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Validation of three viable-cell counting methods: Manual, semi-automated, and automated

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ABSTRACT

A viable cell count is essential to evaluate the kinetics of cell growth. Since the hemocytometer was first used for counting blood cells, several variants of the methodology have been developed towards reducing the time of analysis and improving accuracy through automation of both sample preparation and counting. The successful implementation of automated techniques relies in the adjustment of cell staining, image display parameters and cell morphology to obtain equivalent precision, accuracy and linearity with respect to the hemocytometer. In this study we conducted the validation of three trypan blue exclusion-based methods: manual, semi-automated, and fully automated; which were used for the estimation of density and viability of cells employed for the biosynthesis and bioassays of recombinant proteins. Our results showed that the evaluated attributes remained within the same range for the automated methods with respect to the manual, providing an efficient alternative for analyzing a huge number of samples.

1. Introduction

A viable cell count is crucial for the study of eukaryotic cells for different purposes such as the management of cell cultures in biological research, the titration of cell populations in diagnostics, and in-process controls in industrial bioprocesses [7,13,5].

Among the well-known viable cell count methods developed so far, manual counting with a hemocytometer has been the most commonly used method due to its low cost and versatility [4]. This method depends on the analyst's ability to evaluate different cell attributes regardless of the cell type; in addition allows using different staining techniques according to the purpose of the analyses (Table 1). However, the procedure is time consuming, which precludes the analysis of a large amount of samples at one time and is subject to inter-user variation depending on the degree of expertise of the analyst [6,8].

Currently, the arising of automated cell counting instruments has provided the possibility of analyzing a huge number of samples in a shorter time [6,10], which represents also an economic advantage considering an estimated cost of $4 USD per manually counting sample, thus the return on investment can be easily covered in a short time for laboratories processing large-scale analyses, in addition to the benefits of reducing variability associated to human-error.

Nowadays there are several automated cell-count systems available such as Cedex HiRes System (Roche), Luna™ (Logos Biosystem) and Cellometer™ Auto T4Cell Viability Counter (Peqlab), TC10™ and TC20™ (Bio-Rad), Countess® Automated Cell Counter (Invitrogen) and Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter). In general, automated cell count instruments consist of a digital camera to obtain images and the analyses are performed through specialized software that requires a minimal user involvement [10]. Although automated instruments facilitate the process of analyzing samples, they are constrained by the availability of a few compatible staining options, and may be imprecise in differentiating some types of cells due to technical limitations in their hardware and software [12,8].

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Table 1
Comparison of technical parameters between the viable cell counting methods evaluated in this study.

Cell counting system	Auto-sample	Staining options	Size range (μm)	Sample volume (μL)	Analysis time (min)	Concentration range (cells/mL)	Viability range	Imaging technology
Hemocytometer[a,b,c]	No	Erythrosin B, Nigrosin, Safranin, Methylene blue and Trypan blue	Undefined	50	Concentration sample-dependent	2.5×10^5–8.0×10^6	0–100	Microscope objective 40×
Countess[d]	No	Trypan blue	8–60	20	<1	1×10^4–1×10^7	0–100	Camera 2.3× objective and 3.1 Mega pixel
Vi-CELL® XR[e,f]	Yes	Trypan blue	2–70	500	<2.5	5×10^4–1×10^7	0–100	Auto-focus routine firewire camera 1394 × 1040CCD array

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[f] Bioprocessing Feature of the Beckman Coulter® Vi-CELL™. Technical Note. Beckman Coulter®. B2004-6330. 2004.

The semi-automated Countess® from Invitrogen (Carlsbad, CA) and the fully automated Vi-CELL® XR from Beckman Coulter (Fullerton, CA) (Table 1) are two instruments currently employed in viable cell-counts at many laboratories worldwide, that along with the hemocytometer, perform viable cell-counts based on the trypan blue exclusion technique. This technique is capable of revealing damaged cells, since the dye penetrates through their damaged membrane resulting in blue stained cells that are regarded as non-viable, whereas non-stained cells are assumed to be intact and are considered as viable [9,11,1].

