Cell Cycle and DNA Content Analysis Using the BD Cycletest Assay on the BD FACSVerse™ System

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Application Note

Contents

Summary

Measuring the DNA content of cells is a well established method to monitor cell proliferation, cell cycle, and DNA ploidy. The BD Cycletest™ Plus DNA reagent kit provides a set of reagents for isolating and staining cell nuclei from tissue specimens or cell suspensions, which can be analyzed on a BD flow cytometer. This study focuses on the use of the BD Cycletest Plus kit on the BD FACSVerse™ flow cytometer. This system uses BD FACSuite™ software for all functions from setup to reporting and includes the BD FACSuite research assay modules, one of which is the BD Cycletest assay module. Designed for use with the BD Cycletest Plus reagent kit, the BD Cycletest assay in BD FACSuite software provides acquisition, analysis, and reporting functions to generate cell cycle and DNA content data using a BD FACSVerse system. This application note describes proof of principle experiments that demonstrate the utility of the BD Cycletest Plus reagent kit and BD Cycletest assay to estimate the cell cycle profile of Jurkat E6-1 cells cultured in the presence or absence of serum. In addition, the ploidy and DNA index from two human cancer cell lines were also estimated to demonstrate the function of a user-defined assay on a BD FACSVerse system.

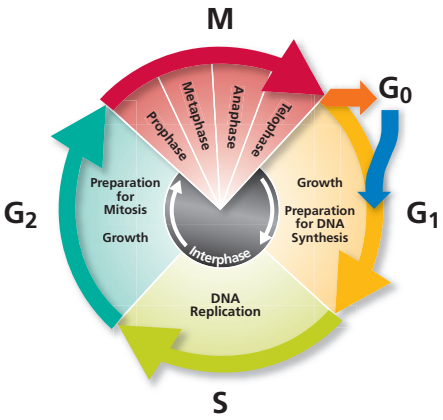


Figure 1. Cell cycle phase distribution for a diploid cell.

Introduction

Measuring DNA content of cells is a well established method for monitoring cell proliferation, cell cycle, and DNA ploidy. Proliferating cells progress through various phases of the cell cycle (G_0, G_1, S, G_2, and M phase) as shown in Figure 1.

At different stages of the cell cycle, cell nuclei contain different amounts of DNA. For example, after receiving signals for proliferation, diploid cells exit the resting state Gap 0 (G_0) phase and enter the Gap 1 (G_1) phase. At this stage, the diploid cells maintain their ploidy by retaining two complete sets of chromosomes (2N). As the cells enter the synthesis (S) phase, DNA replication starts, and in this phase, cells contain varying amounts of DNA. The DNA replication continues until the DNA content reaches a tetraploid state (4N) with twice the DNA content of the diploid state. Tetraploid cells in the G_2 phase start preparing for division and enter the mitosis (M) phase when the cells divide into two identical diploid (2N) daughter cells. The daughter cells continue on to another division cycle or enter the resting stage (G_0 phase). Based on DNA content alone, the M phase is indistinguishable from the G_2 phase, and G_0 is indistinguishable from G_1. Therefore, when based on DNA content, cell cycle is commonly described by the G_0/G_1, S, and G_2/M phases.

DNA ploidy is an indication of the number of chromosomes in a cell. Due to anomalies in DNA replication, some cell populations such as cancer cells can have abnormal DNA content, and therefore, a different ploidy. Flow cytometry can measure DNA content of cells, which reveals not only the information on cell position in the cell cycle but also the ploidy and DNA content of a given cell population. The DNA content is generally expressed as a DNA index, which is the quantity of DNA in the test cell population in relation to that in normal diploid cells. A DNA index of 1.0 indicates normal diploid cells in the G_0/G_1 phase.

