Exploring factors causing differences between cell counting methods

Introduction

Cell counting has been one of the most important and routinely performed assay for biological research development. There are many cell counting methods that have been introduced since the invention of the hemacytometer for manual cell counting. Cell counting method differences can be attributed to systematic error that occurs for each method and other various sources (Figure 1).

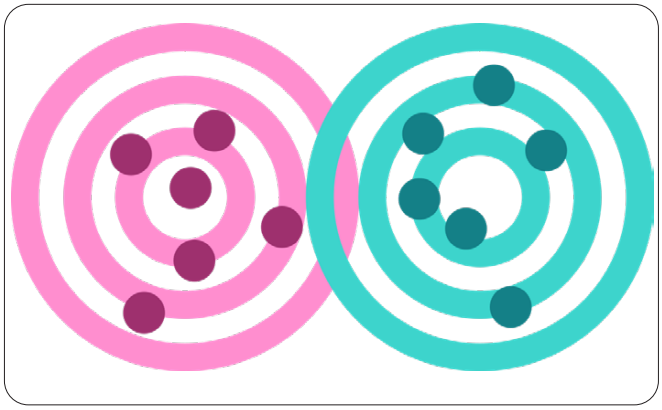


Figure 1: Visual representation of the comparison of cell counting targets between two cell counting methods

Systematic error is the "component of measurement error that in replicate measurements remain constant or varies in a predictable manner" (1). Assume that the exact same cell sample is measured by method 1 and 2, as long as the methods are different, there will be a systematic error that exists for each method, even if the cell counting process is perfect or the Poisson noise is minimized (Figure 2).

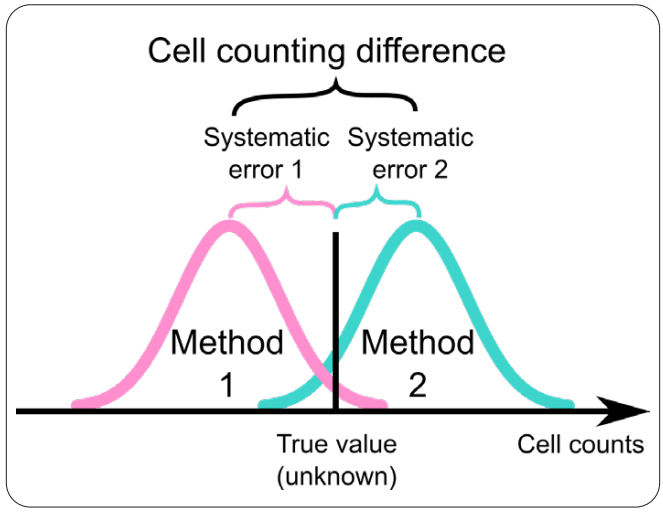


Figure 2: Illustration of distributions of cell counting measurements from two different cell counting methods, and the difference between these two methods.

The difference between two cell counting methods can be characterized as the combination of systematic errors, assuming that they remain constant for each method. Any change of the parameters in the entire cell counting process can be considered a new cell counting method. In this white paper, we will discuss methodologies to determine cell counting differences, the potential sources of cell counting differences, recommendations on increasing the confidence of the cell counting results if there are no live cell reference materials, finally, we will present two case studies showing cell counting method comparison.

revvity

Methods to determine differences between cell counting methods

There are three methodologies to determine the differences between cell counting methods: simple method with two-sample t-test (2), ISO Cell Counting Standard Part 2 analysis with method-to-method bias calculation (3-6), and Bland-Altman comparative analysis (6-9).

Simple method with two-sample t-test

The two-sample t-test is a simple, straightforward, and well-acknowledge method for comparing two methods. Table 1 shows an example of method comparison using two-sample t-test. Without the prior knowledge of distributions of cell counts, it is advised to compare two sets of data with two-tailed, two-sample, unpaired t-test with unequal variance and determine the p-value, which indicates the significance of the difference.

Table 1: Example of method comparison using two-sample t-test.

