

Comparing Automated and Manual Cell Counts for Cell Culture Applications

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In biomanufacturing based on animal cell culture, cell enumeration and viability determination are often accomplished using the trypan blue dye-exclusion method (1). This method is based on the fact that viable cells exclude the dye and remain visually clear, whereas nonviable cells are stained blue.

Historically, cell counts using the technique have been performed manually using a hemacytometer. Cell culture samples are mixed (after dilution in an isotonic buffer, if necessary) with known volumes of trypan blue dye, then loaded into a hemacytometer and counted under a microscope (usually at 100× magnification). It has long been recognized that cell counts obtained using this procedure are subject to inter- and intraanalyst variability that arises from subjective assessments and cell staining, sample dilution, and



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hemacytometer loading. Two parameters that greatly affect the accuracy of cell enumeration are the number of hemacytometer chambers loaded (replicates) and the number of cells counted within each chamber (2).

Instruments that automate the trypan blue cell counting method are now available (e.g., the Vi-CELL XR cell viability analyzer from Beckman Coulter, www.beckmancoulter.com, and the Cedex HiRes from Innovatis, www.innovatis.com). Such instruments feature liquid handling systems that automate sample aspiration and reagent handling. Cells are aspirated from sample cups, then mixed with trypan blue dye, and loaded into a flow cell. A digital camera captures images of those cells, and processing algorithms are applied to the results for distinguishing between viable and nonviable cells. In

addition to cell counts and viabilities, estimates of cell diameters and circularity can also be obtained this way. Following cell diameters can be quite instructive, especially in baculovirus–insect–cell culture systems: A significant increase in cell diameter after addition of virus to a culture indicates successful infection (3).

The automated liquid handling system significantly reduces variabilities that can arise from manual handling, and automation of image processing eliminates subjectivity. In addition, because of the extended dynamic range, samples can be analyzed up to 10^7 cells/mL without dilution. This improves data reproducibility and reliability, which is significant because many manufacturing decisions are made based on viable cell counts (VCCs)

PRODUCT FOCUS: PRODUCTS OF INSECT AND MAMMALIAN CELL CULTURE

PROCESS FOCUS: PRODUCTION

WHO SHOULD READ: THOSE INVOLVED IN PROCESS DEVELOPMENT AND SCALE-UP

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LEVEL: INTERMEDIATE

and/or culture viability. Automated methods also transfer smoothly from process development to CGMP manufacturing environments by eliminating operator variability.

When a new measurement method becomes available, even if it simply automates the traditional method, a statistical comparison is required to establish comparability between both methods. The pitfalls of performing regression analysis and using the product-moment correlation coefficient have been well demonstrated (4). Difference plots (also known as *Bland-Altman plots*) combined with the appropriate use of a transformation method (5) are now well accepted as a tool to compare different measurement techniques. Here we summarize data and provide conclusions from our recent study comparing cell counts and viabilities obtained using both automated and manual methods.

MATERIALS AND METHODS

Cells and Media: We used three cell lines in this work. *Spodoptera frugiperda* Sf-9 and Sf-21 insect cells

(catalog numbers 11496-015 and 11497-013 from Invitrogen, www.invitrogen.com) were routinely maintained in shake flasks containing SF900II SFM (Invitrogen catalog number 10902-088). The flasks were shaken at 125 rpm in a humidified incubator at 27 °C. We cultured an NS0 murine myeloma cell line that secretes a monoclonal antibody in shake flasks and bioreactors with HyQ_CDM4NS0 medium (catalog number SH30579 from HyClone, www.hyclone.com) supplemented with insulin (catalog number 4506 from Serologicals, www.serologicals.com).

Manual Cell Counts: An amount of 0.4% trypan blue dye solution equal to 10% of sample volume was added. After gentle mixing, samples were loaded into a hemacytometer for counting under a microscope at 100× magnification. We appropriately diluted the samples with phosphate buffered saline (PBS) when necessary before addition of trypan blue to ensure that about 200–500 cells would be counted over 10 squares.

