Application note No. 3017 Rev. 1.4

NucleoCounter® NC-3000™

Annexin V Assay using the NucleoCounter® NC-3000™ System

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 externalisation of phosphatidylserine using Annexin V-CF488A conjugate or Annexin V-FITC. We recommend using the Annexin V-CF488A that is a photo stable dye and therefore will provide superior results due to a better signal to noise ratio. Hereafter the Annexin V conjugate will be referred to as Annexin V-CF488A.

Introduction

This note describes a method for detection of translocalisation of phosphatidylserine to the outer membrane layer indicating early apoptosis. Phosphatidylserine is in healthy, non-apoptotic cells predominantly located on the internal leaflet of the plasma membrane facing the cytosol. Early in the apoptotic process, while the cell membrane is still intact, the phosphatidylserine is translocated to the outer layer of the membrane. Annexins are group of cellular proteins that bind to phospholipids in a calcium-dependent manner, and a member of this group; Annexin V has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like phosphatidylserine and shows minimal binding to phosphatidylcholine and sphingomyeline.

By conjugating a fluorescent label to Annexin V it is possible to identify and quantify apoptotic cells. Annexin V will also bind to phosphatidylserine on late apoptotic and necrotic cells but as the membrane integrity on these cells has been lost, these can be distinguished from early apoptotic cells by the use of an impermeant dye such as propidium iodide (PI).

In this note it is explained how to use the NucleoCounter® NC-3000™ to measure the percentage of necrotic and apoptotic cells. Cells are stained using fluorescently labeled Annexin V and PI, the latter to distinguish between the early and late apoptotic/ necrotic cells. In order to detect all cells, the total cell population is also stained with Hoechst 33342.

Principle

Using image analysis, the NucleoCounter® NC-3000™ system automates quantification of early apoptotic cells based on Annexin V binding and PI exclusion. Cells are stained with an Annexin V-CF488A conjugate along with Hoechst 33342. Just before analysis cells are mixed with PI to stain nonviable cells.

After staining, cells are loaded into a NC-Slide A2™. Samples are analyzed using the NucleoCounter® NC-3000™ system. The total cell population will be stained with Hoechst 33342 emitting violet light. Apoptotic cells stained with Annexin V-CF488A will, besides being stained with Hoechst 33342, also fluoresce green. Nonviable/late apoptotic cells will be stained with Hoechst 33342 and PI, emitting violet and red light and in some cases also green light due to Annexin V CF488A staining. The fluorescence intensity of Annexin V-CF488A positive cells versus the fluorescence intensity of PI positive cells (nonviable cells) is shown in a scatter-plot displayed on the PC screen. Only cells with nuclei, i.e. cells which are stained with the DNA stain Hoechst 33342, are shown in the scatter plot. Cells positive for Annexin V-CF488A, but which exclude PI may be regarded as apoptotic. The relative frequency of PI positive (non-viable cells) can be determined by creating a gate in the scatter plot containing the PI positive cells. Both the PI single stained cells and the Annexin V-CF488A and PI double stained cells should be included in the gate. A third gate for double positive cells may be made to show the frequency of late apoptotic cells. It is recommended to include an untreated control.

The advantages of this assay are that it is simple and rapid. However, the assay may not always be specific for apoptosis. E.g. trypsination may cause disturbance of the phosphatidylserine leading to false positives, therefore the Annexin V assay is best suited for suspension cells. Moreover, using another apoptosis assay to confirm the results obtained is highly recommendable.

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

Materials

- Cells to be stained[1,2]
- Phosphate buffered saline (PBS) [1]
- Annexin V-CF488A conjugate[3,4]
- Annexin V binding buffer[3,5]
- Hoechst 33342, 500 µg/ml. E.g. **Solution 15**
- Propidium Iodide, 500 µg/ml. E.g. **Solution 16**
- NC-Slide A2™

