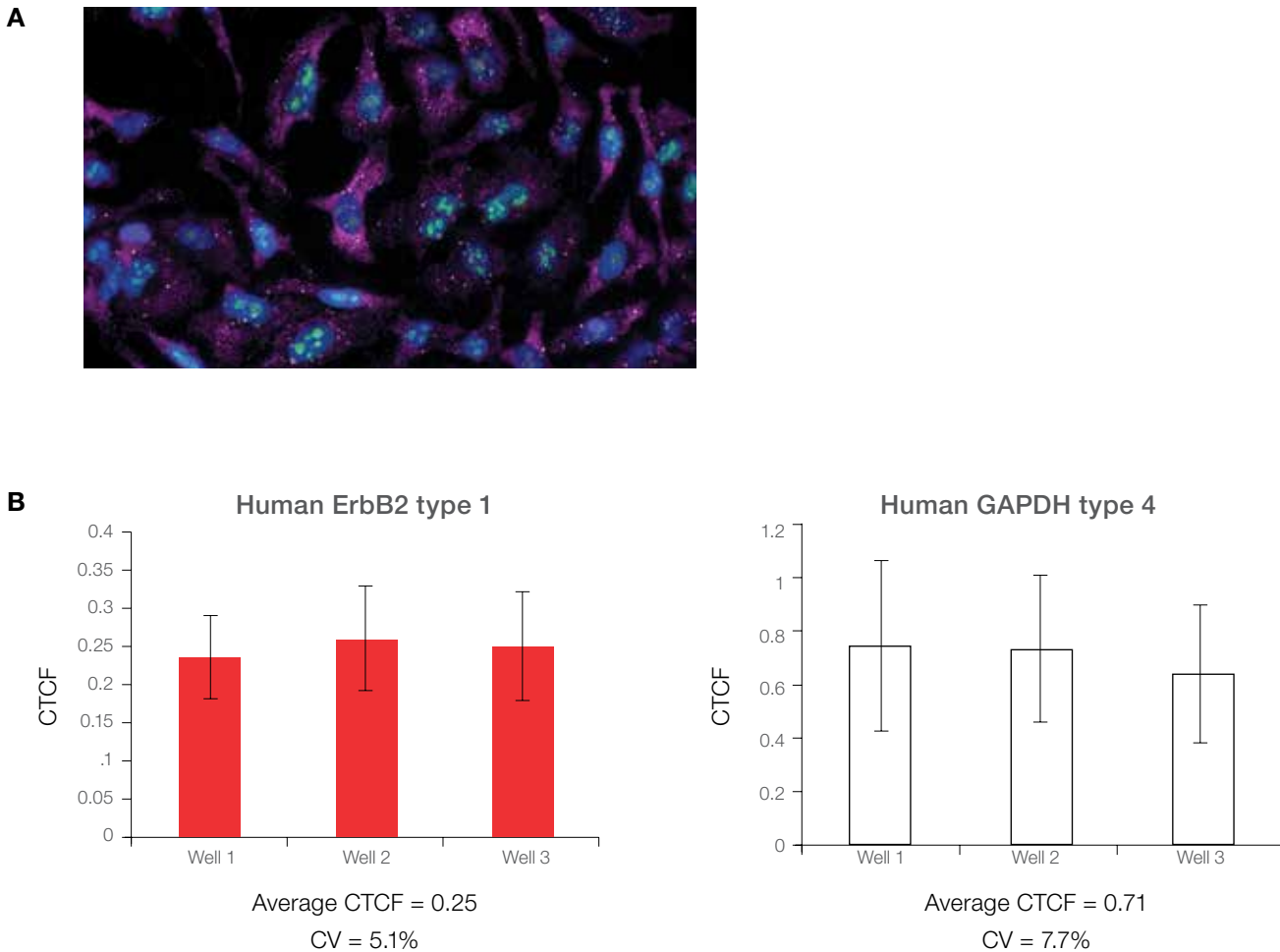


**Figure 13. Donor reproducibility in detecting beta-2 microglobulin, CD8a, and GAPDH mRNAs in C57BL/6 splenocytes using ViewRNA Cell Plus Assay.** The mRNA signals were quantified by counting the number of dots per cell in 45 cells per sample. The error bars represent the standard deviation.

