Application note No. 3021. Rev. 1.3

NucleoCounter® NC-3000™

Caspase assay using the Fluorochrome-labeled inhibitor of caspases assay (FLICA) FAM-DEVD-FMK (caspase 3/7), FAM-LETD-FMK (caspase 8) or FAM- LEHD-FMK (caspase 9)

Product description

The NucleoCounter® NC-3000™ system enables the user to perform advanced cell analyses of a broad range of mammalian cells.

Application

This protocol for the NucleoCounter® NC-3000™ system enables the user to measure caspase activity using the Fluorochrome-labeled inhibitor of caspases assay (FLICA).

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 regulated 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. On 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 note describes a method for quantifying the fraction of cells with caspase activity. Caspases are a family of cysteine aspartic acid specific proteases. The caspases mediate cell death and play essential roles in apoptosis, necrosis and inflammation. Caspases are regulated at a post-translational level, and can thus be rapidly activated. The recognition site for caspases is marked by three to four amino acids followed by an aspartic acid residue, with the cleavage occurring after the aspartate. Caspases are activated by proteolytic cleavage of a precursor, and since the cleavage site within precursors matches the specificity of caspases, sequential activation of precursors by activated caspases can occur.
A way to measure the activity of caspases is to use a caspase specific inhibitor sequence linked to a fluorescent probe known as Fluorochrome Inhibitor of Caspases Assay (FLICA). The non-cytotoxic caspase specific inhibitor is cell permeant and passes through the intact plasma membrane and covalently binds to the reactive cysteine residue on the large subunit of the active caspase heterodimer. Unbound caspase inhibitor diffuses out of the cell and is washed away, thus there is no interference from pro-caspases or inactive forms of the enzyme. The fluorescence thereby gives a direct measure of the amount of active caspase in the whole living cell.

In this note it is explained how to use the NucleoCounter® NC-3000™ to detect cells with caspase activity using the Fluorochrome-labelled Inhibitor of Caspases Assay.

Principle

Using image analysis, the NucleoCounter® NC-3000™ system enables fast determination of apoptotic cells

Gydevang 43 • DK-3450 Allerod • Denmark • Phone: (+45) 48 13 10 20 • Fax: (+45) 48 13 10 21 • Support: contact@chemometec.com • Web: www.chemometec.com

based on caspase activity. When using carboxyfluorescein FLICA reagent (FAM-FLICA), cells with caspase activity will fluoresce green after binding of the inhibitor. The total cell population is stained with Hoechst 33342 which fluoresces violet. Nonviable cells are stained with propidium iodide (PI), and will fluoresce red. After staining, cells are loaded into a NC-Slide A2™. Samples are analyzed using the NucleoCounter® NC-3000™ system. A scatter plot shows PI stained cells (nonviable cell population) versus cells with caspase activity (carboxyfluorescein fluorescence). Only cells with nuclei, i.e. cells which are stained with the DNA stain Hoechst 33342, are shown in the scatter plot. Gating can be used to divide the cells into caspase positive and caspase negative and viable and nonviable populations.

We recommend always including an untreated control in the experiment.

This assay is simple and reliable. Still using another apoptosis assay (e.g. the DNA fragmentation assay) to confirm the results obtained is recommendable.

Materials

- Cells to be stained[1, 2]
- Phosphate buffered saline (PBS)[1]
- Apoptosis wash buffer (diluted to working concentration with distilled H_2O)[3]
- Reconstituted FLICA reagent (add 50 µl DMSO to lysophilized reagent) [3,4]
- Hoechst 33342, 500 µg/ml. E.g. **Solution 15**
- Propidium Iodide, 500 µg/ml. E.g. **Solution 16**
- **NC-Slide A2™**

[1] Provided by the user.

[2] An untreated control should be included. Preferable, use logarithmically proliferating cells as control.

