

Guava Technologies

Validating the Guava[®] ViaCount[®] CDR[™] Assay

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Abstract

Accurate cell counts and viability assessments of homogeneous cell suspensions can be accomplished by a variety of methods. The Guava ViaCount assay provides cell count and viability data for cell suspensions from a variety of cell lines, quickly and easily. However, cell samples containing clumpy aggregates present a challenge by any method, manual or automated. We have developed the Guava ViaCount CDR assay to address this problem. The assay uses Guava ViaCount CDR Cell Dispersal Reagent to gently disaggregate clumps, and Guava ViaCount Reagent to stain cells for count and viability assessment. The assay was optimized and validated using CHO-K1 cells that were adapted to suspension culture in serum-free medium. Significant improvement in the count accuracy was attained for this assay, with good linear correlation to hemacytometer counts ($R^2 > 0.93$) over a broad range of cell densities ($5 \times 10^5 - 1 \times 10^7$ cells/mL) and culture conditions (50 - 95% viable, 17 - 87% aggregation). Assay results were highly reproducible, with % CV for replicate cell count values ranging from 3 - 7% and % CV for replicate viability measurements of 1 - 2%.

Introduction

There are many different ways to count cells and assess their viability. Many laboratories rely on manual counting, viewing stained cells through a microscope on a hemacytometer. The method is inexpensive but laborious, and can take time. Results can show significant variation from reader to reader, due to differences in the interpretation and scoring of cell and debris morphologies and staining patterns. Instrument-based systems are available, such as the impedance counter or flow cytometer. There are instruments that automate the manual methods, incorporating sample preparation and

scoring by image analysis. Instrument-based methods are speedy and save labor, with higher reproducibility in measurements; however, they require more capital investment and staff training to yield accurate results.

Many different combinations of stains have been used in both manual and instrument-based systems. A variety of cell stains for viability determination are available, including colorimetric stains such as trypan blue, and fluorescent dyes such as acridine orange and ethidium bromide, which rely upon differential permeability of cells in a healthy vs. dead or dying state. The Guava ViaCount assay distinguishes between viable and non-viable cells based on the differential permeability of DNA-binding dyes in the ViaCount Reagent. One dye is membrane permeable and stains all nucleated cells; fluorescent signal from the nuclear staining dye is detected by photomultiplier tube 2 (PM2). The other dye will only penetrate cells with compromised membrane integrity (i.e. non-viable cells) and is detected by photomultiplier tube 1 (PM1). Events are counted if they emit nucleated cell fluorescent signal and the forward light scatter (FSC) intensity is appropriate for that of a particle the size of a cell. Debris events with low FSC signal are not counted. Non-viable cells will emit fluorescent signal from the dead cell stain.¹ Absolute cell counts are obtained on the Guava instrument systems by knowing the exact sampling volumes. The cell suspension is drawn by positive displacement into the capillary flow cell of known dimensions at a precisely controlled rate for measured periods of time.

Guava ViaCount provides an absolute cell count and viability data on cell suspensions prepared from a variety of sources: cultured cell lines, blood and tissues. Obtaining an accurate and reproducible cell count and viability assessment from a homogeneous, single cell suspension is straightforward for all the methods described above. However, it is a challenge

to get a good result for samples containing cell aggregates by any method. Cell aggregates are not homogeneous and vary in their size distribution. They contain mixtures of live and dead cells and debris, in varying amounts. Aggregates are clumpy so the cells will not be evenly distributed in a monolayer for easy imaging or viewing at the microscope. At best, the operator or imaging system must adjust through different planes of focus to view cells in a clump for counting. It is difficult to get an accurate count because cells in the clump will not lie in parallel planes of focus, and cells may be missed or counted twice.

Impedance or light scatter in flow cytometry would base the cell number in an aggregate on a particle size measurement. If the instrumentation had suitable software algorithms to determine cell numbers from such measurements, heterogeneity of clump composition (cells and debris of different sizes) and shape would make this measurement a rough estimate, at best. If there was no accommodation for calculating cell number from aggregate size, the clump would be counted as a single event, and the cell count would be grossly underestimated if the sample were highly aggregated. Depending on the size of aggregates, clumps could clog or be excluded from the fluidic systems of automated cell counters, hampering data collection.

Assessment of viability is complicated in cell aggregates, because they contain mixtures of live and dead cells. Certain stains or substrates may have difficulty penetrating to cells in the core of the clump. For automated imaging systems using trypan blue, this could be a problem, causing an overestimation of culture viability. Flow cytometers cannot accurately assess viability in aggregated samples due to difficulties in interpreting viability staining results. Fluorescent viability stains often penetrate cell aggregates efficiently; however, fluorescence quenching can complicate signal interpretation by dimming signal from dead cells in the clump. For simpler automated systems, detection of any dead cell stain with a particle will brand that event as a dead cell. Thus, an aggregate containing a mixture of live and dead cells will be scored as a dead cell, leading to an underestimate of culture viability and cell count. Impedance counters cannot perform viability assessments.

