ADVANCED INSTRUMENTS

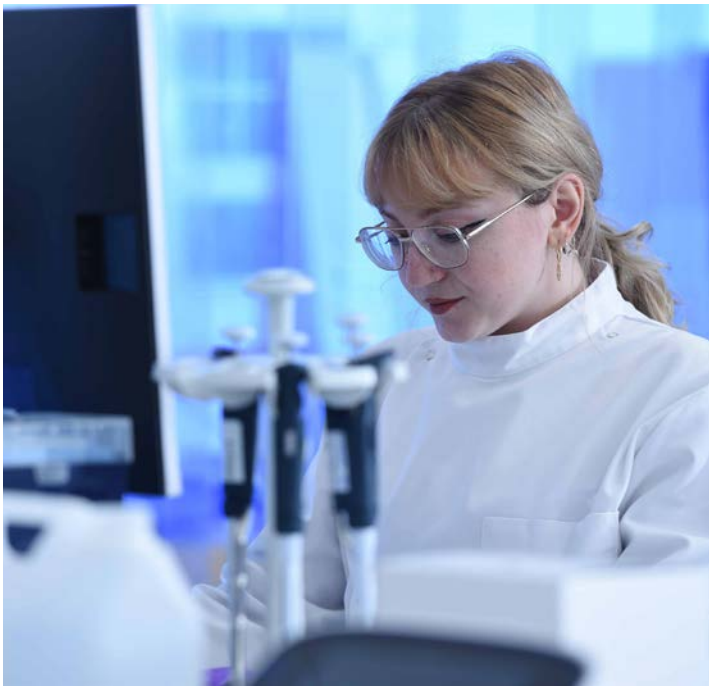
Scientific Resource

# Validation of ICON™ Automated Cell Counting and Viability Assay

**Caitlin Davies, Stefan Borasinski**

## Abstract

Determination of cell viability and concentration using trypan blue exclusion is essential for biotherapeutic workflows, enabling optimisation of protocols, monitoring of changes to the culture environment, and selection of the best clones for culture progression. Automated and semi-automated counters have begun to alleviate the cell counting bottlenecks of the biopharmaceutical industry, yet still often assess one sample at a time, require cross-referencing to other databases or workbooks, and rely on manual calibration. The ICON™ instrument has been designed to assist high-throughput workflows by conducting up to 24 viable cell counts at a time without the need for manual calibration or gating. This study aimed to validate ICON's Viability Assay by measuring cell samples with a range of expected concentrations and viabilities, while simultaneously comparing its performance to that of a well-known commercially available cell counter. ICON consistently displayed lower variation between repeats, increased precision, and saved time when compared to the competitor cell counter, making it the ideal choice for high-throughput cell line development workflows.



## Introduction

Cell counting and viability assays are crucial to the cell line development process, from optimisation and standardisation of cell culture methods to monitoring of cell health, both throughout single cell seeding, clonal selection, expansion, and subsequent shaking culture stages (Thunyaporn, Doh and Lee, 2021).

Automated and semi-automated cell counters use thresholding algorithms to analyze a digital image of the dye exclusion, thereby reducing bias and saving time (Fagète, Steimer and Girod, 2019). However, this usually requires some degree of human input through either instrument calibration or gating of the image detection, increasing the likelihood of user variability.

Powered by STUDIUS™ data management software, the Trypan Blue Viability Assay on the ICON instrument uses artificial intelligence to detect dye exclusion, overcoming

the user bias and time associated with visual adjustment of detection parameters. The neural network-based assay requires just 20 µL of sample and can measure up to 24 samples at a time in under four minutes, making it ideal for both static and suspension culture plates. ICON also includes low volume determination of IgG titer, allowing viable cell counts to be uniquely combined with titer measurements via STUDIUS. This results in the automatic calculation of normalized titer and specific productivity, parameters that are integral for selection of high value clones with ideal protein yield and quality (Chen *et al.*, 2017). Furthermore, STUDIUS can combine Cell Metric® and VIPS™ confluency and clonality data with ICON viability and titer data to make additional rapid, unbiased, and holistic clone selection decisions.

The aim of this study was to assess ICON's ability to accurately detect the viability and concentration of a range of cell samples, and to compare its performance to a popular commercially available cell counter. The following analysis of ICON's artificially intelligent detection deems it notably more accurate and efficient than other cell counting methods.


**Viability slide view in STUDIUS**


**ICON Instrument with STUDIUS software**

## Method

A range of cell suspensions were used to investigate ICON's ability to out-perform an established commercially available automated cell counter in the detection of sample concentration and viability. As two of the most popular cell lines used in biotherapeutic development, CHO and HEK cells were used throughout the validation process, including both suspension and adherent cultures; this ensured ICON's performance could be assessed across different morphologies and growth conditions. Network validation was performed prior to validation of the complete application but has been outlined for context.

### Materials

The following cell lines were used for the whole validation process:

- FreeStyle CHO-S (Gibco R800-07) suspension cell line cultured in CD OptiCHO (Gibco 12681029) + 8mM GlutaMAX (Gibco 35050061).
- Expi293F (Gibco A14527) suspension cell line cultured in Expi Expression Medium (Gibco A1435102).
- 293T (ECACC 12022001) adherent cell line cultured in DMEM High Glucose (Sigma D5796) + 10% Fetal Bovine Serum (FBS) (Gibco 10270106).

Further materials required:

- 1x Phosphate Buffered Saline (PBS) (Gibco 10010023).
- 0.4% Trypan Blue solution (Gibco 11538886) (filtered and diluted with 1x PBS to produce a 0.125% solution for ICON).
- ICON Cell Counting Slides (Advanced Instruments RS-3010).

**ICON Cell Counting Slide containing eight samples**

## Network Validation

The Trypan Blue Viability Assay is powered by artificial intelligence, specifically via an object detection neural network. To train the network on the difference between live and dead cells, 27 images containing a combined total of approximately 23,000 trypan blue stained CHO-S, Expi293F and 293T cells, at a range of sample viabilities, were hand annotated by experienced reviewers and then exposed to the network.

In addition to the training dataset, a further 16 images per cell line containing a combined total of approximately 16,500 stained cells were likewise annotated as live or dead. These additional images were treated as a ground truth (i.e., known to be correct by direct observation) against which to validate the performance of the network. Performance was evaluated via two methods: effectiveness of cell detection (as given by mean average precision) and accuracy of cell count.

Mean average precision (mAP) is a metric used to assess the performance of object detection algorithms via precision and recall. In this case, 'precision' refers to the proportion of correct detections over all detections made:

$$\frac{\textit{true positives}}{\textit{true positives + false positives}}$$

Whereas 'recall' measures the proportion of correct detections over all possible detections in the ground truth:

$$\frac{\textit{true positives}}{\textit{true positives + false negatives}}$$

The precision values are plotted against the recall values to form a precision-recall curve. mAP can then be determined by averaging the areas underneath the resulting precision-recall curves for live and dead detection. Since precision and recall values will always fall between 0 and 1, mAP values will also fall within this range, where a value closer to 1 indicates a network with high effectiveness of cell detection.

Cell count accuracy was calculated as the mean percentage difference between ground truth and network counts of live and dead cells, where a positive percentage difference indicates over-detection by the network, and a negative percentage difference indicates under-detection.

## Trypan Blue Viability Assay Validation

### Sample Preparation

For each cell line, two initial $1x10^7$ cells/mL samples were prepared at ~100% viability and ~0% viability respectively, with the expected 0% samples coming from cultures that had been left in an incubator without subculturing for approximately two weeks. To confirm these cells were as close to the expected viability as possible, samples were analyzed on both instruments and the resulting instrument images visually assessed. A microscope and hemocytometer were also used to observe the cells further, if required. These two samples were mixed proportionally to produce five expected viability samples (from 0% to 100%). Each viability sample was then mixed with 1x PBS to form a dilution series of $1x10^5$ cells/mL to $1x10^7$ cells/mL. This resulted in 25 conditions per cell line (Table 1).

| Concentration cells/mL | Dilution Fraction | Viability % | | | | |
|---|---|---|---|---|---|---|
| $1x10^7$ | 1.00 | 0 | 50 | 70 | 90 | 100 |
| $7x10^6$ | 0.70 | 0 | 50 | 70 | 90 | 100 |
| $5x10^6$ | 0.50 | 0 | 50 | 70 | 90 | 100 |
| $3x10^6$ | 0.30 | 0 | 50 | 70 | 90 | 100 |
| $1x10^5$ | 0.01 | 0 | 50 | 70 | 90 | 100 |

**Table 1: Range of expected viabilities (0% to 100%) and concentrations ($1x10^5$ cells/mL to $1x10^7$ cells/mL) to be tested for each cell line. Dilution fraction refers to the ratio by which the cell concentration has been reduced from the original $1x10^7$ cells/mL sample e.g., $5x10^6$ / $1x10^7$ = 0.50.**

### Inter and Intra Sample Variation Method and Analysis

Each condition sample was mixed thoroughly then divided into two 20 µL samples for ICON and the competitor counter respectively.

