# Cell counting accuracy and precision

## Why it matters and how to achieve it

### Introduction

Cell counting is a cornerstone of cell biology and related research today, whether a scientist is simply splitting cells as a part of routine cell culture or preparing cell samples for experiments downstream. Accurate cell counting and cell viability can be accomplished with a variety of methods that range from manual cell counting with a hemocytometer and microscope to using complex instrumentation like flow cytometers. There are many other automated cell counting options, including Invitrogen™ Countess™ 3 and Countess™ 3 FL Automated Cell Counters.

### Sample preparation

Accurate counting starts with a solid foundation in sample preparation. Optimal sample preparation can be broken down into two phases. In the first phase, steps are required to create the sample of interest, typically in a larger volume to be further processed for downstream purposes. In the second phase, the steps from the first phase are performed to create the counting sample. These steps can vary significantly depending on the sample source, which could be an immortalized suspension cell line, an adherent cell line, frozen stock, or a primary cell sample.

The first-phase steps are highly dependent on the sample type and downstream application, so they will not be included here. However, attention to detail in the preparation of counting samples can be critical. Below are the top three recommendations (Figure 1).

- **Create a homogenous cell suspension**—Do not vortex cell samples. Mix gently but thoroughly by pipetting, inversion, or finger flicking immediately before removing an aliquot from the parent sample. Allowing a sample to settle prior to pipetting may result in the formation of a concentration gradient, potentially causing inconsistent counts.
- **Avoid debris introduction**—Do not vortex trypan blue solution. It is notorious for containing small particulate matter, even when freshly opened. Such particulates will passively accumulate at the bottom of the tube, leaving most of the vial clean and ready for use if mixing and vortexing are avoided. Do not freeze trypan blue solution, as this will dramatically increase the amount of precipitate present.
- **Achieve a uniform focal plane**—After mixing the cell sample (10 µL) and trypan blue solution (10 µL), pipette 10 µL of the stained cell suspension into the counting chamber. Allow it to settle for ~30 seconds to achieve a uniform focal plane.

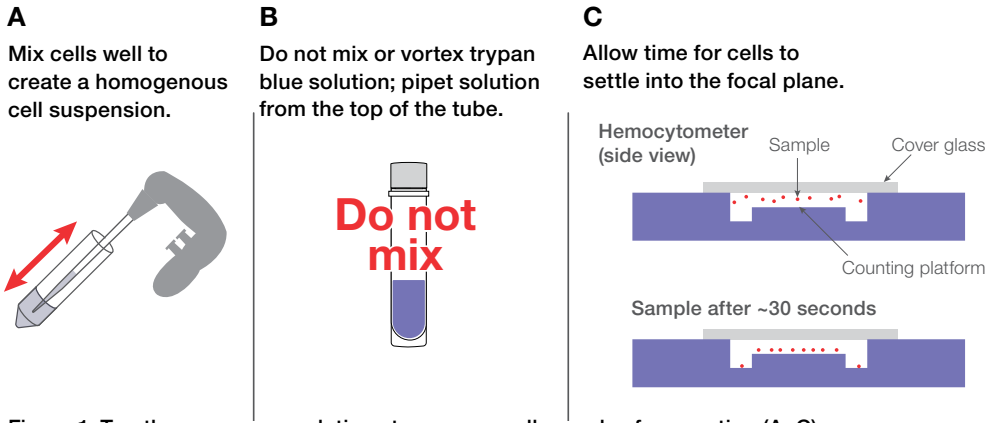


Figure 1. Top three recommendations to prepare cell samples for counting (A–C).

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## Instrument setup

Whether you are using a Countess device, another automated cell counter, or a microscope and a hemocytometer, instrument setup is critical in order to realize accurate and consistent counts. Below are the top three instrument settings to consider to achieve accurate counts. Figure 2 demonstrates two of the three critical instrument settings that are required for accurate and precise counts.

