WHITE PAPER

Practical cell counting method selection to increase the quality of results

Based on ISO cell counting standards

Introduction

The importance of cell counting has increased significantly in the last decade due to the major advances in the fields of cell and gene therapy, biologics production, and regenerative medicine. This has necessitated the development of a standardized approach to cell counting assays. In support of this widespread need, the U.S. Food and Drug Administration (FDA), in collaboration with the National Institute of Standards and Technology (NIST), and the International Organization for Standardization (ISO), have launched a joint effort to regulate cell counting methods and improve the confidence in cell counting measurements

ISO has Published two cell counting standards to improve cell counting results

A wide range of biological samples are dynamic and heterogeneous requiring varying formulations, and specific bioprocessing steps for cell and gene therapies. For the determination of further experimentation and to select the proper dosage of cell and gene-based therapies, cell counting requires a unique and complex method to provide accuracy.

As there are no ground truth reference materials for live cells to ensure the accuracy of cell counting, it is recommended to adhere to the two recently published *ISO cell counting standards: ISO 20391 – 1:2018 Biotechnology – Cell Counting – Part I: General Guidance on Cell Counting Methods*[1] *and ISO 20391 – 2:2019 Biotechnology – Cell Counting – Part II: Experimental Design and Statistical Analysis to Quantify Counting Method Performance.*[2]

The important aspects of the ISO cell counting standards have been distilled to six key factors that can influence the selection of the appropriate fit-for-purpose cell counting method and the quality of the cell counting results. These key factors are divided into three specific categories: Knowledge Collection, Cell Counting Method, and Operation Assurance (Figure 1). Attention to these details will allow researchers to adhere to the ISO recommendations and qualify high-quality cell counting measurements, and in doing so, sites can meticulously document their rationale, process, and data for reporting compliance.

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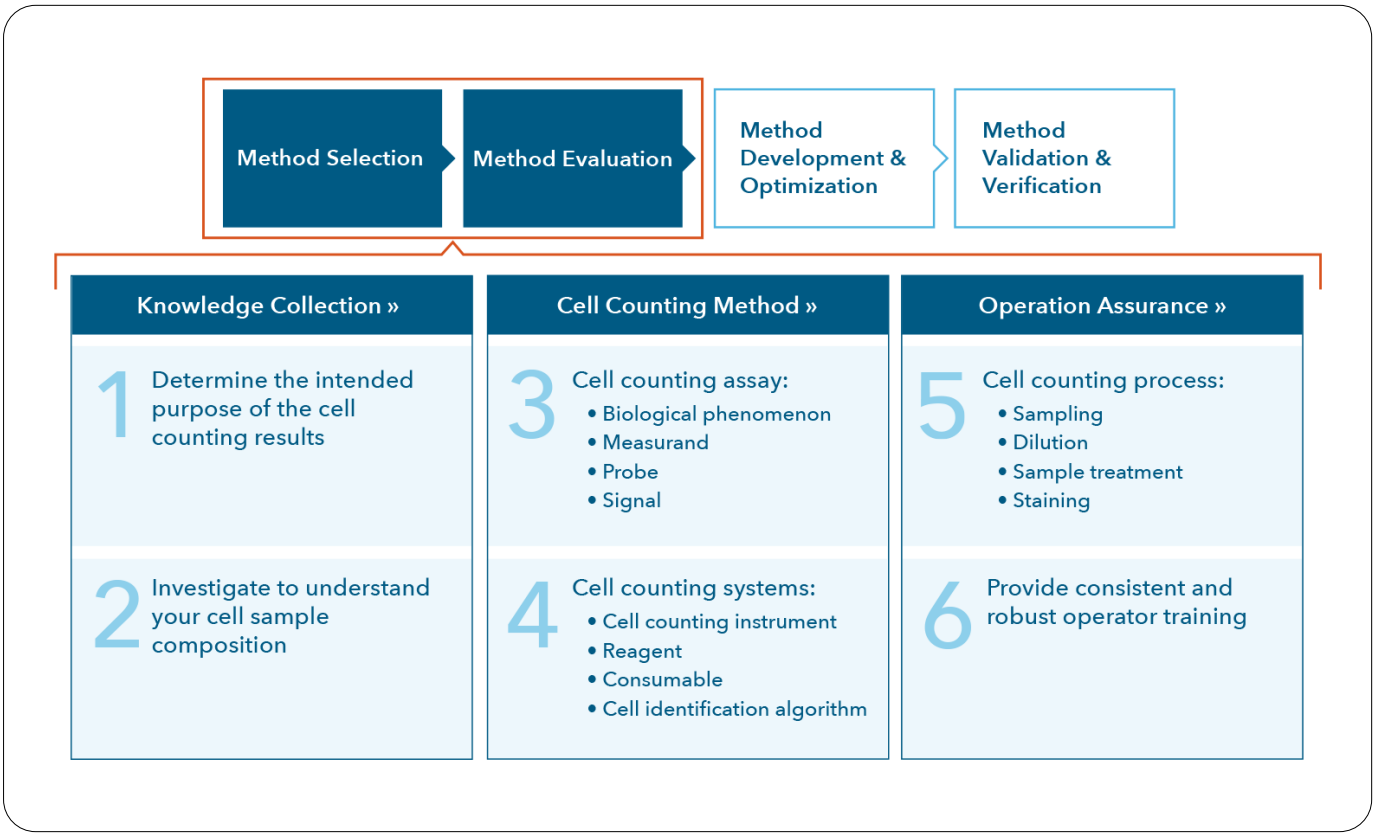


