

## Application note No. 200. Rev 1.0

# Determination of SCC in Milk using the NucleoCounter® SCC-100™ System

## Introduction

This Application Note is intended to give an overview of the NucleoCounter SCC-100 System and its use for the counting of somatic cells in milk. It concerns description of materials and methods used in the determination of SCC in milk as well as in-depth analysis of the analytical performance of the NucleoCounter SCC-100. Finally some specialised applications of the NucleoCounter SCC-100 are discussed.

## Counting of Somatic Cells in Milk

Currently there exist several methods for the determination of somatic cell count in milk (SCC). The different methods offer various properties and performance, but common for all methods is that none of them is capable of determining the 'true' cell count. Ideally the true cell count of a given sample can be determined, but there are several theoretical and practical problem associated with this task, which make it impossible to implement, especially when automation and speed of analysis are necessary.

Firstly due to the random position of cells in the sample media any result will only reflect an estimate of the true cell count unless the entire sample has been analysed. Since the precision of any counting method is dependent on the number of cells that have been counted there is a practical limits to the number of cells and thus the volume of milk needed to be analysed when a certain precision is sought.

Secondly the selectivity of a method, expressed as its reliability when identifying a cell when one is present and dismissing any object or particle in the milk which might resemble or be a fraction of a somatic cell when it is not, is one factor determining the accuracy of any method for the counting of somatic cells in milk. The issue of selectivity becomes even more complicated when considering that majority of milk samples are analysed at a location far away from the animal producing the milk, generally with a considerable time elapsing since milking and even intentional chemical and/or physical alteration of the milk. The reason is that milk is a biological media with active enzymes and micro organisms, which can alter the properties of the milk and/or the somatic cells.

As a solution to this problem there is a general agreement in the milk industry of accepting the manual microscopy method defended in IDF/ISO standard 148 or equivalent, as the reference method for the determination of 'true' somatic cell count of a milk sample. Despite of this there are several aspects of this method that limit its applicability from a practical point of view, such as lengthy procedure and intensive training of the operator in order to maintain stable and objective selectivity or accuracy.

## NucleoCounter SCC-100

The NucleoCounter SCC-100 is developed for the task of obtaining precise and objective count of the number of somatic cells in a volume of milk, where several of the key issues discussed above have been addressed in the construction of the instrument and in the development of the method concerning the use of the instrument. The NucleoCounter SCC-100 does not directly implement the reference method for the counting of somatic cells in milk and does therefore not produce results that are to be considered as reference results or 'true' SCC. On the other hand, several of the inherent properties of the NucleoCounter SCC-100 make it suitable as a secondary reference. Most notably the objective selectivity of the NucleoCounter SCC-100 combined with the sublime long-time stability of the various mechanical,

## Technology that counts

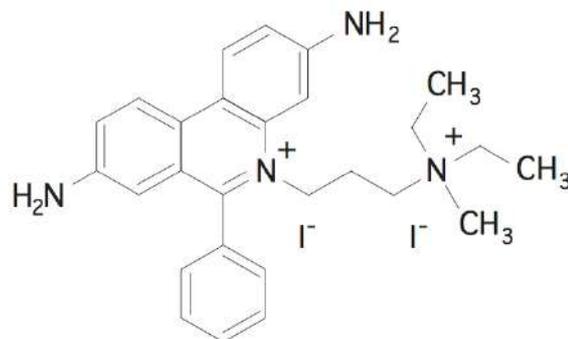
electrical, optical and chemical components of the system assures virtually identical performance throughout the long life-time of each instrument.

Furthermore the production of the instruments assures a high degree of uniformity among different instruments. Thus as a unique feature the NucleoCounter SCC-100 offers virtually identical results when considering the measurement of the same sample, regardless of which instrument is used, at which location the instrument is placed, who operates the instrument and at which time the analyse is performed, provided that the sample has not altered its property.

## Principle of Operation

Determination of SCC in milk with the NucleoCounter SCC-100 System is based on the acquisition of an image of fluorescence signals originating from stained double stranded DNA, followed by the identification and counting of somatic cells in a built-in computer. The sample is prepared by mixing it with a reagent and the sampling is done with a disposable sample cassette containing immobilised DNA staining dye, mixing system and measurement chamber. The results are presented on the display of the instrument as well as on an external printer or computer, which can be used to record the results and optionally generate reports from the results.

The NucleoCounter instrument detects red fluorescent light emitted at or above 600 nm when excited with green light around 525 nm. Natural milk shows little or none fluorescence at these wavelengths and thus the signals detected originate from a dye propidium iodide (PI), added to the sample prior to analysis. PI is a molecule, which can intercalate in the double stranded DNA molecule in a manner which allows one PI molecule to be bound to the dsDNA for every 4 to 5 base pares. The number of base pairs of a mammalian cells is more than  $10^9$ , and therefore a great number of PI molecules can be concentrated in the nuclei of a cell, resulting in a localised dense concentration of PI molecules compared to the solution surrounding the cell. Further the fluorescence properties of PI when bound to DNA change such that the fluorescence efficiency of each molecule increases by a factor of between 30 and 40 further making the distinction between PI in solution and PI bound to DNA more clear.



**Figure 1** The structure of propidium iodide molecule (PI)

## Reagent C

One property of somatic cells is that a cell will resist to the introduction of foreign substances, such as PI molecules. To assure free access to the DNA molecules it is therefore necessary to disrupt or lyse the cell membrane. For this task the NucleoCounter SCC-100 System includes a reagent solution, Reagent C, which among other components contains an efficient lysis agent, Triton X-100. Prior to analysis the sample and Reagent C are mixed in equal volumes and in that process, which takes only few seconds, the cells are lysed, rendering immediate access for PI to the DNA.

## SCC-Cassette

After the mixing of the milk sample and Reagent C, a portion of the mixture is loaded into the SCC-Cassette. The SCC-Cassette is a disposable sampling and measuring device that contains immobilised PI. The inclusion of PI in the cassette reduces the PI consumption to a minimum and additionally it provides a protection for the user against the potentially hazardous DNA staining dye.



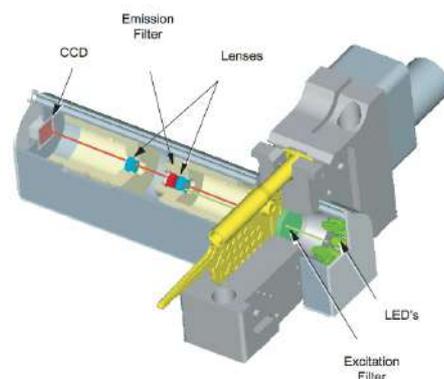
**Figure 2** SCC-Cassette containing immobilised propidium iodide

The cassette is loaded with the solution by pressing the piston while the tip of the cassette is immersed in the liquid. When the solution is loaded into the cassette it immediately dissolves the immobilised PI and as the solution passes through the flow system of the cassette it is mixed thoroughly allowing fast staining of any DNA present.

When the solution has reached the sample compartment the fluorescent image is collected. The sample compartment, which is in the clear window in the upper region of the cassette, is in principle two parallel plates of plastic with a gap of about 100µm between them. When the sample compartment is filled the gap defines the thickness of the sample being analysed. The sample thickness is one of the parameters, which define the volume being analysed. It is therefore of great importance to control or determine the thickness. During the production of each SCC-Cassette the gap, or sample thickness, is carefully measured and written on the cassette in a code, which is read by the instrument during each measurement (see the two black circles on the lower left edge of the cassette in Figure 2). Thus the exact thickness of the sample that is analysed is used to determine the volume analysed in each measurement.

