Application note No. 3001.

NucleoCounter® NC-3000™

Two-step cell cycle analysis

Product description

The NucleoCounter® NC-3000™ system enables the user to perform automated cell counting and analyses of a broad range of eukaryotic cells.

Application

This protocol for the NucleoCounter® NC-3000™ system enables the user to rapidly quantify DNA content of mammalian cells allowing determination of G_0/G_1, S and G_2/M cell cycle phases. The protocol facilitates detaching, permeabilization, disaggregation and homogenous staining of the cell population in two simple steps without any trypsination, washing and centrifugation.

Introduction

The cell cycle represents the most fundamental and important process in eukaryotic cells. Being an ordered set of events, culminating in cell growth and division into two daughter cells, 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 (CDK) are major control switches for the cell cycle, causing the cell to move from G_1 to S or from G_2 to M phases. In a given population, cells will be distributed among three major phases of cell cycle: G_1 /G_0 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA), and G_2/M phase (two sets of paired chromosomes per cell, prior to cell division). DNA content can be measured using fluorescent, DNA-selective stains that exhibit 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.

Because cell cycle dysregulation is such a common occurrence in neoplasia, the opportunity to discover new targets for anticancer agents and improved therapeutics 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.

Principle

Using image analysis, the NucleoCounter® NC-3000™ system automates DNA content measurements. In this application note, a method for cell cycle analysis is described which can measure DAPI stained cells.

After a simple five-minutes sample preparation cells are loaded into either of two types of ChemoMetec slides: the 2-chamber NC-Slide A2™ or the 8-chamber NC-Slide A8™. Samples are analyzed using the NucleoCounter® NC-3000™ system and cellular fluorescence is quantified and DNA content histograms are displayed on the PC screen.

It should be stressed that this method may lead to an underestimation of M-phase cells. Lacking a nuclear envelope, M–phase cells may disintegrate into chromosome fragments and aggregates. If this is a problem it is recommended to make an ethanol fixation of the cell sample utilizing the protocol “Fixed Cell Cycle – DAPI Assay“.

Procedures

Materials

- Cells to be stained*
- Phosphate buffered saline (PBS)*
- **Solution 10** (Lysis buffer)
- **Solution 11** (stabilization buffer)
- **Solution 12** (500 µg/ml DAPI)
- **NC-Slide A2™** or **NC-Slide A8™**

* provided by the user

Prior to analysis prepare an appropriate amount of **Solution 10** containing 10 µg/ml DAPI. E.g., add 20 µl of **Solution 12** to 980 µl of **Solution 10** and mix.

- Do not store **Solution 10** supplemented with DAPI for more than 1 day.
- The optimal concentration of DAPI may vary between cell types. Do not exceed 20 µg/ml DAPI.

Protocol for cells in suspension

Important notes:
For proper staining it is crucial to keep the cell density within the range of $1x10^6$ to $2x10^6$ cells/ml. In case of limited amounts of cells the procedure can be scaled down, e.g for $2x10^5$ to $4x10^5$ cells use 100 µl of **Solution 10** and **Solution 11** in steps 1 and 2.

1. Harvest $5x10^5$ to $1x10^6$ cells by centrifuging 5 min. at 400 *g* at room temperature. Wash once with PBS, remove PBS completely and thoroughly resuspend cells in 250 µl **Solution 10** supplemented with 10 µg/ml DAPI.
2. Incubate cells at 37° C for 5 minutes, add 250 µl **Solution 11** and proceed to step 3.
3. Engage the NucleoCounter® NC-3000™ by starting the NucleoView™ NC-3000 software.
4. Depending on the number of samples a 2-chamber slide (**NC-Slide A2™**) or an 8-chamber slide (**NC-Slide A8™**) can be used.
    a. **NC-Slide A2™**: Load ~30 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**2-Step Cell Cycle Assay**", sample unit **NC-Slide A2** and press RUN.
    b. **NC-Slide A8™**: Load ~10 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**2-Step Cell Cycle Assay**", sample unit **NC-Slide A8** and press RUN.
5. Cellular fluorescence is quantified and DNA content histograms are displayed on PC screen. Markers in the displayed histograms can be used to demarcate cells in the different cells cycle stages (see figure below).

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

Important notes:

