

# Validating the Guava<sup>®</sup> ViaCount<sup>®</sup> Assay for Use in a Manufacturing Setting

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## APPLICATION NOTE

**ABSTRACT** In this Applications Note, we describe the processes used by our customers to validate the Guava ViaCount assay and adopt the Guava Personal Cell Analysis (PCA<sup>™</sup>) system into a Good Manufacturing Process (GMP) bioproducts manufacturing setting. After an initial evaluation of the Guava PCA system, which indicated that the system would work with their cells of interest, they validated the performance of the Guava ViaCount assay, comparing results with the standard manual hemacytometer counts using trypan blue exclusion. They adopted the ViaCount assay and the Guava PCA system for cell counting and viability assessment into their manufacturing group by developing documented procedures for: 1) instrument monitoring and validation using Guava Check<sup>™</sup>; 2) performing the ViaCount assay; and 3) training.



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**Guava Technologies**

# Validating the Guava<sup>®</sup> ViaCount<sup>®</sup> Assay for Use in a Manufacturing Setting

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## Abstract

In this Applications Note, we describe the processes used by our customers to validate the Guava ViaCount assay and adopt the Guava Personal Cell Analysis (PCA<sup>™</sup>) system into a Good Manufacturing Process (GMP) bioproducts manufacturing setting. After an initial evaluation of the Guava PCA system, which indicated that the system would work with their cells of interest, they validated the performance of the Guava ViaCount assay, comparing results with the standard manual hemacytometer counts using trypan blue exclusion. They adopted the ViaCount assay and the Guava PCA system for cell counting and viability assessment into their manufacturing group by developing documented procedures for: 1) instrument monitoring and validation using Guava Check<sup>™</sup>; 2) performing the ViaCount assay; and 3) training.

## Introduction

Many laboratories continue to use the classical hemacytometer counting with trypan blue exclusion for assessing cell concentration and viability, respectively. For a biotechnology or pharmaceutical company, the scale of cell culture can expand in magnitude from R & D to the manufacturing stage. As the cell culture work increases, the need to find an automated cell counting system to provide cell number and viability data, with speed, accuracy and reproducibility becomes compelling. Laboratory managers want a system that is user friendly, which will record data and allow storage and retrieval. The Guava Personal Cell Analysis (PCA<sup>™</sup>) system meets these needs with the Guava ViaCount assay for cell count and viability assessment.

Adopting a new instrument and assay into the GMP environment requires some quality testing and process development. The Customer Solutions group at Guava Technologies can offer assistance with the installation qualification, operation qualification and performance qualification (IQ, OQ and PQ) of the Guava PCA systems. We offer a quality process guide and perform instrument IQ, OQ and PQ during instrument installation and user training. Here, we describe the process one of our customers used to adopt the Guava ViaCount assay into their GMP manufacturing setting. They validated the performance of the Guava ViaCount assay, comparing results with the standard manual hemacytometer counts using trypan blue exclusion. In their validation process, they verified the performance of the Guava PCA and the ViaCount assay for counting and viability accuracy, reproducibility and operator-to-operator variation. They developed documented standard operating procedures (SOPs) for instrument monitoring and validation using Guava Check<sup>™</sup>, performing the ViaCount assay and training, and adopted the Guava PCA system for use in their manufacturing group.

## Materials and Methods

### Cell lines and culture conditions

Cells used in the validation were culture-expanded, adherent human mesenchymal cells (hMC). Cells were grown in defined medium in a humidified 5% CO<sub>2</sub> incubator at 37° C. Cells were trypsinized using standard methods to prepare cell suspensions for counting. Typically the cultures ranged from approximately  $1 \times 10^6$  to  $4 \times 10^7$  cells/mL and 70% to 99+%.

viability for freshly harvested cultures. Samples from twelve (12) cultures were used in this validation process.

