Application note No. 302

Total cell count and viability of yeast in beer samples using the NucleoCounter® YC-100™ System

Product description

The NucleoCounter® YC-100™ System is comprised of the NucleoCounter® YC-100™ instrument, NucleoCassette™ and Reagent Y100. An optional but recommended part of the system is the NucleoView™ software.

The NucleoCounter® YC-100™ is developed as a stand-alone instrument, but it is recommended that the NucleoCounter® YC-100™ instrument is connected to a computer using the NucleoView™ software, which offers a variety of features such as storage of the results and export and printing of the data. In addition the NucleoCounter® YC-100™ System can be configured with an external printer for documentation.

Application

This Application Note for the NucleoCounter YC-100 System enables the user to obtain absolute volumetric cell count (total cell count) and viability determination of yeast in beer (*Saccharomyces cerevisiae*). This Application Note concerns the handling of beer samples, measurements and interpretation of results.

Introduction

The NucleoCounter YC-100 System allows fast, reliable and objective determination of cell count in a sample of beer. The NucleoCounter YC-100 counts individual cells in suspension using imaging technology.

It is recommended that users validate the NucleoCounter YC-100 method individually for different yeast strains and different application against their own preferred method of reference.

Principle

The NucleoCounter YC-100 counts individual cells in suspension by detecting fluorescence signals of stained DNA in the cell nuclei. The staining dye used is propidium iodide. The dye is immobilised in the NucleoCassette prior to analysis.

The cell membrane of viable cells is virtually impermeable with regard to propidium iodide while non-viable, dead and to a certain extent apoptotic cells, are permeable to propidium iodide.

The determination of the total concentration of cells requires that the membrane of viable cells are made permeable to propidium iodide. This is achieved by mixing cell suspension with Reagent Y100. Reagent Y100 permeates cell walls and membranes, thereby allowing the staining of all cells in the suspension. Reagent Y100 has the ability to dissolve small cell aggregates, but not to dissolve or dissociate cells that are in the process of reproductive division.

After Reagent Y100 treatment (generally less than 30 seconds), approximately 50 µl of the cell suspension is loaded into the NucleoCassette containing sufficient amounts of propidium iodide for the staining of the cellular DNA. The NucleoCassette is placed in the NucleoCounter YC-100 instrument where the cells are automatically enumerated within 30 seconds, yielding an absolute volumetric cell count.

The determination of the concentration of non-viable cells by the propidium iodide exclusion method is based on preparing a cell suspension by suitable dilution. A sample of the cell suspension is loaded into the NucleoCassette where cells with membranes permeable to the nuclei stain are stained and subsequently detected and counted.

Sample Preparation

In the preparation of a beer sample for the measurement on the NucleoCounter YC-100 System, a number of factors must be considered. The most important factors which have to be considered are the following:

Cell concentration

The cell concentration of the cell suspension loaded into the NucleoCassette must be within the measurement range of the NucleoCounter YC-100 instrument. Further the precision of the determination is dependent on the number of cells counted, closely following Poisson distribution for the counting of random events. Therefore it is recommended that the cell concentration in the suspension loaded into the NucleoCassette is at least $1x10^5$ cells/ml. At this cell concentration the precision of the measurement, expressed as the relative standard deviation of repeated measurements, is approximately 10%. Precision expressed as relative standard deviation of repeated measurements of 5% or better is obtained at cell concentration in the suspension loaded into the NucleoCassette of $5x10^5$ or higher.

Chemical properties of sample media

Wort is a chemically complex solution and there are at least two properties, which must be considered when preparing a beer sample for the measurement using the NucleoCounter YC-100 System.

Firstly the nuclei staining dye loses its fluorescent property at pH below 4, therefore sample with pH less then 4 must be treated with a buffer prior to analysis.

Secondly several of the components of the wort can influence the efficiency of Reagent Y100 and thus requiring prolonged time of reaction. Therefore the reaction time must be verified for each application based on the NucleoCounter YC-100 System.

Optical properties of sample media

Wort is normally a coloured solution and this colour can affect the fluorescence intensity detected from the cells. Depending on the colour of the wort it is therefore necessary to determine the minimum dilution required to obtain correct determination of cell concentration and to use dilution equal or greater than this in any application based on the NucleoCounter YC-100 System.

