Validation of an automated cell counting method for cGMP manufacturing of human induced pluripotent stem cells

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) must be manufactured as advanced therapy medicinal products (ATMPs) for innovative tissue replacement clinical applications. Yet, production of hiPSCs under current Good Manufacturing Practice (cGMP) presents many hurdles, such as the large-scale cell expansion needed to reach therapeutically-relevant hiPSC doses. For the monitoring of this phase, a fast and reliable cell counting method should be used. Conventional manual cell counting by the hemocytometer method is dependent on the operator's expertise and is time-consuming. Therefore, automation of sample preparation and analysis is needed to improve precision and rapidity of hiPSC cell counting. We investigated whether an automated cell counting method could be validated for use with hiPSCs, in comparison with a reference cell counting method included in the European Pharmacopeia, 10th edition. The proposed method was the fluorescence imaging-based NucleoCounter NC-100 system, whereas the reference method was manual cell counting using a Bürker hemocytometer. The validation strategy complied with EudraLex cGMP regulations for ATMP manufacturing and ICH Q2(R1) indications for validation of analytical methods. The use of the NucleoCounter NC-100 system for automated cell counting was validated, focusing on accuracy, specificity, intra- and inter-operator reproducibility, range and linearity, showing higher precision than the manual method. The automated method can be used more effectively than the manual one for hiPSC cell counting. Thus, this piece of work paves the way for all cGMP facilities that want to pursue hiPSC manufacturing for clinical use.

1. Introduction

As of 2021, human induced pluripotent stem cells (hiPSCs) are fulfilling the promise of the ideal starting cell type for the development of new tissue replacement approaches in regenerative medicine, with a growing number of ongoing clinical studies (https://hpscreg.eu/) [1, 2]. The most targeted pathologies are degenerative diseases of the retina [3], cardiomyopathies [4] and central nervous system disorders [5].

Moving from the research setting to the clinic, hiPSCs must be manufactured as advanced therapy medicinal products (ATMPs) as defined by the European regulations, under current Good Manufacturing Practices (cGMP) [6, 7]. This entails a radical shift of paradigm for many aspects spanning from hiPSC generation and expansion to differentiation into mature cell types with specialized functionalities, raising crucial issues that must be addressed [8, 9].

Concerning the hiPSC expansion phase, a consistent, rapid and reliable cell counting method is necessary as in-process control of cell growth kinetics. Furthermore, to enable product release, the determination of a proper cell dose using an accurate cell counting method is critical. Hemocytometer-based manual counting is the reference method and the only one described in the European Pharmacopeia (EP 10th ed. Par 2.7.29 cell count). Yet, it heavily depends on the analyst, it is time-consuming and hard to standardize [10, 11]. On the contrary, the recently developed automated cell counting methods reduce the analyst-dependent results variability and analysis time, offering a methodology that can be easily validated to serve as an analytical procedure for ATMP manufacturing [12, 13].

Guidelines for validation of analytical procedures are contained in the International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use,

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Validation of Analytical Procedures: Text and Methodology Q2(R1). Specifically, EP, 10th edition: European Directorate for the Quality of Medicines & HealthCare, ISO 20391 Biotechnology - Cell counting and EudraLex, Volume 4, Annex 15 and Part IV - GMP requirements for Advanced Therapy Medicinal Products (https://ec.europa.eu/health/documents/eudralex/vol-4_it) also provide indications for optimal validation strategies in the frame of cGMP production of ATMPs, since cell enumeration has a strong impact on cell-based therapy in terms of assessment of potency and efficacy.

Indeed, it is of paramount importance, and required by the regulatory bodies, to determine, among other features: (i) the accuracy of the proposed analytical procedure with respect to the method of reference; (ii) its precision under same (within single run) or different analytical conditions (between different runs); (iii) its ability to provide results that correspond to the amount of analyte in a linear fashion; (iv) its range of reliability.

Each new ATMP has to be analyzed for every fit-for-purpose need. In our case, the cell count methodology chosen has to be validated, because each product has inherent properties that can alter the results of analytical procedures or impede its application, and the suitability for the intended purpose has to be demonstrated. To this aim, we validated the use of the automated cell counting system NucleoCounter NC-100 with research-grade hiPSCs, manufactured following protocols translatable to the clinical cGMP environment.

2. Material and methods

2.1. Aim and design of validation protocol

The aim of the study was to validate an automated cell counting method for use with human induced pluripotent stem cells (hiPSCs) in view of clinical manufacturing in compliance with current Good Manufacturing Practices (cGMP). Validation was planned following the guidelines by the International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use, Validation of Analytical Procedures: Text and Methodology Q2(R1). EP 10th ed. Par 2.7.29 cell count and ISO 20391 related to cell counting for biotechnology, and EudraLex, Volume 4, Annex 15 and Part IV - GMP requirements for Advanced Therapy Medicinal Products were also taken into account. Validation was conducted on different hiPSC batches ($n = 3$) to account for biological variability. These research-grade hiPSC batches were generated by reprogramming of long-living mesenchymal stromal cells isolated from cord blood [14]. For each batch three runs of analysis were performed by each analyst ($n = 2$). Samples for the three runs were prepared independently. Evaluation of validation was performed on the mean of three runs, each resulting from the mean of three hiPSC batches.

2.2. Prerequisites and setting: instrument validation, reagents and personnel

The Cell Factory of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico is a cGMP facility for ATMP production based in Milan, Italy, certified in 2007 by the Italian Drug Agency (Agenzia Italiana del Farmaco – AIFA) in compliance with European cGMP regulations (first authorization number 120/2007 of July 5, 2007) [15]. As required by the cGMP guidelines, equipment, instrument, and other devices used in our cGMP facility were subjected to installation qualification (IQ), in accordance with the manufacturer's specifications and to operational qualification (OQ), demonstrating the instrument functions according to the operational procedures. Instrument maintenance is strictly monitored as previously described [15]. Instruments and software were compliant with EudraLex, Volume 4, Annex 11: Computerized Systems. Reagents and materials used for Quality Control (QC) tests were of appropriate quality and used according to instructions. Their identification and verification were performed upon receipt or before use as a

standard procedure in our facility. Personnel had the appropriate qualifications and adequate practical experience relevant to the intended operations and received initial and periodic training relevant to their tasks. Risk management was conducted in accordance with Annex 20 of the PIC/S GMP Guide and ICH Q9 guideline, regarding the use of research-grade hiPSC and reagents in place of GMP-grade ones.