**Manual cell count and viability**

Manual counts were done using a Neubauer-type counting chamber. Cell samples were stained with trypan blue by mixing 50 µL of 0.4% trypan blue solution (Sigma Catalog No. T8154) with 50 µL of cell suspension. Cell samples at high concentration were pre-diluted as necessary, before staining with trypan blue. Duplicate stained samples were prepared from each culture for counting.

Samples were transferred to both sides of the counting chamber and scored at the microscope using transmitted light to distinguish between live (colorless) and dead (blue) cells. Total cell concentration and % viability were derived from counts using appropriate dilution factors and hemacytometer calculation factors.<sup>1,2</sup> Total cell concentration was calculated including both live plus dead cells; % viability was calculated using the formula [(Live cells/mL ÷ Total cells/mL) x 100].

**Guava ViaCount assay**

Human MC samples were stained following the procedure recommended in the Guava ViaCount package insert.<sup>3</sup> Culture samples were typically at concentrations around 1x10<sup>6</sup> to 1x10<sup>7</sup> cells/mL; therefore, 20 µL of cell suspension was mixed with 380 µL of Guava ViaCount reagent (Catalog no. 4000-0040) (20-fold dilution) in a 1.5 mL microcentrifuge tube. Samples were mixed well and incubated for at least 5 minutes (and not longer than 30 minutes) at room temperature, protected from light. Test samples were ready for data acquisition on the Guava PCA. Each sample was prepared in duplicate.

Data were acquired on the Guava PCA using CytoSoft™ software as described in the *Guava PCA User's Guide*. Before data acquisition, proper instrument performance was verified by running the Guava Check application with Guava Check reagents (Catalog No. 4500-0020) according to the recommended procedure described in the product package insert<sup>4</sup>, the *Guava PCA User's Guide*, and relevant SOPs. For Guava ViaCount samples, 1000 events

were acquired per test, and test samples were acquired twice.

**Operator-to-operator variability**

For operator-to-operator results comparisons, four hMC cultures were used for testing. Each operator made two samples from each culture: 1) a 1:1 dilution of the original culture sample, and 2) undiluted, original culture. From these culture samples, each operator prepared test samples, staining duplicate samples with trypan blue and duplicate samples with Guava ViaCount reagent, as described above. Each operator scored the trypan blue-stained samples manually using a hemacytometer, and acquired data using the Guava PCA for the ViaCount-stained samples.

**Standard operating procedures**

Upon acceptance of the Guava PCA system for cell counting and viability assessments, they developed documented procedures for instrument monitoring, ViaCount assay performance and training for use in the clinical manufacturing facility.

**Daily instrument performance check.** After the instrument is turned on, they perform a series of cleanings using the Quick Clean function. First a 20% bleach solution is used, followed by a Quick Clean with Coulter Clenz solution (we recommend Guava ICF (Catalog No. 4200-0140) as an alternative), then a Quick Clean with deionized water. After the cleaning, a Guava Check bead sample is prepared, as described in the product package insert. The operator performs the Guava Check application using this sample, records the data in a logbook and describes the acceptable/non-acceptable results. The SOP describes contingencies for non-acceptable performance. If the results are acceptable, the system is cleaned again as described above before beginning the assays.

The instrument logbook is a convenient place to record the maintenance and service history for the Guava PCA system. In addition to documenting routine Guava Check assays and Clean and Shut Down procedures for maintenance, problems and corrective measures can be logged. For example, if the flow cell

was damaged or irretrievably clogged, and the corrective action to replace the flow cell was performed, this could be recorded in the instrument logbook, with operator initials and date.