Figure 1: The six key factors for selecting the optimal cell counting method.

The six key factors that can impact the cell counting method selection and the quality of measurement

1. Determine the intended use of the cell counting result to obtain a fit-for-purpose cell counting method.
2. Investigate the cell sample composition to understand cell morphology to differentiate various cell types from debris, chemical impurities, and suspension medium.
3. Understand the assay principles and select the appropriate cell counting assay, such as total, live, dead cell count, viability, or cell population analysis.
4. Investigate the capabilities and select the appropriate cell counting systems, where the system consists of reagents, consumables, instrumentation, and software algorithms.
5. Treat cell counting as a whole process, including sampling, diluting, and staining.
6. Provide continuous operator training and support to secure consistent cell counting results.

Knowledge collection

Biological samples are dynamic and many require varying formulations and specific bioprocessing steps for cell and gene therapies. For the determination of further experimentation and to select the proper dosage of cell and gene-based therapies, cell counting requires a unique and complex method to provide accuracy. To appropriately select the appropriate fit-for-purpose cell counting method, researchers must first determine the intended use of the cell counting results, such as tumor digestion for single cell-based transcriptome analysis, mouse tissue processing for cytotoxicity assays, or isolation of human PBMCs for immunophenotyping analysis, etc. Additional investigation is necessary to understand the sample composition including morphology and media composition to differentiate various cell types from particle debris, chemical impurities, and suspension medium. The knowledge collected for the first two key factors can be used to hone in on the possible cell counting methods to select and evaluate.

Determining the intended use

Determining the intended purpose of the measurements directly impacts the selection of the cell counting method. For example, a research lab may aim to document and publish novel biological processes or effects, and thus may seek to accumulate knowledge and incorporate best practices about the cell counting measurement quality early on and as a group. Meanwhile, cell therapy manufacturers may need to focus on meeting FDA requirements while being able to transfer assays from discovery to development, clinical trial sites, and manufacturing, ideally leveraging all assay improvements throughout the company. If manufacturing is the final goal, the cell counting method will be dependent on the final product. For example, human bone marrow stromal cells for regenerative therapies have very different needs compared to tumor-infiltrating lymphocytes (TIL-T cells). Careful consideration of the intended use of the cell counting method must be employed to ensure the proper course of action is taken for the most robust and reliable results.

The next critical component to determining intended use is what readout will be measured by the assay. This can be as simple as live or dead cell counts, but can also include measurements such as cell concentrations (alive or dead), percent viability, and biomarker or protein expression data. These various metrics require different conditions for optimization, especially when considered in the context of the sample type. The enormous range of biological sources for cell counting samples cannot be understated. From cultured or purified cell lines, whole tissues, isolated or cryopreserved blood samples, single-cell suspensions, there are a plethora of considerations to choosing a cell counting method for each of these sample types.

Understanding the sample composition

The knowledge collection process should include information gathering to understand the composition of the specific cell samples to be measured. Perhaps most paramount is ascertaining what your cell samples visually look like, which is determined by what biological components are present. In general, a cell sample may contain various cell types, impurities such as debris or red blood cells (RBC), and the different suspension media used, all of which likely will affect cell counting procedures.[3] In the example provided, the cell sample contains three cell types, where only PBMCs are typically considered the measurands, consequently, the cell counting method selected should be able to differentiate PBMCs from RBCs and platelets (Figure 2).

