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Review article / Обзорная статья

Recommendations for Validation of Automated Viable Cell Counting Methods (Review)

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Abstract

Introduction. The quality of viable cell-based products (such as biomedical cell products and advanced therapy medicinal products) must be maintained during the full production cycle to ensure their safety and efficacy for patients. The minimum required number of viable cells is one of the quality control criteria in the final product release specifications. This study looks into the process of validation of automated viable cell counting methods.

Text. The study reviewed the latest data on specific validation characteristics for automated cell counters as compared to manual counting methods. We identified the main problems with the validation methods. Based on the review of scientific and regulatory literature, we identified the key validation parameters, methods of their evaluation and measurement, and reporting of results. We described the validation algorithm for an automated cell counter, including such steps as the selection of reference standards, selection of the number of experimental points, experimental design, mathematical evaluation of the obtained results, and determination of the acceptance criteria.

Conclusion. Based on the data reviewed, the authors developed recommendations for the validation of automated viable cell counting procedures.

Keywords: validation, cell counter, hemocytometer, viability, cell lines

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Marina A. Vodyakova and Nikita S. Pokrovsky wrote the text of the article, gathered and reviewed literature data. Ekaterina V. Melnikova proposed the study design. Vadim A. Merkulov reviewed and approved the final version of the article.

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Рекомендации по валидации методик автоматического подсчета жизнеспособных клеток (обзор)

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Резюме

Введение. Качество продуктов на основе жизнеспособных клеток (таких, как биомедицинские клеточные продукты и высокотехнологические лекарственные препараты) должно поддерживаться на протяжении всего цикла производства, чтобы гарантировать их эффективность и безопасность при использовании пациентами. Минимально необходимое количество жизнеспособных клеток является одним из критериев контроля качества при выпуске конечного продукта. Исследование посвящено анализу процесса валидации методик автоматического подсчета жизнеспособных клеток.

Текст. В рамках данного исследования были рассмотрены актуальные данные об особенностях валидации автоматических счетчиков клеток относительно ручного подсчета. Были определены основные проблемы при валидации. На основе научных и регуляторных источников были выделены ключевые параметры процесса валидации, методы их оценки, измерения и представления результатов. Был описан алгоритм валидации автоматического счетчика клеток, включающий шаги по подбору стандартных образцов, выбору количества экспериментальных точек, разработке дизайна эксперимента, математической оценке полученных результатов и определению критериев приемлемости.

Заключение. На основании изученных данных в работе представлены результаты в виде рекомендаций по валидации методик автоматического подсчета жизнеспособных клеток.

Ключевые слова: валидация, счетчик клеток, гемоцитометр, жизнеспособность, клеточные линии

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. М. А. Водякова и Н. С. Покровский осуществили написание текста статьи, сбор и обработку литературных данных. Е. В. Мельникова предложила дизайн исследования. В. А. Меркулов утвердил окончательную версию статьи.

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INTRODUCTION

The monitoring of cell line growth kinetics by counting viable cells is performed during research, development, and manufacturing of viable human cell-based products (such as biomedical cell products and advanced therapy medicinal products) to ensure the selection of an effective therapeutic cell dose. Both the concentration and viability of cells are key parameters for ensuring a standardized manufacturing process. These parameters are important not only for cell passaging at a constant seeding density, for ensuring optimal cell storage conditions, for maintaining maximum viability after thawing, and standardization of cell assays to obtain comparable data, but they also provide useful information for evaluation of cell culture performance [1]. Cell counting in cell cultures is usually performed once a day, which is consistent with the mammalian cell doubling time during the exponential growth phase. This is sufficient for establishing the overall growth profile of the cell line. More frequent counting can be carried out, if necessary, for certain cell cycles [2].

Traditionally, cell viability is assessed by dye exclusion methods, with trypan blue being one of the most common dyes, which selectively stains non-viable cells. Viable cells, on the other hand, have intact cell membranes and therefore cannot take up the dye from the medium [3]. This type of cell viability analysis involves manual staining followed by direct cell counting based on visual inspection under the microscope or using electronic cell counters [2].

The hemocytometer is considered a gold standard for measuring cell concentration and viability because of its low cost and versatility. However, counting with a hemocytometer takes a lot of time, and the results may vary depending on the analyst's skills. This method is also very tedious and not suitable for the development and production of cell products, in which cell characteristics must be evaluated on a daily basis, and parallel analysis of a large number of samples is hampered [1].

