chemometec

Application Note 005 Ver.1

Total cell count and viability of microcarrier cell cultures

Product description

The NucleoCounter® system is comprised of the NucleoCounter®, NucleoCassettes™, Lysis buffer and Stabilizing buffer. An optional part of the system is the NucleoView™ software.
The NucleoCounter® is developed as a stand-alone instrument. Optionally the NucleoCounter® can be connected to a computer using the NucleoView™ software, which offers a variety of features such as storage of the results and export of the data. It is recommended that NucleoView™ software be used for this application.

Application

This protocol for the NucleoCounter® system enables the user to obtain absolute volumetric cell count and thereby estimate the total number of cells on non-porous microcarriers. The protocol was developed for epithelial cells grown on non-porous microcarriers, but may be useful for other adherent cell lines cultured on similar non-porous surface type microcarriers.

It is recommended that users themselves validate the described cell counting method against their preferred method of reference for estimating the total concentration of cells, e.g. crystal violet staining of released nuclei with manual microscopy counting of cells (Appendices A and B).

Introduction

When adherent anchorage dependant cells are cultured on macrostructures, such as microcarrier beads, counting of the cells becomes difficult due to cell-to-surface and cell-to-cell adherence. Even after trypzination some cell aggregates are not fully released from the solid support and dispersed into solution. Using the NucleoCounter® system it is possible to determine the total concentration of cells in a sample without enzymatic treatment.

Principle

In order to determine the total concentration of cells, the cell suspension is treated with the Lysis buffer A100. The objective of the Lysis buffer is to permeate the cell plasma membranes, thereby allowing the nuclei to be released and fixed prior to detection. The Lysis buffer A100 has the ability to release the nuclei and to dissociate minor cell clusters of mammalian cells. The cells are then stabilized with the stabilizing buffer Reagent B. The stabilizing buffer also elevates pH to ensure consistent and reliable fluorescent detection. Propidium iodide, which is coated on the inside of the NucleoCassette™ is used as a fluorescent stain.

The cells may also be detached from the solid support using alternative protocols for detaching cells such as trypzination. The detached cell suspension can then be analysed following the standard protocol (Application Note 001), and hence viability can also be determined.

After treatment of the cell lines incubation, approximately 50 µL of the cell lyzate is drawn into the NucleoCassette™ that contains sufficient amounts of propidium iodide for staining. The NucleoCassette™ is placed in the NucleoCounter® where the cells are automatically enumerated yielding an absolute volumetric count.

Procedures

Total cell count

The normal NucleoCounter protocol for cells in suspension (Application Note 001) is used on a representative sample containing microcarriers.

Ensure that the sample material is mixed well before pipetting, in order to obtain a representative portion of sample.

1. Add 1 part of representative sample cell suspension to 1 part of Lysis buffer Reagent A100 in a polypropylene tube. (E.g. 300 µL sample cell suspension with media or PBS and 300 µL Lysis buffer.)
2. Mix thoroughly (vortex for 5-10 seconds).
3. Add 1 part (e.g. 300 µL) of Stabilizing buffer Reagent B to the mixture within 30 seconds.
4. Mix thoroughly (use a vortex mixer).
   Note: The samples may be stored for up to several days at room temperature before analysis on the NucleoCounter®.
5. Mix thoroughly and draw a sample of the stabilized lyzate by inserting the tip of the NucleoCassette™ into the lyzate and pressing the piston.
6. Insert the NucleoCassette™ into the NucleoCounter®, close the lid and press RUN.
   After approximately 30 seconds the total concentration of nuclei (C_t) in the stabilized lyzate is displayed on the NucleoCounter® and on the computer if one is connected to the NucleoCounter.
   Note the multiplication factor (M_t) from the dilution, in order to calculate the total concentration of cells in the sample.
   To assure statistically reliable cell counts with the NucleoCounter®, it is recommended that the measured concentration of cells should be within the limits of 1×10^5 cells/mL to 2×10^6 cells/mL.

Note:

It is the number of cells pr. mL in the stabilized lyzate drawn into the NucleoCassette™, which is displayed. Therefore, in order to calculate the number of mammalian cells pr. mL in the original suspension, the displayed number must be multiplied by the multiplication factor (M_t). The multiplication factor is normally 3, assuming the suspension of cells has not been diluted prior to treatment with Lysis buffer and Stabilizing buffer and the procedure above has been followed.

The microcarriers in the sample do not interfere with the counting, since the measuring chamber of the NucleoCassette™ is approximately 100 µm thick. The samples can be stored for up to several days at +4°C or room temperature after the addition of stabilizing buffer Reagent B.

