Application note No. 0021

Determination of SCC in Milk using the NucleoCounter® SCC-400™ System

Introduction

This Application Note is intended to give an overview of the NucleoCounter SCC-400 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-400. Finally some specialised applications of the NucleoCounter SCC-400 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 problems 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 microorganisms, 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-400

The NucleoCounter SCC-400 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-400 allows the determination of somatic cell count with sample throughput as high as 200 samples per hour and the NucleoCounter SCC-400 does this without compromising the quality of the measurement.

The inherent properties of the method of the NucleoCounter SCC-400 technology assures high reproducibility among NulceoCounter instruments, due to high degree of uniformity among different

instruments. Thus as a unique feature the NucleoCounter SCC-400 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. This property is evident in the fact that the NucleoCounter SCC-400 instrument does not need to be adjusted due to alteration in its characteristics, producing equivalent results throughout its lifetime.

Further the NucleoCounter SCC-400 analysis large volumes of each milk sample, thus producing results with low reproducibility error. Good reproducibility means that the SCC-400 offers high stability when determining somatic cell count of a single sample measured in a short period of time.

Principle of Operation

Determination of SCC in milk with the NucleoCounter SCC-400 System is based on the acquisition of a number of images of fluorescence signals originating from stained double stranded DNA, followed by the identification and counting of individual somatic cells in a computer. The sample is prepared by mixing it with a reagent and the sample is introduced into the measurement chamber of the instrument by a simple flow system. The results are presented in a list in the software application, which also generates an electronic report from the results.

Reagent C400

One property of somatic cells is that a living cell will resist to the introduction of foreign substances, such as nuclei dye. 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-400 System includes a reagent solution, Reagent C400, which among other components contains an efficient lysis agent, Triton X-100. Prior to analysis the sample and Reagent C400 are mixed in precise volumes of one part sample and two parts reagent and in that process, which only takes few seconds, the cells are lysed, rendering immediate access for the nuclei dye to the DNA. The purpose of the reagent is two-fold, firstly to disrupt the membranes of the somatic cells, exposing its nucleolus to staining, and secondly it contains the nuclei stain used to identify cell.

Instrument

The NucleoCounter SCC-400 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 image. To enhance the repeatability of the determination the results are based on a number of images, actually up to 12 images of each sample.

The imaging system of the NucleoCounter SCC-400 offers several advantages in the identification and counting of somatic cells in a milk sample. Apart from the large view it allows an extremely simple and ridged construction with only few movable parts, which is the motor that drives the pump and a single valve. 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 (CMOS image sensor).

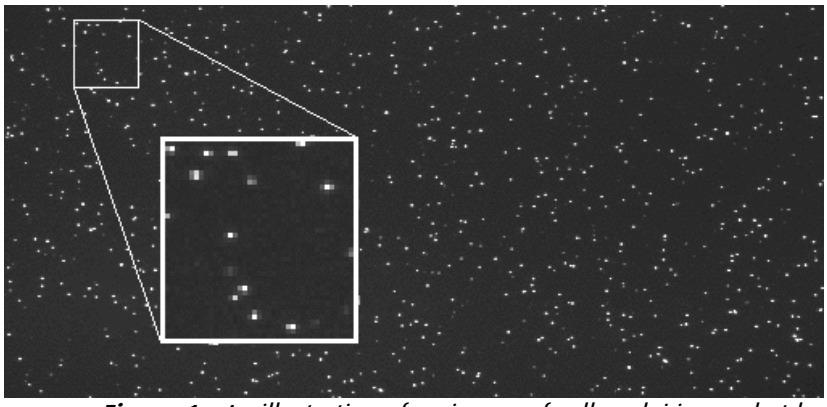

Figure 1 *An illustration of an image of cell nuclei imaged at low magnification*

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 1. The low resolution erases virtually any morphological information of 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 camera (pixels).

When identifying somatic cells in a sample of milk in the NucleoCounter SCC-400, 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 the stained DNA of the nucleus. 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-400.

Figure 1 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.