To prepare the ICON Cell Counting Slides, 80 µL of filtered 0.125% trypan blue was added to the 20 µL sample and mixed thoroughly to establish a homogeneous cell suspension. 20 µL of this mixture was loaded into three ICON Cell Counting Slide channels by gently pipetting into each channel well. Since several images are taken across the whole slide channel by ICON, gentle pipetting is required to avoid bubbles that may cause inaccurate network calculation through inconsistent channel volume or displacement of cells. The concentration and viability of each channel was recorded three times using STUDIUS.

For the competitor automated cell counter slide preparation, the 20 µL sample was mixed with the recommended ratio and concentration of trypan blue then mixed thoroughly to establish a homogeneous cell suspension. Three channels of the instrument specific slides were loaded appropriately. Cell shape, size, and brightness thresholds along with overall image brightness were manually adjusted before every measurement across all cell lines according to the instrument user guide. The concentration and viability of each channel was recorded three times manually.

Inter-channel variation refers to the variation between the results of three separate channels filled from the same condition sample. Intra-channel variation refers to the variation between results from three separate recordings of the same channel. Therefore, for each of the 25 conditions per cell line, nine concentration values and nine viability values were collected.

Precision analysis was based off linear regression and % coefficient of variation (% CV) calculations, where an $R^2$ value ≥0.9 and a % CV of ≤5% were considered optimal.

The intra- and inter-channel coefficient of variation (CV) was calculated by dividing the standard deviation of each of the three repeats of the three replicate channels by the respective means. The resulting CV values were then expressed as a

percentage (% CV) and used to show the relative dispersion of data points around the mean to determine assay precision. The lower the % CV, the higher the precision.

Consequently, each of the 25 conditions produced three inter-variation % CV values and three intra-variation % CV values. Therefore, for each individual cell line, 150 % CV values were calculated for concentration (75 for intra-variation and 75 for inter-variation), and similarly 150 for viability. This can be further broken down to 30 % CV values per dilution fraction (15 for intra-variation and 15 for inter-variation) and similarly 30 per expected viability for a single cell line.

This was then repeated for the competitor results.

#### Stability Across 24 Samples Method and Analysis

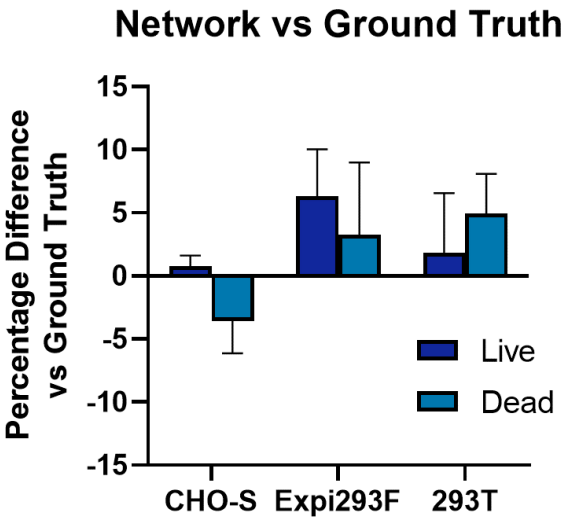
Using only the $5x10^6$ cells/mL samples from each cell line, 400 µL of filtered 0.125% trypan blue was added to 100 µL of the five expected viability condition samples and mixed thoroughly. 20 µL of this mixture was loaded into each of the 24 ICON Cell Counting Slide channels (three slides) and the viable cell count of each channel was recorded a single time using STUDIUS.

For each set of 24 inter-channel measurements, the average cell viability and concentration were calculated alongside % CV. This resulted in 10 inter-channel % CV values per cell line (five for viability and five for concentration).

## Results

### Network Validation

The Viability network can provide counts within an accuracy of ±1-7% across all cell lines. This tightens to between ±1-3.5% in CHO-S samples, where the morphological difference between dead and live cells is most pronounced (Figure 1).




| Cell Line | Live Cells | Dead Cells | Total |
|---|---|---|---|
| CHO-S | 4921 | 5069 | 9990 |
| Expi293F | 3509 | 1963 | 5472 |
| 293T | 285 | 830 | 1115 |

**Figure 1:** Average percentage difference between the Viability network and ground truth cell counts. N=16 (SD displayed). The table depicts the number of cells labelled to determine ground truth across the 16 images per cell line.

For mAP, the Viability network scores between 0.85 and 0.90 across the three cell lines (Figure 2), serving to reinforce the impression made by visually assessing ICON images (see Visual Image Analysis), that the network is extremely competent at evaluating cell viability.

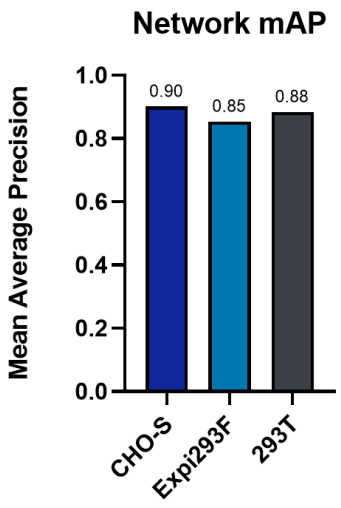



**Figure 2: Mean average precision for dead and live cell detection across each cell line.**

## Inter and Intra Sample Variation

Findings indicate that ICON displays high precision across both inter-channel measurements for both cell concentration and viability. Across all cell lines, concentration and viability assessments, ICON's average $R^2$ value was 0.9725, whereas the competitor's was just 0.8864. Furthermore, 86% of ICON's total % CV values were ≤5%, as opposed to only 62% of the competitor's. A more in-depth analysis and explanation of results is as follows:

### Cell Concentration

Concentration linearity was determined by plotting the dilution fraction against the average measured cell concentration for each sample (Figure 3). A simple linear regression was then conduced for each linearity and the $R^2$ value determined to quantify the model's 'goodness of fit' by the proportion of variance within the data. An $R^2$ value of 1 represents a perfect linear relationship between variables, meaning the closer an $R^2$ value is to 1, the better fit the regression model has around the data and the more variance in the data can be explained by the independent variable. For ICON, $R^2$ values varied between 0.93 and 0.98, whereas the competitor had lower $R^2$ values ranging between 0.81 and 0.93. A graph displaying the concentration linearity and $R^2$ values for all cell lines combined is included in the appendix (Figure 4).

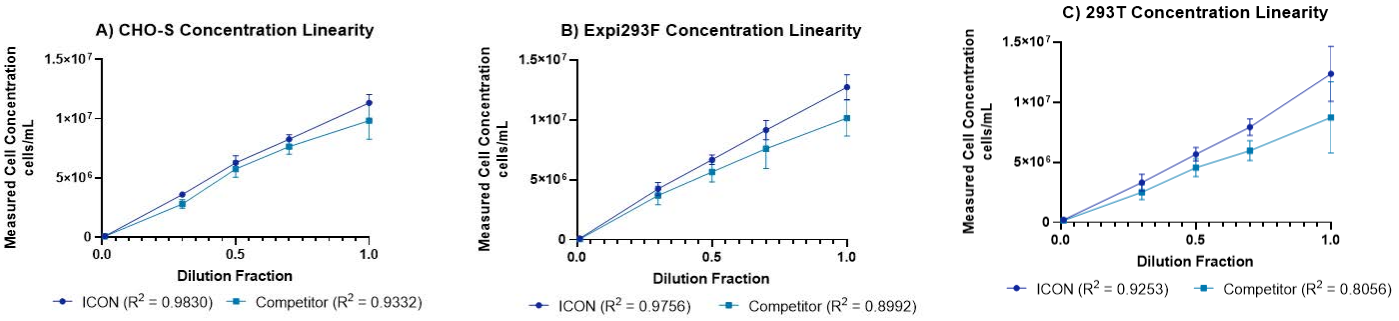



**Figure 3: Concentration linearity of A) CHO-S, B) Expi293F and C) 293T cell lines, where n=45 per dilution fraction ± SD. N.B. Expected concentration linearity is not displayed as this has been previously assessed during network validation.**

Each of the 15 individual % CV values per dilution fraction across all viabilities were then plotted to assess cell concentration detection (Figure 5). Across both inter- and intra-channel measurements and for all cell lines, ICON consistently displayed lower % CV values than the competitor counter, with tighter variance. Although the 0.01 dilution fraction tended to display higher % CV values and greater spread for both instruments, ICON's % CV values remained below 33%, while the competitors reached 57%. Over the four remaining dilution fraction conditions, % CV values reached 11% for ICON, as opposed to 71% for the competitor. A graph displaying concentration % CV values for all cell lines combined is displayed in the appendix (Figure 6).