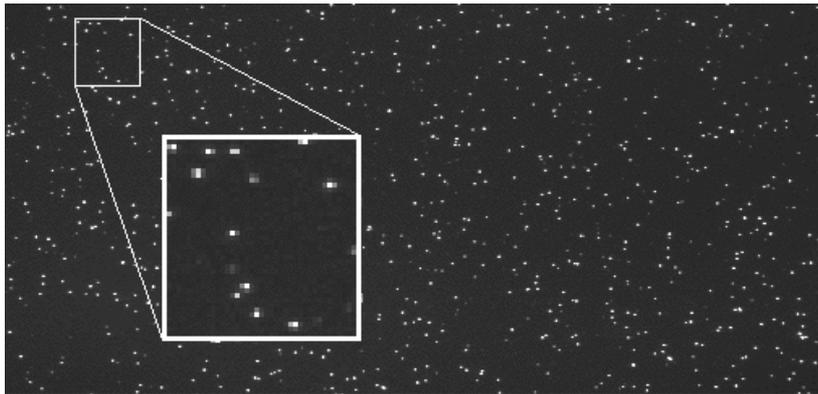
## Instrument

The NucleoCounter SCC-100 instrument is a custom-built fluorescence imaging system. In principle it resembles a microscope but it does not produce an enlarged image, as a microscope would, but rather the collected image is a slight reduction of the original. The purpose of this is to increase the effective view area, or area of sample being imaged in a single measurement. An illustration of the various components of the fluorescence imaging system of the NucleoCounter SCC-100 is given in Figure 3.



**Figure 3** The fluorescence imaging system of the NucleoCounter SCC-100

The imaging system of the NucleoCounter SCC-100 offers several advantages in the identification and counting of somatic cells in a milk sample. Apart from the large view are it allows an extremely simple and ridged construction with only one movable part, which is the motor that drives the piston of the cassette once it is inserted into the instrument. All other parts of the system, including the lenses that focus the collected light, are securely fastened to avoid any alteration of the properties of the system. In addition it allows the use of durable components for the light source (Light Emitting Diodes) and detection (Charge Coupled Device).



**Figure 4** *An image of cell nuclei stained with propidium iodide*

The use of reduction, compared to magnification in traditional microscopy, is clearly apparent in the spatial resolution of the collected image, as illustrated in Figure 4. The low resolution erases virtually any morphological information associated to the imaged cells, but instead it condenses virtually all of the emitted energy from each cell onto one or only few detection elements of the CCD (pixels) .

When identifying somatic cells in a sample of milk in the NucleoCounter SCC-100, it is not necessary to rely on any morphological information to distinguish somatic cells from other objects. In order to be visible in the fluorescence image any object must emit fluorescence at the specific wavelengths, and in the absence of any substantial natural fluorescence in milk, virtually only signals which are recorded are those originating from PI bound to double stranded DNA. Apart from cells, bacteria can also be present in a milk sample. Bacteria do contain DNA, but since the signal intensity corresponds to the amount of DNA present, the fact that somatic cells contain DNA in an amount that is several orders of magnitude larger than in bacteria, the bacterial DNA is not visible in the NucleoCounter SCC-100.

Figure 4 further illustrates that the task of identifying and counting cells can be easily accomplished by a simple computational treatment of the image. Any foreign object, giving rise to a fluorescence signal, not resembling somatic cells in size is easily excluded from the analysis.

## External Printer

For documentation the NucleoCounter SCC-100 can be connected to an external printer. For each analysis the printer generates a printout, as illustrate by the example in Figure 5. It is recommended to use the printout as documentation of the results generated by the NucleoCounter SCC-100.

```

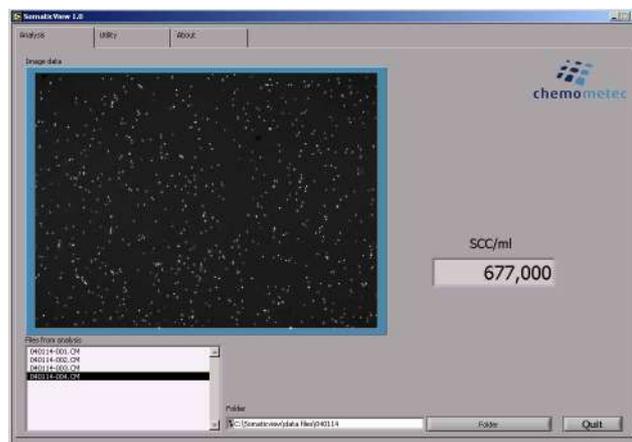
ChemoMetec
NucleoCounter
S/N 000-00 v3.00
-----
Date      07-Jan-2004
Time      09:47:19
Number    #0109
-----
Sample ID
-----
SCC/ml    710,000
Sign.

```

**Figure 5** A printout from NucleoCounter SCC-100

## External Computer

Optionally the NucleoCounter SCC-100 can be connected to an external computer. The software application SomaticView can be used to collect and display data from the instrument. Apart from storing of result and generation of reports the SomaticView does not add to the analytical capability or performance of the NucleoCounter SCC-100.



**Figure 6** NucleoCounter SCC-100 results in SomaticView

## Materials and Methods

The NucleoCounter SCC-100 System is compact and simple in construction and operation, compared to other methods for the determination of SCC in milk. Apart from milk, it is necessary to use a few accessories for the analysis, such as reagent, sample container, and volume dispenser.

### Milk Samples

Any milk sample measured using the NucleoCounter SCC-100 System must be a natural milk sample of good quality. Samples must be homogeneous and free of particles. The NucleoCounter SCC-100 System can measure milk samples at temperatures between 10 and 40 °C, but to assure good mixing, samples should be heated, either to room temperature or preferably to the melting point of milk fat prior to analysis - please observe that temperature has effect on the density and vapour pressure of the milk and the effect of this must be considered for the determination of volumetric mixing ratio of milk and reagent.

Any chemical or physical modification or alteration of a milk sample, including preservation, heating, cooling and storing, can influence the estimated SCC in the sample. It is therefore of outmost importance to verify and estimate the effect of any such modification or alteration of the milk sample, on results obtained by NucleoCounter SCC-100.

Further the NucleoCounter SCC-100 System is developed for the purpose of determining SCC in 'normal' raw milk sample, e.g. fat content approximately between 3 and 9%, protein approximately between 3 and 5% and SCC below 2,000,000 cells/ml. Its performance when measuring samples of other nature, such as colostrum milk, formilk or hindmilk, must therefore be validated carefully.

Since the fundamental property of the NucleoCounter SCC-100 System is its reproducibility it must be primary focus of any application that conditions are kept as constant as possible for all measurements. This will of course not eliminate any effect milk handling might have on the results of the NucleoCounter SCC-100, but it should reduce any variations in the estimated SCC that are caused by variations in milk handling.

## Reagent C

The lysis buffer Reagent C has two main properties, firstly to establish chemical conditions which optimise the lysing of the cells, such as pH and ion strength, and secondly to introduce the lysing agent to the sample. In Reagent C the lysing agent is the detergent Triton X-100.

Reagent C is supplied in a 500 ml container. To aid in the pipetting of the reagent and to prevent the contamination of the entire volume of the reagent it is recommended that a 50 to 100 ml container for Reagent C be used when preparing the lysate solution.

After thorough mixing of Reagent C and milk sample the sample is ready to be loaded into the SCC-Cassette after about 30 seconds. The lysate solution remains stable for considerable time, generally more than 30 minutes, somewhat dependent on the quality of the milk sample. Reagent C is supplied by ChemoMetec A/S.

