Application note 006 Ver. 1.0

Protocol for enzymatic nuclei dissociation of multi-layered hepatocytes - Total cell counts

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.

Application

This protocol for the NucleoCounter® system enables the user to obtain absolute volumetric cell counts (Total cell counts) and thereby estimate the total number of cells grown in multi-layers. The protocol was developed for use on hepatocytes grown in alginate beads, but may be useful for other adherent cell lines cultured in multi-layers or macroporous structures.

It is recommended that NucleoView™ software be used for this application.

Introduction

When cells are grown in macroporous bead-like structures or in multi-layers, counting of the cells becomes difficult due to cell-to-cell adherence. Even after lysis and acidic fixation of the released nuclei, some cells aggregates are not fully dispersed. Therefore the estimate of the total cell concentration is not valid.

The protocol described below is experimental and is not validated against other precise methods for nuclei counting, since no such methods are readily available. The protocol has been tested on hepatocytes (Hep G2 cells) grown in alginate beads, but it may be useful for dissociation of nuclei from other types of cells that are grown in beads or multi-layers.

ChemoMetec recommends that users themselves validate the method against their preferred method of reference for estimating the total concentration of cells (e.g. crystal violet staining with manual estimation of cell concentration).

Principle

In order to determine the total concentration of mammalian cells, the cell suspension is treated with the Lysis buffer A100. The objective of the Lysis buffer is to permeate the plasma membranes, thereby allowing the nuclei to be stained with propidium iodide, which is coated on the inside of the NucleoCassette™. The Lysis buffer A100 has the ability to dissociate minor cell clusters of mammalian cells. The cells are then stabilized with the Stabilizing buffer, Reagent B.

For the applications where epithelial cells are grown in alginate beads, an optional protocol for dissolving alginate beads is presented. Alginate beads are calcium dependant sugar-based spherical structures where the cells may grow in the macroporous structures. The alginate beads can be dissociated by the addition of EDTA (or citrate) that binds the calcium.

Larger cell clusters may then be dissociated by the addition of a portion of the pepsin working solution. The suspension is incubated for up to 90 minutes in order to fully release the nuclei from larger clusters. It is recommended that each

application (and each cell line) is investigated individually and that users initially vary incubation times, in order to determine the optimal protocol.

Pepsin (porcine gastric mucosa) is a 35 kDa proteolytic enzyme that has pH optimum from 2 to 4. It is irreversibly inactivated at pH > 6 and is stable up to 60°C. It preferably cleaves C-terminals to the amino acids Phe, Leu, Glu.

By its proteolytic activity Pepsin helps the dissociation of cell nuclei under the conditions presented in cell suspensions that have been lysed and stabilized using Lysis buffer Reagent A100 and Stabilizing buffer Reagent B.

After enzymatic 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. Using the NucleoCounter® system it is possible to determine the total concentration of cells.

Procedures

Pepsin working solution

(enough for approx. 80 analyses)

1. Mix 12.5mL of ChemoMetec Lysis buffer Reagent A100 and 12.5mL of Stabilizing buffer Reagent B in a 50mL polypropylene tube
2. Add 0.50 gram of Pepsin powder (Sigma Chemicals, P-7000, or equivalent) and stir until powder is dissolved.
3. Leave the pepsin working solution overnight (alternatively >4 h) at maximum +5° C, in order for the protein to be fully dissolved.
4. If any undissolved particles are visible at RT, the solution may be filtrated through a 1-2 µm low protein binding particle removal filter.
5. Store the solution at maximum +5° C. The solution should be used within 4 days from preparation.

Protocol for EDTA treatment of alginate beads (optional)

6. Spin down the alginate beads at approx. 300G for 3min.
   (beads are glass clear spheres with a diameter of approx. 0.5 mm)
7. Remove supernatant without removing any beads.
   The remaining volume of beads should be approx. 300µL
8. Add 2mL of 37°C 20mM EDTA (sodium salt), 0.15M NaCl. Vortex gently.
9. Incubate for 10min at 37°C.
10. Add 8mL of Hank´s Balanced Saline Solution (HBSS) or Phosphate Buffered Saline (PBS). Spin down cells at approx. 300G for 5 min.
11. Remove supernatant without removing any cells (pellet). Remaining volume should be approx. 0.25mL
12. Add 0.75mL HPBB or PBS to dissolve cells in approx. 1.00mL.
    Vortex for a few seconds.