2.3. Sample preparation

The hiPSC were expanded onto hESC-qualified Matrigel (354277; Corning, Corning, NY, USA)-coated culture surfaces in complete TeSR-E8 (05990; STEMCELL Technologies, Vancouver, BC, Canada) at 37 °C, 20% O_2, 5% CO_2. To obtain single cell suspensions, hiPSC were incubated with accutase (L0950; Biowest, Nuaillé, France) for 5 min at 37 °C and collected in D-PBS without Ca^{2+} and Mg^{2+} (ECB4004L; Euroclone, Pero, Italy). The hiPSC were pelleted at 300 ×g for 10 min and resuspended in D-PBS for cell counting. Samples were diluted to comply with the analytical ranges of the cell counting method to be used.

2.4. Manual cell count

A Bürker hemocytometer was used for manual cell counting. Description of its principles is contained in EP, 10th edition: European Directorate for the Quality of Medicines & HealthCare and detailed elsewhere [13]. A volume of 10 μL of cell suspension was loaded into each chamber of the hemocytometer for a total of two samplings. For each sampling, counts were performed in duplicate by two analysts under a microscope with a 20X objective (Nikon Eclipse 50i; Nikon, Tokyo, Japan). Bright field images were taken with the same microscope. Viable cells were counted based on morphology. Cell counts were accepted in the 50,000–550,000 cells/mL range. The cell concentration of hiPSC suspensions outside this range was adjusted accordingly.

2.5. Automated cell count

Automated cell count was conducted on a NucleoCounter NC-100 (Chemometec). The functioning principles of this instrument are based on Propidium Iodide (PI) incorporation and were described in a previous paper [16]. For total cell count, 100 μL of cell suspension was pretreated with a mixture of 100 μL of lysis buffer and 100 μL of stabilizing buffer (Chemometec, Allerod, Denmark) before measurement. Viable cell count was calculated after determination of non-viable cells analyzed without pretreatment. The proprietary software automatically calculated the starting cell concentration. Counts were performed in one replicate or in duplicate by each of two analysts, as specified below for determination of accuracy and precision. Cell counts were accepted in the 5,000–2,000,000 cells/mL instrument range. The cell concentration of hiPSC suspensions outside this range was adjusted accordingly.

2.6. Specificity

D-PBS without Ca^{2+} and Mg^{2+} (Euroclone) was analyzed by the automated cell counting method as the matrix of the hiPSC samples to account for any interference on readings by any possibly present contaminating particles. Misidentification by the manual method was not considered a risk and thus not evaluated.

2.7. Linearity

Linearity was addressed using hiPSC samples ($n = 5$ each run) generated by 1:2 serial dilutions. For each hiPSC batch, serial dilution triplicates were independently prepared for the three runs of validation. Two starting cell concentrations were used: 2×10^6 cells/mL and 4×10^6 cells/mL. Concentration of total cells was considered. Direct proportion of measured values to expected values was verified by linear

regression, reported as intercept, slope and determination coefficient (R^2) values. The goodness of fit of the model was evaluated by a residual analysis via a residuals versus fits plot to detect non-linearity, unequal error variances and outliers. Expected values were calculated for each hiPSC batch and starting cell concentration. For the inference, the two subsequent samples in each dilution series triplicate resulting in the lower coefficient of variation (CV) among their measured values were identified. Based on the mean of their cell counts, all expected values were determined taking into account the dilution factors. Acceptance criterion was adjusted $R^2 \geq 0.9$.

2.8. Range

To evaluate the interval of hiPSC concentrations for which the automated cell counting method has a suitable level of linearity, the values obtained for linearity validation were re-analyzed, comparing measured and expected values individually calculated for each independent run. A range CV ($CV_{RA} \leq 20\%$) was applied as acceptance criterion for a suitable level of linearity.

2.9. Accuracy

Accuracy was assessed as closeness of agreement between the automated (proposed method) and manual (reference method) cell counts across the range of the former analytical procedure. The same sample preparations were used for the measurement by the two methods. The number of viable cells was considered. Accuracy CV (CV_A), expressed as percentage and calculated as the ratio of the standard deviation to the mean was used to measure the dispersion between the values measured by the two analytical cell counting methods. Accuracy error (E_A) was calculated as the difference between the candidate and reference values expressed as absolute value. Accuracy degree ($\%_A$) was calculated as the ratio of candidate to reference values and expressed as percentage. Acceptance criteria were set to $CV_A \leq 10\%$, $E_A \leq 150,000$ cells/mL, $90 \leq \%_A \leq 110\%$.

2.10. Precision

Precision of the automated method was assessed as closeness of agreement between the following series of measurements obtained across the range of the proposed analytical procedure: (i) values obtained from two subsequent samplings of the same hiPSC suspension preparation by the same analyst (repeatability); (ii) values obtained from two subsequent samplings of the same hiPSC suspension preparation each by a different analyst (intermediate precision). For the manual method, a different series of measurements were considered: (i) values obtained from the same sampling of the hiPSC suspension by two different analysts (repeatability); (ii) values obtained from two subsequent samplings of the same hiPSC preparation under the same operating conditions, each performed by two different analysts (intermediate precision). The number of viable cells was considered. Precision was expressed as CV of either repeatability (CV_{RE}) or intermediate precision (CV_{IP}) measurements. Means and standard deviations were also reported. Acceptance criteria for the proposed method were set to $CV_{RE} \leq 5\%$ and $CV_{IP} \leq 5\%$.

2.11. Statistics

Generated data were analyzed as described above, following indications by the aforementioned IHC guidelines. Statistical analysis was conducted using a spreadsheet software as outlined in detail in the previous paragraphs.