Automated Cell Counts: We also used a Vi-CELL XR cell viability

Table 1: Vi-CELL automated operation and analysis parameters

Parameter	Sf-9, Sf-21	NS0
Minimum cell diameter (µm)	8	7
Maximum cell diameter (µm)	50	50
Minimum circularity	0.0	0.0
Cell brightness (%)	85	85
Cell sharpness	100	100
Viable cell spot brightness (%)	75	75
Viable cell spot area (%)	5.0	5.0
Decluster degree	Low	Low
Aspirate cycles	1	1
Trypan blue mixing cycles	2	3

analyzer (Beckman Coulter) for sample analysis. Briefly, a 0.6-mL cell culture sample was aliquoted to each sample cup, then placed on the autosampler, logged, and analyzed using the Vi-CELL software. The machine aspirates the sample material, mixes 0.5 mL of sample with an equal volume of 0.4% trypan blue, then fills the flow

Figure 1: Repeated measures of viable cell counts using manual and automated methods (original scale)

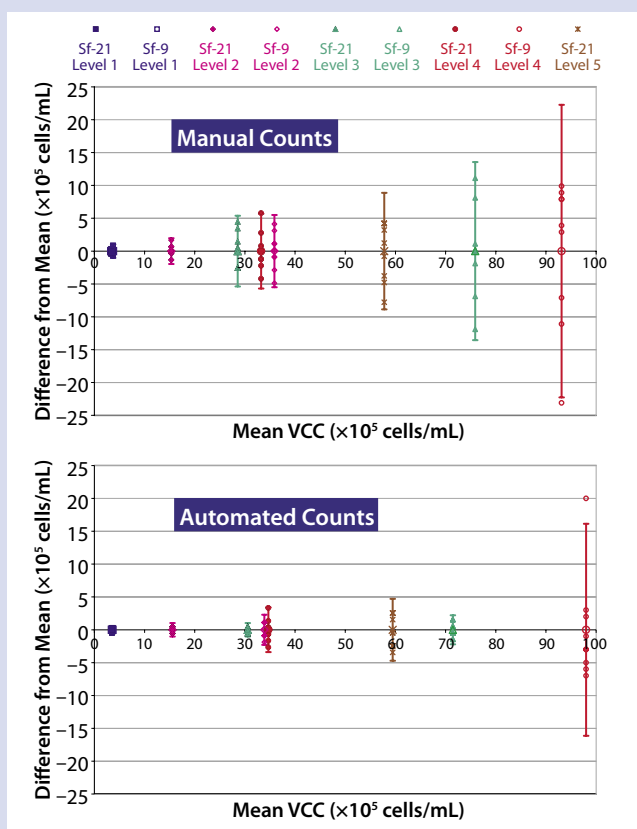


Figure 2: Repeated measures of viable cell counts using manual and automated methods (percent difference scale)