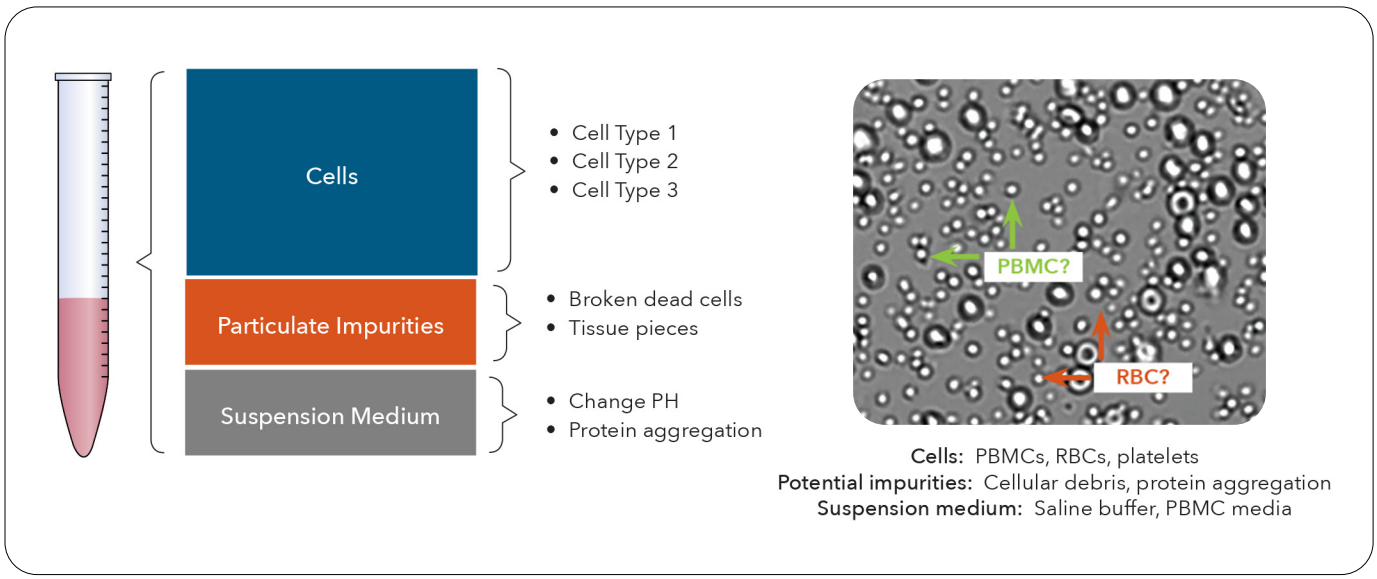


Figure 2: Cell sample composition. A fresh human PBMC sample is collected from a whole blood sample and processed by Ficoll separation and reveals the cells of interest (PBMCs), but also platelets and RBCs, cellular debris, protein aggregates, and various types of culture media.

Cell morphology has a direct impact on the way cells are counted. Variation in cell size and the tendency for some cells to stick together can dictate the selection of the cell counting method (Figure 3). Part of the method selection should ensure that cellular debris, protein aggregation, and buffer or media selection does not interfere with the staining process or the quality of cell counting. The examples provided in Figure 4 illustrate the types of debris or additional cell types that can be present in cell samples, aiding in the appropriate selection of a cell counting method to identify the intended measurands.

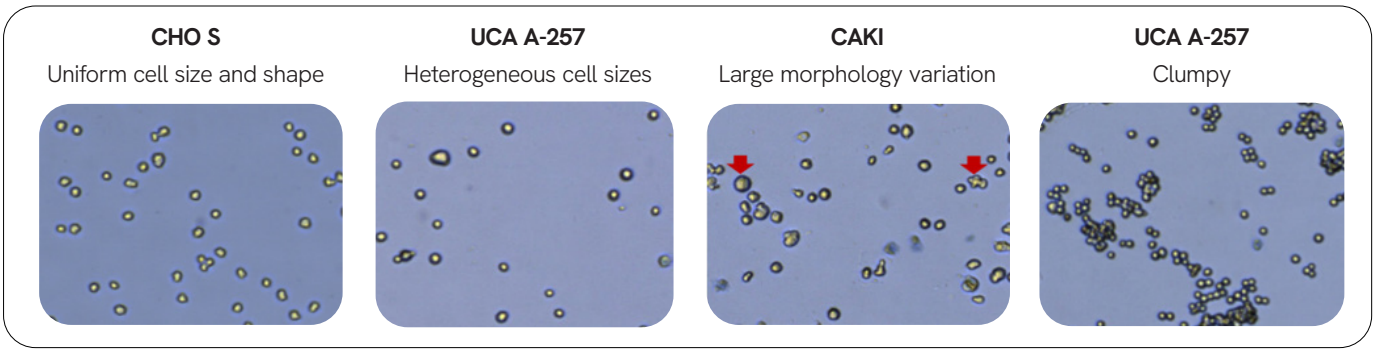


Figure 3: Various cell morphologies observed in different cell types.