Note that the cells have then been concentrated by a factor from the original solution.

13. Dispense 300µL cell suspension in an Eppendorf tube.
14. Add 300µL Lysis Buffer Reagent A100. Vortex briefly.
15. Add 300µL Stabilizing Buffer Reagent B. Vortex briefly.
    From this point cells may be stored at +5°C for several days before continuing.

16. Add 300µL Pepsin working solution to the 900µL of lysed and stabilized cell suspension. Vortex briefly.
    Incubate at 37°C for 2h. Vortex vigorously and pipette up/down every 15min.
    Count cells on NucleoCounter® every 15min, including at 0 min of incubation.
    Remember that the dilution factor in NucleoView™ should be set to 4.

17. Extract cell counts from the NucleoView™ software, e.g. by using the Export data utility. Exported data may be imported into a spreadsheet program. Results may be plotted as shown in Figure 1.

    For hepatocytes a correction factor (F) say 0.7 to 1 may be used for correlating single-nuclei counts to the whole cell counts from manual counting methods. A portion of the growing hepatocytes may be multi-nucleated, hence leading to a difference between the nuclei counts and whole cell counts.

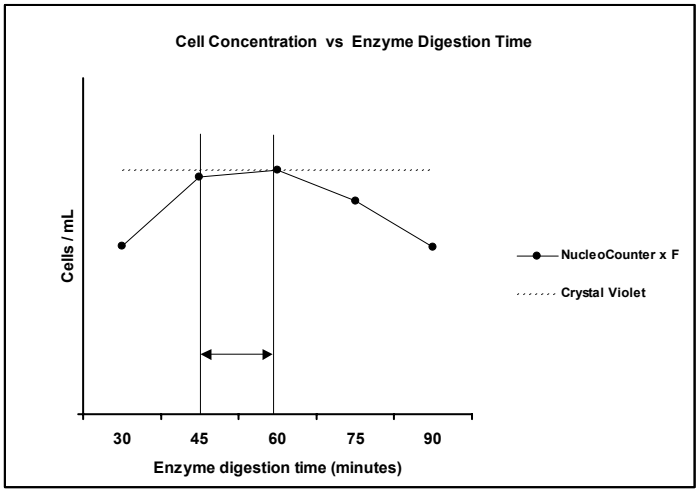

Figure 1: NucleoCounter® data plotted against enzymatic incubation times. Dashed line indicates the initial concentration of cells by manual crystal violet counting method. Note that a correction factor between 0.7 to 1 may be used for correlating single-nuclei counts to the whole cell counts from manual counting methods. The late decrease in cellcounts is due to slight over digestion of the nuclei, leading to disruption and lowered fluorescence signal intensities.

18. Determine the optimal incubation time as the time between the two highest counts (see figure 1). This time should be used for the routine analysis for the cells. This may vary from cell type to cell type, and also with the culturing method.

