

Application Note No. 994-4005

Xcyto[®] 5

Cell Cycle Assay: For rapid measurement of G₀/G₁, S and G₂/M cell cycle phases using the Xcyto[®] 5 system

Product description

The Xcyto[®] 5 image cytometer and XcytoView™ software integrate image acquisition, image analysis, feature extraction and data presentation in one step for seamless quantitative data of individual cells and cell populations within heterogenous samples.

Application

This application note describes a protocol to perform automated cell cycle analysis on a broad range of eukaryotic cells. As cell cycle represents the most fundamental and important process in eukaryotic cells, this assay provides an easy and fast way to evaluate overall cell health.

Introduction

The cell cycle consists of an ordered sequence of stages that occur in a cell in preparation for cell division. The most basic function of the cell cycle is to accurately duplicate the vast amount of DNA in the chromosomes and segregate the copies precisely into two genetically identical daughter cells. These processes define the two major phases of the cell cycle, Synthesis (S) and Mitosis (M).

The cell cycle is tightly regulated by defined temporal and spatial expression, localization, and destruction of several cell cycle regulators. Cyclins and cyclin-dependent kinases (CDKs) are major control switches for the cell cycle, causing the cell to move from G₁ to S or from Gap 2 (G₂) to Mitosis (M) phases. In any given population, cells will be distributed among three major phases of cell cycle: G₁/G₀ phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amounts of

DNA), and G₂/M phase

(two sets of paired chromosomes per cell, prior to cell division). DNA content can be measured using fluorescent, DNA-selective stains that produce emission signals proportional to DNA mass. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain but is also possible using live cells and a cell-permeant nucleic acid stain.

In neoplasia, cell cycle dysregulation is a common occurrence, thus providing the opportunity to discover new targets for anticancer agents and improved therapeutics, which has been the focus of intense interest. The cell cycle assay has applicability to a variety of areas of life science research and drug development, including cancer biology, apoptosis analysis, drug screening, and measuring health status of cell cultures, e.g. in bioreactors.

Using image analysis, Xcyto[®] 5 automates DNA content measurements. In this application note, we describe methods for cell cycle analysis, which can measure fluorescently stained cells using DAPI. DAPI is a competent dye for measurement of the cell cycle stage. Firstly, the intensity of fluorescence of DAPI stained cell is a linear function of DNA-content. Secondly, DAPI binds specifically to double-stranded DNA and, accordingly, there is no requirement to remove RNA from the sample prior to measuring DNA content. This is a prerequisite for other dyes commonly used for measurements of cellular DNA content, such as propidium iodide.

The Xcyto[®] allows for two different cell cycle

assays: 1) The robust Cell Cycle Assay of Fixed Cells, offering the possibility of storing cells to be analyzed for several weeks; and 2) The rapid

Two-step Cell Cycle Assay with a 5-minute robust and simple protocol.

Procedure: Xcyto® Cell Cycle of Fixed Cells Assay

The Cell Cycle of Fixed Cells Assay possesses the possibility to store cells for several weeks. Thus, after ethanol fixation the cells can be left in the fridge for a period prior to analyzing the sample.

The following protocol was developed using Jurkat, HeLa, U2OS and MCF-7 cells with a standard concentration of DAPI (Solution 12) of 1 µg/ml, but it can be adapted for most mammalian cell types. Growth medium, cell density, cell type and other factors may influence labelling. We recommend testing a concentration range of the Annexin V conjugate to estimate the optimal conditions for your preferred cell model.

Materials needed

- Cell sample to be stained in suspension ^{1, 2}
- Phosphate buffered saline (PBS) ¹
- Fixative: 70 % ethanol ¹
- DAPI (500 µg/ml, Solution 12, Cat. No. 910-3012)
- Xcyto® 2-Chamber Slide Type 100-A (Cat.no. 942-0010)

Protocol

Important notes: For proper staining, it is crucial to keep the cell density within the concentration specified. In case of a limited number of cells, the procedure can be scaled down accordingly.

In advance, prepare 15 ml tubes with 4.5 ml 70 % EtOH and chill to -20°C.