**Cell counting using Guava ViaCount.** The SOP for cell counting and viability assessment instructs the user how to prepare cell samples with the Guava ViaCount reagent, making dilutions ranging from 1:5 to 1:160, as required. The use of control samples yielding known results is desirable. These can include samples of known concentration and/or viability. Mixtures of heat-killed dead cells with viable, healthy cells in known ratios can provide convenient controls. In addition to a description of steps for data acquisition, instructions include a discussion of how to detect problems, such as flow cell clogs, and how to alleviate them. ViaCount data are automatically recorded in a spreadsheet; the SOP describes how to print out a report and document the data. The user would create a designated data folder initially, then download the data to secondary and tertiary storage according to quality systems requirements. The data can be ported conveniently to a database for monitoring trends or other QC/QA analysis.

To perform an assay, the operator prepares two dilution tubes per sample and acquires data from each tube twice. This yields four replicate counts per culture sample. For acceptance of the counting results, an average is calculated from the replicate counts, and all four counts must be within  $\pm 15\%$  of the average. If a value falls outside the acceptance range, new samples are prepared and assayed. Counts that require validation by a second operator are performed as described above; however, for acceptance, replicate counts from both operators must be within a  $\pm 20\%$  range of the average for all counts.

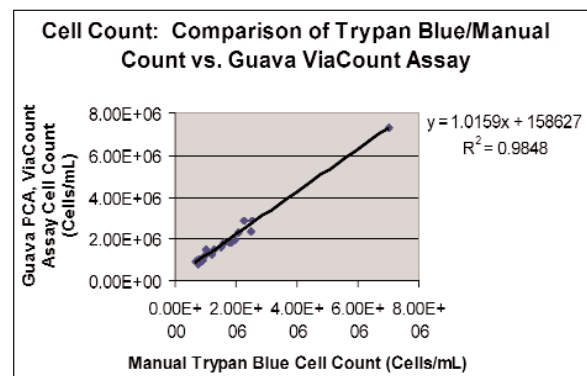
**Training.** New users should be trained one-on-one, to allow hands-on experience. Enough time is allotted so that each step for proper instrument usage and sample preparation can be demonstrated fully. Typically, it can take 3 to 4 hours to train a naive user to perform the Guava ViaCount assay on the Guava PCA system competently. Samples are prepared, which can demonstrate potential problems and errors. For example, prepare dilutions that are too

dilute to illustrate problems with event collection rate and assay time out. Samples that are too concentrated illustrate problems with non-linearity of counting. New users should be trained using cell samples that will be encountered in their work, so they can see how real samples behave in the ViaCount assay. For example, prepare and run different cell lines under different culture conditions or freshly harvested vs. cryopreserved samples, so that new operators can see the differences.

After the trainer demonstrates the proper methods, the trainee prepares and runs samples, independently under trainer observation. The Guava Check kit is handy for use in training. It is easy to prepare samples using the Guava Check reagents to provide known targets for count accuracy and precision when assessing competency after training. The trainee's results are compared to the trainer's results and target values, and they offer to buy them lunch if their %CV results (for Guava Check) are lower than the trainer's.

## Results and Discussion

**Good agreement between manual trypan blue exclusion counts and Guava ViaCount.** In the validation testing eight (8) different hMC cultures were assayed with Guava ViaCount and trypan blue exclusion on hemacytometers. The averages from replicate counts are plotted in Figure 1. The Guava PCA system counts showed excellent correlation to the manual counts, with a linear best fit slope of 1.02 and correlation coefficient ( $R^2$ ) of 0.98.



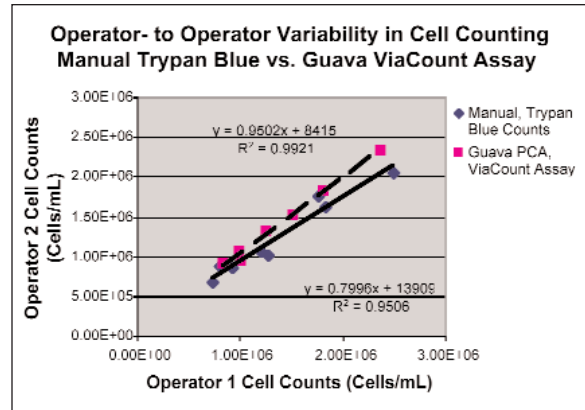
**FIGURE 1:** Comparison of total cells/mL results from manual hemacytometer counts vs. Guava ViaCount. The values plotted are average values from replicate counts of 8 different hMC culture samples.