We developed the Guava ViaCount CDR assay to address the problems associated with determining an accurate cell count and viability from samples containing cell aggregates. Clumps are gently disaggregated by treatment with Guava ViaCount CDR Cell Dispersal Reagent, which contains protease, DNase and collagenase activities, and cells are stained with ViaCount Reagent for cell count and viability assessment on the Guava PCA. We validated the assay using CHO-K1 cells, adapted for growth in suspension culture. About 1200 samples were assayed, using 26 different CHO-K1 cultures with a range of viabilities, “clumpiness” and cell densities. For our predicate method, we performed manual cell counts using a hemacytometer, because most laboratories are familiar with this method. We wanted to evaluate cell count and viability results before and after disaggregation. Trypan blue exclusion was not used for viability assessment because we did not expect trypan blue to penetrate cell aggregates efficiently. Cell samples for microscopic evaluation were stained with ethidium bromide and acridine orange (EB-AO) because these dyes permeated the clumps and stained cells efficiently (see Figure 1). The results of our assay validation study are presented here.

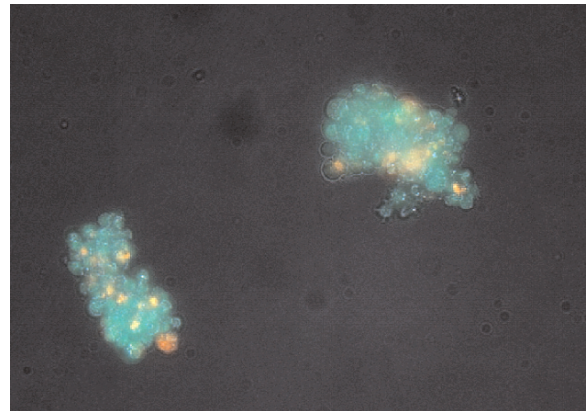


FIGURE 1: CHO-K1 cell aggregates from a culture adapted to grow in suspension in serum-free medium. The sample was stained with ethidium bromide and acridine orange, which penetrate to cells within the interior of the aggregate. Live cells appear green, fluorescing from acridine orange intercalated into cellular nucleic acid. Ethidium bromide is excluded from live cells. Dead cells appear orange, fluorescing from ethidium bromide, which can pass through the compromised cell membrane and intercalate into nuclear DNA. View captured through fluorescence microscope (1000x magnification); most of the cells in the aggregates are out of the plane of focus and difficult to count.

Materials and Methods

Scope of validation study

This validation study involved comparing cell counts and viability assessments of clumpy CHO-K1 suspension cultures, obtained before and after disaggregation treatment using Guava ViaCount CDR Cell Dispersal Reagent. For count and % Viability accuracy, the results obtained on the Guava PCA using CytoSoft software were compared to manual hemacytometer counts of EB-AO stained cells from the same culture. Optimal ViaCount CDR assay conditions were obtained for each culture sample by using a range of ViaCount CDR concentrations and incubation times. Twenty-six (26) different culture samples were tested in the validation, covering a range of cell densities, viabilities and level of aggregation. A summary of the test sample characteristics is presented in Table 1.

TABLE 1. Test Sample Characteristics:

CELL LINE	CHO-K1, adapted to suspension culture; N = 26 test cultures	
CELL DENSITY	Total cell concentration ranged from approx.: 1×10^6 to 1×10^7 (mean = 4.42×10^6 , median = 3.47×10^6 by manual count after disaggregation)	
VIABILITY	% Viabilities ranged from approx.: 50% to 95%: (by manual count before disaggregation)	
	% Viability	Distribution of Test Cultures
	90-100%	7/26 = 27%
	80-90%	8/26 = 31%
	70-80%	6/26 = 23%
	Below 70%	5/26 = 19%
AGGREGATION	% of Cells in Clumps in the Sample (estimate): 17% to 87% (mean = 45%, median = 47%)	

Count linearity was verified over a range of cell culture densities (5×10^5 to 1×10^7 cells/mL) for the ViaCount CDR assay. Assay reproducibility was determined by preparing replicates with 5 different culture samples.

Cell lines and culture conditions

We adapted an adherent CHO-K1 cell line (ATCC, Cat. No. CCL-61) to suspension culture. The adherent line was cultured in Hams F12 (F12K) medium, supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. The cell line was adapted to suspension culture by subculturing cells in a spinner flask containing 50:50 (v/v) mixture of CD CHO medium (Invitrogen, Cat. No. 10743) and complete F12K medium. The culture was gradually weaned to growth in CD CHO medium.² Adaptation of the adherent culture to suspension growth took about two weeks. CHO-K1 cells were grown in a humidified 5% CO₂ incubator at 37° C. For high viability cells, the cultures were split 1:5 (1 part cell suspension, 4 parts CD CHO Medium) every 2 or 3 days. To obtain cell samples with lowered viability, CHO-K1 cells were split 1:2 (1 part cell suspension, 1 part CD CHO Medium) every 3 days until the desired viability and cell concentration were attained.