In order to determine the suitability of each of these viable cell count methods, they were evaluated in their performance towards the compliance of measurable attributes such as: specificity, accuracy, precision, linearity and range, through a validation. For this purpose we standardized the sample preparation, and adjusted the settings in order to obtain viable cell-counts using CHO-K1 and U937 cells that are employed for the biosynthesis and bioassays of recombinant proteins respectively. The suitability of these methodologies was evaluated through a validation according to the ICH Q2R1 guideline [2,3].

2. Materials and methods

2.1. Materials

CHO-K1 cells (CRL-CCL 61) and U937 (CRL-1593.2) were acquired from ATCC® (Manassas, VA). RPMI 1640 medium, PBS 1X pH 7.4 and 0.4% trypan blue solution were purchased from Gibco®-Life technologies™ (New York, GI). ViaCheck™ Control beads (concentration: 1, 4 and 8×10^6 beads/mL, and viability: 0, 50, 75, 90, and 100%) were acquired from Bangs Laboratories, Inc. (Fishers, IN). Countess® Automated Cell Counter and Countess® cell counting chamber slides were acquired from Invitrogen (Carlsbad, CA). Vi-CELL® XR Cell Viability Analyzer, Vi-CELL® XR Quad Pak Reagent Kit and Coulter CC Size standard Mix kit (2, 5, 10, 20 and 43 μm latex Beads) were purchased from Beckman Coulter (Fullerton, CA). Trypan blue powder was obtained from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum was acquired from PAA Laboratories (Linz, AT). DM222 A11- Liquid media were purchased from Irvine Scientific (Santa Ana, CA).

2.2. Sample preparation

We employed concentration standards of 1, 4 and 8×10^6 beads/mL, (concentrations stated in the manufacturer's certificate of analysis "COA") that did not require further preparation; additionally in order to have five points for constructing a concentration curve, we prepared two extra concentration standards of 2×10^6 and 6×10^6 beads/mL, by mixing 500 μL of the 4×10^6 beads/mL standard with 500 μL of 1X PBS, and 750 μL of the 8×10^6 beads/mL standard with 250 μL of 1X PBS, respectively.

For viability, we used standards of 0, 50, 75, 90 and 100% viability (viabilities stated in the manufacturer's COA) that did not require further preparation, in addition, to construct a five point viability curve we prepared a 25% viability standard was by mixing 500 μL of the 50% viability standard with 500 μL of the 0% viability standard.

To analyze cell concentration, cells were diluted in their corresponding media at the same concentrations of standards (1, 2, 4, 6 and 8×10^6 cells/mL), while to evaluate cell viability, non-viable cells were obtained by inducing thermal stress at 70 °C throughout 180 min, unviability was confirmed through the staining pattern and morphology of stressed cells in the hemocytometer; thereafter, stressed cells were mixed with viable cells at different proportions in order to obtain comparable results to viability standards.

2.3. Manual counts

50 μL of sample was mixed with 50 μL of 0.4% trypan blue by gently pipetting, and then 20 μL of the mix were loaded into each chamber of the hemocytometer. Counts were performed by triplicate by one analyst under a 40× objective according to the standard methodology [6].

2.4. Semi-automated cell count

In order to standardize the Countess method, we tested different concentrations of trypan blue (from 0.4% to 1.0%) to determine the best staining conditions to perform the readings, being 0.8% the concentration that worked better for our analyses (Fig. 1). The focus of the instrument was calibrated using particle size standards of 5, 10 and 20 μm. A cell count was performed by mixing 20 μL of sample with 20 μL of 0.8% trypan blue solution, afterwards the mixture was loaded into the chamber slide. A cell-count protocol was customized accounting for sensitivity, circularity, maximum and minimum size, and was applied for readings of both standards and samples (Table 2).

