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Abstract. Cell counting is a fundamental procedure in living cell culture-based experiments and protocols in which the cell number quantification is required. The number of cells is one of the parameters necessary to investigate several cell culture features requiring to be monitored as function of time, such as cell viability, proliferation, growth, fitness and metabolism. Aim of this paper is contributing to declare a comprehensive uncertainty budget for cell counting through metabolic assays according to the EURACHEM/CITAC Guide *Quantifying Uncertainty in Analytical Measurement*.

1. Introduction

The aim of this paper is to contribute to declare a comprehensive uncertainty budget for cell counting by metabolic assays according to the EURACHEM/CITAC Guide *Quantifying Uncertainty in Analytical Measurement* [1].

The lack of traceability of diagnostic and clinical measurement justifies the consideration on the quantification of cells number as a measurement service requested by National Metrology Institutes (NMIs). NMIs raise the issue of quantification of cell population in both bi-dimensional (2D) cell cultures and three-dimensional (3D) cells systems mimicking real tissues [2].

Cell counting is a fundamental procedure in living cell culture-based experiments and protocols in which the cell number quantification is required. The cell number is one of the parameters necessary to investigate several cell culture features such as cell viability, proliferation, growth, fitness and metabolism which require a monitoring as function of time [3]. Cell counting is the quantification of the quantity of cells/organisms in a bioreactor which is any limited volume where a cell culture evolves. Cell number can be determined by removing cells from the bioreactor and counting them with either a hemocytometer or an electronic cell counter, or by cell image analysis, or by assaying a metabolic activity of the cells within the bioreactor (e.g. intracellular enzymes activity) [4], when the activity is proportional to the number of cells explicating that activity.

Metabolic assays measure a metabolic activity accounting for vital cells only and quantify the number of cells under the hypothesis that all the active cells show a metabolic activity and that the mean of these cell activities is stable. There are at present some metabolic reaction-based methods for monitoring animal cell number (giving information on cell proliferation) over time [5, 6]. However, most of them requires the use of substances that are cytotoxic [5], or results in cell lysis to measure the metabolic product [5, 6, 7]. The method chosen to be analyzed in this work is a metabolic method based on the reduction of resazurin into the end product resorufin. It is not cytotoxic and do not require any cell damaging steps [8]. The cell counting methods based on metabolic activities are

indirect methods and require a high reproducibility in experiments to calibrate the method and in experiments with samples. The identification of standard measurement units for fluorescence intensities and absorbance are certainly a significant prerogative for methods unification in order to increase their traceability, regardless of the adopted measurement instrument.

This paper presents a brief description of the measurement method, states a realistic model equation and evaluates the uncertainty of a CellTiter-blue® proliferation assay for use to evaluate the cell number and estimate the cell proliferation in 2D cell culture.

2. Materials and Measurement method

2.1. *Cell seeding and cell culture medium*

The human mesenchymal stem cells (hMSCs) were purchased from Lonza Group Ltd (Basel, Switzerland). They are bone marrow derived-hMSCs from donor. hMSCs were expanded and maintained in non-differentiating cell growth αMEM (alpha modified Minimum Essential Medium - Lonza, Wokingham, UK) with FBS 10% v/v (Fetal Bovine Serum, Lonza, Wokingham, UK), L-glutammine 2 mM, penicillin 100 U/ml and streptomycin 100 g/ml (Lonza Wokingham, UK). Cells were grown in monolayers in Petri dish (plastic vessels) for cell culture and fresh medium was replaced every 3-4 days until cells reached about the 80% of confluence(area occupied by cells/total area). Cells were then washed once with 1X Phosphate Buffer Saline (PBS), detached with 0.25% Trypsin – 0.53 mM Ethylenediaminetetraacetic acid (EDTA) solution, counted by means of a hemocytometer and suspended at several concentrations (cells/ml) in 100 µl non-differentiating αMEM growth medium to be seeded and cultured statically in 96 well microplates. Then, cells are placed in an incubator at 37°C with 5% CO_2 over night to let them to adhere to the bottom of the wells. Cells were managed in laminar flow hood (class II) under sterile conditions.