Manual cell count and viability

Manual cell counts were done using a Neubauer-type counting chamber (Brite-Line hemacytometer, Hauser Scientific, Part No. 1492) at the epi-fluorescence microscope (Leica DM LB). Cell samples were stained with ethidium bromide and acridine orange (EB-AO).³

EB-AO was prepared as a 100X stock solution by dissolving 50 mg of ethidium bromide (Molecular Probes, Cat. No. E-1305) and 15 mg of acridine orange (Molecular Probes, Cat. No. A-1301) in 1 mL of 95% ethanol (Rossville Gold Shield). After the dyes were dissolved, distilled water was added to 50 mL. The solution was filtered through a 0.45 or 0.2 micron syringe filter unit to remove particulates. The 100X EB-AO stock solution was divided into 1 mL aliquots and stored frozen at -20° C, protected from light. The 100X EB-AO solution contains 1 mg/mL ethidium bromide and 0.3 mg/mL acridine orange.

To stain cells for counting, the 100X EB-AO was diluted 100-fold with Dulbecco's phosphate

buffered saline (PBS), pH 7.2 (JRH Biosciences, Catalog No. 59321) (1 part 100X EB-AO, 99 parts PBS) to make a 1X working solution of EB-AO. Untreated cell samples were diluted as necessary to a concentration of 1×10^6 to 5×10^6 cells/mL in PBS or other isotonic medium. Equal volumes of cell suspension (25 μ L) and 1X EB-AO (25 μ L) were mixed in a microcentrifuge tube for staining. For the validation study, replicate samples (N = 2) were prepared for manual count from each culture.

For manual counts of disaggregated cells, 100 μ L of CHO-K1 cells were mixed with 100 μ L of 0.2X Guava ViaCount CDR Cell Dispersal Reagent (Guava Technologies, Catalog No. 4700-0050). The 0.2X ViaCount CDR was made by diluting ViaCount CDR with PBS (i.e. 1 part ViaCount CDR, 4 parts PBS). The mixture was gently vortexed and incubated at 37° C for indicated time periods (10, 20 or 30 minutes). At the indicated time point, 25 μ L of treated CHO-K1 cells was mixed with 25 μ L of 1X EB-AO in a microcentrifuge tube. Replicate samples were prepared for each time point (N = 2). The test sample was returned to the incubator for further reaction. Stained cell samples were ready for hemacytometer counting.

For cell count and viability determination, a small amount (20 μ L) of EB-AO-stained cell suspension was placed under the coverslip of the hemacytometer. The hemacytometer was placed on the fluorescence microscope stage. The cells were observed initially under visible light, adjusting the light intensity and diaphragm of the microscope to reduce the light level as much as possible while keeping the hemacytometer grid visible. Then the cells were viewed in epi-fluorescence mode using the fluorescein filter set (Leica, I 3 filter set: BP 450-490 nm, LP 515 nm). Cells were counted using a multichannel manual tally counter, scoring green fluorescing cells as live and orange fluorescing cells as dead. For untreated, clumpy CHO-K1 samples, cells in clumps were scored, as well (see below). Cell concentrations were calculated from hemacytometer counts.^{4,5} Viabilities were calculated as percentages: %Viability = [(Live cell count/Total cell count) x 100]. Total cell count equaled the sum of the live cell count plus dead cell count.

Assessing the level of aggregation

While performing the manual cell count and viability assessment of CHO-K1 samples before disaggregation, an assessment of the level of aggregation was made by analyzing the cell clumps in the sample. An estimate of the number of cells per clump was made by visual inspection, and a tally of the number of clumps per size category was recorded. Clumps were scored in the following size categories: 3-5 cells, 6-10 cells, 10-20 cells and >20 cells. A weighted average value per category was derived by surveying the clumps in the category. The weighted average values were used to calculate the number of cells in clumps per category. The sum of the number of cells in clumps from all the categories equaled the total number of aggregated cells for the sample. This value was converted to cell concentration using appropriate dilution factors and hemacytometer calculation factors. The level of aggregation in the culture was characterized by the % of Cells in Clumps, which equaled [(Total Aggregated Cells per mL / Total Cells per mL) x 100]. Total Cells per mL value comes from a cell count from the same culture after disaggregation treatment.