Flow System

The flow system of the NucleoCounter SCC-400 has the purpose of transporting a portion of the sample mixture to the sample compartment where it is held while the image is taken. The flow system consists of an intake pipette, the flow compartment, e.g. the cuvette, a valve, a pump and outlet to waste. The schematics of the flow system are shown in Figure 2.

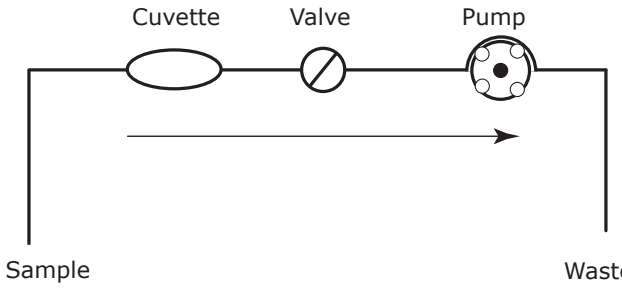

Figure 2 *A schematic illustration of the flow system of the SCC-400*

External Computer

The NucleoCounter SCC-400 is operated by an external computer. This computer controls the valve and pump of the flow system, collects images for cell count analysis and displays and stores the results. For detailed description of the NucleoCounter SCC-400 software please refer to documentation supplied with the system.

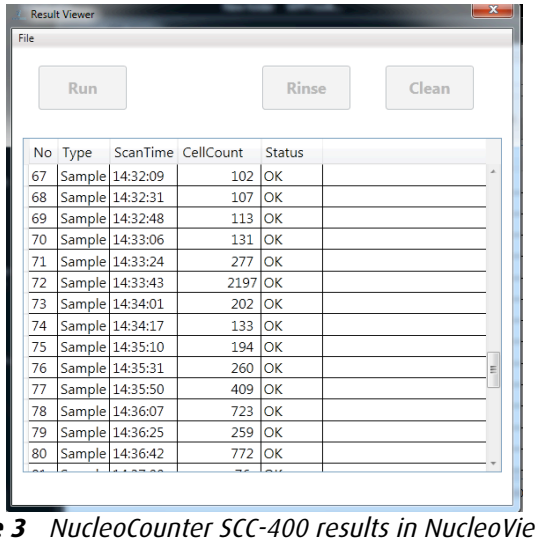

Figure 3 *NucleoCounter SCC-400 results in NucleoView SCC-400*

Materials and Methods

The NucleoCounter SCC-400 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-400 System must be a natural milk sample of good quality. Samples must be homogeneous and free of particles. The NucleoCounter SCC-400 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-400.

Further the NucleoCounter SCC-400 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-400 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-400, but it should reduce any variations in the estimated SCC that are caused by variations in milk handling.

Reagent C400

The lysis buffer Reagent C400 has three main properties, firstly to establish chemical conditions which optimise the lysing of the cells, such as pH and ion strength, secondly to introduce the lysing agent to the sample and thirdly it contains the nuclei dye needed to stain the lysed cells. In Reagent C400 the lysing agent is the detergent Triton X-100.

Reagent C400 is supplied in 500 mL or 2 L containers. 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 C400 and milk sample the sample is ready to be loaded into the SCC-400 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 C400 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. It is recommended to use Eppendorf tubes, 1.5 mL Safe-Lock micro test tubes, shown in Figure 4, supplied by Eppendorf AG, generally available through suppliers of laboratory equipment.

Figure 4 *Eppendorf 1.5 mL Safe-Lock tube*

Volume Dispenser

Both accuracy and precision of results of the NucleoCounter SCC-400 System depend on the preparation of the sample reagent solution. Variation in volume dispensing of either reagent or milk 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 controlled volumes of reagent and milk. 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 5.

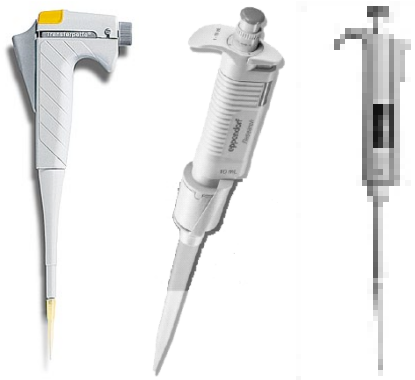

Figure 5 *Examples of manual pipettes supplied by Brand GmbH, Eppendorf AG and Thermo Electron Corporation*

NucleoCounter SCC-400 Instrument

The detection of fluorescent signal, signal processing and result presentation takes place in the NucleoCounter SCC-400 instrument. For the operation of the NucleoCounter SCC-400 instrument please refer to the "NucleoCounter SCC-400 User's Guide". The NucleoCounter SCC-400 instrument is supplied by ChemoMetec A/S.

