# Maximize research capacity with cell counting efficiency

Optimize your workflows and lab budgets by choosing the best cell counter for your application

THE QUEEN'S AWARDS FOR ENTERPRISE: INTERNATIONAL TRADE 2021

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# Introduction

**The increasing emphasis on both inter- and intra-lab reproducibility in research means obtaining accurate cell counts is more important than ever. Cell counting is a fundamental quality control procedure, but traditional manual counting can be time-consuming, error-prone and highly subjective.**

Automation addresses many of these challenges and the latest image-based automated cell counters are essential in delivering cell concentration and viability data with greater accuracy, reliability, and higher throughput than ever before. Additionally, cutting-edge optics and software can now detect individual cells within clusters, helping to transform your laboratory workflows and productivity.

In this eBook, we take a look at Logos Biosystems' range of automated cell counters that address all your cell counting needs. Through case studies, understand their different technical features and applications,

## Contents

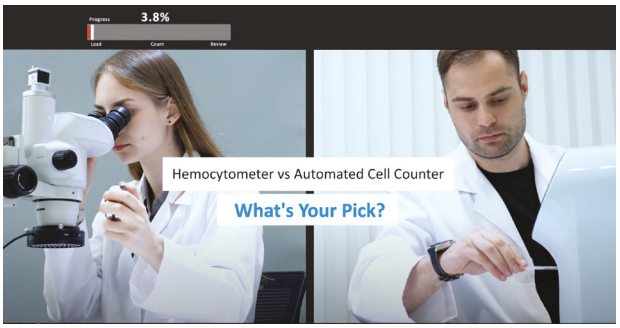
| Cell counter | Key features | Recommended applications |
|---|---|---|
| **LUNA-II™** | Brightfield, trypan blue viability, Erythrosin B (eco-friendly option) | General cell counting needs |
| **LUNA-III™** | Machine-learning<br>Reanalysis Feature<br>Brightfield, trypan blue viability<br>Erythrosin B (eco-friendly option) | General cell counting needs |
| **LUNA-FL™** | Dual-fluorescence for accurate viability assessment | GFP analysis, stem cell research |
| **LUNA-FX7™** | High-throughput, bioprocessing, 21 CFR Part 11 compliance | GMP labs, large-scale cell culture |

***Table 1. Key features and recommended applications for four of Logos Biosystems' leading automated cell counters.***

and see how expanding cell counting capacity with the latest automated solutions can reduce budgets and improve workflows in labs of all shapes and sizes.

**A versatile cell counting solution for every lab**

Logos Biosystems' award-winning family of LUNA™ automated cell counters are grouped into three main categories – brightfield cell counters, fluorescence cell counters, and microbial cell counters. Each type carries features common to the whole family of instruments – such as a small benchtop footprint and advanced optics and software – as well as unique features tailored to more specific applications. For example, the brightfield cell counters feature an eco-friendly cell staining option and consistent, accurate results to increase lab efficiency for general counting needs, whilst the LUNA-FX7™ dual-fluorescence and brightfield cell counter includes advanced algorithms, higher throughput, precise autofocus, and software that is 21 CFR Part 11/GMP-compliant for bioprocess monitoring in large-scale cell culture.




To help you understand just what a difference automated cell counters can make to your lab in terms of accuracy and time-saving compared with manual counting using a hemocytometer and microscope, view this video.

**How to enhance accuracy and consistency in cell counting**

The LUNA™ family of automated cell counters can readily distinguish viable and non-viable cells from debris. They also feature de-clustering algorithms in their software that enable the counting of individual cells within clumps or clusters of cells. Accurate cell viability assessment is crucial to the success of downstream workflows, especially in bioprocessing, and automated cell counters eliminate both human error and the potential for variability between operating staff.

Achieving accurate and consistent cell counting using the LUNA™ family of instruments is facilitated by the following key features:

• **Validation slides** provide a superior reference material ensuring stability and consistency in cell viability counting compared with more traditional validation beads. In the following application note,

discover how the ISO 20391 cell counting standard led to the development of validation slides, and learn how they allow for the verification of total cell count and viability using both brightfield and fluorescence optics of a cell counter.