Observation	Method 1	Method 2
1	3.67E+06	3.25E+06
2	3.73E+06	3.73E+06
3	3.49E+06	3.71E+06
Mean	3.63E+06	3.56E+06
Difference		6.83E+04
t-test p-value		0.72
Significance (p<0.05?)		Not significant

ISO Cell Counting Standard Part 2 analysis with method-to-method bias calculation

In the ISO Cell Counting Standard Part 2 (4), researchers will need to test a range of independently prepared cell concentration series that is “fit-for-purpose”, generate multiple replicates, and make multiple observations from each replicate by two or more methods. Finally, the results are used to generate cell counting quality parameters to compare two or more methods with proportionality plots, proportionality constants for each method, and method-to-method bias and its significance based on the proportionality results (Figure 3).

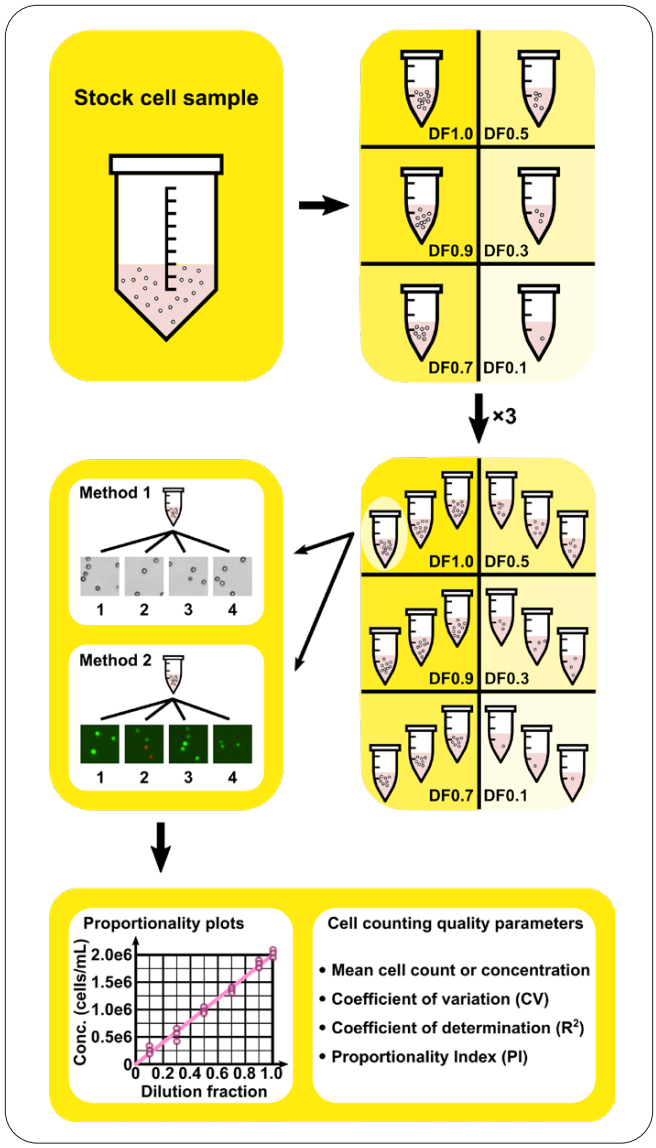


Figure 3: ISO Cell Counting Standard Part 2 protocol. Adapted from Huang Y, Bell J, Kuksin D, Sarkar S, Pierce LT, Newton D, Qiu J, Chan LLY. Practical application of cell counting method performance evaluation and comparison derived from the ISO Cell Counting Standards Part 1 and 2. Cell and Gene Therapy Insights 2021;7:937-960.

The proportionality plot is the mean cell concentration with respect to the dilution fraction and the proportionality constant is the slope of the proportional fit for each method. The method-to-method bias is the percent difference between two methods calculated with the proportionality constants. The method-to-method bias calculation was proposed and presented by the National Institute of Standards and Technology (NIST) (5). To facilitate data analysis, NIST has developed a Counting Method Evaluation Tool application (COMET app.) to generate proportionality plots, compare the proportionality constants, and calculate the method-to-method bias and its significance.

