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Cluster Count Analysis and Sample Preparation Considerations for the Vi-CELL BLU Cell Viability Analyzer

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Introduction

Cell lines such as human embryonic kidney (HEK) cells may form both weakly clustered agglomerates and more difficult to separate aggregates under certain culture conditions (1). Software algorithms may have difficulty counting aggregates and agglomerates properly which can lead to inaccurate cell counts. To mitigate this, sample preparation methods may need to be optimized to eliminate or reduce larger aggregates and agglomerates before taking a sample for analysis (2). The Vi-CELL BLU Cell Viability Analyzer has integrated software features that allow clumps to be more accurately counted and are described in more detail in our “Cell Type settings considerations for counting clumpy cells on the Vi-CELL BLU cell viability analyzer” application note.

If software mitigation features are not sufficient to divide and count small aggregates and agglomerates, the Vi-CELL BLU Cell Viability Analyzer software will quantify the remaining larger clusters and flag them in images with a red square (Figure 3 below). The cells within are not counted due to the inaccuracy of enumerating large, sometimes three-dimensional cell clusters, however, the software will quantify and report the number of larger clusters. By reviewing this cluster count data, you may examine the extent of clustering for a particular sample. Because large aggregates may have negative effects on suspension cell cultures for reasons such as poor cell growth due to decreased metabolic diffusion, and reduced transfection and transduction efficiencies leading to lower titers due to the inability of plasmids and viral particles to reach the interior cells (3), it may be preferred to discontinue a culture. If clustering is determined to be unacceptable but counts are still desired, sample preparation procedures may need to be established.

For cell lines with more difficult to separate aggregates such as some HEK293 cultures under certain conditions, additional dissociation optimization is a way to deliver well-dispersed suspensions to the Vi-CELL BLU Cell Viability Analyzer and enable more accurate counts. In this application note, we describe the effect of different dissociation media and trituration by pipetting on the degree of clustering, viability, and viable counts of adherent HEK293 cells. We also provide a side sample preparation technique to obtain more accurate counts on the Vi-CELL BLU Cell Viability Analyzer without having to change existing dissociation methods for passaged cultures.

In the first experiment, three enzymatic dissociation media were tested: trypsin, trypsin with EDTA, and TrypLE. In addition, trituration by pipetting up and down with a 1000 µL air displacement pipette was tested for each dissociation medium on divided samples. In the second experiment, a side sample was taken after initial trypsin treatment and treated with TrypLE.

Dissociation Media Optimization Protocol:

1. HEK293 cells were grown in T75 cm2 flasks to >70% confluency in DMEM with 10% FBS.
    - Two flasks each were prepared to test trypsin, TrypLE, and trypsin-EDTA as dissociation treatment.
2. Culture media was removed, and monolayer was rinsed with 5 mL of fresh Ca^{2+}, Mg^{2+} free DPBS.
3. 2 mL of dissociation media was added and the flask was gently rocked to ensure the monolayer was fully coated. Cultures were incubated at 37°C and 5% CO_2 for 2 minutes.

    Note: Gently rocking media over cells will ensure the monolayer is fully coated in dissociation media, however, tapping or shaking the flask too vigorously may cause large clusters of cells to be removed at once.
4. After the 2-minute incubation and cells were thoroughly detached from the flask, 10 mL of warmed DMEM with 10% FBS was added and gently mixed by pipetting up and down 5 times using a 10 mL serological pipette.
5. Next, the 12 mL cultures were divided by transferring 3 mL into four 5 mL tubes each.
    - Two tubes were designated as non-triturated controls, and two were designated as triturated samples.
        - For the triturated samples, 1000 µL of suspension was pipetted up and down 10 times with a 1000 µL air-displacement pipette.
6. Tubes were inverted gently 3 times to mix and five 200 µL replicates were distributed to Vi-CELL BLU Cell Viability Analyzer counting tubes and read on the Vi-CELL BLU Cell Viability Analyzer.
    - Samples were read by alternating between tubes and replicates for the triturated samples and non-triturated controls. *For example, triturated tube 1-replicate 1, non-triturated tube 1-replicate 1, triturated tube 2-replicate 1, non-triturated tube 2-replicate 1.*