3. Results

3.1. Validation strategy

The validation of cell count is essential for a reliable dose quantification of ATMPs. Following the outline contained in IHC Q2(R1), the validation of the automated counting method by NucleoCounter NC-100 was performed assessing its characteristics in the following order: specificity, linearity, range, accuracy and precision. The reference method for accuracy was manual cell counting by Bürker hemocytometer, as described in the EP 10th ed. Par 2.7.29 cell count. Accuracy and precision were then evaluated within the defined range of the proposed analytical procedure to assess its characteristics relevant to the intended use. Cell counting process, including sample preparation, measurement and data analysis was compliant to ISO 20391.

Two analysts were involved in all validation steps to take into account inter-operator variability. Three research-grade hiPSC batches previously generated and fully characterized for self-renewal and pluripotency [14] were used as surrogate cell samples for all the validation experiments. The cGMP guidelines specific to ATMPs allow the use of surrogate material for process validation in case of starting material shortage. The use of research-grade hiPSC for the validation of an analytical method was subject to a risk analysis that ensured its suitability for the intended purposes, in reason of the adequate comparability of the surrogate cell sample to the cell product that will be subject to manufacturing process validation.

The cells were grown and prepared under protocols translatable to cGMP settings for future use of the analytical procedure in hiPSC manufacturing for clinical use. Evaluation of the methods' characteristics was performed in three runs for each hiPSC batch by each analyst. The validation strategy is summarized in Fig. 1.

3.2. Specificity, linearity and range

In the absence of inherent impurities of the samples, specificity of the NucleoCounter NC-100 was investigated on the matrix of the hiPSC suspension. No contaminating particles/unspecific events were detected.

To assess linearity across the entire range of measurement of the NucleoCounter NC-100, two different starting hiPSC concentrations were used (Fig. 2A for schematics). Total cells were considered for validation of linearity to rule out any bias related to decrease of viability during extensive manipulation and time-demanding analysis of many samples.

Measured values were directly proportional to estimated values in both experimental conditions for all hiPSC batches, as shown by linear regression performed on values of serial dilution samples starting from 2 (Fig. 2B) and 4 (Fig. 2C) million cells per mL. Determination coefficient (R^2) was higher than 0.98 for all runs of validation. For a deeper understanding of the goodness of the regression model, residual analysis by residuals versus fits plot was performed on 2 (Fig. 2D) and 4 (Fig. 2E) million cells per mL serial dilutions. Globally considered, the plots showed a satisfactory dispersion of the residuals, compatible with linearity, absence of unequal error variances and outliers. Adjusted R^2 was also taken into account as a less biased estimator than ordinary R^2, resulting again in values higher than 0.98 for all runs of validation starting from 2 (Fig. 2F) and 4 (Fig. 2G) million cells per mL.

Range of hiPSC automated cell counting by the NucleoCounter NC-100 was determined based on the linearity data applying the acceptance criteria of $CV_R < 20\%$ (Additional file 1). The runs of validation of the three hiPSC batches were considered separately and resulted in a range of 0.19–5.06 million cells/mL.

3.3. Accuracy

The protocol of validation to assess accuracy (A) is illustrated in
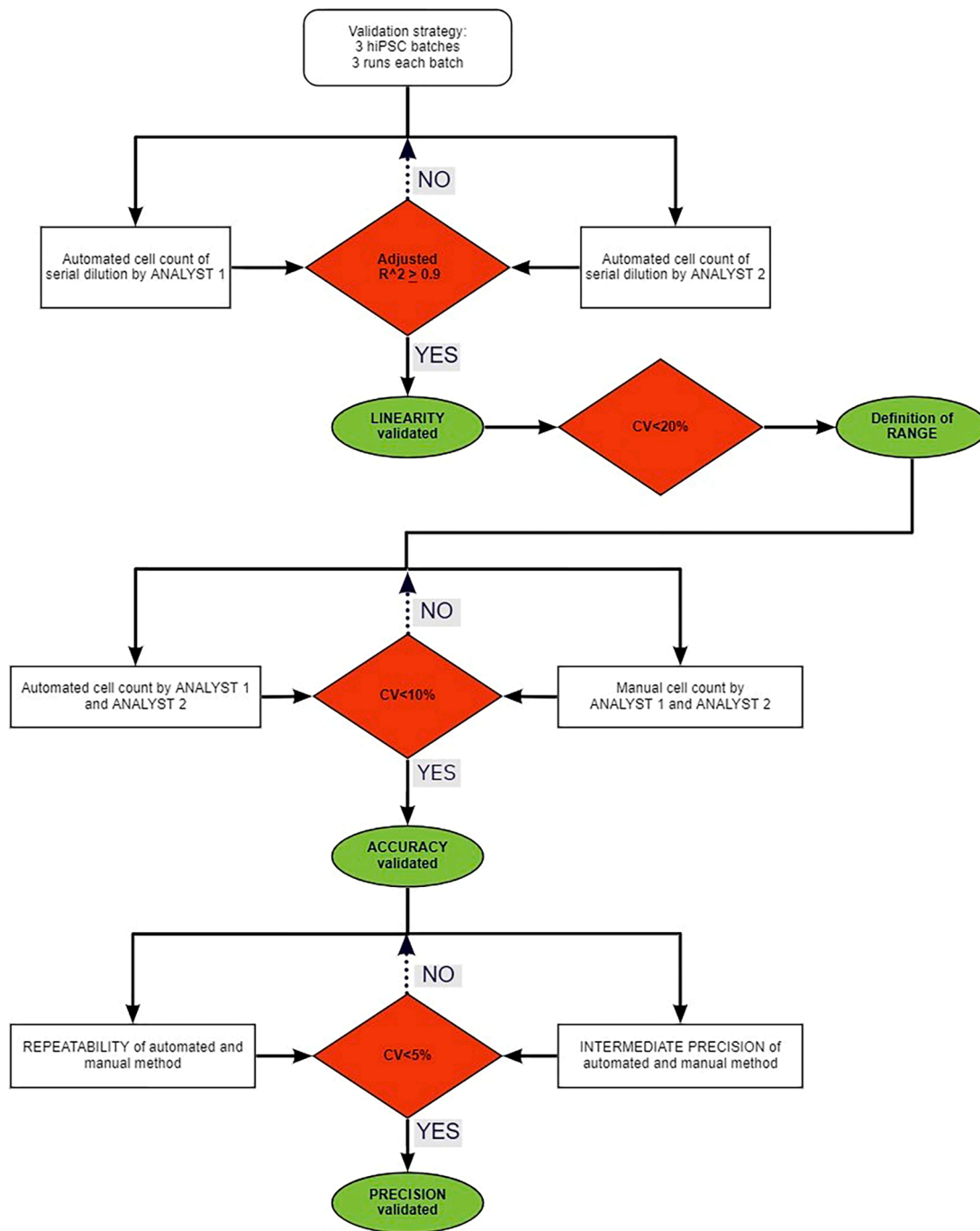
Fig. 1. Validation strategy. Flowchart summarizing the strategy adopted for the validation of the automated cell counting method. In accordance with ICH Q2(R1), three runs of validation were performed for each hiPSC line ($n = 3$) by two analysts. Acceptance criteria specified in the red rhombuses were applied to the final mean of all runs. CV, coefficient of variation. Created with Miro.com.

