Detection of Chemotherapy-Induced Apoptosis in Human Breast Cancer

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Abstract

Caspase-cleaved keratin 18 (K18) is used as a biomarker of apoptosis to measure chemotherapy-induced cell death. The M30-FITC antibody can be used as a method of detection for caspase-cleaved K18, giving it potential as a prognostic and predictive tool in cancer treatment. This study tests the M30-FITC antibody for use with the human breast cancer cell lines MCF7, SKBR3, and MDA-MB-231 in flow cytometry, with the goal of optimizing the M30-FITC assay for use in human whole blood. The assay was evaluated for use with two different apoptotic pathways: first induced by the chemotherapy docetaxel (Taxotere), and then by the tumor inhibitor staurosporine. Analysis indicates that the M30-FITC antibody requires a specific caspase cleavage product to be produced during apoptosis, which the mechanism of docetaxel-induced apoptosis (mitotic catastrophe) does not appear to produce. Staurosporine treatment appears to induce apoptosis in a manner compatible for use with the M30 antibody, and is sufficient to induce apoptosis in MCF7, SKBR3, and MDA-MB-231. These treated cells are also suitably detected in spiked human whole blood, indicating the potential for clinical relevance of the assay.

Introduction

Circulating tumor cells (CTCs) are cells that have detached from a primary tumor and entered the bloodstream, playing a critical role in the metastasis of cancer[1]., CTCs aide in the progression of disease and makes cancerous growth unpredictable and problematic to contain. Therefore evaluation of CTCs can yield important information about the cancer growth and potentially about responses to treatment[2]. When treated with some chemotherapy agents, cancer, cells undergo apoptotis indicating response to treatment. Apoptosis can occur by different mechanisms, depending on the type of cell and the type of chemotherapy to which they were exposed and is characterized by blebbing of the membrane, cell shrinkage, and condensation of chromatin. A family of cysteine proteases, called caspases, are an essential part of this apoptotic pathway. These caspases fall into two groups: “initiator” caspases (caspases-8 and -9), which can either self-cleave or activate the second type, “effector” caspases (caspases-3, -6, and -7)[3]. Together, caspases catalyze the process of apoptosis through regulation of other proteins in the cell.

The chemotherapy agent, docetaxel, used to induce apoptosis in the first part of this study, initiates its apoptotic mechanism through the process of mitotic catastrophe, which inhibits the depolymerization of microtubules in the cytosol[4]. The other toxin in this experiment, the kinase inhibitor staurosporine, is not used as a chemotherapy due to its high toxicity, but was used to model a specific apoptotic pathway. It utilizes caspase-3, one of the main caspases involved in apoptosis, in its mechanism of programmed cell death[5]. This study investigates apoptosis specifically in three different human breast cancer cell lines: MCF7, SKBR3, and MDA-MB-231, as these cell lines exemplify some of the most common types of breast cancer: estrogen receptor positive, Her2 positive, and triple negative, respectively. Of these cells, the apoptotic mechanism in MCF7 is the most well-documented, and will thus be the focus of discussion.

The ability to effectively test for apoptotic CTCs in patient blood would result in a clinically relevant, non-invasive method to monitor the early stages of cancer progression, as well as the recurrence of treated cancers. Currently, CTCs are difficult to detect due to their

infrequency of occurrence—only 1-10 CTCs may be present per mL of whole blood[2]—and are therefore an inefficient method of evaluating cancer development. Flow cytometry is one common method of CTC detection, and involves the detection of fluorescent biomarkers in apoptotic cells. Cells can be fluorescently stained in a sample of blood, and then isolated and characterized by the cytometer, which uses spectroscopy to detect the fluorophores on the cells. Three-colour flow, used in this project, allows the simultaneous detection of intracellular components and surface markers[8] by using differently-coloured labels on specific antibodies. Flow cytometry is also useful to differentiate between apoptosis and necrosis, the distinction between which is important for identification of which cells died in response to the applied chemotherapy, and which underwent a natural death due to other factors[9].

Creation of fluorescent antibodies for flow cytometry is a process by which fluorescent dyes are conjugated to antibodies targeted for proteins of interest in the cell. The modified antibodies then bind to their target, the antigen, and fluoresce when detected by the cytometer's laser. Epithelial cell adhesion molecule (EpCAM), conjugated with the red protein-pigment phycoerythrin (PE), is one of these commonly used fluorescent markers. As it is expressed on most epithelial cells, but no other types[10], it is useful for detection of epithelial tumor cells in the blood. In conjunction with this, other fluorescently-tagged antigens are used to detect apoptosis. In this experiment, AnnexinV-fluorescein isothiocyanate (AnnexinV-FITC), Cytokeratin-PE (CK-PE) and 7-Aminoactinomycin D (7AAD) were used to characterize apoptosis and necrosis. AnnexinV binds to the surface marker phosphatidylserine, which is translocated from the inner to the outer cellular membrane during the early stages of apoptosis. The dye with which it is conjugated, FITC, is a green fluorophore and is easily distinguishable from the red PE[11]. CK is an extremely useful marker for representing cell progression through apoptosis, as they are released from apoptotic cells during intermediate apoptosis[12]. Epithelial cells, and thus epithelial cancers, have relatively large concentrations of both soluble and insoluble cytokeratins[13]. 7AAD is a nuclear marker, and thus is one of the last markers to be detected in apoptosis. As 7AAD cannot pass through an intact cell membrane, it will not fluoresce until the nuclear envelope has broken open, indicating the final stages of apoptosis and entry into necrosis[14].

While the protocols for the aforementioned antibodies have all been optimized, the associated assays require the use of several antibodies to test for apoptosis in epithelial cells—EpCAM to detect the epithelial cells, and AnnexinV to detect early apoptosis, for instance. Another less common assay, which uses an M30 antibody, can perform both these tasks with use of only one antibody and could provide another method of study for cell death in chemotherapy-treated cells. The M30 antibody, conjugated with FITC for this experiment, detects caspase-cleaved keratin 18 (K18), a biomarker of epithelial apoptosis[15]. In previous studies, M30 antigen levels have been correlated with other established prognostic markers[13], indicating the potential for M30 to be successful in characterization of apoptosis. Treatment of normal, tumor-free rats with a kinase inhibitor (AZD1152) yielded no change in baseline values of M30, while treatment of tumor-bearing rats produced a three-fold increase in M30 antigen levels in plasma, compared to controls[16]. These findings suggest the prospective uses of M30 as a more prevalent antigen in the detection of apoptotic CTCs in whole blood, an ability which is critical in monitoring cancer progression and evaluating the effectiveness of administered chemotherapies[2]. This experiment will examine the efficacy of the M30 antibody and apoptosis assay to determine its value as a potential biomarker of chemotherapy-induced cell death in human breast cancer cells.