To overcome these limitations, automated cell counters that combine trypan blue staining and digital imagery were developed. There are a few automated cell counters on the market, e.g. Cedex (Roche, Switzerland), Luna (Logos Biosystems, South Korea), TC10 and TC20 (Bio-Red, USA) and others. Automated cell counting devices usually have a digital camera for imaging, and the analysis is performed with specialized software with minimal help from the analyst. The automated counting method also allows counting of a larger number of cells per unit of time, compared to manual counting. Thus, they are characterized by high repeatability and accuracy, because they provide operator-independent results; they also greatly reduce the time of analysis, have improved productivity and performance [1]. Disadvantages of automatic counters are that they do not allow for simultaneous staining with different dyes and may lead to inaccuracies in differentiating some types of cells due to technical restrictions of their hardware and software [4].

The combination of specific features, advantages, and disadvantages of automated cell counters hampers their integration into research, laboratory, and production activities. The accuracy and repeatability of results both depend, to a great extent, on careful setting-up and calibration of the instrument, imaging parameters, as well as thorough sample preparation. Moreover, the use of automated counters during production of cell products requires validation of the counting procedures. Considering the above, the aim of this study was to develop a validation technique for the cell counting procedure using automated counters.

Key parameters of the procedure validation

When validating an automated cell counting procedure, it is important to consider factors that affect the measurements results, such as sample preparation, instrument calibration, as well as parameters and potential errors of the instrument, and the human factor. It is also essential to thoroughly homogenize the cell sample before sampling and perform parallel experiments in manual and automated modes in order to minimize differences in cell viability [4].

According to general monograph OFS.1.1.0012.15 "Validation of analytical procedures" of the Russian Pharmacopoeia, XIV edition[1], an automated cell counting procedure falls under the "quantitative determination of the drug substance and specified components" type. Validation of test procedures of this type requires determination of such parameters as specificity, range, linearity, accuracy, and precision – both for the total number of cells and for viable cells only.

Specificity is an unambiguous identification of cells in a sample, their quantification and differentiation from the surrounding medium and impurities. Specificity can be of two types – positive and negative. To determine positive specificity, one specifically measures the analyte (cells or beads with certain concentration and viability). The presence of some amounts of impurities or degradation products, theoretically, should not affect the measurement results. Negative specificity could be demonstrated by measuring cell medium or buffer and should yield zero viability and concentration.

Range is a scope of a sample's concentration values (or viability values) with a guaranteed acceptable level of accuracy and intermediate precision. Besides, the minimum concentration range is usually determined based on values from 80 % to 120 % of the nominal concentration, or from 80 % of the lower concentration to 120 % of the upper concentration.

The linearity of the test procedure is confirmed by a linear dependence of the number of cells on the sample's concentration within an established range. The result of the linearity measurement is presented as a coefficient of determination (R^2).

Accuracy, just like linearity, is studied within an established range. For quantitative methods, accuracy is determined by comparing the results obtained with the automated counting method with the results obtained with an orthogonal method (a well-studied method which is based on a different measurement principle)[2] with known accuracy and precision. In the case of cell counting, this would be manual counting on a hemocytometer. The accuracy of the procedure can also be assessed by measuring a well-characterized reference standard. The result of such accuracy assessment is presented as a percentage ratio of the results obtained by the validated and orthogonal procedures (recovery, Δ%). In addition, the conclusion on the accuracy of the procedure could also be made based on the established precision, specificity, and linearity.

Precision is a parameter reflecting the degree of similarity between several consecutive measurements obtained by the procedure in question. Precision is comprised of repeatability, intermediate precision, and reproducibility. Intermediate precision assesses the influence on the measurements results of such factors as the analyst, equipment, and environmental factors. Reproducibility is only used in standardization of compendial procedures and establishes their repeatability in different laboratories. The results for this parameter are presented as standard deviation (SD) and coefficient of variation [CV, also known as relative standard deviation (RSD)] [5].

In spite of the fact that cell counters are widely used in the biopharmaceutical industry, there are relatively few papers on the validation of such procedures (Table 1). In addition, some papers discuss non-conventional validation parameters. For example, Bottová et al. [6] validated a counting procedure for dendritic cells in a prostate cancer immunotherapy cell product (DCVAC/PCa) based on their size and shape, for which they used an automated cell counter Vi-CELL XR (Beckman Coulter, USA).