The BD Cycletest Plus reagent kit provides a set of reagents to easily isolate and stain cell nuclei from fresh or previously frozen cell suspensions. Briefly, the procedure involves lysing the cell membrane with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with Ribonuclease A, and stabilizing the nuclear chromatin with spermine. No sample purification or cleanup step is needed before staining. Propidium iodide (PI) is used to stain the DNA of isolated nuclei in a stoichiometric fashion. PI bound to DNA can be excited by a 488-nm laser and detected using the 586/42 detector. The emitted fluorescence intensity can be measured using a flow cytometer such as the BD FACSVerse system.

The BD FACSVerse system is a high-performance flow cytometer that is designed to support easy-to-use, task-based workflows. The system streamlines every stage of operation from automated setup through data analysis and reporting. The system includes unique features such as a flow sensor option for volumetric counting, automated procedures for setting up the instrument and assays, and configurable user interfaces that provide maximum usability for researchers. These functions are integrated to provide simplified use for routine applications while simultaneously providing powerful acquisition and analysis tools for more complex applications. In addition, the BD FACS™ Universal Loader option (the Loader) is available, which enables use of either tubes or multiwell plates for samples, with or without barcoding for sample identification and tracking.

Based on the BD Cycletest Plus reagent kit, the BD Cycletest assay in BD FACSuite software provides a specific assay module that contains all the acquisition, analysis, and reporting functions necessary for generating data to estimate cell cycle phase distribution.

BD-defined assays also can be used as a starting point for creating custom experiments and assays with user-defined statistics using the expression editor feature of BD FACSuite software. These user-defined assays can then be run in batch acquisition mode, in a worklist, or deployed to other BD FACSVerse cytometers throughout the researcher's laboratory or to external collaborators.

Objective

The objective of this application note is to show proof of principle experiments that demonstrate the ease of use of the BD Cycletest Plus reagent kit and BD Cycletest assay in conjunction with the BD FACSVerse system for:

- Determination of the cell cycle profiles of control and serum starved Jurkat cells
- Ploidy and DNA index calculation for two human cancer cell lines

Methods

Kits

Product Description	Vendor	Catalog Number
BD Cycletest Plus DNA reagent kit	BD Biosciences	340242

Reagents and Materials

Product Description	Vendor	Catalog Number
BD™ DNA QC Particles	BD Biosciences	349523
BD Falcon™ round-bottom tubes, 12 x 75 mm	BD Biosciences	352052
BD Falcon conical tubes, 50 mL	BD Biosciences	352070
BD Falcon conical tubes, 15 mL	BD Biosciences	352097
BD Falcon round-bottom tube with strainer cap, 35 µm	BD Biosciences	352235
BD Falcon tissue culture plate, 6 well	BD Biosciences	353046
BD FACSuite™ CS&T research beads	BD Biosciences	650621 (50 tests) 650622 (150 tests)
BD Vacutainer® tubes with heparin	BD Medical	367874

BD FACSVerse Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Fluorochrome
488	D	560 LP	586/42	Propidium Iodide

Software

Product Description	Catalog Number
BD FACSuite Research Assay Software v1.0	651363

Cell Lines

Cell Line	Source	Designation	Culture Medium
Jurkat, Clone E6-1	ATCC	TIB-152	RPMI 1640 medium + 10% FBS
MOLT-4	ATCC	CRL-1582	RPMI 1640 medium + 10% FBS
K-562	ATCC	CCL-243	IMDM + 10% FBS

Methods

Preparation of Cells for Cell Cycle Determination

1. Jurkat cells, in exponential growth phase, were seeded into a 6-well plate at 2.5 x 10^5 cells/mL and cultured in RPMI 1640 medium (ATCC, No. 30-2001) with and without 10% FBS (ATCC, No. 30-2020) for 20 to 24 hours.
2. Cells were harvested from the plate into 50-mL conical tubes and centrifuged (400*g*, 5 min) at room temperature (RT).
3. The pellet was lysed, and the isolated nuclei were stained with propidium iodide (PI) as outlined in the BD Cycletest Plus DNA reagent kit technical data sheet (TDS) included with the kit.