Fig. 3A. Bürker hemocytometer cell counting method was performed following standard procedures, as shown in Fig. 3B. The manual method allowed for discrimination of cells from any possible contaminating impurity by morphology evaluation, while the automated method provided a fluorescence image representative of the analyzed sample, which allowed for quality check of the prepared hiPSC suspension by assessment of a homogeneous dispersion of single cells (Fig. 3C).

The NucleoCounter NC-100 showed a suitable accuracy in the majority of the performed runs with final mean values of the defined validation parameters meeting the acceptance criteria: $CV_A = 9.7\%$, $E_A = 0.11$ million cells and $\%_A = 109.1\%$ (Fig. 3D).

3.4. Precision

Taking into account the principles of operation of the NucleoCounter NC-100, its precision was evaluated as repeatability (RE) by the same analyst and intermediate precision (IP) by two different analysts (Fig. 4A). As summarized in Fig. 4B, the automated cell counting method showed a $CV_{RE} < 5\%$ for each independent run, with a final average $CV_{RE} = 3.1\%$, which satisfactorily met the defined acceptance criteria. To note, each of the two analysts involved in the validation obtained a $CV_{RE} < 5\%$. Intermediate precision resulted in a $CV_{IP} \leq 5\%$ for each hiPSC batch evaluated as a triplicate of independent runs, which thus led to a final average $CV_{IP} = 4.7\%$ (Fig. 4C).

Fig. 2. Linearity of automated hiPSC cell counting method. (A) Schematics summarizing the experimental approach for assessment of linearity; 1:2 serial dilutions were performed to prepare three independent set of samples from a common starting cell concentration to be analyzed by two analysts; created with BioRender.com. Plots showing the mean value of three runs of validation for the hiPSC batches used, starting from 2 (B) and 4 (C) million cells/mL; linear regression is also represented. Residuals versus fits plots showing distance of measured from estimated values for each data point of each validation run for the hiPSC batches used, starting from 2 (D) and 4 (E) million cells/mL. Tables reporting adjusted R^2 of the applied linear regression models for the hiPSC batches used, starting from 2 (F) and 4 (G) million cells/mL. hiPSC, human induced pluripotent stem cells; R^2, coefficient of determination.

a

b

c

d

hiPSC batch	Method	Run 1	Run 2	Run 3	Mean
#1	NC	1.2E+06	2.0E+06	8.7E+05	
	HCM	1.1E+06	2.3E+06	9.3E+05	
	Accuracy error	4.2E+04	2.6E+05	6.2E+04	1.2E+05
	Accuracy recovery	104%	89%	93%	95%
	CV	2.6%	8.6%	4.9%	5.4%
#2	NC	3.5E+05	9.6E+05	8.4E+05	
	HCM	2.9E+05	6.4E+05	6.7E+05	
	Accuracy error	5.9E+04	3.2E+05	1.7E+05	1.8E+05
	Accuracy recovery	121%	149%	125%	132%
	CV	13.3%	27.9%	15.9%	19.0%
#3	NC	1.9E+05	2.7E+05	2.7E+05	
	HCM	1.7E+05	2.9E+05	2,8E+05	
	Accuracy error	1.8E+04	1.9E+04	7.9E+03	1.5E+04
	Accuracy recovery	110%	93%	97%	100%
	CV	7.0%	4.8%	2.0%	4.6%
Final mean	Accuracy error	1.1E+05			
	Accuracy recovery	109%			
	CV	9.7%			