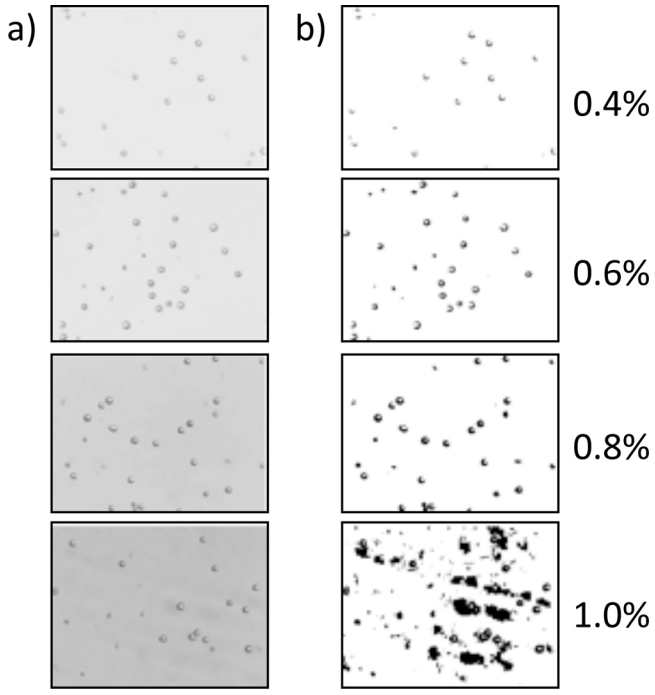

Fig. 1. Comparison of images acquired by the Countess® camera at different trypan blue concentrations: (a) Presents the images as were acquired by the instrument camera, while (b) Represents images as they were analyzed by the software. At the concentration of 1.0% the background noise interfered with the capability of the instrument to perform more accurate and precise counts.

2.5. Automated cell counts

For the analysis in the Vi-CELL® XR, 1 mL of diluted samples were loaded into the carrousel (no manual mixture of the samples with trypan blue is required since it is carried out automatically by the instrument). The parameter settings were established according to the recommended by the manufacturer of the standards and were applied for standards and samples (Table 2).

Table 2
Customized parameters for Vi-CELL® XR and Countess®.

Instrument		Control protocol[a]	Viability protocol[a]
Vi-CELL® XR (Cell viability Analyzer v2.04)	Parameter		
	Minimum diameter (μm)	2	5
	Maximum diameter (μm)	50	50
	Images #	50	50
	Aspirate cycles	2	2
	Trypan blue mixing cycles	3	3
	Cell brightness (%)	70	85
	Cell sharpness	75	100
	Viable cell spot brightness (%)	55	60
	Viable cell spot area (%)	1	3
	Minimum circularity	0.9	0.9
	Decluster degree	Low	Low

Instrument	Protocol	Settings
Countess® (Software v2.05)	Sensitivity	6
	Minimum size (μm)	8
	Maximum size (μm)	15
	Cicularity (%)	100

[a] Parameters settings recommended by the manufacturer of the standards.

2.6. Validation protocol

The validation protocol was designed to evaluate linearity, range, specificity, accuracy, and repeatability-precision of each method.

2.6.1. Linearity and range

Linearity and range of the three methods were evaluated using concentration standards, viability standards and cells prepared at the same concentrations and percentages of the standards, as mentioned in preparation of samples. Samples were prepared and analyzed by triplicate, and the results were collected and plotted by linear fit against the expected concentrations in the range from 1 to 8×10^6 units/mL using Minitab® v.15.0 statistical software (Minitab Inc., State College, PA). Linearity of viability was determined by the same procedure used for the range from 0 to 100% for beads or cell lines. Intercept, slope and correlation coefficient (R^2) values were reported.

2.6.2. Specificity

This parameter was evaluated on the matrix (media or buffer employed in the preparation of samples) in order to demonstrate specificity against cell-like particles that may interfere with the readings in the Countess® and Vi-CELL® XR instruments. Specificity was evaluated only for the automated methods since their hardware and software are likely to misidentify cell-like particles and consider them as cells.

2.6.3. Accuracy

Accuracy of the three methods was assessed using concentration standards (1, 2, 4, 6 and 8×10^6 units/mL), viability standards (0, 25, 50, 75 and 100%) and the two cell lines prepared at the same concentration and percentages of the control beads. Samples were prepared by independent triplicates for each cell-count method. The recovery percentages were related to theoretical values.