7. Samples were read using the default Vi-CELL BLU Cell Viability Analyzer settings and the Mammalian cell type.

8. The procedure was repeated for a total of 2 flasks for each dissociation medium.

Materials:	Manufacturer	Catalog	Lot	Exp
DMEM	Gibco	11995-065	2661720	5/30/2024
FBS	Gibco	A52094-01	2629066RP	1/30/2028
Trypsin	Gibco	15050-065	2500310	2/28/2025
TrypEDTA	Gibco	25200-072	2518029	8/30/2024
TrypLE Express	Gibco	12605-028	2537714	1/30/2025
DPBS	Gibco	14190-144	2293643	12/31/2023

Table 1: Reagent List

Dissociation Media Optimization Results:

Mean cluster counts were significantly lower for trypsin-EDTA and TrypLE treated samples compared to trypsin treated samples ($p<0.05$; Student's t-test) indicating more effective dissociation using trypsin-EDTA and TrypLE. Cluster counts were similar for trypsin-EDTA and TrypLE treated samples indicating similar performance of both media. Trituration of trypsin-EDTA and TrypLE treated samples was able to significantly lower cluster counts ($p<0.05$; Student's t-test) indicating the presence of more weakly clustered agglomerates that were dissociated by pipetting, however, trituration was not able to break up aggregates from trypsin treated samples. Trypsin-EDTA and TrypLE shared similar viable cell densities, with no significant statistical difference between groups ($p>0.05$; Student's t-test). Trituration improved viabilities in all cases likely due to a more effective counting algorithm in the absence of agglomerates and aggregates.

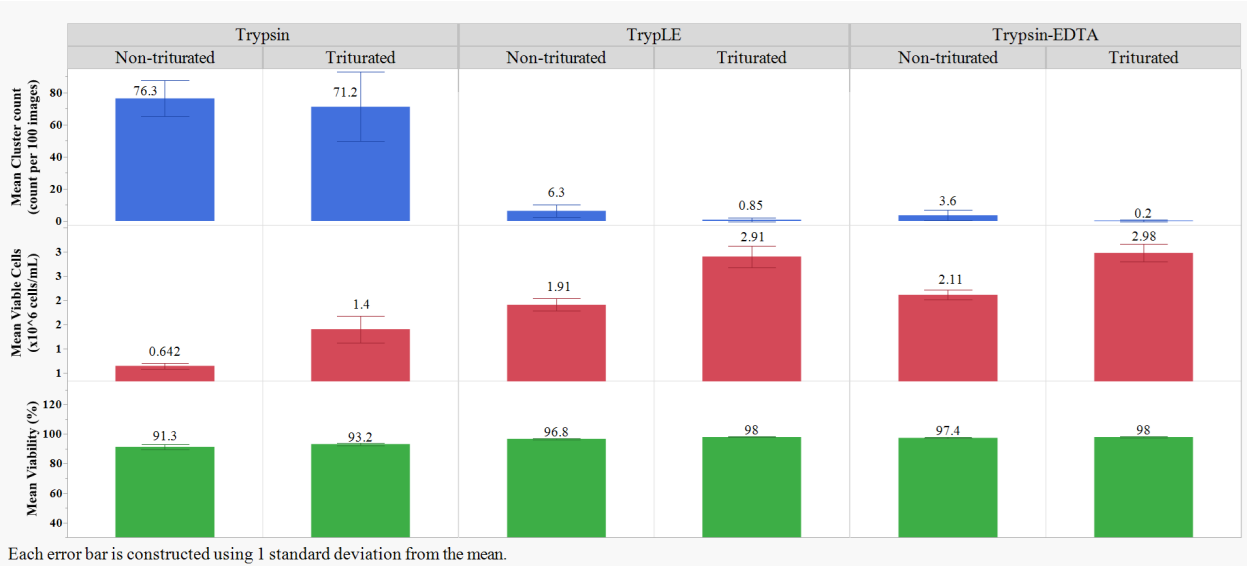

Figure 1: Mean cluster count, mean viable cell density and mean viability results for three dissociation media and trituration on divided samples. Results are the average of all replicates from all tubes and flasks for each group. Data was analyzed with JMP version 16.1.0.