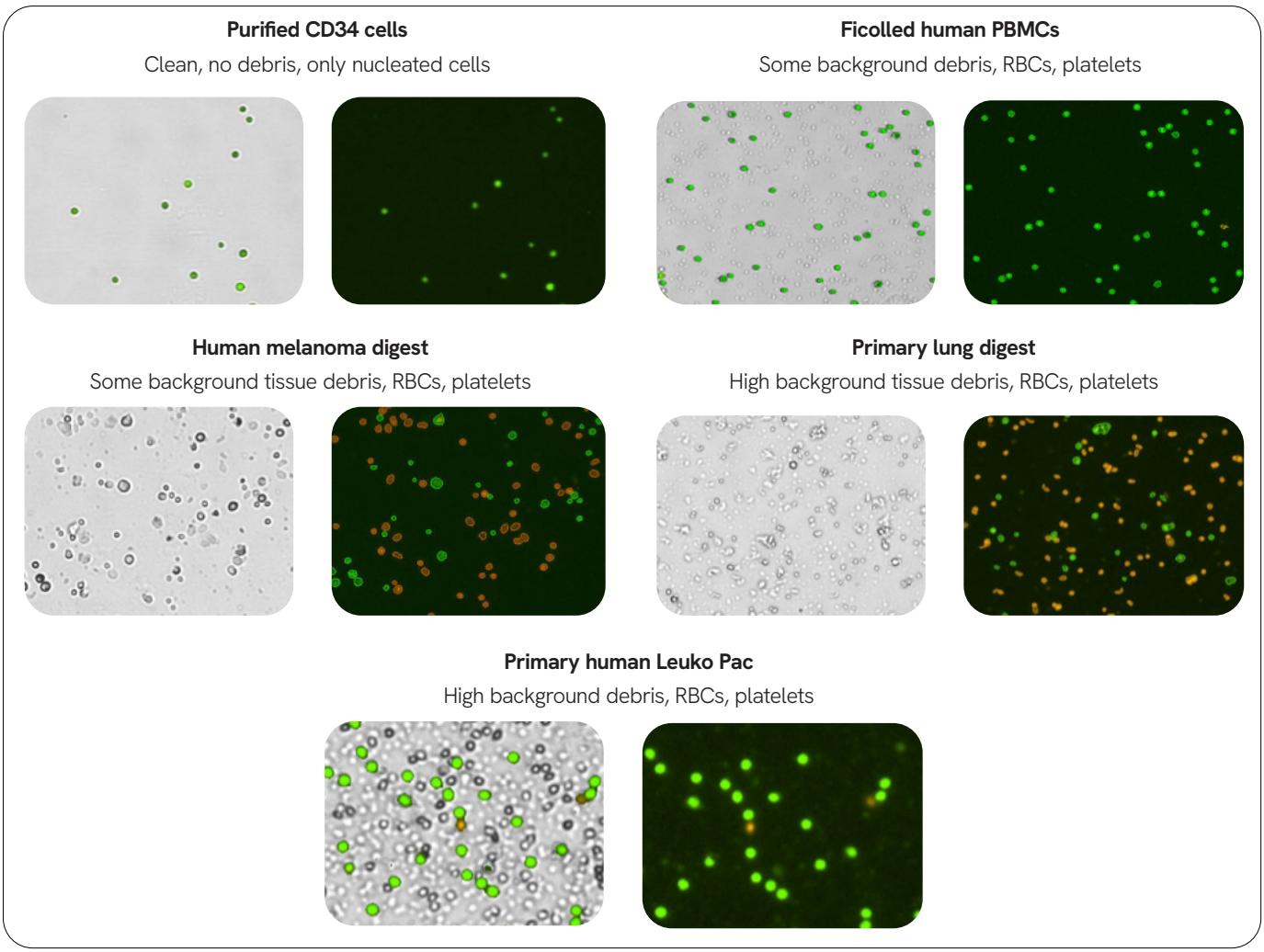


Figure 4: Commonly observed variation, debris, and additional cell types in samples. In each pair of examples shown above, a brightfield and fluorescent overlay image shown on the right with a fluorescent image of cells stained with acridine orange and propidium iodide shown on the right.

The example images (Figure 4) demonstrate fluorescent nuclear staining using acridine orange and propidium iodide as a live/dead dye mixture to fluorescently label only nucleated cells in green and red fluorescence respectively. This method is a safeguard where only nucleated cells will fluoresce and nonspecific particles such as debris, red blood cells, and platelets will not. This process is ideal for correctly counting the cells for primary samples and proves that unpurified primary cell samples unquestionably require the use of fluorescent stains to identify the target cells of interest.