• **Reusable slides** help avoid the expenses associated with disposable slides and eliminate errors in the use of haemocytometers, Logos Biosystems have produced the LUNA™ Reusable Slide L12008 specifically for use with the LUNA™ family of automated cell counters. In the following application note, understand how cell counting with reusable slides under both brightfield and fluorescence optics compared favourably with a hemocytometer and disposable slides.

• **CountWire™ software** is integrated with the LUNA™-FX7 automated fluorescence cell counter and makes provision for cell counting to be fully compliant with the FDA's 21 CFR Part 11. In the following whitepaper, learn how the software's features and functions satisfy the requirements of subparts B and C (electronic records and electronic signatures) of 21 CFR Part 11.

**Advanced cell counting in action**

Through the case studies highlighted below, discover how the most advanced instrument in the LUNA™ family of cell counters – the LUNA-FX7™ – can be harnessed in advanced research at the cutting-edge of next-generation sequencing and cell bioprocessing.

• **Single-cell RNA-seq.** All single-cell sequencing protocols depend upon accurately quantifying cells before processing, and accurate cell counts are therefore a vital first step. Quantifying single-cell sequencing samples requires counting of all sample types including dissociated tissues, separated nuclei, whole blood and cultured cell lines. In the following application note, discover how the LUNA-FX7™ not only provides accurate counts for many diverse sample types, but also allows for verification of sample quality.

• **Nuclei quality in single cell genomics.** The rapidly evolving field of single cell genomics research requires the precise assessment of nuclei quality to maximize the probability of successful downstream workflows. In this application note, learn how the LUNA-FX7™ and LUNA-FL™ automated cell counters were used successfully to assess isolated nuclei from a range of different cell types that were stained with different combinations of four fluorescent dyes.

• **Bioprocess analysis.** The LUNA-FX7™ cell counter incorporates a bioprocess monitoring feature that can record and analyze an individual batch of bioprocessing activities. Cell counting in this mode automatically generates growth rates, cell doubling time and trend charts. In the following application note, understand how the bioprocess mode was used to monitor the cell growth of three suspension cell lines using all three different counting modes available on the instrument. Learn how this enabled reliable forecasting of bioprocess production timelines.

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Application Note

automated cell counters, cell counting accuracy, cell counter validation slides, ISO 20391 standard, cell counting reference materials, cell counting techniques, LUNA cell counter series, cell counting validation, cell viability assessment

# How to Validate Automated Cell Counters: Benefits of Using Validation Slides over NIST Standard Beads

Automated cell counters (https://logosbio.com/compare-cell-counters/) have gained popularity thanks to their ability to provide fast and precise cell counting results. Regardless of these benefits, it is essential to validate the accuracy to ensure reliable cell counting results. Beads are frequently used for the validating devices such as flow cytometers and automated cell counters.

However, there are various issues associated with using beads, which led to the development of Cell Counter Validation Slides (https://logosbio.com/cell-counting-consumables/?cell_counting_acc_category=validation) for validating the LUNA™ cell counter series. In this article, we will explore the cell counting standard and the advantages of utilizing Cell Counter Validation Slides for validating the LUNA™ cell counter series.

## International Cell Counting Standard

The ISO 20391 standard was developed to provide guidance on accurate cell counting and to ensure the reliability of cell counting results. The standard outlines a protocol for validating the quality of a cell counting measurement through serial dilution experiments, which can be used when reference materials are not available. However, this process can be time-consuming and complicated, requiring intricate calculations to determine the coefficient of determination and proportionality. To overcome this challenge, reference materials are recommended to ensure measurement traceability, enable comparison, and verify the measurement process. The most efficient approach is to compare the mean analytical result to a reference value obtained from certified reference materials, eliminating the need for complicated calculations. Nonetheless, choosing the appropriate reference materials can still present challenges.