1. Harvest 2×10^6 cells by centrifugation for 5 minutes at 500 g at room temperature
2. Wash cells once in 1 ml PBS
3. Resuspend in 0.5 ml PBS and loosen cell pellet by vortexing
NOTE: It is important to have a single cell suspension when mixing cells with EtOH
4. Transfer cell suspension into tubes containing ice-cold 70 % EtOH, vortex vigorously, and incubate cells in fixative for minimum 2 hours at 4°C
5. Spin ethanol-suspended cells for 5 minutes at 500 g, decant EtOH thoroughly, wash with PBS, and resuspend in 1 ml PBS
NOTE: Cell pellet may be loose. Make sure that cells are not lost during the washing step
6. Add DAPI to a final concentration of 1 µg/ml
7. Mix thoroughly by pipetting or gently vortexing
8. Incubate cells at 37°C for 5 minutes in the dark
9. Load Xcyto® 2-Chamber Slide Type 100-A with 50 µl of cell suspension
10. Engage Xcyto® 5 image cytometer by opening XcytoView™ Software
11. Eject sample tray and place slide on tray
12. Select the *Cell Cycle* protocol in the Protocol Browser or drop-down menu; both are available from the Main Window

¹ Provided by the user

² Preferable, use logarithmically proliferating cells as control

13. In the data acquisition control section in the main window, select *Sample media*, enter *Sample ID*, and press the *Run* icon
OPTIONAL: Create user-defined stop criteria in the acquisition limits section, available in the main window, by the number of either counts or views. Criteria can be edited when limits are disabled
 - a. To define a gate to use for the counts limit, wait for the file to be displayed in the plot manager, create a gate or use a template-defined gate, select the name in the drop-down menu and enable the counts limit
14. Cells are automatically imaged, segmented and analyzed. Numerical cell data is presented in the plot manager
NOTE: Inspection of the gates, "Cells", "G0/G1", "S" and "G2/M", is crucial and may require adjustment for proper analysis

Procedure: Xcyto® Two-Step Cell Cycle Assay

The Two-Step Cell Cycle Assay facilitates detaching, permeabilization, de-clumping and homogenous staining of cell population in two simple steps without trypsinization, wash or centrifugation. Preparation of samples only takes 5 minutes.

The following protocol was developed with Jurkat A3, HeLa, U2OS and MCF-7 cells with an optimized concentration of DAPI ([Solution 12](#)) of 10 µg/ml, but it can be adapted for most mammalian cell types. Growth medium, cell density, cell type and other factors may influence labelling. We recommend testing a concentration range of DAPI to estimate the optimal conditions for the used cell model.

Materials needed

- Cell sample to be stained in suspension ^{3, 4}
- Phosphate buffered saline (PBS) ³
- Lysis 3 buffer ([Lysis 3](#), Cat. No. 910-0012)
- Stabilization buffer ([Solution 11](#), Cat. No. 910-3011)
- DAPI (500 µg/ml, [Solution 12](#), Cat. No. 910-3012)
- Xcyto® 2-Chamber Slide Type 100-A (Cat.no. 942-0010)

Protocol for cells in suspension

IMPORTANT: For proper staining it is crucial to keep the final cell density at 2×10^6 cells/ml. In case of limited numbers of cells, the procedure can be scaled down, e.g. for 4×10^5 cells use 100 µl of [Lysis 3](#) and [Solution 11](#) in steps 2 and 3.

Prior to analysis prepare an appropriate amount of [Lysis 3](#) containing 10 µg/ml DAPI. E.g., add 20 µl of [Solution 12](#) to 980 µl of [Lysis 3](#) and mix. Do not store [Lysis 3](#) supplemented with DAPI for more than one day. The optimal concentration of DAPI may vary between cell types. Do not exceed 20 µg/ml DAPI.

1. Harvest 1×10^6 cells by centrifugating 5 minutes at 500 g at room temperature
2. Wash once with PBS, remove PBS completely and thoroughly resuspend in 250 µl [Lysis 3](#) supplemented with 10 µg/ml DAPI

³ Provided by the user

⁴ Preferable, use logarithmically proliferating cells as control

3. Incubate cells at 37°C for 5 minutes in the dark, add 250 µl **Solution 11** and proceed to analysis
4. Analyze immediately or preferably within 15 minutes!
5. Load the Xcyto® 2-Chamber Slide Type 100-A with 50 µl of cell suspension
6. Engage Xcyto® 5 image cytometer by opening XcytoView™ Software
7. Eject sample tray and place slide on tray
8. Select the *Cell Cycle* protocol in the Protocol Browser or drop-down menu; both are available from the Main Window
9. In the data acquisition control section in the main window, select *Sample media*, enter *Sample ID*, and press the *Run* icon
OPTIONAL: Create user-defined stop criteria in the acquisition limits section, available in the main window, by the number of either counts or views. Criteria can be edited when limits are disabled
 - a. To define a gate to use for the counts limit, wait for the file to be displayed in the plot manager, create a gate or use a template-defined gate, select the name in the drop-down menu and enable the counts limit
10. Cells are automatically imaged, segmented and analyzed. Numerical cell data is presented in the plot manager
NOTE: Inspection of the gates, “Cells”, “G0/G1”, “S” and “G2/M”, is crucial and may require adjustment for proper analysis

Protocol for cells attached to T-flasks, cell culture plates or micro-carriers

This protocol is for cells grown in 6-well culture plates, *i.e.* ~9.5 cm² per well. The procedure can be scaled up or down, e.g. for a T25 flask (25 cm²) use 660 µl of **Lysis 3** and **Solution 11** in steps 1 and 2, respectively. Examples of other formats are given in Table 1.