Cell aggregation

In samples where cells form aggregates it is important to verify the efficiency of Reagent Y100 to dissolve the cell aggregate. This is done by inspecting the cell suspension after the mixing with Reagent Y100 in a microscope. If there is a proven method for dissolving cell aggregates, such as homogenisation or ultrasonic treatment, it is recommended that it should be used.

Viability of the cells

When determining the viability of the sample it is important that any sample preparation does not affect the viability status of the sample. Therefore the effect of chemical or physical factors such as ion concentration, pH, temperature or

centrifugation on the permeability of the cells for propidium iodide must be verified. For dilution of cell suspension intended for the determination of non-viable cells it is recommended to use PBS (Phosphate Buffered Saline) solution.

Dilution error
Since the determination of cell concentration by the NucleoCounter YC-100 System generally requires substantial dilution of the sample it is important to apply reliable methods of dilution. Especially when diluting a sample of pitching yeast, which contains substantial amount of gas that make volumetric measurements difficult. It is recommended to use suitable methods, such as analytical scale to weigh the sample and diluent (e.g. weighing accurately 1.00g of sample and adding 99.0g of diluent to obtain 1/100 dilution). Generally all equipment and tools used for dilution should be verified at regular intervals to comply with any laboratory standards of quality.

Reagent Y100 concentration
The formulation of Reagent Y100 is adapted to conditions where the sample is diluted 10-fold or more in Reagent Y100 prior to reaction. This generally assures completion of reaction within 30 seconds. The reaction time used in any particular application should be verified to assure reliable results. In particular if the dilution with Reagent Y100 is less then 10-fold, great care should be given to the reaction time.

Measurement Procedure - Total Count

Procedure for the determination of total cell concentration

1. Dispense 450µL of Reagent Y100 into suitable container, e.g. a 1.5mL polypropylene tube.

   Note:
   This volume is only appropriate if the dilution obtained is adequate to remove any chemical or optical sample interferences. This has to be determined for each application.

2. Mix the sample suspension thoroughly by inverting/shaking the sample tube to ensure homogeneity.

3. Transfer 50µL of the sample cell suspension to the reagent solution. Mix the solution thoroughly by vortexing or by pipetting vigorously for a few seconds.

   Note:
   The total volume of the mixture is 500µL, resulting in a 10-fold dilution of the sample. Incubation time of 30 seconds is generally sufficient to ensure complete cell membrane permeability but longer reaction time may be necessary in particular application.

   After the mixing with Reagent Y100 the cells suspension may be stored at room temperature or in refrigerator for prolonged time before analysis. It is important to mix the suspension thoroughly before aspirating any volume, or before loading the NucleoCassette.

4. To determine the cell concentration in the sample/Reagent Y100 suspension load NucleoCassette with the suspension and measure on the NucleoCounter YC-100 instrument as described in the User's Guide supplied with the system.

Note:
The NucleoCounter YC-100 instrument presents the estimated cell concentration of the sample suspension loaded into the NucleoCassette. To obtain the determination of the cell concentration of the cell sample the result must be multiplied by the total dilution of the sample, both dilution with any diluent and Reagent Y100.

5. If the cell concentration of the sample suspension is above the upper detection limit of the NucleoCounter YC-100 instrument it is necessary repeat the steps above, e.g. achieving an additional 10-fold dilution each time.

Note:
In routine application it is recommended to define a suitable one-step dilution, depending on the general cell concentration of the sample. This will improve reliability of the application and reduce sample handling.

6. If the cell concentration of the cell suspension is below the desired limit, e.g. determined by detection limit or requirements to precision, then the concentration of cells in the analysed sample may be increased by centrifugation of suitable volume of either the cell sample or the sample/Reagent Y100 suspension, followed by re-suspension of the cell pellet in less volume of suitable diluent before counting.

Note:
The precision of the determination of cell concentration is dependent on the number of individual cells counted by the NucleoCounter YC-100 instrument. To obtain relative precision of 10% or better the cell concentration loaded into the NucleoCassette should be above $1x10^5$ and to obtain relative precision of 5% or better the cell concentration should be above $5x10^5$ cells/ml.