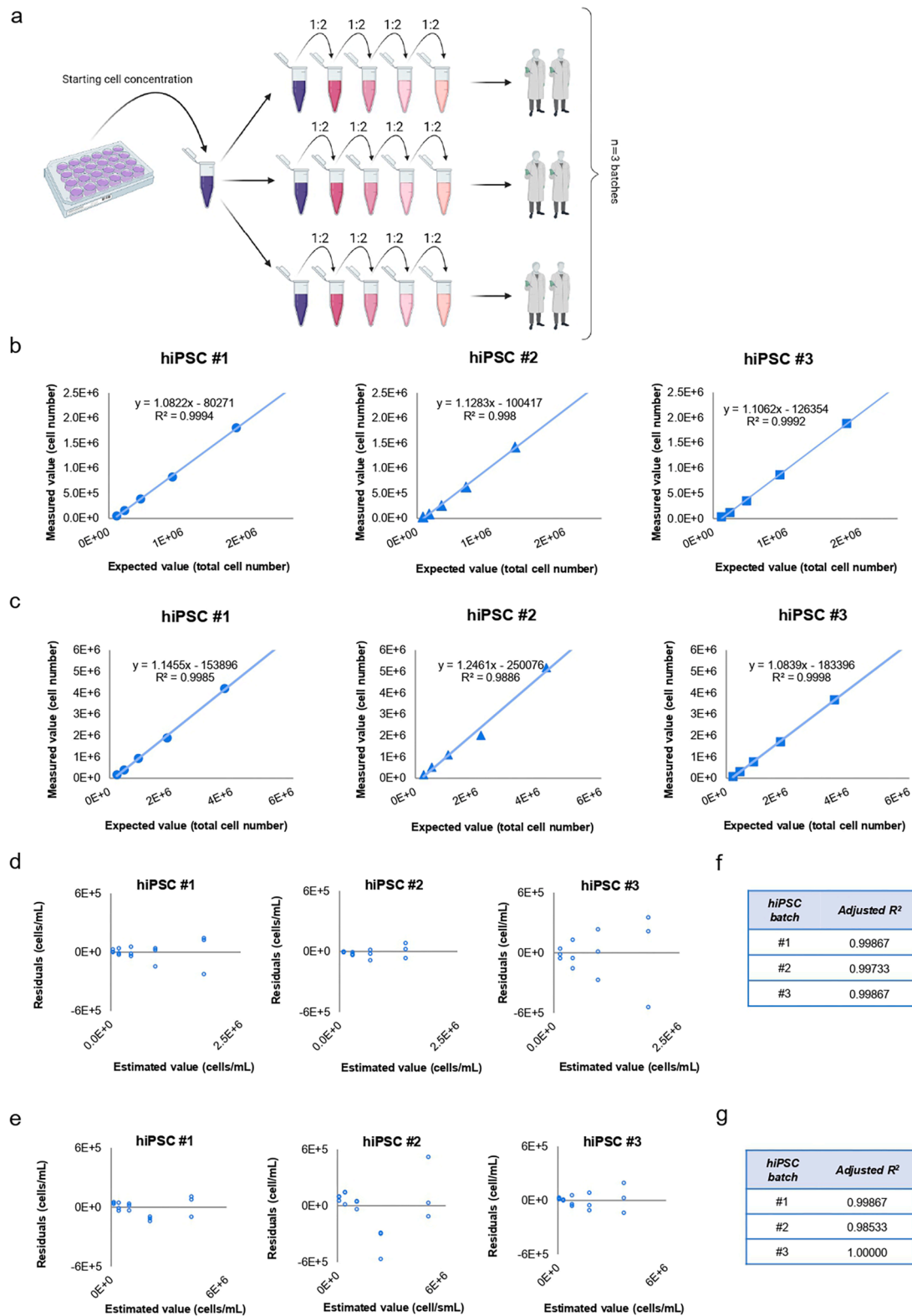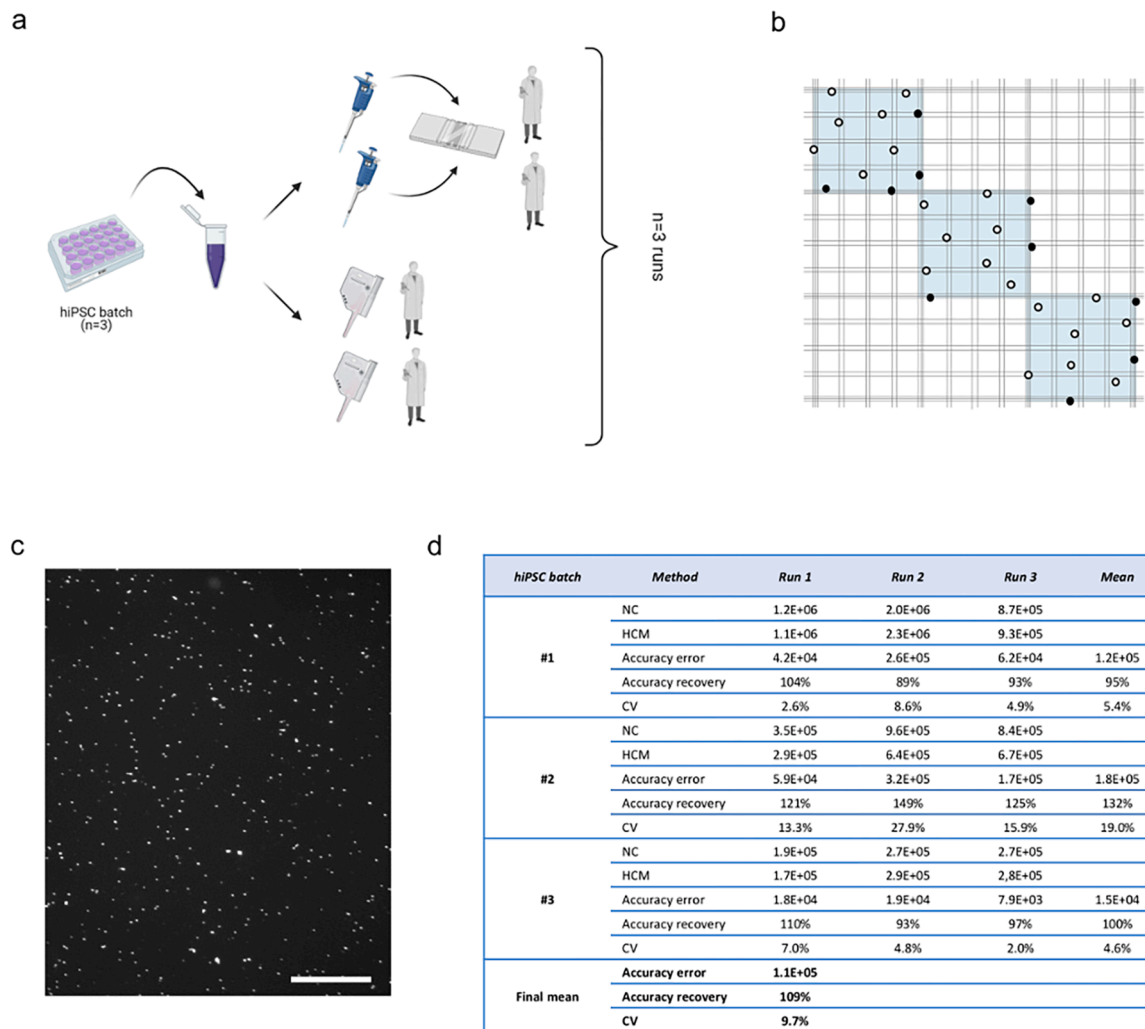**Fig. 3.** Accuracy of automated hiPSC cell counting method. (A) Schematics summarizing the experimental approach for assessment of accuracy; three runs of validation were performed by two analysts comparing the manual (top) with the automated (bottom) method; counts were performed in duplicate; created with BioRender.com. (B) Schematics of the manual counting method strategy; three squares (blue) of the Bürker chamber were considered for cell count, excluding the cells localized at the right and bottom sides of the squares (black dots). (C) Representative output image of the analyzed cell suspension produced by the Nucleo-Counter NC-100 software; scale bar is 1 mm. (D) Table reporting the measured values and means of three runs of validation for the hiPSC batches used and associated accuracy error, recovery, coefficient of variation (CV). hiPSC, human induced pluripotent stem cells; HCM, hemocytometer; NC, NucleoCounter.