- Uniform, consistent lighting
- Correct focus
- Consistent gating (not possible with manual counting)

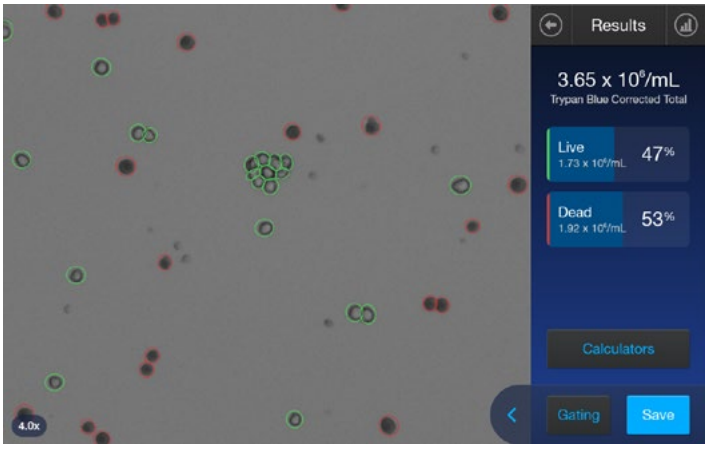
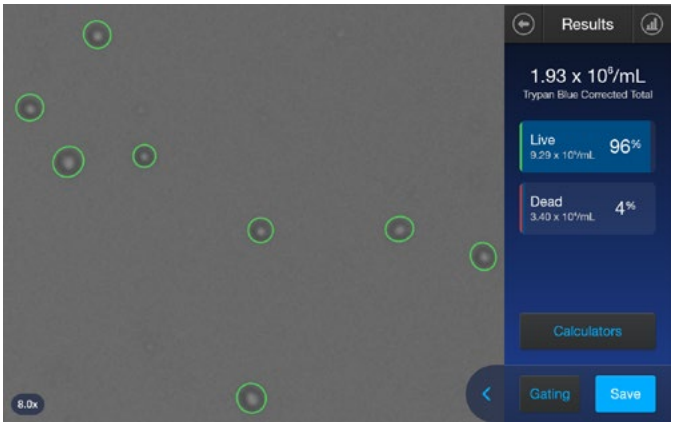



**Figure 2. Segmentation ability of the Countess 3 Cell Counter demonstrated in a bright-field image.**

When using a Countess 3 or Countess 3 FL instrument, we recommend selecting the default instrument profile, as this ensures all gating settings are maximized at the beginning of cell counting. Countess 3 and Countess 3 FL instruments are equipped with autofocus and auto-lighting features that optimize focus and lighting for each sample, thus removing variability that can negatively affect your counting result. Figure 3 demonstrates uniform lighting and focus with human and mouse PBMC samples. These primary samples lack small particulates and other debris that are commonly found in PBMC preparations. With previous Invitrogen™ Countess™ instruments, it was commonly recommended that smaller objects be gated out to help minimize the effect of debris on count accuracy (Figure 4). Gating is largely unnecessary with Countess 3 and Countess 3 FL instruments due to the advanced focus and image analysis algorithms developed with artificial intelligence (AI). However, customers who want to tailor their count parameters can adjust various settings, including size, brightness, and circularity.

**A.** hPBMCs




**B.** mPBMCs

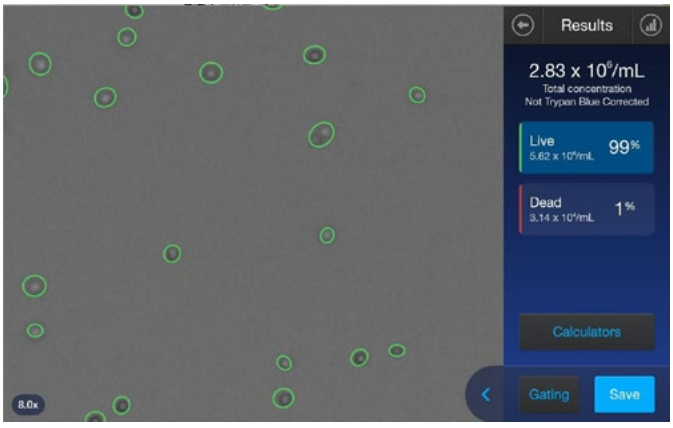



**Figure 3. Successful counting of two peripheral blood mononuclear cell (PBMC) samples: (A) human and (B) mouse.** The cells were counted efficiently due to uniform lighting, focus, and staining with very little debris. The default settings of the Countess 3 Automated Cell Counter were used to obtain these results.