The average of the 15 % CV values for each dilution fraction was also calculated and presented alongside standard deviation (Table 2). Similarly, although the 0.01 dilution fraction samples had a higher average % CV than all other dilution samples due to being the lowest cell concentration, ICON's standard deviation remained at or below 6.71, while the competitor's reached 16.45. The average % CV values for the four remaining dilution fraction conditions were much lower, with standard deviation reaching 4.06 for ICON, as opposed to 22.61 for the competitor.

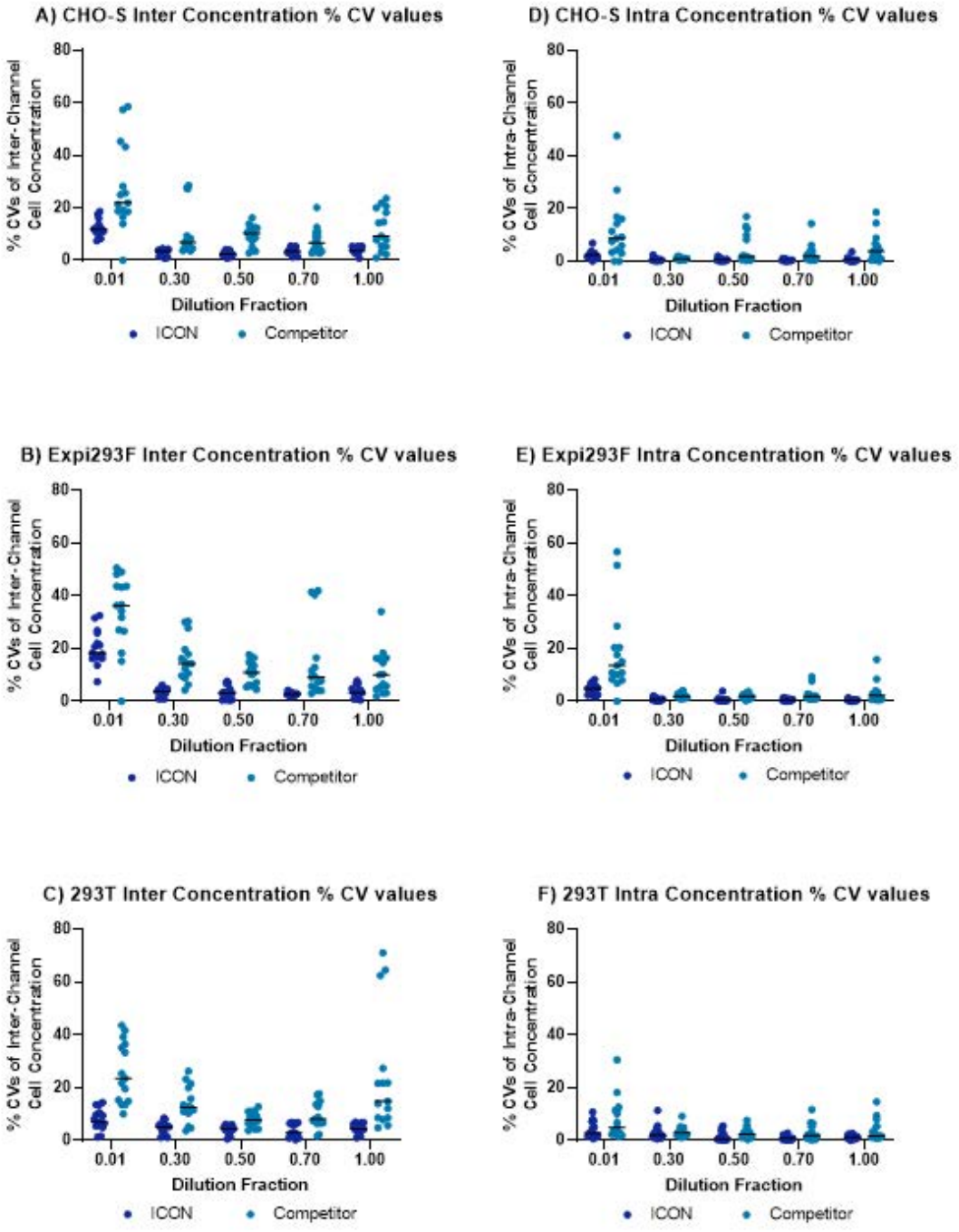
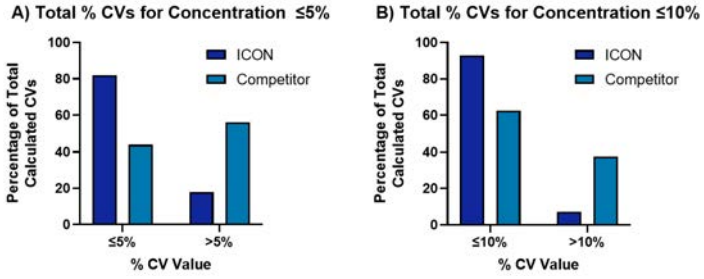



**Figure 5:** Individual % coefficient of variation values of inter-channel concentration for A) CHO-S, B) Expi293F and C) 293T cell lines, and of intra-channel concentration for D) CHO-S, E) Expi293F and F) 293T cell lines. n=15 % CV values per dilution fraction per instrument where each % CV is calculated from three sample repeats. Medians are displayed as black horizontal lines.

| Cell Line | Dilution Fraction | Average % CV Cell Concentration ± Standard Deviation | | | |
|---|---|---|---|---|---|
| | | Inter-channel % CV | | Intra-channel % CV | |
| | | ICON | Competitor | ICON | Competitor |
| CHO-S | 0.01 | **12.29** ± 3.14 | **27.57** ± 16.45 | **2.45** ± 1.53 | **11.71** ± 12.30 |
| | 0.30 | **2.90** ± 1.25 | **10.37** ± 9.18 | **0.64** ± 0.57 | **1.01** ± 0.49 |
| | 0.50 | **2.62** ± 1.01 | **9.62** ± 3.86 | **0.64** ± 0.53 | **4.78** ± 5.70 |
| | 0.70 | **3.41** ± 1.30 | **7.40** ± 4.78 | **0.45** ± 0.30 | **2.96** ± 3.52 |
| | 1.00 | **3.79** ± 1.25 | **11.68** ± 7.76 | **0.64** ± 0.92 | **4.97** ± 5.39 |
| Expi293F | 0.01 | **20.29** ± 6.71 | **33.62** ± 14.25 | **4.52** ± 2.06 | **18.59** ± 15.97 |
| | 0.30 | **3.53** ± 1.53 | **15.84** ± 8.14 | **0.76** ± 0.51 | **2.16** ± 0.96 |
| | 0.50 | **3.10** ± 2.52 | **10.62** ± 4.59 | **0.72** ± 0.89 | **1.95** ± 0.96 |
| | 0.70 | **2.78** ± 0.64 | **14.69** ± 14.21 | **0.60** ± 0.45 | **2.55** ± 2.51 |
| | 1.00 | **3.73** ± 2.19 | **11.09** ± 8.48 | **0.43** ± 0.32 | **3.18** ± 4.06 |
| 293T | 0.01 | **7.81** ± 4.02 | **25.74** ± 11.49 | **4.16** ± 2.98 | **8.22** ± 7.96 |
| | 0.30 | **4.57** ± 2.43 | **13.54** ± 6.75 | **2.92** ± 2.72 | **3.37** ± 2.01 |
| | 0.50 | **4.13** ± 1.67 | **7.85** ± 2.82 | **1.60** ± 1.84 | **2.90** ± 1.95 |
| | 0.70 | **3.68** ± 3.68 | **9.62** ± 5.05 | **0.93** ± 0.77 | **3.08** ± 3.16 |
| | 1.00 | **4.51** ± 1.91 | **24.42** ± 22.61 | **1.28** ± 0.78 | **3.70** ± 4.01 |

**Table 2:** Average % CV of inter- and intra-channel cell concentration across all viabilities and cell lines ± standard deviation. n=15 % CV values per dilution fraction where each % CV is calculated from three sample repeats.