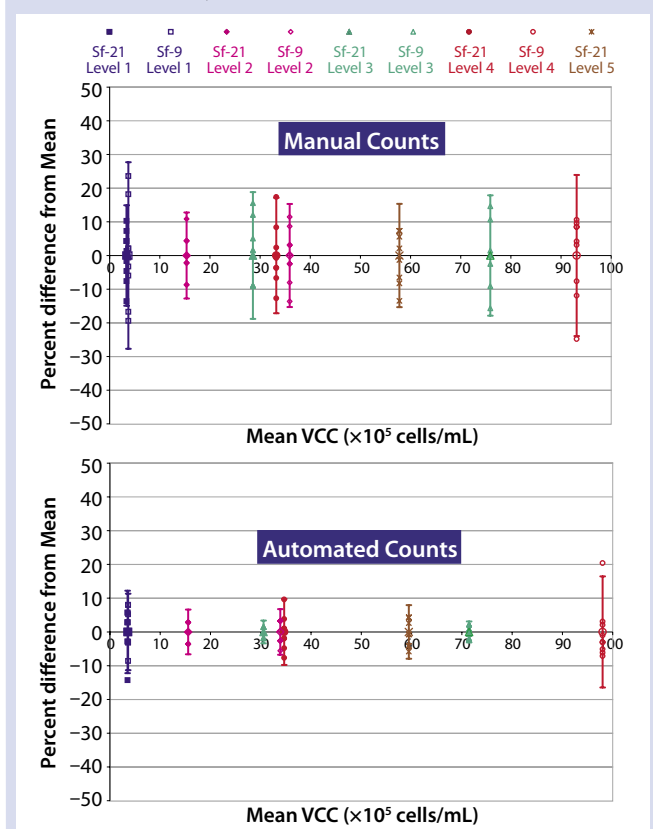
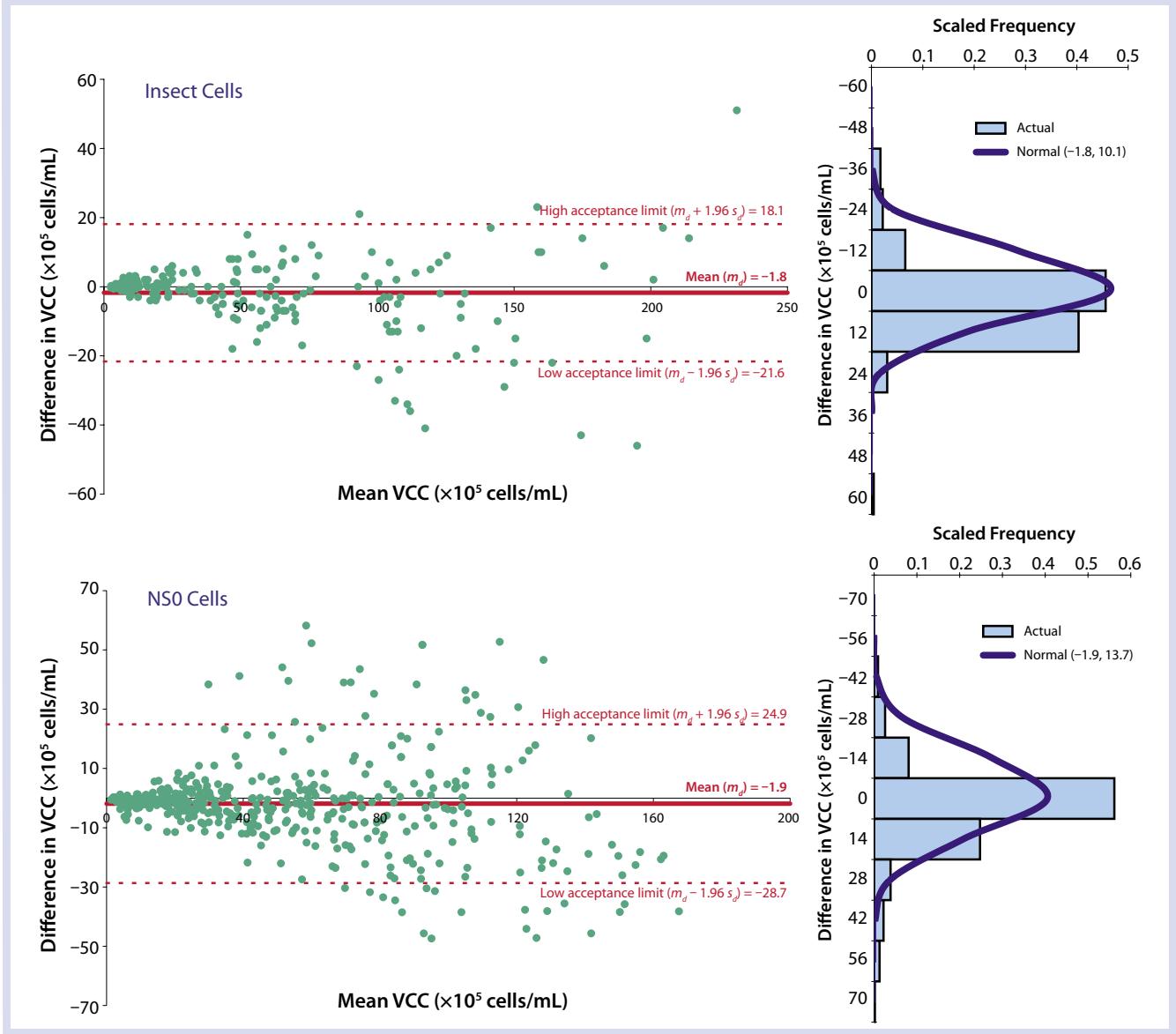