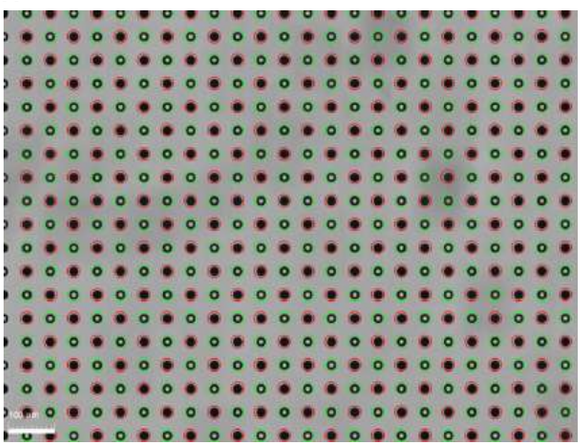
## Challenges with Using Validation Beads as a Reference Material

Validation beads, such as NIST (National Institute of Standards and Technology) beads, are commonly used as reference materials in various analytical methods involving particle size and counting. These beads have a known size and concentration and are frequently utilized as calibration standards for flow cytometry, microscopy, and other similar techniques. However, NIST beads are not available in fluorescent form making them unsuitable for testing viability measurements, although cell counters can provide both total cell count and viability measurements. While commercial fluorescent beads can serve as an alternative, errors during pipetting and evaporation during long-term storage may compromise their accuracy, requiring careful handling and repeated testing. Consequently, there is currently no commercially available reference material for viability assessment. Users who opt to produce their reference materials in-house would have to rely on manual counting, which is relatively imprecise and requires a large number of samples. Alternatively, flow cytometry could be used, although obtaining absolute count results can be challenging and this method is not user-friendly. Therefore, there is a need to find an alternative method for viability assessment.

## Using Validation Slides as a Reference Material

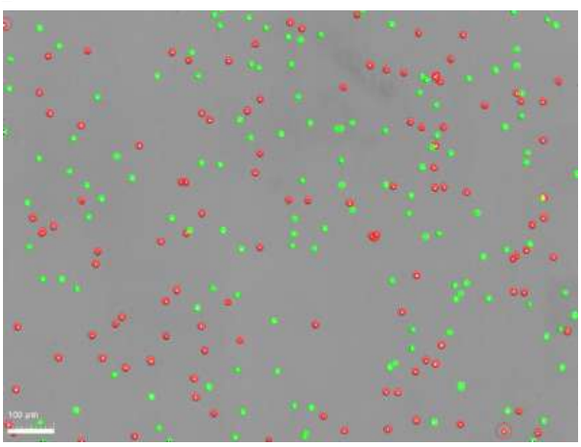
Using reference materials with stability and consistency is essential to ensure accurate results in cell counting. One such option that provides this level of quality control is the use of validation slides. These slides contain pre-spotted patterns (https://logosbio.com/cell_counting_acc/cell-counter-validation-slide-bf-ii/) or pre-fixed fluorescent beads (https://logosbio.com/cell_counting_acc/cell-counter-validation-slide-fl/) with a known number of objects, eliminating issues such as concentration manipulation caused by sample loading, evaporation during storage, or uneven distribution. Furthermore, validation slides allow for the verification of total cell count and viability using both brightfield and fluorescence optics of a cell counter. Cross-validation with other equipment is also straightforward, and their stability ensures that measurement values remain consistent even with long-term storage or repeated use. By incorporating validation slides, researchers can save time and resources that would otherwise be spent conducting additional experiments to ensure accuracy.

**A**



*Cell counter validation slide – BF II*

**B**



*Cell counter validation slide – FL*

## How to Assess the Accuracy Using the Cell Counter Validation Slides

The Cell Counter Validation slide protocol simplifies and streamlines the process of validating the accuracy of cell counters by providing a simple and efficient alternative to using beads. This method saves time and offers an easy approach to validate the accuracy of cell counting results.

To use the Cell Counter Validation slide protocol, follow these simple steps:

1. Check the cell concentration and viability values on the label of the Cell Counter Validation slide.
2. Insert the slide into the cell counting device.
3. Start counting using the 'IQOQ' protocol.
4. Record the total cell concentration and viability values.

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*application note*

# Using the LUNA™ Reusable Slide for Accurate Cell Counting with Automated Cell Counters

## INTRODUCTION

Cell counting is an essential step in routine cell maintenance and for obtaining accurate and consistent experimental results. In recent years, automated cell counters have become more popular because affordable instruments have become increasingly available at competitive prices. However, many researchers still use the hemocytometer to avoid the expenses associated with the use of disposable slides. To overcome this problem, Logos Biosystems designed a reusable slide (LUNA™ Reusable Slide, L12008) to be used with the LUNA™ family of automated cell counters. Here, we compare the cell counting results of the LUNA™ Reusable Slide, a hemocytometer, and disposable slides.