The intra- and inter-channel % CV values for concentration were then combined to produce a total of 450 % CV values per instrument across all cell lines, and the percentage of those 450 values at a precision threshold of ≤5% and ≤10% (when rounded to the nearest integer) was calculated (Figure 7). The number of % CV values at a precision threshold of ≤5% was calculated to be 369, meaning 82% of a total 450 triplicates had a precision of ≤5% CV for ICON, compared to only 44% for the competitor. This increased to 93% for ICON, but only 62% for the competitor at a precision threshold of ≤10%. Results by individual cell line with inter- and intra-variation separated are displayed in the appendix (Tables 3, 4 and 5).




| % CV Value | Number of Total Calculated CV Values | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 369 (82%) | 198 (44%) |
| ≤10% | 418 (93%) | 281 (62%) |

**Figure 7:** Percentage of total % CV values that show a precision of A) ≤5% CV and B) ≤10% CV across all cell lines for inter- and intra-channel concentration (N=450). The table depicts the total number of % CV values at each threshold with the percentage included in parentheses.

### Viability

Viability linearity was determined by plotting the expected viability against the average measured viability for each sample (Figure 8). A simple linear regression was then conduced for each linearity. For ICON, $R^2$ values varied between 0.98 and 0.99, whereas competitor $R^2$ values ranged between 0.81 and 0.95. A graph displaying the viability linearity and $R^2$ values for all cell lines combined is included in the appendix (Figure 4).

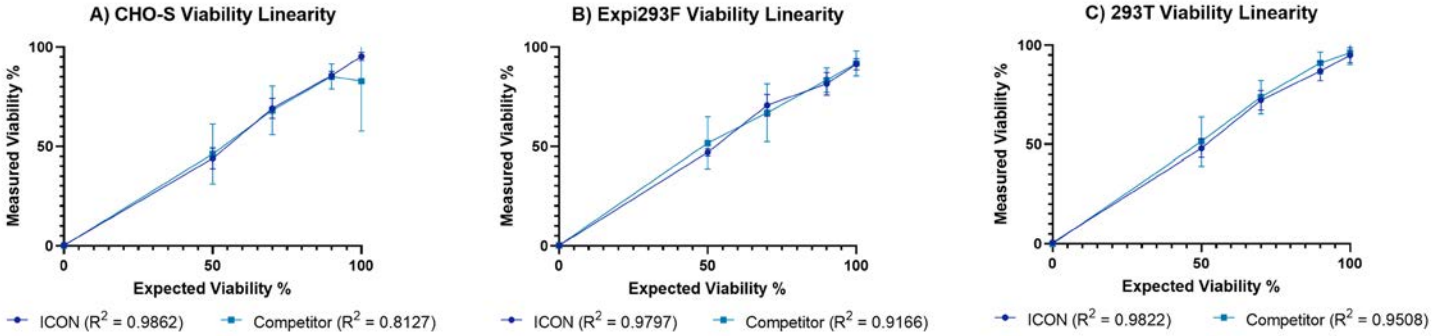



**Figure 8:** Viability linearity for A) CHO-S, B) Expi293F and C)293T cell lines across different cell counting methods, where n=45 per viability ± SD. N.B. Expected viability linearity is not displayed as this has been previously assessed during network validation.

Each of the 15 individual % CV values per expected viability across all dilution fractions were then plotted to assess viability detection (Figure 9). Across both inter- and intra-channel measurements and for all cell lines, the 0% viable samples had the highest % CV values, for both instruments.

Since coefficient of variation measures the relative dispersion of data points around their mean:

$$CV = \frac{Standard\ Deviation}{Mean}$$

a data set with a mean close to 0 will result in a % CV that approaches infinity. Therefore, a small difference in values for low numbers can result in a large % CV, when compared to the same difference in value for higher numbers as demonstrated in Table 6:

| Channel 1 Viability (%) | Channel 2 Viability (%) | Channel 3 Viability (%) | Mean | Standard Deviation | % CV |
|---|---|---|---|---|---|
| 0.00 | 0.00 | 1.00 | 0.33 | 0.57 | 173.00 |
| 50.00 | 50.00 | 49.00 | 49.67 | 0.57 | 1.00 |

**Table 6:** Example of % CV calculation over different triplicate viability conditions, each with a single value out by 1.

Therefore, it is more appropriate to infer instrument precision from the higher viability conditions, as shown in the 50% viable and above samples. Here, ICON's % CV values remained below 35%, while the competitors reached 94%. A graph displaying viability % CV values for all cell lines combined is displayed in the appendix (Figure 10).

The average of the 15 % CV values for each expected viability was also calculated and presented alongside standard deviation. Again, the 0% samples produced a higher average % CV since they have a mean close to 0. Therefore, 50% to 100% viable samples are displayed in Table 7, with 0% samples included in the appendix (Table 8).

Of the four higher viability conditions in Table 7, ICON had a highest average % CV of 6.68, compared to a highest average of 20.93 for the competitor. Similarly, ICON's standard deviation remained at or below 11.02 for ICON, as opposed to 30.06 for the competitor.

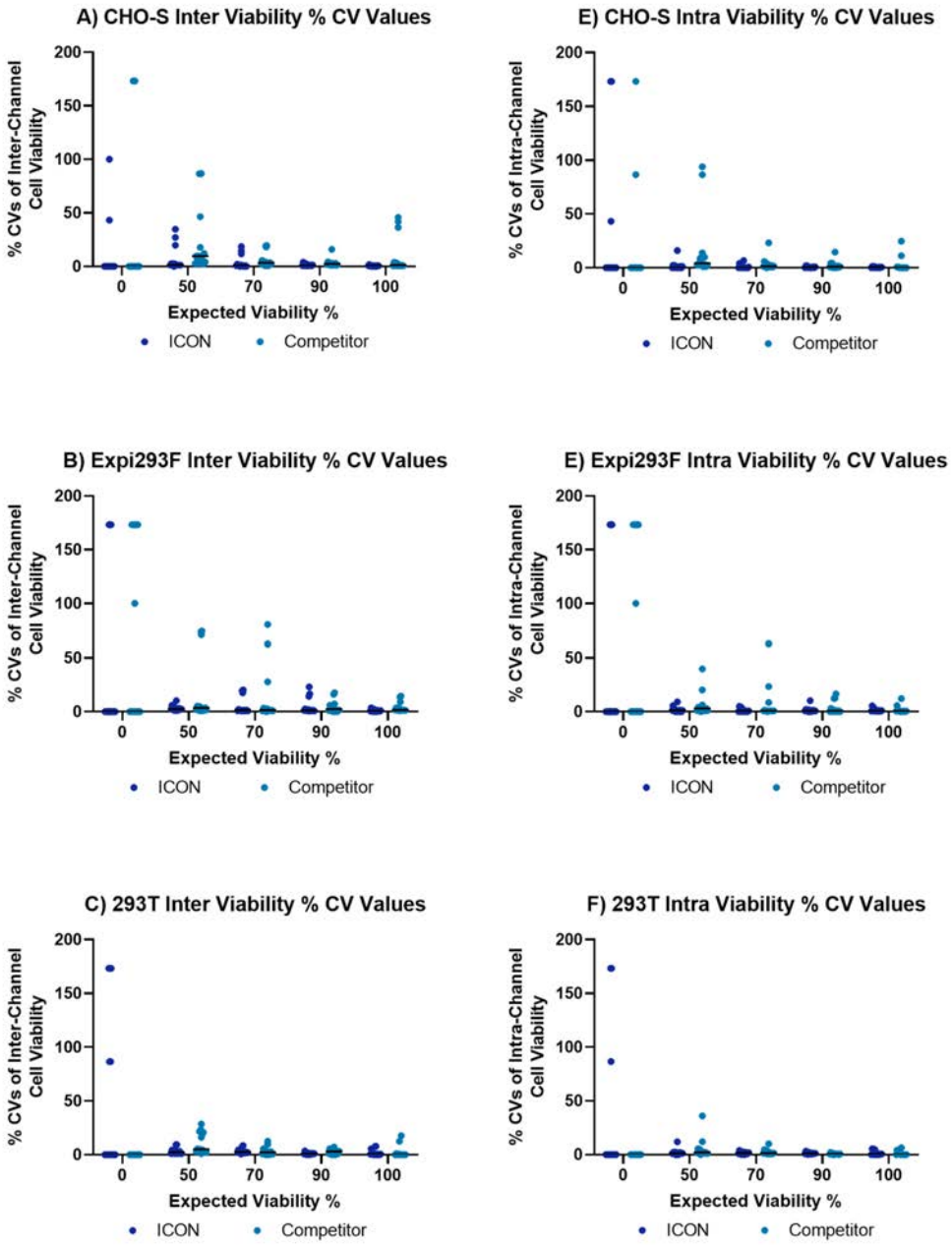
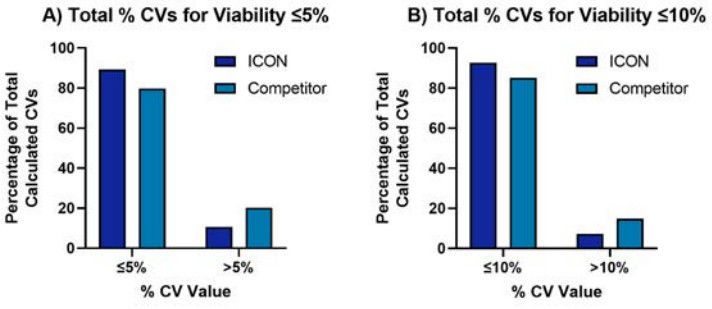