## Sample Container

The sample container used, must be clean and dry and able to comfortably hold the volume of reagent and milk solution. The form of the sample container should allow the tip of the SCC-cassette to be immersed at least 10 mm into the solution.

It is recommended to use Eppendorf tubes, 1.5 ml Safe-Lock micro test tubes, shown in Figure 7, supplied by Eppendorf AG, generally available through suppliers of laboratory equipment.



*Figure 7 Eppendorf 1.5 ml Safe-Lock tube*

## Volume Dispenser

Both accuracy and precision of results of the NucleoCounter SCC-100 System depend on the preparation of the sample reagent solution. Variation in volume dispensing of either reagent or milk sample have direct influence on the measurement. Therefore it is of great importance that the equipment and the method used for the preparation are carefully validated and maintained. The preparation of the milk lysate involves the dispensing of equal volumes of reagent and milk and it is therefore recommended to use the

same dispenser for both milk and reagent. It is recommended to use a fixed or variable volume pipette with disposable tip, such pipettes supplied by several producers of laboratory equipment, such as Brand GmbH, Eppendorf AG and Thermo Electron Corporation, generally available through suppliers of laboratory equipment. These pipettes are available in several models, manual or battery operated. Some examples of commercially available pipettes are illustrated in Figure 8.



**Figure 8** Examples of manual pipettes supplied by Brand GmbH, Eppendorf AG and Thermo Electron Corporation

## SCC-Cassette

SCC-Cassette serves two purposes. Firstly it contains immobilised propidium iodide (PI) nuclei staining fluorescent dye which gives rise to the detected signal and a flow system mixing the sample and PI. Secondly it contains the sample compartment where fluorescent image is recorded. For the use of the cassette please refer to the NucleoCounter SCC-100 User's Guide. The SCC-Cassette is supplied by ChemoMetec A/S.

## NucleoCounter SCC-100 Instrument

The detection of fluorescent signal, signal processing and result presentation takes place in the NucleoCounter SCC-100 instrument. For the operation of the NucleoCounter SCC-100 instrument please refer to the "NucleoCounter SCC-100 User's Guide". The NucleoCounter SCC-100 instrument is supplied by ChemoMetec A/S.

## External Printer

The external printer, which is a part of the NucleoCounter SCC-100 System. The use of the printer is recommended as documentation of the measured results. For the operation and maintenance of the external printer please refer to the "NucleoCounter SCC-100 User's Guide" and Technical Bulletin supplied with the printer. The external printer is supplied by ChemoMetec A/S.

## SomaticView

SomaticView is a dedicated software application for the acquisition and registration of results obtained by the NucleoCounter SCC-100. SomaticView does not add nor enhance any analytical performance to the NucleoCounter SCC-100 System. For further description of SomaticView please refer to "SomaticView User's Guide", SomaticView is supplied by ChemoMetec A/S.

## Preparation of Lysate Solution

The preparation of the lysate solution is the single most important factor, with respect to operator influence on the result of the NucleoCounter SCC-100. Any error in the amount of either reagent or milk sample is directly reflected in the estimation of somatic cell count (SCC). The dispensing of both reagent

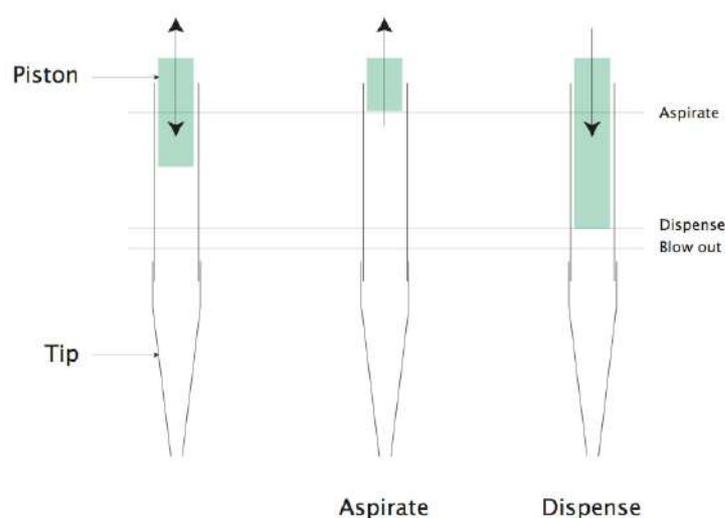
and milk are associated with two factors that are relevant, the mean volume, and the variation from the mean volume.

The mean volume affects the accuracy or reproducibility of the results (fixed error), while variations from the mean affect the precision or repeatability of the results (random error). With respect to error in mean volume, it is important to emphasise that the final result depends on the ratio of the volume of the milk to the volume of the reagent and thus if the error in mean of both volumes is the same than the two errors cancel each other. In other words, it is more important that the volumes of milk and reagent are equal rather than they are exactly a specified volume.

## Pipetting

A pipette is a precision tool for measuring out known volumes of a liquid. The pipette consists of a cylinder within which a piston moves, causing displacement of air in the cylinder. On the end of the cylinder a disposable tip is attached, which is immersed into the liquid being pipetted.

In neutral position the piston is spring-loaded in its topmost position (aspirate in Figure 9). Pressing the piston down reduces the volume in the cylinder, thus forcing any air or liquid out of the tip. At a position the piston meets resistance (dispense in Figure 9), marking the position at which the desired volume has been dispensed. Pressing the piston as far as it goes (blow out in Figure 9) can be used to empty the tip of liquid.



**Figure 9** Illustration of the operation of a pipette

The following recommended procedure for the pipetting of reagent and milk is based on the use of a manual pipette, with the target volume of 0.5 ml. Motorised pipettes and/or other volumes might require modification of the procedure.

Step	Action	Remarks
1	Place a new tip on the pipette	A new tip reduces the risk of contamination
2	Press the piston of the pipette down to the dispense position	The pipette is now ready to aspirate
3	Hold the pipette in a vertical position and immerse the tip few mm into the solution being pipetted	The pipette must be in a vertical position in order to aspirate the correct volume

- |    |                                                                                                                                      |                                                                                                                            |
|----|--------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|
| 4  | Release the piston slowly until it returns to its aspirate position                                                                  | Fast aspiration can create droplets and trap bobbles of air in the tip. Aspiration should last for approximately 3 seconds |
| 5  | Press the piston of the pipette slowly until it reaches the dispense position returning the aspirated volume back into the container | Dispensing the first volume wets the inside of the tip. Keep the piston at the dispense position                           |
| 6  | Hold the pipette in a vertical position and immerse the tip few mm into the solution being pipetted                                  | The tip has been wetted. Ready to aspirate                                                                                 |
| 7  | Release the piston slowly until it returns to its aspirate position                                                                  | Hold pipette still for a moment, when aspirated, to allow pressure to equilibrate                                          |
| 8  | Raise the pipette out of the solution. Hold the tip against the inside walls of the container for about 2 seconds                    | Draws liquid off the outside of the pipette                                                                                |
| 9  | Move the pipette slowly to the Eppendorf tube                                                                                        | Use gliding movements to avoid spilling from the tip                                                                       |
| 10 | Place the tip on the inside wall of the Eppendorf tube                                                                               | Liquid will flow down along the wall                                                                                       |
| 11 | Press the piston of the pipette slowly until it reaches the dispense position returning the aspirated volume into the Eppendorf tube | Dispensing should last for approximately 3 seconds.                                                                        |
| 12 | Hold the tip against the inside walls of the Eppendorf tube for about 2 seconds                                                      | Allows the remainder of the liquid to run off the tip                                                                      |
| 13 | Carefully raise the pipette from the Eppendorf tube                                                                                  | Remove the pipette from the tube without further touching the walls of the tube                                            |
| 14 | Eject the tip                                                                                                                        | Discard used tips                                                                                                          |

In short the procedure for the pipetting of either Reagent C or milk includes the following three steps:

- a** An initial aspiration of a liquid, dispensed directly back to the container thus wetting the walls of the pipette tip.
- b** A second aspiration of the liquid.
- c** Dispensing of that liquid into the sample tube.