NucleoView SCC-400

NucleoView SCC-400 is a dedicated software application for the operation of the NucleoCounter SCC-400. For further description of NucleoView SCC-400 please refer to " NucleoCounter SCC-400 User's Guide ", NucleoView SCC-400 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-400. 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 important that the volumes of milk and reagent are accurate and precise.

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 6). 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 6), marking the position at which the desired volume has been dispensed. Pressing the piston as far as it goes (blow out in Figure 6) can be used to empty the tip of liquid.

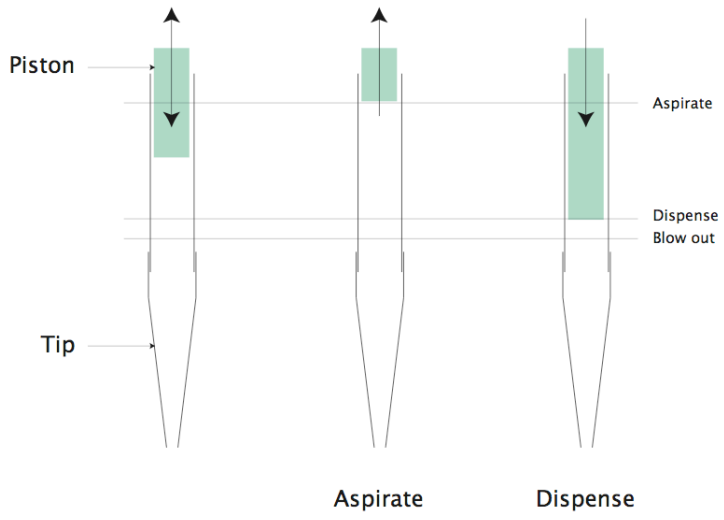

Figure 6 *Illustration of the operation of a pipette*

The following recommended procedure for the pipetting of reagent and milk is based on the use of a manual pipette. 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 pipettee 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-400 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, both Reagent C400 and the milk being kept at room temperature. 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 Mean: $\bar{x} = \frac{\sum x_i}{n}$ Standard deviation: $s = \sqrt{\frac{\sum \left(x_i - \bar{x}\right)^2}{n-1}}$

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 C400 and milk. In addition the mean for the volume of Reagent C400 must be twice the mean for the volume of milk, e.g. the reagent - sample ration is 2 to 1. 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 C400 at 20°C has been determined to 1031 kg/m^3 ± 0.5 kg/m^3, 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^3 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^3 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^3 at 20°C.

Mixing of Milk and Reagent C400

Upon the addition of Reagent C400 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 C400 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). In order to carry out such test it is necessary to prepare large volumes of sample lysate for this test.

Precision Mode

NucleoCounter SCC-400 can be operated in two precision modes. In *Normal Precision Mode* (NPM) about 18µL of reagent-sample mixture are analysed and in *High Precision Mode* (HPM) about 36µL are analysed when the SCC of a sample is approximately below 550,000 at SCC above that same volume as in NPM is analysed. This corresponds to working factor of 170 and 85 respectively. The impact on precision is that in HPM the precision is approximately 40% better than in NPM. For the setting of precision mode please refer to NucleoCounter SCC-400 User's Guide for instructions.

