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# Optimizing a High-Throughput Fluorescence In Situ Hybridization and Flow Cytometry-Based System for the Detection of Cottontail Rabbit Papillomavirus

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# Optimizing a High-Throughput Fluorescence In Situ Hybridization and Flow Cytometry-Based System for the Detection of Cottontail Rabbit Papillomavirus

## **Abstract**

Human papillomaviruses are the most frequent known cause of cervical cancer. They preferentially infect epithelial cells, and their life cycles are dependent upon the differentiation of infected cells. Cottontail Rabbit Papillomavirus (CRPV) has been a reliable animal model for papillomavirus study, and has provided germane insight into human papillomavirus biology. Despite the usefulness of CRPV in this regard, single-cell analysis of infected cell cultures has proven challenging due to the latent, non-lytic nature of the virus. The purpose of this study was to develop a highthroughput method for the single-cell detection and analysis of CRPV-infected cells. This method utilizes fluorescence in situ hybridization (FISH) followed by flow cytometric (FC) analysis. This report describes the initial characterization of an FISHFC protocol designed to detect the highly abundant 28S ribosomal RNA in RK-13 (rabbit) cells.

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OPTIMIZING A HIGH-THROUGHPUT FLUORESCENCE IN SITU  
HYBRIDIZATION AND FLOW CYTOMETRY-BASED SYSTEM FOR THE  
DETECTION OF COTTONTAIL RABBIT PAPILLOMAVIRUS

By

Matthew Raines

A Senior Thesis Submitted to the

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with Honors in Biology

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## **Abstract**

Human papillomaviruses are the most frequent known cause of cervical cancer. They preferentially infect epithelial cells, and their life cycles are dependent upon the differentiation of infected cells. Cottontail Rabbit Papillomavirus (CRPV) has been a reliable animal model for papillomavirus study, and has provided germane insight into human papillomavirus biology. Despite the usefulness of CRPV in this regard, single-cell analysis of infected cell cultures has proven challenging due to the latent, non-lytic nature of the virus. The purpose of this study was to develop a high-throughput method for the single-cell detection and analysis of CRPV-infected cells. This method utilizes fluorescence in situ hybridization (FISH) followed by flow cytometric (FC) analysis. This report describes the initial characterization of an FISH-FC protocol designed to detect the highly abundant 28S ribosomal RNA in RK-13 (rabbit) cells.

## **Introduction**

### *Papillomavirus background*

Papillomaviruses infect human and other mammalian epithelial cells, and complete their life cycles in a differentiation-dependent manner (20). They are known to selectively infect epithelial cells to the exclusion of the underlying dermal layer (8). Infections cause warts and condylomata, and have been established as the main cause of cervical cancer (14). Due to the species-specific nature of papillomavirus infections, several animal models have been developed to study papillomavirus infections and pathogenesis. Cottontail Rabbit Papillomavirus (CRPV) serves as a robust model for gaining insight into the latent life cycle of human papillomaviruses (24).

### *FISH and flow cytometry to detect latent viruses*

By their very nature, latent, non-lytic viruses are difficult to detect at the single-cell level in cell culture. Previously described methods of *in vitro* papillomavirus detection include western blot (21), northern blot (5), immunofluorescence (13), immunoblotting (9), PCR of methylated DNA (23), and loop-mediated isothermal amplification (11). While these methods have provided fecund information about papillomaviruses, none preserves the cells in a semi-intact state.

Solution-based fluorescence in situ hybridization (FISH), however, is unique in its preservation of semi-intact cells (3). FISH involves two major steps: the intracellular hybridization of fluorochrome-conjugated oligonucleotide probes specific for target DNA or RNA, and visualization of the resulting hybrid (10). Bauman and Bentvelzen (3) described the first known application of FISH to an RNA target in a suspension of intact cells. This technique, followed by flow cytometric

analysis, was able to detect a low number of mRNA transcripts, and the authors anticipated that as few as 10 mRNA transcripts per cell could be detected. Since then, Stowe, et al. (19) have reported a method for detecting viral infection at the single cell level using FISH and flow cytometry (FC). This group investigated EBV gene expression in EBV-positive and EBV-negative cell lines using fluorochrome-conjugated probes targeting the EBV small RNA 1 (EBER1) and provided quantitative analyses of infected cells by flow cytometric analysis. In this way, the group was able to assess the number of EBV-infected cells in a sample. Crouch et al. (6) reported a similar method of FISH and flow cytometry for detecting EBV infection at the single cell level. They employed a single oligonucleotide probe, which also targeted EBER1. This technique was sensitive enough to detect one EBV-positive Raji cell in a negative culture of nine thousand.

With modifications, these FISH-FC models could provide a high-throughput system for the detection of papillomaviruses. Narimatsu and Patterson (15) have developed a similar combined technique for cervical cancer screening. Their FISH-FC technique achieved sensitive detection of oncogenic HPV transcripts in HeLa and SiHa cells and, therefore, provides the potential for more effective diagnosis.

A combined protocol that preserves the integrity of cells in solution is necessary in order to obtain information about infected sub-populations via flow cytometry. Such information would be used to analyze cellular phenotypic markers which may be altered upon papillomavirus infection. In order to distinguish papillomavirus-infected from uninfected cells, however, a probe must be employed that targets a transcript in infected monolayer cell cultures. Choosing a high-

abundance viral mRNA would ostensibly reduce the relative impact of background and nonspecific fluorescence.

*E1<sup>E4</sup> transcripts as probe target*

In order to effectively distinguish infected from uninfected cells, Stowe, et al. (19) chose to target viral transcripts of relatively high abundance in latently infected cells. In order to recognize non-lytic CRPV-infected cells in solution, a ubiquitous CRPV transcript should be targeted by an antisense probe.

Culp and Christensen (7) found that infection of RK-13 cells with CRPV showed similar early infection kinetics to HPV-11 infection. They also found that among a number of PV infections, early E1<sup>E4</sup> transcripts (which code for the E4 protein) are most prevalent during HPV-11 infection. While the abundance of the E4 protein has been reported, its function remains to be fully elucidated (20). Nakahara, et al. (14) reported that the HPV-16 E1<sup>E4</sup> gene function is pleiotropic, producing a protein that functions both early and late in the viral life cycle. They discovered that one early role involves the replication of viral DNA.