Methods

Cell Culture

Human cell lines MCF7, SKBR3, and MDA-MB-231 were cultured in T-75 flasks for adherent cells, and maintained in RPMI 1640 media, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glutamate. To prevent overgrowth, cells were split approximately every three days and kept at approximately 50% optical density. For cell splitting, the media was removed, cells were washed with 5 mL Dulbecco's phosphate-buffered saline (DPBS), and incubated at 37° C for three minutes in 1 mL Trypsin. The flask was tapped against the counter to loosen any adherent cells, and 9 mLs of RPMI, warmed to 37° C, was added. Cells were then brought into a single-cell suspension and a fraction was discarded into 10% bleach. Fresh RPMI was added to bring the total volume of the flask back to 12 mL and cells were replaced in the incubator at 37° C.

Drug Treatment

For treatment with docetaxel or staurosporine, cells were collected with Trypsin and counted using Trypan blue. Cells were plated in six-well plates, at 500,000 cells/well, and incubated overnight at 37° C. Cell media was then replaced with media containing the appropriate treatment at a concentration varying from 0.0625 to 2 μM for a duration ranging from 30 min to 24 hrs.

Apoptosis Assays

Cells from each cell line were collected independently, washed in DPBS, resuspended in DPBS, and added to Epi tubes in 100 uL aliquots. Cells were stained according to manufacturer's specifications. AnnexinV-FITC was used in conjunction with EpCAM-PE, CK-PE, and 7AAD as a control. The M30-FITC CytoDEATH antibody from Peviva was used as an experimental variable. For the M30 assay, methanol was used as a fixative and 1% PBSF was used as an incubation buffer.

Flow Cytometry

All samples were assayed on a BD Accuri™ C6 flow cytometer, with 10,000 cells per sample collected for analysis. Apoptotic cells are defined as AnnexinV-FITC$^+$, 7AAD$^+$, or M30-FITC$^+$, necrotic cells as CK-PE$^+$ or M30-FITC$^+$, and EpCAM-PE was used as an epithelial marker. Control tubes of untreated and treated cells stained with each individual antibody were run before each group of experimental tubes, to compensate and establish a baseline to determine positivity.

Results

M30-FITC as an Apoptotic Marker in Docetaxel-Induced Apoptosis

The first undertaking in this experiment was to evaluate the potential of M30-FITC to be used as an apoptotic marker, using a previously-optimized AnnexinV apoptosis assay. A secondary goal was to test if the staining of any antibodies was affected by methanol fixation: methanol fixation is required for M30-FITC staining, but is not standard for any of the other antibodies used. The cell line MCF7 was initially used for this experiment, as it was shown in

previous experiments to be compatible with the chemotherapy docetaxel, and the required treatment concentration was known: MCF7 was treated for 30 min with 50 μM docetaxel. Both fixed and unfixed cells were stained with AnnexinV-FITC, CK-PE, 7AAD, and M30-FITC individually. Unfixed cells were used as a control to which to compare the fixed, treated cells. The increase in apoptosis from the untreated cells to the treated cells (Fig. 1) determines that the cells responded successfully to the docetaxel treatment: 11.5% apoptosis and 2.3% necrosis in the untreated cells increased to 42.4% apoptosis and 29.4% necrosis in the treated cells as evaluated by AnnexinV-FITC and 7AAD fluorescence. This is in agreement with the goal of ~50% apoptosis in treated cells. Staining of the cells (Fig. 2) indicated that fixation with methanol does not affect the use of any necessary antibodies. However, M30-FITC has little induction in treated cells, with levels of apoptosis for treated cells closely reflecting the levels of apoptosis in untreated cells, despite the knowledge that the treated cells were in fact more apoptotic than the untreated cells, as evidenced by the AnnexinV-FITC assay. Similar results were obtained for the SKBR3 and MDA-MB-231 cell lines.

Permeabilizaton of Docetaxel-Treated MCF7 Cells

One hypothesis as to why M30-FITC had such little induction was the possibility of poor permeabilization acting as a barrier to effective binding. To test this hypothesis, MCF7 cells, treated with 50 μM docetaxel as in the first experiment, were permeabilized with Tween20 before antibody staining, according to standard protocol. Cells were shown to be ~50% apoptotic/necrotic with a Trypan blue cell count before fixation with methanol. Trypan blue was used to measure apoptosis, as opposed to AnnexinV, to conserve both time and resources. Trypan blue has previously been shown to be a comparable substitute to the AnnexinV apoptosis assay. However, minimal apoptosis (1.6%) was detected in treated by M30-FITC staining, which was equivalent to the amount of apoptosis present in the healthy, untreated cells (Fig. 3). These results suggest that, while apoptosis is being induced by docetaxel in MCF7, the M30-FITC antibody is incapable of recognizing it.

Staurosporine-Induced Apoptosis in MCF7

The hypothesis that M30-FITC was effectively recognizing apoptosis, but not apoptosis induced by the chemotherapy docetaxel, was then put under investigation. The kinase inhibitor staurosporine was selected as the new toxin to be tested, as literature searches suggested its compatibility with the M30 antibody[5]. In its first experiment, a serial dilution of staurosporine was done overnight (24 hours), starting with 2 μM and decreasing to 0.125 μM. The treated cells were stained with M30-FITC and run on the cytometer. Not only was M30-FITC capable of recognizing apoptosis, it appeared to stain in a dose-dependent manner, detecting increasing amounts of apoptosis corresponding to the increases in concentration of staurosporine (Fig. 4). This suggests that the mechanism of apoptosis induced by docetaxel is indeed not compatible for use with the M30 antibody, but the apoptotic mechanism brought about by treatment with staurosporine produced the appropriate caspase cleavage product required for M30 binding.