It is of great importance that all pipetting is done under identical conditions, including the movement of the piston. This will improve the precision in the dispensing of the Reagent C and milk.

Note that the blow-out position of the pipette is not used to dispense the remains of the liquid from the tip, as is done in normal pipetting, since the surface tension of the liquids normally cause some of the liquid to form a film on the inside of the tip. By initially wetting the pipette tip the volume of any such film is compensated for allowing a volume equal to the displacement of the pipette to be dispensed, without the use of the blow-out action.

### ***Verification of Dispensing Volume***

Before taking the NucleoCounter SCC-100 System in use in an application for the determination of somatic cells in milk the performance of the pipetting must be evaluated. This evaluation must involve at least 10 pipetting of both reagent and milk sample, where the milk sample must be representative for the milk being used in the application. Since the pipetting requires certain routine each person performing the application should perform such evaluation as a part of the preparation or training for the application.

Each dispensed volume should be weighed on an analytical scale with the resolution of 0.1 mg. The mean weight of the volumes as well as the standard deviation are calculated according to Equation 1, where  $x_i$  is the measured weight of a dispensed volume.

$$1 \quad \text{Mean: } \bar{x} = \frac{\sum x_i}{n} \quad \text{Standard deviation: } s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

For a successful verification of the pipetting the standard deviation must be equal to, or less than 0.5% of the mean for both Reagent C and milk. In addition the mean for the volume of Reagent C and the mean for the volume of milk must be approximately equal. Having measured the weight of the pipetted volumes it is convenient to use this to compare the actual volumes and for that purpose it is necessary to know the density of the liquids. The density of Reagent C at 20°C has been determined to 1031 kg/m<sup>3</sup> ± 0.5 kg/m<sup>3</sup>, determined in equilibrium with ambient air.

Regarding milk, it is somewhat difficult, since its density is dependent on the composition of the milk (mainly fat) and the history of the sample. For a cow milk sample it can be estimated that the density varies from between 1027 to 1033 kg/m<sup>3</sup> at 20°C (range of 0.6%) decreasing by approximately 0.7% upon heating to about 40°C.

Assuming that the density of milk is approximately distributed according to the normal Gaussian distribution, then the error in assuming milk has the average density of 1030 kg/m<sup>3</sup> contributes to the random distribution by about 0.2% or less, when repeating the test using milk of varying composition. For convenience it is therefore recommended to determine the mean volume in the dispensing of 2 or more milk samples, showing variation in fat content, and to use the average of these determination with the density of 1030 kg/m<sup>3</sup> at 20°C.

The difference in the volume of Reagent C and milk should preferably be less than the significant level of the determination of the respective means. Assuming no more than 2.5µl (0.5%) standard deviation of the pipetting of 500µl volume, and 10 replicate determination of the volume for each of Reagent C and milk, absolute difference between the mean volumes should be less than about 2.2µl in 95% of the tests. In other words the absolute difference of the mean volume of Reagent C and milk should only exceed 2.2µl in 1 test out of 20 tests. If tests indicate that the difference in the volume exceeds this, then its possible influence on the accuracy or reproducibility of the results should be considered and preferably compensated for.

## Mixing of Milk and Reagent C

Upon the addition of Reagent C and milk to the Eppendorf tube, the tube should be closed. The content of the tube is then mixed, either by inverting the tube about 10 times, or by placing it on a vortex mixer for about 2 seconds. This should be adequate mixing for normal raw cow milk samples, but if samples other than raw cow milk are measured, then the mixing efficiency should be verified.

## Reaction Time

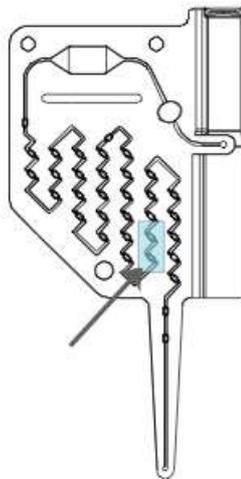
Reagent C is developed with the aim of obtaining fast lysing of somatic cells, while the cells are stable in solution for considerable time. Under normal conditions the lysing is completed in about 30 seconds after mixing but this can vary depending on condition and property of the milk sample. The lysed cells are stable for at least 10 minutes, and depending on the quality of the sample, as long as 2 hours or even more. The stability of the lysed cells in each application should be verified in order to assure stable measurements. This is done by performing repeated measurements on the same sample lysate, noting any development in the cell count with time, which exceeds significantly the repeatability error (e.g. ±2 standard deviations of the Poisson distribution). Please prepare large volumes of sample lysate for this test, recommended at least 0.2 ml per loaded SCC-Cassette to reduce the effect of particle separation (see below).

## Loading of the SCC-Cassette

The detection of cells in the NucleoCounter SCC-100 instrument is inherently resistant to foreign objects in the in collected image, as long as these do not resemble somatic cells in size and fluorescence intensity. On the other hand any contamination does not improve the analytical performance of the system and therefore, each SCC-Cassette should be inspected for particles and severe scratches in the window before use. Particles should be removed, preferably by compressed air, and if that does not work then the windows should be wiped gently using soft cloth or tissue. When cleaning the cassette with a cloth or tissue, it is of great importance not to damage the surface of the window.

The recommended method for loading the cassette is to grab hold of the cassette between the thumb and index finger of the left hand (assuming the operator is right handed) close to the centre of the cassette, with the cylinder to the right hand side. Then place one finger of the right hand beneath each of the flanges on the top of the cylinder, and the thumb on the top of the piston.

To load the sample into the SCC-Cassette immerse the cassette into the lysate solution, and press the piston gently down. Please avoid pressing the piston with great force, since this can form air bobbles in the flow system, which can influence the mixing of lysate solution and reagent and thus affect the results of the measurement. The piston should be pressed down, until the top of the piston is in level with the flanges on the cylinder. This should load the adequate amount of lysate into the cassette, such that the front of liquid should reach the region indicated in Figure 10.



*Figure 10 After loading of the SCC-Cassette the solution should reach the position marked with the shaded region*

After loading with lysate solution the SCC-Cassette is ready to be measured. It is not recommended to wait more than few seconds from the loading of the cassette, until it is measured, to prevent the cells from settling down in the relatively narrow flow system of the cassette.

## Particle Separation

Approximately 50µl of lysate solution is loaded into the SCC-Cassette when the piston is pressed down. If the sample is free of particles then this volume can be assumed to be representative for the lysate. On the other hand, some liquid is also drawn in the thin slit formed when the two parts of the SCC-Cassette are assembled. This slit draws up some liquid due to the capillary force but since the width of the slit is small, the liquid is not necessary representative for the lysate solution, since particles such as fat globules, protein micelles and somatic cells can to some degree not enter the slit.

The effect of this separation is that the concentration of the particles increases in the lysate solution. Through experiment this effect has been estimated to result in as much as 0.3% increase in SCC each time a cassette is loaded from a 1000µl volume of lysate solution. The consequence of an effect of this

magnitude is only weakly significant compared to the precision in each measurement, but nevertheless it should be considered and it is not recommended to load more than 4 cassette from a 1000µl lysate solution. If it is intended to load more than 4 cassettes from a single solution it is recommended to increase the volume of the lysate solution.