Guava ViaCount assay

The Guava ViaCount assay was performed on samples from untreated, clumpy CHO-K1 cultures, as described in the package insert for Guava ViaCount Reagent (Guava Technologies, Catalog No. 4000-0040).⁶ For typical cell samples at a concentration of 10^6 to 10^7 cells/mL, 20 μ L of cell suspension was mixed with 380 μ L of Guava ViaCount Reagent (20 fold dilution) in a 1.5 mL microcentrifuge tube. Samples were mixed well and incubated for about 5 minutes at room temperature, protected from light. Test samples were ready for data acquisition on the Guava PCA. Each sample was prepared in replicate (N = 3) and acquired twice.

Guava ViaCount CDR assay

The Guava ViaCount CDR assay was performed as described in the package insert for Guava ViaCount CDR Cell Dispersal Reagent.⁷ For the validation study, a range of ViaCount CDR concentrations (see Table 2) and incubation times were used in order to

obtain data under optimized assay conditions for the different CHO-K1 culture samples. In brief, 100 μL of CHO-K1 cells was mixed with 100 μL of ViaCount CDR solution and 300 μL of Guava ViaCount Reagent in a microcentrifuge tube. The sample was mixed by vortexing and placed on a tube rotator in a 37° C incubator. Test samples were incubated for 10, 20 or 30 minutes. At the end of the incubation period, 500 μL of ViaCount Reagent was added. The sample was incubated for an additional 5 minutes at room temperature, protected from light exposure. Samples were ready for data acquisition on the Guava PCA. The ViaCount CDR concentration and incubation time for the optimal assay condition yielded a maximal total cell count (i.e. most complete disaggregation) and lowest % Viability value (i.e. minimized loss of dead cells by digestion).

TABLE 2. Concentrations and preparation of ViaCount CDR reagent

VIACOUNT CDR CONCENTRATION	VIACOUNT CDR	PBS BUFFER
1X	1 part	0 part
0.8X	4 parts	1 part
0.6X	3 parts	2 parts
0.4X	2 parts	3 parts
0.2X	1 part	4 parts

Count linearity study for the ViaCount CDR assay. Six (6) different CHO-K1 culture samples of medium to high viability (70% to 90+%) were obtained and tested as follows. For each experiment, a culture was diluted with PBS to yield 5 different test concentrations ranging from 5×10^5 to 1×10^7 cells/mL. Three (3) replicate samples were prepared from each test concentration. Replicate aliquots were treated according to the ViaCount CDR assay protocol using the solutions at 0.8X to 1X ViaCount CDR concentration and 20 minutes for the disaggregation incubation period. Upon completion of the assay, samples were acquired on the Guava PCA.

For manual counts, replicate aliquots (N = 3) from each CHO-K1 test concentration were disaggregated with ViaCount CDR, stained with EB-AO and counted at the fluorescence microscope using a hemacytometer, as described previously.

Reproducibility study for the ViaCount CDR assay.

Five (5) different CHO-K1 culture samples of medium viability (68% to 88%) were obtained and tested as follows. Five (5) replicate samples were prepared and treated according to the ViaCount CDR assay protocol using the solution at 0.8X ViaCount CDR concentration and 20 minutes for the disaggregation incubation period. Upon completion of the assay, data from each sample was acquired on the Guava PCA in triplicate, yielding 15 data points per culture sample. % CV results for the cell counts and viability were calculated.

Data Acquisition

Proper instrument performance was verified by running the Guava Check application with Guava Check reagents (Catalog No. 4500-0020). Data was acquired on the Guava PCA using CytoSoft software as described in the *Guava PCA User's Guide* and respective package inserts. For Guava ViaCount, 1000 events were acquired.

Results and Discussion

The approach for accurate, reproducible cell counts of clumpy cultures. Performing accurate cell counts and viability assessments on samples with cell aggregates is difficult. The simplest approach is to disaggregate the sample before attempting an assay. The most effective methods to disaggregate cell clumps involve enzymatic treatment to digest the proteins, nucleic acids, and other agents that cause cells to stick together. All enzymatic treatments will digest dead cells preferentially over live, healthy cells. Live cells can resist enzymatic treatment by repairing digestive effects. Dead cells cannot repair enzymatic damage. In addition, dead cells have lost membrane integrity, allowing enzymes to leak into the cells and digest cells from the inside and outside.