Optimization of Staurosporine in MCF7

Now that an effective apoptotic inducer had been recognized, the assay needed to be optimized in order to determine the concentration of staurosporine required to induce approximately 50% apoptosis in the cells. From the previous experiment, 1 μM staurosporine was anticipated to be sufficient for inducing 50% apoptosis, so another serial dilution, from 1 μM to 0.0625 μM was performed. The M30-FITC antibody again was able to stain in a dose-

dependent manner, and produced clear populations of apoptotic and healthy cells (Fig. 5). One problem that arose in this assay was a high amount of clumping among the apoptotic cells. Clumping is fairly common when large amounts of apoptosis are induced, so to combat this in future experiments, precautions were taken during cell culture. Rather than grow a single large population of cells for treatment, several smaller populations were cultured and then amalgamated after treatment (ie. Instead of one six well plate with 500,000 cells/well, two six well plates with 250,000 cells/well were used), with the intention of preventing overcrowding of the cells. Clumping was not encountered again. This experiment indicated that an overnight treatment of 1 μM staurosporine was sufficient to induce approximately 50% apoptosis, the desired amount for future assays. This concentration was also sufficient in SKBR3 and MDA-MB-231.

Discussion

Circulating tumor cells are indicators of cancer cell dissemination in blood, and identification and detection of any chemotherapy induced changes in them might be useful for predicting responses to chemotherapy. The M30-FITC antibody is capable of recognizing apoptosis of human breast cancer cells in whole blood, and thus has potential as an apoptotic identifier, but it also has several associated issues that might need to be optimized prior to it being a useful tool in the clinical setting. Most notably, its specific requirement for the epitope formed after caspase cleavage of K18 makes it incompatible with the chemotherapy docetaxel, which is a commonly used treatment. Docetaxel induces apoptosis through mitotic catastrophe, which prevents microtubule destabilization and does not produce the appropriate caspase cleavage product. Thus, the M30 antibody is limited in its application, as the cells to which it could be applied would have to be carefully selected to ensure the necessary cleavage product was present.

Despite these limitations, the M30 antibody seems apt at detecting apoptosis in a laboratory setting, and provides a quicker assay than many alternatives. M30 specifically detects epithelial apoptosis with a single antibody, rather than needing several antibodies to detect epithelial cells and apoptosis, such as EpCAM and 7AAD. The use of M30 could facilitate identification of apoptotic tumor cells by offering a relatively simple and speedy assay for laboratory use. Furthermore, this study has confirmed the ability of staurosporine to induce apoptosis in MCF7, SKBR3, and MDA-MB-231, at a treatment concentration of 1 μM for 24 hours. While it was initially thought that the cell line MCF7 might lack caspase-3, other studies have determined alternate pathways that can produce this cleavage product in MCF7, pro-caspase-6 or caspase-9, for example[6, 7]. Our studies confirm the likelihood of an alternate pathway, and per this study, MCF7 appears to be a good candidate for use with staurosporine, and the dose-response data collected suggests that staurosporine is effective at inducing apoptosis in MCF7. The next logical step in the evaluation of the M30 antibody's usefulness would be to test its ability to stain cells spiked in whole blood, as opposed to spiking the blood with previously-stained cells. As the ultimate goal of this endeavor is to create a laboratory relevant assay for M30.

References

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Figure Legend

Figure 1: Apoptosis in treated and untreated MCF7 cells. (**A**) An untreated sample of MCF7 cells shows 11.5% apoptosis and 2.3% necrosis. This cellular death is likely due to the natural cell cycle and cells initiating apoptosis due to mistakes during replication or other common factors. These baseline levels establish the distinction between apoptosis in the normal cells and apoptosis due to treatment. (**B**) A sample of the same cells that had been treated with 50 μM docetaxel for 30 minutes shows an increase to 42.4% apoptosis and 29.4% necrosis. This increase indicates the success of the docetaxel treatment in initiating apoptosis in MCF7. Apoptosis was evaluated by the fluorescence of the AnnexinV-FITC and 7AAD antibodies.

Figure 2: Staining of fixed untreated and 50 μM docetaxel treated MCF7. (**A, B**) Staining with 7AAD and CK-PE in fixed, untreated cells indicates that CK-PE needs to be titrated down in concentration to be brought into scale. 7AAD and CK-PE staining in fixed cells treated with 50 μM docetaxel shows similar results. 7AAD staining is not affected by methanol fixation. (**C**) Fixed, untreated cells stained with CK-PE and M30-FITC also indicate that CK-PE must be titrated down for use with this assay. (**D**) Fixed, treated cells stained with CK-PE and M30-FITC show little induction of M30-FITC. (**E, F**) Staining with 7AAD and EpCAM-PE both in untreated and treated cells demonstrates that EpCAM-PE efficacy is not affected by methanol fixation. (**G**) Staining with EpCAM-PE and M30-FITC show 12.3% apoptosis in the fixed, untreated sample, which is consistent with the data from the AnnexinV assay—13.7% apoptosis and necrosis. (**H**) EpCAM-PE and M30-FITC staining in the fixed, treated sample, show little induction of M30-FITC: only 12.5% apoptosis is shown, the same as in the untreated sample.

Figure 3: Permeabilization in fixed MCF7. (**A**) Staining of untreated MCF7 with M30-FITC shows apoptosis present in the healthy population of cells to be 2.0%. (**B**) Staining of 50 μM docetaxel treated MCF7 with M30-FITC shows little/no induction of M30—the 1.6% apoptosis shown is due to apoptosis from the natural cell cycle as indicated by the baseline provided by (A). Note that the population of cells in the lower left (LL) quadrant has shifted up when compared to the corresponding quadrant in (A). This is likely due to auto-fluorescence caused by permeabilization and is not a confound for the results. (**C**) Reiteration of the data from (A) shown as a histograph. Cells in M1 (1.9%) are M30-FITC positive. (**D**) Reiteration of the data from (B). M30-FITC appears to detect apoptosis from cell cycle death, but not docetaxel-induced apoptosis.