Preparation of Cells for Ploidy Determination

1. Whole blood was collected from normal donors into BD Vacutainer tubes (heparin).
2. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood following the instructions from the Ficoll-Paque PREMIUM Medium TDS.[1]
3. MOLT-4 cells were cultured in RPMI 1640 medium and K-562 cells were cultured in IMDM (ATCC, No. 30-2005), both supplemented with 10% FBS. Cells in exponential growth phase were harvested for DNA content analysis.
4. PBMCs, MOLT-4, and K-562 cell suspensions were harvested by centrifugation (400*g*, 5 min, RT). The cell pellets were washed twice with 1 mL of the Buffer Solution provided in the BD Cycletest Plus reagent kit and then resuspended in 1 mL of Buffer Solution.
5. The concentration of cells in each cell suspension was determined using the Trypan Blue exclusion method.
6. Both MOLT-4 and K-562 cell suspensions were spiked with PMBCs at a ratio of 10:1. The mixed cell sample then was centrifuged (400*g*, 5 min, RT) and processed according to the BD Cycletest Plus DNA reagent kit TDS included in the kit.

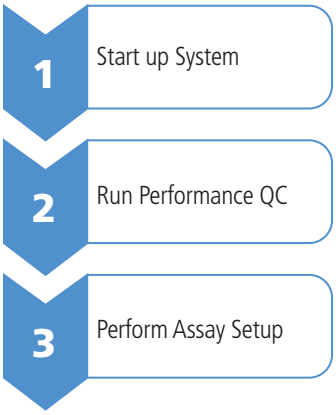


Figure 2. Workflow for instrument setup.

Instrument Setup

The basic workflow for the BD FACSVerse instrument setup is shown in Figure 2. Performance quality control (PQC) was performed using BD FACSuite CS&T research beads as outlined in the *BD FACSVerse System User's Guide*.[2] The BD Cycletest assay was then set up following the instructions in the *BD FACSuite Software Research Assays Guide*.[3]

BD Cycletest Assay

Data was acquired using a BD FACSVerse system and BD FACSuite software using the BD Cycletest assay. As shown in Figure 3, a worklist was created from the assay and the samples were acquired automatically using the Loader with an acquisition criterion of 20,000 events for each tube. BD DNA QC particles[4] were also run initially to set up the BD FACSVerse cytometer for DNA analysis according to instructions in the *BD FACSuite Software Research Assays Guide*.[3] During acquisition preview, gates for nuclei were adjusted in the FSC-A vs SSC-A and Propidium Iodide-W vs Propidium Iodide-A plots. The propidium iodide-A voltage was adjusted to set the mean of the singlet peak of the G_0/G_1 population at 50,000 in the histogram. The data was analyzed and a report was automatically generated.

The report generated from the BD Cycletest assay included the following plots with gates and histograms, including markers for the test and control samples.

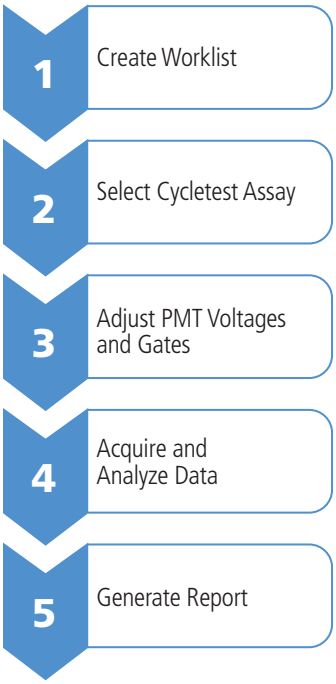


Figure 3. BD Cycletest assay workflow.