Trypsin is commonly used and is effective at disaggregating cells; however, we found it difficult to control the rate of digestion reproducibly and obtained inaccurately high viability results when using trypsin. We tested other commercially available disaggregation reagents (Accutase and Accumax, Innovative Cell Technologies, Inc.; No-Zyme, JRH Biosciences) but

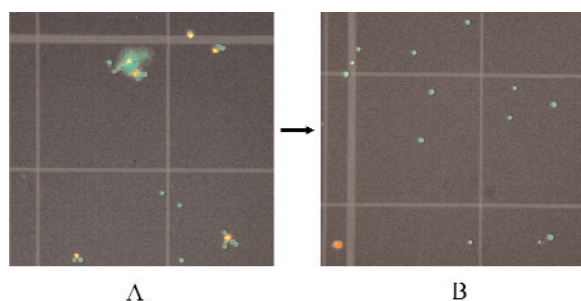


FIGURE 2: CHO-K1 sample before (A) and after (B) treatment with ViaCount CDR to disaggregate cell clumps. Cells were stained with EB-AO and placed on a hemacytometer for viewing and counting (400X magnification). Treatment with ViaCount CDR gently disaggregates clumps, yielding a more homogeneous cell suspension for easier and more accurate counting.

found it difficult to disaggregate the clumps reproducibly with these reagents. Guava ViaCount CDR contains a concentrated formulation of enzymes with various digestive activities (collagenase, DNase, etc.). The reagent was formulated to disaggregate cells gently, yet yield reproducible results for cell count and viability when used on clumpy cell samples in the ViaCount CDR assay (see Figure 2).

We chose to compare cell counts and viability assessments from manual hemacytometer counts using EB-AO staining rather than trypan blue. As shown in Figures 1 and 2, EB-AO penetrates readily into the cell aggregate, and the green vs. orange stain is easier to distinguish and score at the microscope than the varying shades of blue with trypan blue. Although there would be error associated with the manual counts due to the difficulties in focusing on cells in clumps through the microscope, we chose to use the hemacytometer counts on untreated, clumpy cells in order to avoid digestive loss of dead cells, when comparing total cell counts and viability measurements for accuracy.

Skewing of results due to preferential loss of dead cells was a concern for enzyme treated samples. We could not perform a direct manual count on a sample prepared using the ViaCount CDR assay protocol. The cell sample would be too dilute to obtain an accurate count on the Neubauer-type hemacytometer. To maintain a sufficiently high cell density for manual counting, the sample volume was limited. This restriction necessitated a protocol where the cells may have been exposed to a higher relative con-

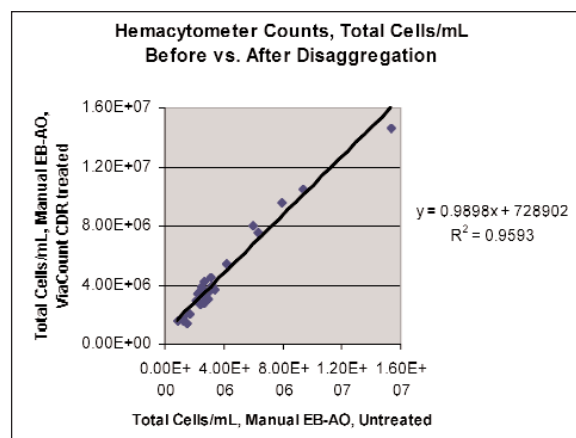


FIGURE 3: Comparison of Total Cells/mL results from manual hemacytometer counts for CHO-K1 cultures, before and after treatment with ViaCount CDR to disaggregate clumps. Cells were stained with EB-AO and counted at the fluorescence microscope.

centration of ViaCount CDR for hemacytometer counting than that used for samples run on the Guava PCA system. However, manual count results correlated well between untreated and disaggregated samples, because there was not much loss of dead cells, and any residual clumps were small and could be scored more easily. Figure 3 shows a plot comparing Total Cells/mL results from manual counts done before and after treatment with ViaCount CDR to disaggregate cells. There was an excellent correlation, with a slope of 0.99 and correlation coefficient R^2 of 0.96. Good linear correlations were obtained when comparing Live Cells/mL and % Viability results for manual counts done before and after cell disaggregation (Figures 4 and 5). Slope for the Live Cells/mL plot was 1.08 with a correlation coefficient R^2 of 0.89. For the % Viability comparison, slope was 0.97 with a correlation coefficient R^2 of 0.94. Although the results matched very well, these manual counts were laborious and difficult to perform, especially for the untreated, clumpy samples.

Clump disaggregation improves accuracy of cell counts and viability assessments for ViaCount. The Guava PCA system counts particle events, discriminating size using forward light scatter analyzed on a log scale. Due to the log scale display, single cell events are not well resolved from aggregates. Without treatment with ViaCount CDR, clumps are generally counted as single cells, leading to serious undercounting and poor accuracy for cell cultures containing

