A Cell Counting Process and Its Quality Assessment According to ISO 20391

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The cells used in regenerative medicine and cell therapy must be of stable quality. To understand their status, the number of cells is counted during the cell production process. However, it was difficult to evaluate the quality of the measurement process because a standard counting method had not been determined. Therefore, the ISO 20391-2 international standard was developed. This standard describes the dilution fraction experimental design and statistical methods in cell counting and shows how to evaluate the cell counting process using quality indicators.

Yokogawa's CQ1 Confocal Quantitative Image Cytometer can acquire fluorescence and bright-field images and count the number of cells in the images. We counted cell numbers in each dilution fraction of TF-1 suspension cells by using CQ1 and assessed the quality of the process in accordance with ISO 20391-2. We also compared the performance of CQ1 with that of devices from other companies and found that Yokogawa's CQ1 delivered far more precise counting. This paper describes these results and Yokogawa's standardization efforts in the biotechnology field.

INTRODUCTION

Animal and human cells have been used for researches such as unraveling cell structures and functions and evaluating the medicinal effects of drugs. Today, cell products are manufactured for medical purposes such as regenerative medicine.

When manufacturing such cell products, stable quality is critical, and therefore control items are set for various quality factors that are evaluated during culture processes and on completion of production. The number of cells (cell concentration in a culture medium) is the most important among such control items. The number of cells is generally measured by sampling a part of a culture medium and counting the cells in it. Specifically, the measurement consists of the following steps: (1) sampling from a culture medium, (2) preparing a diluted sample, (3) injecting the diluted sample into a hemocytometer (an apparatus of the same size as a slide glass used for counting the number of cells), and (4) counting the number of cells (Figure 1). The procedure of preparing a diluted sample involves a weighing error, which largely depends on the expertise of operators and on the facility equipment. The number of cells is counted by an automated cell counter or visually by an operator under a microscope. In either case, the counted number includes measurement errors. In addition, the detailed procedure for cell counting depends on the facility or operator. Therefore, it is necessary to define the precision of cell counting but there is no standard method of cell counting or procedure for error assessment. This has been a challenge in process control and quality assessment in cell manufacturing[1].

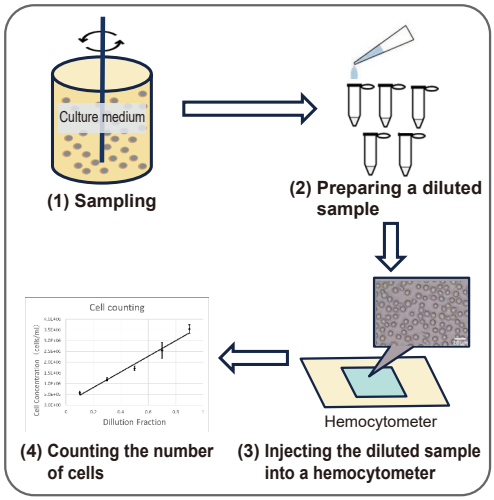

Figure 1 Major processes of cell counting

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YOKOGAWA'S EFFORTS FOR INTERNATIONAL STANDARDIZATION ON CELLS

In 2013, the International Organization for Standardization (ISO) established the TC276 Committee for international standardization in the biotechnology field[2]. The Forum for Innovative Regenerative Medicine (FIRM) is an industry group launched in 2011 which is working to achieve regenerative medicine. FIRM is also a deliberative council of TC276 in Japan.

Yokogawa has been a member of FIRM since 2013, and participates in activities for standardization in the biotechnology field. Yokogawa has also been engaged in preparing the ISO 20391-2 International Standard on cell counting, as a member of the ISO/TC276 deliberative council.

Outline of ISO 20391-2

The ISO issued ISO 20391-2 "Experimental design and statistical analysis to quantify counting method performance" in 2019 after reviewing international standards on cell counting[3]. This has made it possible to assess the measurement methods for calculating the number of cells. ISO 20391-2 sets a proportionality index (PI) for visualizing the quality of cell counting processes. Multiple procedures are indicated for calculating PI, allowing cell manufacturers to choose an appropriate procedure according to their purpose and situation. The quality of cell counting processes can be compared mutually if two processes share the same cell species, experiment design, and procedure of calculating PI.

