Application note No. 0256. Rev. 1.1

NucleoCounter® NC-250™

Vitality assay: Analysis of the level of cellular thiols using the NucleoCounter® NC-250™ system

Product description

The NucleoCounter® NC-250™ 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-250™ system enables the user to detect changes in the intracellular level of (reduced) thiols. Such changes may occur in apoptotic cells or cells undergoing other pathological processes. As the intracellular reducing power available to the cell is an indicator of overall health status, this assay provide a very easy and fast way to evaluate cell vitality.

Introduction

This application note describes a method for investigating apoptosis and cell health by determining the level of free thiols such as reduced glutathione. The tripeptide glutathione exists in two forms, a reduced state (GSH) and in an oxidized state; glutathione disulfide (GSSG). In the reduced state the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to unstable molecules such as free radicals. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form GSSG. GSH can be regenerated from GSSG by the enzyme glutathione reductase. (See ***Figure 1***.)

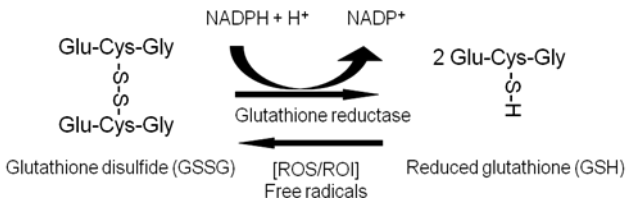

Figure 1. *Glutathione redox cycle. The flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase reduces GSSG to GSH. When the cell encounters free radicals, the antioxidant GSH reduces the free radicals, thereby itself becoming oxidized to GSSG.*

GSH is the most abundant low molecular weight thiol in animal cells; thus, its oxidation status largely determines the thiol-disulfide status of the cell by thiol-disulfide interchange reactions. Moreover, GSH is involved in many cellular processes including quenching of free radicals, drug detoxification, cell signaling, and cell proliferation. Alterations in the concentration of intracellular GSH have been demonstrated as a common feature of many diseases including AIDS, neurodegenerative diseases, and cancer.

A decrease in cellular GSH concentration is an early hallmark in the progression of cell death in response to different apoptotic stimuli. Studies have shown a correlation between cellular GSH depletion and the progression of apoptosis[1]. The decrease in GSH level in connection to apoptosis seems to be attributed to two mechanisms; A) direct GSH oxidation promoted by radicals and B) export of GSH through an ATP-dependent plasma membrane transport system which is triggered by the initiation of apoptosis. When GSH is depleted, the cytosol is shifted from a reducing to an oxidizing environment, which may lead to a further depletion of GSH.

This assay provides a very easy method to quantify the amount of free thiols at the single cell level. The stain VitaBright-48™ (VB-48™), a component of **Solution 6**, immediately reacts with thiols forming a fluorescent product. By quantifying the fluorescence it is possible to determine the level of cellular thiols, and thus determine cell health.

Principle

In this application note, a method for measuring the cellular level of thiols is described. The cells to be investigated are mixed with **Solution 6**. The solution contains two different reagents: propidium iodide (PI) for staining the dead cells and VB-48™ which stains viable cells in an intensity-dependent manner reliant on their level of thiols. A high fluorescence intensity of a particular cell indicates that the cell has a high level of thiols such as GSH.

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

The stained cells are immediately loaded into a NC-Slide: either the 2-chamber NC-Slide A2™ or the 8-chamber NC-Slide A8™. Samples are analyzed using the NucleoCounter® NC-250™ system. A fluorescence intensity histogram showing the distribution of thiol levels in all cells are displayed on the PC screen. By comparing histograms of treated cells to controls the fraction of cells with low vitality (e.g. apoptotic or stressed cells) can be determined.

References

[1] See Coppola S, Ghibelli L. *GSH extrusion and the mitochondrial pathway of apoptotic signalling*. Biochem Soc Trans. 2000;28:56–61 and references herein.

Procedures

If the cell line to be investigated is adherent or semi-adherent, then start by getting all cells into suspension using the preferred method of your laboratory (e.g. trypsin/EDTA treatment). Although the NucleoCounter® NC-250™ is able to count aggregated cells, the accuracy is higher for single cell suspensions.

Materials needed

- Cells to be stained [1, 2]
- **Solution 6** (VB-48™ · PI)
- **NC-Slide A2™** or **NC-Slide A8™**

[1] Cells provided by the user.
[2] An untreated control should be included. Preferable, use logarithmically proliferating cells as control.

1. Pipette a representative cell sample from the cell suspension into a microcentrifuge tube. Add one volume of **Solution 6** into 19 volumes of the cell suspension. E.g., if the volume of the cell suspension is 190 µl then add 10 µl **Solution 6**. Mix by pipetting.

2. Engage NucleoCounter® NC-250™ by starting the accompanying software.

3. 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 NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-250™ and select "**Vitality protocol**" and 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 NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-250™ and select "**Vitality protocol**" and sample unit **NC-Slide A8™** and press RUN.