Measurement Procedure - Non-viable

Procedure for the determination of non-viable cell concentration

1. Dispense 450µL of diluent into suitable container, e.g. a 1.5mL polypropylene tube.

Note:
This volume is only appropriate if the dilution obtained is adequate to remove any chemical sample interferences. This has to be determined for each application.
Further, the diluent must not affect the viability or permeability of cells.

2. Mix the sample suspension thoroughly by inverting/shaking the sample tube to ensure homogeneity.

3. Transfer 50µL of the sample cell suspension to the diluent solution. Mix the solution thoroughly by vortexing or by pipetting vigorously for a few seconds.

4. To determine the non-viable cell concentration of the sample suspension load the NucleoCassette with the suspension and measure on the NucleoCounter YC-

100 instrument as described in the User's Guide, supplied with the system.

Note:
The NucleoCounter YC-100 instrument presents the estimated cell concentration of the sample suspension loaded into the NucleoCassette. To obtain the determination of the cell concentration in the cell sample the result must be multiplied by the total dilution of the sample.

5. If the non-viable cell concentration of the sample suspension is above the upper detection limit of the NucleoCounter YC-100 instrument it is necessary repeat the steps above, e.g achieving an additional 10-fold dilution each time.

   Note:
   In routine application it is recommended to define a suitable one-step dilution, depending on the general cell concentration of the sample. This will improve reliability of the application and reduce sample handling.

6. If the non-viable cell concentration of the cell suspension is below the desired limit, e.g. determined by detection limit or requirements to precision, then the concentration of cells may be increased by careful centrifugation of suitable volume of the cell sample, followed by re-suspension of the cell pellet in a less volume of suitable diluent before counting.

   Note:
   The precision of determination of cell concentration is dependent on the number of individual cells counted by the NucleoCounter YC-100 instrument. To obtain a relative precision of 10% or better the cell concentration loaded into the NucleoCassette should be above $1x10^5$ and to obtain a relative precision of 5% or better the cell concentration should be above $5x10^5$ cells/ml.

Optical Interferences

The following table lists the recommended minimum dilution of various beer samples in order to eliminate the effect of colour on the detection of fluorescent objects.

Beer Type	Minimum Dilution
Pilsner	4 fold
Ale	10 fold
Stout	20 fold

The dilution above should only be considered as guidelines. The necessary minimum dilution for different beers or wort must be determined individually.

Sample Interferences

The following table lists the recommended minimum addition of Reagent Y100 to various beer samples (no prior dilution) in order to obtain satisfactory and fast lysing of yeast cells.

Beer Type	Minimum Y100 Addition
Pilsner	10 fold

Ale	10 fold
Stout	20 fold

The dilution with Reagent Y100 listed above should only be considered as guidelines. The necessary minimum addition of reagent for different beers or wort must be determined individually. Also the reaction time needed under various conditions should be determined.

Nuclei Staining

The nuclei dye Propidium Iodide (PI) binds to double stranded DNA whereby its fluorescence efficiency is increased by a factor of about 30. The selectivity in binding is very high, which in combination with the high number of binding sites on the DNA molecules in the nuclei makes the staining of cell nuclei extremely selective.

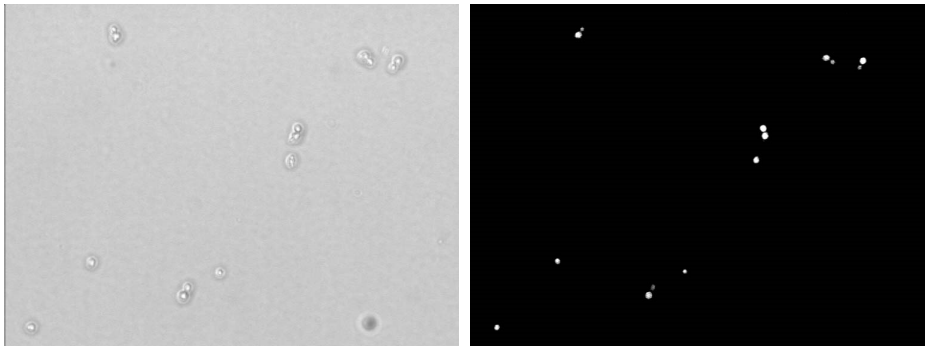

Figure 1 *Left* - A visual microscopy image of yeast cells (*Saccharomyces cerevisiae*) after Reagent Y100 treatment. *Right* - A fluorescent microscopy image of the same sample, showing the staining of cell nuclei. Note that the object in the lower right-hand corner of the visible image does not show any fluorescence.