To compare the characteristics of the proposed method with the reference method, precision was determined also for the manual cell counting method. Assessment of precision for the Bürker hemocytometer followed the schematics in Fig. 4D. Repeatability was defined as intra-assay precision, considering repeated counts of the same sample by different analysts. All runs had a $CV_{RE} < 20\%$, and a final mean $CV_{RE} = 7.1\%$ higher than the value obtained for the automated cell counting method (Fig. 4E). Intermediate precision was defined as inter-assay precision, considering counts of different samplings of the same hiPSC preparation, performed by two analysts. All hiPSC batches led to a $CV_{IP} < 20\%$, and a final mean CV_{IP} of 10.3%, again higher than the proposed method (Fig. 4F).

4. Discussion

Reliable cell counting methods are needed for standardization of quality control activities, ensuring straightforward manufacturing processes. Furthermore, they guarantee reproducible preparation of cell product doses over time in the same facility or between different facilities. Precise cell concentration of the final product of a ATMP manufacturing process is also crucial to perform consistent and reliable

potency assays for the evaluation of therapeutic efficacy [17, 18].

Following EudraLex indications, analytical procedures for cell counting described in the EP 10th are considered validated, whereas their suitability for the intended purpose should be verified. These methods are: hemocytometer-based manual counting; automated particle counters based on conductivity variation and flow cytometry. Although these methods have the advantage of being already included in the EP 10th, other imaging-based automated methods dramatically reduce analyst-associated variability, provide visual representation of cell suspensions, and allow scalability. Hematocytometer has been the gold standard in cell analysis for over a century, however, starting from 1963, automated counting methods have been developed to allow high-throughput analysis, while maintaining accuracy [19]. Indeed, automation is pivotal in cell manufacturing applications, as it increases reproducibility and reduce time consumption, paving the way to industrial translation [20]. More specifically, the counting time using an hematocytometer has been found to increase almost linearly with sample concentration [21], ranging from around 30 seconds with 4×10^4 cells/mL to around 3 min with 8×10^5 cells/mL [22]. On the other hand, the analysis time using an automated cell counter is independent on cell concentration. Specifically, it is 30 seconds for the automated cell
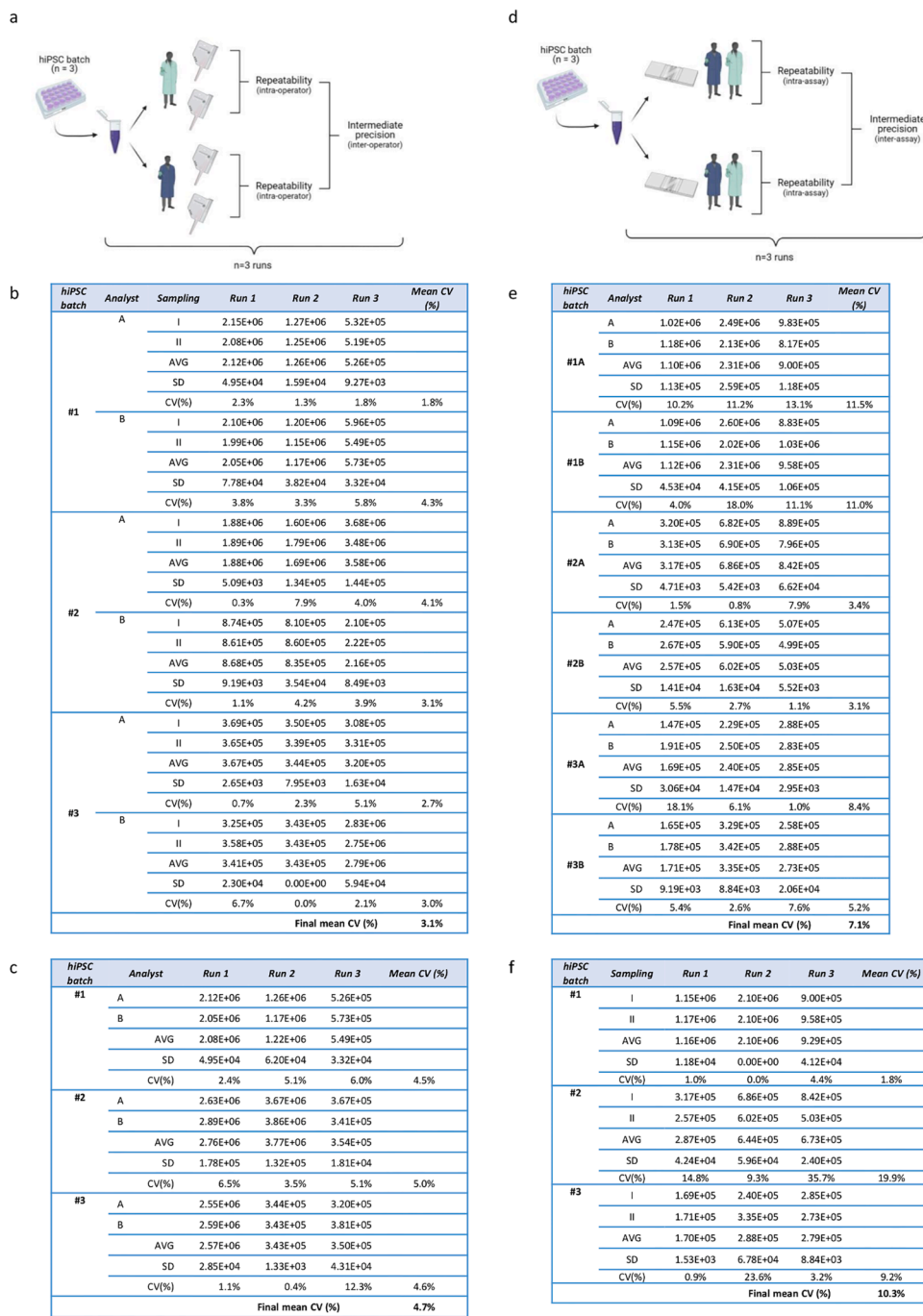
Fig. 4. Precision of automated and manual hiPSC cell counting method. (A) Schematics summarizing the experimental approach for assessment of precision by the automated method; three runs of validation were performed by two analysts; counts were performed in duplicate; created with BioRender.com. Tables reporting the measured values and parameters for evaluation of repeatability (B) and intermediate precision (C) of the automated method. (D) Schematics summarizing the experimental approach for assessment of precision by the manual method; three runs of validation were performed by two analysts; counts were performed in duplicate; created with BioRender.com. Tables reporting the measured values and parameters for evaluation of repeatability (E) and intermediate precision (F) of the manual method. hiPSC, human induced pluripotent stem cells; AVG, average; SD, standard deviation; CV, coefficient of variation.