While the full importance of the E4 protein remains unclear, the use of the E1<sup>E4</sup> transcript as an indicator of the early viral life cycle has been established. Culp and Christensen (7) reported the kinetics of CRPV and HPV-11 viral entry. While a small number of virions entered monolayer cell cultures a few hours after infection, they found an increase in HPV-11 E1<sup>E4</sup> viral transcripts 48-120 hours post-infection across a number of cell lines. This was observed despite a 24-hour wash step to remove any unattached virions from the supernatant. Use of a neutralizing antibody effectively reduced the number of HPV-11 E1<sup>E4</sup> viral transcripts when added as late as 48 h p.i. This suggests that increases in E1<sup>E4</sup>



expression over time is due to the slow entry of virions into cells. While papillomaviruses display relatively slow entry kinetics and low initial transcript production, the E1<sup>E4</sup> transcript can serve as an indicator of the early stages of infection. Cells infected with CRPV will therefore be detectable with a fluorochrome-conjugated oligonucleotide probe to the early E1<sup>E4</sup> transcript.

In order to develop the system by testing effective probe entrance and in situ binding, an oligoprobe that targets a ubiquitous RNA transcript is required. Additionally, if multiple cell lines are to be studied, a nucleotide sequence that is highly conserved across species would provide the ideal target. Ribosomal RNA is certainly ubiquitous as a normal living cell contains millions of ribosomes in its cytoplasm (1). Additionally, the role of the 28S rRNA in ribosomal translocation across the endoplasmic reticulum was established early in evolution, and has been highly conserved (18). The 28S rRNA should therefore provide a highly conserved and ubiquitous RNA target across mammalian species.

## **Materials and Methods**

### *RK-13 Cell Culture*

A rabbit kidney epithelial cell line (RK-13) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Minimum Essential Media (MEM) with additives, all from Gibco (Carlsbad, California). These additives included 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Cell cultures were grown at 37 °C with 5% CO<sub>2</sub>. Cells were passaged via enzymatic digest with TrypLE Express (Gibco,

Carlsbad, California) at ratios ranging from 1:2 to 1:9. These experiments utilized cells from passage 192 to passage 200.

### *Probes*

28S rRNA was chosen as a ubiquitous target transcript in RK-13 cells. A relevant antisense probe sequence was determined using MacVector<sup>®</sup> sequencing software (Cary, IN). The following oligomer was chosen for its physical parameters on MacVector software and synthesized by Integrated DNA Technologies (Coralville, IA):

5'- ACA TAC ACG AAC CGA CTC CTC/3Cy5Sp/ -3' A sense probe

complementary to the antisense strand was also synthesized by IDT and utilized as a negative control: 5'- TGT ATG TGC TTG GCT GAG GAG/3Cy5Sp/ -3' A BLAST search (2) verified that neither probe was complementary to other known sequences in the rabbit genome.

An antisense probe to the CRPV E1<sup>E4</sup> transcript was optimized by Culp and Christensen (7) and utilized in this experiment. The following oligomer was provided by Integrated DNA Technologies (IDT): 5'-6-FAM d(TGA AAA TGG CTG AAG CTC CCC) BHQ-1-3' All probes were provided with phosphorothioate internucleoside modifications in order to prevent intracellular nuclease digestion.

### *Solution-Based Fluorescence In Situ Hybridization*

RK-13 cell monolayers were dissociated with TrypLE Express for approximately 10 minutes at 37°C. Samples were centrifuged at 300x g for 5 minutes, then suspended in PBS. 10<sup>6</sup> cells were used for each experimental sample. Samples were again centrifuged as above, followed by exposure to an alcohol-based fixing and

permeabilizing agent (ABF), as described by Stowe et al. (19), containing 50% water, 40% ethanol, and 10% glacial acetic acid. Samples were incubated at room temperature for 1 hour.

Following permeabilization, samples were washed in 2X SSC and centrifuged at the aforementioned conditions. A hybridization solution (Sigma, St. Louis, MO) containing 5 µg/ml probe (antisense or sense) for each sample was heated to 80°C for 3 minutes, then cooled on ice. Hybridization mixtures (50 µl) were then applied to appropriate samples and incubated at 40°C for 30 minutes.

Wash solutions were preheated to 55°C. Each sample was washed in 2X SSC for 5 minutes at 55°C. Next, each sample was washed in 0.1X SSC for 10 minutes at 55°C. Samples were then centrifuged and suspended in PBS with 1% BSA for flow cytometric analysis.

#### *Flow Cytometric Analysis*

Fluorescence and light scatter of cells was analyzed via flow cytometry on a FACS Calibur equipped with argon (488 nm) and red diode (635 nm) lasers (BD, Franklin Lakes, NJ). Cells were analyzed in FACS Sheath Solution (BD), and 20,000 events per sample were analyzed, unless otherwise noted. Cy5 fluorescence was detected using the FL4 channel, while FITC and propidium iodide signals were detected using FL1 and FL2 channels, respectively. Data analysis was performed using CellQuest Pro software (BD).

#### **Results**

The initial hybridization of the 28S rRNA antisense probe resulted in retention of the probe within target cells as evidenced by a large mean fluorescence intensity (MFI)

(data not shown). This suggested a successful hybridization, and the following components of the system were analyzed in order to determine the robustness and sensitivity of the developed FISH-FC method.

#### *Effects of Permeabilization*

Following permeabilization and hybridization with ABF, samples retained similar cell size and granularity as an unpermeabilized sample not exposed to probe (Figure 1).

The average mean side scatter among ABF-permeabilized samples was 184.92, compared to 149.18 for an unpermeabilized sample. The average mean forward scatter among these samples was 574.01, compared to 559.71 for an unpermeabilized sample.

A different method which involved fixing with 2% paraformaldehyde, followed by permeabilization with 0.2% Triton X-100 in TBS (TX), was also employed. This method was used in order to investigate the possibility that permeabilization-based artifacts enhanced nonspecific binding. Exposed samples produced an average mean side scatter (76.60) that differed markedly from an unpermeabilized sample (149.18), indicating a decrease in cell granularity (Figure 1). The average mean forward scatter among these samples (561.31), however, did not differ substantially from the unpermeabilized sample (558.71). This indicates that the population's average cell size remained relatively unchanged.

Samples permeabilized with ABF and infiltrated by propidium iodide (PI), had a mean fluorescence intensity (MFI) of 256.94 (Figure 2). While a subpopulation of unfixed cells (M2, 61.09% of total) exposed to PI also provided a strong positive

signal (187.16), a subpopulation of this sample (M1, 24.56% of total) provided a response (5.61) similar to the negative control (3.16).