2.6.4. Precision (repeatability)

Repeatability was evaluated, as the relative standard deviation (RSD) among runs, for all counting methods using sextuplicates of control beads and samples prepared at a unique concentration of 1×10^6 units/mL. Samples were prepared on the same day by the same analyst.

3. Results and discussion

It is well standardized that the 0.4% trypan blue concentration is the most appropriate to provide the better visualization of damaged cells by the human eye through a 40× microscope objective, towards determining viable and non-viable cell counts with a hemocytometer. Vi-CELL® XR also employs the same trypan blue concentration since it provides an appropriate resolution for image acquisition by the Auto-focus camera; however, for this comparative validation exercise Countess® required an adjustment in staining in order to obtain a better resolution of stained cells from the background, for a posterior differentiation among viable and non-viable cells. We observed that as the trypan blue concentration increased from 0.4 to 1.0% the cell staining improved, as shown by the images acquired by the instrument's camera (Fig. 1a); however, since the instrument's software processes the images as 1-bit binary images, when the trypan blue concentration was 1.0%, the background noise intensity interfered with the capability of the instrument to properly distinguish cells from the background (Fig. 1b); nevertheless, no significant background noise interference was observed between 0.4 and 0.8%, being the latter the one we chose since it was the concentration that provided a better differentiation of stained cells