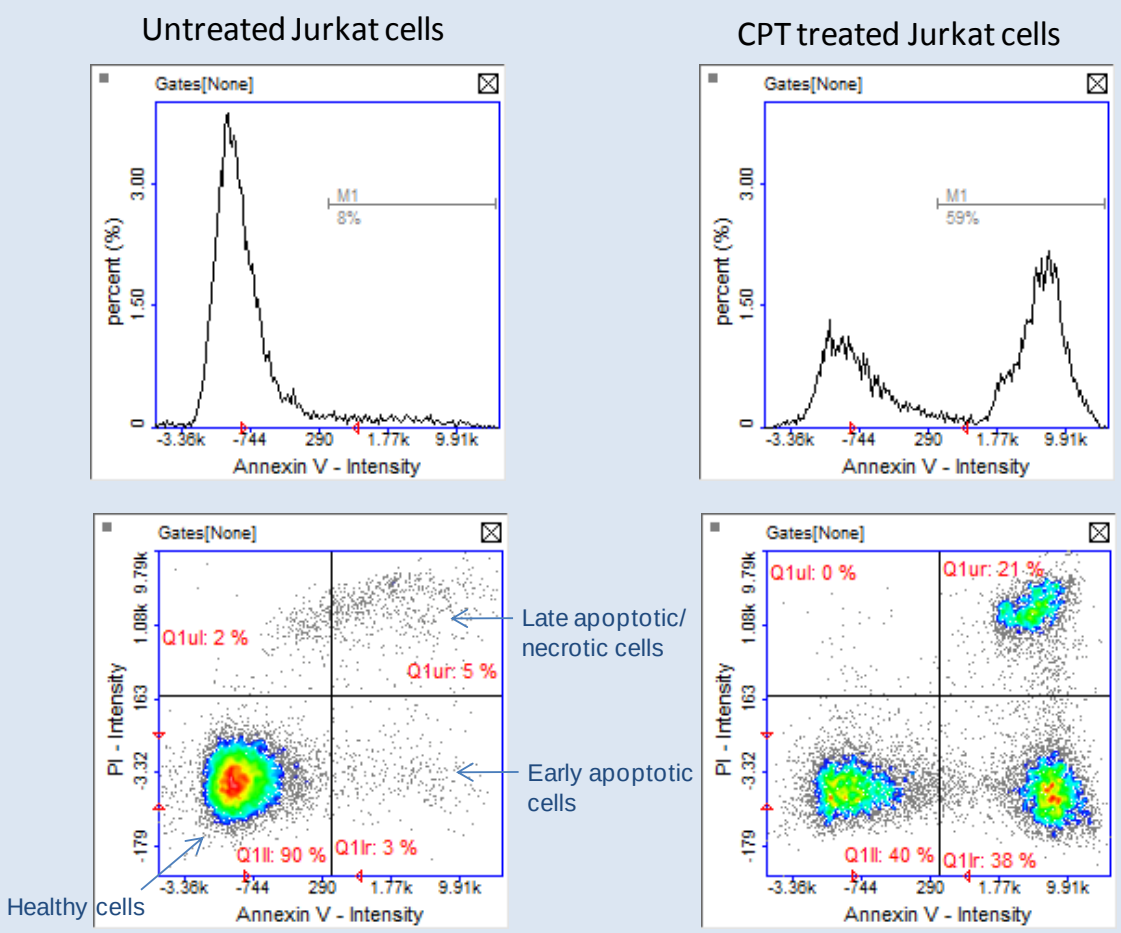
[1] Provided by the user.
[2] An untreated control should be included. Preferable, use logarithmically proliferating cells as control.
[3] Recommended manufacturer: www.biotium.com
Annexin V-CF488A conjugate (Catalog no. 29005)
Annexin V binding buffer (Catalog no. 99902)
[4] Do not freeze! Annexin V CF488A conjugate is photo sensitive. Perform all staining procedures in dark.
[5] Remember to dilute the Annexin V binding buffer to a 1X working solution using deionised H_2O.

Protocol

Follow manufacturer's protocol for staining cells with Annexin V. 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). It may be a good idea also to include a positive control e.g. using staurosporine for inducing apoptosis.

1. Harvest cells after the incubation period and determine cell concentration e.g. by using a Via1-Casette™.
2. (Optional step) Centrifuge cells at 400 *g* for 5 minutes, discard supernatant, and resuspend pellet in 300 µl PBS.
3. Centrifuge cells at 400 *g* for 5 minutes.
4. Remove the supernatant carefully by pipetting without disturbing the cell pellet.
5. Resuspend ca. 2-4x10^5 cells in 100 µl Annexin V binding buffer. It is important that the cell concentrations of all samples are within the specified range in order to enable comparison between samples.
6. Add 2 µl Annexin V-CF488A conjugate.
7. Add 2 µl Hoechst 33342 (final concentration: 10 µg/ml) and mix by pipetting.
8. Incubate cells at 37 °C for 15 minutes using a heating block. This step is crucial, thus comply with the specified incubation time and temperature!
9. Spin down stained cells at 400 *g* for 5 min. at room temperature. Remove supernatant by pipetting.
10. Resuspend cell pellet in 300 µl Annexin V binding buffer by pipetting, centrifuge at 400 *g* for 5 min. and remove supernatant by pipetting.
11. Repeat step 10.
12. Resuspend cell pellet in 100 µl Annexin V binding buffer supplemented with 10 µg/ml PI (e.g. 2 µl **Solution 16** to 100 µl binding buffer) and analyze immediately.
13. Engage NucleoCounter® NC-3000™ by starting the accompanying software.
14. Load 30 µl of each of the cell suspensions into the chambers of the 2-chamber slide (NC-Slide A2™). Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select "**Annexin V Assay**" and press RUN.

After image acquisition and analysis scatter plots and histograms showing information about Annexin V-CF488A 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 apoptotic and late apoptotic/necrotic cells (see examples below).



Jurkat cells stained with Hoechst 33342, Annexin V-CF488A 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 Annexin V Assay. Histograms (upper panel) show the Annexin V-CF488A intensity of the cell population. Scatter plots (lower panel) show the Annexin V-CF488A intensity versus the intensity of Propidium iodide (PI). Note that in the untreated control sample only very few early apoptotic cells (Annexin V positive and PI negative) are observed.

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.

Inappropriate Hoechst-staining of cells:

The Annexin V assay depends on successful Hoechst-staining. In order to get adequate Hoechst staining it is crucial to follow the protocol carefully. Thus, in step 8 it is mandatory to incubate at 37 °C for 15 minutes using a heating block. Moreover, it is important to used Hoechst-33342 and not other variants such as Hoechst-33258 and Hoechst 34580.

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

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.

Copyright