ABF and TX sufficiently permeabilized cell samples for allow probe entrance (Figure 3). Compared to a sample not exposed to probe, all probe samples (including sense probe samples, the negative control) provided a strong positive signal.

Additionally, the use of TX increased the MFI for the antisense probe almost four-fold, and over four-fold for the sense probe sample.

#### *Optimization of 28S rRNA probe concentration*

A series of probe concentrations were administered in order to determine the lowest concentration of probe that provided the strongest MFI. The antisense probe provided a positive signal that increased with probe concentration from 0.1 – 5 µg/ml (Figure 4). The sense probe (a negative control), however, provided a stronger signal than the antisense probe at all concentrations. The antisense probe provided a steadily increasing MFI with probe concentration that was nearly saturated at 1 µg/ml (Figure 1d). Future hybridizations were thus performed at a probe concentration of 1 µg/ml.

#### *Hybridization procedures and nonspecific binding*

Nonspecific binding of the sense probe was rampant at all probe concentrations assayed (Figure 3d). In order to reduce nonspecific binding, a blocking buffer containing 1X Denhardt's solution and 5 µg/sample of herring sperm DNA was utilized. While this buffer was employed in order to block nonspecific binding, the MFI increased for samples exposed to both probes in the presence of blocking buffer (Figure 5).

In order to assess whether the nonspecific binding was attributable to hybridization conditions or characteristics of the 28S rRNA sense probe, the CRPV antisense probe was applied to CRPV-uninfected RK-13 cells under hybridization conditions. As Figure 6 displays, the CRPV probe bound cells as frequently as the 28S rRNA antisense probe, which was the positive control.

#### *Wash procedures and nonspecific binding*

In order to reduce post-hybridization nonspecific binding, an additional wash solution was implemented and the temperature of wash solutions was increased from 55° to 60°C. While this was done in an attempt to reduce nonspecific binding, an increase in MFI resulted in both the antisense (from 591.73 to 977.61) and sense (from 1122.83 to 1392.54) samples under such conditions (Figure 7).

The wash and hybridization procedures were further modified by performing all incubations in a shaking water bath or incubator. Figure 8 reveals that such conditions did not significantly change the mean fluorescence intensity for the antisense probe sample (from 460.45 to 435.79) or the sense probe sample (from 1547.06 to 1615.94).

In an attempt to degrade and thus remove nonspecifically bound single-stranded probe, DNase was added to samples following the wash steps, and prior to flow cytometric analysis. There was, however, no significant difference in MFI between antisense and sense samples that received DNase and those that did not (Figure 9). This was consistent for both forms of permeabilization (ABF and TX).

#### **Discussion**

The detection of latent viral infection by FISH-FC analysis provides a potentially successful high-throughput method (19). The intention of this investigation was to

optimize a system that would allow for such analyses of multiple cell lines from different mammalian species infected with CRPV. Multiple components of the originally prepared protocol were to be optimized to develop a more effective system. An initial FISH-FC analysis of the 28S rRNA antisense probe revealed a large MFI compared to a sample not exposed to probe, indicating successful probe entrance (data not shown). Further trials involved performing FISH-FC with a 28S rRNA sense probe as a negative control. A BLAST analysis revealed that this sense probe did not exhibit specificity for known rabbit nucleotide sequences other than the template of the 28S rRNA. This single genomic copy therefore should have allowed for the specific binding of just one probe per cell. This signal would be insignificant compared to the binding of the 28S rRNA antisense probe, which would bind the ubiquitous 28S rRNA. Instead, binding of the sense probe produced a stronger MFI than the antisense probe in each trial (see Figures 3-9). Nonspecific binding thus prevented the implementation of this system in the desired manner.

It was first postulated that the RK-13 samples were agglutinated with probe during hybridization, thus rendering the wash steps insufficient, and resulting in nonspecific binding. To address this, a FISH/flow analysis was run with probes varying in concentration from 0.1 – 5  $\mu\text{g/ml}$ . This concentration range indeed revealed that MFI increased with probe concentration (Figure 4). It also revealed that the sense probe (negative control) provided a similar or stronger MFI than the antisense probe (positive control) even at the lowest probe concentration (0.1  $\mu\text{g/ml}$ ).

In efforts to prevent nonspecific binding, a blocking solution was added to the hybridization mix. This included 1X Denhardt's solution and 5  $\mu\text{g}$  of salmon sperm

DNA per sample. This application, however, did not reduce nonspecific binding of the sense probe (Figure 5). At this point it was postulated that nonspecific binding might be a necessary byproduct of the hybridization process, and that the wash steps required improvement in order to interrupt nonspecific binding.