Image Examples

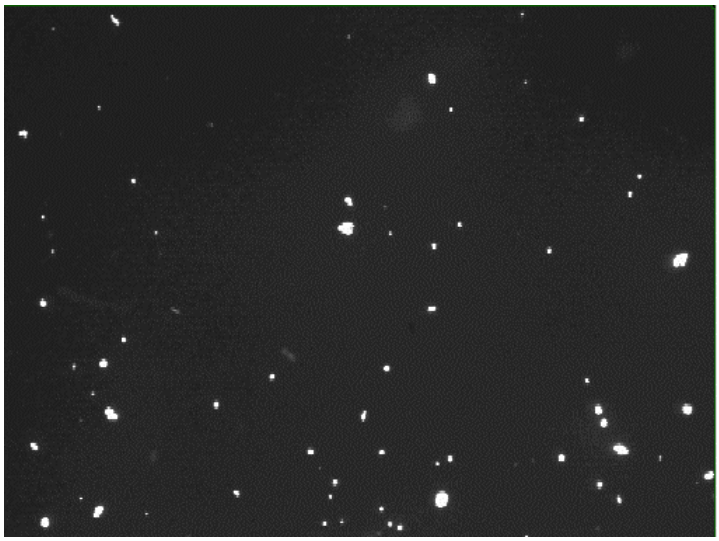

Image 1: NucleoCounter® image before enzymatic treatment (t=0min) of hepatocytes after dissolving alginate beads. Note that large particles consisting of many cells are visible.

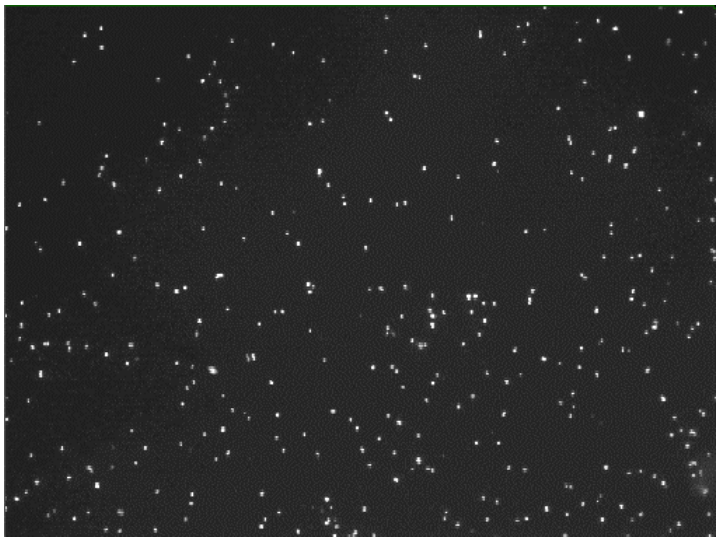

Image 2: NucleoCounter® image after 30minutes of enzymatic treatment (t=30min) of hepatocytes after dissolving alginate beads. Note that large particles consisting of many cells have been reduced in number, and that single nuclei are visible in larger numbers than without enzymatic treatment (see image1). All objects have fluorescence intensities similar to whole mammalian cells, and are uniform in size, indicating minimal nuclei disruption (data not shown). The nuclei may be manually monitored during the incubation by fluorescence microscopy with appropriate filters for propidium iodide.

Note

To assure reliable cell counts with the NucleoCounter®, it is recommended that the total concentration of cells in the cell lyzate should be within the limits of $1 \cdot 10^5$ cells/ml to $2 \cdot 10^6$ cells/ml.

If the concentration of cells in the cell lyzate is below $1 \cdot 10^5$ 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 enzyme digestion. Alternatively, repeated measurements may be performed and the average counts

used. Note that recommended minimum volume of lyzate sample is 0.5mL per tube before analysis.

If the concentration of cells in the enzymatically treated cell lyzate is above $2 \cdot 10^6$ cells/ml, the suspension of cells can be further diluted with "Reagent C100" (made of 1 part Reagent A100 + 1 part Reagent B) prior to analysis without compromising the enzymatic activity.

Note that the pepsin activity will decrease as pH is raised, if PBS or growth media is used for dilutions instead of Reagent C100. It is recommended that Reagent C100 be used for further dilutions when continuing enzymatic treatment.

Viability

It is not possible to calculate the viability of untreated multi layer cells using the NucleoCounter®. If cells can be released into suspension, the normal protocol for the NucleoCounter® may be used for viability determination. The present protocol enables the user only to estimate the total hepatocyte multi-layer cell count (total nuclei count) using the NucleoCounter®. Cell viability in multi-layers may be roughly estimated by manual microscopy staining methods.

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.

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.

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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Technology that counts