After image acquisition and analysis scatter plots and histograms showing information about e.g. VB-48™ fluorescence intensity will be displayed on the PC screen. Moreover, the mean and standard deviation of VB-48™ fluorescence of the living cells (PI negative) are shown in a result box. Cells with a low level of thiols (e.g. apoptotic cells) also have a low intensity score, and are thus found in the lower end of the plots. By placing gates or markers in the plots, the user can divide the cell population into two (or more) subpopulations (See examples below).

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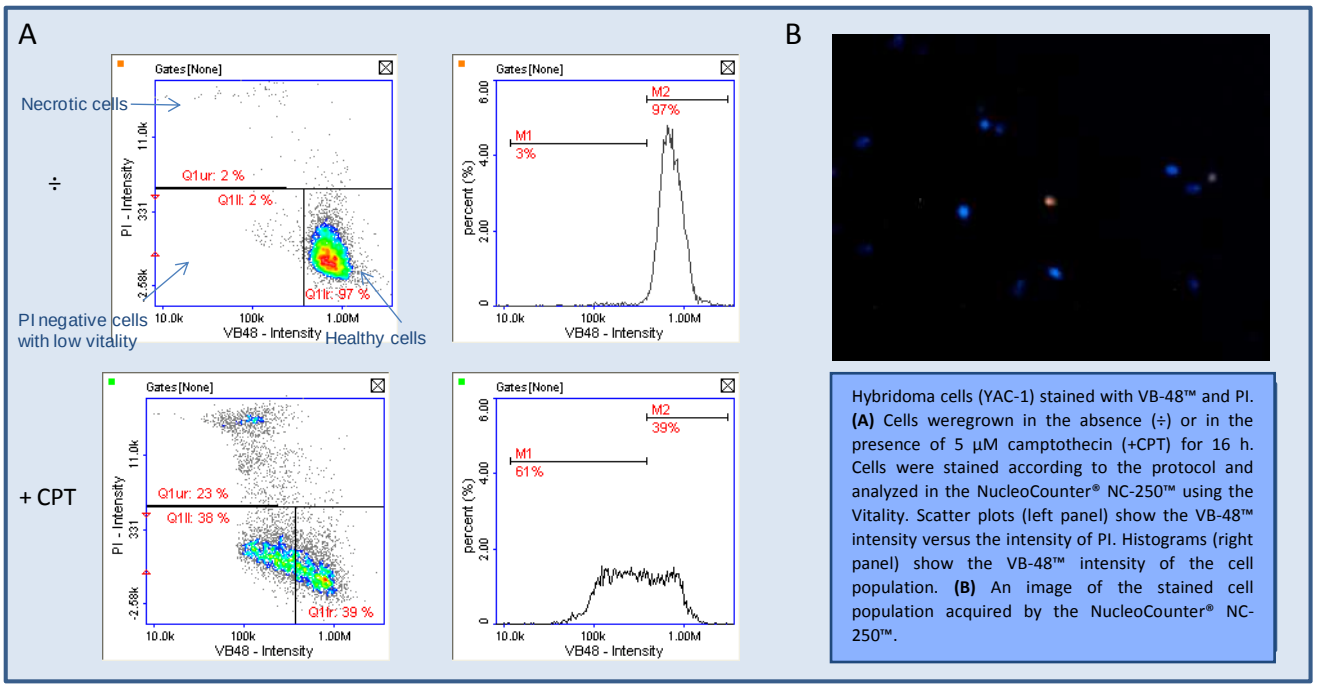
Note

To assure reliable results, it is recommended that the total cell concentration of the cell suspension should be in the range of $5{\cdot}10^4$ cells/ml to $5{\cdot}10^6$ cells/ml. If the concentration of cells is below $5{\cdot}10^4$ cells/ml then the cell concentration may be increased by centrifugation followed by resuspension of the pellet using growth media or PBS. The resuspended cell sample is then treated as described above.

If the total cell concentration is above $5{\cdot}10^6$ cells/ml, the cell suspension can be diluted with growth media or PBS to achieve the desired concentration. The diluted cell sample is then treated as described in the procedure.



Hybridoma cells (YAC-1) stained with VB-48™ and PI. **(A)** Cells weregrown in the absence (÷) or in the presence of 5 µM camptothecin (+CPT) for 16 h. Cells were stained according to the protocol and analyzed in the NucleoCounter® NC-250™ using the Vitality. Scatter plots (left panel) show the VB-48™ intensity versus the intensity of PI. Histograms (right panel) show the VB-48™ intensity of the cell population. **(B)** An image of the stained cell population acquired by the NucleoCounter® NC-250™.

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 coverslip. 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.

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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-250™ 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-250™ system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® NC-250™ system depend on correct use of the reagents, NC-Slide and the NucleoCounter® NC-250™ instrument and might depend on the type of cells being analyzed. Refer to the NucleoCounter® NC-250™ 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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