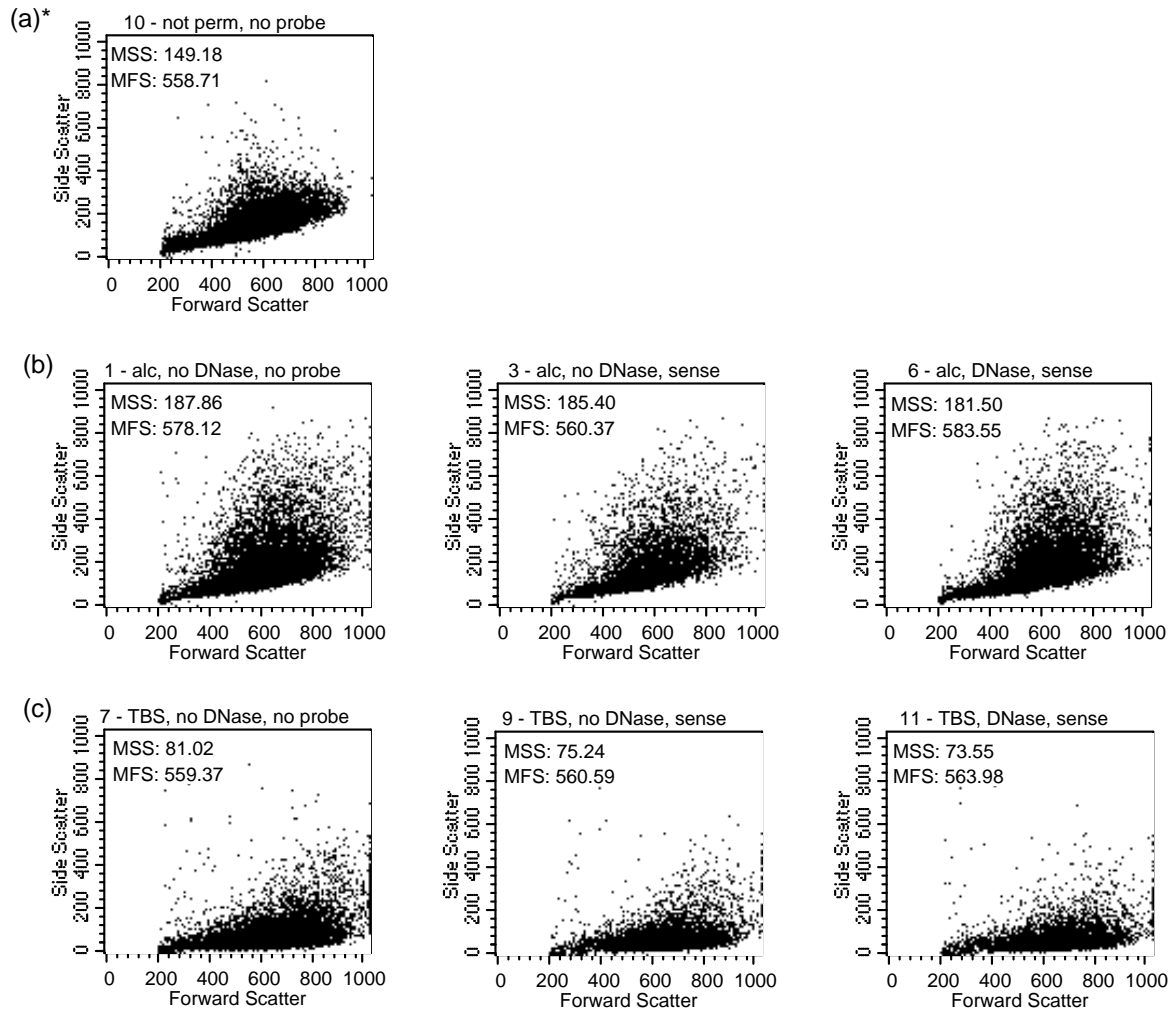
The wash temperature, number of applications, and duration of wash were all analyzed. Increasing the temperature (40°C to 55°C and 60°C), adding an intermediate wash step (0.5X SSC, 0.1% Triton X-100), and increasing the wash duration with this additional 10-minute step resulted in a greater MFI for both 28S rRNA antisense and sense samples (Figure 7). In order to more vigorously draw excess probe into the supernatant to be discarded (following centrifugation), samples were then administered wash steps in shaking water baths. This did not, however, reduce the MFI of sense probe samples (Figure 8).

Given the possibility that the ABF was creating artifacts that promoted nonspecific binding, a different permeabilizing agent was employed. Use of the TX fixing and permeabilizing protocol, however, did not reduce nonspecific binding, and actually increased the MFI for both 28S rRNA antisense and sense samples (Figure 3). Since the potentially deleterious components of the FISH/flow protocol had been analyzed prior to this point without reducing nonspecific binding, a relatively drastic addition was made to the protocol. An application of DNase was provided to each sample following the wash steps in an attempt to cleave nonspecifically bound single-stranded probe. This showed no appreciable effect on MFI in 28S rRNA antisense or sense samples (Figure 9).



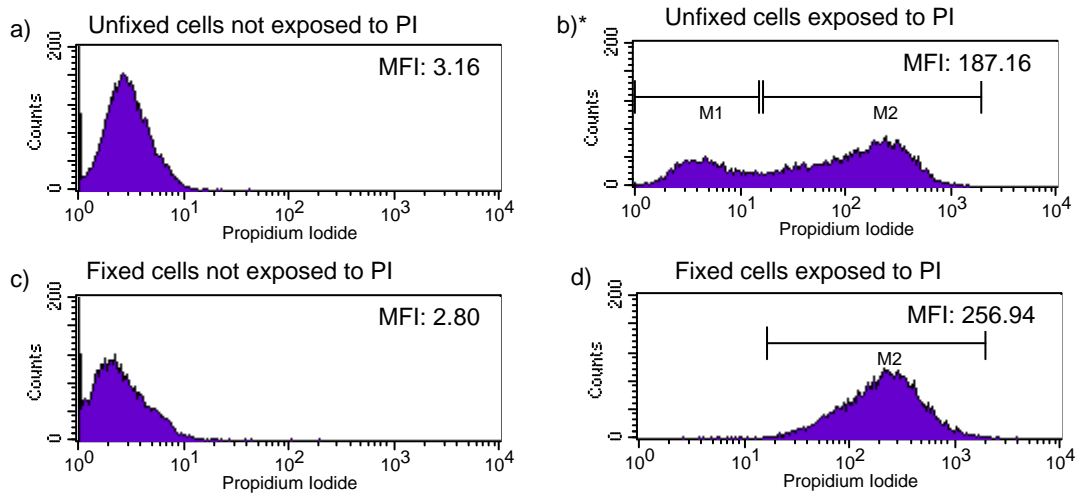
Since the CRPV transcript is to be the eventual probe target, it was important to assess if a CRPV antisense probe would experience the same nonspecific binding as the 28S rRNA probe. CRPV-uninfected RK-13 samples underwent hybridization with the CRPV antisense probe alongside samples exposed to the 28S rRNA antisense and sense probes. The uninfected samples exposed to a CRPV antisense probe showed a strong positive signal similar to that of the 28S rRNA antisense probe (Figure 6). It is unlikely that both the 28S rRNA sense probe (optimized by MacVector software and investigated via a BLAST search) and the CRPV antisense probe (previously chosen selectively by Culp and Christensen [7]) require further optimization to prevent nonspecific binding. It is therefore likely that the problem lies with the insufficient blocking or removal of nonspecifically bound probe. Future studies could focus on novel blocking agents and wash methods (to remove nonspecifically bound probe) so that this system can be employed to study aspects of CRPV infection at the single-cell level. Additionally, potential roles of the phosphorotioate internucleoside treatment in nonspecific probe removal could be investigated.