Side Sample Dissociation

1. HEK293 cells were grown in T75 cm^2 flasks to >70% confluency in DMEM with 10% FBS.
    - Three flasks were prepared to test the side sample dissociation method.
2. Culture media was removed, and the monolayer was rinsed with 5 mL of fresh Ca^{2+}, Mg^{2+} free DPBS.
3. Next, 2 mL of trypsin media was added and the flask was gently rocked to ensure monolayer was fully coated. Cultures were incubated at 37°C 5% CO_2 for 2 min.
4. After the 2-minute incubation, 10 mL of warmed DMEM with 10% FBS media was added and gently mixed by pipetting up and down 5 times using a 10 mL serological pipette.
5. The 12 mL culture was divided by transferring 3 mL each into four 5 mL tubes.
    - Two of the tubes were designated as controls, and two as side samples.
    - The two control tubes were inverted 3 times and three 200 µL samples were taken from each tube and read on the Vi-CELL BLU Cell Viability Analyzer. They were designated as untreated controls that represent an existing passage procedure. The remaining volume was designated as treated controls.
    - The two side sample tubes were also inverted 3 times and then all 4 tubes (two 2400 µL control tubes and two 3000 µL side sample tubes) were spun down at 500 rcf for 3 minutes to pellet cells.
6. After centrifugation, the culture medium was removed and cells were resuspended in either 400 µL DMEM with 10% FBS for the 2 treated control tubes, or 500 µL TrypLE for the 2 side sample tubes and then pipetted up and down 10 times with a 1000 µL air displacement pipette to resuspend cells.
7. After a 2 min incubation at room temperature, samples were resuspended to initial volumes (2400 µL for treated controls or 3000 µL for side samples).
8. Samples were pipetted up and down 10 times with a 1000 µL air displacement pipette to mix and then tubes were inverted 3 times.
9. Next, five 200 µL replicates were taken from each tube and read on the Vi-CELL BLU Cell Viability Analyzer alternating between treated control and side sample tubes and between replicates for a total of 10 reads per condition. *For example, treated control tube 1-replicate 1, side sample tube 1-replicate 1, treated control 2-replicate 1, side sample tube 2-replicate 1.*
10. All samples were read using the default settings and the Mammalian cell type. The procedure was repeated for a total of 3 flasks.

Side Sample Results:

Cluster counts for TrypLE treated side samples were significantly lower than both untreated and treated controls ($p<0.05$; Student's t-test). Trypsin treated controls with additional processing steps and TrypLE treated samples had significantly higher VCD than untreated controls ($p<0.05$; Student's t-test). Higher VCD for treated controls is likely from additional mixing and pipetting steps causing agglomerates to dissociate. Additional processing significantly reduced viability for treated controls vs untreated controls slightly by 1.5% ($p<0.05$; Student's t-test). TrypLE treatment significantly improved viability percentage compared to untreated and treated controls ($p<0.05$; Student's t-test) likely from more accurate counts in the absence of agglomerates and clusters.