Figure 4: Dose-response in staurosporine-treated MCF7. M30-FITC proved capable of detecting a dose-dependent response in increasing concentrations of staurosporine treatment. Untreated MCF7 cells were defined as M30-FITC negative and set as a baseline to 0%, while percent apoptosis in treated samples was detected by the fluorescence of M30-FITC. The highest concentration of staurosporine, 2 μM, yielded 41. 63% apoptosis. The drug was titrated from that concentration to concentrations of 1, 0.5, 0.25, and 0.125 μM, with levels of apoptosis corresponding to 39.57, 27.16, 16.68, and 11.11% apoptosis, respectively.

Figure 5: Optimization of staurosporine in MCF7. (**A**) Untreated, unstained cells were defined as M30-FITC negative. (**B**) Untreated, M30-FITC stained cells show 11.7% apoptosis. This apoptosis is indicative of the baseline amount of apoptotic cells in the MCF7 culture. (**C**) Stained cells treated with 0.0625 μM staurosporine yielded 20.3% apoptosis. (**D**) 0.125 μM treated cells were 43.2% apoptotic. There was also a low yield of cells; treated cells were particularly clumpy, likely due to the high levels of apoptosis, and had to be filtered before assaying. (**E**) Cells treated with 0.25 μM docetaxel also had a high propensity to clump, with 31.7% apoptosis. (**F**) Cells

treated with 0.5 μM docetaxel had 43.7% apoptosis and a moderate amount of clumping. (**G**) The 1 μM treatment induced 47.5% apoptosis and moderate clumping. The 1 μM staurosporine treatment was selected for further experiments, as it yielded approximately 50% apoptosis.