## Appendix



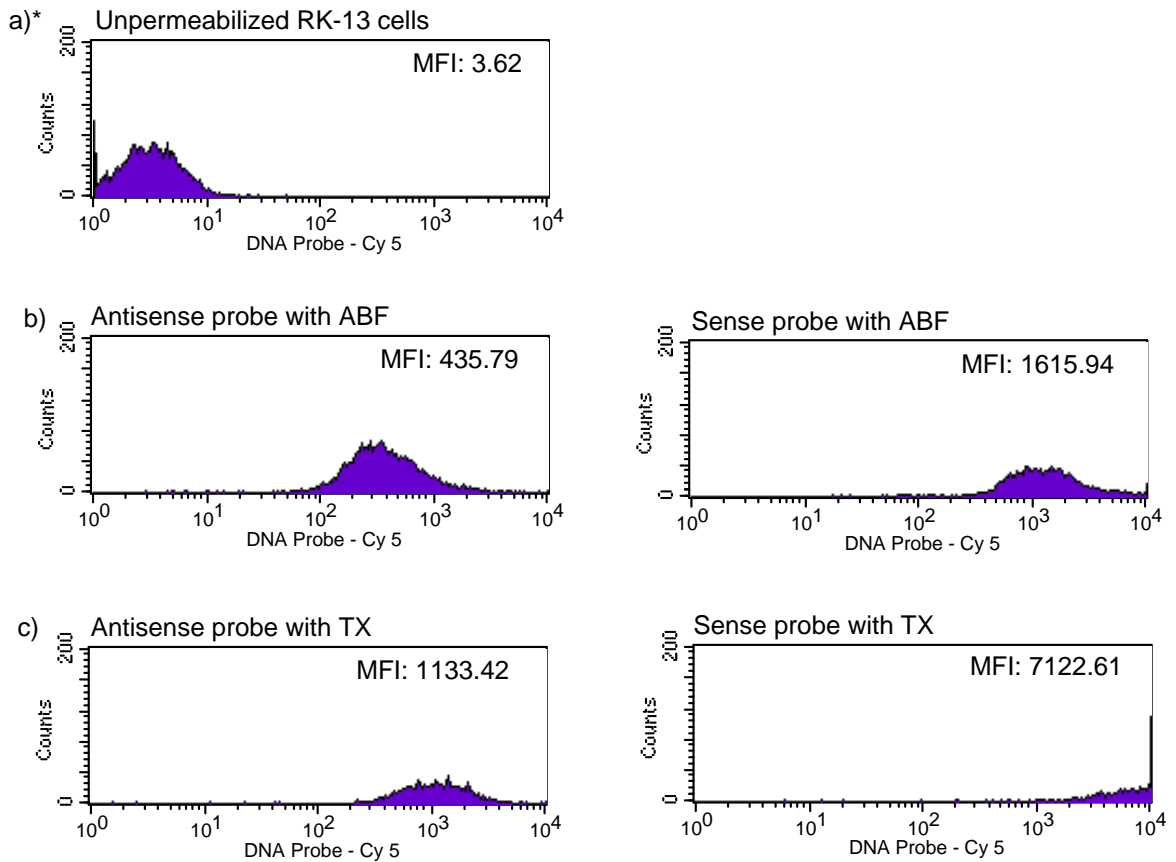
**Figure 1.** *The effects of differential fixation and permeabilization on RK-13 cell phenotype.* (a) Unfixed cell population. (b) Samples fixed and permeabilized with an alcohol-based fixative. (c) Samples fixed with 2% paraformaldehyde, then permeabilized with 0.2% Triton X-100 in TBS.

\*Sample (a) was prepared and analyzed under the same conditions as (b) and (c), but during a different experiment.



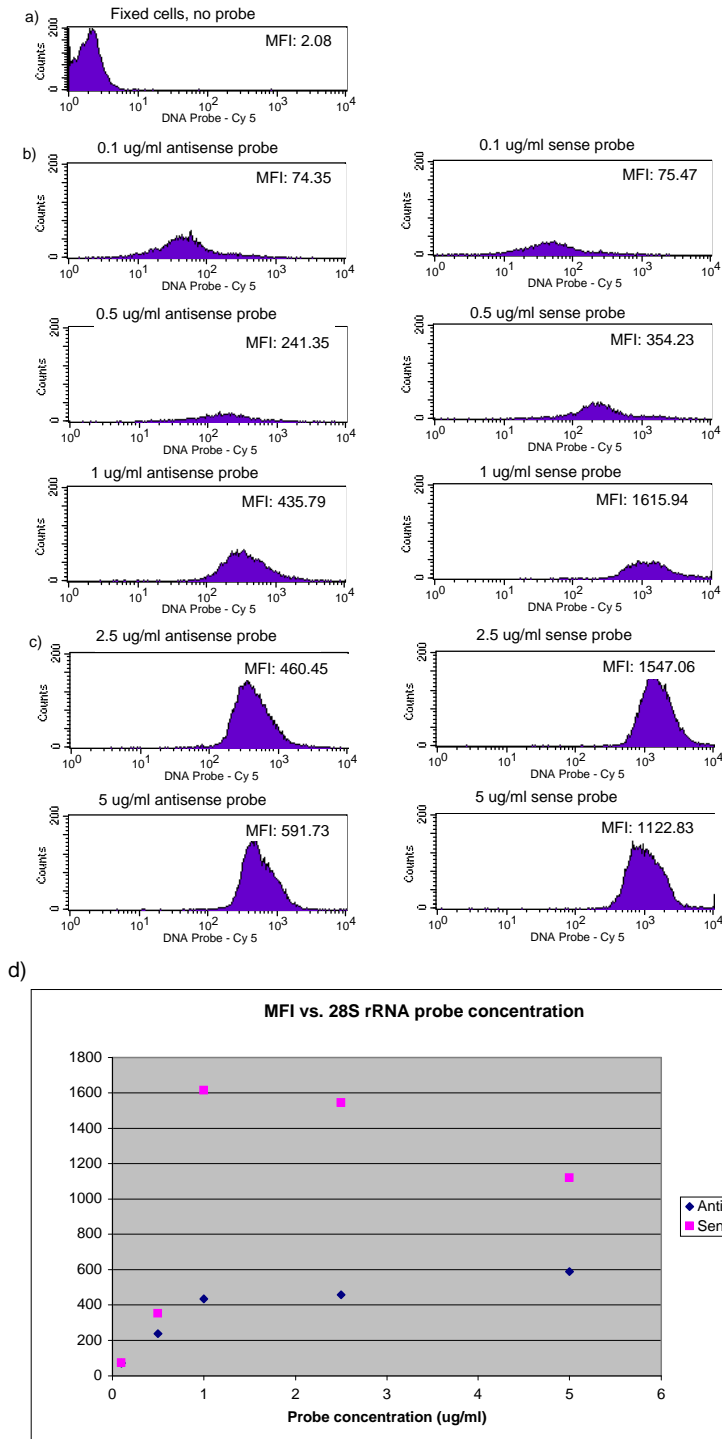
**Figure 2.** *RK-13 cell membrane integrity assessed by propidium iodide.* (a,b) Samples were not fixed, but were exposed to the hybridization and wash steps. (c,d) Samples were fixed, then exposed to the hybridization and wash steps. (b,d) Samples were exposed to 5 ul of propidium iodide following the wash steps and prior to flow cytometric analysis.