1. SSC-A vs FSC-A with a gate for nuclei
2. Propidium Iodide-W vs Propidium Iodide-A with a gate for the singlet nuclei population
3. Propidium Iodide-A histogram with markers of G_0/G_1, S, and G_2/M phases to identify cell cycle phases, and markers of sub G_0/G_1 and >4N to identify events not included in the normal cell cycle phase distribution but observed by flow cytometry

In addition, a summary of assay results with the following statistics for test and control samples was automatically calculated in the report:

- Total number of events
- Singlet events
- % sub G_0/G_1
- % G_0/G_1
- % S
- % G_2/M
- % >4N

The data from the BD Cycletest assay can also be exported and analyzed using third-party software such as ModFit LT™ to calculate cell cycle phase distribution.

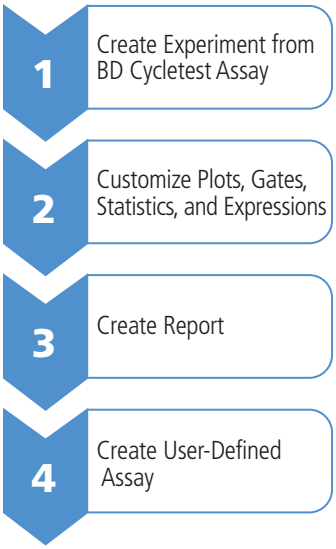


Figure 4. Workflow for creating a user-defined assay from a BD-defined assay.

User-Defined Assay
The BD Cycletest assay was used as a starting point to create a user-defined assay to automatically calculate statistics such as mean and %CV of propidium ioidide-A stained populations of interest. In addition, using the expression editor, the DNA index was calculated as the ratio of the mean fluorescence intensity (MFI) of the test sample G_0/G_1 population to the MFI of the normal reference G_0/G_1 population. Ploidy of the test sample was then calculated based on the DNA index and the ploidy of the normal reference. Figure 4 outlines the workflow to create a user-defined assay from the BD Cycletest assay. This user-defined assay was then used to acquire the data from human cancer cell lines K-562 and MOLT-4 along with PBMCs spiked as a normal reference control. The samples were automatically acquired by running the user-defined assay in the worklist.

Results and Discussion

Cell Cycle Phase Distribution

After acquisition and data analysis, a report was automatically generated by the BD Cycletest assay (Figure 5). In the report, three plots for each sample are displayed, along with the assay results summary for side-by-side comparison of statistics from the samples. The plots include an FSC-A vs SSC-A plot to display the total number of nuclei events acquired, a Propidium Iodide-W vs Propidium Iodide-A plot to distinguish singlet nuclei from doublets and aggregates, and a histogram of Propidium Iodide-A from the singlet gate to display the phases of the cell cycle.

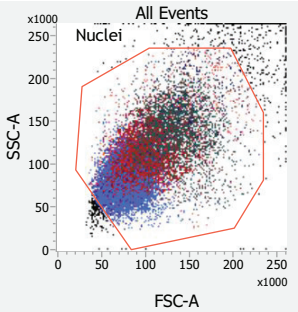
Jurkat cells were used to demonstrate an application of the BD Cycletest reagent kit and the BD Cycletest assay. Jurkat is a pseudodiploid human T-cell leukemia line with a model chromosome number of 46 in the majority of the cell population.[5] These cells proliferate under normal culture conditions (RPMI 1640 + 10% FBS). Data presented on page 2 of the lab report for Jurkat (control) cells shows that this particular proliferating culture, when cultured in serum-containing medium, contained 51.11% of cells in the G_0/G_1 phase, 26.51% in the S phase, and 18.95% in the G_2/M phase.