ASSESSMENT OF CELL COUNTING PROCESSES USING CQ1

The quality of cell counting processes of TF-1 floating cells (human leukemia cell lines) was assessed in this study following the procedure shown in ISO 20391-2. TF-1 cells are used for evaluating the activity of medicines and cell growth factors. Yokogawa's CQ1 Confocal Quantitative Image Cytometer was used to acquire images of hemocytometers with diluted samples injected, and the number of cells in the image was counted automatically.

CQ1 Confocal Quantitative Image Cytometer

The CQ1 Confocal Quantitative Image Cytometer is an integrated microscope with a built-in confocal scanner unit, and enables time lapse analyses of live cells and 3D fluorescence imaging of cell aggregations, in addition to acquiring bright field images and phase contrast images. It also enables high-throughput screening using microplate stackers and construction of a system connected to an external incubator for long time lapse analyses. Furthermore, various quantitative analyses are possible by combining CQ1 with machine learning and deep learning using the CellPathfinder analysis software and label-free analyses (Figure 2). Multi-well plates, as well as slide glasses, are accepted as measurement objects. Thus, CQ1 is highly versatile equipment for cell evaluations in general. In this study, CQ1 was used to acquire unstained images of hemocytometers with diluted samples and to quantify the number of cells in the images.

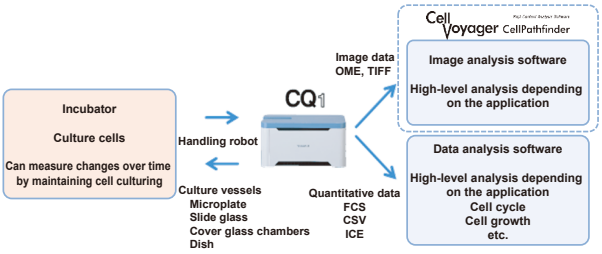

Figure 2 Measurement objects and expandability of CQ1

Preparation of Diluted Samples and Evaluation of Pipetting Errors

Five types of samples were prepared by diluting a cultured medium of TF-1 with phosphate-buffered saline (PBS). Table 1 shows the amounts of culture medium and PBS in each sample. All samples were prepared with the same volume. Repeating this procedure, three sets of samples were prepared for each dilution rate (Figure 3). In this study, two types of TF-1 culture fluid were used for assessing the cell counting process at different cell concentrations. The samples with the culture medium of higher cell concentration are called stock H, and the other samples are called stock L.

Table 1 Preparation of diluted samples

Diluted samples	1	2	3	4	5
Dilution rate	0.9	0.7	0.5	0.3	0.1
TF-1 culture fluid (µl)	90	70	50	30	10
PBS (µl)	10	30	50	70	90
Total (µl)	100	100	100	100	100

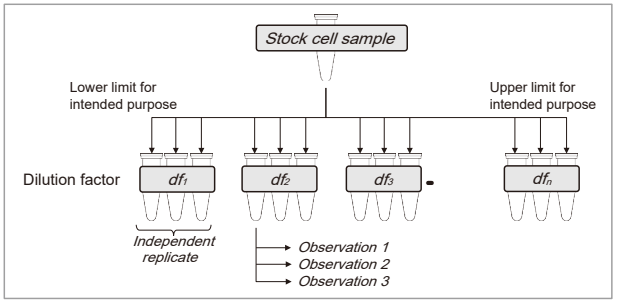

Figure 3 Image of preparing diluted samples

The weight of each sample prepared above was measured and the pipetting error was evaluated following the procedure of ISO 20391-2. While the determination coefficient for quality judgment of weighing errors was set to be 0.980 or greater, both samples of stock H and stock L satisfied this criterion (0.998). Thus, the assessment moved on to the next step.

Measurement of the Number of Cells

The diluted samples were injected into hemocytometers (C10228, ThermoFisher) and cell images were acquired using CQ1. Three images were acquired from three different fields of view for a diluted sample, to make three measurements for a sample. The acquired cell images were binarized and the number of oval bright spots was counted as the number of cells (Figure 4). Then, the cell concentrations (cells/ml) in the diluted samples were calculated based on the volume obtained from the area of the field of view and the depth of the hemocytometer (100 µm).