**Figure 9:** Individual % coefficient of variation values of inter-channel viability for A) CHO-S, B) Expi293F and C)293T cell lines, and of intra-channel viability for D) CHO-S, E) Expi293F and F) 293T cell lines. n=15 % CV values per expected viability per instrument where each % CV is calculated from three sample repeats. Medians are displayed as black horizontal lines.

| Cell Line | Expected Viability % | Average % CV Cell Viability ± Standard Deviation | | | |
|---|---|---|---|---|---|
| | | Inter-channel % CV | | Intra-channel % CV | |
| | | ICON | Competitor | ICON | Competitor |
| CHO-S | 50 | **6.68** ± 11.02 | **20.93** ± 28.75 | **1.71** ± 4.04 | **16.99** ± 30.06 |
| | 70 | **3.65** ± 6.05 | **6.16** ± 6.76 | **1.10** ± 1.90 | **3.42** ± 5.71 |
| | 90 | **1.29** ± 1.02 | **4.05** ± 4.88 | **0.53** ± 0.63 | **2.26** ± 3.62 |
| | 100 | **0.56** ± 0.47 | **9.33** ± 16.70 | **0.46** ± 0.48 | **2.57** ± 6.77 |
| Expi293F | 50 | **3.29** ± 2.37 | **16.87** ± 29.16 | **1.69** ± 2.48 | **6.18** ± 10.48 |
| | 70 | **4.82** ± 7.43 | **12.54** ± 25.13 | **1.09** ± 1.65 | **6.96** ± 16.60 |
| | 90 | **4.61** ± 7.17 | **4.52** ± 5.56 | **1.53** ± 2.52 | **3.36** ± 5.54 |
| | 100 | **1.05** ± 1.01 | **4.04** ± 4.58 | **1.37** ± 1.42 | **1.63** ± 3.28 |
| 293T | 50 | **3.22** ± 2.81 | **10.7** ± 9.62 | **2.06** ± 2.88 | **5.09** ± 9.08 |
| | 70 | **3.21** ± 2.20 | **3.22** ± 3.61 | **1.56** ± 1.09 | **2.27** ± 2.47 |
| | 90 | **1.18** ± 0.84 | **2.71** ± 2.08 | **1.27** ± 0.85 | **0.58** ± 0.56 |
| | 100 | **1.80** ± 2.79 | **3.06** ± 5.97 | **1.28** ± 1.97 | **0.89** ± 1.86 |

**Table 7:** Average % CV of inter- and intra-channel cell viability across all concentrations and cell lines ± standard deviation. n=15 % CV values per expected viability where each % CV is calculated from three sample repeats.

The intra- and inter-channel % CV values for viability were then combined to produce a total of 450 CV values per instrument across all cell lines, and the percentage of CV values at a precision threshold of ≤5% and ≤10% (when rounded to the nearest integer) was calculated (Figure 11). The number of total CV values at a precision threshold of ≤5% was calculated to be 402, meaning 89% of a total 450 triplicates had a precision of ≤5% for ICON, compared to only 80% for the competitor. This increased to 93% for ICON and 85% for the competitor at a precision threshold of ≤10%. Results by individual cell line with inter- and intra-variation separated are displayed in the appendix (Tables 3, 4 and 5).




| % CV Value | Total Number of Calculated % CV Values | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 402 (89%) | 359 (80%) |
| ≤10% | 417 (93%) | 383 (85%) |

**Figure 11:** Percentage of total % CV values that show a precision of A) ≤5% CV and B) ≤10% CV across all cell lines for inter- and intra-channel concentration (N=450). The table depicts the total number of % CV values at each threshold with the percentage included in parentheses.

## Stability Across 24 Samples

For each set of 24 inter-channel measurements, the average cell viability and concentration was calculated alongside % CV (Table 9). For viability, as with the inter- and intra-channel variation assessment, the 0% viable samples have been included in the appendix (Table 10). % CV values for the remaining 50% to 100% viable samples across all cell lines fluctuated from 0.51% to 3.21%. For concentration, the lowest % CV values were from the CHO-S cell line, ranging from 3.18% to 5.18%, whereas the Expi293F and 293T cell lines ranged from 5.56% to 10.04% and 6.73% to 8.19% respectively. Consequently, of the 30 calculated % CV values, 27 (90%) were ≤10%.

| Cell Line | Expected Viability % | Measured Cell Concentration (cells/mL) | | Measured Cell Viability (%) | |
|---|---|---|---|---|---|
| | | Average | % CV | Average | % CV |
| CHO-S | 0 | $6.96 \times 10^6$ | 3.18 | 0.01 | - |
| | 50 | $5.89 \times 10^6$ | 5.18 | 41.73 | 2.62 |
| | 70 | $5.70 \times 10^6$ | 4.88 | 69.07 | 1.87 |
| | 90 | $5.34 \times 10^6$ | 4.35 | 85.91 | 0.91 |
| | 100 | $5.32 \times 10^6$ | 4.38 | 98.43 | 0.51 |
| Expi293F | 0 | $6.29 \times 10^6$ | 6.38 | 0.09 | - |
| | 50 | $6.25 \times 10^6$ | 5.56 | 47.97 | 1.93 |
| | 70 | $6.43 \times 10^6$ | 6.76 | 68.16 | 1.55 |
| | 90 | $6.28 \times 10^6$ | 10.04 | 81.15 | 1.26 |
| | 100 | $6.42 \times 10^6$ | 6.70 | 90.59 | 0.65 |
| 293T | 0 | $6.11 \times 10^6$ | 7.94 | 1.35 | - |
| | 50 | $5.48 \times 10^6$ | 8.19 | 50.97 | 3.21 |
| | 70 | $6.23 \times 10^6$ | 6.73 | 77.99 | 1.44 |
| | 90 | $4.87 \times 10^6$ | 8.08 | 89.16 | 3.16 |
| | 100 | $4.27 \times 10^6$ | 7.99 | 93.42 | 1.13 |

**Table 9:** Average inter-channel cell concentration and viability measured on ICON across three cell lines at an expected cell concentration of $5 \times 10^6$ cells/mL, with corresponding coefficients of variation. n=24 per expected viability.

## Visual Image Analysis

Figures 12 to 14 display example images taken from the 'Stability Across 24 Samples' ICON assays to illustrate the labelling capability of ICON's neural network across the different cell lines, as confirmed via mAP scores. Cells identified as 'dead' from the trypan blue stain are automatically circled red by the network, whereas cells identified as 'live' are circled green.