This method of determining the total number of cells in the culture is most accurate when cultures are evenly suspended and when using culture conditions that help prevent aggregation of microcarriers and cells.

The method described above is based on the same principles as the crystal violet counting method (see Appendix A) described by Sanford et al. (1), and later modified by van Wezel (2). The results are expected to correlate well to this method for most common cell culture types grown on non-porous type microcarriers.

Viability of supernatant cells

The normal NucleoCounter protocol for cells in suspension (Application Note 001) is used on sample supernatant that does not contain microcarriers.

Supernatant total cell count

1. Leave micro carrier suspension for 1 minute in order to sediment carriers. Transfer sample supernatant to a new polypropylene tube.
2. Add 1 part of sample supernatant to 1 part of Lysis buffer Reagent A100 in a polypropylene tube. (E.g. 300-µL-sample cell suspension with media or PBS and 300 µL Lysis buffer.)
3. Mix thoroughly (vortex for 5-10 seconds).
4. Add 1 part (e.g. 300 µL) of Stabilizing buffer Reagent B to the mixture within 30 seconds. Mix thoroughly (use a vortex mixer).
   Note: The samples can then be stored for up to several days at room temperature or +4°C.
5. Start analysis by drawing a volume of the stabilized lyzate by inserting the tip of the NucleoCassette™ into the lyzate and pressing the piston.
6. Insert the NucleoCassette™ into the NucleoCounter®, close the lid and press RUN. After approximately 30 seconds the total concentration of nuclei in the supernatant ($\mathbf{C_{st}}$) is displayed on the NucleoCounter® and on the computer if one is connected to the NucleoCounter. Note the multiplication factor ($\mathbf{M_t}$) from the dilution, in order to calculate the total concentration of cells in the supernatant.
   To assure statistically reliable cell counts with the NucleoCounter®, it is recommended that the measured concentration of cells should be within the limits of 1×10^5 cells/mL to 2×10^6 cells/mL.

Supernatant non-viable cell count

7. Mix sample supernatant thoroughly. Start analysis by drawing a volume of sample by inserting the tip of the NucleoCassette™ into the supernatant and pressing the piston.
8. Insert the NucleoCassette™ into the NucleoCounter®, close the lid and press RUN.
   After approximately 30 seconds the concentration of non-viable cells ($\mathbf{C_{nv}}$) in the supernatant is displayed on the NucleoCounter® and on the computer if one is connected to the NucleoCounter. Note the multiplication factor ($\mathbf{M_t}$) from the dilution, in order to calculate the concentration of non-viable cells in the sample.
   To assure statistically reliable cell counts with the NucleoCounter®, it is recommended that the measured concentration of cells should be within the limits of 1×10^5 cells/mL to 2×10^6 cells/mL.

Note:
In many types of non-porous carrier cultures mostly viable cells are attached to the surface of the carriers, and hence the non-viable cell count of the whole sample is approximately the same as the supernatant non-viable cell count.

When counting the number of non-viable cells, the cells are not pre-treated with Lysis buffer and Stabilizing buffer and they are thereby not diluted. Thus, the cell count displayed on the NucleoCounter and/or the NucleoView software on the attached computer, is the actual number of non-viable cells pr. mL in the suspension. If the suspension of cells has been concentrated or diluted the appropriate multiplication factor ($\mathbf{M_t}$) must be used to calculate the correct concentration of non-viable cells in the original cell suspension.

Alternatively cells may be detached from the carrier surface by enzymatic treatments, e.g. trypzinization (see Appendix B, steps 1 – 6) or collagenase treatment, and then the viability of the culture may be estimated using the normal NucleoCounter® protocol for cells in suspension (Application Note 001).

Viability

It is possible to calculate the approximate viability of the carrier culture after having obtained the total concentration of cells ($\mathbf{C_t}$) and the concentration of non-viable cells in the supernatant ($\mathbf{C_{nv}}$). Assuming that the cells have been counted as described above the calculation of viability is as follows.

$$\%\text{viability} = \frac{C_t \cdot M_t - C_{nv} \cdot M_{nv}}{C_t \cdot M_t} \cdot 100\%$$

%Viability	The percentage of viable cells in the original cell suspension.
$\mathbf{C_t}$	The total concentration of cells in the NucleoCassette (the displayed result of the total cell count).
$\mathbf{C_{nv}}$	The concentration of non-viable cells in the NucleoCassette (the result displayed when counting the non-viable cells).
$\mathbf{M_t}$	The multiplication factor used for the total cell count (most often 3).
$\mathbf{M_{nv}}$	The multiplication factor used for the non-viable cell count (most often 1).