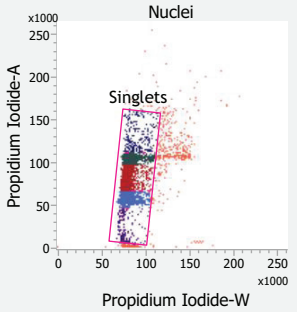
BD Cycletest v1.0: Lab Report

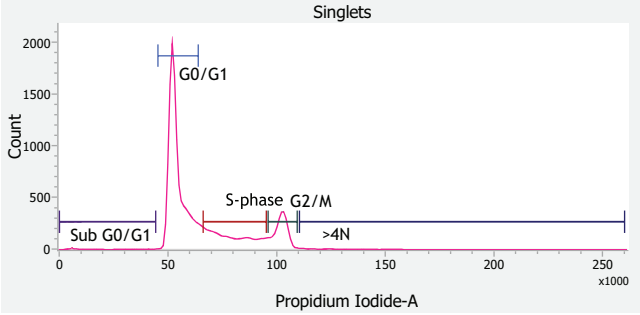
Cytometer Name: BD FACSVerse
Cytometer Serial #:123456789
Software Name & Version: FACSuite Version 1.0.0.1477
Operator Name: BDAdministrator
Report Date/Time: 06-Jul-2011 00:57:11

Cycletest: Test Sample- Jurkat (Serum Starved)

Sample ID	1.0	Acquisition Date	06-Jul-2011
Events Acquired	20000	Acquisition Time	00:57:00
Sample Type	Jurkat		







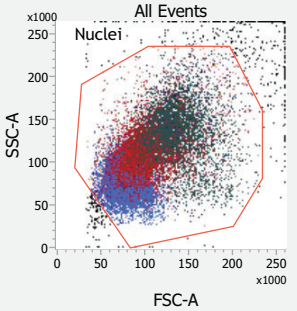
Cytometer Name: BD FACSVerse
Cytometer Serial #:123456789

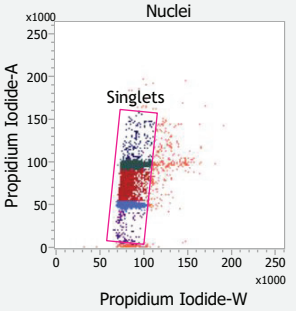
Software Name & Version:
FACSuite Version 1.0.0.1477

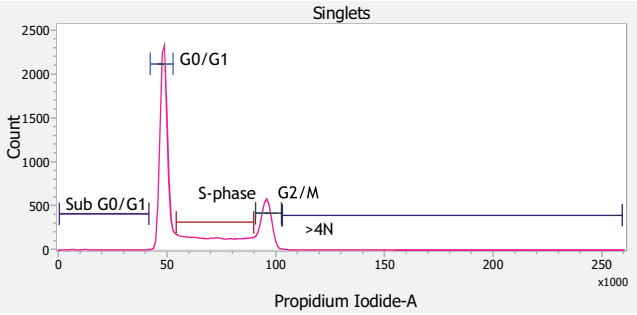
Operator Name: BDAdministrator
Report Date/Time: 06-Jul-2011 00:57:11

Cycletest: DNA Control- Jurkat (Control)

Sample ID	1.0	Acquisition Date	06-Jul-2011
Events Acquired	20000	Acquisition Time	00:57:32
Sample Type	Jurkat		







Cytometer Name: BD FACSVerse
Software Name & Version: FACSuite Version 1.0.0.1477
Operator Name: BDAdministrator
Cytometer Serial #:123456789
Report Date/Time: 06-Jul-2011 00:57:11

Results Summary

Label	Test Sample	DNA Control
Nuclei events	18999	19344
Singlet events	18480	18883
% sub G0/G1	0.88	0.61
% G0/G1	60.05	51.11
% S	20.70	26.51
% G2/M	13.54	18.95
% >4N	1.26	0.84

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Figure 5. BD Cycletest assay report showing cell cycle distribution of proliferating and serum starved Jurkat cells.