Figures

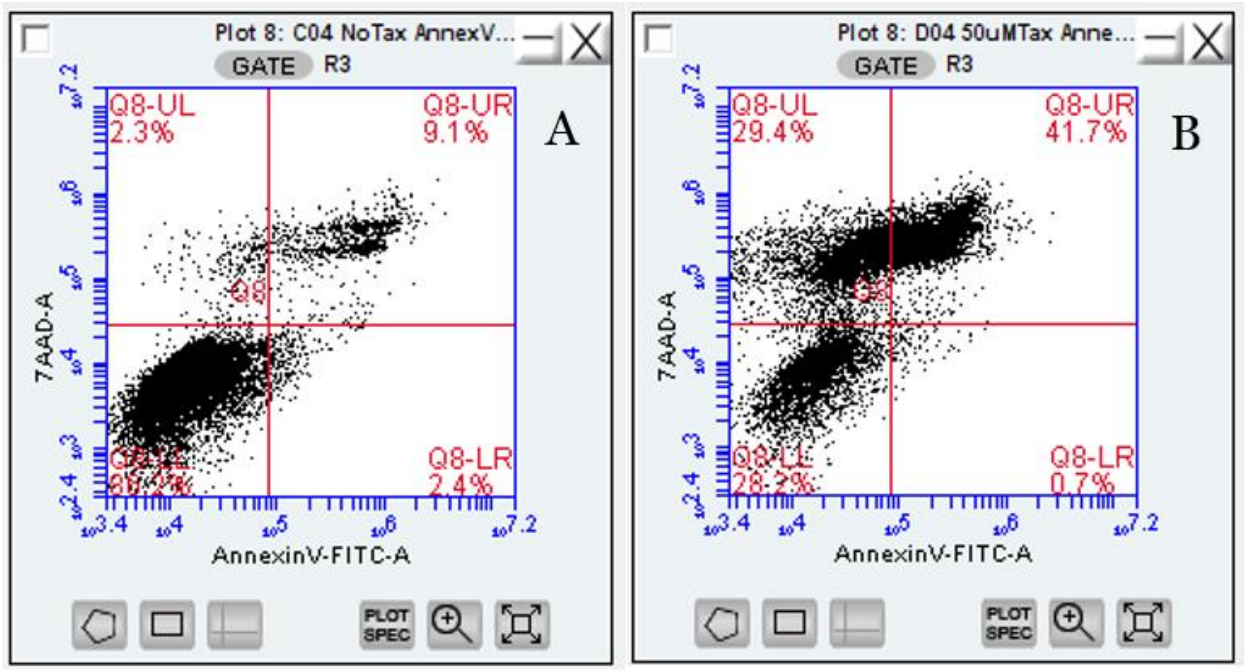
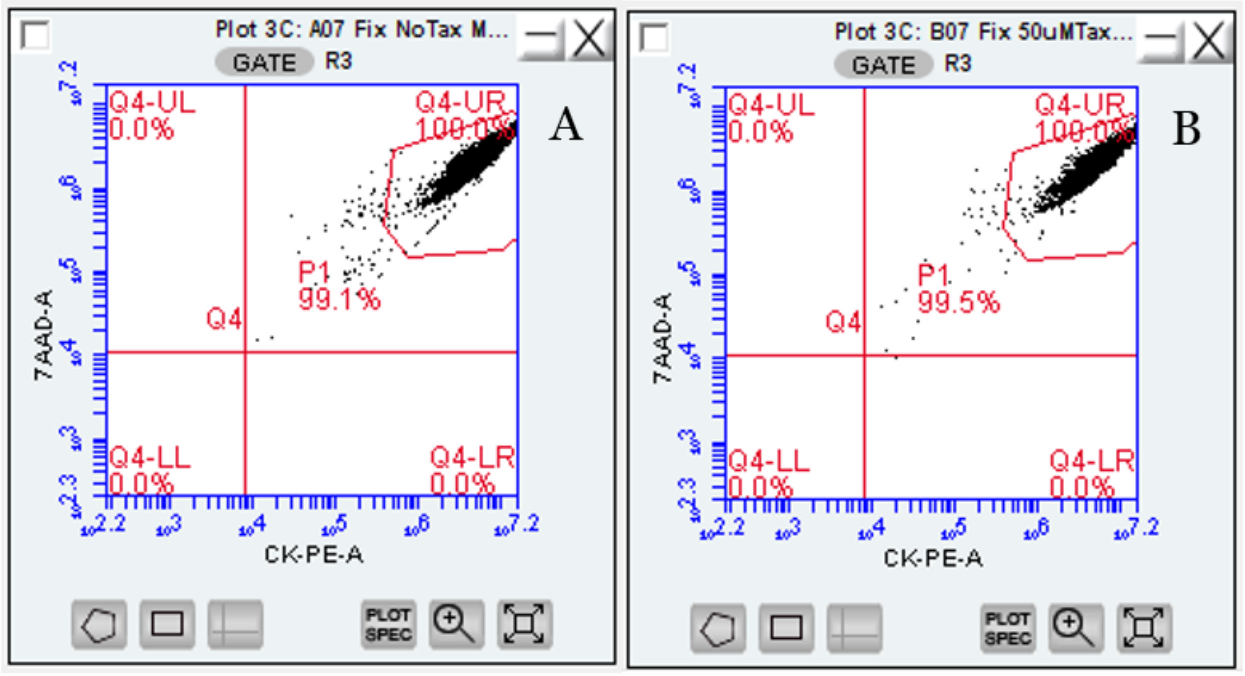
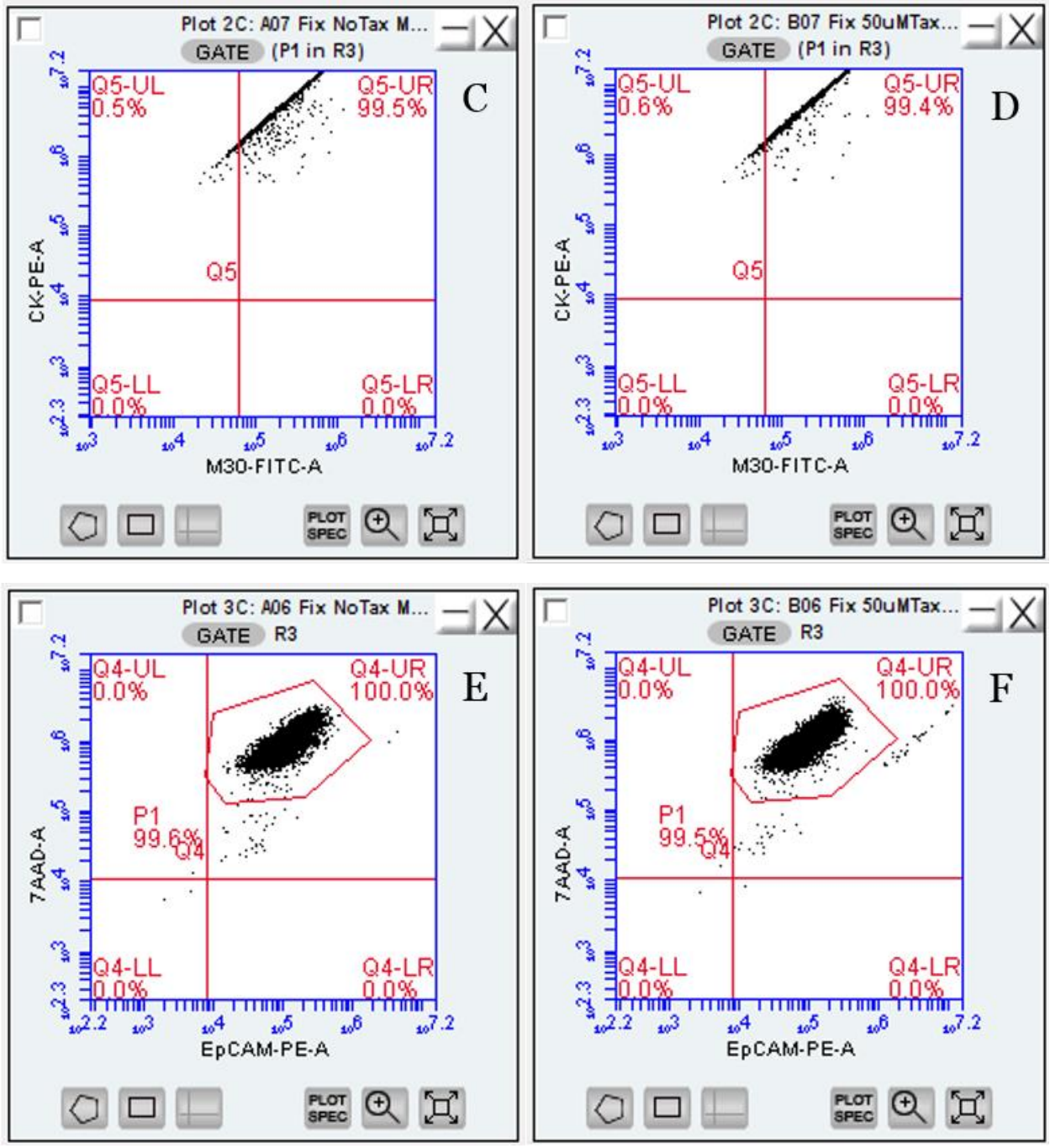