Furthermore, some validation studies use different sets of parameters, e.g., one study [8] compared a hemocytometer, an automated cell counter TC20 (Bio-Rad, USA), and a flow cytometer BD FACSCalibur (BD Biosciences, USA) using such parameters as precision, accuracy, and time of analysis, on HeLa (uterine adenocarcinoma cells) and Jurcat (human T-lymphocytes) cell lines. The counting of polystyrene beads loaded into the hemocytometer was performed 10 times according to an individual plan to calculate CV for a given hemocytometer area. The variability was shown to be affected by the area and sample concentration. The hemocytometer CV between measurements was greater than 10 % most of the time at concentrations lower than 0.1 million/mL, and was related to the area in the concentration range of up to 0.45 million/mL. Seven different analysts performed cell counting on 2 different hemocytometers; inaccuracy was observed from analyst to analyst, and the CV between analysts was 7.1–15.6 %. Next, they compared counting of one chamber with 6 different TC20 devices, and the calculated CV was 2.4 %. The manual counting took about 3 minutes or more depending on the concentration of the test sample, while automated counting took only about 20 to 30 seconds.

Another study [9] analyzed factors that affect the accuracy of measurement on a TC20 automated cell counter (Bio-Rad, USA) in comparison with Goryaev chamber, using human mesenchymal stem cells (MSC) from bone marrow (FetMSC cell line). As was shown in the paper, the presence of protein components of the conditioned medium and trypan blue particles introduced errors in the measurements, therefore a modification of the procedure was proposed. This new procedure removes cells smaller than 10 μm in size from the counting pool and gives more accurate cell counting results.

Due to lack of public information on the topic, the authors of this paper proposed a validation algorithm based on the current knowledge in academic literature [4–9], which consists of several steps, such as selection of reference standards, selection of the number of experimental points, experimental design, mathematical evaluation, and acceptance criteria.

[1] State Pharmacopoeia of the Russian Federation, XIV edition; 2018.

[2] Q2(R2) Validation of analytical procedures. Ich harmonised guideline (Draft version). International council for harmonisation of technical requirements for pharmaceuticals for human use. 24.03.22.

Table 1. Examples of experimental studies on the validation of automated cell counters

	Cadena-Herrera D. et al. [4]		Huang L. C. et al. [7]
Validated equipment	**Countess (Invitrogen, USA)**	**Vi-CELL XR (Beckman Coulter, USA)**	**Cedex (Roche, Switzerland)**
Reference method	**Hemocytometer**		**–**
Tested cell lines	CHO-K1 and U937		CG8711 and CG1940
Standard	ViaCheck™ Control beads (Polysciences, USA) (*concentration*: 1, 4, 8 million/mL, *viability*: 0, 50, 75, 90, and 100%)		Certified Cedex calibration beads (Roche, Switzerland) at a *concentration* of 5.045 million/mL
Range	**Measurement parameters**		
	Concentration: 5 points (1, 2, 4, 6, 8 million/mL) in triplicate *Viability*: 5 points (25, 50, 75, 90, 100%) in triplicate		*Concentration*: 6 points (0.3125 million/mL – 10 million/mL for cells, 0.3125 million/mL – 5 million/mL for beads) *Viability*: 6 points (0, 50, 70, 80, 90, 100%)
	Result		
	Concentration: from 1 to 8 million/mL *Viability*: from 0 to 100 %		*Concentration*: 0.3125 million/mL – 10 million/mL for cells, 0.3125 million/mL – 5 million/mL for beads *Viability*: from 0 to 100 %
Linearity	**Measurement parameters**		
	Concentration: 5 points (1, 2, 4, 6, 8 million/mL) in triplicate *Viability*: 5 points (25, 50, 75, 90, 100%) in triplicate		*Concentration*: 6 points (0.3125 million/mL – 10 million/mL for cells, 0.3125 million/mL – 5 million/mL for beads) *Viability*: 6 points (0, 50, 70, 80, 90, 100%)
	Result		
	Concentration: $R^2 \geq 0.99$ *Viability*: $R^2 \geq 0.98$		*Concentration*: $R^2 \geq 0.999$ *Viability*: $R^2 \geq 0.995$
Accuracy	**Measurement parameters**		
	Concentration: 5 points (1, 2, 4, 6, 8 million/mL) in triplicate *Viability*: 5 points (25, 50, 75, 90, 100 %) in triplicate		*Concentration*: measurement of bead samples with a concentration of 5.045 million/mL *Viability*: measurement of various proportions of viable and non-viable cells (0, 50, 70, 80, 90, 100 % viable cells)
	Result		
	Concentration: from 99 to 105 % *Viability*: from 99 to 105 %		*Concentration*: from 91.3 to 105.1 % *Viability*: from 95.3 to 106.4 %
Precision	**Measurement parameters**		
	Concentration 1 million/mL in 6 replicates. All the samples were prepared on the same day by the same analyst		*Concentration* Repeatability: triplicate analysis by two analysts on three different days. Intermediate precision: three independent analyses in triplicate by two analysts. Additionally, three independent analyses in triplicate on another similar instrument *Viability* Repeatability: triplicate analysis of samples with 85 % and 75 % viability. Intermediate precision: three independent analyses in triplicate by two analysts. Additionally, three independent analyses in triplicate on another similar instrument
	Result		
	RSD 11,04 – 14,3 %	RSD 2,27 – 5,28 %	*Concentration* Repeatability: RSD 1.11–5.92 % Intermediate precision: RSD 12.7–15.8 % *Viability* Repeatability: RSD 0.40–2.11 %
	For hemocytometer: RSD 0.75 – 8.06 %		
Specificity	**Measurement parameters**		
	Measurement of the cell medium background, bead buffer, impurities		Measurement of the cell medium background, bead buffer, impurities. Measurement of various proportions of viable and non-viable cells (0, 70, 100 % viable cells)
	Result		
	No influence of the medium on the measurement results		The contribution of the medium is less than 0.1 % of the total measurement. The method does not allow to distinguish cells from other particles of the same size