Cell counting method

Many researchers may assume cell counting methodology represents the instrument used to perform the assay. Outside of sample preparation, the quality of the cell counting results is dependent on the cell counting system, which includes not only the instrument, but also the assay, reagents, consumables, and software analysis algorithms.

Choosing a cell counting assay

Once the knowledge collection phase is complete, researchers can consider the intended use and sample composition to determine which cell counting assay is appropriate. There are four categories of cell counting assays relating to how the cells or measurands are assessed: direct total, indirect total, direct differential, and indirect differential (Table 1).

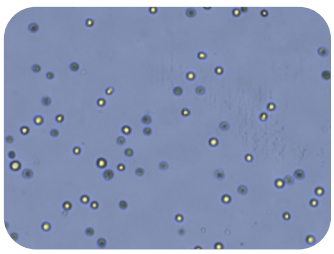
- **Direct cell counting** is the detection of independent signal(s) for each cell.
- **Indirect cell counting** is the measurement of a signal from a population of cells, which is relayed back to the cell number through a mathematical model.
- **Total cell count** includes the count of all cells, regardless of attributes.
- **Differential cell count** refers to a distinguished subset of cells that is identifiable from other cell subpopulations by some measurable attribute.

Table 1: Different counting categories for cell counting measurands.

Cell counting categories	Cell counting assay example
Direct Total	Brightfield counting all cells in hemacytometer
Indirect Total	MTT or MTS assays to measure viable cells
Direct Differential	Fluorescent cell counting with live cells stained with acridine orange, dead cells stained with propidium iodide
Indirect Differential	Luciferase assay to measure cytotoxicity of T cells killing tumor cells

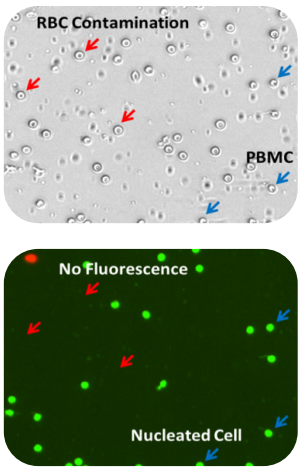
A cell counting assay should be selected to best measure the intended measurands in the target cell sample, and probes should be selected based on the cell sample, application, and biological phenomenon. Researchers have been increasingly moving towards direct cell counting methods for assessing cell concentrations. A recent publication in PLOS ONE (Chan et al. 2013) demonstrated that utilizing an indirect counting method such as MTS or MTT assays can yield misleading results because the ATP content can be affected by various drug treatments induced on the target cells.[4]

There are several significant factors to consider when choosing a counting assay. Researchers should consider not only the counting method category but also the biological phenomena, ideal probe, and the optimal signal to count the cells of interest. Measurands can include live cells, dead cells, and combinations of both, while biological phenomena might be cell membrane permeability, metabolic activity, the presence or absence of biomarkers, and more.



What are the objects intended to be measured?	Live and dead cells
What is the biological phenomenon you are measuring?	Cell membrane-permeable
What is the probe of choice?	Trypan blue
What are the signals detected?	Live (refractive light), dead (blue color)

Figure 5: Example assay principles for trypan blue stained CHO cells.



What are the objects intended to be measured?	Live and dead cells
What is the biological phenomenon you are measuring?	Cell membrane-permeable
What is the probe of choice?	Acridine orange and propidium iodide
What are the signals detected?	Live (green fluorescence), dead (red fluorescence)

Figure 6: Example assay principle for isolated human PBMCs. Images show brightfield (top, left) and fluorescent stained with acridine orange / propidum iodide (bottom, left).

The consideration of both measurands and biological phenomena will then be used to determine the probe of choice for cell counting. The choice of probe and the resulting signal should be carefully considered, not just in terms of the sample biology, but also concerning other potential sample traits such as the presence of debris, nonspecific cell types, and sample media. A simple example is shown in Figure 5, where the measurands are both live and dead cells, and the biological phenomenon is cell membrane permeability. To detect the colorimetric visual signals from dead cells the selected probe is trypan blue.

Figure 6 presents an example where the measurands are both live and dead cells, and the biological phenomenon is cell membrane permeability. In this case, the fluorescent probes were selected to specifically identify PBMCs from the contaminating cell types and cellular debris (Figure 4) and to easily differentiate the live and dead cells within the sample (Figure 6).