Bland-Altman comparative analysis

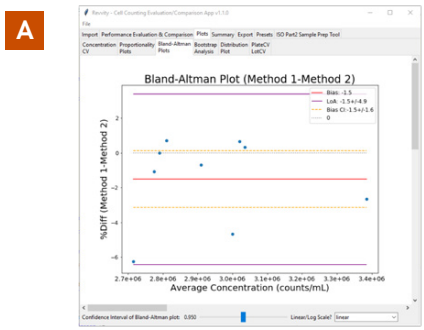
Bland-Altman comparative analysis is a method to assess the agreement or comparability between two quantitative measurements, which has been popularized by Bland and Altman in medical statistics (7-8). The agreement between two measurements is evaluated by the mean difference plot (Bland-Altman plot) and the construction of limits of agreement within the plot.

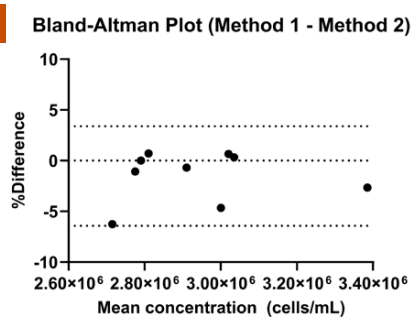
Three key results are generated: bias is the measured average percent difference between two methods; limit of agreement (LoA) is the coverage range of the bias that is approximately $\pm 2\sigma_D$ (standard deviation) from the bias at 95% confidence interval; and confidence interval of the bias (Bias CI), which is the uncertainty of the bias at $\pm 2\sigma_E$ (standard error) from the bias at 95% confidence interval. It is important to note that percent difference (%difference) is used instead of absolute difference, which is to account for heteroscedasticity of the difference.

Bland-Altman comparative analysis is employed to provide clear visualization of differences for each pair of observations/replicates, which is easy to demonstrate the bias (%difference), its range, and its significance. The method utilizes a simple calculation, and it is well accepted in the medical field. In order to use the Bland-Altman analysis, we will need to assume that the %difference does not change with concentration, it is not skewed, follows a normal distribution, and equal weight is assigned for each pair of observations/replicates (9).

Revvity has created a cell counting performance evaluation and comparison application (cell counting app.) that can directly perform Bland-Altman comparative analysis and calculate the bias between two cell counting methods, which supports exported results from Revvity instruments (Figure 4a). Alternatively, Bland-Altman comparative analysis can be simply done with commercial data analysis software or Excel (Figure 4b, 4c).

Figure 4c demonstrate an example of the Bland-Altman analysis using Excel. First, the mean and percent differences from the pair-wise results are obtained from two cell counting methods. The bias or estimated percent difference is calculated as the average of the percent differences and used to generate a mean difference plot. Significance of the difference is based on 95% confidence level where limits of agreement is calculated with $LoA = Bias \pm 1.96 \times \sigma_D\ (95\%\ CI)$ and the confidence interval of bias is calculated with $BiasCI = Bias \pm 1.96 \times \frac{\sigma_D}{\sqrt{n}}\ (95\%\ CI)$ and used to determine if the bias is significant.





	Method 1	Method 2	Mean	%Difference
2	2.79E+06	2.79E+06	2.79E+06	0.00%
3	2.90E+06	2.92E+06	2.91E+06	-0.69%
4	2.63E+06	2.80E+06	2.72E+06	-6.26%
5	3.34E+06	3.43E+06	3.39E+06	-2.66%
6	3.03E+06	3.01E+06	3.02E+06	0.66%
7	2.76E+06	2.79E+06	2.78E+06	-1.08%
8	3.04E+06	3.03E+06	3.04E+06	0.33%
9	2.82E+06	2.80E+06	2.81E+06	0.71%
10	2.93E+06	3.07E+06	3.00E+06	-4.67%
11	Bias			-1.52%
12	Standard Deviation (σ_D)			2.50%
13	Limit of agreement (LoA)		-6.42%	3.39%
14	95% Confidence Interval of Bias (Bias CI)		-3.15%	0.12%