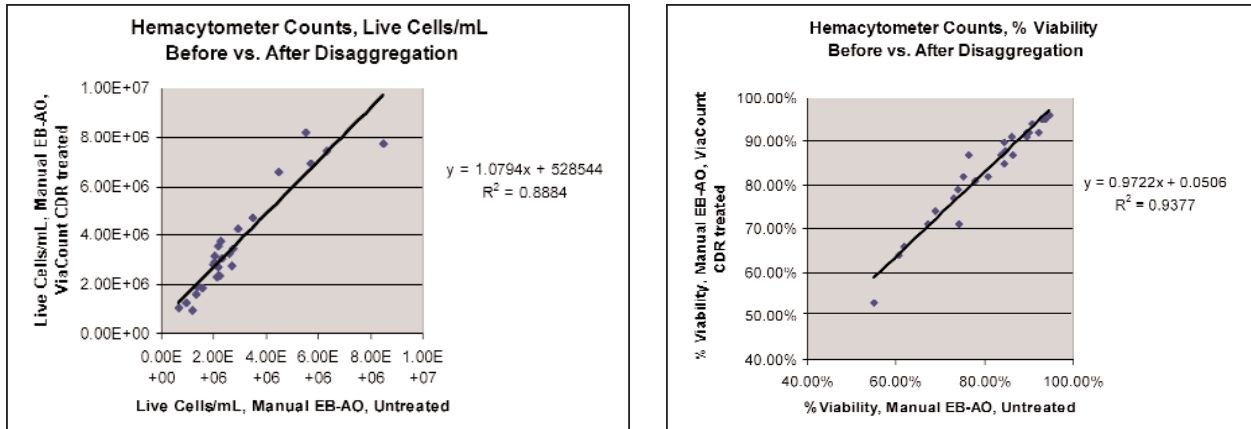
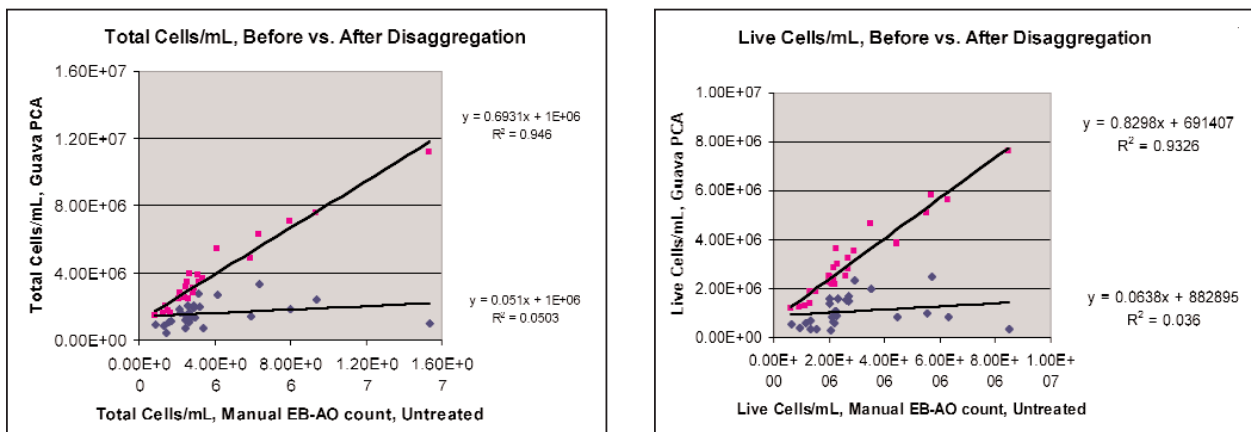


FIGURE 4 & 5: Comparison of Live Cells/mL results (LEFT) and % Viability results (RIGHT) from manual hemacytometer counts, before and after treatment with ViaCount CDR to disaggregate clumps. Cells from CHO-K1 cultures were stained with EB-AO and counted at the fluorescence microscope.

significant aggregation. This can be seen in Figures 6, 7 and 8. Aliquots of clumpy CHO-K1 cultures were disaggregated and stained in the ViaCount CDR assay, or left untreated and stained with ViaCount reagent for direct data acquisition and analysis on the Guava PCA system. Another untreated sample from the same culture was stained with EB-AO and counted manually using a hemacytometer.

In Figure 6, we have plotted the Total Cells/mL values of the Guava results for the untreated and ViaCount CDR assayed samples against the manual count results. The cells counts obtained on the Guava PCA system from the untreated, clumpy ViaCount stained samples do not correlate with the manual counts because the clumps were being counted as single cell

events (blue diamonds; slope = 0.05 and $R^2 = 0.05$). After disaggregation, a significant improvement was seen, with improved linear correlation between the ViaCount CDR assay result and manual counts (pink squares; slope = 0.69 and $R^2 = 0.95$). There was an undercounting of total cells after treatment in the ViaCount CDR assay at high cell densities. We suspected that there was some level of residual small cell clumps present in these samples after ViaCount CDR treatment. When we analyzed the level of aggregation in the 26 test cultures, we found a higher percentage of cells in aggregates in cultures at higher cell densities (graph not shown, slope = 0.56 and $R^2 = 0.76$), whereas there was no correlation between the level of aggregation and the viability of the culture (graph