The % Viability results showed good correlation between manual trypan blue exclusion and Guava ViaCount, with a slope of 0.95 and  $R^2$  of 0.90 (data not shown). The trypan blue exclusion results showed a slightly higher viable cell count than Guava ViaCount. This discrepancy was more evident with cultures in poorer condition (lower viability). Guava ViaCount reagent allows the identification of apoptotic cells, distinct from healthy live cells and dead cells,<sup>5</sup> whereas trypan blue does not. Many apoptotic cells do not stain blue and appear viable by trypan blue exclusion, leading to an overestimate of culture viability. Mascotti et al. reported seeing an overestimate of viability with trypan blue exclusion when comparing counts of bone marrow hematopoietic precursors done with acridine orange and propidium iodide (PI).<sup>6</sup> Altman et al. reported seeing erroneous culture viabilities with trypan blue when comparing cells assayed with fluorescein diacetate and PI.<sup>7</sup> Thus, the results obtained were not surprising and they found the correlation acceptable.

**Less operator-to-operator variability when counting cells with Guava ViaCount.** Two operators stained and counted replicate samples from four (4) different hMC cultures using Guava ViaCount and trypan blue exclusion. The average values of replicate counts from Operator 1 and Operator 2 for the same culture samples are plotted on the graph shown in Figure 2. A better correlation and consistency between operators was seen with Guava ViaCount than with trypan blue manual counts. The slope of the correlation plot for Guava ViaCount results (pink squares, hatched linear regression trend line) was closer to 1.0 (slope = 0.95) than that for manual counts (slope = 0.80). There was less variability in the Guava ViaCount results ( $R^2 = 0.99$ ) than the manual counts ( $R^2 = 0.95$ ).

When counting the same sample several times on the Guava PCA system, they obtain good precision, with <10% difference in the range of results. Typically, the difference in replicate counts of the same sample is 5% or less.

**Adoption of the Guava system in their clinical manufacturing facility.** The results of the validation study showed that the Guava system could provide comparable counts to their current method, with improved precision and less operator-to-operator variability.



**FIGURE 2:** Comparison of operator-to-operator cell count results when performing manual, hemacytometer counts with trypan blue exclusion and Guava ViaCount assays. Four (4) different hMC culture samples were stained and counted in replicate by two different operators using each method. The average values of replicate counts for the same culture sample are plotted on the graph from Operator 1 and Operator 2 for correlative comparison. The blue diamonds (solid linear regression trendline) show the correspondence between Operator 1 and Operator 2 cell counts obtained by manual, hemacytometer counts using trypan blue. The pink squares (hatched linear regression trendline) show the correspondence between Guava ViaCount results for Operator 1 and Operator 2.

They developed SOPs for instrument monitoring and maintenance, counting and training so that their manufacturing group could use the system. Standard of performance specifications for results acceptance were set, based on the results from the validation study and accepted laboratory practice.

In their SOP for cell counting, replicate cell counts (N = 4) must fall within  $\pm 15\%$  of the average count value for a single operator count. For counts that require validation by a second operator, replicate counts from both operators must be within a  $\pm 20\%$  range of the average for all counts. If a value falls outside the acceptance range, new samples are prepared and assayed.