IMPORTANT: For proper staining, it is crucial to scale **Lysis 3** and **Solution 11** correctly.

The number of cells seeded needs to be optimized for each cell type to have a sufficient number of cells for the analysis, while avoiding the cells arrest in the G1-phase due to a high cell confluence. We recommend seeding a total of 2.5×10⁵ - 5×10⁵ cells/well in a 6-well culture plate the day before the experiment and use the well most suited for the experiment.

1. Remove culture medium, wash once with 3 ml of PBS, remove PBS completely and add 250 µl of **Lysis 3** supplemented with 10 µg/ml DAPI
2. Incubate cells at 37°C for 5 minutes in the dark, resuspend cells thoroughly by pipetting and add 250 µl of **Solution 11** and proceed to analysis
3. Analyze immediately or preferably within 15 minutes!
4. Load the Xcyto® 2-Chamber Slide Type 100-A with 50 µl of cell suspension
5. Engage Xcyto® 5 image cytometer by opening XcytoView™ Software
6. Eject sample tray and place slide on tray
7. Select the *Cell Cycle* in the Protocol Browser or drop-down menu; both are available from the Main Window
8. In the data acquisition control section of the main window, select *Sample media*, enter *Sample ID*, and press the *Run* icon
OPTIONAL: Create user-defined stop criteria in the acquisition limits section, available in the main window, by the number of either counts or views. Criteria can be edited when limits are disabled
 - a. To define a gate to use for the counts limit, wait for the file to be displayed in the plot

manager, create a gate or use a template-defined gate, select the name in the drop-down menu and enable the counts limit

9. Cells are imaged, segmented and analyzed automatically. Numerical cell data is presented in the plot manager

NOTE: Inspection of the gates, "Cells", "G0/G1", "S" and "G2/M", is crucial and may require adjustment for proper analysis

During image acquisition and analysis, results will be displayed in the Plot Manager, when the analysis of the first view is complete. For every analyzed view, result data will be added incrementally to the results displayed.

Cellular fluorescence is quantified by an intensity-based analysis of the DAPI signal, and DNA content histograms are displayed in the plot manager. Use marker gates in the displayed histograms to mark cells in the different cells cycle stages (see figure 1 below).

No. of wells	4	6	12	24	48
Culture area (cm ² /well)	~ 22.0	~ 9.5	~ 3.8	~ 1.9	~ 1.0
Lysis 3 with DAPI (μl)	580	250	100	50	30
Solution 11 (μl)	580	250	100	50	30

Table 1: Volumes of Lysis 3 with DAPI and Solution 11 required for different types of cell culture plates.