Results

The results of the NucleoCounter SCC-400, when estimating the number of somatic cells per volume of milk (SCC) are based on the counting of identified cells in a volume of about 3µL of reagent - milk solution in each image, amounting to about 18µL or 36µL depending on the selected precision mode. Since the reagent and milk are mixed in the volume ratio 2:1 then it is assumed that milk constitutes 33.33% 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 images, a is the view area, t is the sample thickness, m is a factor depended on the precision mode (6 or 12) and f_{Milk} is the fraction of milk in the lysate solution (e.g. 0.3333):

2 $$Cells/\mu L_{Milk} = \frac{n}{v_{\mu L} * \left(f_{Milk}\right)} = \frac{n}{(a*t*m)_{\mu L} * \left(f_{Milk}\right)} \approx \frac{n}{18_{\mu L} * 0.3333} = {}^{n}\!/_{\mu L}$$

In the evaluation of the results, the exact volume being analysed, (a*t*m) 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 and the thickness of the sample t, determined during the production of each sample compartment.

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 cannot resolve signal from two cells, located close to each other, 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 polynomial. The observed cell count is therefore corrected for deviation from linearity in order to present correct SCC.

Precision

The precision of the NucleoCounter SCC-400 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-400 instrument, 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 under same conditions. According to the Poisson distribution, the expected value is equal to n (mean $\mathbf{x} = n$) and the expected standard deviation of repeated experiments is the square root of n (standard deviation $\mathbf{s} = \sqrt{n}$). 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 **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 then **x**=400 cells and **s**=√400=20 cells (e.g. **s** amounts to 5% of **x**). In the following the properties of the normal distribution will be used on several occasion to estimate the properties of the NucleoCounter SCC-400 System with respect to repeatability and reproducibility error.

Coefficient of Variation

Since the relationship between **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 2,400 cells are counted in *Normal Precision Mode* (18µL of sample mixture), it correspond to SCC of 400,000 cells/mL, but the square root of 400,000 is equal to 632 (or 0.2%) while the standard deviation **s** is equal to 49, **n** is equal to 2,400 and thus the relative error, CV% is 49/2,400*100% = 2.04% or 8,160 cells/mL. The ratio between **s** and **x** is of course 2.04% regardless of which units the results are presented in.

The correlation between **x** and **s** implies that **s** increases with increasing **x**, but since **s** is the square root of **x**, then the ratio of **s** to **x** decreases with increasing **x** as illustrated in Figure 7. The figure illustrates that when 2,000 cells are counted (SCC 166,000/mL) the relative standard deviation is about 2.2% and when 6,000 (SCC 500,000/mL) cells are counted it is about 1.3%.

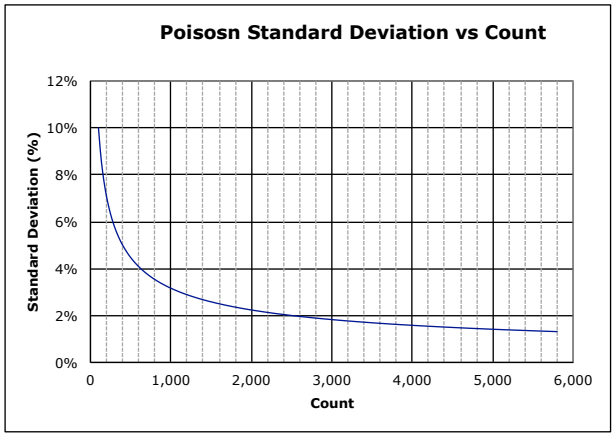

Figure 7 *Relative standard deviation of the Poisson distribution versus the number of counted objects in the range 100 to 6,000 objects*

NucleoCounter SCC-400 Instrument

Significant factors relating to the NucleoCounter SCC-400 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. Thus it can be concluded that the contribution of the NucleoCounter SCC-400 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.

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-400 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 sample-reagent 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-400 System can be compromised in quality.

Repeatability Error

When the samples are prepared by carefully following the procedure described previously the effect of counting random events, is by far the dominant source of precision error. In the following it will therefore be used to describe the precision of the NucleoCounter SCC-400 system. We therefore have the approximation of the repeatability error, $S_{SCC\text{-}400}$, in the following equation:

3 $$s_{SCC-400} \approx s_{Poisson} = \sqrt{n} \quad ; \quad n : Counted\ Cells$$

Coefficient of Variation

It is customary to express the repeatability error as coefficient of variation (*CV*) or the relative error, defined as the ratio between standard deviation and expected value. From Equation 3 we have that the standard error is correlated to the square root of counted cells, and as the number of counted cells is directly correlated to the cell concentration we can write the *CV* as in Equation 4.