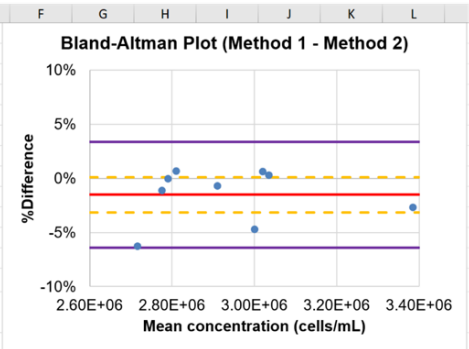


Figure 4: Example Bland-Altman comparison analysis using in-house application (a), commercial software (b), and Excel (c).

Potential sources of cell counting differences

Other sources of cell counting differences can be identified following the cause and effect diagram of variability in a cell counting assay (Figure 5) (10).

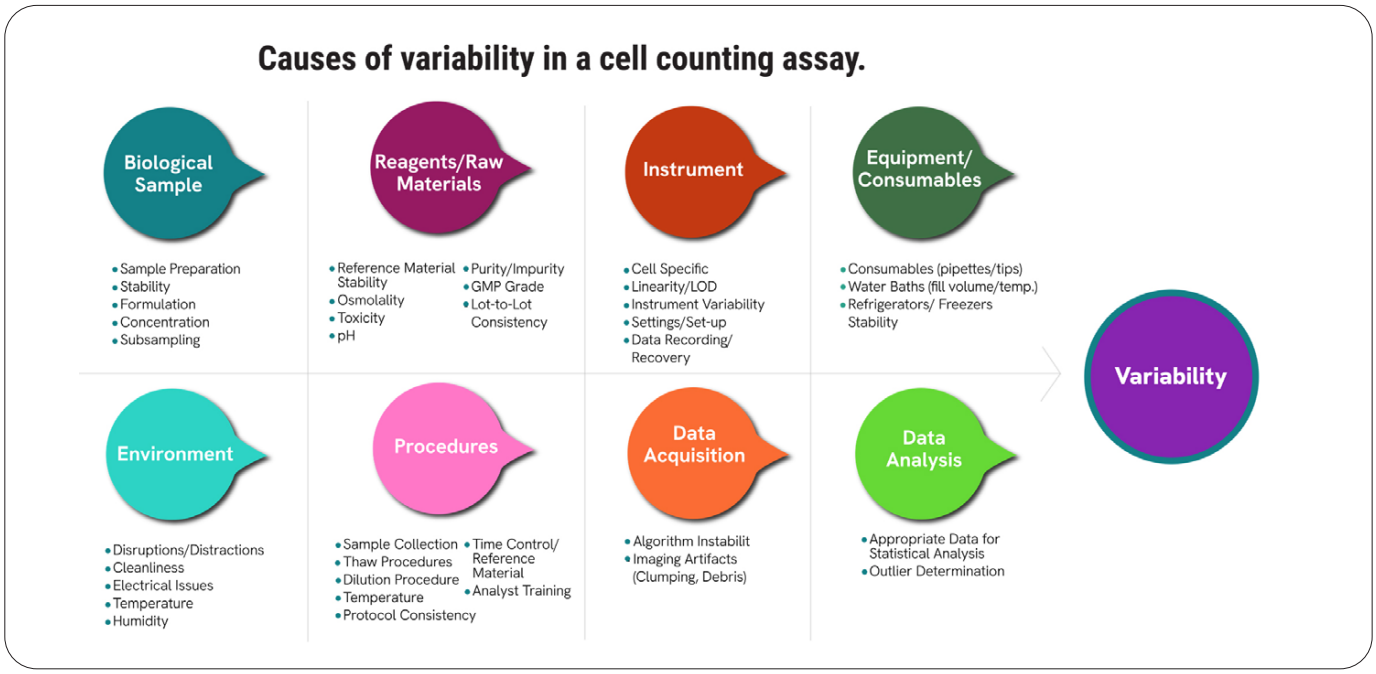


Figure 5: Potential sources of cell counting variation that can cause cell counting difference. Adapted from Simon, C. G., Jr, Lin-Gibson, S., Elliott, J. T., Sarkar, S., & Plant, A. L. (2016). Strategies for Achieving Measurement Assurance for Cell Therapy Products. Stem cells translational medicine, 5(6), 705-708.