Same consideration must be given to conditions, such as evaporation, spillage or contamination that can have effect on the concentration of somatic cells in the milk or in the milk reagent lysate solution.

## Measuring on the Instrument

Before the instrument is used, please verify that the external printer is connected and operational and/or that SomaticView is running on a computer connected to the instrument. It is recommended to use at least either the printer or SomaticView to document the results. When the SCC-Cassette has been loaded insert it into the instrument, making sure that it is fully inserted. Then the measurement should be performed as described in the User's Guide for the NucleoCounter SCC-100.

## Results

The results of the NucleoCounter SCC-100, when estimating the number of somatic cells per volume of milk (SCC) is based on the counting of identified cells in a volume of about 2µl of reagent - milk solution. Since the reagent and milk are mixed in the volume ratio 1:1 then it is assumed that milk constitutes 50% of the volume of the solution analysed ( $f_{Milk}$ ). The initial correlation between the number of counted objects and SCC is therefore as given in Equation 2, where  $n$  is the number of counted cells in the image,  $a$  is the view area,  $t$  is the sample thickness and  $f_{Milk}$  is the fraction of milk in the lysate solution (e.g. 0.5):

$$2 \quad Cells/\mu l_{Milk} = \frac{n}{v_{\mu l}} * 2 = \frac{n}{(a * t)_{\mu l} * (f_{Milk})} \approx \frac{n}{2_{\mu l} * 0.5} = n/\mu l$$

In the evaluation of the results, the exact volume being analysed, ( $a * t$ ) is used, while the approximate expression will be used for convenience in relation to statistical evaluation. The view area  $a$  is determined for each instrument during production (on average about 22 mm<sup>2</sup>) and the thickness of the sample  $t$ , determined during the production of each SCC-Cassette, varies typically between 0.090 and 0.100 mm. The resulting volume is therefore between 1.98 and 2.2 mm<sup>3</sup> (1mm<sup>3</sup>=1µl).

To arrive at the number of cells per ml of milk the result of Equation 2 is multiplied by 1,000µl/ml.

## Linearisation

The cells in the fluorescent image are randomly distributed. Therefore in view of the large thickness of the sample compartment, compared to the size of cell, then coincidence of cells can occur, primarily when the number of cells per image is high. In these situation the analysis of the image can not resolve signal from two cells, resulting in systematic under-determination of the number of cells. Since the degree of under determination depends only on the number of cells counted, this effect can be compensated for, by the use of an empirical function, in this case a ploynomium of second order. In the NucleoCounter SCC-100 this phenomena becomes apparent when more than about 1,600 cells are present in the image (SCC of 1,600,000) increasing in effect to about 5% at 2,000 cells in the image (SCC of 2,000,000). The observed cell count in this range is therefore corrected for deviation from linearity in order to present correct SCC.

## Precision

The precision of the NucleoCounter SCC-100 System, or its ability to repeat results performed under 'identical' conditions, is determined by several factors. These factors can be identified in several groups, each group consisting of factors independent of the factors of the other groups. The groups are firstly the counting of randomly distributed objects, secondly factors concerning the NucleoCounter SCC-100

instrument, thirdly factors concerning the SCC-Cassette and its use, and finally factors concerning the sample and sample preparation.

## Poisson Statistics

The statistical behaviour of counting random objects is generally described by the Poisson distribution. Assuming that  $n$  cells have been counted in a volume of milk in a single experiment, then the Poisson distribution describes the expected distribution of repeated measurements of the same sample. According to the Poisson distribution, the expected value is equal to  $n$  (mean  $\bar{x} = n$ ) and the expected standard deviation of repeated experiments is the square root of  $n$  (standard deviation  $s = \sqrt{n}$ ). Therefore the true value for the number of cells per volume of milk can be expected to be close to the observed value  $n$ , and the measure of the closeness to the true value is the standard deviation  $s$ .

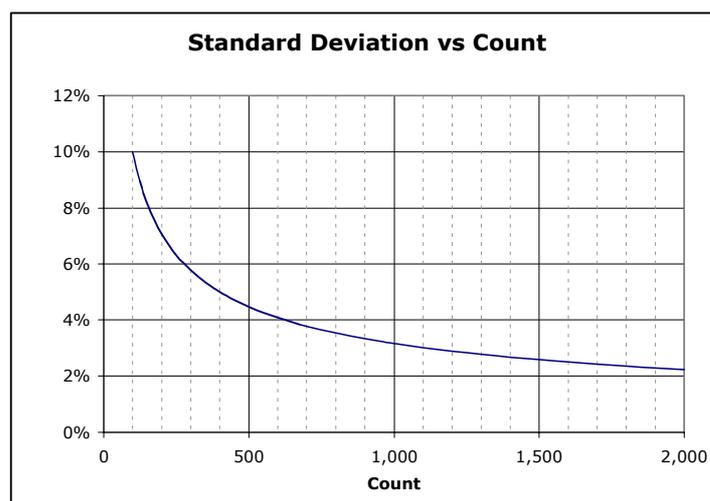
### *Poisson Distribution vs Normal Distribution*

For high number of cells counted (e.g. more than 30) the probability distribution of the Poisson distribution is closely approximated by the normal distribution described by  $\bar{x}$  and  $s$ . Under these conditions we can therefore use the normal distribution, for instance to determine levels of significance. For example if we have counted 400 cells in 1 $\mu$ l then  $\bar{x}$ =400 cells/ $\mu$ l and  $s$ = $\sqrt{400}$ =20 cells/ $\mu$ l (e.g.  $s$  amounts to 5% of  $\bar{x}$ ). In the following the properties of the normal distribution will be used on several occasion to estimate the properties of the NucleoCounter SCC-100 System with respect to repeatability and reproducibility error.

### *Coefficient of Variation*

Since the relationship between  $\bar{x}$  and  $s$  is not a direct one, it is important to note that  $s$  is derived directly from the number of counted cells and not the cell concentration, e.g. if 400 cells are counted in one  $\mu$ l this correspond to 400,000 cells/ml, but the square root of 400,000 is equal to 632 while the standard deviation  $s$  is equal to 20,000 cells/ml ( $s=1,000\sqrt{400}$ ). The ratio between  $s$  and  $\bar{x}$  is of course 5% regardless of which units the results are presented in.

The correlation between  $\bar{x}$  and  $s$  implies that  $s$  increases with increasing  $\bar{x}$ , but since  $s$  is the square root of  $\bar{x}$ , then the ratio of  $s$  to  $\bar{x}$  decreases with increasing  $\bar{x}$  as illustrated in Figure 11. The figure illustrates that when 100 cells are counted then the relative standard deviation is 10%, when 1,000 cells are counted the relative standard deviation is about 3.2% and when 2,000 cells are counted it is about 2.2%.



**Figure 11** Relative standard deviation of the Poisson distribution versus the number of counted objects in the range 100 to 2,000 counts

### *Sample Mean*

According to the Poisson distribution it makes no difference if the counts originate from a single measurement or from two or more measurements of the same sample. Thus if a sample containing about 1,000 cells/ $\mu$ l (SCC 1,000,000) is measured 10 times, then the average should be close to 1,000 cells/ $\mu$ l

but the standard deviation of the average is 1.0%. This is of course the same result as the estimation of the variance of the mean according to the normal distribution, where in this example  $\text{Mean} = \Sigma n / 10$  and  $s_{\text{Mean}} = s / \sqrt{10}$ . This illustrates that repeating the measurement will reduce the uncertainty, e.i. increase the precision, of the estimated SCC.