Figure 3: Difference in VCC as a function of mean VCC for insect (TOP) and NS0 cells (BOTTOM) — with distributions shown to the right of each



cell and captures images of them before using a proprietary image-processing algorithm to count and differentiate between viable and nonviable cells. We used the default SF-21 cell type included with the software for analyzing insect cell culture samples and the default settings for CHO cells with some modifications for our NS0 cell culture samples. Table 1 summarizes the Vi-CELL parameters defining both cell types.

Statistical Analyses: We performed two sets of studies: a repeatability study, in which the objective was to assess the inherent variability for each method; and a comparability study, in which the objective was to assess how well the manual and automated methods agreed. For both studies, we used a similar

statistical approach to quantify the repeatability of each method or the comparability of the two.

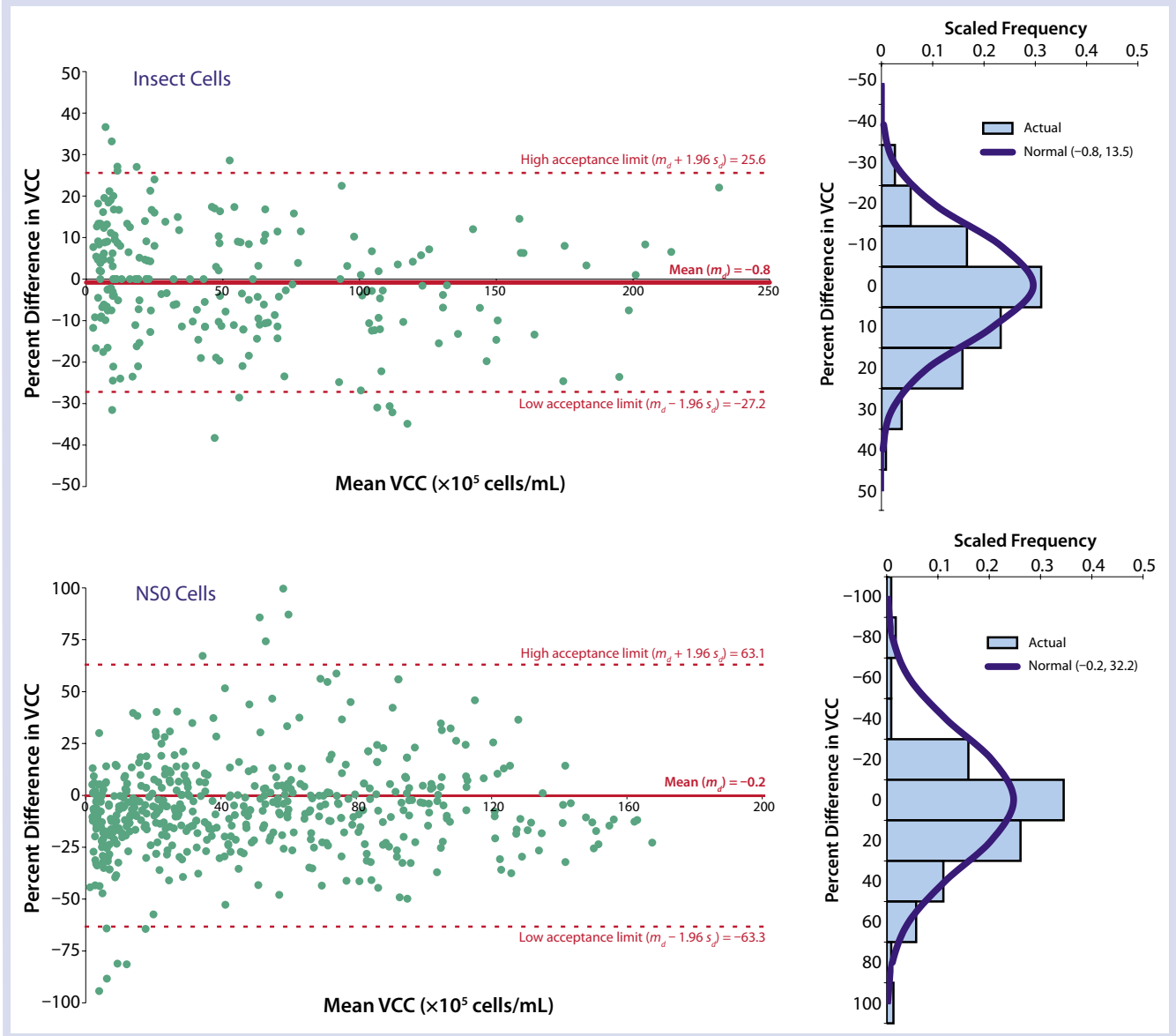
For our repeatability study, three analysts sampled shake flasks containing insect cells grown to various cell densities (levels) and obtained cell counts using both the manual and automated methods. Furthermore, each analyst obtained three manual and automated counts at each level, which produced a total of nine cell counts for each level using each method.