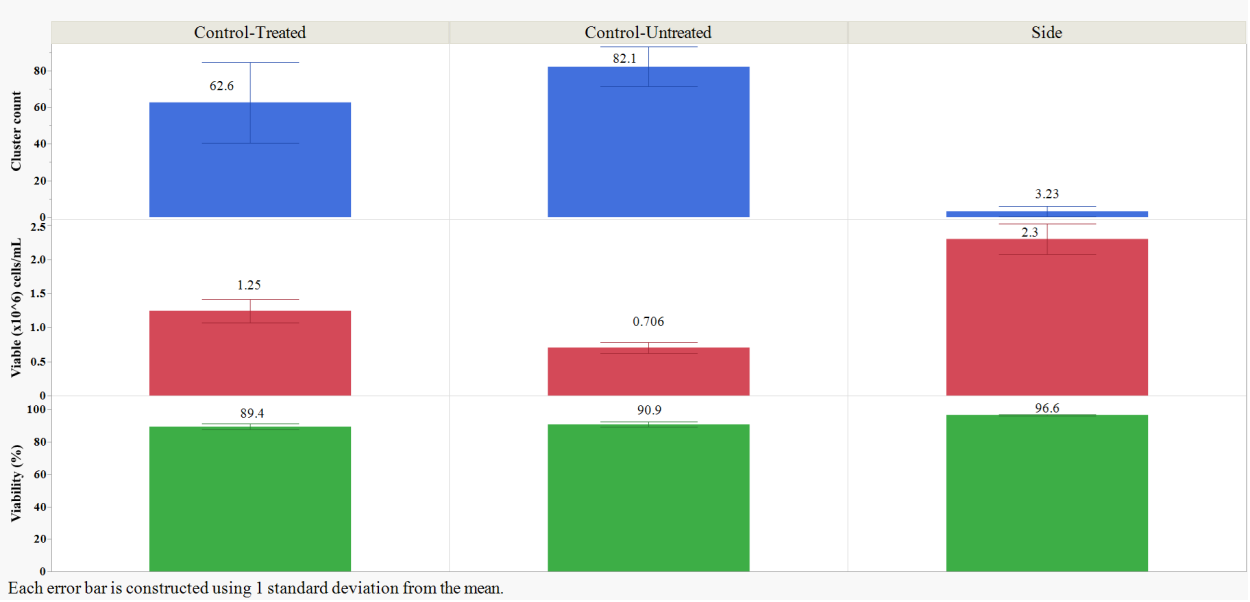

Figure 2: Mean cluster count, mean viable cell density and mean viability results for TrypLE treated side samples, untreated controls and media treated controls. Results are the average of all replicates from all tubes and flasks for each group. Data was analyzed with JMP version 16.1.0.

A review of images displayed large excluded clusters and some smaller clusters for trypsin treated samples (Figure 3 left image). TrypLE treated side samples (Figure 3 right image) displayed few large excluded clusters and smaller clusters overall.

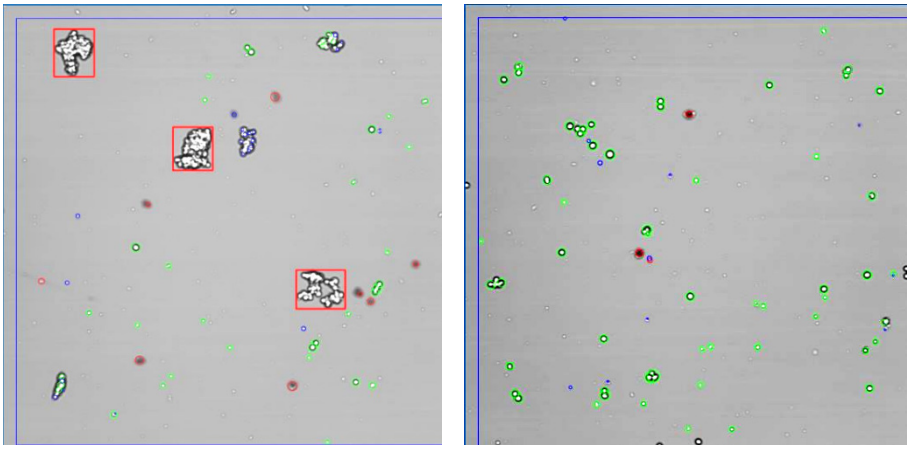

Figure 3: Image examples for the trypsin control (left) and TrypLE treated side sample (right).

Discussion:

As demonstrated, the choice of dissociation method can significantly affect viability, viable cell counts and degree of clustering. Gentle trituration by pipetting may break up weakly clustered agglomerates and allow for a more accurate count without compromising viability. For larger and more difficult to separate aggregates that do not respond well to trituration by pipette, treatment with alternative dissociation reagents such as Trypsin with EDTA or TrypLE may allow for a more dispersed suspension. Lastly, a side sample technique may be used as an option to continue using an existing dissociation method while enabling more accurate counts on the Vi-CELL BLU Cell Viability Analyzer.

Please reach out anytime for assistance with your Vi-CELL BLU Cell Viability Analyzer application needs.

References:

1. Iuchi et al. Cytotechnology Feb 2020; 72(1): 131-140. Different morphologies of human embryonic kidney 293T cells in various types of culture dishes
2. ISO 20391-1:2018 Biotechnology – Cell Counting – Part 1: General guidance on cell counting methods
3. Tsap. Y et al. Biotechnology Progress 2000; 16(5): 809-814. Biomass and Aggregation Analysis of Human Embryonic Kidney 293 Suspension Cell Cultures by Particle Size Measurement
4. Drummen, N. Cell Type settings considerations for counting clumpy cells on the Vi-CELL BLU cell viability analyzer. Beckman Coulter application note. Content ID: 2023-GBL-EN-101699

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2023-GBL-EN-101754-v2