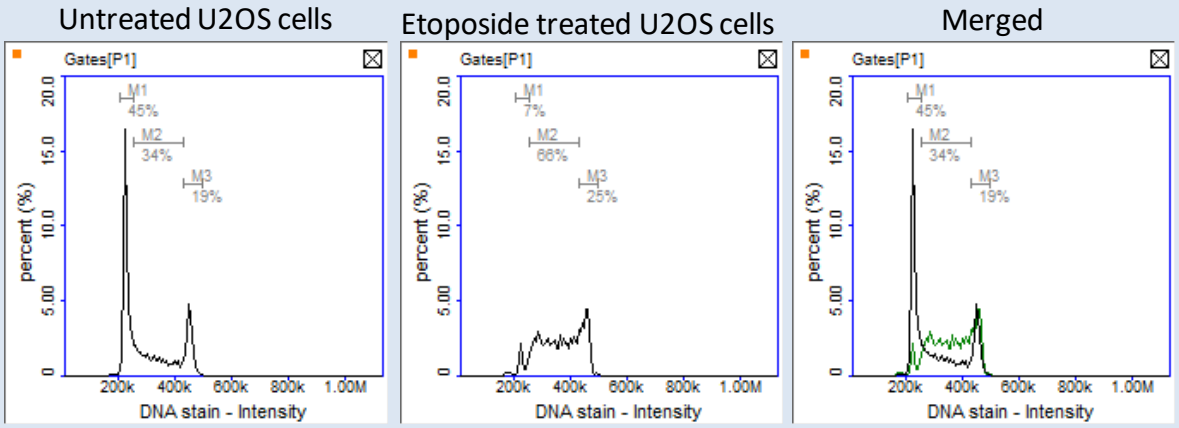
For proper staining it is crucial to use the amounts specified in steps 1 and 2. This procedure specifies the amount required for cells growing in 6-well culture plates having a growth area of ~9.5 cm^2 per well. The procedure can be scaled up or down. Accordingly, for a T25 flask (25 cm^2) use 660 µl of **Solution 10** and **Solution 11** in steps 1 and 2. Examples of other formats are given in Table 1.

The number of seeded cells needs to be optimized for each cell type to have a sufficient number of cells for the analysis and at the same time avoiding the cells to arrest in G_1 due to high confluences of the cells. A starting point could be to seed out a total of $2.5x10^5$ to $5x10^5$ cells/well in a 6-well culture plate the day before the experiment.

1. Remove culture medium, wash once with 3 ml of PBS, remove PBS completely and add 250 µl of **Solution 10** supplemented with 10 µg/ml DAPI
2. Incubate cells at 37° C for 5 minutes, resuspend cells **thoroughly by pipetting**, add 250 µl **Solution 11** and proceed to step 3.
3. Engage the NucleoCounter® NC-3000™ by starting the NucleoView™ NC-3000 software.
4. Depending on the number of samples a 2-chamber slide (**NC-Slide A2™**) or an 8-chamber slide (**NC-Slide A8™**) can be used.
    a. **NC-Slide A2™**: Load ~30 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**2-Step Cell Cycle Assay**", sample unit **NC-Slide A2** and press RUN.
    b. **NC-Slide A8™**: Load ~10 µl of each of the cell suspensions into the chambers of the slide. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**2-Step Cell Cycle Assay**", sample unit **NC-Slide A8** and press RUN.
5. Cellular fluorescence is quantified and DNA content histograms are displayed on PC screen. Markers in the displayed histograms can be used to demarcate cells in the different cells cycle stages (see figure below).

Table 1: volumes of Solution 10 with DAPI and Solution 11 required for different types of cell culture plates

No. of wells	4	6	12	24	48
Culture area , cm^2/well	~22.0	~9.5	~3.8	~1.9	~1.0
Solution 10 with DAPI, µl	580	250	100	50	30
Solution 11, µl	580	250	100	50	30



U2OS cells were grown in the absence (left panel) or in the presence of 10 µM etoposide for 5 hours at 37° C (middle panel). Cells were analysed on the NC-3000 system using the 2-step Cell Cycle Assay. Markers in the displayed histograms were used to demarcate cells in the different cells cycle phases. In the untreated sample G_1/G_0 cells represent the majority of the total population (~45%), whereas the majority of the cells are in S-phase in the Etoposide treated sample (~66%). Right panel is a merge of the untreated and etoposide treated samples (the green line represents the etoposide treated cells)

Troubleshooting

Inappropriate loading of the NC-Slides:

Due to variations in chamber volumes the exact amount needed to fill the chamber may vary. Make sure that the chamber is completely filled and that no excess liquid spreads into other chambers or onto the top of the cover slip. Furthermore, avoid introduction of air bubbles into the chambers. Insufficient filling and air bubbles may cause cell movement compromising the quality of the image analysis.

Intensity of G_1/G_0 peak varies from sample to sample:

If the intensity of the G_1/G_0 peak varies between samples (using the same cell type) it is recommended to add extra **Solution 12** (DAPI) to each of the samples and incubate another 5 minutes at 37° before re-analyzing in the NucleoCounter® NC-3000™. E.g. by adding 5 µl of **Solution 12** to 495 µl of cell sample the DAPI concentration is increased from 5 µg/ml to 10 µg/ml. Note that an increase in DAPI concentration in general will enlarge the CV (Coefficient of Variation) of the G1/G0 peak.

Handling and storage

For handling and storage of ChemoMetec instruments and reagents and NC-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 NC-Slides refer to the corresponding product documentation and the NucleoCounter® NC-3000™ user's 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 NucleoCounter® NC-3000™ system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® NC-3000™ system depend on correct use of the reagents, NC-Slide and the NucleoCounter® NC-3000™ instrument and might depend on the type of cells being analyzed. Refer to the NucleoCounter® NC-3000™ user's 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

ChemoMetec A/S reserves the right to introduce changes in the product to incorporate new technology. This application note is subject to change without notice.

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