2.2. *CellTiter-Blue® proliferation assay*

The CellTiter-Blue (CTB) proliferation assay was purchased from Promega Corporation. It is a fluorimetric and metabolic assay used for determining the number of living cells in a biological *in vitro* system. It is based on a redox reaction: the reduction of resazurin (dark blue in color, redox dye with a slight intrinsic fluorescence) into the end product resorufin (pink, highly fluorescent molecule, $\lambda_{ex/em}$ 579/584 nm), made by redox enzymes. Only living cells can perform this reaction because nonviable cells rapidly lose metabolic activity and do not generate any fluorescent signal [8]. O'Brien et al. in 2000 proposed the resazurin dye, the original name of the Alamar Blue dye, to assess the mammalian 2D cell cultures cytotoxicity [9]. CellTiter-Blue reagent is a buffered solution containing highly purified resazurin (substrate of the reaction). The resaruzin molecule can penetrate cells by passing the cell membrane and into the cytoplasm is reduced by cytosolic, microsomal and mitochondrial redox enzymes producing the fluorescent resorufin (reaction product). Resorufin diffuses out of cells back to the culture medium which alone does not reduce resazurin [10]. After cell adhesion, resazurin is added as 10% v/v to the cell culture growth medium in the well containing the cells (adherent on the bottom of the well). Fluorescence intensity of the resorufin was measured after a contact time (0.5 to 6 hours) of resazurin with cells.

Important to note that in the specific case the entire liquid phase (cell culture growth medium containing resazurin) volume was used to measure fluorescence intensity, i.e. 100 µl. However, cell culture can be establish in a different vessel (such as a Petri dish) where the liquid phase volume is much higher. In that case, the volume of resorufin used for fluorescence intensity measurement (typically 100 µl) is taken from the liquid phase (previously mixed to be homogeneous) and placed in a well of a multiplate (typically a 96 well plate) for the measurement. Two volumes are therefore distinguished: the total liquid phase volume (V_{LP}) in which the reaction occurs and the volume of the aliquot of liquid phase placed in the well (V_w) in which the measurement is made. In the following measurand definition, both volumes V_{LP} and V_w will be taken into account, even if in the specific measurements they are the same entity.

2.3. *Fluorescence measurement system*
The GloMax®-Multi Microplate Multimode Reader (Promega Corp.) was used to measure fluorescence intensity. Excitation was performed at 525 nm wavelength while emission was measured in the range 580-640 nm by selecting a pre-set program for CTB. Microplates with 96 wells were used.

3. Measurand definition
The cell culture, of which the number of cells (N) is measured, is in a certain volume of liquid phase (made by growth medium in which the resazurin is diluted) in cell culture vessel, such as a well of a microplate.

The number of cells N in the cell culture liquid phase volume is proportional to the molar concentration of the reaction product (resorufin) in the liquid phase $C_{Prod,t}$ available in the well at contact time τ and to the volume of the liquid phase in the cell culture vessel V_{LF}.

In the following equation, K_1 defines the specific metabolic activity of the cell and depends by the contact time (τ) between resazurin and cells

$$N = K_1(\tau) \cdot C_{Prod,t} \cdot V_{LF} \quad (1)$$

The product concentration at the contact time τ in the liquid phase is proportional to the fluorescence intensity emitted by the molecules of product $I_{f,Prod,t}$. K_2 is influenced by the liquid phase volume in the measurement well (V_W).

$$C_{Prod,t} = K_2(\mathrm{V}_W) \cdot I_{f,Prod,t} \quad (2)$$

The cell culture medium and the reaction product resorufin could emit in the same spectrum region and lead to modifications of the fluorescence intensity. It is, hence, necessary to measure the blank (b) in absence of cells but with times and treatments analogous to those of the samples (s) with cells.

$$I_{f,Prod,t} = (I_{f,s,t} - I_{f,b,t}) \quad (3)$$

Hence, the measurand equation can be written as:

$$N = K(I_{f,s,t} - I_{f,b,t})V_{LF} \quad (4)$$

Where $K = f(K1,K2)$ and is therefore influenced by τ, V_W and from the metabolic activity of the cells.

4. Analysis of quantities influencing the measurement
Each input quantity x_i appearing in equation (4) (V, K, I and the dilution in the measurement procedure) has been considered as quantitiy influencing the measurement. The estimation of the associated standard uncertainties $u(x_i)$ and of the systematic effects have been given, as shown in table 5.1. A detailed description of each input quantity follows.

4.1. Fluorescence
The fluorescence intensity is determined by the instrument used for the measurement, hence the fluorescence measurement needs to be characterized with respect to the adopted measurement system. Here the GloMax®-Multi Microplate Multimode Reader was adopted.