It is worth noting that there are slight behavioral differences with each nuclear fluorescent probe, and many require long incubation times to properly stain dead cells. Additionally, an incorrect probe choice can harm cell counting accuracy, such as in the case of trypan blue. While this is one of the most commonly used probes to measure live/dead cells and cell viability, it can have adverse morphological effects on dead primary immune cells such as PBMCs or mouse splenocytes.[5] Trypan blue can cause cells to rupture, decreasing dead cell count, and increasing cell viability artificially.[6] Ultimately, probe choice is extremely context-specific and must be made with respect to all previous considerations of the cell counting method.

Key factors for appropriate probe selection

- Trypan blue is the most common dye to measure cell viability. However, if cell viability is not high, trypan blue can affect the morphology of the dead cells, over-estimating viability.[7]
- When a cell sample contains other nonspecific particles such as RBCs, platelets, and debris, use fluorescent probes to ensure accurate counts.[8]
- All nuclear fluorescent probes have slightly different behaviors except for PI, most of the dyes require longer staining time to properly stain the dead cells, specifically with DAPI and 7-AAD.[9]
- For dyes requiring a longer staining time, shelter by keeping in the dark to avoid photobleaching.

Questions to consider when selecting your cell counting assay

- What is the measurand (objects intended to measure)?
  - Live cells only
  - Dead cells only
  - Live and dead cells
  - Total cells
  - Other measurands (i.e. CD4 positive cells)
- What is the biological phenomenon you are measuring?
  - Cell membrane permeability
  - Metabolic activity
  - Existence or lack of surface/intracellular biomarkers (e.g. transduction/transfection efficiency)
  - Replicability
  - Other biological phenomena

- What is the probe of choice?
  - Trypan blue
  - Fluorescent nuclear stains for membrane-permeable dead cells
    - *Propidium Iodide,* DAPI, 7AAD, *Dead Nuclear Blue/ Green/ Red/Far Red, DRAQ 7*
  - Fluorescent nuclear stains for total cells
    - *Acridine Orange, Hoechst 33342, Total Nuclear Blue/ Green/ Red/Far Red, DRAQ 5*
  - Fluorescent metabolic activity stains
    - *CFDA AM, Calcein AM, CFDA, CMFDA, CFSE*
  - Combination stains for live and dead cells
    - *AO/PI, Hoechst/PI, Calcein/PI, AO/DAPI*
  - Other probes
    - *Annexin V-FITC, Caspase 3/7, JC-10, ROS marker*
  - No probe
- What are the signals detected?
  - Refractive light in brightfield imaging
  - Dark spots in brightfield imaging
  - Blue spots in brightfield imaging
  - Blue/Green/Red/Far Red fluorescence
  - Impedance

Choosing an automated cell counting system

An automated cell counting system can be used to detect the biological phenomena as well as the signals from your measurands (cells) and probes. By considering all of the different components of the cell counting system including reagents, consumables, and software algorithms, an optimal selection can be made for the specified cell counting assay. Table 2 demonstrates specific examples of cell counting system solutions offered by Revvity.

Table 2: Examples of cell counting systems for selection.

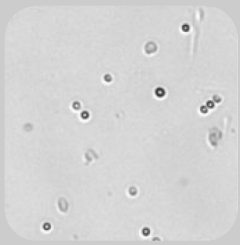
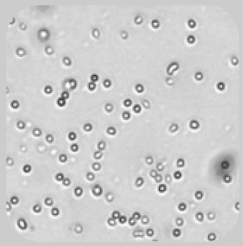
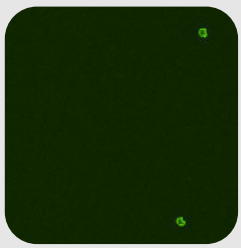
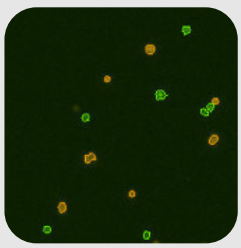
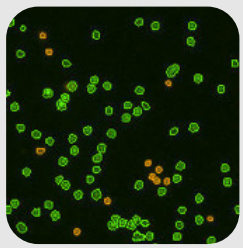
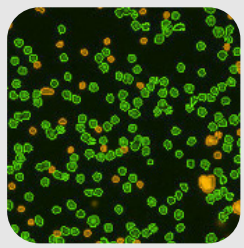
Instrument	Reagent(s)	Consumable	Cell ID algorithm
Impedance			
Coulter Counter	Buffer	Buffer container	Total Count
Brightfield Only			
Cellometer Auto T4 (advanced cell counter)	Trypan Blue	Slides	BF, TB, chamber height
Brightfield + Fluorescence			
Cellometer K2 (advanced cell counter)	Trypan Blue, AO/PI	Slides	13 Total: FL only, FL1 + FL2, dual FL, etc.
Cellometer X2 (yeast cell counter)	Trypan Blue, AO/PI	Slides	13 Total: FL only, FL1 + FL2, dual FL, etc.
Cellometer Auto 2000 (all-in-one counter)	Trypan Blue, AO/PI	Slides	13 Total: FL only, FL1 + FL2, dual FL, etc.
Cellometer Spectrum (advanced counter with 2 color cell-based assays)	Trypan Blue, AO/PI, Hoechst/PI, etc.	Slides	13 Total: FL only, FL1 + FL2, dual FL, etc.
Cellaca MX High-throughput Cell Counter	AO/PI, Hoechst/PI, PI only, etc.	Chamber plate	13 Total: FL only, FL1 + FL2, dual FL, etc.