The Y100 lysis buffer renders the membrane of the cells permeable to PI. Thus after the treatment with Y100 all cells are stained. This allows the determination of the total cell concentration in a sample.

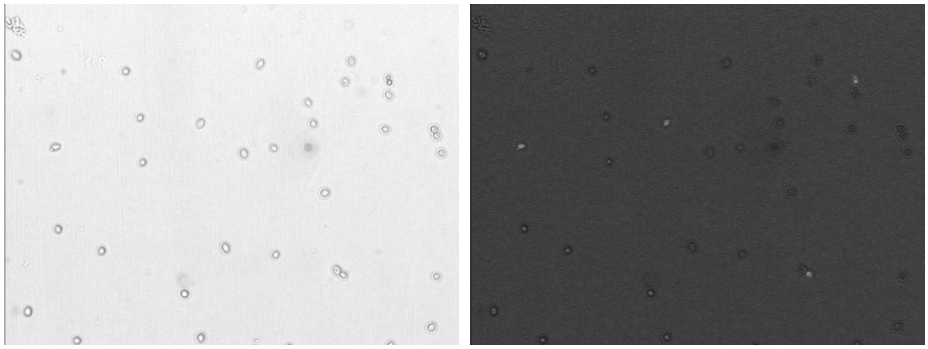

Figure 2 *Left* - A visual microscopy image of yeast cells (*Saccharomyces cerevisiae*) in PBS. *Right* - A fluorescent microscopy image of the same sample, showing the staining of cell nuclei as bight objects. Note that only four of the cells visible to the left emit fluorescence. The membrane of the cells not stained with PI was impermeable PI.

In a solution, not affecting the permeability of the cells, such as isotonic saline solution, viable cells will not be stained with PI. Non-viable cells, on the other hand, will have permeable cell membrane allowing PI to stain the cellular DNA. This allows the determination of the concentration of non-viable cells in a sample.

Viability

To calculate the viability of a yeast sample, first determine the total and non-viable

Calculation

cell concentration. Then use the following equation, where **C** denotes the concentration as reported by the NucleoCounter Instrument, and **D** is the total dilution with diluent and reagent, to calculate the viability:

$$\text{Viability}\% = \frac{C_{Total} * D_{Total} - C_{Dead} * D_{Dead}}{C_{Total} * D_{Total}} * 100\%$$

The fraction of dead cells is calculated by subtracting the **Viability%** from 100%.

The NucleoView software application can be used to calculate the viability of a sample. Please refer to the documentation supplied with the NucleoView for further information.

NucleoView - Image Example

The NucleoView software application can be used to collect results and image from the NucleoCounter instrument. The collected data can be used for documentation and also for the evaluation of the effect of sample preparation. Further, it is possible to place a NucleoCassette measured on the NucleoCounter instrument in a fluorescent microscope, fitted with appropriate filters for the detection of Propidium Iodide (excitation around 525 nm, emission around 605 nm) for visual inspection.