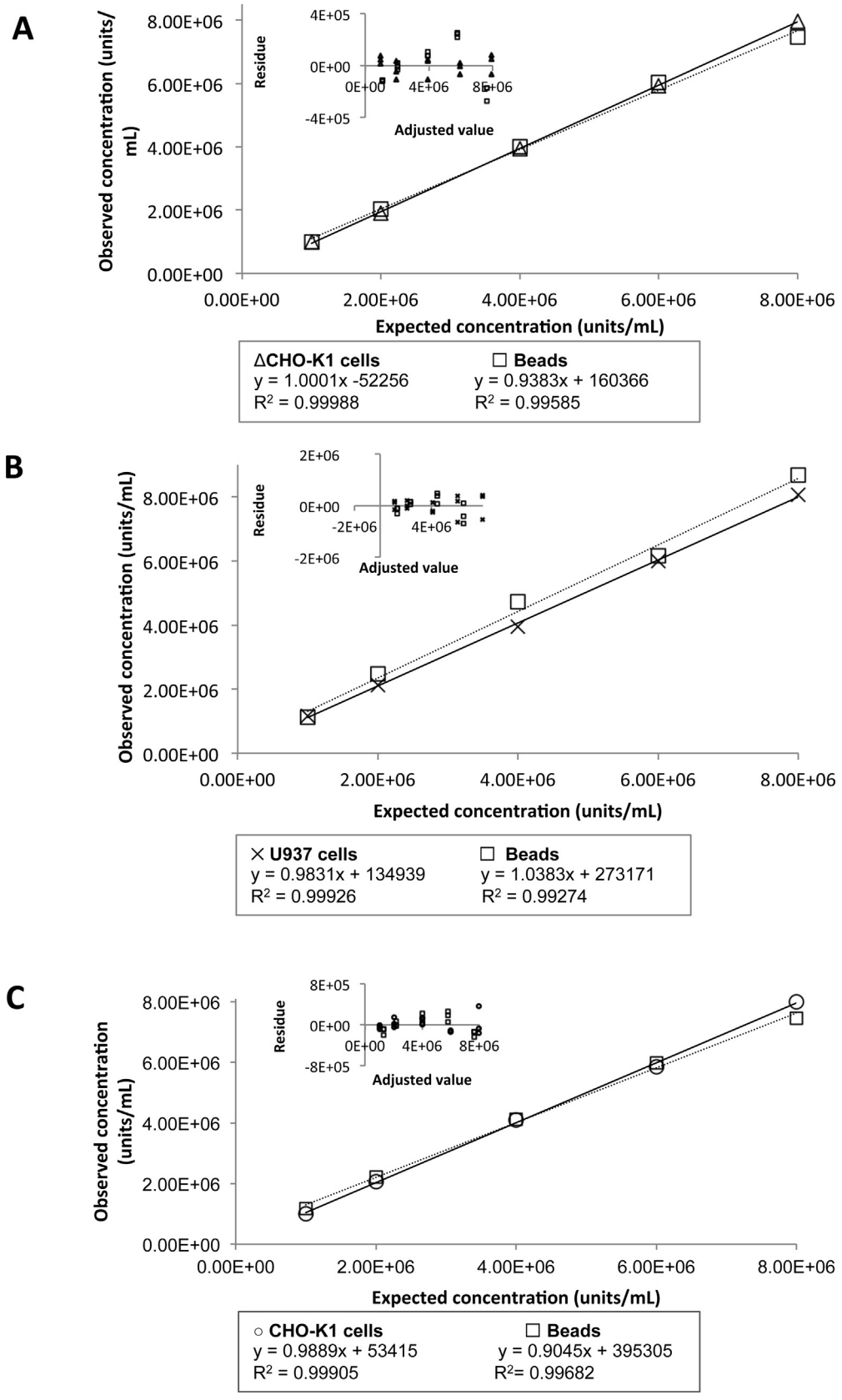

Fig. 2. Concentration linearity obtained for: (A) CHO-K1 cells by hemocytometer, (B) U937 cells by Countess®, and (C) CHO-K1 cells by Vi-CELL® XR; all compared versus concentration beads.

in binary images and allowed the software to perform the analysis with more precision and accuracy than the other trypan blue concentrations.

3.1. Linearity and range

3.1.1. Hemocytometer

Curves of cell concentration and cell viability obtained for both cell lines and beads using the hemocytometer, fitted using a linear model within the studied ranges (concentration: 1×10^6 to 8×10^6 units/mL and viability: 0–100% for the three methods) (Fig. 3). R^2 values were ≥0.99 in all cases, and the slopes varied from 0.938 for the concentration curves of beads to 1.002 for the viability of CHO-K1 cells (Table 3).

3.1.2. Countess®

Cell concentration and cell viability curves obtained from the Countess® fitted with a linear model within the studied ranges (Fig. 2). R^2 values for the concentration curves were ≥0.99 in all the cases, while for viabilities the R^2 values varied between 0.98 and 0.99 (Table 3). However, slopes showed differences among the bead counts ($m = 1.038$) and cell counts for CHO-K1 cells ($m = 0.80$) this difference might be attributable to the difference in size and morphology of CHO-K1 cells with respect to the beads (cells are larger than the beads) that influenced the capability of the instrument to disregard cells during countings. It was observed that as the cell titration increased, the difference with respect to the expected value also increased. This variability suggests that the capabilities in the hardware and software of the instrument that are influenced by the adjustment of circularity, size range and sensitivity settings, and the accurate range recommended by the manufacturer (1×10^4 to 4×10^6 units/mL) require to be customized for each cell line.

3.1.3. Vi-CELL® XR

R^2 values obtained from cell counts and viability measurements within the studied ranges using Vi-CELL® XR were >0.99 in all cases (Fig. 2). Linearity evaluated through this method was similar to the obtained from the hemocytometer as denoted by their R^2 values; furthermore results between cells and beads were similar (Table 3). Despite that the Vi-CELL® XR uses an automated imaging system controlled by software, this instrument provided more consistent results than Countess®; this consistency might be associated with an improved imaging technology and the flexibility in the adjustment of input parameters such as brightness, circularity, size, cell spot area, dilution factor, and the capability to differentiate cells within clusters allowing a more extensive major control for analyzing samples.

3.2. Specificity

Media and buffer were analyzed by the Countess® for matrix effects which displayed an alert stating that the measurements were below the lower limit that the instrument is capable of measuring (1×10^4 units/mL) as expected for this parameter since the samples lacked cells. On the other hand, specificity measurements conducted with the Vi-CELL® XR provided counting values of 0.00 units/mL and 0.0% viability, indicating no interference of matrix effect with the readings and that this method is specific as well. In summary, Countess® and Vi-CELL® XR were demonstrated to be specific for detecting both cells and beads without interference of media or buffer matrix.