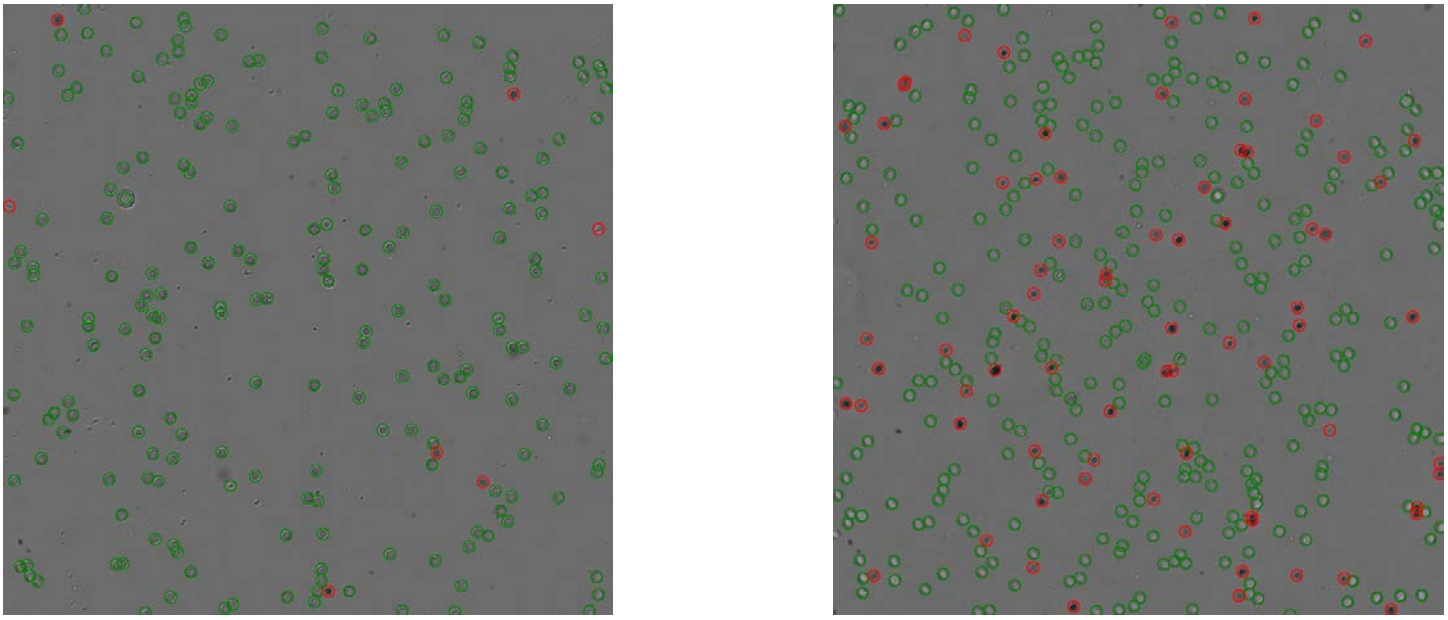


**Figure 12:** Network detection of 293T cells at a concentration of approximately 5x10$^6$ cells/mL at ~90% viability (left) and ~75% viability (right). Red circles denote where ICON's neural network has identified trypan blue stained dead cells, whereas green circles denote live cells.

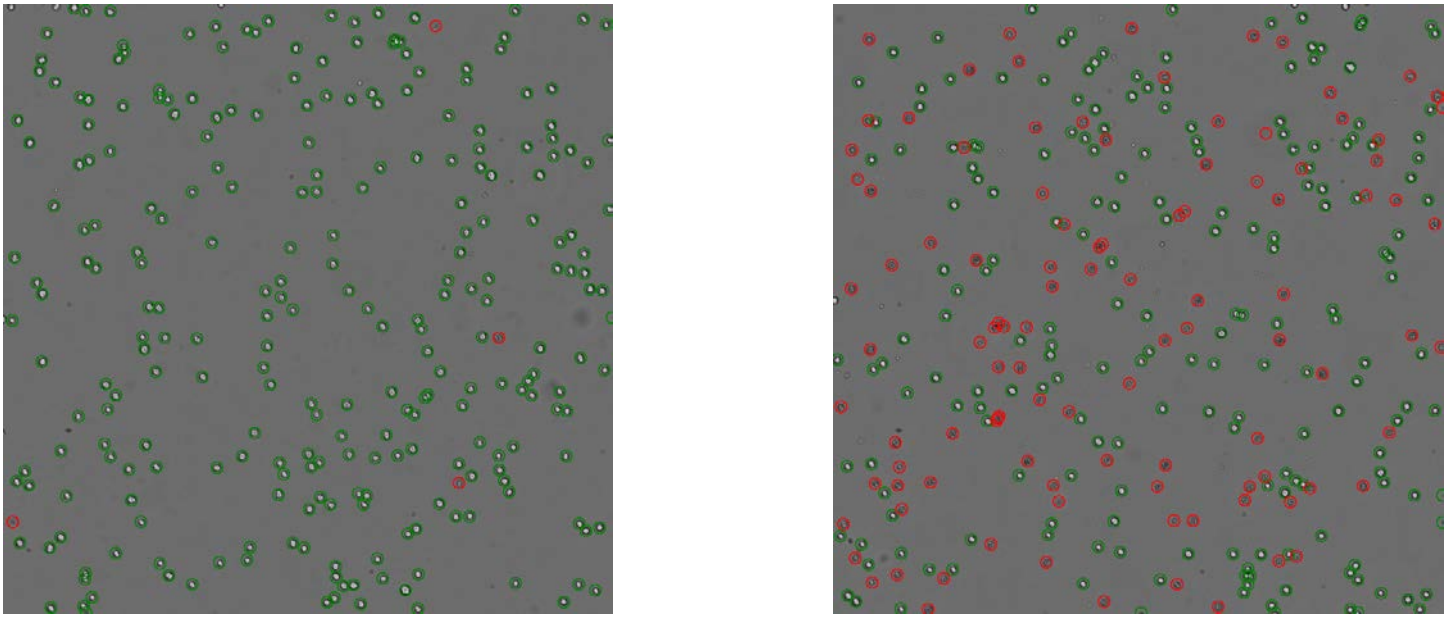


**Figure 13:** Network detection of CHO-S cells at a concentration of approximately 5x10$^6$ cells/mL at ~90% viability (left) and ~75% viability (right). Red circles denote where ICON's neural network has identified trypan blue stained dead cells, whereas green circles denote live cells.

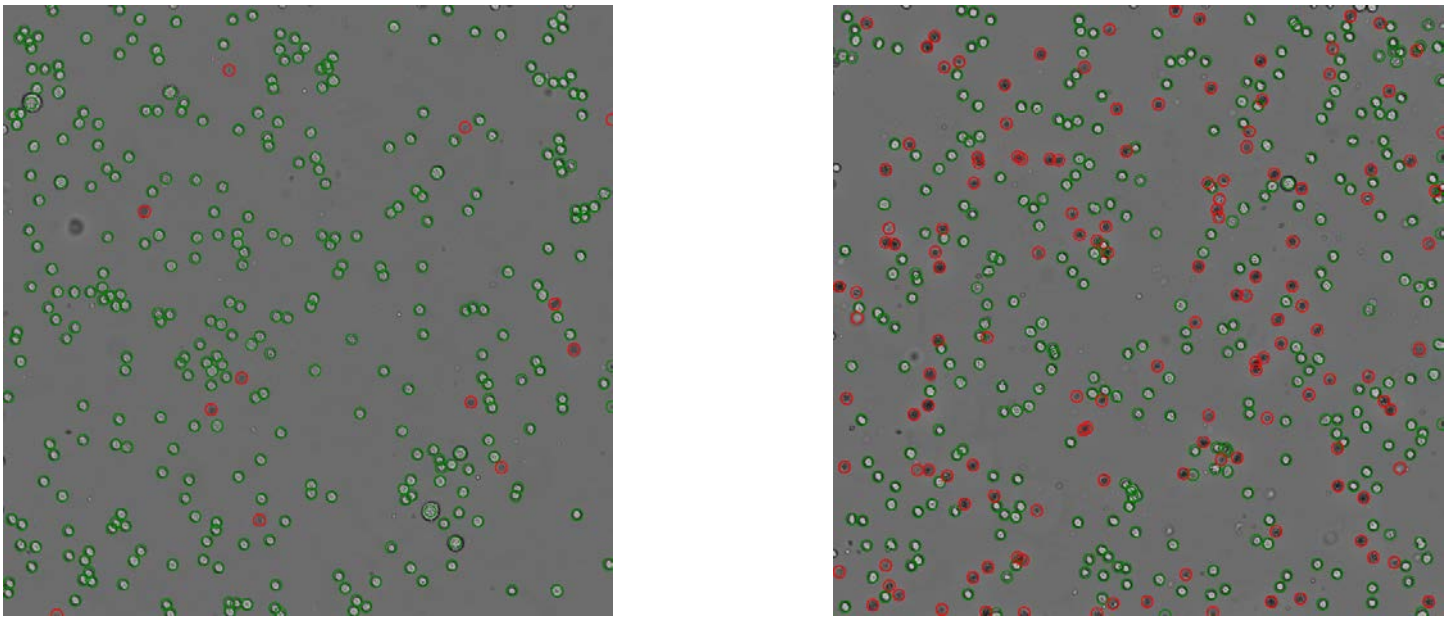


**Figure 14: Network detection of Expi293F cells at a concentration of approximately 5x10$^6$ cells/mL at ~90% viability (left) and 75% viability (right). Red circles denote where ICON's neural network has identified trypan blue stained dead cells, whereas green circles denote live cells.**

### Time Savings

The time to load and measure 24 samples on ICON was approximately 4 minutes 30 seconds (decreasing to just over three minutes when timing the assay only), whereas the competitor automated cell counter took approximately 30 minutes 25 seconds. Overall, using ICON could save an estimated 9½ hours per month compared to other commercial automated cell counters when measuring an average of 24 samples per day (such as a single 24 deep well shaking plate). Furthermore, this does not account for the time saved on data management and analysis by using the STUDIUS platform.