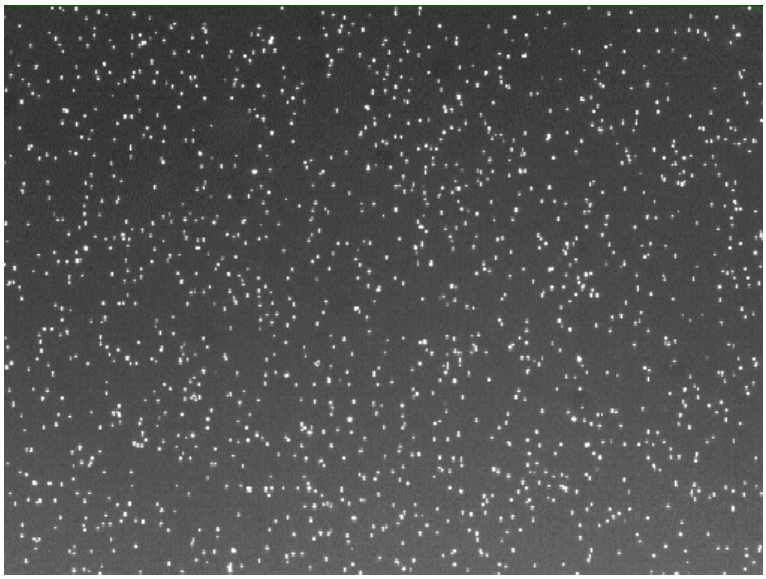

Figure 3 NucleoCounter YC-100 raw image of a yeast cells suspension (*Saccharomyces cerevisiae*) after Reagent Y100 treatment using 100-fold dilution (10 fold dilution in PBS and 10 fold dilution in Regent Y100), as presented in the NucleoView software application. Cell concentration of the sample is approx. 1×10^8cells/mL.

The figure above shows a typical image as collected by the NucleoCounter instrument. It shows each cell as a bright object on dark background. The objects have all similar fluorescence intensity and are uniform in size. The cells are uniformly distributed in the image.

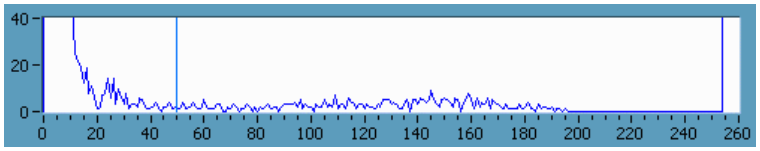

Figure 4 A typical NucleoCounter YC-100 object fluorescence intensity data plotted in a frequency histogram ("object intensity histogram") in the NucleoView software application.

The Object Intensity Histogram shown above illustrates the results of the analysis of a typical yeast sample. The horizontal axis represents the signal intensity of the identified objects, while the vertical axis represents the number of objects having corresponding intensities. Objects determined to be yeast cells, are fluorescent objects that fall above the "gate", illustrated by the vertical discriminator line at signal intensity 50. Several of the cells will normally have intensities at or above the saturation of the detection system (signal intensity 255) illustrated by the high number of objects having this signal intensity.

Signals below the discriminator are discarded as cells. These signals represent the electrical and optical noise of the system as well as possible DNA fragments and are normally not present at high frequencies above signal intensity of 40.

Histograms which show features not similar to those shown above indicate problems with the sample or the sample preparation. A typical cause of such a problem is insufficient dilution.

Cell Concentration

The NucleoCounter YC-100 instrument counts the cells captured in an image representing about 1µl of the solution loaded into the NucleoCassette. Since the analysed volume is fixed to about 1µl this determines the detection limit of the instrument, which is equivalent to cell concentration of 5 cells/image ($5x10^3$ cells/ml). Similarly the resolution of the image defines the upper limits of detection to 2,000 cells/image ($2x10^6$ cells/ml). The measurement range of the NucleoCounter YC-100 is illustrated in the figure below.

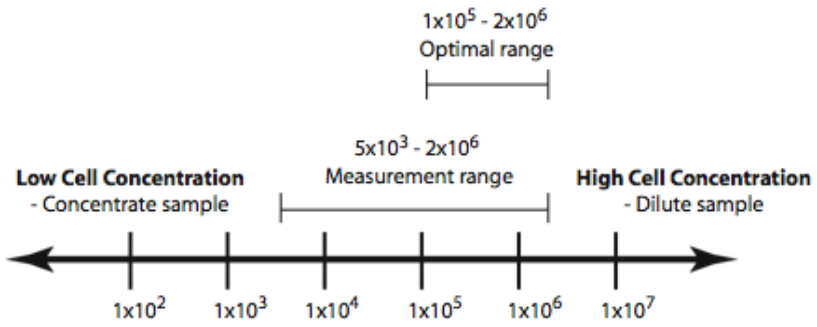

Figure 5 Schematic illustration of the measurement range of the NucleoCounter YC-100 instrument. The cell concentration is the concentration of the solution loaded into the NucleoCassette and does not refer to the cell concentration of the cell sample. The range marked "Optimal range" refers to the range where coefficient of variation is better than 10%

The precision of the determination of the cell concentration is dependent on the number of cells counted. It is normally assumed that the counting of random events follows the Poisson distribution, according to which the expected standard deviation is equivalent to the square root of the number of counted cells. The relative precision, expressed as Coefficient of Variation (CV) is therefore:

$$CV\% = \frac{\sqrt{n}}{n} * 100\% = \frac{1}{\sqrt{n}} * 100\%$$

In cell counting a precision of about 10% or less is generally suitable. According to the

equation above this implies that the number of cells counted should be more than 100. Therefore the "Optimal range" in Figure 3 is defined as the range above $1x10^5$. If the NucleoCounter instrument reports a cell concentration above this limit, then the precision is expected to be better than 10%.