FIGURES 6 & 7: Correlation for Total Cells/mL results (LEFT) and Live Cells/mL results (RIGHT) between manual hemacytometer counts and the Guava PCA system, before vs. after cell disaggregation. For manual counts, clumpy CHO-K1 cells were stained with EB-AO and counted directly using a hemacytometer. In each graph, the upper plot (pink squares) shows the results after ViaCount CDR assay to disaggregate clumps. The lower plot (blue diamonds) shows results counting clumpy cultures with ViaCount alone.

not shown, slope = 0.0007 and $R^2 = 0.02$). If we considered only the culture samples at cell densities below 5×10^6 cells/mL ($N = 21$), the slope of the linear fit trendline becomes 1.10 ($R^2 = 0.82$), indicating good accuracy for these samples. Cultures at lower cell densities contain lower levels of aggregation, making it easier to attain a homogeneous single cell suspension treatment with ViaCount CDR. The desire for complete disaggregation of clumps must be balanced with the preservation of dead cells; using higher concentrations of enzyme or longer incubation times causes digestive loss of dead cells preferentially. Loss of dead cells by over-digestion contributes to the problem of obtaining lower total cell counts than expected.

A comparison of the Live Cells/mL values for the untreated and ViaCount CDR assayed samples obtained with the Guava PCA system and the manual count results is presented in Figure 7. Again, a poor correlation was found for the cells counts obtained on the Guava PCA system from the untreated, clumpy ViaCount stained samples and the manual counts because the clumps were being counted as single cell events (blue diamonds; slope = 0.06 and $R^2 = 0.04$). After disaggregation, a good correlation was seen between the ViaCount CDR assay results and manual counts (pink squares; slope = 0.83 and $R^2 = 0.93$). Again, there was a slight undercounting of live cells after treatment in the ViaCount CDR assay for the higher density samples. If we consider the cultures at a live cell concentration below 4×10^6 cells/mL ($N = 20$), which should contain lower levels of aggregation, the agreement with manual results improves (slope = 1.14 and $R^2 = 0.82$). If an accurate live cell count was desired, with no interest in a total cell count or viability determination, the enzyme concentration or incubation time could be extended to ensure complete disaggregation of cell clumps; loss of the dead cells would not be a concern.

In Figure 8, we have plotted the % Viability values of the Guava results for the untreated and ViaCount CDR assayed samples against the manual count results. The % Viability obtained on the Guava PCA system from the untreated, clumpy ViaCount stained samples show surprisingly good linear correlation with the manual % Viability (blue diamonds; slope = 1.3 and $R^2 = 0.89$); however, the result is less accu-

rate, with significant underestimation of the culture viability. This is due to skewed counting of clumps as dead cell events. A clump would have to contain 100% live cells for it to be scored as a viable cell event; however, any detectable dead cell stain signal associated with a clump would cause the event to be scored as a dead cell. This problem would be exacerbated in lower viability cultures, where the probability of dead cell inclusion in cell clumps

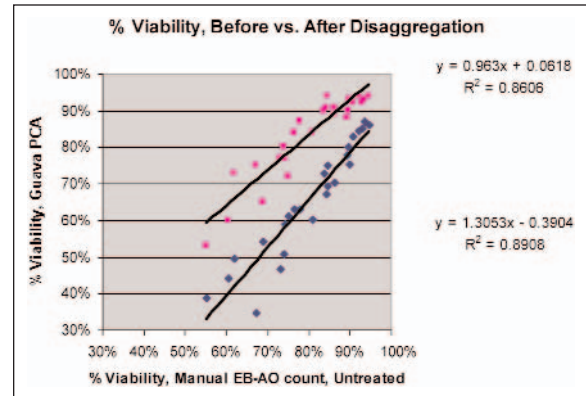


FIGURE 8: Correlation for % Viability results between manual hemacytometer counts and the Guava PCA system, before vs. after cell disaggregation. For manual counts, clumpy CHO-K1 cells were stained with EB-AO and counted directly using a hemacytometer. Upper plot (pink squares) shows results after ViaCount CDR assay to disaggregate clumps. Lower plot (blue diamonds) shows results counting clumpy cultures with ViaCount alone.