b

hiPSC batch	Analyst	Sampling	Run 1	Run 2	Run 3	Mean CV (%)
#1	A	I	2.15E+06	1.27E+06	5.32E+05	
		II	2.08E+06	1.25E+06	5.19E+05	
		AVG	2.12E+06	1.26E+06	5.26E+05	
		SD	4.95E+04	1.59E+04	9.27E+03	
		CV(%)	2.3%	1.3%	1.8%	1.8%
	B	I	2.10E+06	1.20E+06	5.96E+05	
		II	1.99E+06	1.15E+06	5.49E+05	
		AVG	2.05E+06	1.17E+06	5.73E+05	
		SD	7.78E+04	3.82E+04	3.32E+04	
		CV(%)	3.8%	3.3%	5.8%	4.3%
#2	A	I	1.88E+06	1.60E+06	3.68E+06	
		II	1.89E+06	1.79E+06	3.48E+06	
		AVG	1.88E+06	1.69E+06	3.58E+06	
		SD	5.09E+03	1.34E+05	1.44E+05	
		CV(%)	0.3%	7.9%	4.0%	4.1%
	B	I	8.74E+05	8.10E+05	2.10E+05	
		II	8.61E+05	8.60E+05	2.22E+05	
		AVG	8.68E+05	8.35E+05	2.16E+05	
		SD	9.19E+03	3.54E+04	8.49E+03	
		CV(%)	1.1%	4.2%	3.9%	3.1%
#3	A	I	3.69E+05	3.50E+05	3.08E+05	
		II	3.65E+05	3.39E+05	3.31E+05	
		AVG	3.67E+05	3.44E+05	3.20E+05	
		SD	2.65E+03	7.95E+03	1.63E+04	
		CV(%)	0.7%	2.3%	5.1%	2.7%
	B	I	3.25E+05	3.43E+05	2.83E+06	
		II	3.58E+05	3.43E+05	2.75E+06	
		AVG	3.41E+05	3.43E+05	2.79E+06	
		SD	2.30E+04	0.00E+00	5.94E+04	
		CV(%)	6.7%	0.0%	2.1%	3.0%
					Final mean CV (%)	**3.1%**

e

hiPSC batch	Analyst	Run 1	Run 2	Run 3	Mean CV (%)
#1A	A	1.02E+06	2.49E+06	9.83E+05	
	B	1.18E+06	2.13E+06	8.17E+05	
	AVG	1.10E+06	2.31E+06	9.00E+05	
	SD	1.13E+05	2.59E+05	1.18E+05	
	CV(%)	10.2%	11.2%	13.1%	11.5%
#1B	A	1.09E+06	2.60E+06	8.83E+05	
	B	1.15E+06	2.02E+06	1.03E+06	
	AVG	1.12E+06	2.31E+06	9.58E+05	
	SD	4.53E+04	4.15E+05	1.06E+05	
	CV(%)	4.0%	18.0%	11.1%	11.0%
#2A	A	3.20E+05	6.82E+05	8.89E+05	
	B	3.13E+05	6.90E+05	7.96E+05	
	AVG	3.17E+05	6.86E+05	8.42E+05	
	SD	4.71E+03	5.42E+03	6.62E+04	
	CV(%)	1.5%	0.8%	7.9%	3.4%
#2B	A	2.47E+05	6.13E+05	5.07E+05	
	B	2.67E+05	5.90E+05	4.99E+05	
	AVG	2.57E+05	6.02E+05	5.03E+05	
	SD	1.41E+04	1.63E+04	5.52E+03	
	CV(%)	5.5%	2.7%	1.1%	3.1%
#3A	A	1.47E+05	2.29E+05	2.88E+05	
	B	1.91E+05	2.50E+05	2.83E+05	
	AVG	1.69E+05	2.40E+05	2.85E+05	
	SD	3.06E+04	1.47E+04	2.95E+03	
	CV(%)	18.1%	6.1%	1.0%	8.4%
#3B	A	1.65E+05	3.29E+05	2.58E+05	
	B	1.78E+05	3.42E+05	2.88E+05	
	AVG	1.71E+05	3.35E+05	2.73E+05	
	SD	9.19E+03	8.84E+03	2.06E+04	
	CV(%)	5.4%	2.6%	7.6%	5.2%
				Final mean CV (%)	**7.1%**