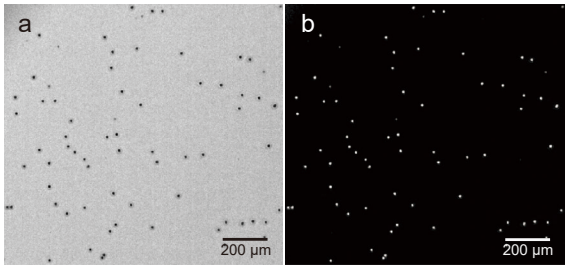

Figure 4 Example of unstained cell image used for measurement
(a: Image acquired by CQ1, b: Image after binarization)

Assessment of Cell Counting Process

(a) Calculation of coefficient of determination (R^2)

From the relation between the calculated cell concentrations and dilution rates, a regression line was obtained and the coefficient of determination was calculated (Figure 5 and Figure 6). The R^2 value was 0.979 when stock H was used, and 0.975 when stock L was used (Table 3). A high correlation was shown between the dilution rate and the measured cell count.

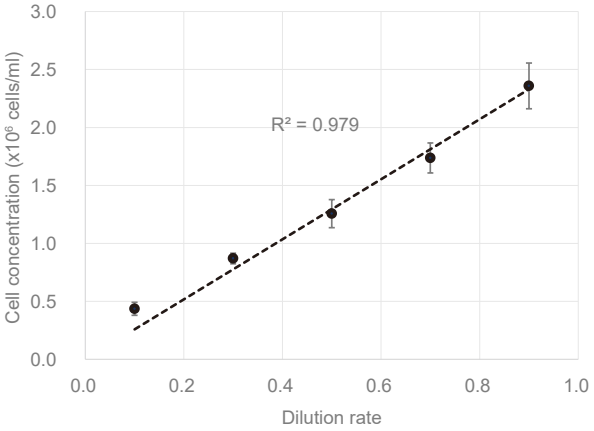

Figure 5 Relation between dilution rate and cell concentration for the diluted samples prepared from stock H: Measurement with CQ1

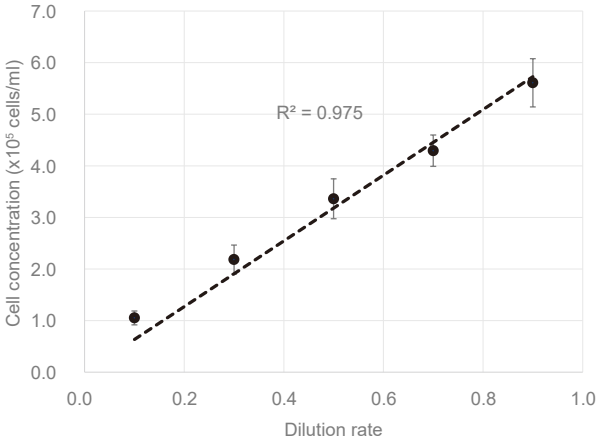

Figure 6 Relation between dilution rate and cell concentration for the diluted samples prepared from stock L: Measurement with CQ1

(b) Proportionality Index (PI)

PI is the degree of deviation of measured values from the regression line, and shows the quality of the cell counting process. ISO 20391-2 describes multiple formulas for calculating PI. In this study, Equation (1) was used to express the magnitude of differences of each data average from the regression line. Table 2 explains the abbreviations used in the equation. With the formula used in this study, a smaller value of PI means less deviation of measured value for a diluted sample from the regression line, or higher quality of measurement process. The result of cell counting in this study shows nearly the same values of PI for the samples from stock H and from stock L (Table 3).

$$PI = \sum_i \left| \frac{e_i^{\text{smoothed}}}{\lambda_{df_i}^{\text{proportional}}} \right| \quad \cdots (1)$$