The following analysis was performed independently for both methods: First, the mean and standard deviation of VCCs for each level were calculated using the nine samples at that level. Then, the difference between each sample's VCC and the

level mean VCC was calculated. Finally, the mean (m_d) and standard deviation (s_d) describing the difference in VCC from the level mean were calculated for each level. The mean of those differences should be zero. If the differences were normally distributed, 95% of them would lie within the acceptance limits of $m_d \pm 1.96 \times s_d$. The value $1.96 \times s_d$ is also known as the coefficient of repeatability and provides an estimate of the variability inherent in the process of sampling and obtaining a cell count. Because m_d is always zero for the repeatability study, the acceptance limits are the same as the coefficient of repeatability.

For our comparability study, we grew insect and NS0 cells to various cell densities. Cell counts and

Figure 4: Percent difference from mean VCC as a function of mean VCC for insect (TOP) and NS0 cells (BOTTOM) — with distributions shown to the right



viabilities for each culture sample were obtained using both the manual and automated methods. We calculated the difference or error between the two methods of measurement for each sample by subtracting the automated cell count or viability from the corresponding manual cell count or viability. Then the mean (m_d) and standard deviation (s_d) of the differences were calculated. The mean of those differences provides an indication of any systematic bias when comparing the two methods, and the standard deviation of the differences allows for construction of a 95% confidence interval on the mean. And just as in the repeatability study, we also derived acceptance limits corresponding to $m_d \pm 1.96 \times s_d$. We

analyzed the insect and NS0 cell culture data separately.

