1 Cell Culture

1. Suspend 0.5 x 10^6 MCF-7 breast cancer or PE01 ovarian cancer cells in 25 ml RPMI/PS/10% FCS and transfer to a 75-cm^2 tissue culture flask. Place in a 5% CO_2 humidified incubator at 37 °C.
2. Allow cells to grow to confluence, feeding twice per week by aspirating the spent media, washing with 20 ml PBS, and then adding 25 ml fresh tissue culture media.
3. When cells become confluent, remove spent tissue culture media and wash cells in 10 ml PBS. Remove excess PBS.
4. Add 5 ml Trypsin/EDTA to each flask and place back in the incubator until the cells have become detached (see Note 5).
5. Transfer cell suspension to a sterile Universal container using a sterile pipet; pour 20 ml RPMI/PS/10% FCS (to deactivate the Trypsin) into the flask in order to wash it out and then pool with the cell suspension in the Universal container (total volume of 25 ml).
6. Centrifuge at 600 g for 5 minutes.
7. Pour off the media and resuspend the pellet in 5 ml RPMI/PS/10% FCS. Syringe with a 21G needle three times to break up the pellet, and then make up to 25 ml with RPMI/PS/10% FCS
8. Count total number of cells using a hemocytometer.
9. Transfer aliquots of 1 x 10^6 cells to FACS tubes (regardless of volume) and then centrifuge again at 600 g for 5 minutes.
10. Resuspend pellet in 100 µl citrate buffer, cover tubes, and store at –40°C prior to analysis.

2 Preparation of Samples for Analysis

1. Thaw Solutions A and B, along with the frozen whole cell suspensions (in 100 µl) (but not heated) in a water bath at 37° before preparing and staining nuclei for DNA analysis. Allow Solution C to thaw at room temperature before placing on ice.
2. Digest cell suspensions down to nuclei by adding 450 µl of 0.003% Trypsin solution (Solution A), mix and leave at room temperature for 10 minutes (see Notes 6 and 7).
3. Prevent further degradation by adding 0.05% (w/v) Trypsin inhibitor solution and 0.01% (w/v) RNase A (Solution B) in a final volume of 375 µl, mix, and leave for 10 minutes.
4. Finally, stain cells by adding 416 µl/ml ice-cold propidium iodide/1.16 mg/ml spermine tetrahydrochloride solution (Solution C) in a final volume of 250 µl and leave the samples on ice in the dark for an additional 10 minutes prior to analysis.

3 Acquisition of Flow Cytometric DNA Histograms

1. This protocol assumes that the user is familiar with the principles and practices of flow cytometry and is able to run samples according to the operator's manual pertaining to the instrument being used.
2. For the purpose of collecting data, all plots must be formatted for "Acquisition".
3. Plot a two-parameter dot-plot of Forward Light Scatter (FLS) vs. Side Scatter (SS).
4. Plot a single-parameter FL2 (area) histogram with linear x-axis to illustrate relative DNA content (propidium iodide fluorescence is usually assigned to the FL2 channel; see Note 8).

5. Plot a two-parameter dot-plot of FL2 (area) vs. FL2 (width) to monitor doublets (see Sub-heading 3.4).
6. Select the signal threshold (the point at which a signal will be accepted as a positive event) to FL2 and then set an appropriate value to gate out debris (a value of 20 should suffice in the first instance).
7. No compensation is required since only one fluorochrome (propidium iodide) is present.
8. Introduce the sample and set the machine to “Run”. Using the appropriate settings panel, adjust both FLS and SS photo multiplier tube (PMT) voltages so that the majority of dots in the first two-parameter dot-plot (FLS vs. SS) are contained roughly within the center of the box.
9. Adjust the FL2 PMT voltage up or down until the peak appears in the graph. The voltage can then be fine-tuned so that the main peak is approximately one quarter of the way along the x-axis within the linear graph. This voltage will allow sufficient space along the x-axis for the G_2/M peak (which will have twice as much DNA per cell) to be held in the graph and not over-spill the end. The histogram can also be monitored for any tetraploid cells that may be present.
10. After setting up the machine, 10,000 ungated events are collected. Data files are stored in an appropriate folder for subsequent retrieval/analysis using cell cycle software provided with the machine being used.

4 Analysis of Flow Cytometric DNA Histograms

1. Provisional analysis of data can be conducted in a manner similar to data acquisition with all histograms being formatted for “Analysis” rather than “Acquisition”.
2. Plot a two-parameter dot-plot of FL2 (area) vs. FL2 (width), open the first data file, and set the "gates" around the majority of cells contained within the FL2 (area) vs. FL2 (width) dot-plot. Define as "gate 1".
3. Plot a single-parameter FL2 (area) histogram with linear x-axis to represent relative DNA content. Format the histogram by deselecting the default "ungated" events and choose "gate 1".
4. Place cursors around the first (G_0/G_1), intermediate (S), and second (G_2/M) populations of cells in a manner appropriate to your machine and choose the appropriate statistics of interest. Successful analysis should yield the appropriate proportion of cells in the G_0/G_1-phase, S phase, and G_2/M phases of the cell cycle.
5. Accurate cell cycle analysis must be performed using dedicated software supplied with the instrument being used. Users are expected to be familiar with such software and the appropriate statistical models to use for such analysis. For the purposes of this protocol, analysis was carried out using the ModFit software provided with the FACSCalibur flow cytometer.
6. Open the ModFit program and select the appropriate **FILE**.
7. Choose the parameter for analysis; in this case select Fl2A for relative DNA content.
8. Define “gate 1” by selecting FL2A (x) and FL2W (y). Drag each of the points of the gate (R1) to include the entire cell population of interest.
9. Choose a specific **MODEL** to analyze the data or use the suggested model according to specified parameters, such as whether samples were fresh or frozen or paraffin embedded; of diploid, aneuploid or tetraploid DNA content; whether aggregates were present; or if there is a visible G_2/M fraction. The model can also account for the presence of internal standards should they be included.

10. Check the position and **RANGE** of the markers that are automatically placed on the histogram and adjust their position if necessary (this may be the case, particularly if the S-phase fraction becomes relatively high).
11. Calculate the relative cell cycle distributions using the **FIT** option. Repeat the process for all other samples, making sure that the cell population of interest is within the defined “gate”. Adjust the gate if necessary.
12. Data can then be tabulated and exported to a suitable presentation package, for example, Excel.