Table 2 Abbreviations and symbols used in PI calculation

Abbreviated term or Symbol	Description *
PI	Proportionality index
i	Index for target dilution fraction
β_1	Scalar coefficient estimated from the proportional model fitting
df_i	Targeted dilution fraction
R^2	Coefficient of determination
$e_i^{smoothed}$	Smoothed residual when target dilution fraction is used in the analysis of proportionality
$\lambda_{df_i}^{proportional}$	Estimated cell count at df_i using β_1 obtained from proportional model fit $\lambda_i^{proportional}$

* Compiled based on ISO 20391-2 Section 3.2

Table 3 R^2 and PI in cell counting using CQ1

Sample	Stock H (high concentration)	Stock L (low concentration)
R^2	0.979	0.975
PI	11.0	11.2

Assessment of Cell Counting Using Devices from Other Companies

Cell counting was also carried out using automatic cell counters from other companies (devices from other companies). These devices count the number of cells based on the images of hemocytometers and calculate the cell concentration automatically. The diluted samples used were the same as those used for the measurements with CQ1. Measurement was repeated three times for each diluted sample, and then R^2 and PI were calculated. Equation (1) was used for calculating PI. Figure 7 shows the measurement result of the diluted samples prepared from stock L (the result of the diluted samples prepared from stock H is not shown). R^2 and PI are shown in Table 4.

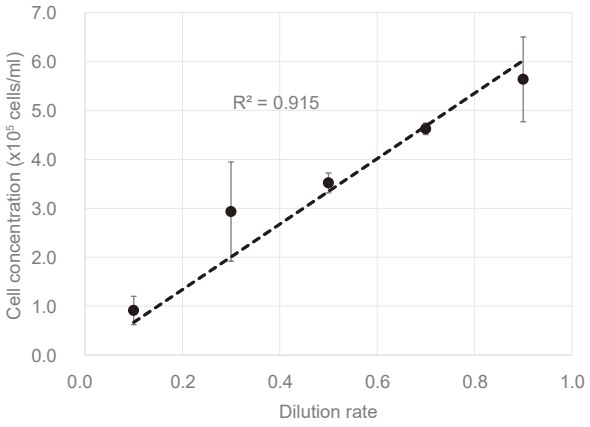

Figure 7 Relation between dilution rate and cell concentration of diluted samples prepared from stock L: Measured with devices from other companies

Table 4 R^2 and PI in cell counting using the devices from other companies

Sample	Stock H (high concentration)	Stock L (low concentration)
R^2	0.980	0.915
PI	11.2	14.1

DISCUSSION

Assessment of Cell Counting Using Stocks of Various Cell Concentrations

The PI values obtained by using CQ1 for the cell counting process were nearly the same for the two cases of stock H and stock L, while the numbers of cells in the diluted samples from stock H and stock L were about 10^6 and 10^5, respectively. The weighing errors estimated from weight measurements of the samples were equally small for stock H and stock L. Thus, CQ1 enables precise measurements even for samples of relatively low concentration with the number of cells of the order of 10^5.

Comparison of Cell Counting by CQ1 and Devices from Other Companies

The cell counting process was assessed using CQ1 and devices from other companies, with the same cell species, experiment design, and formula to calculate PI. Thus, it was possible to compare precision between the two measurements. The PI values from both measurement processes were nearly the same when the samples from stock H were used. However, when samples from stock L were used, the value of PI measured with CQ1 was lower than that measured with the devices from other companies (Table 3 and Table 4). The weighing error was the same for the two cases, because the diluted samples used for measurement were the same. Thus, the difference in the PI value was considered to represent the difference in measurement errors of each device. When devices from other companies were used, large dispersion of data was seen at several dilution rates, and the average of measured values deviated from the regression line by about 30% in the worst case (Figure 7). These results show that the selection of measurement devices impacts the quality of the cell counting process.

CONCLUSION

In this paper, the cell counting process was assessed in accordance with ISO20391-2, and CQ1 was shown to be capable of measuring the number of cells with equivalent or better precision than the devices from other companies. In constructing a cell counting process for cell manufacturing, it is essential to select measurement devices taking the cell quality and measurement time permissible for the process into account and assessing the quality of the cell counting method based on the indices including PI indicated in ISO20391-2.

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