### Intra-assay variability (96-well plate format)

To assess intra-assay variability using the multi-well plate format, we visualized human ERBB2 mRNA (type 1), GAPDH mRNA (type 4), Let-7a miRNA (type 1), and Ki-67 protein (Anti-Ki-67 eFluor 660 antibody) in HeLa cells from three different wells in a 96-well plate. We quantified the corrected total cell fluorescence (CTCF) from 15 cells per well per target. Cell fluorescence was measured by selecting the cell of interest and using ImageJ software to

measure the area, integrated density, and mean gray value. Background was determined by taking measurements from three regions surrounding the cell of interest (adjacent to the cytoplasm). CTCF was then calculated using the following formula:  $CTCF = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})$ . Our results demonstrate that the ViewRNA Cell Plus Assay yields very good well-to-well reproducibility with CV values for all targets less than 8% (Figure 14).

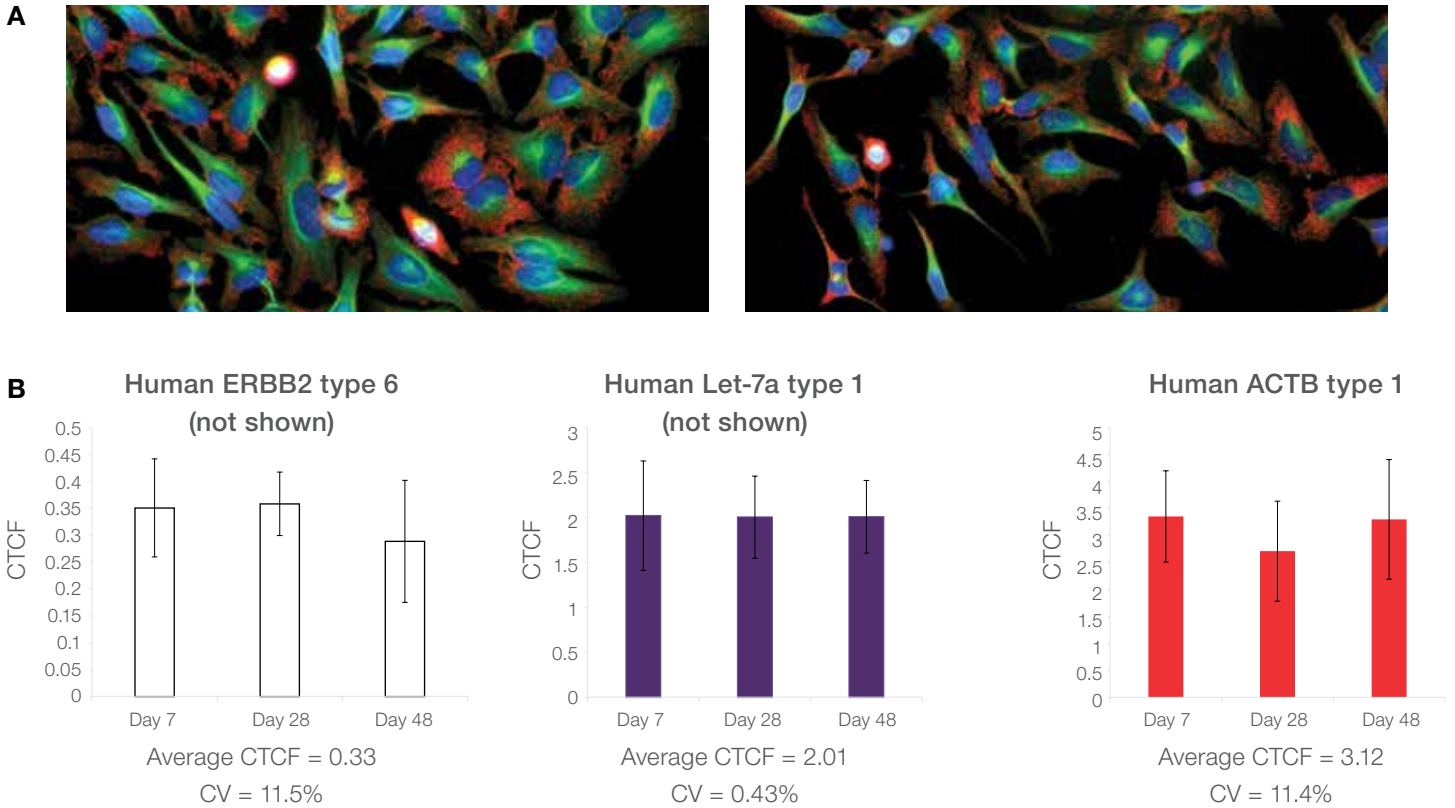


**Figure 14. Intra-assay reproducibility of detecting expression of human ERBB2 and GAPDH mRNA with Ki-67 protein in HeLa cells using the 96-well plate format in ViewRNA Cell Plus Assay. (A)** Photomicrograph of human ERBB2 mRNA, human GAPDH, and Ki-67 protein. White: ERBB2 mRNA. Pink: GAPDH mRNA. Green: Ki-67 protein. Blue: DAPI-stained nuclei. **(B)** Quantitation of human ERBB2 and human GAPDH mRNA signal is expressed as CTCF. The error bars represent the standard deviation.