## MATERIALS AND METHODS

**Comparing cell counting accuracy of the LUNA™ Reusable Slide and a hemocytometer**

To determine the accuracy of cell counts done with the LUNA™ Reusable Slide, cell counts were compared to those obtained manually with a hemocytometer. Cells were serially diluted and mixed with an equal volume of 0.4% trypan blue stain. 10 µL of the mixture was loaded into the LUNA™ Reusable Slide and a glass hemocytometer with Neubauer counting grids (Marienfeld). To obtain cell concentrations with the glass hemocytometer, the hemocytometer was imaged with the iRiS™ Digital Cell Imaging System and the cells within the four corner squares of the Neubauer counting grids were counted. Cell samples in the LUNA™ Reusable Slide were counted with the LUNA-II™ Automated Cell Counter. All experiments were performed in triplicate.

**Evaluating cell counting accuracy of the LUNA™ Reusable Slide with brightfield and fluorescence automated cell counters**

The performance of the LUNA™ Reusable Slide for brightfield and fluorescence cell counting was evaluated by using two stains, trypan blue and acridine orange/propidium iodide (AO/PI), respectively. For brightfield counting, cells mixed 1:1 with trypan blue were loaded into the LUNA™ Reusable Slide or disposable slides and counted with the LUNA™, LUNA-FL™, and LUNA-II™ automated cell counters. For fluorescence cell counting, 2 µL AO/PI was mixed with 18 µL cell suspension and 10 µL was loaded into the LUNA™ Reusable Slide or disposable slides and counted with the LUNA-FL™ Automated Fluorescence Cell Counter. All experiments were performed in triplicate.


## RESULTS

### Cell counting accuracy of the LUNA™ Reusable Slide

To determine the cell counting accuracy of the LUNA™ Reusable Slide, the total cell concentration results obtained with the LUNA™ Reusable Slide were compared to those obtained manually with the hemocytometer. The LUNA™ Reusable Slide results were highly correlated (R2 > 0.999) with the hemocytometer results at the tested concentrations (Fig. 1). The cell concentration data for the LUNA™ Reusable Slide were more consistent at higher concentrations (>2 x 106 cells/mL) than the hemocytometer. The fact that the LUNA-II™ counts a larger area within the LUNA™ Reusable Slide than was manually counted with the hemocytometer may account for the difference.

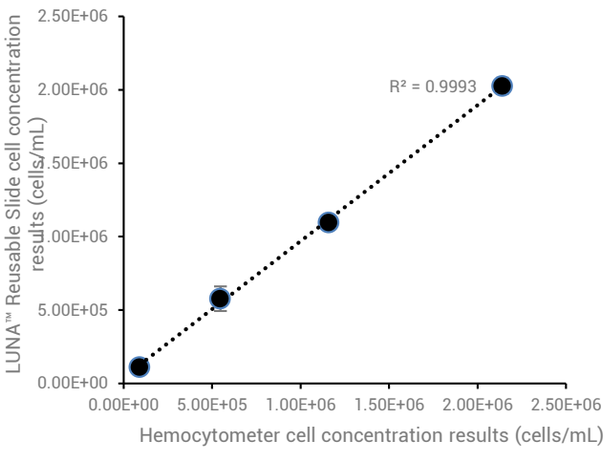



*Fig 1. Comparison of the cell count results of the LUNA™ Reusable Slide and a hemocytometer.*

### Cell counting performance of the LUNA™ Reusable Slide for brightfield counting

To assess the compatibility of the LUNA™ Reusable Slide for automated brightfield counting, cells stained with trypan blue were loaded into the LUNA™ Reusable Slide and disposable slides and counted with the LUNA™, LUNA-II™. As shown in Fig. 2, there was no significant difference in the total, live, or dead cell concentrations among the slides or devices.