Low Cell Concentration
If the cell concentration reported by the NucleoCounter YC-100 instrument is below the lower measurement limit, or preferably below the lower optimal limits, then the sample should be concentrated (e.g. by centrifugation, followed by re-suspension in less volume).

High Cell Concentration
If the cell suspension measured by the NucleoCounter YC-100 instrument contains concentration of detectable cells above the upper measurement limits then the sample must be diluted, using a suitable diluent, before a result can be obtained.

Sample Dilution

The following table shows the result of the measurement of diluted cell suspension as presented by the NucleoCounter YC-100. The dilution in each column is the total dilution of the sample, including both diluent and any reagent. Results written in bold typeface indicate the "Optimal range" of the NucleoCounter YC-100 instrument, e.g. where the precision is 10% or better, expressed as one standard deviation.

	Dilution			
Cell Concentration	1/10	1/100	1/1,000	1/10,000
$1x10^4$	$<5x10^3$	$<5x10^3$	$<5x10^3$	$<5x10^3$
$5x10^4$	$5x10^3$	$<5x10^3$	$<5x10^3$	$<5x10^3$
$1x10^5$	$1x10^4$	$<5x10^3$	$<5x10^3$	$<5x10^3$
$5x10^5$	$5x10^4$	$5x10^3$	$<5x10^3$	$<5x10^3$
$1x10^6$	**$1x10^5$**	$1x10^4$	$<5x10^3$	$<5x10^3$
$5x10^6$	**$5x10^5$**	$5x10^4$	$5x10^3$	$<5x10^3$
$1x10^7$	**$1x10^6$**	**$1x10^5$**	$1x10^4$	$<5x10^3$
$5x10^7$	$>2x10^6$	**$5x10^5$**	$5x10^4$	$5x10^3$
$1x10^8$	$>2x10^6$	**$1x10^6$**	**$1x10^5$**	$1x10^4$
$5x10^8$	$>2x10^6$	$>2x10^6$	**$5x10^5$**	$5x10^4$
$1x10^9$	$>2x10^6$	$>2x10^6$	**$1x10^6$**	**$1x10^5$**
$5x10^9$	$>2x10^6$	$>2x10^6$	$>2x10^6$	**$5x10^5$**
$1x10^{10}$	$>2x10^6$	$>2x10^6$	$>2x10^6$	**$1x10^6$**

The table below shows the expected precision (CV) under the conditions of the table above.

	Dilution			
Cell Concentration	1/10	1/100	1/1,000	1/10,000
$1x10^4$				
$5x10^4$	45%			
$1x10^5$	30%			
$5x10^5$	14%	45%		
$1x10^6$	10%	30%		
$5x10^6$	5%	14%	45%	

$1x10^7$	3%	10%	30%	
$5x10^7$		5%	14%	45%
$1x10^8$		3%	10%	30%
$5x10^8$			5%	14%
$1x10^9$			3%	10%
$5x10^9$				5%
$1x10^{10}$				3%

Handling and storage

For handling and storage of the instrument, reagents and NucleoCassette 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 NucleoCounter reagents and NucleoCassettes refer to the corresponding product documentation. For other reagents refer to the safety data sheet from the manufacturer of the reagents and chemicals required for this protocol. It is recommended to wear suitable eye protection and protective clothes and gloves at all times when handling reagents and biologically active materials.

Limitations

The NucleoCounter YC-100 System is FOR RESEARCH USE ONLY - NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter YC-100 System depend on correct use of the reagents, NucleoCassette and the NucleoCounter YC-100 instrument. The results might depend on the strain of cells being analysed. Refer to the NucleoCounter YC-100 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. The use of the NucleoCounter YC-100 System must be supervised by a technically qualified individual, experienced in the handling of 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 results obtained by, handling of or contact with the above product.

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