4 $$CV_{SCC-400} \approx s_{Poisson} / n = \sqrt{n} / n = 1 / \sqrt{n} \quad ; \quad CV_{\%} = 100 * CV_{SCC-400}$$

Figure 8 illustrates the behaviour of CV as a function of counted cells.

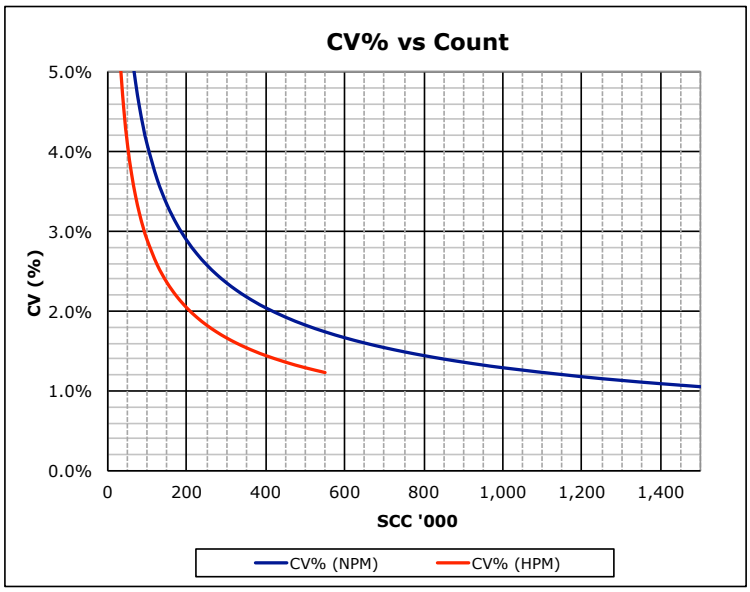

Figure 8 *Estimated Coefficient of Variation of NucleoCounter SCC-400,* ***showing results obtained with instrument in NPM (blue) and HPM (red)***

Figure 8 illustrates the expected relative $s_{SCC\text{-}400}$ (CV%) as a function of the cell concentration on the NucleoCounter SCC-400 instrument. Firstly Figure 8 shows the CV% decreases as the SCC increases.

Secondly it shows the effect of measuring samples in Normal Precision Mode (NPM) and High Precision Mode (HPM), where the CV% when in HPM (red line in Figure 8) is reduced by approximately 40% in the SCC range up to about 550,000 above that range repeatability in NPM and HPM is uationual as equal volume of milk is analysed.

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-400 System according to this definition can be determined on the basis of $\mathbf{CV\%}_{\text{SCC-400}}$ as expressed in Equation 5.

5 $$r = 2.83 * CV\%_{SCC-400}$$

Accuracy

The accuracy of the NucleoCounter SCC-400 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-400 System.

Reproducibility

When a number of NucleoCounter SCC-400 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-400 Systems, e.g. reproducibility.

When comparing two NucleoCounter SCC-400 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-400 System.

Sensitivity

The two potential sources of differences in sensitivity between NucleoCounter SCC-400 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 light emitting diode (LED) and an optical filter. Likewise the detection system consists of glass lenses for the collection and focusing of the light, an optical filter and an image sensor (CMOS). 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 fluorescence detection. The effect of error in the sensitivity adjustment on the counting of cells has been determined through experiment to be less than ±0.5% in range (expressed as standard deviation about 0.1%).

The long-term stability of the sensitivity of the NucleoCounter SCC-400 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-400 System should be carefully characterised against a working reference method in order to optimise the quality of the individual system.

One factor that can give rise to difference in response between two NucleoCounter SCC-400 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-400 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 SCC-400measurement compartment and secondly on the compensation for the effective view area of the optical detection system. The thickness of each sample compartment is determined during production. The determination of the effective view area of each NucleoCounter SCC-400 instrument is done during production. The reliability of this determination, and thus the compensation is estimated to be better than about ±0.5% (expressed as standard deviation about 0.1%).