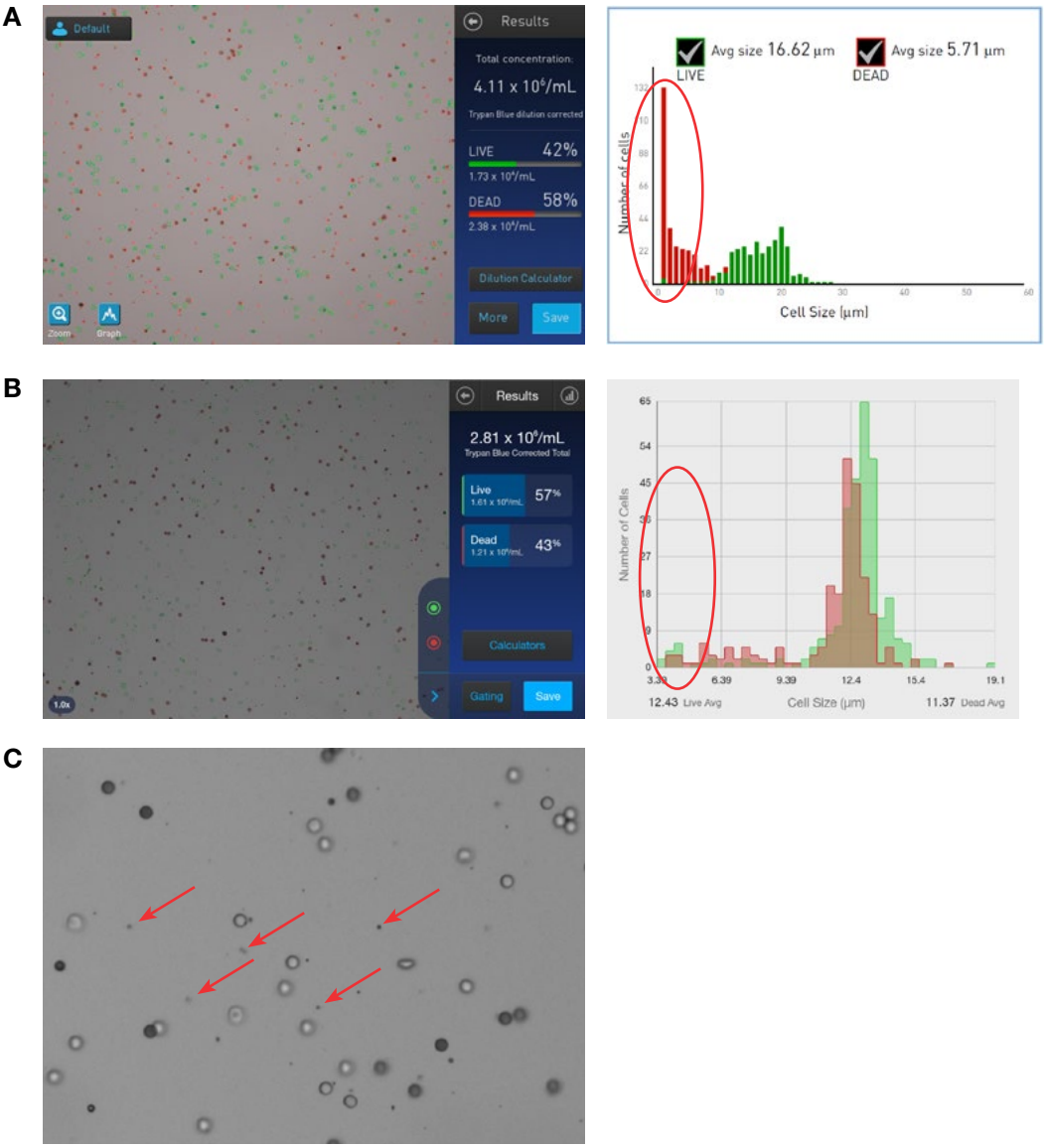



**Figure 4. Comparison of cell counts performed with Invitrogen™ Countess™ II and Countess 3 cell counters using the default instrument settings. (A)** A significant number of small particulates were counted using the Countess II instrument, so gating was required to remove them. **(B)** The small particulates were avoided using the Countess 3 instrument, and gating was not required. **(C)** Red arrows denote small particulates commonly observed in samples from primary cells and particulates due to precipitation of samples from trypan blue solution.