\*MFI is reported for gated M2 population.

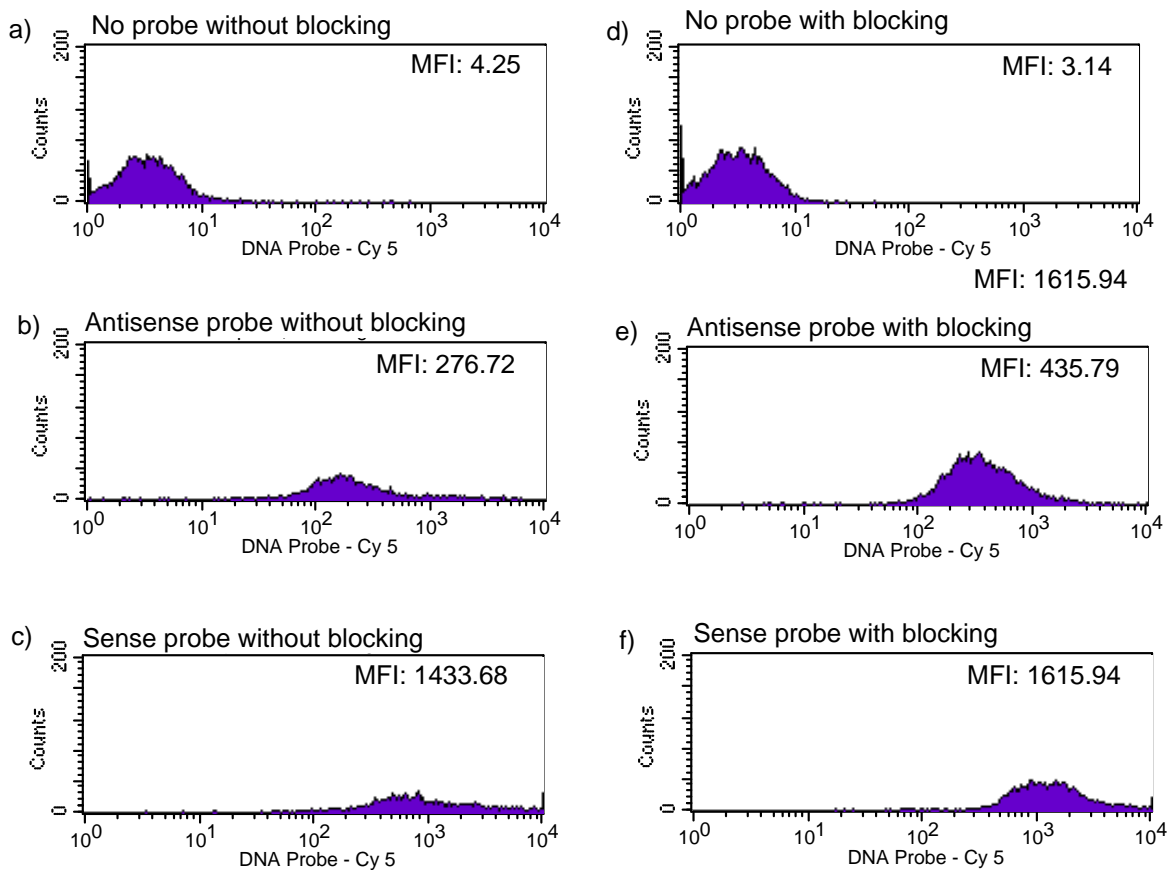


**Figure 3.** The effects of differential fixation and permeabilization on 28S rRNA probe (1 ug/ml) binding in RK-13 cells. (a) Unfixed and unpermeabilized cell population. (b) Samples fixed and permeabilized with ABF. (c) Samples fixed with 2% paraformaldehyde, then permeabilized with 0.2% Triton X-100 in TBS.

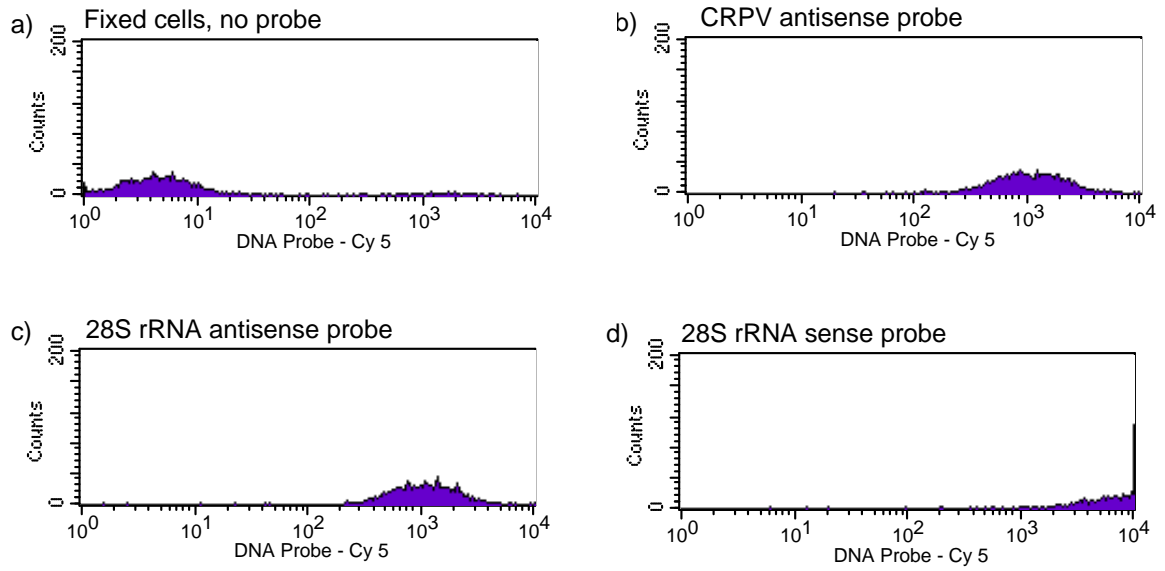
\*Sample (a) was prepared and analyzed under the same conditions as (b) and (c), but during a different experiment.