RESULTS AND DISCUSSION

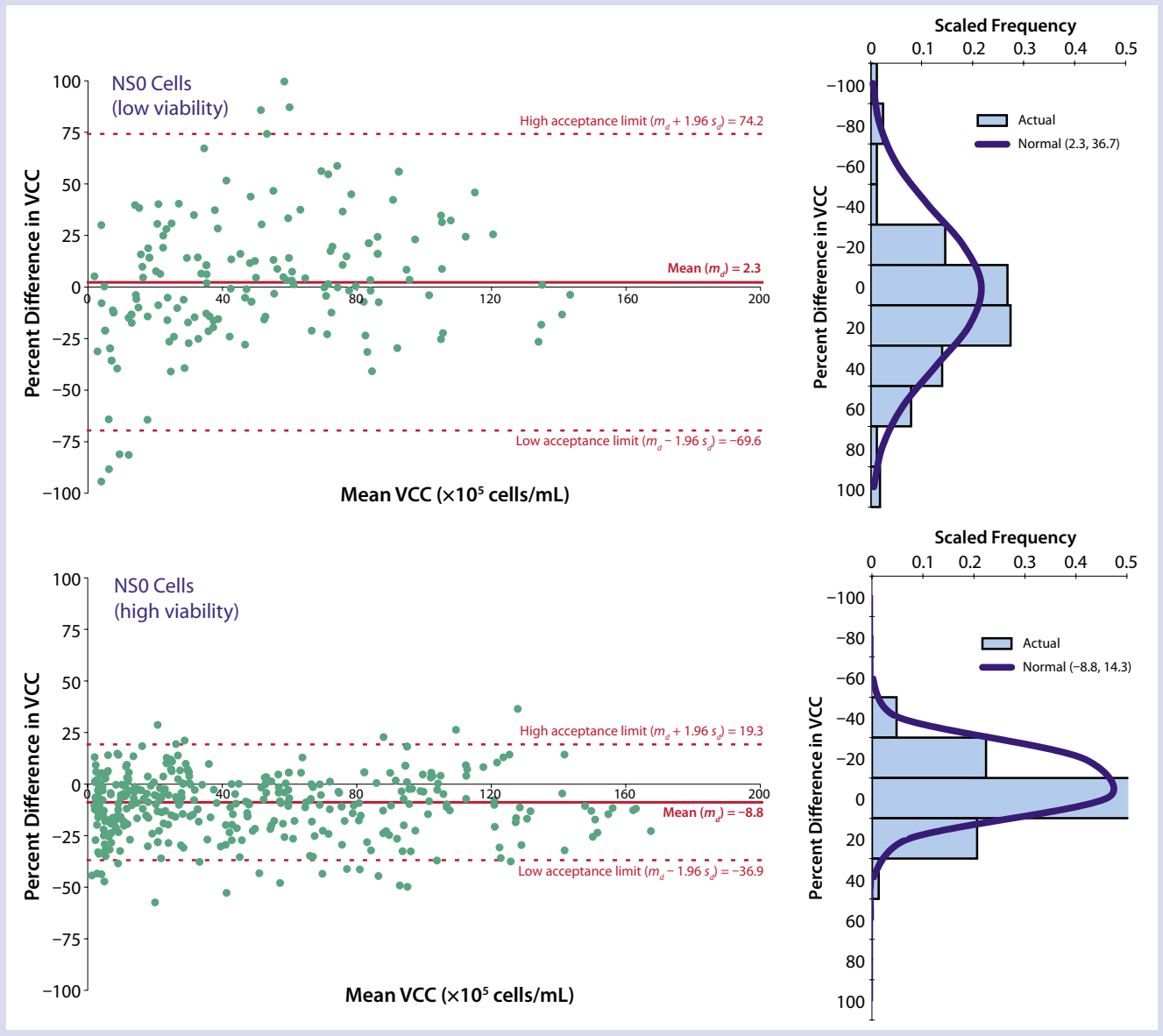
For the **repeatability study**, we grew insect cells in shake flasks to various cell densities: about 3.0, 15, 30, 35, and 60×10^5 cells/mL for Sf-21 cells (levels 1–5 for Sf-21) and 4.0, 35, 75, and 95×10^5 cells/mL for Sf-9 cells (levels 1–4 for Sf-9). Statistical analyses were carried out as described above.

Figure 1 compares the difference of each sample from the mean VCC for each level, with bars showing acceptance limits. As expected, m_d is zero at each level, and 95% of the differences are within the acceptance limits at each level. However, the difference from level mean increases as

the mean VCC increases for both manual and automated counts, suggesting that the differences are not normally distributed.

Because those differences are proportional to the mean, we transformed the data for further analyses. In such situations, logarithmic or percent differences typically exhibit constant variance over the entire range. We chose to analyze percent difference from mean in our study. Figure 2 compares the percent difference of each sample from the mean VCC for each level. No systematic trend of those differences with the mean can be observed when the percent difference from mean VCC is plotted as a function of mean VCC. The acceptance limits we calculated

Figure 5: Percent difference from mean VCC as a function of mean VCC for (top) low- and (bottom) high-viability NS0 cells — with distributions right



using all the differences at all levels are 18% and 9% for the manual and automated counts, respectively.

The coefficient of repeatability for the manual method is as expected, in the 15–25% range. Beckman Coulter claims a counting accuracy of $\pm 6\%$ using well-defined particles such as polystyrene beads (6). That level of accuracy is difficult to obtain in practice with cell culture samples, which are more heterogeneous because of their wider size distribution and the presence of cellular debris. In fact, Beckman recommends acceptance limits of $\pm 10\%$ for system suitability tests using 10- μm “concentration control” beads. Taking those factors into consideration, the 9% coefficient of repeatability obtained for our

automated counting method using cell culture samples is reasonable and justifies the 10% acceptance limits for system suitability using the concentration control samples.