Figure 1

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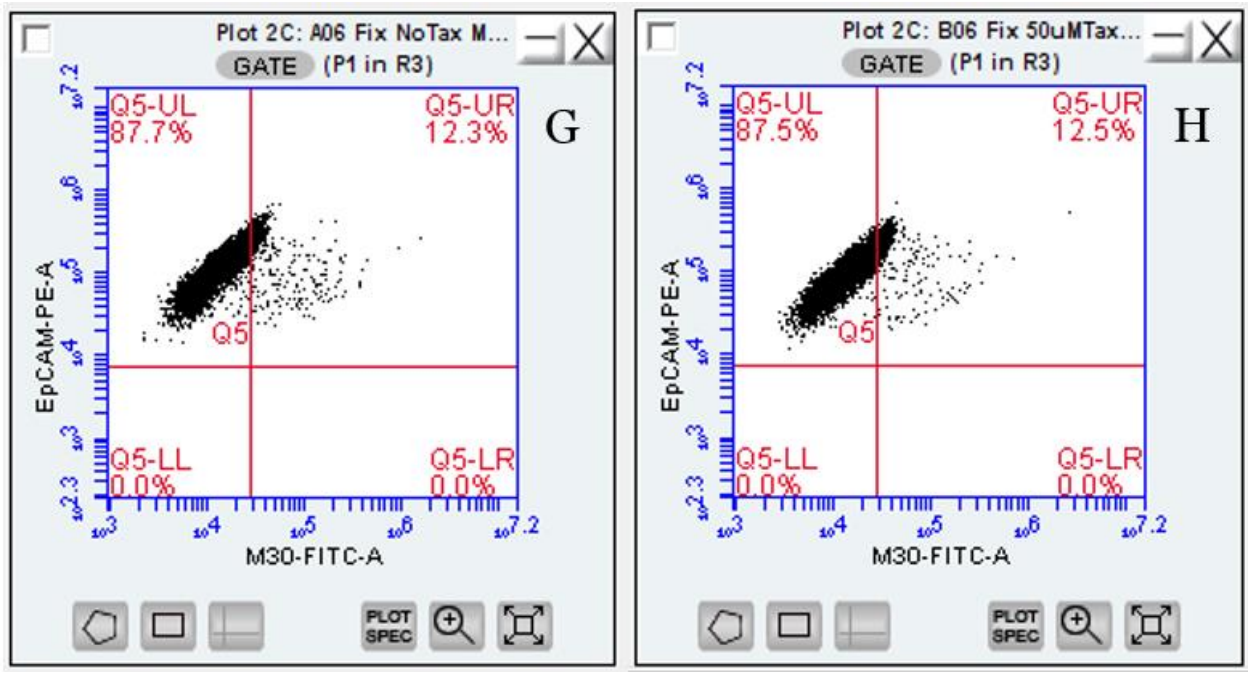
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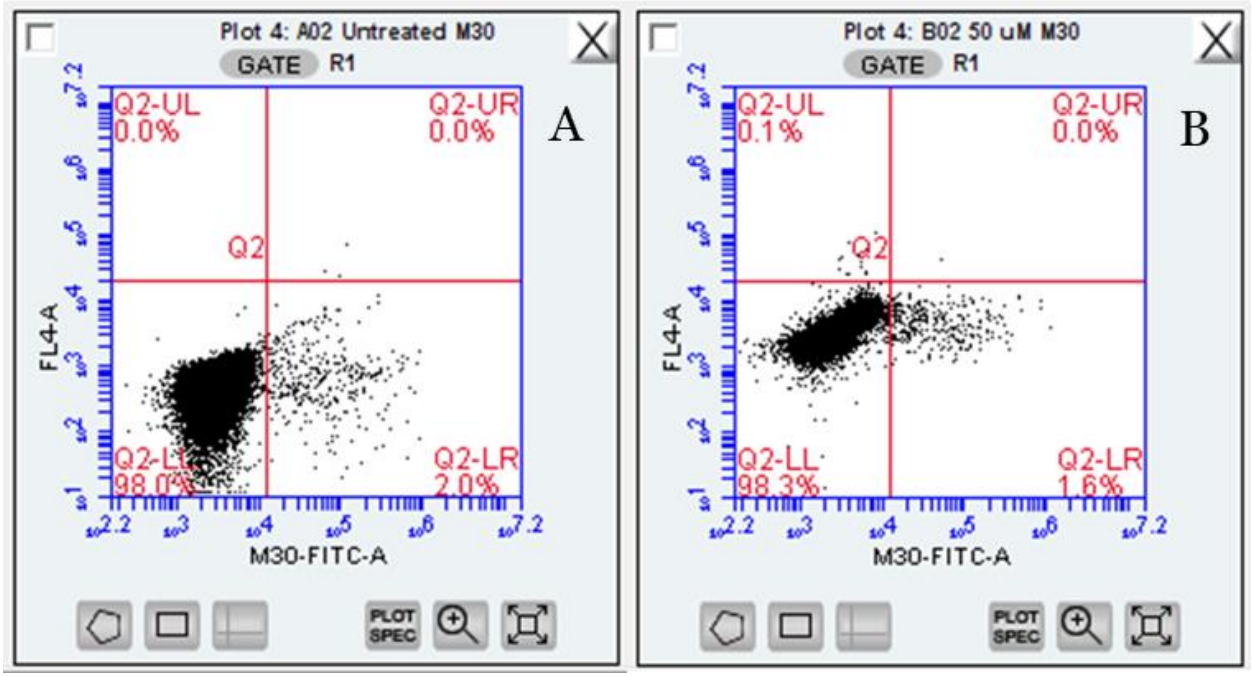
Figure 2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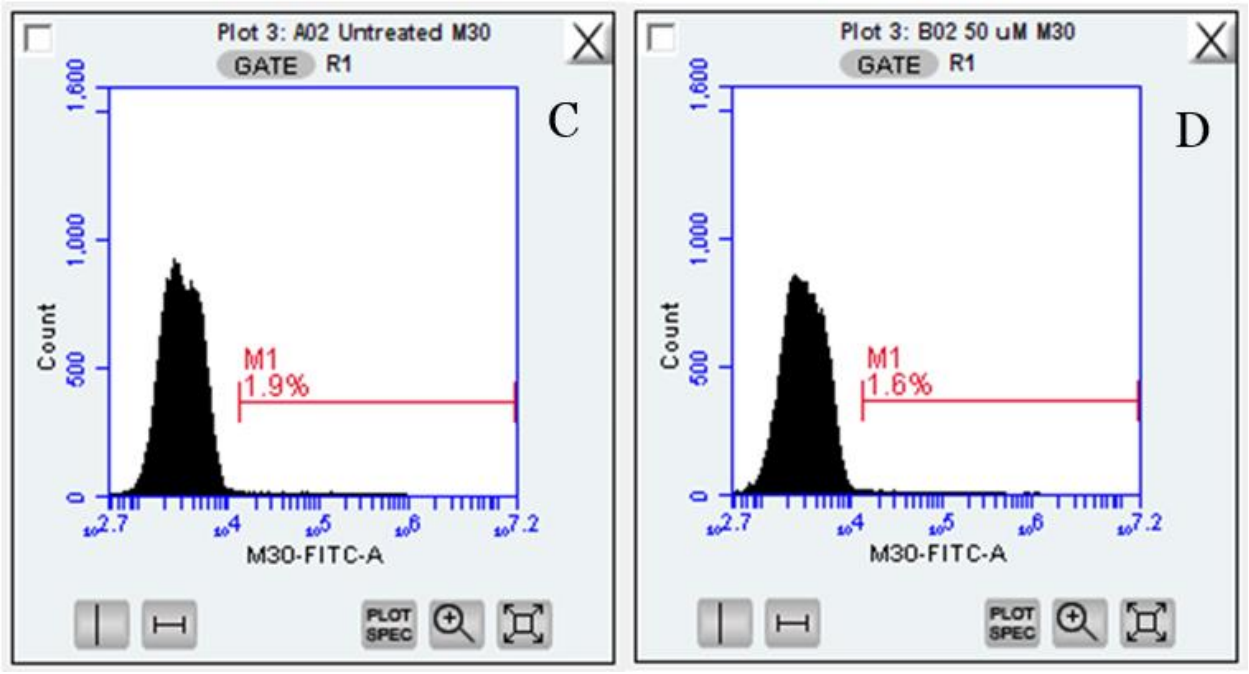