Some of the parameters are highlighted in Table 2.

Table 2. Different sources of variation and examples.

Source of variation	Examples
Biological sample	Counting of different cell types
	State of the sample
Reagent/raw materials	Different stains/dyes to measure cell count and viability
Instrument	Instrument with different cell counting principles ▪ Image-based ▪ Flow-based ▪ Impedance-based ▪ Metabolic activity-based
	Instrument calibration
Equipment/consumables	Differences in consumable
	Differences in pipettors used
Environment	Change in temperature, humidity, pH, etc. causing variation in cells, reagents, raw materials, consumables, instruments, etc.
Procedures	Sampling, mixing, and diluting can cause variations dependent on the operators
Data analysis	Different image analysis algorithms may cause variation in cell counting results

Recommendation to increase the confidence of cell counting results

Since there is currently no live cell reference material, leading to no accuracy definition for cell counting results, it is important to include other cell counting methods to increase the confidence of the cell counting results. For example, when comparing a flow-based cell counter and an image-based cell counter like Cellaca™ MX or Cellaca™ PLX (Revvity, Lawrence, MA), it is important to include a hemacytometer or other automated cell counters to gain a better idea of the cell counting difference. The measured difference does not indicate which method is more accurate, but just stating the difference as a matter of fact. Based on different considerations, a list of acceptance criteria that the cell counting methods may be more fit-for-purpose.

Case studies

In the following section, we will present two case studies relating to specific parameters affecting cell counting differences.

Case Study 1 – Method comparison

In this example, we compared two cell counting methods – Cellaca MX and Cellaca PLX, both using acridine orange and propidium iodide (AO/PI) dual-fluorescence assay. First, a stock concentration of fresh Jurkat cells was collected. Next, the truncated ISO Cell Counting Standard Part 2 protocol was performed.

1. Prepare 4 independent dilution concentrations from stock, instead of serial dilutions to eliminate propagating pipetting error
2. Prepare 3 replicate tubes for each dilution, total of 12 tubes
3. Perform 3 observations of each tube using each method that you want to compare

An example of sample preparation table for the truncated ISO Cell Counting Standard Part 2 protocol was presented in Table 3.

Table 3: Sample preparation table for independent diluted replicate Jurkat cell samples.

Tubes	Working stock of cells (µL)	Media (µL)	Total volume (µL)	Dilution fraction (DF)	Replicate	Cell type
1	200	0	200	1.0	1	Jurkat
2	150	50	200	0.75	1	Jurkat
3	100	100	200	0.5	1	Jurkat
4	50	150	200	0.25	1	Jurkat
5	200	0	200	1.0	2	Jurkat
6	150	50	200	0.75	2	Jurkat
7	100	100	200	0.5	2	Jurkat
8	50	150	200	0.25	2	Jurkat
9	200	0	200	1.0	3	Jurkat
10	150	50	200	0.75	3	Jurkat
11	100	100	200	0.5	3	Jurkat
12	50	150	200	0.25	3	Jurkat

Note: The sequence of the tubes should be randomized during image acquisition.

The method comparison results were presented below. First, the proportionality plots (Figure 6) showed comparable linear regression lines of mean total cell concentrations versus dilution fractions for both Cellaca MX and Cellaca PLX cell counting methods.