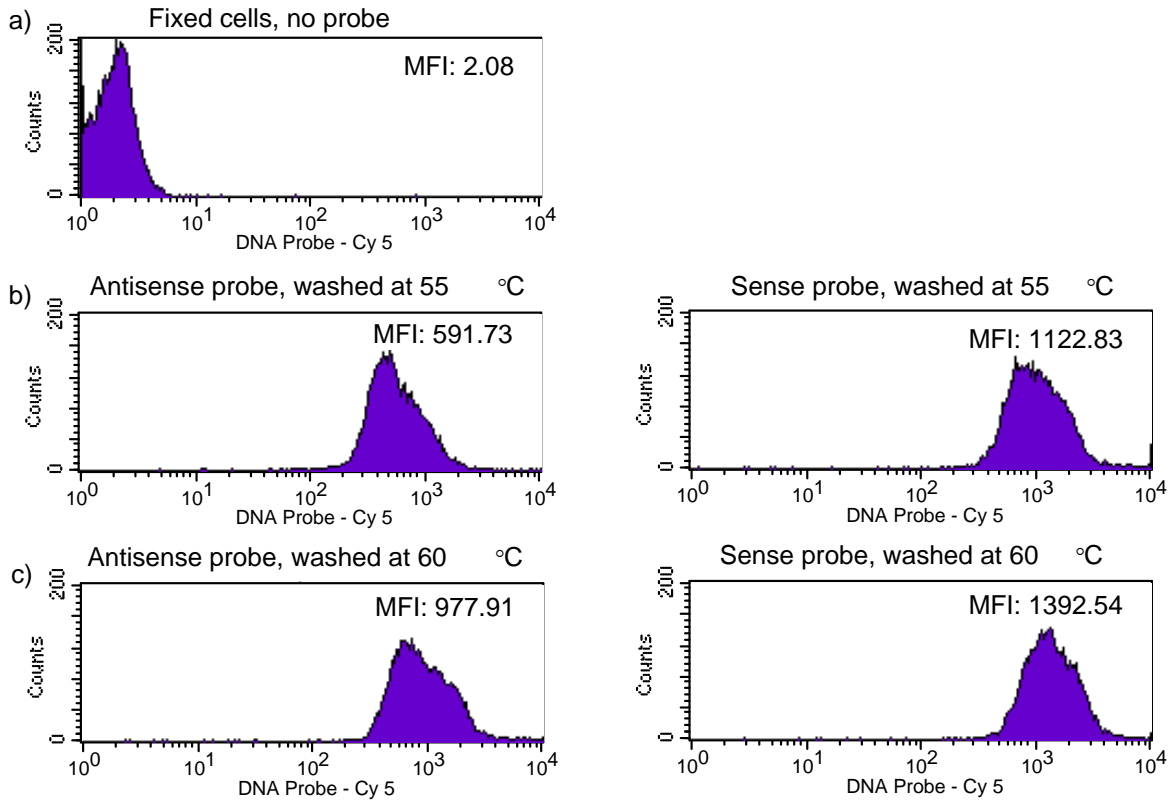
**Figure 4.** *Permeabilized RK-13 cells hybridized with varying concentrations of 28S rRNA antisense and sense DNA probes. (a) Baseline fluorescence of samples that underwent hybridization conditions without being exposed to probe. (b) Samples hybridized with 0.1 – 1  $\mu$ g/ml antisense or sense probe. Note: sample preparation resulted in low cell populations, producing a flow cytometric count less than 20,000. (c) Samples from a different experiment where samples received 2.5 or 5  $\mu$ g of probe. (d) Graph of probe concentration versus mean fluorescence intensity.*



**Figure 5.** *The effects of blocking buffer on nonspecific binding.* (a-c) Samples underwent standard hybridization conditions (with no probe, 28S rRNA antisense probe, or 28S rRNA sense probe) without exposure to a blocking buffer. (d-f) Samples underwent hybridization conditions (with no probe, antisense, or sense probe) in the presence of a blocking buffer. The blocking buffer consisted of 5  $\mu$ g of herring sperm DNA per sample in 1X Denhardt's solution.