In addition, the effect of serum starvation on the cell cycle distribution of these cells was investigated. Serum starvation is a widely used method to synchronize cells in a culture into the G_0/G_1 phase.[6] Jurkat cells were cultured in RPMI medium without serum for 24 hours. As shown on page 1 of the lab report, in the absence of serum, 60.05% of cells were in the G_0/G_1 phase, 20.70% in the S phase, and 13.54% in the G_2/M phase. Overall, with serum starvation, there was a 17.5% increase in cells in the G_0/G_1 phase, 22% decrease in cells in the S phase, and 28.5% decrease of cells in the G_2/M phase.

These results indicate that, as expected, the Jurkat cells used in this study were responsive to serum starvation, as shown by an increase in the percentage of cells in the G_0/G_1 phase, and decrease in percentages of cells in the S and G_2/M phases.

DNA Index and Ploidy Estimation

The BD Cycletest assay was customized to create a user-defined assay so that it could be used to estimate the DNA index (DI) and ploidy of two human cancer cells lines, K-562 and MOLT-4. To determine the DNA index and ploidy, normal cells were mixed with the cancer cell lines and used as a reference. The DNA index was obtained by dividing the MFI of the test sample G_0/G_1 population by the MFI of the normal reference G_0/G_1 population. Since most human PBMCs are non-dividing diploid cells resting in G_0/G_1, they were used as "the normal reference G_0/G_1 population" for calculating the DNA index for the K-562 and MOLT-4 cancer cell lines. The ploidy of the sample was then calculated by multiplying the DNA index by the ploidy of the control (diploid for PBMCs).

Figure 6 shows the data from K-562 and MOLT-4 spiked with PBMCs. The histogram plots of Propidium Iodide-A from singlet populations show markers for G_0/G_1 for PBMCs and G_0/G_1 for the cancer cells (K-562 or MOLT-4 cells). In addition, the statistics tables provide the MFI of PI-A of the marked populations, with the CVs and the DNA index and ploidy calculated using the BD FACSuite expression editor function.

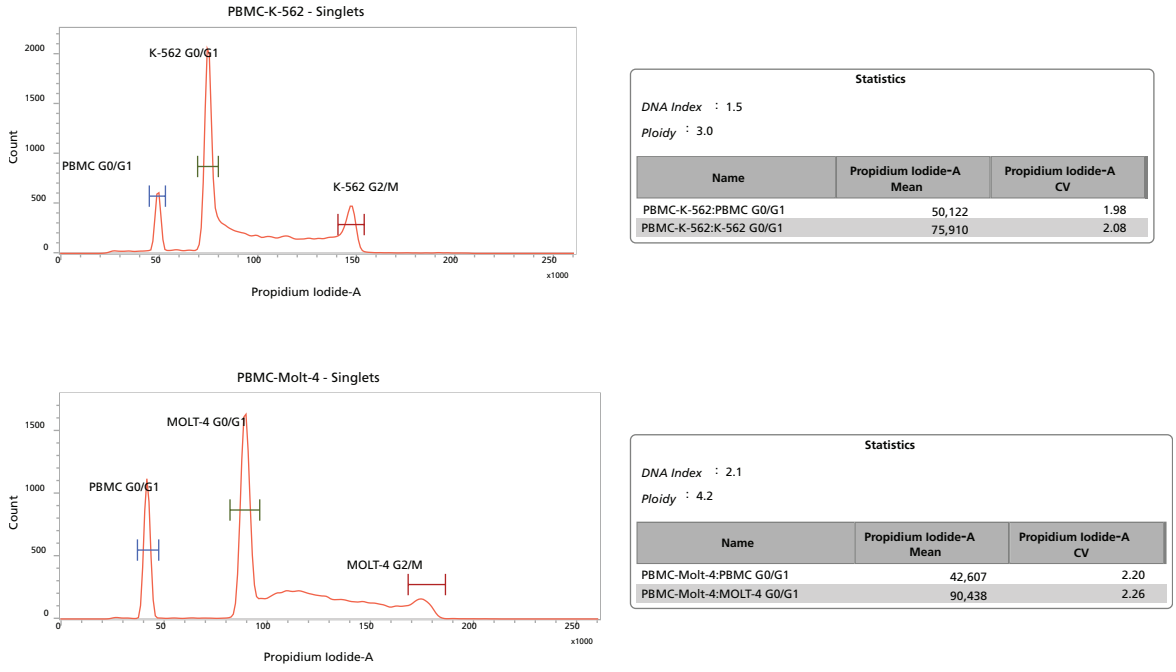

Figure 6. DNA content analysis of K-562 and MOLT-4 cell lines spiked with PBMCs.