The fluorescence measurement can be influenced by: repeatability and reproducibility of the measurement, the position of the well within the plate, the plate re-positioning into the measuring system and the sample volume loaded in the well.

For the sample volume loaded in the well, an analysis of the fluorescence intensity sensitivity needs to be carried out to evaluate the sensitivity coefficients.

The repeatability of the fluorescence measure in a single well has been evaluated lower than 0.5% over the whole fluorescence intensity range of interest. The reproducibility evaluated among the 96

wells on the same plate has been found lower than 1%, even though results from 5-10% of wells were outliers. Thus, it is necessary to work in triplicate in order to eventually identify outliers.

The plate placement into the plate reader could contribute to the reproducibility. Hence, the standard deviation of repeated measurements has been calculated.

The system measures the fluorescence intensity due to excitation produced by an incident ray entering the well. The effective optical path length of the incident beam is influenced by the liquid level in the well, thus the fluorescence intensity has been measured as function of the volume filling the well V_W. The fluorescence intensity is highest in the range around 100 µl. This volume minimizes the contribution to the uncertainty of the filling-the-well liquid volume fluorescence intensity.

To summarize, the fluorescence intensity uncertainty has a component of repeatability of instrument measure (0.5%) and a component of reproducibility due to the well positions in the plate (1%). A further contribution is due to the volume of liquid filling the well and can be evaluated separately as function of the available sample volume. Sensitivity coefficients of sample and blank fluorescence intensity ($I_{f,s,2.5h}$ and $I_{f,b,2.5h}$) are calculated by derivatives of eq. (4) with respect to $I_{f,s,2.5h}$ and $I_{f,b,2.5h}$.

4.2. Liquid volumes

The type of liquid phase, namely the cell culture medium, is one of the most influencing factors that can interfere on the reaction rate by increasing or decreasing the cell growth. Hence, two different cell culture media added each one with two different FBS quantities have been analyzed. The difference of fluorescence intensity of the different media as function of the contact time τ, i.e., contact between resazurin and cells, for several different cell concentrations (cell number/ml) seeded on wells, has been evaluated. The volume of the liquid phase in the cell culture vessel V_{LF} and the volume filling the measurement well V_w have been measured by a calibrated micropipette. V_{LF} is affected by the residual volumes during liquid replacements, evaporation during the contact time and micropipette uncertainty. In the specific tests the cell culture vessel was the well itself, thus V_W and V_{LF} are the same volume. A 3% total uncertainty has been calculated. The sensitivity coefficient of V_{LF} has been calculated by the derivative of eq (4) with respect to V_{LF}.

4.3. Slope

The slope of calibration curve (K) have been calculated by the regression analysis of fluorescence intensity experimental data and the nominal value of cells in culture. The nominal value of cells in culture is estimated from the cell seeding concentration measured by detaching and counting the cell by means of an hemocytometer, e.g., Neubauer chamber.

The linear regression minimizes the objective function at the estimated K value [11]:

$$K = \frac{\sum_{i=1,n} \frac{N_i\left(I_{f,s,t,i} - I_{f,b,t,i}\right)V_{LF,i}}{\sigma_{y,i}^2}}{\sum_{i=1,n} \frac{\left[\left(I_{f,s,t,i} - I_{f,b,t,i}\right)V_{LF,i}\right]^2}{\sigma_{y,i}^2}} \tag{5}$$

The uncertainty of K [11] is:

$$u(K) = \sqrt{\frac{1}{\sum_{i=1,n} \frac{\left[\left(I_{f,s,t,i} - I_{f,b,t,i}\right)V_{LF,i}\right]^2}{\sigma_{y,i}^2}}} \tag{6}$$

where $\sigma_{y,i}$ is the uncertainty of each experimental point and combines the uncertainty of all the measured quantities, i.e., number of cells, volume and fluorescence intensities. Cell counting relative uncertainty, evaluated as the reproducibility of the Neubauer chamber's cell counting, has been calculated to be 5%.

The K uncertainty decreases when the ratio between cell number N and available liquid volume V_{LF} increases and depends on the number of experimental points. The experimental conditions in which K is calculated should be as similar as possible to the experimental conditions in which the unknown samples are measured. The K uncertainty has been calculated to be 2.2% for time of contact larger than 1 hour and 3% at 0.5 hours of contact. The sensitivity coefficient of K has been calculated by derivative of eq (4) with respect to K.