### Day-to-day single-user reproducibility (8-well chamber slide format)

To assess day-to-day variability, we examined human ERBB2 mRNA (type 6), human Let-7a miRNA (type 1), and beta-actin (ACTB) mRNA (type 1) in addition to alpha-tubulin protein in HeLa cells using ViewRNA Cell Plus Assay. Experiments were performed by the same user on three different days using 8-well chamber slides. The reagents used for each experiment were from the same manufactured lot. However, the passage number of the

HeLa cells used for the second and third experiments was increased by three and six passages, respectively. The average CTCF was quantified from seven cells counted per experiment. Our data indicate that no significant differences were obtained from experiments performed on different days (Figure 15). Statistical significance was determined using the Student's *t*-test, which resulted in a *P* value greater than 0.18 for this experiment. Thus, ViewRNA Cell Plus Assay produces very consistent day-to-day performance with CV values for all targets less than 12% and no statistical difference in signal levels.



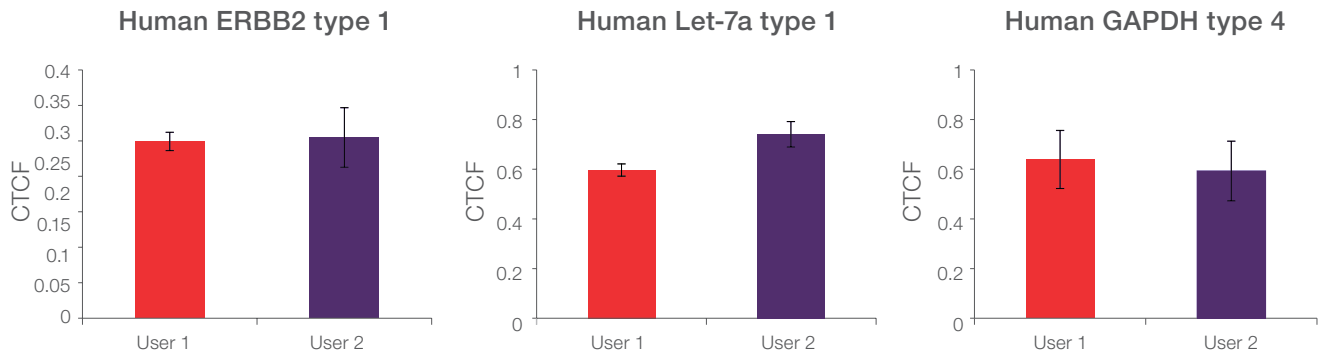
**Figure 15. Day-to-day reproducibility by a single user using ViewRNA Cell Plus Assay. (A)** Photomicrographs of ACTB mRNA (red), HPRT1 mRNA (white), and alpha-tubulin protein (green) in HeLa cells cultured for 7 days (left) and 48 days (right). Nuclei are stained with DAPI (blue). **(B)** Quantitation of human ERBB2 mRNA, human Let-7a miRNA, and human ACTB mRNA is expressed as CTCF. The error bars represent the standard deviation.