Figure 3

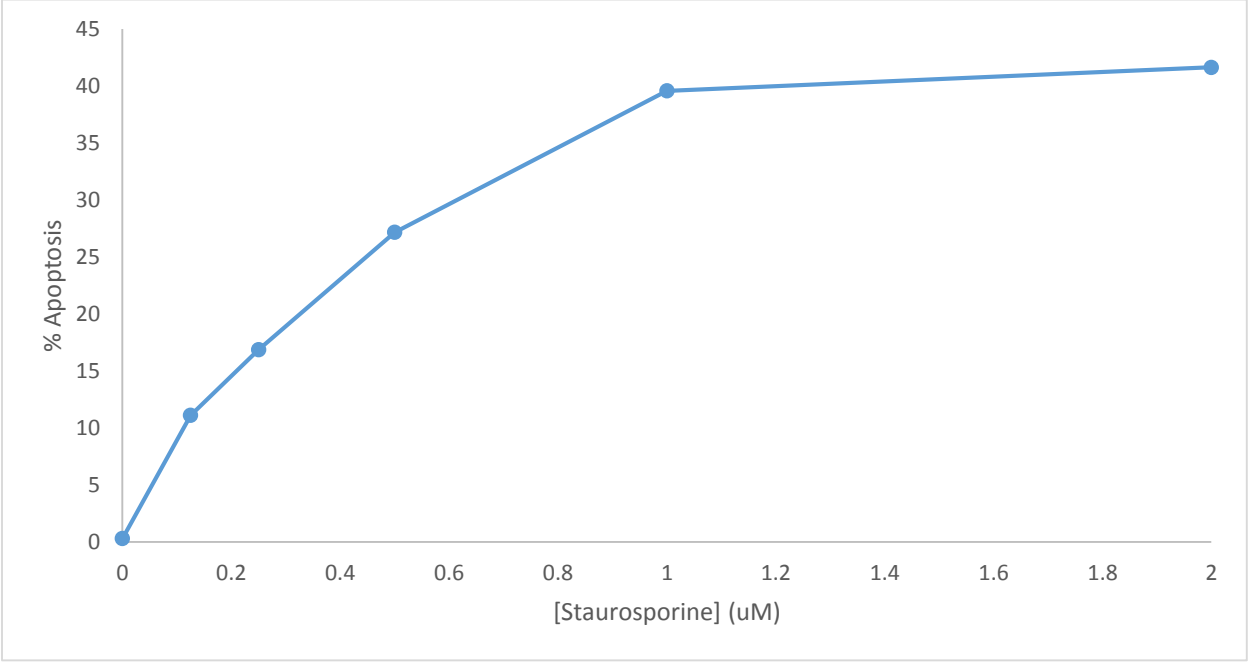
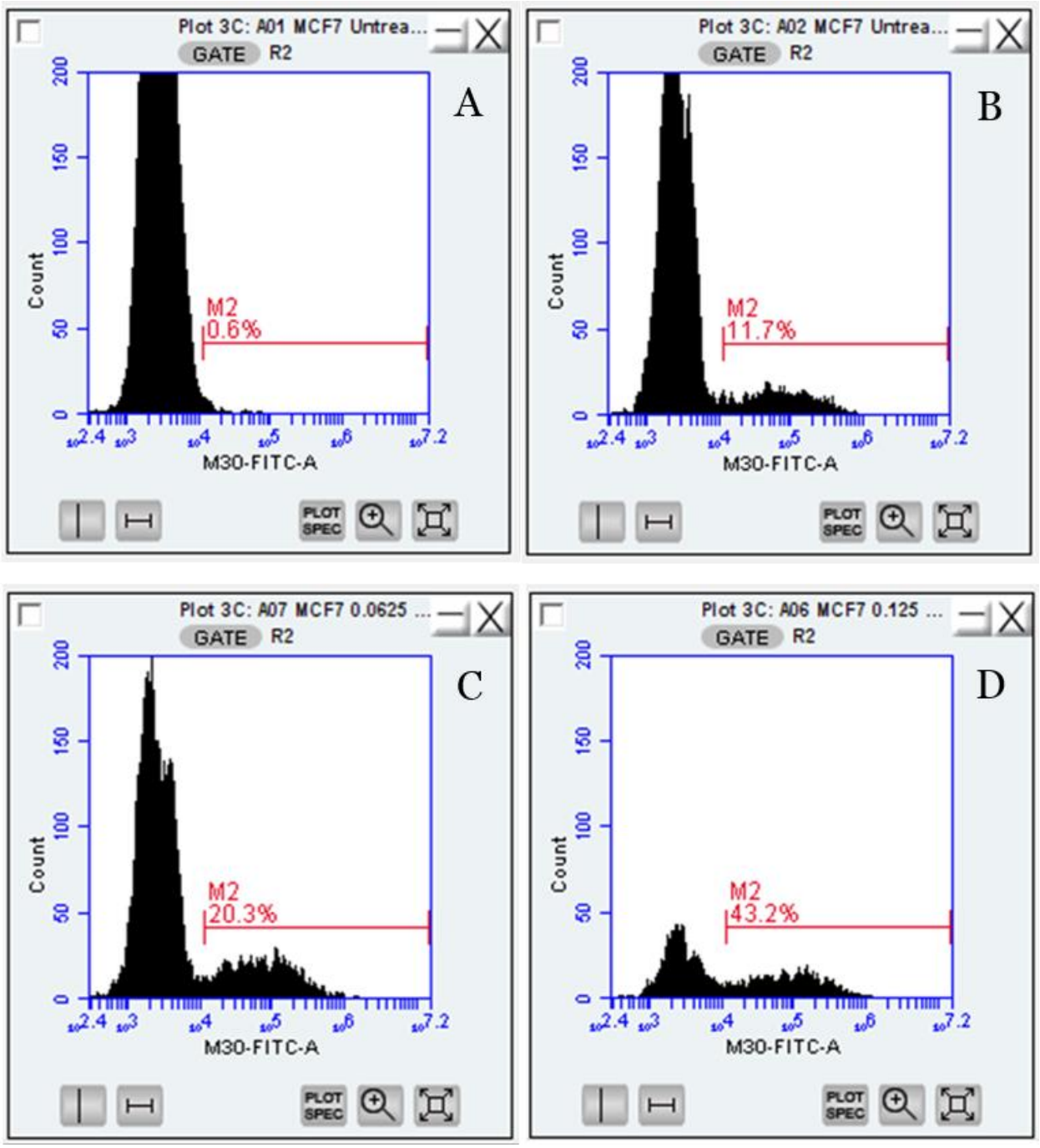