Correspondingly it is possible to determine the number of cells that have to be counted in order to obtain certain precision expressed as the standard deviation of the mean, for instance, 3% requires the counting of at least 1,100 cells, 2% requires the counting of at least 2,500 cells, and 1% requires the counting of at least 10,000 cells.

## **NucleoCounter SCC-100 Instrument**

Significant factors relating to the NucleoCounter SCC-100 instrument, that can affect the repeatability of the measurement have not been identified, which of course is not a proof against that such a factor exists rather it indicates that the magnitude of such a factor is small. Experiments concerning the measurement of the same cassette repeatedly show no significant difference from one measurement to the next. Thus it can be concluded that the contribution of the NucleoCounter SCC-100 instrument to the precision of the system can be ignored, and in any case would such behaviour be included in other factors significant in relation to the precision.

## **SCC-Cassette**

The distance between the sample compartment windows determines the thickness of the sample being analysed. The production methods used in the production of the SCC-Cassette result in a considerable variation of this distance. In order to compensate for this the distance between the sample compartment windows of each SCC-Cassette is measured during production, and this value printed on the cassette by means of one two or three round markings or dots along the left edge of the SCC-Cassette. These markings are read by the NucleoCounter SCC-100 instrument during measurement of each SCC-Cassette and interpreted as sample compartment thickness, and combined with the view area of the instrument it is used to accurately determine the volume of lysate being analysed.

It is therefore important that the markings on the SCC-Cassette are not damaged and that they appear as solid black regions. Please inspect the conditions of the markings on each cassette and in case of damaged or incomplete coverage please use a black marker pen to repair these marking, since otherwise an incorrect compensation of sample compartment thickness can be applied.

Since the representation of the value when written on the cassette has the resolution of 2µm, this is a source of a random error of about 0.6µm, since the precise values can be considered evenly spaced in the 2µm intervals. Given the average sample compartment thickness of about 100µm this amounts to about 0.6% random error.

## **Sample and Sample Handling**

There are several aspects of sample properties and sample handling which can influence the repeatability of the somatic cell count determined by the NucleoCounter SCC-100 System but the magnitude of the various factors are difficult to estimate beforehand. The composition and quality of the milk sample being used, such as its fat content, its age and storage conditions, and the manner in which it is treated, such as mixing and heating can have influence on the results from repeated measurement of the same sample. For instance can poor mixing between samplings result in a repeatability or reproducibility error, e.g. due to sedimentation of cells and/or separation of fat.

Since the effect of many of these factors is eliminated, or greatly reduced, upon mixing of the milk sample and the reagent, they might show up differently depending on how the experiment is conducted. When a single measurement is done on each lysate solution then any effect from the sample and sample handling will show up in each measurement. On the other hand, if two or more measurements are carried out on each solution, then this effect will only become visible when comparing results of different solutions made up from the same milk sample.

The preparation of the reagent and milk solution can introduce an error that shows up when comparing results obtained from the measurement of different lysate solutions. This error is mainly associated with uncorrelated variations in the volumes of reagent and milk used to form the lysate solution.

As a consequence it is important to emphasise, that the sample used must be of a good quality and it must be treated in a careful and reproducible manner. Otherwise the results obtained by the NucleoCounter SCC-100 System can be compromised in quality.

### Repeatability Error

The observed result of each measurement ( $SCC_{Observed}$ ) can be expressed as in Equation 3 as the expected or 'true' value for that sample when measured on the NucleoCounter SCC-100 System ( $SCC_{SCC-100}$ ) (not to be confused with the 'true' value of the sample, see *Accuracy* later) and a random contribution  $\varepsilon$  assumed to follow the normal probability distribution with mean 0 and standard deviation  $s$ .

$$3 \quad SCC_{Observed} = SCC_{SCC-100} + \varepsilon$$

The repeatability error is a measure of the precision of a NucleoCounter SCC-100 System and defines the variations from a 'true' value e.g. estimated as the mean value of repeated measurement of the same or identical milk sample. From the above, we can assume that repeatability error standard deviation  $s_{SCC-100}$  contains contributions from two sources, the NucleoCounter SCC-100 System ( $s_r$ ) and the sample and/or sample handling ( $s_s$ ), as expressed in Equation 4. The  $s_{SCC-100}$  is dominated by the random behaviour of counting  $s_{Poisson}$  and in addition it contains contribution from several aspects of the NucleoCounter SCC-100, mainly random error associated with the SCC-Cassettes  $s_{SCC-Cassettes}$ .

$$4 \quad s_{SCC-100}^2 = s_r^2 + s_s^2 = s_{Poisson}^2 + s_{SCC-Cassette}^2 + s_s^2$$

In the following the different sources of error will be briefly discussed. For simplicity the error is calculated relative to the counting of somatic cells per  $\mu\text{l}$  of milk. In order to express the error in terms of cells per ml the respective standard deviation is multiplied by 1,000 $\mu\text{l}/\text{ml}$ .

#### Poisson - $s_{Poisson}$

The standard deviation of the Poisson distribution,  $s_{Poisson}$  in units of counted cells is expressed in Equation 5. Previously it has been given that this error is equal to the square root of the number of counted cells.

$$5 \quad s_{Poisson}^2 = n \quad ; \quad n = \text{counted cells}$$

#### NucleoCounter and SCC-Cassette - $s_{SCC-Cassette}$

The uncompensated variations in the thickness of the sample compartment are estimated to be about 0.6%, e.g. square distribution with range  $2\mu\text{m}$  with average of about  $95\mu\text{m}$ , as expressed in Equation 6. Since its effect is on the volume of lysate analysed the effect in terms of counted cells is expected to be directly proportional to the number of counted cells or a given fraction of the counted cells thus expressed as  $f_{SCC-Cassette} * n$ .

$$6 \quad s_{SCC-Cassette}^2 = (f_{SCC-Cassette} * n)^2 \approx (0.006 * n)^2 \quad ; \quad n = \text{counted cells}$$

#### Sample preparation and Sample handling - $s_s$

The contribution of sample preparation and sample handling to  $s_{SCC-100}$ , can not be defined but has to be estimated or determined. It is difficult to identify and determine the different significant factors contributing, except possibly the effect of variation in the volumes of dispensed reagent and milk sample as discussed previously.

The variation in dispensing should be determined for each application, but typical values for the pipetting of 500 $\mu\text{l}$  should be around 0.5%. Thus assuming no correlation between the random error of pipetting reagent and milk,  $s_s$  can be expressed as in Equation 7 where  $s_{sGH}$  is any sample or sample handling error except the pipetting error.  $s_{sGH}$  will generally relate to factors that are directly correlated to volume and

therefore it can be assumed to be proportional to the number of counted cells (e.g. a fraction), in the same way as does the cassette and pipetting errors.

$$7 \quad s_s^2 = s_{S\&H}^2 + s_{Pipetting}^2 = (f_{S\&H} * n)^2 + 2 * (f_{Pipetting} * n)^2 \quad ; \quad n = \text{counted cells}$$

The combining of Equations 4 to 7 results in an approximation of  $s_{SCC-100}$  as a function of the number of counted cells, assuming that pipetting error is of the order of 0.5%.