## Conclusions

The Viability network is extremely competent at identifying live and dead cells across all cell lines tested. It is most accurate at assessing CHO-S viability, although it near equally excels on 293T and Expi293F samples. Cell counts were accurate between 1-7% of expert human review across cell lines, with the best performance being accurate between 1-3.5% in the analysis of CHO-S samples.

Of the whole application validation, linear regression analysis shows high precision and low variability in ICON, with an average $R^2$ value across the three cell lines of 0.96 and 0.98 for concentration and viability respectively, which is evidently higher than the average competitor values of 0.88 and 0.89.

ICON also displays highly consistent and reliable measurements with 82% of 450 triplicate repeats having a coefficient of variation ≤5% for concentration and 89% for viability. This again is significantly higher than the competitor instrument, where only 44% and 80% of CV values were ≤5% for concentration and viability respectively. When using a precision threshold of ≤10%, an impressive 93% of ICON's % CV values fall under this category for both concentration and viability, as opposed to just 62% and 85% respectively for the competitor counter.

Consequently, ICON easily out-performs the competitor cell counter and is extremely competent at identifying cell concentration and viability. The ability to use low sample volumes of 20 µL and run up to 24 samples at once with minimal requirement for user calibration or gating allows cell line development workflows to be streamlined and accelerated. Furthermore, the tracking and management of viable cell count data in STUDIUS allows rapid assessment of samples with high confidence levels. In conclusion, ICON and STUDIUS Viability assessments overcome labour and time intensive bottle necks within workflows, from conducting more counts faster without sacrificing precision or accuracy, to eliminating manual data assessment and tracking, thereby allowing assured selection of the best clones.

## References

Chen, A., Leith, M., Tu, R., Tahim, G., Sudra, A. and Bhargava, S., 2017. Effects of diluents on cell culture viability measured by automated cell counter. PLOS ONE, 12(3).

Fagète, S., Steimer, C. and Girod, P., 2019. Comparing two automated high throughput viable-cell counting systems for cell culture applications. Journal of Biotechnology, 305, pp.23-26.

Thunyaporn, R., Doh, I. and Lee, D., 2021. Multi-volume hemacytometer. Scientific Reports, 11(1).

# Appendix

## Concentration Linearity

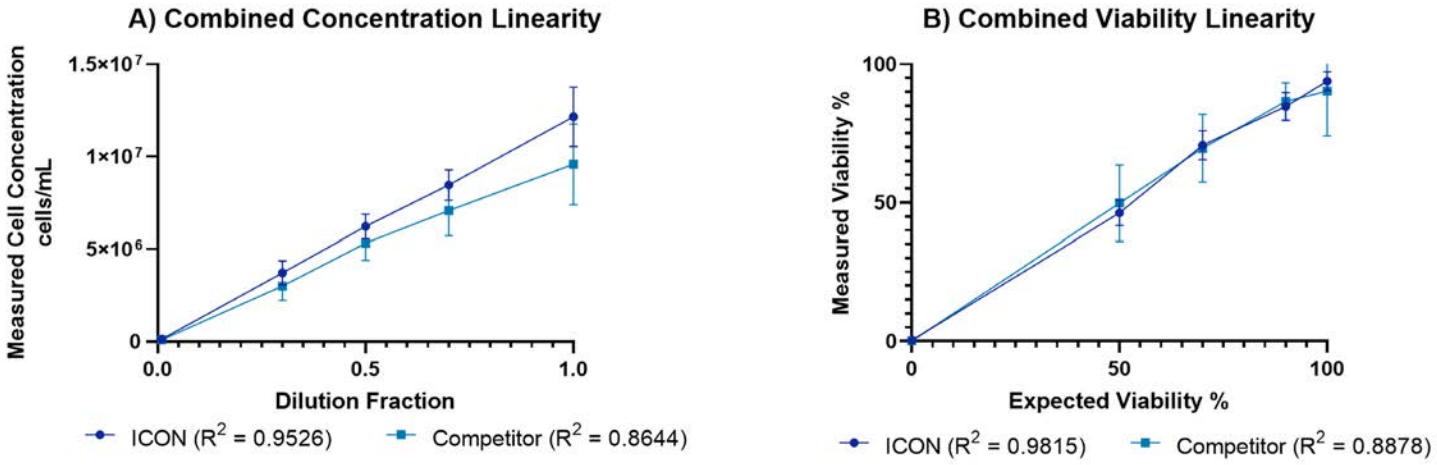



**Figure 4:** Linearity of A) Concentration and B) Viability measurements for CHO-S, Expi293F and 293T cell lines combined (n= 135 ± standard deviation).

## Overall Coefficient of Variation for Concentration

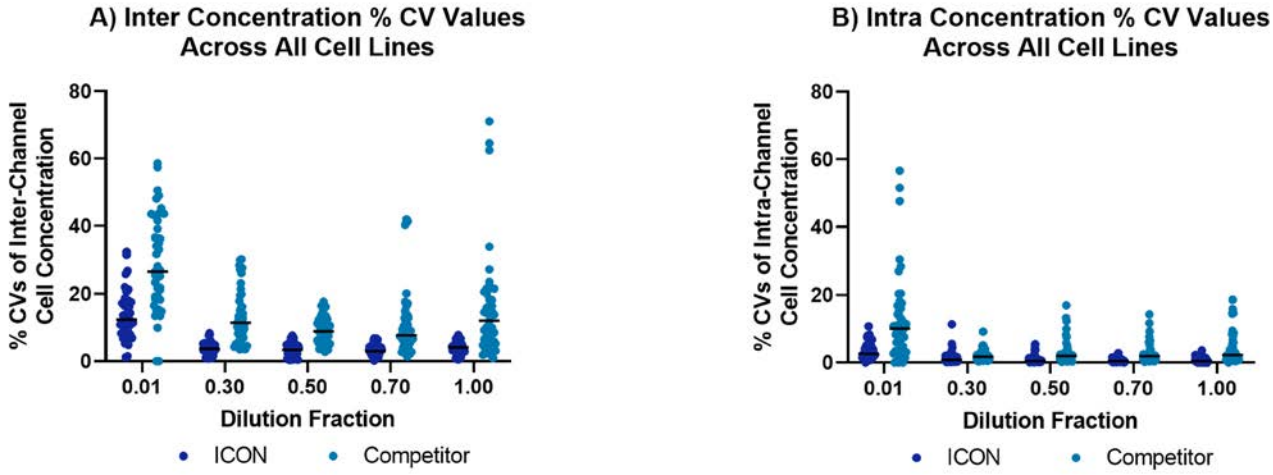



**Figure 6:** Individual % coefficient of variation values for A) Inter-channel concentration for all cell lines combined and B) Intra-channel concentration for all cell lines combined. n=45 % CV values per dilution fraction per instrument where each % CV is calculated from three sample repeats. Medians are displayed as black horizontal lines.