Figure 4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Count
M2
11.7%
M30-FITC-A
Plot 3C: A07 MCF7 0.0625 ...
GATE R2
C
Count
M2
20.3%
M30-FITC-A
Plot 3C: A06 MCF7 0.125 ...
GATE R2
D
Count
M2
43.2%
M30-FITC-A
PLOT SPEC

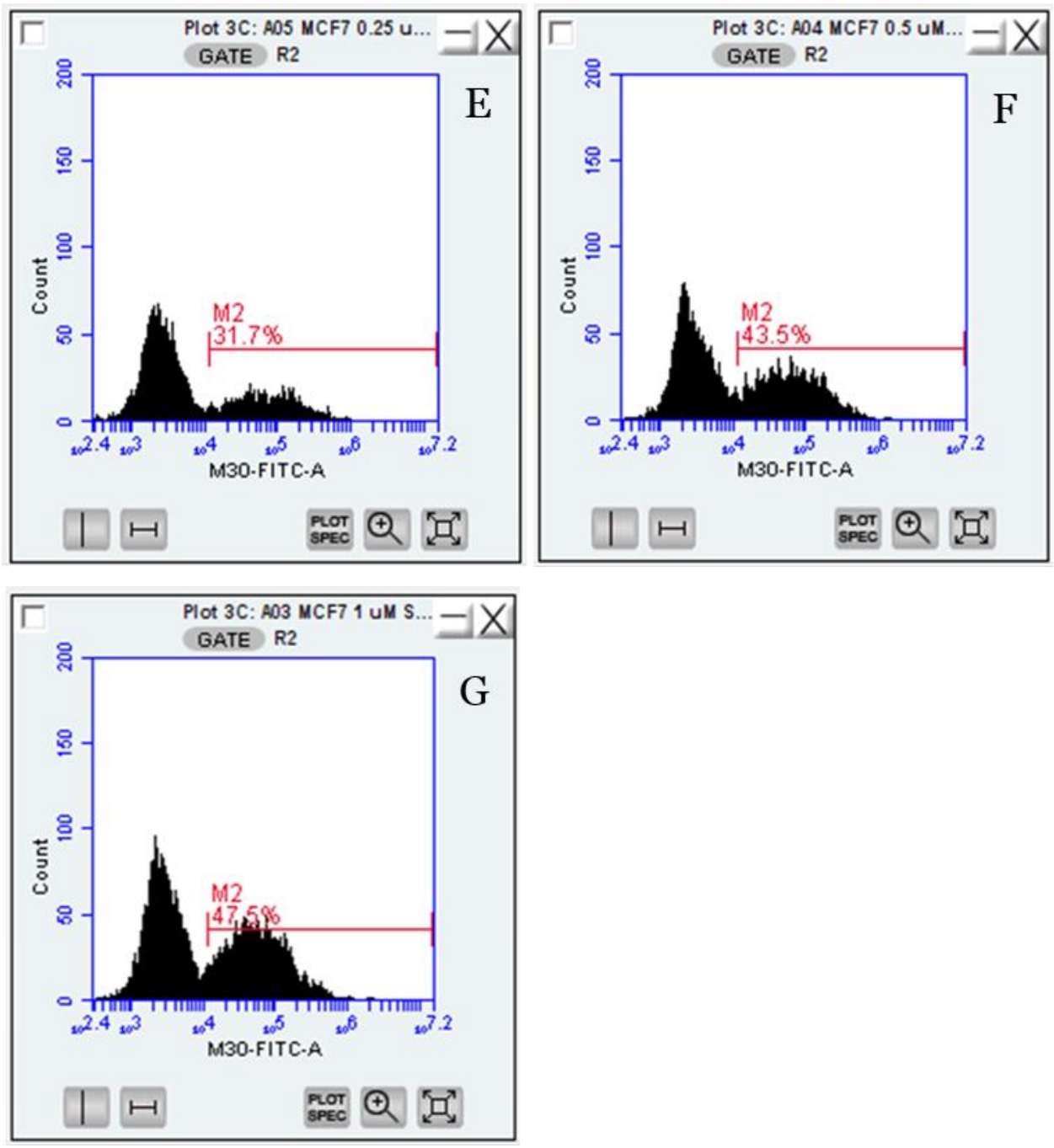
Plot 3C: A05 MCF7 0.25 u...
GATE R2
E
Count
M2
31.7%
M30-FITC-A
Plot 3C: A04 MCF7 0.5 uM...
GATE R2
F
Count
M2
43.5%
M30-FITC-A
Plot 3C: A03 MCF7 1 uM S...
GATE R2
G
Count
M2
47.5%
M30-FITC-A
PLOT SPEC

Figure 5