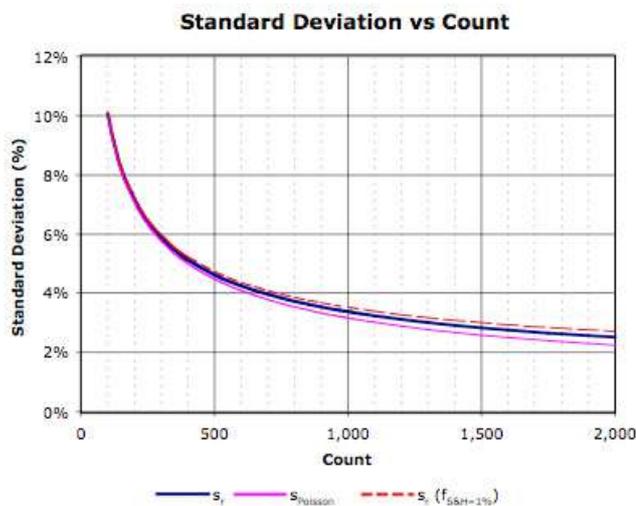
$$8 \quad s_{SCC-100}^2 \approx (n + f_{SCC-Cassette}^2 * n^2) + (f_{S\&H}^2 * n^2 + 2 * f_{Pipetting}^2 * n^2) \approx n + (0.000136 + f_{S\&H}^2) * n^2$$

The second approximation of Equation 8 refers to the approximation that  $f_{SCC-cassette} \approx 0.006$  (0.6%) and  $f_{pipetting} \approx 0.005$  (0.5%) but if the actual value for  $f_{pipetting}$  is known it should be entered into Equation 8 instead. Please note that the  $s_{SCC-100}$  in Equation 8 is expressed in somatic cell count per  $\mu\text{l}$  of milk and to get the corresponding standard deviation in somatic cells per ml of milk this value is multiplied by 1,000 $\mu\text{l}/\text{ml}$ .

From Equation 2 we have that the result of the NucleoCounter SCC-100 System in cells per  $\mu\text{l}$  is approximately equal to the number of counted cells. For calculation we can therefore replace  $n$  in Equations 4 through 8 with the results from the NucleoCounter SCC-100 divided by 1,000 $\mu\text{l}/\text{ml}$ .

## Coefficient of Variation

It is customary to express the repeatability error as coefficient of variation (CV) or the relative error. From Equation 8 it is apparent that the relative error is a function of the number of counted cells, but further that the error is not directly related to the number of counted cells. Figure 12 illustrates the behaviour of CV as a function of counted cells.



**Figure 12** Estimated repeatability error of NucleoCounter SCC-100,  $s_{Poisson}$  (solid thin),  $s_{SCC-100}$  without  $s_{S\&H}$  (solid thick) and  $s_{SCC-100}$  with  $s_{S\&H}$  equal 1% (dashed)

Figure 12 illustrates the expected behaviour of the relative  $s_{SCC-100}$  (CV) as a function of the number of cells counted (in the NucleoCounter SCC-100 System approximately equal to 1/1000 SCC). Firstly Figure 12 shows the effect of the random behaviour of counting (Poisson distribution), which is the dominating source of repeatability error. Secondly  $s_{SCC-100}$  calculated under the assumption that the cassette error amounts to 0.6% and pipetting error of both of the pipetting of reagent and milk is 0.5%, while error associated with the sample and sample handling is set to zero. Thirdly the expected repeatability error with sample and handling error of 1% is illustrated.

## Repeatability

Repeatability  $r$  of a method is often determined as the difference between single measurements of the same sample on the same instrument at the 95% significance level, in other words a difference between duplicate measurements exceed in no more than 5% of the tests. The Repeatability of the NucleoCounter SCC-100 System according to this definition can be determined on the basis of  $s_{SCC-100}$  as defined in Equations 4 through 8, as expressed in Equation 9.

$$9 \quad r = 2.83 * s_{SCC-100} = 2.83 * (s_r^2 + s_s^2)^{1/2}$$

## Accuracy

The accuracy of the NucleoCounter SCC-100 System is difficult to assess in the absence of a known 'true' value for the number of cells per volume of a milk sample, while the accuracy compared to a reference method can be determined. On the other hand since the reference method itself has significant reproducibility error (variations in results, when the same or identical milk samples are measured by several operators, using different equipment) such comparison can be difficult to perform as to give a global estimate of the accuracy of the NucleoCounter SCC-100 System.

## Reproducibility

When a number of NucleoCounter SCC-100 Systems, operated by different operators, measure the same or identical milk sample, there will certainly be observed differences between the observed results. These differences can either be caused by sources relating to the random behaviour of the individual systems, e.g. precision or repeatability, or sources relating to the fixed properties of the different NucleoCounter SCC-100 Systems, e.g. reproducibility.

When comparing two NucleoCounter SCC-100 systems reproducibility would be identified as consistent difference in the results obtained by the two systems. The most likely source of such differences would be differences in sensitivity or response and instrumental bias, but other sources such as differences in linearity or specificity could also apply to a method such as the NucleoCounter SCC-100 System.

## Sensitivity

The two potential sources of differences in sensitivity between NucleoCounter SCC-100 systems are detection and volume assessment. Variations in detection sensitivity can be caused by either the excitation system or the detection system. The excitation system consists of a number of light emitting diodes (LEDs) and an optical filter. Likewise the detection system consists of two glass lenses for the collection and focusing of the light, an optical filter and a charged coupled device (CCD) camera. All components are stable with respect to environmental factors, and they are also considered highly stable with respect to durability.

During production the overall sensitivity of each instrument is carefully adjusted using DNA-PI intercalation fluorescence. The effect of error in the sensitivity adjustment on the counting of cells has been determined through experiment to be less than  $\pm 0.5\%$  in range (expressed as standard deviation about 0.1%).

The long-term stability of the sensitivity of the NucleoCounter SCC-100 has not yet been determined since it requires a number of instruments to be used under normal conditions for a long period of time. It is therefore recommended that each NucleoCounter SCC-100 System should be carefully characterised against a working reference method in order to optimise the quality of the individual system as a secondary or in-house reference instrument.

One factor that can give rise to difference in response between two NucleoCounter SCC-100 Systems is the sample handling, and primarily the sample preparation, since the ratio of the volumes of reagent and milk will directly affect the results. The presence and magnitude of such effect should be carefully verified on regular basis, as described before, and its effect taken into consideration in reporting of any cell counting

results obtained through the use of a NucleoCounter SCC-100 System. If such careful verification and correction are carried out on a regular basis, this effect is excluded as a reproducibility factor.

## Analysed Volume

The assessment of the volume being analysed is firstly based on the compensation for the thickness of the measurement compartment of the SCC-Cassette and secondly on the compensation for the effective view area of the optical detection system. Any effect relating to the SCC-Cassette can be assumed to be related to the individual cassette and would therefore appear as a random contribution, affecting the repeatability of the system. The determination of the effective view area of each NucleoCounter SCC-100 instrument is done during production. The reliability of this determination, and thus the compensation is estimated to be better than about  $\pm 0.5\%$  (expressed as standard deviation about 0.1%).

## Reproducibility Error

The contribution of any effect having influence on the difference between any unspecified NucleoCounter SCC-100 Systems can be expressed as Reproducibility Error ( $s_R$ ) or the standard deviation of reproducibility. In short  $s_R$  describes the deviation in the comparison of a number of independent NucleoCounter SCC-100 Systems, which is not contributed to Repeatability Error ( $s_{SCC-100}$ ). Therefore the "total" error of the NucleoCounter SCC-100 System,  $s_{NucleoCounter}$  can be expressed by  $s_R$  and  $s_{SCC-100}$  as in Equation 10.

$$10 \quad s_{NucleoCounter}^2 = s_R^2 + s_{SCC-100}^2 = s_R^2 + s_r^2 + s_s^2$$

According to the assumptions above,  $s_R$  can be expected to be small compared to  $s_{SCC-100}$ . Thus  $s_{NucleoCounter}$  is largely dominated by  $s_{SCC-100}$ , which in turn is dominated by the random error of counting (Poisson distribution).