4.4. Dilution

The fluorescence intensity detection limit of the GloMax®-Multi Microplate Multimode Reader is $5 \cdot 10^5$ fluorescence units (or counts). Consequently, the maximum ratio between the number of cells and the volume of available liquid has a limit and for values above the detection limit a dilution of the samples is necessary. For different τ, the limit value has been analyzed. The optimal dilution is with the blank solution. However, the available volumes of the blank solution are rarely sufficient for each dilution. Hence, it has been necessary to dilute also the blank solution to obtain a correct linearity of the calibration curve. The dilution of sample and blank solutions with a solvent similar to the blank, i.e., fresh medium, has given a good response.

4.5. Correlation between input quantities

In order to keep the evaluation simple we did not consider correlation between none of the input quantities.

5. Uncertainty budget

The uncertainty budget of the cell count by CTB assay is reported in table 5.1. It has been calculated for a contact time of 2.5h and low cell number at the experimental conditions of the tests. The Significance Index (SI) is the ratio between the contribution of the analyzed influence quantity and the maximum contribution to the uncertainty provided by one of the influence quantities. SI lower than 1% indicates that the contribution to the uncertainty is negligible, SI upper that 10% indicates that the contribution is relevant, SI=100% indicates the most relevant contribution.

The most relevant contribution was given by K. The K uncertainty can be reduced by increasing the number of measurement points of calibration curve; in addition, the fluorescence intensity may be enhanced calibrating the system by fluorophores standard solutions.

Table 5.1. Uncertainty budget for cell number quantification by CTB assay

Component	*Unit*	*Value*	*Source of uncertainty*	*Standard uncertainty*		*Relative standard uncertainty*	*Sensitivity Coefficient*	*Uncertainty contribution*	*SI**
x	$[x]$	x_i		$u(x_i)$		$u(x_i)/x_i$	$\lvert c_i \rvert$	$[u(x_i) \cdot c_i]^2$	* *see above*
$I_{f,s,2.5h}$	counts	$3.8 \cdot 10^5$	reproducibility	$3.8 \cdot 10^3$	$4.2 \cdot 10^3$	1.1%	$2.7 \cdot 10^{-2}$	$1.3 \cdot 10^4$	35%
			repeatability	$1.9 \cdot 10^3$					
$I_{f,b,2.5h}$	counts	$5.0 \cdot 10^4$	reproducibility	$5.0 \cdot 10^3$	$5.6 \cdot 10^3$	11.2%	$2.7 \cdot 10^{-2}$	$2.3 \cdot 10^4$	60%
			repeatability	$2.5 \cdot 10^3$					
V_{LF}	dm^3	$1.0 \cdot 10^{-4}$	pipette calibration	$1.0 \cdot 10^{-6}$	$1.0 \cdot 10^{-6}$	1.0%	$8.9 \cdot 10^7$	$7.9 \cdot 10^3$	21%
K	dm^{-3}	270	regression	5.9	5.9	2.2%	$3.3 \cdot 10^1$	$3.8 \cdot 10^4$	100%
Cell number (N)		**8910**	***Combined standard uncertainty***		**286**	**3.2%**			

6. Discussion and conclusions

Cell counting methods based on the monitoring of metabolic activities are indirect methods and require high reproducibility in experiments to calibrate instruments and to analyze unknown samples. The identification of standard measurement units for fluorescence intensities is certainly a significant prerogative for comparing the results obtained by using a certain investigation methodology (i.e. CTB assay) in order to increase its traceability, independently from the adopted measurement instrument.

Results of this work on the evaluation of cell number measurement uncertainty by CTB assay in 2D cell cultures, revealed that the main contributions to the uncertainty are: repeatability and reproducibility of the measurement system and a contribution due to the contact time between the substrate of the metabolic reaction and the cells, the measured sample volume and the cellular metabolic activity. Standard uncertainty was found to be around 3.5% in the tested experimental conditions. All the influence quantities gave a relevant contribution to the total uncertainty, it means that to reduce uncertainty by one order of magnitude, the uncertainty of all the quantities must be reduced.

This work is a first approach to the uncertainty evaluation of a metabolic assay in conventional 2D cell cultures and can be the basis for the innovative and recently applied 3D cell culture systems where several other components can influence the measurement of the cell number in order to increase the traceability of those measurements, independently from the adopted measurement instrument.

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