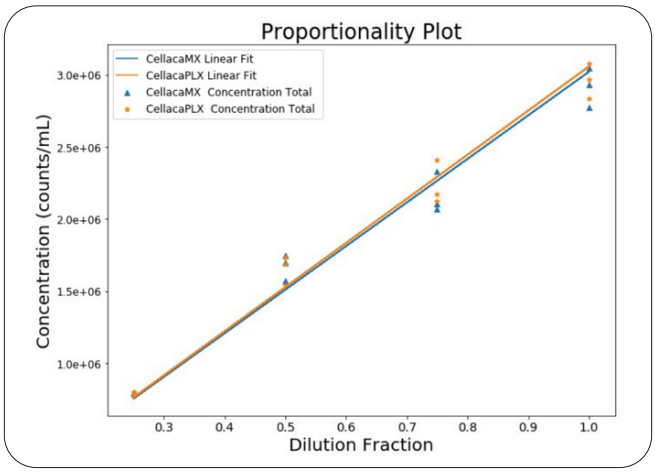


Figure 6: Proportionality plots of Cellaca MX and Cellaca PLX cell counting methods.

The proportionality constants, which were the slopes of the linear regression lines, were shown in Table 4. The total cell concentrations measured by Cellaca MX and Cellaca PLX methods were highly comparable, which only exhibited a 1.3% difference between the two methods.

Table 4: Compilation of the proportionality constant, R2 (coefficient of determination), and proportionality index (PI).

	Total cell concentration (cells/mL)		
	Cellaca MX	Cellaca PLX	%Bias
Proportionality constant	3.02e+06	3.06e+06	-1.3%
R2	0.995	0.996	
PI	0.72	0.53	

In addition, we also performed the Bland-Altman comparative analysis, which also showed comparable results between Cellaca MX and Cellaca PLX methods (Figure 7 and Table 5). The -0.8% bias between the two methods was not significant.

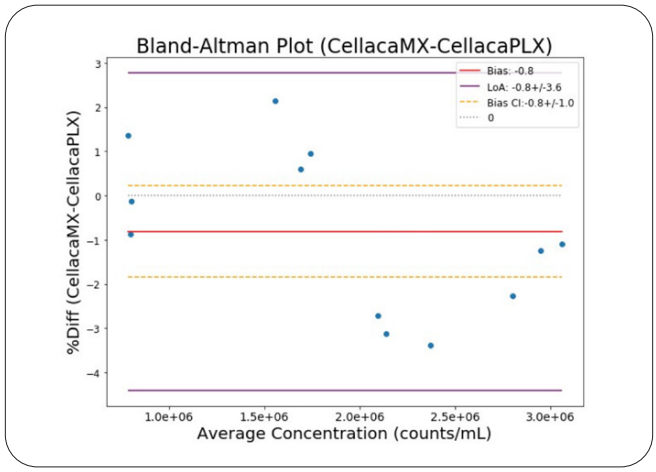


Figure 7: Bland-Altman comparison between Cellaca MX and Cellaca PLX.

Table 5: Compilation of Bland-Altman results from the comparison between Cellaca MX and Cellaca PLX cell counting methods.

	Total cell concentration (cells/mL)
Bias (%)	-0.8
Limit of agreement (%)	-0.8 ± 3.6
95% Confidence interval of bias (%)	-0.8 ± 1.0
Significance of bias	No

Case Study 2 – Procedures – Extra dilution step

In the second example, we compared the effects on cell counting precision when cells are diluted between 2 and 10X (Figure 8).

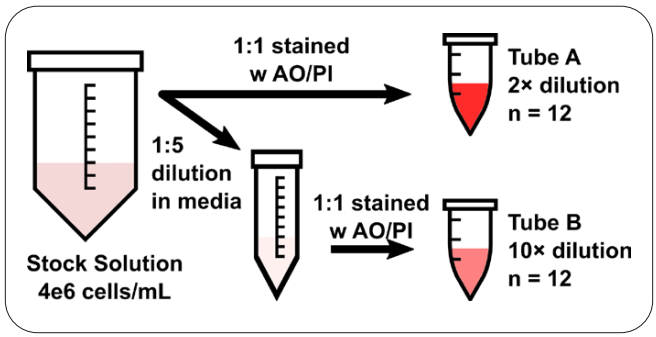


Figure 8: Experimental protocol for comparison of cell counting results between undiluted and diluted cell samples.