## Reproducibility

Reproducibility R of a method is often determined as the difference between single measurements of the same sample on two different instruments at the 95% significance level, in other words a difference between duplicate measurements exceed in no more than 5% of the tests. The Reproducibility of the NucleoCounter SCC-100 System according to this definition can be determined on the basis of  $s_{NucleoCounter}$  as defined in Equation 10, as expressed in Equation 11.

$$11 \quad R = 2.83 * s_{NucleoCounter} = 2.83 * (s_R^2 + s_r^2 + s_s^2)^{1/2}$$

## Applications

The NucleoCounter SCC-100 can be applied in the analysis of somatic cells in milk, whenever there is a need for objective and stable cell counting results. In particular the NucleoCounter SCC-100 is well suited as in-house or secondary reference method as a support for the manual microscopy method. While the microscopy method is the reference method, it is not well suited for daily measurements of a considerable number of samples. By first characterising a NucleoCounter SCC-100 System against a reference method it can be used in routine operation, to obtain fast, stable and reliable results.

In the following some of the possible specialised applications of the NucleoCounter SCC-100 System are discussed and some guidelines to the implementation of these applications are given.

## Comparison to a Reference Method

The reference method for the determination of the number of somatic cells per volume of milk is defined in IDF/ISO standard 148 "Enumeration of Somatic Cells". Generally the purpose of comparing a NucleoCounter SCC-100 System to a reference method is to determine the level of the results as obtained by the NucleoCounter SCC-100 System relative to the reference method in order to establish the NucleoCounter SCC-100 System as a secondary or in-house reference method.

The advantage of using the NucleoCounter SCC-100 System as a secondary reference method is that it is much more convenient to apply and therefore it is possible to arrive at an estimate of SCC for a given milk sample in a fast and reliable manner.

To establish a NucleoCounter SCC-100 System as a secondary or in-house reference method it is necessary to determine the correlation between a reference method according to IDF 148 and the results obtained by the NucleoCounter SCC-100 System in the sample range of interest. The following is the recommended procedure for such comparison.

## **Samples**

For the test use 8 fresh milk samples of good quality. 4 of these samples should have cell count of between 250,000 and 350,000 cells/ml and the remaining 4 samples should have cell count of between 500,000 and 700,000 cells/ml. Each sample is carefully mixed and divided up into two test samples of appropriate volumes. The 16 test samples should be numbered 1 through 16 in random order. The identity of each sample should not be disclosed to the operators of the cell counting methods.

## **Sampling**

Each test sample is prepared and sampled as described in IDF 148. The sampling volumes for both the reference method and the NucleoCounter SCC-100 method should be verified by weighing and any result obtained by either method should be corrected accordingly.

## **Measurements**

For each of the test samples two films are analysed by the reference method according to IDF 148, in total 32 results. If there is more than one operator for the reference method then each operator should perform a complete analysis of all the test samples. No operator should be aware of the results obtained by any other operator or by the NucleoCounter SCC-100 System.

A NucleoCounter lysate is prepared from each test sample, and from each of these two SCC-Cassettes are loaded and measured, resulting in a total of 32 NucleoCounter SCC-100 measurements. The results from the measurement must be recorded on the external printer and preferably also on an external computer using the software application SomaticView.

## **Results**

For each of the results obtained by the reference method both the number of cells counted as well as the estimated SCC for each sample should be reported. The estimated SCC of the NucleoCounter SCC-100 for the corresponding samples should be reported.

### ***Variance Analysis***

To determine the repeatability and sample error of the two methods an analysis of variance (ANOVA) is performed on the results of each method, separately for results from high and low samples respectively. The test is a two factor ANOVA with samples, test samples and repetitions. It is assumed that there is no significant interaction between samples and test samples thus the sources of variation to be estimated are the variation of the SCC of the samples, difference between duplicate test samples and difference between duplicated measurements.

### ***Regression Analysis***

The aim of the regression analysis is to determine the correlation between the two methods. This is done by performing a least square regression of the NucleoCounter SCC-100 results (y data) on the reference method (x data).

For each test sample calculate the average of the two measurements by the reference method and use this value as x-value for each of the NucleoCounter SCC-100 determination (y-values). Determine slope and intercept for the least squares regression as well as the estimated error in the determination of the two

parameters ( $s_{\text{slope}}$  and  $s_{\text{intercept}}$ ). Also determine the residual error independently for the high and the low samples.

## Routine Instruments

To compare a routine method to the NucleoCounter SCC-100 System it is possible firstly to use individual samples, measured over a period of time (see In-house Reference Material below) and secondly to use the procedure for the comparison to the reference method replacing the reference method with the NucleoCounter SCC-100 System (see Comparison to Reference Method above).

## In-house Reference Material

With the NucleoCounter SCC-100 System it is possible to establish objective somatic cell count of milk samples to be used as in-house reference material. Milk is well suited as in-house reference material, firstly since it truly is the very same analyte as is to be measured, including all sources of variation that can affect the results, and secondly since it is readily available in a milk analysing laboratory. The draw back of using milk as in-house reference material is that it has limited chemical and physical stability. Therefore a milk sample used for this purpose is generally altered chemically or physically in order to improve its stability, but this will inevitably introduce a risk of introducing systematic effect in the determination of somatic cell count of such samples, when measured under routine condition.

The NucleoCounter SCC-100 System makes it possible to determine the SCC of any milk sample, and thus transforming such sample instantly into an in-house reference material. The determination of SCC by the NucleoCounter SCC-100 is performed at the same time as the routine determination and without any alteration of the sample. Therefore it is possible to verify the performance of a routine method in a fast and reliable manner.

The method for establishing in-house reference material is largely dependent on the intended use of such material. One frequent purpose of in-house reference material is to measure a sample in connection with the measurement of routine samples in order to verify the short-time analytical performance of a routine instrument, e.g. pilot samples.

## Pilot Samples

The purpose of pilot samples is to prepare a number of identical samples, which can be measured on different instruments and/or at different times in order to monitor routine measurements. Without reliable SCC, pilot samples can reflect the relative response of a routine instrument, but it is necessary to have a reliable and objective determination of SCC in order to be able to also verify the absolute response of a routine instrument. Also under condition where the SCC of a sample is not stable for the duration of the test, such as can be the case when using samples to monitor routine methods over several days, the NucleoCounter SCC-100 System allows the SCC of a sample to be determined at the exact time of analysis.

To use the NucleoCounter SCC-100 System to establish a reliable SCC for a pilot sample, first prepare the sample in the same manner as it will be prepared for the routine measurement, e.g. heating and mixing. Then prepare two test samples for the NucleoCounter SCC-100 from the sample. Finally perform at least two NucleoCounter SCC-100 measurements on each of the two test samples, where the total number of measurements is determined by the requirements to the precision of the determination, e.g. according to Equation 8, where the number of counted cells  $n$  is approximately equal to the number of NucleoCounter SCC-100 measurements times the cell concentration in 1/1,000 of cells/ml.

Determine the variation of SCC within and between each of the test samples in order to verify the sampling and sample preparation method.

Use the value of the pilot sample, as obtained by the NucleoCounter SCC-100 System to monitor the routine method, giving due consideration to the significance level of each determination of both the routine method and the NucleoCounter SCC-100 System, when using the results to adjust the response of a routine method.