An example is shown in Table 1. Operator A acquired four replicate cell counts, yielding an average of  $7.2 \times 10^6$  cells/mL. All of the replicate counts fall within  $\pm 15\%$  of that average value (i.e.  $6.12 - 8.28 \times 10^6$  cells/mL); therefore, these results were acceptable for a single operator count. Operator B prepared replicates from the culture sample and acquired the data shown in Set 1. The average count value was  $7.79 \times 10^6$  cells/mL, with a  $\pm 15\%$  range of  $6.61 - 8.96 \times 10^6$

cells/mL. The third count of  $8.99 \times 10^6$  cells/mL was outside the acceptance range, so Operator B had to prepare new samples for recounting. Results from the new samples (Set 2) were acceptable because all of the replicate counts fell within the  $\pm 15\%$  acceptance range.

To obtain a validated count for this cell culture from the two operators, the cell counts from both passing runs were averaged (i.e. average cell count from data generated by Operator A and Operator B (Set 2) =  $7.42 \times 10^6$  cells/mL). The acceptance range of  $\pm 20\%$  was  $6.31 - 8.53 \times 10^6$  cells/mL. All of the counts fell within the  $\pm 20\%$  range, so the results were acceptable.

**TABLE 1.** Cell counting results for the same culture sample from two independent operators.

	OPERATOR A	OPERATOR B (SET 1)	OPERATOR B (SET 2)
Replicate Cell Counts ( $\times 10^6$ cells/mL)	6.78 7.23 6.99 7.80	7.21 7.56 8.99 7.41	7.36 7.92 7.23 8.01
Average Cell Count ( $\times 10^6$ cells/mL)	7.20	7.79	7.63
Range of $\pm 15\%$ ( $\times 10^6$ cells/mL)	6.12 – 8.28	6.61 – 8.96	6.48 – 8.77
Acceptance?	Yes	No	Yes

**Recordkeeping.** Data from Guava assays are recorded in two file formats. The event data are recorded in a FCS3.0 format file, and results and instrument settings are recorded in a spreadsheet file. The spreadsheet file can be viewed and printed directly using Microsoft® Excel, minimizing data transcription errors. Results can be graphed or analyzed using Excel or other third party analysis programs, as well. Event data files can be recalled with CytoSoft and printed after data acquisition for lab notebook records. The event data can be reanalyzed after data acquisition, if desired and saved under a new filename. A new spreadsheet file with reanalyzed results is automatically saved with the new filename.

## Conclusion

In practice, users have found that the Guava system is quick and easy to use. They can accurately count multiple samples in a fraction of the time it took to perform manual hemacytometer counts. Additional timesavings were realized because the results are highly reproducible and cell counts rarely needed to be repeated between operators. The data are recorded in a spreadsheet file and print out, which can be put directly into the laboratory record, eliminating data transcription errors. For these reasons, this customer performed an assay validation for Guava ViaCount and instituted documented procedures for adoption of the Guava PCA system into their clinical manufacturing process.

The description of their process is intended only as an illustrative example. Quality practices and processes are dictated by the situation and needs in each organization, and evolve over time as new products or services are developed. In our assay development at Guava, we performed tests for accuracy and precision, comparing results to those obtained using alternative (predicate) methods. We tested for operator-to-operator variability, as well as instrument-to-instrument variability. Prepared sample stability was tested, to determine practical limits for time between staining and data acquisition. For the ViaCount assay, count linearity over different cell concentration ranges and culture viability ranges was tested. Each laboratory will need to validate assays using cells and conditions as appropriate, and prepare documented methods, processes and performance specifications relevant to their GMP product development and production requirements. Any assay validation work will apply to the specific version of CytoSoft used. Revision to SOPs, equivalence testing or revalidation may be required if upgrades to software or instrumentation are desired. However, adoption of the Guava system into a GMP environment is straightforward, and customers are putting the Guava ViaCount assay to good use in research, development and manufacturing laboratories.

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## Product Notes

Products sold by Guava Technologies are intended for in vitro research use only. A technically qualified individual should supervise usage. Individuals receiving this information must exercise independent judgments in determining its appropriateness for a particular purpose. Material Safety Data Sheets are available for each product. Contact Guava Technologies Technical Support for the most up-to-date information.

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