3.3. Accuracy

According to the results from the accuracy tests, a global averaged recovery of 99.4% (calculated from the collective recovery values for the two cell lines and beads) was obtained using the hemocytometer (Table 4). For Vi-CELL® XR and Countess® the global averaged recovery values were 104.7% and 99.25%, respectively. Viability readings obtained by the three methods were comparable for concentration percentages (Table 4). Both viability and concentration data fitted within the recovery range of 90–110%. The Countess® presents major variability at higher cell concentrations. This result might be associated with a major degree of cell clusterization at higher cell concentrations, and the instrument is unable to recognize them as independent units and excludes them from the analyses. It is remarkable that as the cell concentration increased the error decreased, because the probability of including a representative number of cells in the sample under the visual field is higher at a major cell density up to the saturation, which determines the linear range of analysis.

Table 3
Concentration and viability linearity results obtained by the three cell counting methods for each cell line and standards.

Attribute	Method	Cell line/standard	R^2	y
Concentration	Hemocytometer	U937 cells	0.9999	$0.996x + 42134$
		CHO-K1 cells	0.9999	$1.000x - 52256$
		Concentration beads	0.9959	$0.938x + 16037$
	Countess®	U937 cells	0.9993	$0.983x + 13494$
		CHO-K1 cells	0.994	$0.802x - 426.83$
		Concentration beads	0.9927	$1.038x + 27317$
	Vi-Cell®XR	U937 cells	0.9999	$0.982x + 19555$
		CHO-K1 cells	0.9991	$0.989x + 53415$
		Concentration beads	0.9968	$0.905x + 39531$
Viability	Hemocytometer	U937 cells	0.9989	$0.982x + 0.2117$
		CHO-K1 cells	0.998	$1.002x + 0.9229$
		Viability standards	0.9995	$0.996x + 0.7791$
	Countess®	U937 cells	0.9804	$0.878x + 5.5657$
		CHO-K1 cells	0.9928	$0.940x - 0.2851$
		Viability standards	0.9873	$0.991x + 1.7119$
	Vi-Cell®XR	U937 cells	0.9984	$0.980x + 1.5634$
		CHO-K1 cells	0.9995	$1.007x + 0.1424$
		Viability standards	0.9991	$1.006x + 1.1386$