For our **comparability study**, we obtained cell counts for 228 insect cell culture samples and 545 NS0 samples using both the manual and automated methods. Statistical analyses were carried out as described above.

Figure 3 plots the difference in VCC as a function of the mean VCC (average of manual and automated counts) for each sample. The center red line represents the mean difference, whereas the upper and lower such lines represent acceptance limits ($m_d \pm 1.96 s_d$). Clearly, the differences in cell counts are smaller at

lower values and larger at higher values. The same trend is observed with both insect and NS0 cells. Figure 3 also shows a distribution of the differences. Normal distributions using the average and standard deviation calculated from each data set are overlaid for comparison, and the results suggest that the underlying distribution is not normal. Just as in our repeatability studies, because the differences are proportional to the mean VCC, we reanalyzed the data using percent difference from mean.

Figure 4 plots those percent difference in VCC as a function of the mean VCC for each sample. The center red line represents the mean percent difference, whereas the upper and lower such lines represent

acceptance limits in the transformed scale. The percent differences do not show any systematic trend as a function of mean VCC. Figure 4 also shows a distribution of the percent difference in VCC compared with an overlay of normal distribution using the average and standard deviation calculated from the data sets. The results suggest that the transformed data (specifically for insect cells) behave more normally than untransformed data.

Statistical Analysis: Considering the acceptance limits of 18% and 9% we obtained in the repeatability study for the manual and automated methods, respectively, our low and high acceptance limits of -27.2% and 25.6% for insect cell counts are as expected. However, the low and high acceptance limits for NS0 cells are -63.3% and 63.1%, respectively. Even though a repeatability study was not performed with NS0 cells, those numbers are still significantly greater than could be reasonably expected.

We reanalyzed the NS0 data after dividing it into sets, one with viability <80% (low-viability cultures) and the other with viability >80% (high-viability cultures). Figure 5 summarizes the results. Very clearly, the spread in percent difference is much higher for the low viability NS0 cultures, as characterized by high acceptance limits, whereas the higher viability NS0 cultures have acceptance limits that are similar to those for insect cells. That indicates a significant discrepancy between cell counts obtained using manual and automated methods at lower viabilities. It most likely comes from increased spread in manual counts, which become more difficult and subjective at lower viabilities. Because of the deterministic image-processing algorithm, it is unlikely that the automated method would deteriorate as rapidly at lower viabilities.

Table 2 summarizes statistics for the mean of the percent difference in VCC for both insect and NS0 cells. Because the 95% confidence interval for the mean includes zero for the insect cells, we cannot reject a null hypothesis that the percent difference in VCC is zero. In other words, the

hypothesis that the two methods are indistinguishable cannot be rejected.

However, in the case of NS0 cells, the 95% confidence interval for the mean percent difference in VCC includes zero for low-viability cultures but does not include zero for high-viability cultures. Thus, for the high-viability cultures, we reject the null hypothesis that the mean percent difference is zero, which implies that the two methods of analyzing cell counts do provide different values. Because the percent difference was computed by subtracting the manual counts from corresponding automated counts, a negative value for the mean indicates that the automated method consistently provides higher cell counts. VCC from the manual method is on average 8.8% lower than that obtained using the automated method for high viability NS0 cultures. Although this may suggest that CHO cell type parameters are suboptimal for analyzing NS0 samples, systematically changing various cell type parameters did not improve the correlation between manual and automated counts. Visual check of the Vi-CELL images suggested that both viable and nonviable cells were being counted properly. Considering that repeatability for the automated method is significantly better than for the manual, we surmise that the automated counts are likely to be more accurate.