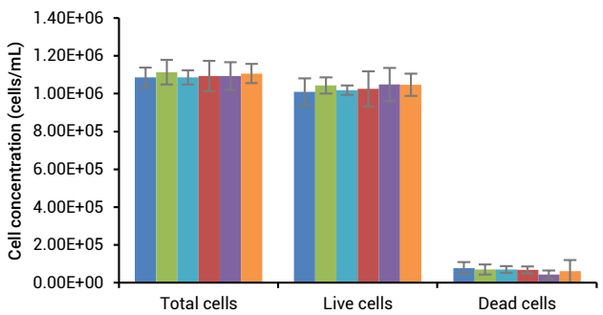
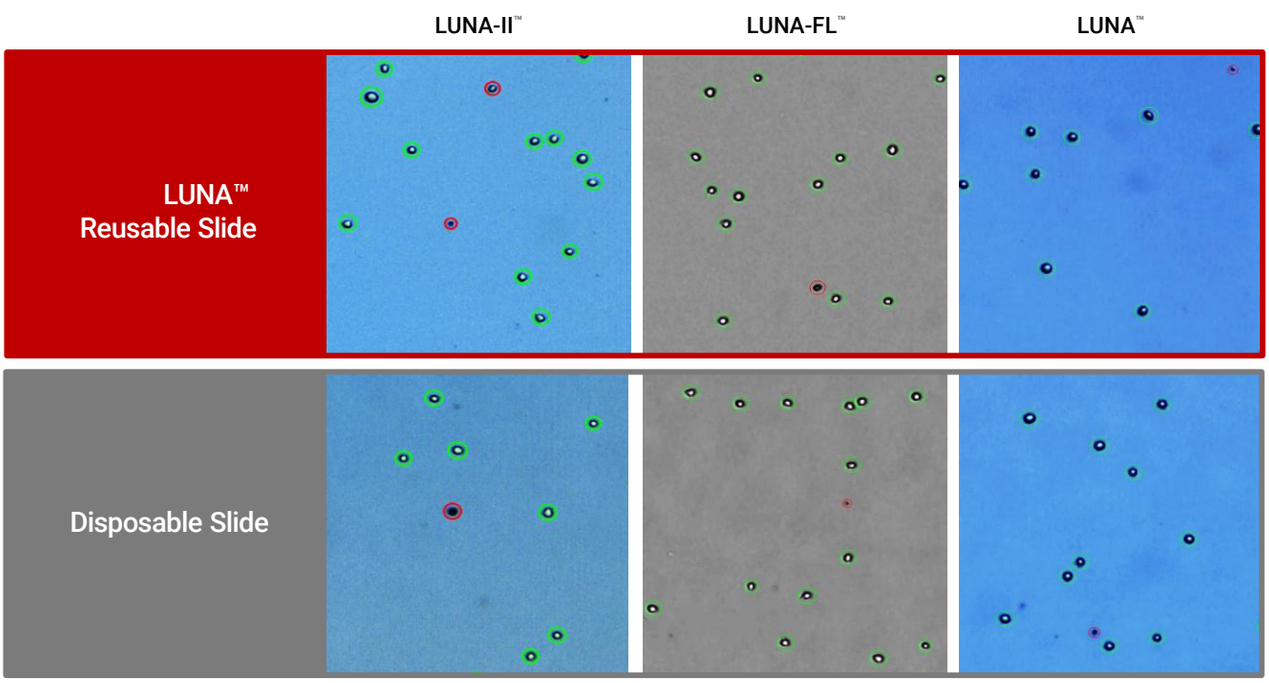



*Fig. 2. Compatibility of the LUNA™ Reusable Slide with automated cell counters by trypan blue staining. (Top) Cell images captured and tagged by the LUNA™ family of automated cell counters; (bottom) cell counting results using the LUNA™ Reusable Slide and disposable slides.*



## RESULTS

**Cell counting performance of the LUNA™ Reusable Slide for fluorescence staining**

To assess the compatibility of the LUNA™ Reusable Slide for automated fluorescence counting, cells stained with AO/PI were counted with the LUNA-FL™. There were no significant differences in any of the parameters (Fig. 3).