Another major consideration for any cell counting instrument is the range without dilution. Some instruments may indicate a large cell concentration range but may require multiple dilutions, which can increase the Coefficient of Variation (CV) of the cell counting results. To combat this, cell counting range is determined in the absence of any dilution steps (Table 3). Demonstration of this concept is observed in an experiment performed by measuring cell count and viability of lymphocytes from thirty different mice using an acridine orange/ propidium iodide fluorescent stain. The Cellometer® Auto 2000 showed that mouse lymphocytes can be properly measured within a range from 1 x 10^5 to 6 x 10^7 cells/mL

without additional dilution steps (Table 3). With these tools, researchers can reliably use automated cell counting instrumentation to measure within this range by avoiding dilution as a confounding factor.

Beyond dilution range, additional influences when choosing a cell counting system include what reagents and consumables are required. While reagents can affect cell counting itself through the type, fluorescence color, or strength of a signal, they also can affect general experimental flow-through cost, availability, and accessibility. Additional consumable considerations include sterility and reusability, as well as shelf-life. Finally, researchers must carefully consider the software used to count cells, as algorithms between systems can vary drastically. The system software should be able to accurately assess the desired measurand, which may include many considerations. Ultimately, the cell counting system chosen will be specific to the sample and desired result and should be made with consideration to long-term consistency, and manufacturing applications.

Table 3: Eliminating dilution steps with the Cellometer Auto 2000.

	Sample #7	Sample #6	Sample #2	Sample #5
Total cell count	49	1419	10635	24245
Live cell concentration	1.07E+05	2.84E+06	3.11E+07	6.75E+07
Viability	63.20%	58.00%	84.40%	80.30%
Brightfield cell image				
Counted live & dead cell image				

Questions to consider when selecting your cell counting system

- What reagents can you use for the intended measurand?
  - What is the availability of the reagents?
  - What is the lead time or shipping duration?
  - What is the cost of the reagent?
  - What color/fluorescence are they?
  - How bright is the signal?
- What consumables are needed to measure the cells?
  - What is the availability of different consumable offers?
    - *Such as depth, for different sizes or spheroid sizes?*
  - What is the shelf-life of the consumables?
  - What is the cost of the consumables?
  - Do the consumables have auto-fluorescence?
  - How clean are they? Are they reusable?
  - How accurate are the dimensions?
- What are the available cell identification algorithms?
  - What imaging modes are available?
  - Is the algorithm able to analyze both brightfield and fluorescent images separately and together?
  - Can the software measure both total cells or viable cells and viability simultaneously from the same cell sample?

- Can the software provide cell counts, size, and morphology?
- How well can the algorithm de-cluster cells that are in close proximity?
- How many preset assays and cell types does the software contain?
- Can the software analyze cell population through visual gating strategies?
- Can the counting parameters be saved for future use?
- How easy is the software to use?

Operation assurance

Cell counting is a process that can strongly dictate the quality of the results. Optimization of the cell counting process involves proper operator training on processes for the cell counting assays, systems, as well as operations. Cell counting operators should be provided training from either the vendor or technical lead in the lab and receive a training certificate, as well as periodic retraining to maintain high quality and robust cell counting results.

Cell counting process

Cell counting success is highly influenced by cell sample preparation, which needs to be performed consistently and carefully to make certain cell counting measurements are of the highest quality. The procedures for preparing the cell sample for cell counting are essential, as improper cell sample handling can increase counting error and standard deviation, especially between operators, which has been published previously. Numerous bioprocessing steps are required when processing from primary patient cell samples to final cell and gene therapy products, where the sampling and dilution steps are critical when preparing cell samples for cell counting.

Important general considerations for sample processing include how the sampling and diluting occurs, which sample treatments are performed, and what staining procedures are implemented for the assay probes. These considerations can be further detailed when designing cell counting protocols ensuring all operators are performing the assay consistently. Cell sampling factors include sample size, frequency, and sample mixing, as well as the proper calibration and usage of sampling tools such as pipettes.

