Application note No. 3002. Rev. 1.5

# NucleoCounter® NC-3000™

# Cell cycle analysis of fixed cells

**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 quantify DNA content of fixed mammalian cells allowing determination of $G_0/G_1$, S and $G_2$/M cell cycle phases.

**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 NC-3000™ system automates DNA content measurements. In this application note, methods for cell cycle analysis are described which can measure fluorescently stained cells using either DAPI, or propidium iodide. DAPI is a competent dye for measurement of the cell cycle stage. Firstly, the intensity of fluorescence integrated over a DAPI stained cell is in stoichiometric relationship to DNA content. Secondly, DAPI preferentially binds to double stranded DNA and the quantum yield of DAPI/RNA complexes is only 20% of that of the DAPI/DNA complex. Hence, using DAPI there is no requirement for removing RNA by RNase treatment prior to DNA content measurements. This is a prerequisite for other dyes commonly used for measurements of cellular DNA content, such as propidium iodide.

After staining cells are loaded into a 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 NC-3000™ system and cellular fluorescence is quantified and DNA content histograms are displayed on the PC screen.



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**Procedures**

***DAPI staining***

Materials

- Cells to be stained*
- Phosphate buffered saline (PBS)*
- Fixative: 70 % ethanol*
- **Solution 3** (1 µg/ml DAPI, 0.1% triton X-100 in PBS)
- **NC-Slide A2™** or **NC-Slide A8™**

* provided by the user

Important notes:
For proper staining it is crucial to keep the cell density within the range of $2x10^6$ to $4x10^6$ cells/ml. In case of limited amounts of cells the procedure can be scaled down, e.g use $2x10^5$ to $4x10^5$ cells in 0.1 ml PBS in step 1.

Protocol

1. Collect cells for fixation
    a. *For cells growing in suspension or hematologic samples.* Harvest cells by centrifuging 5 min. at 500 *g* at room temperature. Wash once with PBS. Count cells (e.g. by using a Via1-Cassette™) and thoroughly resuspend $1x10^6$ to $2x10^6$ cells in 0.5 ml PBS.
    b. *For adherent cells.* Harvest cells by trypsinization and pool the trypsinized cells with cells floating in the medium (latter consist of detached mitotic, apoptotic and dead cells). Centrifuge cells for 5 min. at 500 *g* at room temperature. Wash once with PBS. Count cells (e.g. by using a Via1-Cassette™) and thoroughly resuspend $1x10^6$ to $2x10^6$ cells in 0.5 ml PBS.
2. Add 4.5 ml of 70% ethanol to each of an appropriate number of 10-15 ml centrifuge tubes. Keep on ice.
3. Transfer the cells suspensions (prepared in step 1) into the appropriate tubes containing ice-cold 70% ethanol, vortex rigorously, and keep the cells in the fixative for at least 2 hours.
    a. Important: it is essential to have a single-cell suspension at the time that cells are mixed with ethanol.
    b. Cells can be stored in 70% ethanol for several months at 0-4° C.
4. Centrifuge ethanol-suspended cells for 5 min. at 500 *g*. Decant ethanol thoroughly.
    a. Note: Cell pellet may be loose. Make sure that no cells are lost in this and subsequent washing steps.
5. Suspend cell pellet in 5 ml PBS, leave for 50 sec, and centrifuge 5 min. at 500 *g*.
6. Resuspend cell pellet in 0.5 ml **Solution 3** and incubate for 5 minutes at 37 °C.
7. Engage NucleoCounter® NC-3000™ by starting NucleoView NC-3000™ software.
8. 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 "**Fixed Cell Cycle-DAPI 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 "**Fixed Cell Cycle-DAPI Assay**", sample unit "**NC-Slide A8"** and press RUN.

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).



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***Propidium iodide staining***

Materials

- Cells to be stained*
- Phosphate buffered saline (PBS)*
- Fixative: 70 % ethanol*
- RNase A (10 mg/ml)*
- **Solution 2** (20 µg/ml PI, 0.1% triton X-100 in PBS)
- **NC-Slide A2™** or **NC-Slide A8™**

* provided by the user

Important notes:
For proper staining it is crucial to keep the cell density within the range of $2x10^6$ to $4x10^6$ cells/ml. In case of limited amounts of cells the procedure can be scaled down, e.g use $2x10^5$ to $4x10^5$ cells in 0.1 ml PBS in step 1.