## Key considerations

When comparing cell counting methods and/or replicates, it is common to simply select the “correct” result without considering bias or accounting for the inherent error built into a method. In many cases, a single hemocytometer count is selected as the gold standard without statistical considerations. At the opposite end of cell counting complexity are flow cytometry methods. While they are extremely accurate, many do not realize that reagent titration is required for optimal flow cytometry results.

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Figure 5 demonstrates changing results due to reagent titration. This is an important example of how obtaining an accurate bright-field count with a Countess 3 instrument can have a dramatic downstream effect when using fluorescence flow cytometry.

A similar pattern can be observed in results obtained with samples that contain high or low amounts of debris. The benefit of image-based techniques is the direct sample feedback offered by the created images. Both novice and expert users can believe results obtained through direct observation. As shown in Table 1, repeatable inter-sample and intra-sample counts were observed using the Countess 3 FL instrument. Each sample was drawn from the same stock of Jurkat cells, pipetted into a chamber slide, and read four times on a Countess 3 FL instrument using the default instrument profile. Based on the data, remarkable consistency was observed between replicate counts. Overall agreement between separately pipetted samples was achieved by following the recommendations outlined on page 1. If sample preparation was inconsistent, significant differences would be observed between samples 1 and 2. If instrument focus, lighting, or gating was inconsistent between sample or count replicates, the coefficient of variation (CV, %) would be significantly higher.

## Summary

Regardless of the downstream application, obtaining an accurate and precise cell count is critical. Incorrect cell counts can easily lead to suboptimal culture conditions in simple cell-splitting applications or failed experiments due to incorrect labeling of titration reagents in flow cytometry or imaging applications.

Today's automated cell counters allow scientists to count cell samples more quickly and easily than ever before due to significant advances in hardware and software technologies and AI-based image analysis. However, a steady hand and solid sample preparation practices can be the difference between success and failure.

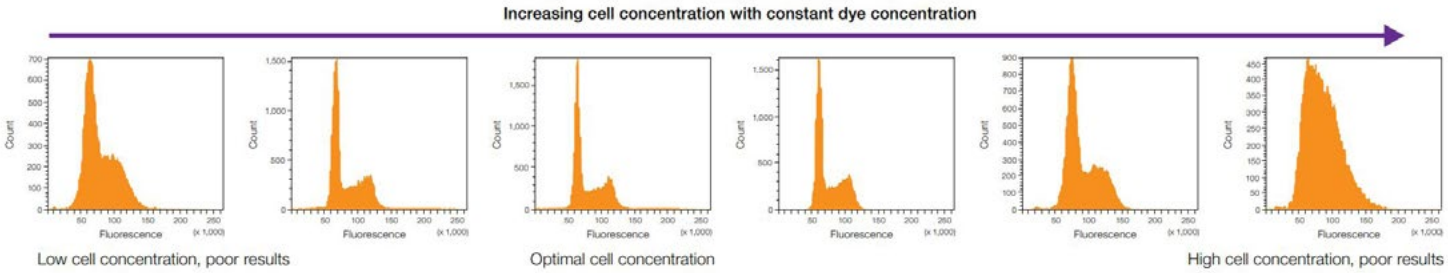


**Figure 5. Suboptimal dye vs. cell concentration examples as shown by flow cytometry.** Different concentrations of live Jurkat cells were labeled with a constant concentration (10 µM) of Invitrogen™ Vybrant™ DyeCycle™ Orange Stain. Using the same concentration of stain produced poor cell cycle histograms for both low and high cell concentrations. Staining with the optimal cell concentration of 1 x $10^6$ cells/mL gave the optimal cell cycle histogram at the same dye concentration.

**Table 1. Consistency between data from replicate cell counts observed with the Countess 3 FL instrument.**

| | Count | Total | Live | Dead |
|---|---|---|---|---|
| Sample 1 | 1 | 409 | 198 | 211 |
| | 2 | 409 | 198 | 211 |
| | 3 | 410 | 197 | 213 |
| | 4 | 403 | 199 | 204 |
| | CV (%) | 0.79 | 0.41 | 1.88 |

| | Count | Total | Live | Dead |
|---|---|---|---|---|
| Sample 2 | 1 | 395 | 198 | 197 |
| | 2 | 392 | 198 | 194 |
| | 3 | 391 | 193 | 198 |
| | 4 | 396 | 197 | 199 |
| | CV (%) | 0.78 | 0.85 | 2.12 |

Find out more at **thermofisher.com/countess**

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