We performed a similar analysis on the viability data. The differences in viability did not show any systematic trend as a function of mean viability (data not included here). Table 3 summarizes the statistics for the mean difference in viability. A 95% confidence interval for the mean includes zero for the insect cells, so our null hypothesis that the mean difference is zero cannot be rejected. However, again with NS0 cells, the 95% confidence intervals did not include zero, so the null hypothesis is rejected, implying that the two methods do provide different values for culture viability. Because the difference was computed by subtracting the viability from manual counts from the corresponding viability from the automated counts, a positive value for the mean indicates

Table 2: Statistics for percent differences in VCC for insect and NS0 cells

Parameter	Insect Cells	NS0 Cells	
		Low Viability	High Viability
Number of samples	228	166	379
Mean	-0.8	2.3	-8.8
Standard deviation	13.5	36.7	14.3
Standard error	0.9	2.8	0.7
Lower 95% confidence level	-2.6	-3.3	-10.3
Upper 95% confidence level	1.0	7.9	-7.4

that the automated method consistently provides lower viabilities.

Comparing the two methods indicates that the final trypan blue concentration is about 0.036% with the manual technique and 0.2% with the Vi-CELL method. Our hypothesis is that the difference in dye concentration leads to the difference in viability data. That was confirmed when we subjected a cell culture sample to a manual count using both the standard (0.036%) and elevated concentration (0.2%), noting that the measured viability was lower by 3–6% (data not shown) when the higher trypan blue concentration was used. Also, this also confirms that the method of sample aspiration and mixing in the automated protocol does not significantly contribute to lowering cell viabilities.

Table 3: Statistics for differences in viability for insect and NS0 cells

Parameter	Insect Cells	NS0 Cells	
		Low Viability	High Viability
Number of samples	228	166	379
Mean	-0.4	4.2	6.2
Standard deviation	3.0	12.1	4.2
Standard error	0.2	0.9	0.2
Lower 95% confidence level	-0.8	2.3	5.7
Upper 95% confidence level	0.0	6.0	6.6

NOT JUST DIFFERENT — BETTER

In assessing repeatability of our manual and automated methods for cell enumeration, we found coefficients of repeatability at 18% for the manual method and 9% for the automated method. These results have reinforced our belief in the superior reproducibility of the automated method for counting cells.

Comparing the manual and automated methods using a large number of insect and NS0 cell samples over a wide range of cell densities indicated no statistically significant difference between the two methods for cell enumeration for insect cells. But the manual NS0 cell counts for high-viability cultures were consistently lower when compared with the automated counts. Similarly, we saw no statistically significant difference between the two methods for assessing viability of insect cell cultures; however, the manual method consistently showed higher viabilities compared with the automated method when NS0 samples were analyzed. That was probably attributable to the higher trypan blue concentrations found with the automated method. These differences in comparability results between insect and NS0 cells suggest process and cell-line specific relationships that should be thoroughly quantified whenever the two methods are used interchangeably (e.g., in process development and CGMP manufacturing of biotherapeutics).

This methodology has been used in our laboratory to convert legacy data obtained using manual cell counting to enable meaningful comparison with newer data generated using more contemporary techniques. 🌐

REFERENCES

1 Freshney IR. *Culture of Animal Cells: A Manual of Basic Techniques*, Fourth Edition. Wiley-Liss: New York, 2000.

2 Nielsen LK, Smyth GK, Greenfield PF. Hemacytometer Cell Count Distributions: Implications of Non-Poisson Behavior. *Biotechnol. Progr.* 7(6) 1991: 560–563.

3 Palomares LA, Pedroza JC, Ramirez OT. Cell Size As a Tool to Predict the Production of Recombinant Protein By the Insect-Cell Baculovirus Expression System. *Biotechnol. Lett.* 23(5) 2001: 359–364.

4 Bland JM, Altman DG. Measurement in Medicine: The Analysis of Method

Comparison Studies. *Statistician* 32, 1983: 307–317.

5 Bland JM, Altman DG. Statistical Methods for Assessing Agreement Between Two Methods of Clinical Measurement. *Lancet* i, 1986: 307–310.

6 BR-9713B. Product Brochure: *Vi-CELL Series Cell Viability Analyzers*. Beckman Coulter: Fullerton, CA, 2004; www.beckmancoulter.com/Literature/BioResearch/BR-9713B.pdf.

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