### Operator variability (96-well plate format)

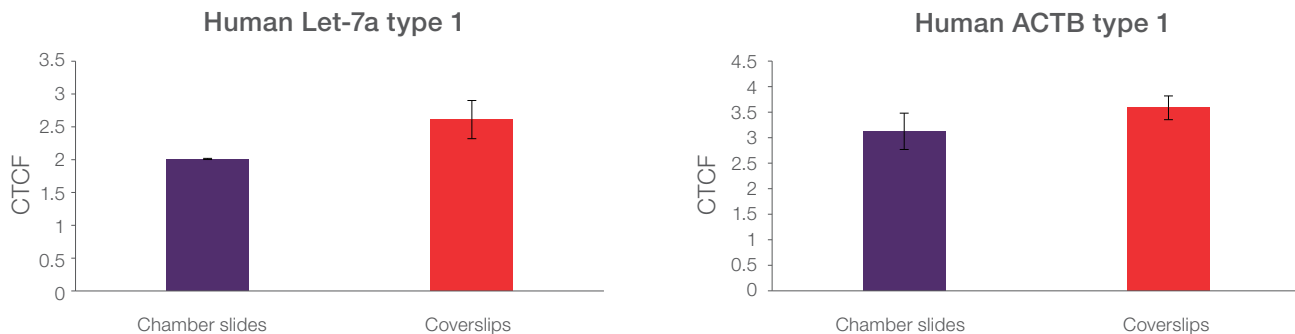
To assess operator variability, two different operators performed the ViewRNA Cell Plus Assay on HeLa cells to detect human ERBB2 mRNA (type 1), Let-7a miRNA (type 1), and GAPDH mRNA (type 4) in addition to alpha-tubulin and Ki-67 proteins. The assay was performed in triplicate wells using the 96-well plate platform. The CTCF of 45 cells was measured across three wells for each user. The reagents used for each experiment were from the same manufactured lot. However, the passage number of the HeLa cells varied by 1–2 passages. Analysis of the average CTCF for each RNA probe did not show a significant difference between users (Figure 16). Statistical significance was determined using the Student's *t*-test, which resulted in a *P* value greater than 0.05 for this experiment. Therefore, ViewRNA Cell Plus Assay enables very consistent performance between different operators with CV values for all targets less than 20% and no statistical difference in signal levels between operators.

### ViewRNA Cell Plus Assay reproducibility across platforms (chamber slides and cover slips)

To assess platform variability, we compared detection of Let-7a miRNA (type 1) and ACTB mRNA (type 1) across experiments performed using chamber slides and coverslips. For chamber slides, we examined the assay performance from three different experiments conducted by the same user on different days. For coverslips, triplicate samples used in a single experiment by the same user were examined. The reagents and cells used for each of the platform experiments were derived from different manufacturing lots and cell passages, respectively. Analysis of the average CTCF for each RNA probe in 21 cells did not show a significant difference between platforms (Figure 17). Statistical significance was determined using the Student's *t*-test, which resulted in a *P* value greater than 0.07 for this experiment, indicating no statistical difference in signal levels between the two platforms. ViewRNA Cell Plus Assay shows consistent platform-to-platform performance with CV values for all targets less than 12% and no statistical difference in signal levels obtained from the different platforms.



**Figure 16. Operator reproducibility in detecting ERBB2 mRNA, Let-7a miRNA, and GAPDH mRNA in HeLa cells using ViewRNA Cell Plus Assay.** Quantitation of human ERBB2 mRNA, human Let-7a miRNA, and human GAPDH mRNA signal is expressed as CTCF. The error bars represent the standard deviation.



**Figure 17. Cross-platform reproducibility in detecting Let-7a miRNA and ACTB mRNA in HeLa cells using ViewRNA Cell Plus Assay.** Quantitation of human Let-7a miRNA and human ACTB mRNA signal is expressed as CTCF. The error bars represent the standard deviation.

## Conclusion

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 ISH provides a method for examining the levels of specific RNA transcripts in individual cells, and 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. Further complicating this analysis, traditional ISH methods lack sensitivity for detecting low-abundance RNA species and have limited multiplexing capability that further constrain its compatibility with other cell assays.

The ViewRNA Cell Plus Assay is a novel method that combines ViewRNA technology—a proprietary FISH and bDNA amplification technique—with antibody-based protein detection to simultaneously visualize RNA and protein 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 immunocytochemistry. Antibody compatibility with this assay must be determined using either the ViewRNA Cell Plus Fixation/Permeabilization Buffer Set or by use within the assay. Antibodies should be titrated for optimal performance in this assay as in immunocytochemistry experiments. As with any microscopic analysis, actual results depend on filter configuration, camera sensitivity, and optimization to obtain the best results.

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