One major example demonstrating the significance of the cell counting process is sample mixing and waiting time. This lag can cause the cells to settle, harming the cell counting results. To measure the effect of cell settling time on cell counting measurements, a 2 mL sample of Jurkat cells was aliquoted into a 15 mL tube. After uniform mixing by inverting the tube ten times and pipetting up and down ten times, approximately 200 µL of cells were removed from the middle of the suspension at 0, 1, 5, and 20 minutes. The analyzed results demonstrate that only five minutes of settling time can cause a 26% reduction in cell concentration (Figure 7). This serves to emphasize how even slight variability in sample processing can have significant effects on cell counting accuracy.

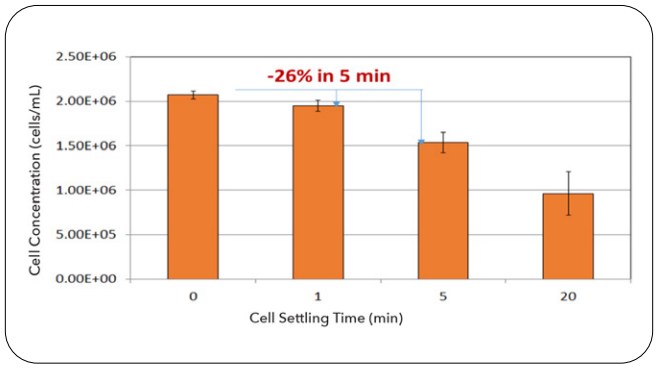


Figure 7: Effect of cell settling time on measured cell concentration.

Protocols that include dilution should consider dilution buffer type (i.e. pH, temperature, etc.), as well as rounds of dilution. Previous publications have found that higher dilution factors generated lower CV; however, too many dilution steps can also increase CV.[10] Sample treatments may include lysing, de-clumping, and any form of purification.

Finally, when assessing staining procedures, careful attention should be paid to the amount of stain, as well as the staining conditions such as temperature, light exposure, and time. As previously mentioned, many probes are time sensitive and researchers must carefully consider their probes and signals through the context of operator processing. All of these steps for sample collection and processing must be performed consistently between all operators, as even minor variations can negatively affect cell counting accuracy and data.

Questions to consider when improving your cell counting process

- How are you sampling your cells?
  - How much do you sample each time?
  - How frequent do you need to sample?
  - How do you mix your cells uniformly?
  - Do you have your pipettes calibrated regularly?
  - Are you using manual or automated pipettes?
- What sample treatments will you perform?
  - Do you need to lyse your cell sample?
  - Do you need to declump your cells?
  - Do you need to purify your cells?
- How are you diluting your cells?
  - What is the buffer?
  - What is the pH of the buffer/media?
  - How many times do you dilute?
- What is your staining procedure?
  - How much stain do you need to use?
  - How long is the staining time?
  - Do you need to incubate at a certain temperature?
  - Do you need to incubate in the dark?

Routine operator training

Cell counting operators should be fully trained both on sample preparation and the selected cell counting systems to guarantee consistent and precise cell counting results. Periodic retraining from the manufacturer or lead user in the laboratory is recommended. Examples of how routine operation assurance should be conducted are described below.

Cell counting process

1. The operators should be trained on the consistent sampling of cells, such as pipetting, sampling volume, location to sample, and mixing prior to sampling.
2. The operators should be trained on the protocols to dilute the cell sample, such as volume to dilute and how many times to dilute.
3. The operators should be trained on sample preparation, such as lysing, declumping, and purification.
4. The operators should be trained on how to properly stain the cell sample, such as nuclear, metabolic, or colorimetric staining.

Cell counting system

1. The operators or manufacturers should perform the Installation Qualification (IQ) and Operation Qualification (OQ) on the instrument, and provide completion documentation.
   a. IQ – To verify the installation of the hardware and software in the selected environment and its documentation
   b. OQ – To verify that hardware and software will function according to its operational specifications in the selection environment
2. The operator should be trained on using the cell counting system specifically on their intended use, meaning training on the operation of the cell counting instrument.
   a. Physical usage of the cell counting instrument
   b. Physical usage of the consumable and reagent
   c. Operation of the cell counting instrument such as software control and analysis

Conclusions

The increasing demand for consistent and accurate cell counting methodology has been met with the generation of the ISO cell counting standards. These include detailed guidelines for selecting and evaluating cell counting methodologies, which have here been distilled to the six key factors to consider for success. Researchers can take advantage of these factors to navigate the process of cell counting methodology selection and evaluation. Adherence to these aspects will result in robust and accurate cell counting results.

References

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