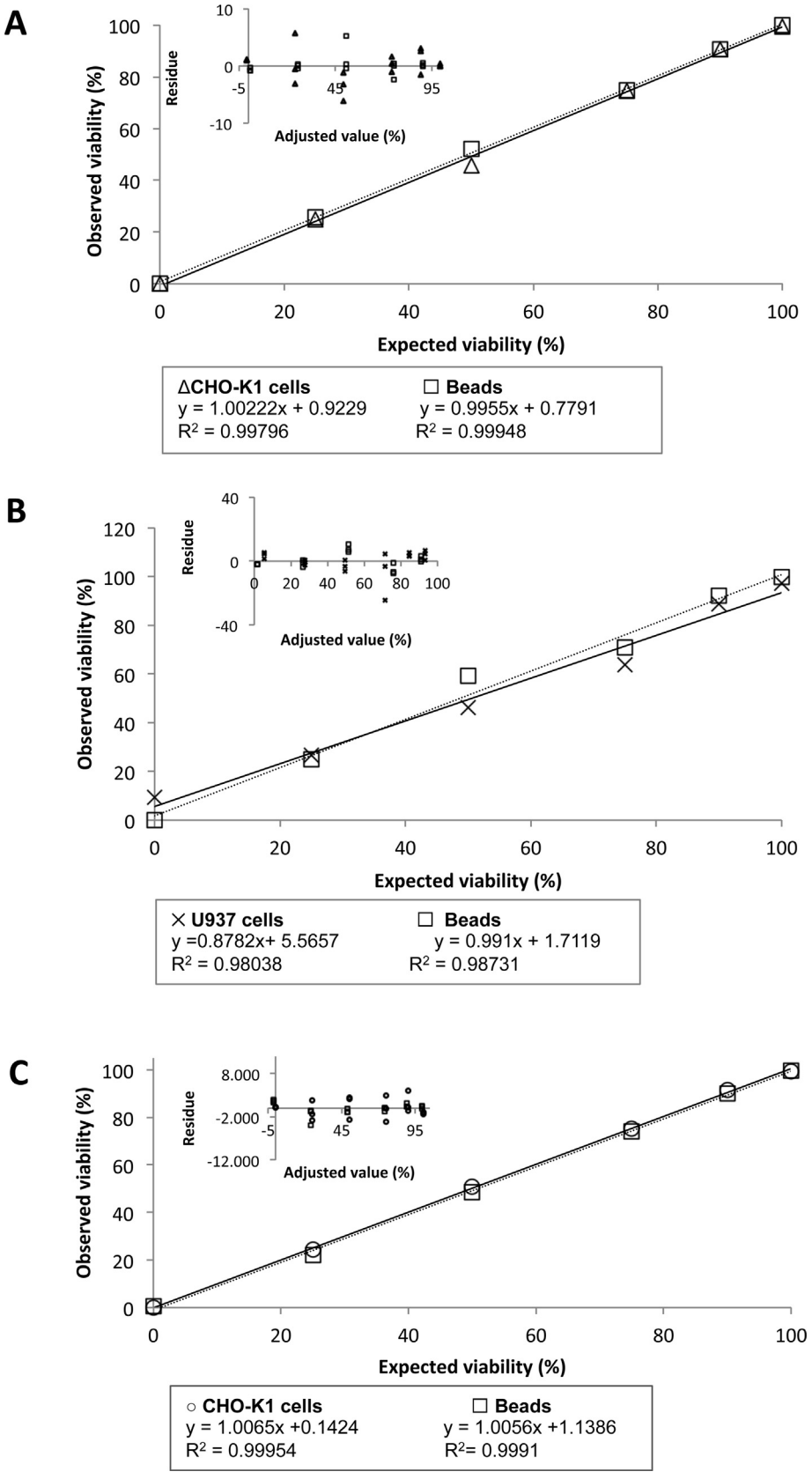

Fig. 3. Viability linearity obtained for: (A) CHO-K1 cells by hemocytometer, (B) U937 cells by Countess®, and (C) CHO-K1 cells by Vi-CELL® XR; all compared versus viability standards.

Table 4
Recovery values obtained by each cell counting method.