c

hiPSC batch	Analyst	Run 1	Run 2	Run 3	Mean CV (%)
#1	A	2.12E+06	1.26E+06	5.26E+05	
	B	2.05E+06	1.17E+06	5.73E+05	
	AVG	2.08E+06	1.22E+06	5.49E+05	
	SD	4.95E+04	6.20E+04	3.32E+04	
	CV(%)	2.4%	5.1%	6.0%	4.5%
#2	A	2.63E+06	3.67E+06	3.67E+05	
	B	2.89E+06	3.86E+06	3.41E+05	
	AVG	2.76E+06	3.77E+06	3.54E+05	
	SD	1.78E+05	1.32E+05	1.81E+04	
	CV(%)	6.5%	3.5%	5.1%	5.0%
#3	A	2.55E+06	3.44E+05	3.20E+05	
	B	2.59E+06	3.43E+05	3.81E+05	
	AVG	2.57E+06	3.43E+05	3.50E+05	
	SD	2.85E+04	1.33E+03	4.31E+04	
	CV(%)	1.1%	0.4%	12.3%	4.6%
				Final mean CV (%)	**4.7%**

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hiPSC batch	Sampling	Run 1	Run 2	Run 3	Mean CV (%)
#1	I	1.15E+06	2.10E+06	9.00E+05	
	II	1.17E+06	2.10E+06	9.58E+05	
	AVG	1.16E+06	2.10E+06	9.29E+05	
	SD	1.18E+04	0.00E+00	4.12E+04	
	CV(%)	1.0%	0.0%	4.4%	1.8%
#2	I	3.17E+05	6.86E+05	8.42E+05	
	II	2.57E+05	6.02E+05	5.03E+05	
	AVG	2.87E+05	6.44E+05	6.73E+05	
	SD	4.24E+04	5.96E+04	2.40E+05	
	CV(%)	14.8%	9.3%	35.7%	19.9%
#3	I	1.69E+05	2.40E+05	2.85E+05	
	II	1.71E+05	3.35E+05	2.73E+05	
	AVG	1.70E+05	2.88E+05	2.79E+05	
	SD	1.53E+03	6.78E+04	8.84E+03	
	CV(%)	0.9%	23.6%	3.2%	9.2%
				Final mean CV (%)	**10.3%**

counting system NucleoCounter NC-100, according to the manufacturer's specifications.

In this framework, the fluorescence-based image cytometer Nucleo-Counter NC-100 is an automated cell counting method that we validated for use with hiPSC samples. The validation protocol was designed to assess specificity, linearity, range, accuracy and precision of the proposed method. Following a similar workflow, Cadena-Herrera and colleagues validated an automated cell counter based on the trypan blue exclusion technique, and optimized dye concentration to reduce background noise [12]. Nevertheless, trypan blue can overestimate viable cells, while fluorometric assays are more accurate [23, 24]. Nucleo-Counter NC-100 allows fast and reliable cell counts based on PI incorporation, detected by an integrated fluorescence microscope [16]. Following the indications contained in the aforementioned regulations

regarding ATMP manufacturing (2.1 Aim and design of the validation protocol; 3.1 Validation strategy), validation of hiPSC counting by this system was performed as a step instrumental to the development and optimization of cGMP-grade hiPSC production process. While the same guidelines were followed for different ATMPs and methods [12, 16], interpretation of the validation strategy may vary, when taking into consideration the hierarchy of the single validation steps [13].

The reference hemocytometer-based cell counting method was used to determine accuracy and was further investigated for precision. For the two analytical procedures, both classified as direct cell counting, precision was investigated differently, based on the peculiarities of the two methods.

According to the results generated, both proposed and reference analytical procedures can be useful cell counting methods during hiPSC

manufacturing. The former guaranteed better repeatability and inter-mediate precision than the latter, which still demonstrated an acceptable degree of precision. Yet, the increased precision of the automated cell counting method is achieved at the expense of the number of hiPSCs to be analyzed. Indeed, the range lower limit of the proposed method is higher than that of the reference method. Therefore, the proposed automated cell counting method can be the most appropriate choice when a lot of samples or many repeated measurements have to be performed, or when working under tight time limitations, or a more reliable cell count is needed, given that sufficient hiPSC amounts are available, such as at the final expansion phases of a manufacturing process. On the contrary, the reference manual cell counting method would be indicated if the hiPSC sample is scarce (below the calculated range lower limit of the automated method) or very few samples need to be analyzed. Another advantage of the automated analytical procedure is the possibility to digitally register and store the data, facilitating compliance with cGMP requirements. Moreover, with an appropriate risk analysis, the validated method may be considered suitable not only for counting hiPSCs, but also for other pluripotent cell types, such as human embryonic stem cells, and potentially to other cell types that present the same attributes and behavior in suspension (such as size, tendency to aggregate, presence of debris, etc.).

5. Conclusions

In conclusion, we verified the suitability of an EP 10th manual cell counting method for QC at initial steps of hiPSC generation under cGMP manufacturing, when the cell number is scarce; we also validated an automated cell counting method, not included in EP 10th, for QC during large-scale cGMP expansion of hiPSCs or at final steps of hiPSC manufacturing, such as dose preparation and freezing, when high cell numbers are achieved. Our work demonstrates that it is possible to fully validate analytical methods also for innovative ATMPs such as hiPSCs and will help other researchers interested in the translation of hiPSC research protocols into cGMP-compliant manufacturing processes.

Statements

Competing interests

The authors declare that they have no competing interests.

Ethical approval and consent to participate

All experiments were performed according to the amended Declaration of Helsinki. Informed consent by cord blood donors used to generate hiPSC lines was obtained for research under resolution n° VII/18653 by Lombardy Region, Italy, and evaluation by the Ethical Committee of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico n° 1982, 14th January 2020.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

P.M., V.P., A.R.O. and M.B. carried out the experiments; S.B. performed risk assessment and provided guidance on validation strategy; A.R.O. and M.B. outlined the validation strategy; M.B. coordinated and supervised the experiments; P.M., V.P., A.R.O. and M.B. contributed to analysis of the data, drafting of the manuscript and representation of results; M.B. performed statistical analysis; M.B. and L.L. revised the manuscript and gave final approval; L.L. provided resource and funding. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00708.

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