Application note No. 3003. Rev. 1.4

NucleoCounter® NC-3000™

DNA fragmentation assay

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 the DNA content of mammalian cells in order to detect apoptotic cells with fragmented DNA (sub-G_1 cells).

Introduction

Cell death can occur by two distinct mechanisms, necrosis or apoptosis. Necrosis occurs when cells are exposed to harsh physical or chemical stress (e.g., hypothermia, hypoxia) while apoptosis is a tightly controlled biochemical process by which cells are eliminated and where the cell is an active participant in its own termination ("cellular suicide"). Apoptosis is one of the main types of programmed cell death which occur in multicellular organisms and is characterized by a series of events that lead to a variety of morphological and biochemical changes, including membrane blebbing, cell shrinkage, alteration of membrane asymmetry and permeability, condensation of chromatin and nucleus, DNA fragmentation, and formation of membrane bound vesicles (apoptotic bodies)

Apoptosis is both a very complex and very important process and dysregulations in the apoptosis machinery may lead to very severe diseases. A growing body of evidence suggests that resistance to apoptosis is a feature of most, if not all types of cancer. In the other hand may hyperactivity of the apoptotic processes also cause diseases such as neurodegenerative diseases as seen in Parkinson's and Alzheimer's.

Reliable detection and monitoring of apoptosis is crucial for the development of treatments for apoptosis-associated diseases and for investigating apoptotic mechanisms in general. A number of methods have been developed to study apoptosis in individual cells. One of the major problems in detecting apoptosis is that many features of apoptotic and necrotic processes overlap and therefore it may be necessary to use several independent assays before drawing definitive conclusions. Each of the available methods has its advantages and limitations making it appropriate for some applications but not others.

This application note describes a method for detection of DNA fragmentation, a key apoptotic event, in individual cells. During apoptosis, calcium- and magnesium-dependent nucleases are activated which degrade DNA. This means that within the DNA there are nicks and double-stand breaks causing fragmentation. This late event of apoptosis is detected using DNA content analysis to measure cell having less than 1 DNA equivalent (so-called Sub-G_1 cells having less than 2C DNA content). The Sub-G_1 method relies on the fact that after DNA fragmentation, small DNA molecules are able to diffuse out of the cells following washing with e.g. PBS. Thus, after staining with a quantitative DNA-binding dye, such as DAPI, cells having lost DNA will take up less stain and will appear left of the G_1 peak in a DNA content histogram.

Principle

Using image analysis, the NucleoCounter® NC-3000™ system automates detection of cells with fragmented DNA (sub-G_1 cells). In this application note, a method for quantifying cellular DNA content is described. Cells are permeabilized with ethanol. During this procedure, the Low Molecular Weight (LMW) DNA inside apoptotic cells leaks out and is removed from the sample during subsequent washing step. The High Molecular Weight (HMW) DNA retained in the cells is stained with the DNA-specific dye, DAPI. After staining 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 the amount of HMW DNA is quantified. Apoptotic cells are seen as a sub-G1 peak in a DNA content histogram displayed on PC screen.

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The advantages of this assay are that it is simple, rapid and cheap. Furthermore, it detects cumulative apoptosis and is applicable to most cell types. However, the assay may not always be specific for apoptosis. Thus, sub-G_1 peak may also represent mechanically damaged cells, cells with different chromatin structure, normal cells with lower DNA content that are in heterogenous cell populations, or aneuploid populations. Also the method may not detect cells induced to apoptosis in G2/M phase (4C DNA content).

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 12 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 weeks 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 the NucleoCounter® NC-3000™ by starting the 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 "**DNA Fragmentation 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

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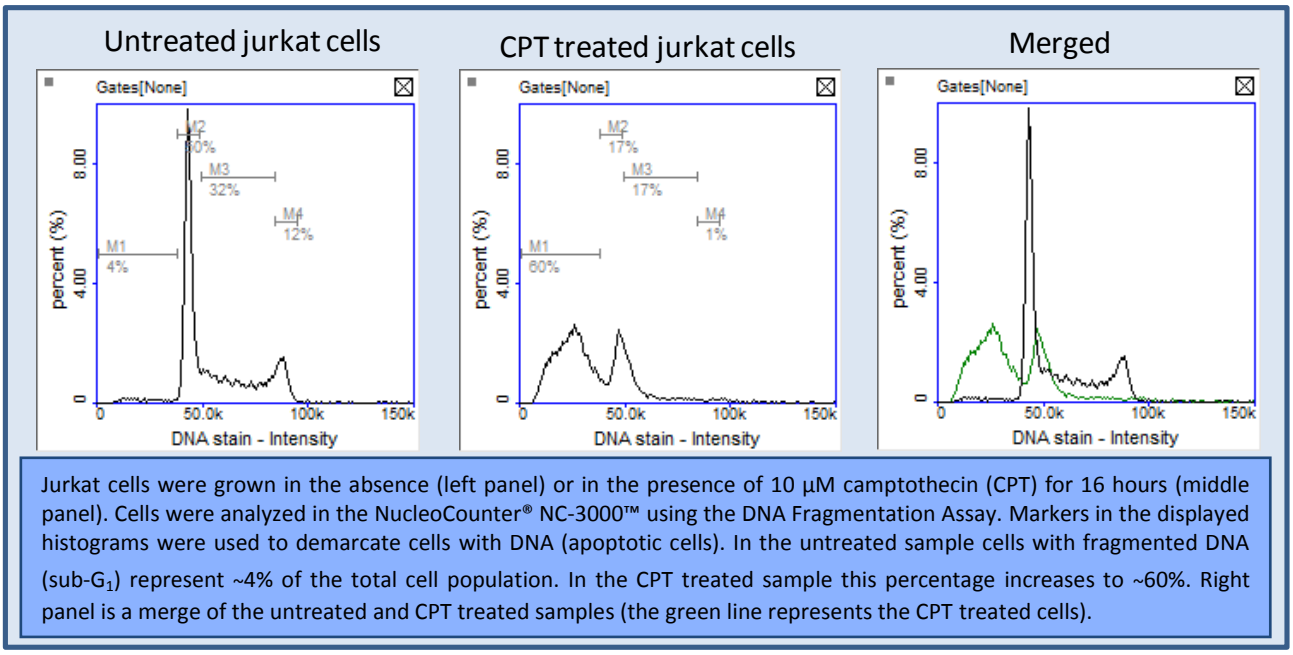
loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**DNA Fragmentation Assay**", sample unit **NC-Slide A8** and press RUN.

9. Cellular fluorescence is quantified and apoptotic cells with fragmented DNA are seen as a sub-G_1 peak in a DNA content histogram displayed on PC screen. Markers in the histogram can be used to demarcate apoptotic cells (see figure below).



Jurkat cells were grown in the absence (left panel) or in the presence of 10 µM camptothecin (CPT) for 16 hours (middle panel). Cells were analyzed in the NucleoCounter® NC-3000™ using the DNA Fragmentation Assay. Markers in the displayed histograms were used to demarcate cells with DNA (apoptotic cells). In the untreated sample cells with fragmented DNA (sub-G_1) represent ~4% of the total cell population. In the CPT treated sample this percentage increases to ~60%. Right panel is a merge of the untreated and CPT treated samples (the green line represents the CPT 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 dilute the samples 2-5 fold with **Solution 3** (DAPI) and incubate another 5 minutes at 37° before re-analyzing in the NucleoCounter® 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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