The propidium iodide exclusion method that is used in the NucleoCounter® is suitable for calculation of viabilities above 80%, but may in many cases be useful down to lower viabilities.

Adherence

It is possible to calculate the approximate percentage of adherent cells of the carrier culture after having obtained the total concentration of cells (C_t) and the total concentration of cells in the supernatant (C_{st}). Assuming that the cells have been counted as described above the calculation of adherence is as follows

$$\%\ \text{adherence} = \frac{C_t \cdot M_t - C_{st} \cdot M_{st}}{C_t \cdot M_t} \cdot 100\%$$

% adherence — The percentage of adherent cells in the original cell suspension.

C_t — The total concentration of cells in the NucleoCassette (the displayed result of the total cell count).

C_{st} — The supernatant total concentration of cells in the NucleoCassette (the displayed result of the supernatant total cell count).

M_t — The multiplication factor used for the total cell count (most often 3)

M_{nv} — The multiplication factor used for the supernatant total cell count (most often 3)

Image Example

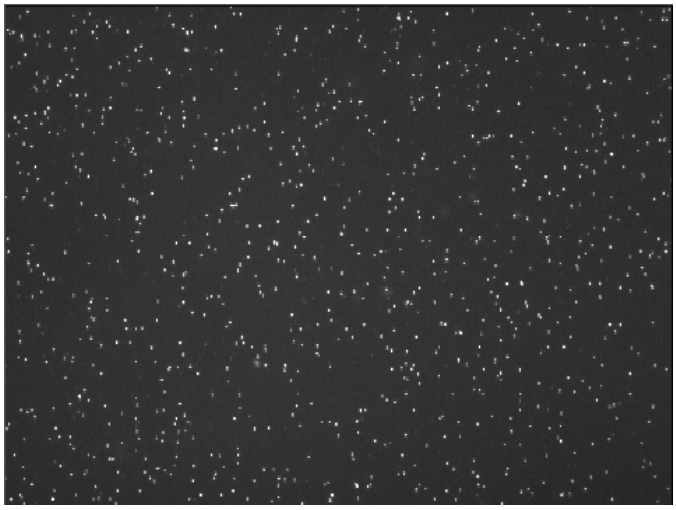

Image 1: Typical NucleoCounter® image when viewed in NucleoView Software. Cells are shown as light dots. Cells have been treated with Reagent A100 and Reagent B before analysis in the NucleoCounter Instrument.

Note:
To assure statistically reliable cell counts with the NucleoCounter®, it is recommended that the measured concentration of cells should be within the limits of 1×10^5 cells/mL to 2×10^6 cells/mL. If the concentration of cells in the cell lyzate is below 1×10^5 cells/mL then the concentration of cells per volume may be increased by centrifugation followed by resuspension of the pellet using PBS or growth media before analysis. Alternatively, repeated measurements may be performed and the average counts used. If the concentration of cells in the suspension is above 2×10^6 cells/mL, cells can be further diluted with PBS or growth media before analysis.

Alternatively a reagent mixture (made of 1 part Reagent A100 + 1 part Reagent B) may be used for diluting the stabilized lyzate prior to analysis.

Correlation Curve

Construction of a correlation curve (optional):

Exported data may be imported into a spreadsheet program. Results may be plotted as

- Crystal Violet (citric acid solution) nuclei count (Cells/mL)
- Optical Density (OD_{600}) against Cell concentration (cells/mL) (see Figure 1)
- Dry Cellular Weight (g/L) against Cell concentration (cells/mL).
- Viability of NucleoCounter® method (percent) against other viabiliy methods (percent)

For different cell types a correction factor may be used for correlating the NucleoCounter® nuclei counts to the cell counts from different manual counting methods. A portion of the growing cells may form clusters that contain two or more cells, hence leading to a difference between the manual cell counts, and the single nuclei counts from the NucleoCounter®.

Example of correlation curve

Preliminary data, Cytopore:

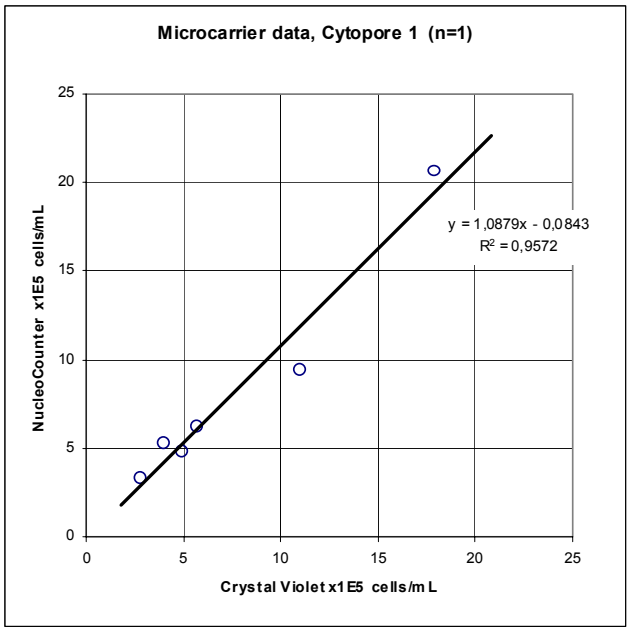

Figure 1: Correlation of Crystal Violet manual cell counts and NucleoCounter® total cell counts. (*CHO cells on commercial macroporous microcarrier*).

Appendix A Crystal violet staining

(reference method for total cell count)

The method was first described by Sanford et al (1) and later modified by van Wenzel et al (2).

1. 1 mL sample of evenly suspended culture is centrifuged (<50×g, 5min.) and the supernatant is discarded.
2. The peleted microcarriers are resuspended in 1 mL 0.1 M citric acid containing 0.1% (w/v) crystal violet, (and possibly 0.1% Triton TX-100). The contents of the tube are mixed well (e.g. with a "Whirl mixer") and then incubated for 1 hr at 37°C. Evaporation of the contents of the tube must be avoided.
3. After incubation the contents of the tube are mixed as above and the released stained nuclei are counted with a haemocytometer. The microcarriers in the sample do not interfere with the counting since they do not enter the measuring chamber. The samples can be stored for up to several days at +4°C.

This method of determining the number of cells in the culture is considered most accurate when cultures are evenly suspended and when culture conditions have avoided aggregation of microcarriers and cells.

Appendix B Trypan blue staining of cells released by trypsinization

1. 1 mL of sample of evenly suspended microcarrier culture is placed in a test tube. After the microcarriers have settled (usually less than 1min) the supernatant is removed.
2. The microcarriers are briefly washed in 2 mL of Ca_2^+, Mg_2^+ -free PBS containing 0.02% (w/v) EDTA, pH 7.6.
3. When the microcarriers have settled this solution is decanted and replaced by 1mL of a1: 1 mixture of 0.25% (w/v) trypsin in Ca_2^+, Mg_2^+ -free PBS and EDTA (0.02%, w/v) in Ca_2^+, Mg_2^+ -free PBS. The pH of this mixture should be 7.6.
4. The tube is incubated at 37°C for 15 min. with occasional agitation.
5. The microcarriers are allowed to settle and the supernatant is transferred to another test tube.
6. The microcarriers are washed with 2 mL culture medium containing serum (5-10%, v/v) and the supernatant is pooled with the first supernatant.
7. The cell suspension is centrifuged (300×g, 5 min., 4°C), the supernatant is discarded and the pellet is resuspended in 2 mL Ca_2^+, Mg_2^+ -free PBS containing 0.05% (w/v) trypan blue.
8. The concentration of cells in the suspension can be counted in a haemocytometer or electronic counter and the concentration of the cells in the culture can be expressed per mL or per cm^2 of microcarrier surface area. Including trypan blue in the re-suspending solution allows estimates of cell viability to be made at the same time. A similar method can be used when using collagenase in combination with some types of microcarriers.

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References:

1. The measurement of proliferation in tissue cultures by enumeration of cell nuclei. Sanford, K.K., Earle, W.R., Evans, V.J. et al., J. Nat. Cancer Inst. 11, p.773-795, (1951)

2. Microcarrier cultures of animal cells. In "Tissue Culture:Methods and Applications" (Kruse,P.F.Pattersson, M.K.,eds) Academic Press, New York, p.372-377, van Wezel, A.L. et al. (1973)

3. Multiparameter analysis and sorting of mammalian cells, Steinkamp JA, Romero A, Horan PK, Crissman HA., Experimental Cell Res., Mar.15; 84(1), p.15-23, (1974)

4. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. Jones KH, Senft JA. J Histochem Cytochem 33, p.77-79 (1985)

5. Rapid assessment of islet viability with acridine orange and propidium iodide. Bank HL. In Vitro Cell Dev. Biol. 24, p.266-273 (1988)

Handling and storage

For handling and storage of ChemoMetec reagents and NucleoCassettes™ 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 NucleoCassettes™ refer to the corresponding product documentation and the NucleoCounter® 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.

Liability disclaims and Limitations

The NucleoCounter® system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® system depend on correct use of the reagents, NucleoCassettes™ and the NucleoCounter®. Refer to the NucleoCounter® user's guide for instructions and limitations and to the reagent material safety data sheet (MSDS) for handling and hazard information.

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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