## Overall Coefficient of Variation for Viability

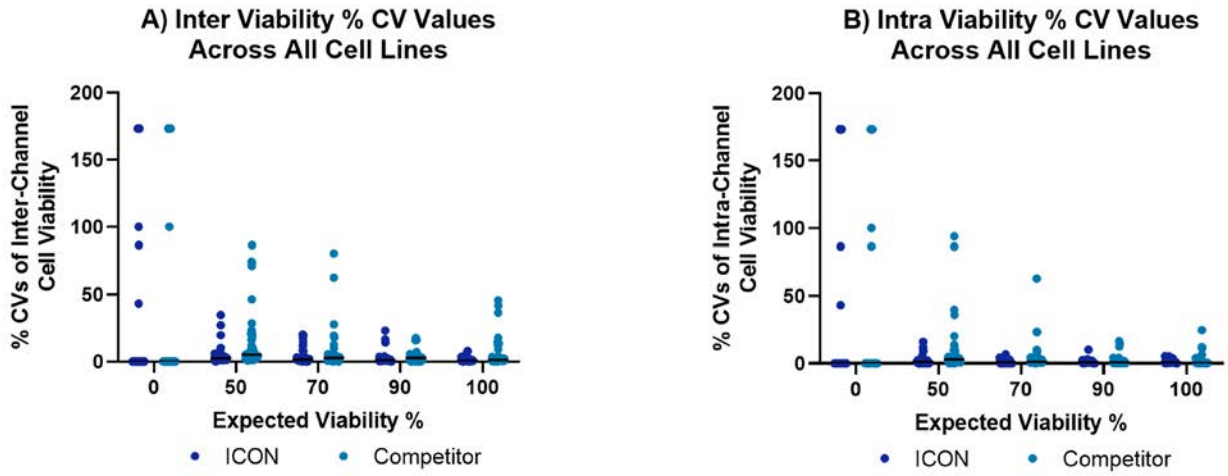



**Figure 10:** Individual % coefficient of variation values for A) Inter-channel viability for all cell lines and B) Intra-channel viability for all cell lines. n=45 % CV values per expected viability per instrument where each % CV is calculated from three sample repeats. Medians are displayed as black horizontal lines.

## Coefficient of Variation for CHO-S

A)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 59 (79%) | 21 (28%) |
| ≤10% | 63 (84%) | 41 (55%) |

B)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 74 (99%) | 38 (51%) |
| ≤10% | 75 (100%) | 48 (64%) |

C)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 67 (89%) | 52 (69%) |
| ≤10% | 67 (89%) | 59 (79%) |

D)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 70 (93%) | 61 (81%) |
| ≤10% | 71 (95%) | 66 (88%) |

**Table 3:** Total number of % CV values at each threshold for A) Inter-channel concentration, B) Inter-channel viability, C) Intra-channel concentration and D) Intra-channel viability for CHO-S only (the total expressed as a percentage is included in parentheses). n=75 % CV values where each % CV is calculated from three raw measurements.

## Coefficient of Variation for Expi293F

A)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 53 (71%) | 13 (17%) |
| ≤10% | 61 (81%) | 30 (40%) |

B)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 70 (93%) | 57 (76%) |
| ≤10% | 75 (100%) | 64 (85%) |

C)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 63 (84%) | 55 (73%) |
| ≤10% | 66 (88%) | 59 (79%) |

D)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 69 (92%) | 58 (77%) |
| ≤10% | 72 (96%) | 61 (81%) |

**Table 4:** Total number of % CV values at each threshold for A) Inter-channel concentration, B) Inter-channel viability, C) Intra-channel concentration and D) Intra-channel viability for Expi293F only (the total expressed as a percentage is included in parentheses). n=75 % CV values where each % CV is calculated from three raw measurements.

## Coefficient of Variation for 293T

A)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 45 (60%) | 10 (13%) |
| ≤10% | 71 (95%) | 30 (40%) |

B)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Concentration | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 69 (92%) | 59 (79%) |
| ≤10% | 73 (97%) | 68 (91%) |

C)

| % CV Value | Number of Calculated Inter-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 62 (83%) | 62 (83%) |
| ≤10% | 70 (93%) | 65 (87%) |

D)

| % CV Value | Number of Calculated Intra-Channel % CV Values for Viability | |
|---|---|---|
| | ICON | Competitor |
| ≤5% | 71 (95%) | 71 (95%) |
| ≤10% | 71 (95%) | 73 (97%) |

**Table 5:** Total number of % CV values at each threshold for A) Inter-channel concentration, B) Inter-channel viability, C) Intra-channel concentration and D) Intra-channel viability for 293T only (the total expressed as a percentage is included in parentheses). n=75 % CV values where each % CV is calculated from three raw measurements.

## Average % CV with 0% Viability Included

| Cell Line | Expected Viability % | Average % CV Cell Viability ± Standard Deviation | | | |
|---|---|---|---|---|---|
| | | Inter-channel % CV | | Intra-channel % CV | |
| | | ICON | Competitor | ICON | Competitor |
| CHO-S | 0 | **9.55** ± 27.39 | **34.64** ± 71.72 | **25.98** ± 60.80 | **17.32** ± 48.55 |
| | 50 | **6.68** ± 11.02 | **20.93** ± 28.75 | **1.71** ± 4.04 | **16.99** ± 30.06 |
| | 70 | **3.65** ± 6.05 | **6.16** ± 6.76 | **1.10** ± 1.90 | **3.42** ± 5.71 |
| | 90 | **1.29** ± 1.02 | **4.05** ± 4.88 | **0.53** ± 0.63 | **2.26** ± 3.62 |
| | 100 | **0.56** ± 0.47 | **9.33** ± 16.70 | **0.46** ± 0.48 | **2.57** ± 6.77 |
| Expi293F | 0 | **34.64** ± 71.72 | **69.00** ± 84.74 | **34.64** ± 71.72 | **64.40** ± 83.58 |
| | 50 | **3.29** ± 2.37 | **16.87** ± 29.16 | **1.69** ± 2.48 | **6.18** ± 10.48 |
| | 70 | **4.82** ± 7.43 | **12.54** ± 25.13 | **1.09** ± 1.65 | **6.96** ± 16.60 |
| | 90 | **4.61** ± 7.17 | **4.52** ± 5.56 | **1.53** ± 2.52 | **3.36** ± 5.54 |
| | 100 | **1.05** ± 1.01 | **4.04** ± 4.58 | **1.37** ± 1.42 | **1.63** ± 3.28 |
| 293T | 0 | **46.19** ± 72.21 | **0.00** ± 0.00 | **28.87** ± 62.68 | **0.00** ± 0.00 |
| | 50 | **3.22** ± 2.81 | **10.7** ± 9.62 | **2.06** ± 2.88 | **5.09** ± 9.08 |
| | 70 | **3.21** ± 2.20 | **3.22** ± 3.61 | **1.56** ± 1.09 | **2.27** ± 2.47 |
| | 90 | **1.18** ± 0.84 | **2.71** ± 2.08 | **1.27** ± 0.85 | **0.58** ± 0.56 |
| | 100 | **1.80** ± 2.79 | **3.06** ± 5.97 | **1.28** ± 1.97 | **0.89** ± 1.86 |

**Table 8:** Average % CV of inter- and intra-channel cell viability across all concentrations and cell lines ± standard deviation. n=15 % CV values per expected viability where each % CV is calculated from three sample repeats.

## Stability Across 24 Samples with 0% Viability Included

| Cell Line | Expected Viability % | Measured Cell Concentration (cells/mL) | | Measured Cell Viability (%) | |
|---|---|---|---|---|---|
| | | Average | % CV | Average | % CV |
| CHO-S | 0 | $6.96 \times 10^6$ | 3.18 | 0.01 | 98.62 |
| | 50 | $5.89 \times 10^6$ | 5.18 | 41.73 | 2.62 |
| | 70 | $5.70 \times 10^6$ | 4.88 | 69.07 | 1.87 |
| | 90 | $5.34 \times 10^6$ | 4.35 | 85.91 | 0.91 |
| | 100 | $5.32 \times 10^6$ | 4.38 | 98.43 | 0.51 |
| Expi293F | 0 | $6.29 \times 10^6$ | 6.38 | 0.09 | 274.86 |
| | 50 | $6.25 \times 10^6$ | 5.56 | 47.97 | 1.93 |
| | 70 | $6.43 \times 10^6$ | 6.76 | 68.16 | 1.55 |
| | 90 | $6.28 \times 10^6$ | 10.04 | 81.15 | 1.26 |
| | 100 | $6.42 \times 10^6$ | 6.70 | 90.59 | 0.65 |
| 293T | 0 | $6.11 \times 10^6$ | 7.94 | 1.35 | 175.59 |
| | 50 | $5.48 \times 10^6$ | 8.19 | 50.97 | 3.21 |
| | 70 | $6.23 \times 10^6$ | 6.73 | 77.99 | 1.44 |
| | 90 | $4.87 \times 10^6$ | 8.08 | 89.16 | 3.16 |
| | 100 | $4.27 \times 10^6$ | 7.99 | 93.42 | 1.13 |

**Table 10:** Average inter-channel cell concentration and viability measured on ICON across three cell lines at an expected cell concentration of $5x10^6$ cells/mL, with corresponding coefficients of variation. n=24 per expected viability.

ADVANCED INSTRUMENTS

Two Technology Way / Norwood, Massachusetts 02062, USA

800-225-4034 | 781-320-9000 | www.aicompanies.com