Note. CHO-K1 – Chinese hamster ovary cells; U937 – human cells, histiocytic lymphoma (pleural effusion); CG8711 and CG1940 – prostate adenocarcinoma cells; RSD – relative standard deviation; R2 – coefficient of determination.

Validation algorithm

1. Reference standard selection

In addition to cell samples, it is recommended to use certified fluorescent beads with a known concentration for validation of an automated counting procedure. The choice of the reference standard depends on the cell line used: the beads should be as close as possible to the cells in terms of such characteristics as size, roundness, density, and affinity to dyes.

2. Selection of the number of experimental points

In their papers on the validation of automated cell counters, researchers often do not explain the rationale for the choice of experimental points, therefore, the recommendations we offer below are based on the generalization of data presented in the scientific literature and guidelines [4–9]. The summarized data are presented in Table 2 as minimum and recommended numbers of experimental points and replicates.

The recommended values given in Table 2 are not the maximum allowable values, because the selection of more points and replicates will give more accurate results. However, the relevance of using more measurements must be justified.

3. Experimental design

After determining the number of control points, specific values of concentration and viability must be established for each experiment. An example of such experimental design is shown in Table 3 [4–9].

The choice of two points in the specificity experiment makes it possible to assess both positive and negative specificity. In addition, it is also possible to use a larger number of experimental points by diluting the samples. When determining concentration, the samples are diluted with buffer or cell medium, and when determining viability, viable and non-viable cells (e.g., treated with ethanol) are mixed in the proportions necessary to obtain the required viability.

Table 2. **Selection of the number of experimental points**

	Minimum number of		Recommended number of	
	Experimental points	Replicates per point	Experimental points	Replicates per point
Specificity	1–3	3	3–5	3–5
Linearity	5	3	8	3–5
Precision (repeatability and intermediate precision)*	3	3	3–5	3–6
	or			
	1	6	1–3	6
Accuracy	Determined by comparing the results of the analysis obtained by another reference method			
	3	3	5	3
	or			
	Determined after establishing precision, linearity, and specificity			
Range	Determined by the smallest and the largest values that have been shown to have acceptable precision and linearity			

Note. * Repeatability analysis is performed by one operator on a single day, intermediate precision analysis is performed in parallel by two operators (or by one operator on different days).

If a reference method or a reference standard with well-known concentration and viability characteristics are not used in the test method validation, the accuracy of the test method can be deduced from the analysis of specificity, linearity, and precision (the accuracy in this case is confirmed if the results for specificity, linearity, and precision meet their acceptance criteria). If such a method or reference standard are available, the accuracy can be determined by comparing the measurement results obtained for the sample and the reference standard.