Protocol

1. Collect cells for fixation
    a. *For cells growing in suspension or hematologic samples.* Harvest cells by centrifuging 5 min. at 500 *g* at room temperature. Wash once with PBS. Count cells (e.g. by using a Via1-Cassette™) and thoroughly resuspend $1x10^6$ to $2x10^6$ cells in 0.5 ml PBS.
    b. *For adherent cells.* Harvest cells by trypsinization and pool the trypsinized cells with cells floating in the medium (latter consist of detached mitotic, apoptotic and dead cells). Centrifuge cells for 5 min. at 500 *g* at room temperature. Wash once with PBS. Count cells (e.g. by using a Via1-Cassette™) and thoroughly resuspend $1x10^6$ to $2x10^6$ cells in 0.5 ml PBS.
2. Add 4.5 ml of 70% ethanol to each of an appropriate number of 10-15 ml centrifuge tubes. Keep on ice.
3. Transfer the cells suspensions (prepared in step 1) into the appropriate tubes containing ice-cold 70% ethanol, vortex rigorously, and keep the cells in the fixative for at least 2 hours.
    a. Important: it is essential to have a single-cell suspension at the time that cells are mixed with ethanol.
    b. Cells can be stored in 70% ethanol for several months at 0-4° C.
4. Centrifuge ethanol-suspended cells for 5 min. at 500 *g*. Decant ethanol thoroughly.
    a. Note: Cell pellet may be loose. Make sure that no cells are lost in this and subsequent washing steps.
5. Suspend cell pellet in 5 ml PBS, leave for 50 sec, and centrifuge 5 min. at 500 *g*.
6. Resuspend cell pellet in 0.5 ml **Solution 2** supplemented with 200 µg/ml Rnase A (final concentration)
    a. E.g. add 10 µl Rnase A (10 mg/ml) to 0.5 ml **Solution 2**.
7. Incubate 30 minutes at 37° C (removal of RNA).
8. Engage NucleoCounter® NC-3000™ by starting NucleoView NC-3000™ software.
9. 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 "**Fixed Cell Cycle-PI 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 "**Fixed Cell Cycle-PI Assay**", sample unit "**NC-Slide A8"** and press RUN.

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).



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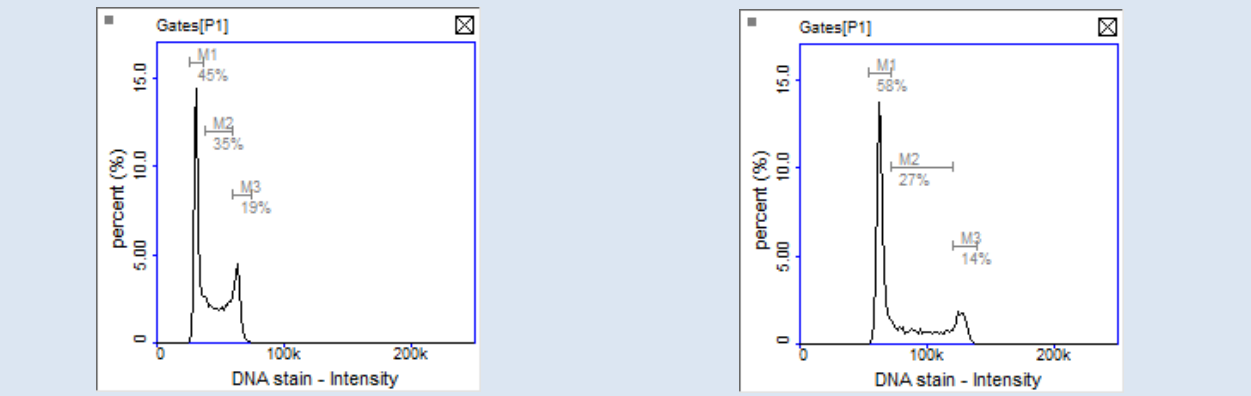


Cell cycle profiles of CHO (A) and U2OS (B) cells. Vegetatively growing cells were analyzed in the NucleoCounter® NC-3000™ using the Fixed Cell Cycle Assay. Markers in the displayed histograms were used to demarcate cells in the different cell cycle phases. In these examples $G_1/G_0$ cells represent the majority of the total population (e.g. ~45% and ~58% for CHO and U2OS respectively).

**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 dilute the samples 2-5 fold with **Solution 3** (DAPI) or **Solution 2** (PI) and incubate another 5 minutes at 37° before re-analyzing in NC-3000™.



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**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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