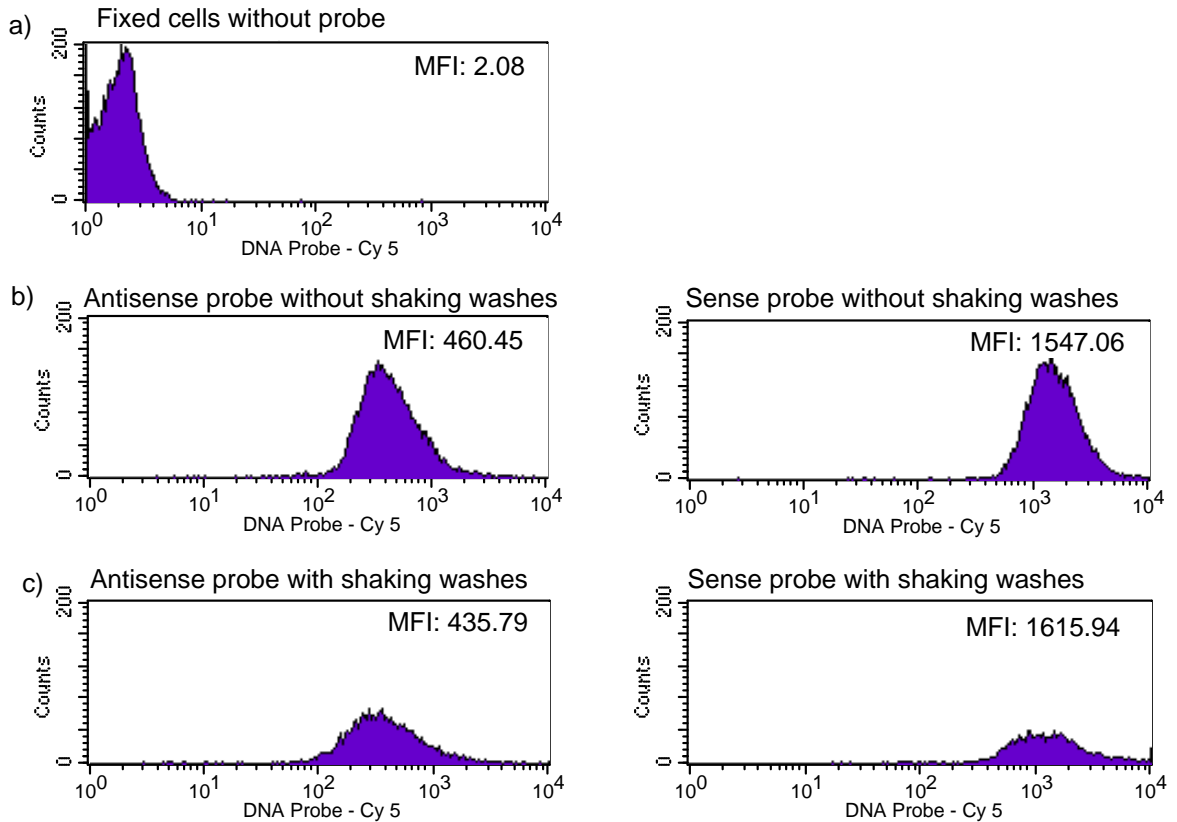


**Figure 6.** *Differential binding of probes in CRPV-negative RK-13 cells. Samples underwent hybridization conditions in the presence of 1 ug/ml of: (a) no probe. (b) CRPV antisense probe. (c) 28S rRNA antisense probe. (d) 28S rRNA sense probe.*

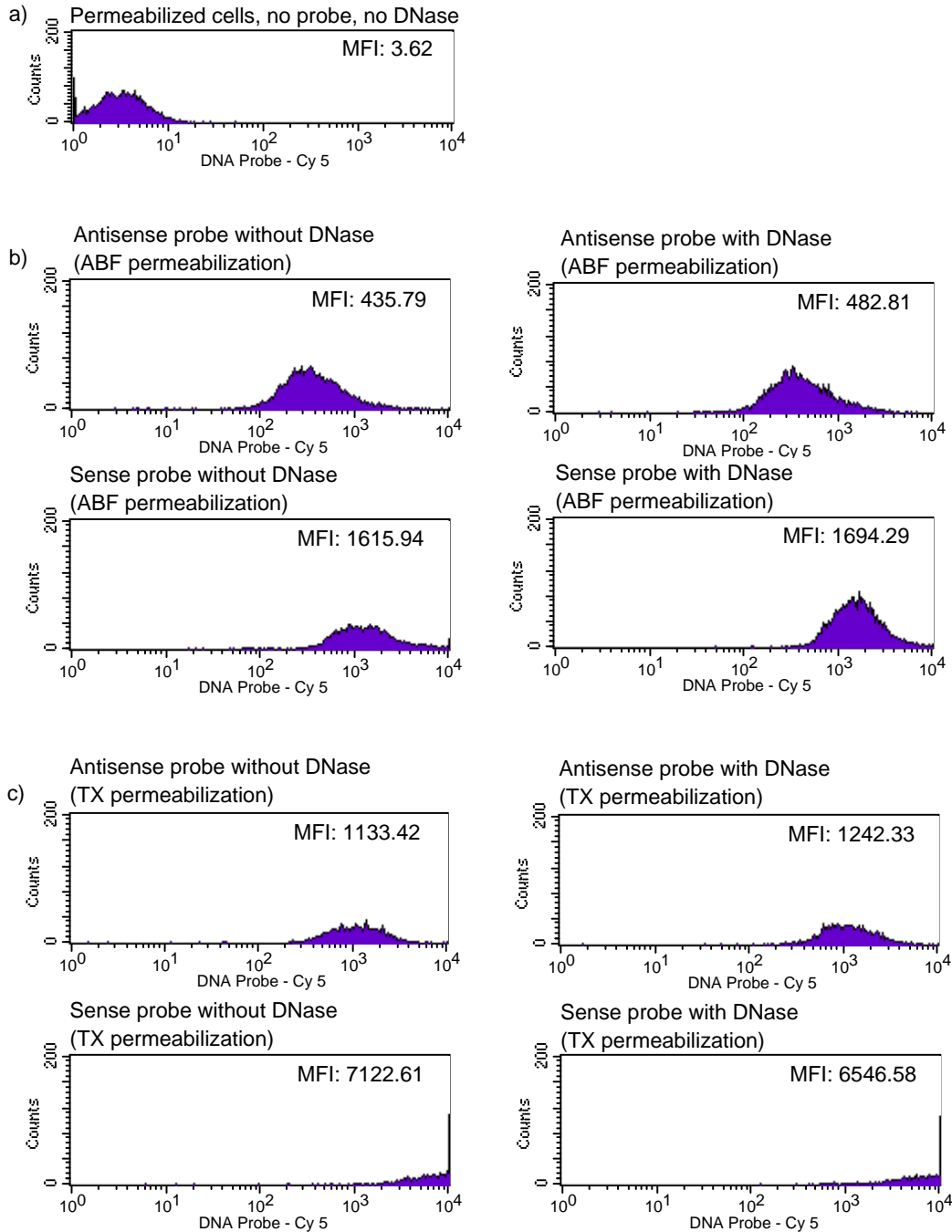


**Figure 7.** The effects of post-hybridization wash temperature and salt concentration on 28S rRNA probe (5 ug/ml) binding in RK-13 cells. Samples received 2X SSC with 0.1% Triton X-100 for 5 min., then 0.1X SSC with 0.1% Triton X-100 for 15 min. (a) Fixed cell sample underwent hybridization and wash conditions but was not exposed to probe. (b) Samples underwent wash steps at 55°C. (c) Samples underwent wash steps at 60°C and received an intermediate wash step with 0.5X SSC (0.1% Triton X-100) for 10 min.





**Figure 8.** *The impact of shaking hybridization and shaking wash steps on 28S rRNA probe binding in RK-13 cells.* Samples underwent hybridization and wash at 55°C. (a) Sample exposed to hybridization conditions without probe. (b) Samples exposed to 2.5 ug/ml of probe without shaking conditions. (c) Samples exposed to 1 ug/ml of probe with shaking conditions. (Note: Samples displayed in (b) were prepared during a different experiment than samples in (c).)



**Figure 9.** The effectiveness of nonspecific probe removal with DNase in different fixative treatments. (a) Sample was not exposed to probe during the hybridization or DNase following the wash steps. The following samples were exposed to 1  $\mu\text{g/ml}$  28S rRNA antisense or sense probe during hybridization. Particular samples received DNase following the wash steps, as indicated in the figure. (b) Samples were permeabilized with ABF. (c) Samples were permeabilized with TX.

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