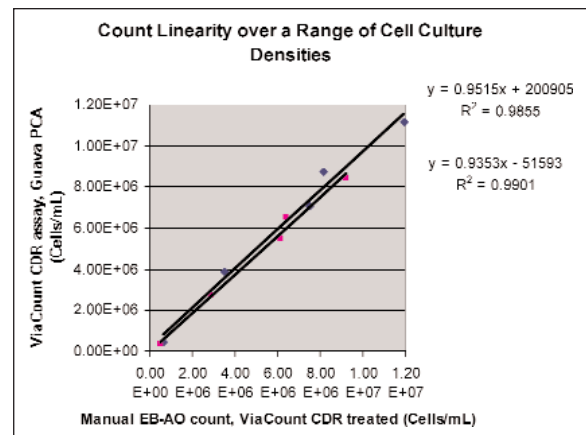


FIGURE 9: Correlation of count linearity over a 24-fold range of cell concentrations. Replicate CHO-K1 samples ($N=3$) were treated according to the ViaCount CDR assay using 1x ViaCount CDR and a 20 minutes incubation, and counted on the Guava PCA system. For manual count, replicate aliquots from the same culture ($N=3$) were disaggregated with ViaCount CDR, stained with EB-AO, and counted on a hemacytometer. Average values for Total Cells/mL are plotted (blue diamonds). Average values for Live Cells/mL are shown (pink squares). Sample concentrations ranged from approximately 5×10^5 to 1.2×10^7 cells/mL.

TABLE 3. Results of the Guava ViaCount CDR precision study

Culture No.	AVERAGE CELL COUNT RESULTS (N=5)			%CV (N=5)		
	Total Cells/mL	Live Cells/mL	% Viability	Total Cells/mL	Live Cells/mL	% Viability
1	2.66 x 10 ⁶	2.22 x 10 ⁶	84%	7%	7%	1%
2	2.50 x 10 ⁶	2.19 x 10 ⁶	88%	3%	4%	1%
3	2.76 x 10 ⁶	2.34 x 10 ⁶	85%	3%	4%	2%
4	3.45 x 10 ⁶	2.85 x 10 ⁶	83%	5%	5%	1%
5	2.66 x 10 ⁶	1.81 x 10 ⁶	68%	3%	4%	2%

would increase. After disaggregation, a significant improvement was seen in the accuracy of viability assessment, with good linear correlation between the ViaCount CDR assay result and manual counts (pink squares; slope = 0.96 and R² = 0.86).

Counts are linear over a broad range of culture densities in the ViaCount CDR assay. The ViaCount CDR assay and manual counts for this linearity study were performed on the six different CHO-K1 culture samples. For the ViaCount CDR assay and manual counting, two sets of test samples at five different concentrations were prepared in triplicate from each culture. A typical result is shown in Figure 9, where averages of the counts are plotted. The determinations for Total Cells/mL and Live Cells/mL were linear over the test range, from about 5 x 10⁵ to 1.2 x 10⁷ cells/mL. The data show high correlation coefficients and excellent correspondence for total cell counts (slope = 0.95, R² = 0.99) and live cell counts (slope = 0.94, R² = 0.99) between the Guava PCA system and manual counts. We do not know the actual limit of the linear range, because we were unable to test higher concentration cultures (we do not have the facilities to culture cells at densities much higher than 10⁷ cells/mL). However, it seems likely that the linear range could extend to higher cell densities, if the assay conditions (e.g. relative concentration of reagents to cells) are maintained.

ViaCount CDR assay yields highly reproducible results. We tested the reproducibility of the ViaCount CDR assay on five different CHO-K1 cultures. For each culture, five replicates were prepared according to the ViaCount CDR assay using 0.8X ViaCount CDR reagent and a 20-minute incubation period. Results are summarized in Table 3.

We achieved great reproducibility in cell counts and viability assessments from the ViaCount CDR assay. Assay precision was high, with %CV ranging from 3 – 7% for Total Cells/mL, 4 – 7% for Live Cells/mL and 1 – 2% for % Viability values.

Conclusion

The Guava ViaCount CDR assay expands the utility of the Guava ViaCount application to cell lines that form aggregates. Performing an accurate and reproducible cell count and viability assessment on clumpy cell lines is difficult by any method. Our assay uses Guava ViaCount CDR Cell Dispersal Reagent to gently disaggregate cell clumps and Guava ViaCount reagent to stain cells. The assay was optimized and validated using clumpy CHO-K1 cells that were adapted to suspension culture in serum-free medium. In our assay development, we optimized for retention of dead cells for more accurate total cell counts and % Viability determinations. The Guava ViaCount CDR assay yielded results with good correspondence and linear correlation to EB-AO hemacytometer counts (R² > 0.93) over a broad range of cell densities (5 x 10⁵ – 1 x 10⁷ cells/mL) and culture conditions (50 - 95% viable, 17 - 87% aggregation). Assay results were highly reproducible, with % CV for replicate cell count values ranging from 3 - 7% and % CV for replicate viability measurements of 1 - 2%. The assay is easy to perform, with rapid data acquisition and analysis on the Guava PCA system. The Guava ViaCount CDR assay provides a more realistic assessment of the status of your clumpy cell cultures, allowing better control over the condition of cells used in your experiments and processes.

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