First, a Jurkat cell sample at approximately 4 × 10^6 cells/mL was stained 1:1 with AO/PI and mixed uniformly to load into cell counting chambers at n = 12. Next, the same Jurkat cell sample was diluted in media 1:5, stained 1:1 with AO/PI, and loaded into cell counting chambers at n = 12, resulting in a final dilution of 10X. The results showed that the extra dilution step generated high concentration bias (-9.4%) for total cell concentrations (Figure 9).

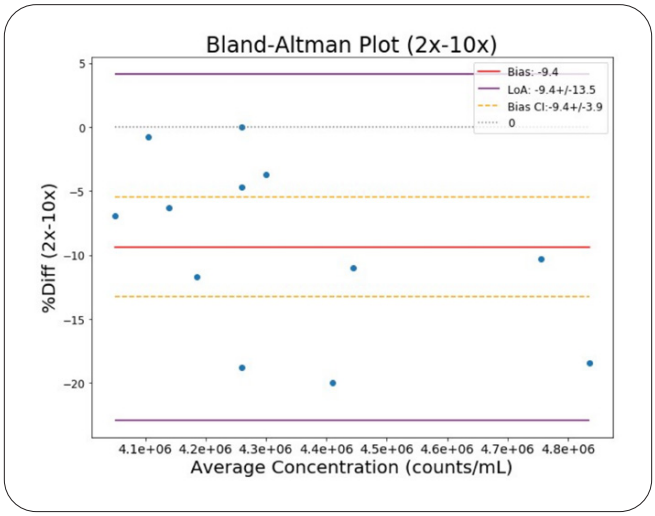


Figure 9: Bland-Altman comparative analysis showing ~9% bias between diluted and undiluted cell samples.

Conclusion

In conclusion, cell counting difference always exists between two different cell counting methods. There are three methods that can be utilized to determine the differences: (1) simple method with two-sample t-test, (2) ISO Cell Counting Standard Part 2 analysis with method-to-method bias calculation, and (3) Bland-Altman comparative analysis. Our research shows that using the Bland-Altman analysis method to determine the bias, construct limit of agreement (LoA), and 95% confidence interval of the bias (Bias CI) between two cell counting methods may help you identify the cell counting difference between two methodologies. The advantages of utilizing Bland-Altman analysis are clear visualization to show the difference for each pair of observations/replicates, easy to demonstrate the bias (% difference), its range, and its significance, straightforward calculation, and widely accepted in the medical field. Finally, we can always follow the cause and effect diagram to allow investigations into specific parameters that can affect the difference between cell counting methods.

References

1. ISO/IEC Guide 99:2007(en) International vocabulary of metrology — Basic and general concepts and associated terms (VIM)
2. Bulmer, M.G., Principles of statistics, Dover Publications, Inc., 1979.
3. ISO. Biotechnology — Cell counting — Part 1: General guidance on cell counting methods. International Organization for Standardization 2018;20391-1:2018.
4. ISO. Biotechnology — Cell counting — Part 2: Experimental design and statistical analysis to quantify counting method performance. International Organization for Standardization 2019;20391-2:2019.
5. Sarkar, S., Lund, S. P., Vyzasatya, R., Vanguri, P., Elliott, J. T., Plant, A. L., & Lin-Gibson, S. (2017). Evaluating the quality of a cell counting measurement process via a dilution series experimental design. Cytotherapy, 19(12), 1509–1521.
6. Huang Y, Bell J, Kuksin D, Sarkar S, Pierce LT, Newton D, Qiu J, Chan LLY. Practical application of cell counting method performance evaluation and comparison derived from the ISO Cell Counting Standards Part 1 and 2. Cell and Gene Therapy Insights 2021;7:937-960.
7. Altman DG, Bland JM, Statistician, 1983; 32: 307–17
8. Bland JM, Altman DG, Lancet, 1986, 327 (8476): 307–10
9. Giavarina D. (2015). Understanding Bland Altman analysis. Biochemia medica, 25(2), 141–1511
10. Simon, C. G., Jr, Lin-Gibson, S., Elliott, J. T., Sarkar, S., & Plant, A. L. (2016). Strategies for Achieving Measurement Assurance for Cell Therapy Products. Stem cells translational medicine, 5(6), 705–708.