Cell lines/ standards	Concentration (units/mL)	Viability (%)	Hemocytometer		Countess®		Vi-CELL® XR	
			Count recovery (%)	Viability recovery (%)	Count recovery (%)	Viability recovery (%)	Count recovery (%)	Viability recovery (%)
U937	1×10^6	25	102.0 ± 2.5	100.9 ± 18.4	117.0 ± 45.6	106.7 ± 15.2	119.3 ± 10.3	105.3 ± 16.5
	2×10^6	50	103.3 ± 5.2	95.3 ± 7.6	106.7 ± 19.0	92.7 ± 17.4	106.8 ± 1.9	106.1 ± 7.2
	4×10^6	75	99.7 ± 2.5	101.0 ± 6.5	98.8 ± 13.5	84.9 ± 49.6	102.8 ± 1.9	101.2 ± 4.0
	6×10^6	90	100.8 ± 1.3	97.0 ± 4.2	100.0 ± 21.9	98.5 ± 3.2	102.1 ± 1.3	99.0 ± 2.1
	8×10^6	100	100.5 ± 1.9	98.7 ± 1.4	100.8 ± 16.6	97.3 ± 7.6	100.3 ± 4.1	98.3 ± 0.4
CHO-K1	1×10^6	25	99.0 ± 7.4	99.1 ± 45.0	92.5 ± 10.8	100.7 ± 15.9	99.3 ± 5.9	97.1 ± 23.9
	2×10^6	50	95.3 ± 8.7	91.3 ± 12.5	78.3 ± 28.0	84.7 ± 12.5	103.2 ± 8.2	101.7 ± 14.4
	4×10^6	75	98.4 ± 5.2	99.4 ± 4.5	79.2 ± 7.8	96.0 ± 15.2	102.4 ± 3.2	100.3 ± 10.1
	6×10^6	90	98.8 ± 1.9	100.7 ± 6.8	75.8 ± 2.1	90.0 ± 2.8	97.6 ± 0.3	101.8 ± 6.9
	8×10^6	100	99.6 ± 2.5	99.4 ± 0.7	82.7 ± 5.5	97.0 ± 6.6	100.1 ± 6.0	99.4 ± 0.7
Beads	1×10^6	25	98.7 ± 1.4	102.4 ± 3.6	113.3 ± 28.7	100.0 ± 24.3	117.7 ± 16.5	88.8 ± 18.9
	2×10^6	50	101.7 ± 3.1	104.5 ± 15.2	123.3 ± 7.2	138.3 ± 13.8	110.7 ± 6.1	97.0 ± 2.2
	4×10^6	75	100.0 ± 1.1	99.7 ± 5.0	118.3 ± 12.9	94.7 ± 18.9	103.2 ± 5.9	98.8 ± 1.5
	6×10^6	90	100.4 ± 0.7	100.6 ± 0.8	102.8 ± 16.7	102.4 ± 5.3	99.8 ± 4.2	100.1 ± 1.3
	8×10^6	100	93.2 ± 1.8	99.9 ± 0.3	108.3 ± 17.9	99.8 ± 1.0	93.2 ± 1.8	99.7 ± 0.8

Table 5
Repeatability obtained by the three cell counting methods evaluated by RSD.

Viable cell counting method	Cell line/standard	Mean(1×10^6 units/mL)	Standard deviation (1×10^5)	%RSD
Hemocytometer	U937	1.05	0.85	8.06
	CHO-K1	0.97	0.27	2.81
	Beads	0.99	0.07	0.75
Vi-CELL® XR	U937	1.14	0.26	2.27
	CHO-K1	0.99	0.55	5.28
	Beads	1.09	0.40	3.68
Countess®	U937	1.04	1.30	12.50
	CHO-K1	0.89	0.99	11.04
	Beads	1.10	0.16	14.30

3.3.1. Precision (repeatability)

The RSD values for the system precision tests using a hemocytometer were 8.06% for U937 cells, 0.75% for concentration beads and 2.81% for CHO-K1 cells (Table 5). Vi-CELL® XR always showed RSD values $\leq$ 5.28%. Countess® presented more variability, since its lower RSD value was 11.04% for CHO-K1, whereas its higher value was up to 14.30% for beads (Table 5).

4. Conclusions

According to the results obtained from this work, we observed that the three methods are suitable to perform viable cell counts. In general, the hemocytometer and Vi-CELL® XR showed similar results in all the evaluated characteristics. Results obtained by the Countess® were more variable than that obtained by the other methods; nevertheless, this variability can be acceptable for considering the Countess® as an alternative to the hemocytometer in processes that involve the analyses of a lot of samples, as long as this variability will not affect the process under study.

Despite the manual method continuing to be the more employed due to the quality of its results and low cost, it requires a certain degree of expertise of the analyst and it is time consuming; therefore, it is difficult to be implemented for large-scale analyses, on the other hand Vi-CELL® XR and Countess® provide the opportunity of analyzing a huge number of samples in a short time with reliable results, after the adjustment of the instruments for cell type and cell culture growth parameters.

Other advantages of using automated systems such as Vi-CELL® XR and Countess® is that their hardware and software comply with the international regulations for working under a good manufacturing practices environment, and the data obtained from the analyses is stored under a security-chronological record (audit trail). Likewise, the manufacturers provide technical support, documentation and recommendations to the personnel at each laboratory to perform installation and operational qualification (IQ/OQ) procedures, which facilitates the process of validation of the instruments.

Conflict of interest

The authors declared no conflict of interest.

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