K-562 is a multipotential, hematopoietic malignant cell line derived from a chronic myelogenous leukemia female patient and is known to be triploid.[7] K-562 cells were mixed with PBMCs at a ratio of 10:1 as outlined in the methods section, and the DNA index was found to be 1.5 as shown in Figure 6. Based upon this DNA index, the ploidy of the K-562 culture used in the experiment was estimated to be triploid.

MOLT-4, a T lymphoma cell line with the hypertetraploid chromosome number,[8] was also mixed with PBMCs (10:1), and the data was acquired using the user-defined assay. The DNA index for the MOLT-4 culture used in this experiment was 2.1, and the ploidy was estimated to be 4.2. The slightly higher ploidy observed in the culture might be due to a small population with DNA content greater than tetraploid.[8]

CVs of the G_0/G_1 populations of PBMCs from both the experiments were close to 2%, which demonstrates the high quality of the DNA histogram and the performance of the BD FACSVerse instrument. Overall, the results of the DNA content analysis indicate that the two human cancer cell lines used in the experiments, K-562 and MOLT-4, were triploid and hypertetraploid, respectively, which is consistent with the existing literature.[7-8]

Conclusions

The BD Cycletest Plus kit and BD Cycletest assay module in BD FACSuite software provide a quick and easy method for researchers to estimate cell cycle distribution using pre-defined templates for acquisition, analysis, and reporting. The data outlined provides an example of the effect of serum starvation on the cell cycle of Jurkat cells using the BD Cycletest assay. Further, using a user-defined assay in BD FACSuite software, we have estimated the DNA index and ploidy for two human cancer cell lines spiked with PBMCs as controls.

References

1. Ficoll-Paque PREMIUM Instructions 28-4039-56 AC.
2. BD FACSVerse System User's Guide. 23-11463-00 Rev. 01.
3. BD FACSuite Software Research Assays Guide. 23-11470-00 Rev. 01.
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5. Jurkat Clone E6-1. ATCC Product information Sheet for ATCC TIB-152.
6. Kues WA, Anger M, Carnwath JW, Paul D, Motlik J, Niemann H. Cell cycle synchronization of porcine fetal fibroblasts: Effects of serum deprivation and reversible cell cycle inhibitors. *Biol Reprod*. 2000;62:412-419.
7. K-562. ATCC Product information Sheet for ATCC CCL-243.
8. Molt-4. ATCC Product information Sheet for ATCC CRL-1582.

Tips and Tricks

1. The DNA samples should be acquired using the low flow rate of the instrument to ensure the best resolution of the DNA data.
2. BD DNA QC particles should be used to set up the BD FACSVerse flow cytometer for DNA analysis following instructions from the *BD FACSuite Software Research Assays Guide*.[3]
3. The markers for the histograms in the BD Cycletest assay are unique for each sample and can be adjusted accordingly to ensure correct cell cycle phase analysis.
4. It is important that the cell number is accurately determined and the correct cell concentration used prior to lysis and staining using the BD Cycletest Plus kit. This ensures an optimal nuclei concentration to have approximately 60 events per second when samples are acquired at a low flow rate.
5. Complete cell lysis is essential for good quality histograms. The lysis procedure should be optimized for different cell types.
6. The BD Cycletest assay can provide an estimation of cell cycle phases. However, modeling software such as ModFit LT™ can be used for more accurate cell cycle analysis.

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