4. Mathematical evaluation of the results

Mathematical evaluation is performed to verify the reliability of the study results and to prove the presence (or absence) of statistical differences in the cell counting results obtained by different methods, instruments, ana-

Table 3. **Experimental design for an automated cell counter validation**

Experiment	Validated parameter	N	R	O	Control points, % of max. concentration	Control points, % of max. viability
1	Specificity	2	3	1	0, 100	0, 100
2	Linearity	8	3	1	15, 30, 45, 60, 75, 90, 100, 120	0, 15, 30, 45, 60, 75, 90, 100
3	Repeatability	3	6	1	100 (×3)	100 (×3)
4	Intermediate precision	3	3	2	100 (×3)	100 (×3)
5	Accuracy	5	3	1	0, 25, 50, 75, 100	0, 25, 50, 75, 100
		or				
		–	–	–	Determined by the results obtained for specificity, linearity, and precision	
6	Range	–	–	–	Determined by linearity assessment	

Note. N – number of control points; R – number of repeated measurements carried out for each control point; O – number of operators conducting measurements (can be replaced by the number of days on which one operator conducts measurements).

lysts, as well as differences in the results obtained on different days or under different conditions. The comparison of the results of the two methods can be evaluated using the paired Student's t-test or by calculating SD of the results obtained relative to the true value or the value obtained using the reference method.

The linearity results are presented as a linear graph of the observed concentrations and viability values against the expected ones, and as a linear regression equation. The R^2 and CV coefficients for each experimental point are also evaluated for compliance with the acceptance criteria. R^2 can be calculated automatically by most statistical software. It is recommended to represent the graph together with the y-intercept, slope ratio, and the residual sum of squared deviations.

CV is determined by the ratio of SD of a series of measurements to their mean value and is expressed in percent.

$$CV = \frac{\sigma}{\mu} \cdot 100\ \%\ [10], \quad (1)$$

where σ is the standard deviation of a series of concentration or viability measurements, μ is the mean value of a series of concentration or viability measurements.

The recovery parameter (Δ%) shows the percentage ratio of the two values. In the case of accuracy determination, Δ% shows the percentage difference between the mean values of measurement series for the method being validated and the reference method.

$$\Delta\% = \left| \frac{\mu_{exp} - \mu_{ref}}{\mu_{ref}} \right| \cdot 100\ \%, \quad (2)$$

where μ_{exp} is the mean value of a series of concentration or viability measurements for the method being validated, μ_{ref} is the mean value of a series of concentration or viability measurements for the reference method.

5. Acceptance criteria

Table 4 provides acceptance criteria for evaluation of validation parameters for an automated cell counting method.

The method is considered validated if all the test parameters meet the acceptance criteria. A deviation in one or more of the parameters must be justified.

CONCLUSION

The development of a cell counting method is one of the key steps in the research, development, and production of cell products. However, the currently recognized reference method of manual counting with a hemocytometer has a number of drawbacks that limit its use in cases where high throughput and counting speed are required. In such cases, an automated counting method using a cell counter can be used as a replacement. However, the introduction of such cell counters for quality control in the production of cell products requires validation. Validation of automated cell counting methods is a complex procedure that considers many aspects, such as proper selection of validation parameters, standards, and the optimal number of replicates. Careful consideration of these aspects ensures the reliability and accuracy of the validation process. The authors have considered the advantages of automated cell counting in comparison with manual counting, analyzed scientific literature and guidelines on this topic, and described such key parameters of the validation process of an automated cell counting method as specificity, range, linearity, accuracy, and precision. Thus, the authors proposed a general validation algorithm based on the selection of reference standards, the number of experimental points, experimental design, mathematical evaluation of the results, and verification that the results meet the acceptance criteria.

Table 4. Acceptance criteria for the estimated validation parameters

Validation parameter	Acceptance criteria
Specificity	Negative: No influence of the medium or buffer on the measurement results
	Positive: CV ≤ 10 %
Linearity	$R^2 \geq 0.95$
	CV ≤ 10 %
Precision	CV ≤ 20 %
Accuracy	Δ% ≤ 20
	or
	Specificity, linearity, and precision results meet the acceptance criteria
Range	Linearity, precision, and accuracy results meet the acceptance criteria

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