[3] Recommended manufacturer: www.immunochemistry.com

Green FLICA™ Caspases 3 & 7 Assay Kit (Catalog no. 93 or 94)
Green FLICA™ Caspase 8 Assay Kit (Catalog no. 99 or 910)
Green FLICA™ Caspase 9 Assay Kit (Catalog no. 912 or 913)

[4] FLICA reagent is photo sensitive. Perform all staining procedures in the dark.

Protocol

Follow manufacturer's protocol.

We have tested the following procedure on a wide range of cell types and cell lines:

Induce apoptosis in cells using the desired method. Prepare a negative control by incubating cells in the absence of an apoptosis inducing agent, but in the presence of vehicle (e.g. DMSO).

1. Harvest the cells after the incubation period.
2. Determine cell concentration e.g. by using a Via1-Casette™ and adjust the cell concentration to $2x10^6$-$5x10^6$ cells/ml.
3. Dilute the reconstituted FLICA reagent 1:5 in PBS, e.g. by adding 200 µl PBS to 50 µl reconstituted FLICA reagent.
4. Add 5 µl diluted FLICA reagent to 93 µl of the cell suspension prepared in step 1. *Each investigator should adjust the amount of FLICA reagent to accommodate their particular cell line and research conditions.*
5. Add 2 µl Hoechst 33342 (final concentration: 10 µg/ml) and mix by pipetting.
6. Incubate cells for 60 minutes at 37 °C. Gently swirl the cells once or twice during incubation.
7. After incubation, wash cells twice in 400 µl 1X apoptosis wash buffer. Spin down at <400 *g*.
8. After last wash resuspend cells in 100 µl apoptosis wash buffer supplemented with 10 µg/ml PI (e.g. 2 µl **Solution 16** to 100 µl wash buffer) and analyze immediately.
9. Load 30 µl of each of the cell suspensions into the chambers of the NC-Slide A2™. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**Caspase Assay**" and press RUN.

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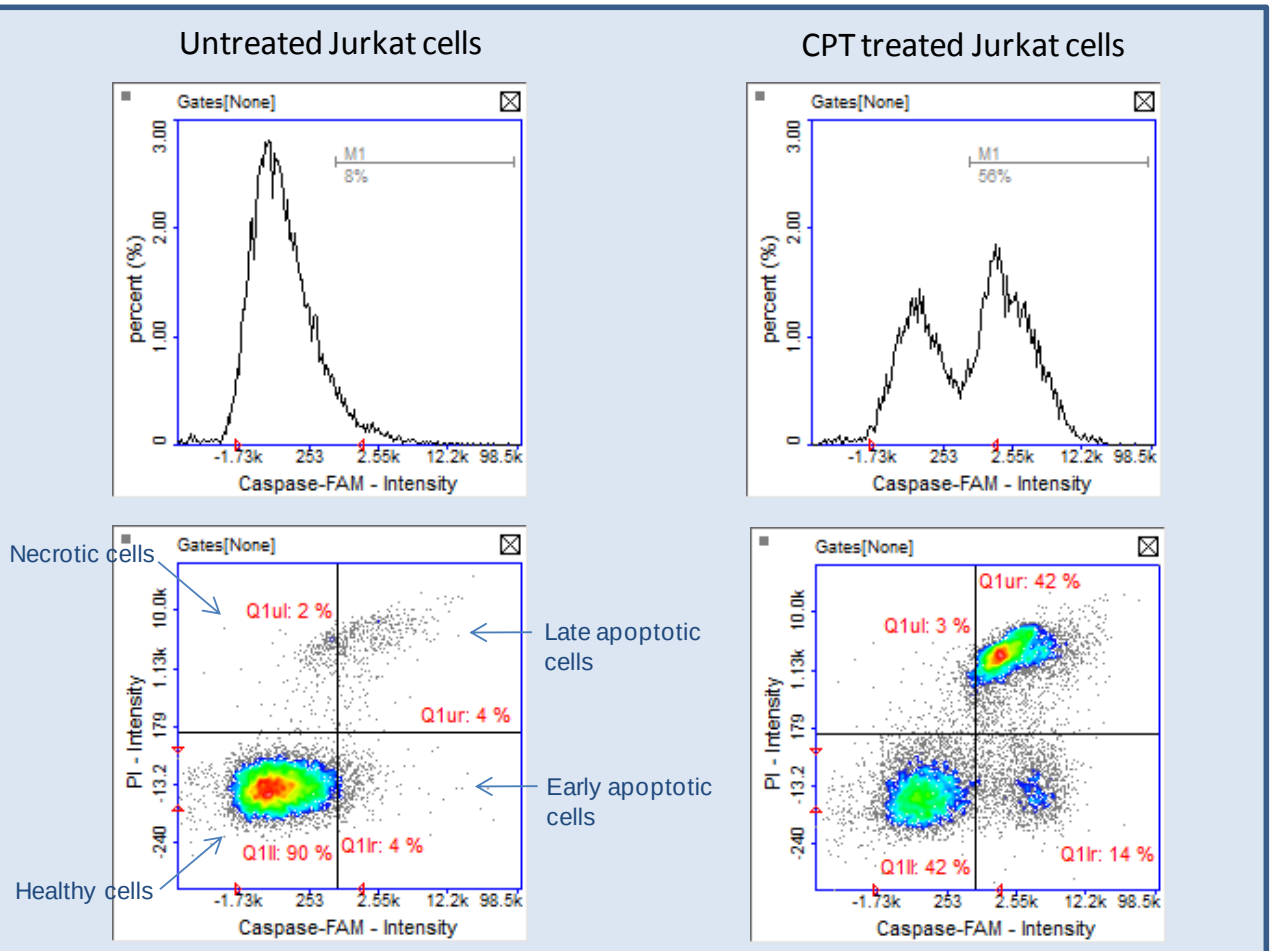
After image acquisition and analysis scatter plots and histograms showing information about Caspase-FAM and propidium iodide fluorescence intensity will be displayed on the PC screen. Using gates in the scatter plot it is possible to determine the ratio of, respectively, early apoptotic, late apoptotic and necrotic cells (see examples below).