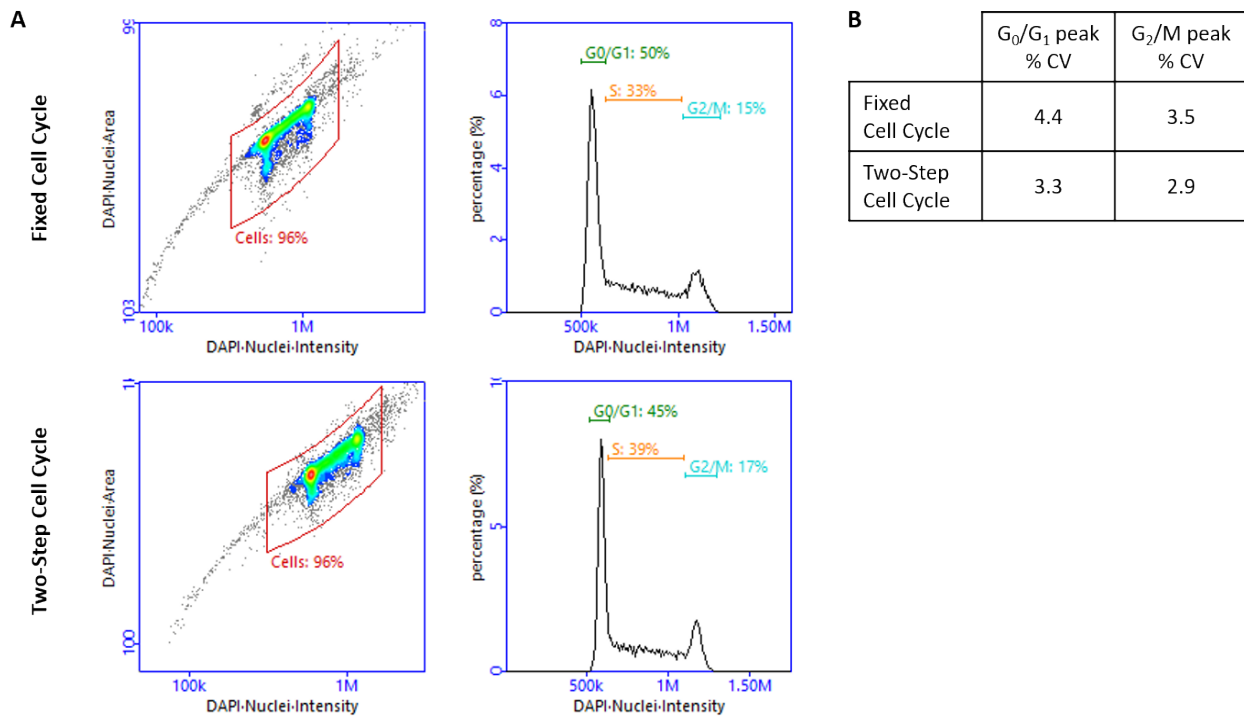


Figure 1. Cell cycle analysis of Jurkat cells. **A)** Jurkat cells were harvested and stained according to the Cell Cycle of Fixed Cells Assay protocol or the Two-Step Cell Cycle Assay protocol. The cells were imaged and analyzed with the Xcyto[®] 5 image cytometer and the XcytoView™ software using the Cell Cycle protocol. Cells were identified by an intensity-based analysis of the DAPI signal and displayed in the masking plots (left panels) along with the gate “Cells”. Histograms (right panels) show the DAPI intensity. The histogram plots (right panels) show events from the gated “Cells” population from the masking plot (left panels). Marker gates in the displayed histograms were used to mark the different cell cycle phases. Inspection of the gates, “Cells”, “G₀/G₁”, “S” and “G₂/M”, is crucial and may require adjustment for proper analysis. **NOTE:** Only single cells are included in the analysis. **B)** The table lists the percentage coefficient of variance (% CV) of the two peak populations for each assay.

Notes

It should be stressed that the “Two-Step Cell Cycle Assay” may lead to underestimation of M-phase cells. Lacking a nuclear envelope, M-phase cells may disintegrate into chromosome fragments and make aggregates. In case of issues with M-phase cells it is recommended to make an ethanol fixation of the cell sample utilizing the “Cell Cycle of Fixed Cells Assay”.

For samples containing small cell types or particles, such as PBMCs, we recommend a settling time of 2-5 minutes after loading the Xcyto[®] slides to avoid movement of sample components during data acquisition. Movement during data acquisition can cause errors in image overlay and thereby quantification of cell data.

Analysis time is highly correlated with cell density of the sample; Lower cell densities increase acquisition and analysis times.

Handling and storage

For handling and storage of ChemoMetec® instruments, reagents and Xcyto® slides refer to the corresponding product documentation. For other reagents, refer to the material data sheet from the manufacturer of the reagents and chemicals.

Warnings and precautions

For safe handling and disposal of the ChemoMetec® reagents and Xcyto® slides refer to the corresponding product documentation and the Xcyto® Software User Guide. For other reagents refer to the safety data sheet from the manufacturer of the reagents and chemicals required for this protocol. Wear suitable eye protection and protective clothes and gloves when handling biologically active materials.

Limitations

The Xcyto® 5 system, consisting of the Xcyto® 5 image cytometer and XcytoView™ software, is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the Xcyto® 5 system depend on correct use of the reagents, Xcyto® slides and the Xcyto® 5 instrument and might depend on the type of cells being analyzed. Refer to the Xcyto® 5 Software User Guide for instructions and limitations.

Liability disclaimer

This application note is for RESEARCH PURPOSES ONLY. It is

not intended for food, drug, household, or cosmetic use. Its use must be supervised by a technically qualified individual experienced in handling potentially hazardous chemicals. The above information is correct to the best of our knowledge. Users should make independent decisions regarding completeness of the information based on all sources available. ChemoMetec A/S shall not be held liable for any damage resulting from handling or contact with the above product.

Product disclaimer

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