Jurkat cells stained with Hoechst 33342, FLICA reagens and PI. Cells were grown in the absence (left panel) or in the presence (right panel) of 10 µM camptothecin (CPT) for 12 hours. Cells were harvested, washed and stained according to the protocol and analyzed in the NucleoCounter® NC-3000™ using the Caspase Assay. Histograms (upper panel) show the Caspase-FAM intensity of the cell population. Scatter plots (lower panel) show the Caspase-FAM intensity versus the intensity of Propidium iodide (PI). Note that in the untreated control sample only very few early apoptotic cells (Caspase positive and PI negative) are observed.

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Trouble shooting

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.

Inappropriate Hoechst-staining of cells:
The caspase assay depends on successful Hoechst-staining. In order to get adequate Hoechst staining it is crucial to follow the protocol carefully. Moreover, it is important to use Hoechst-33342 (which is provided with the FLICA kit) and not other variants such as Hoechst-33258 or Hoechst 34580.

Preparation of 1X Wash buffer
Immunochemistry supply the wash buffer as a 10X concentrate which must be diluted ten times with distilled H_2O. This can be done by e.g. using 1 ml wash buffer to 9 ml distilled H_2O. Diluted wash buffer may be stored at 2- 8 °C for up to 14 days. If the 10X wash concentrate shows precipitation, gently warm the solution to completely dissolve any salt crystals.

Reconstitution of the lyophilized FLICA powder
The FLICA reagent is supplied by Immunochemistry as a lyophilized reagent, which needs to be reconstituted with DMSO before use. Add 50 µl DMSO to dissolve the reagent; mix by swirling or tilting the vial allowing the DMSO to completely dissolve all powder completely. If the reconstituted FLICA is not used immediately, it may be stored at -20 °C for 6 months. After second thaw discard any remaining reagent. If you anticipate using it more than twice, make small aliquots in amber vials or polypropylene tubes and store at -20 °C protected from light.

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
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