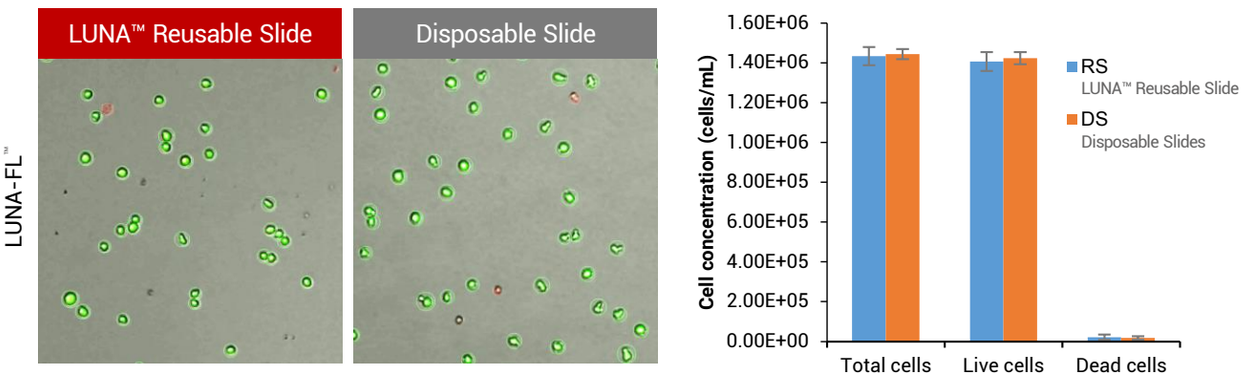



*Fig. 3. Compatibility of the LUNA™ Reusable Slide with automated cell counters by AO/PI staining. (Top) Cell images captured by the LUNA-FL™; (bottom) cell counting results using the LUNA™ Reusable Slide and disposable slides.*

## CONCLUSIONS

Automated cell counting with the LUNA™ Reusable Slide is a good alternative to the hemocytometer for the following re asons:

- No learning curve – similar to using a hemocytometer
- Reusable and cost-effective
- Accurate cell counting results
- Removal of user variability and time consuming calculations
- Compatible with autofocused automated counting (with the LUNA-II™ and LUNA-II YF™)

## REFERENCES

Tucker KG, Chalder S, Al-Rubeai M, Thomas CR. Measurement of hybridoma cell number, viability, and morphology using fully automated image analysis. Enzyme Microb Technol. 16:29–35. (1994)

## • Ordering Information

| | | | |
|---|---|---|---|
| **Automated Cell Counters** | | | |
| Brightfield | L10001 | LUNA™ Automated Cell Counter | 1 unit |
| | L40001 | LUNA-II™ Automated Cell Counter (with built-in printer) | 1 unit |
| | L40002 | LUNA-II™ Automated Cell Counter (without printer) | 1 unit |
| Fluorescence | L20001 | LUNA-FL™ Automated Fluorescence Cell Counter | 1 unit |
| | L30001 | LUNA-STEM™ Automated Fluorescence Cell Counter | 1 unit |
| | L50001 | LUNA-II YF™ Automated Yeast Cell Counter | 1 unit |
| **Slides** | | | |
| Reusable | L12008 | LUNA™ Reusable Slide | 1 unit |
| | L12010 | LUNA™ Reusable Slide Coverslips | 10 units |
| Disposable | L12001 | LUNA™ Cell Counting Slides, 50 Slides | 1 box |
| | L12002 | LUNA™ Cell Counting Slides, 500 Slides | 10 boxes |
| | L12003 | LUNA™ Cell Counting Slides, 1000 Slides | 20 boxes |
| | L12005 | PhotonSlide™, 50 Slides | 1 box |
| | L12006 | PhotonSlide™, 500 Slides | 10 boxes |
| | L12007 | PhotonSlide™, 1000 Slides | 20 boxes |
| **Stains** | | | |
| Brightfield | T13001 | Trypan Blue Stain, 0.4% | 2 x 1 mL |
| | L13002 | Erythrosin B Stain | 2 x 1 mL |
| Fluorescence | F23001 | Acridine Orange/Propidium Iodide Stain | 2 x 0.5 mL |
| | F23002 | Acridine Orange Stain | 2 x 0.5 mL |
| | F23003 | Propidium Iodide Stain | 2 x 0.5 mL |
| **Beads** | | | |
| Brightfield | B13101 | LUNA™ Standard Beads | 2 x 1 mL |
| Fluorescence | F23102 | LUNA™ Fluorescence Calibration Beads | 1 x 0.5 mL |

WHITE PAPER

# Complete Compliance with 21 CFR Part 11

## CountWire™ System for the LUNA-FX7™


With advancing technology and the subsequent increase in electronic record keeping, the demand for electronic data protection and security is more prevalent. To ensure the accuracy, reliability, authenticity, and consistency of electronic records the United States Food & Drug Administration established Title 21 also known as 21 CFR Part 11, to regulate electronic records and electronic signatures.
In general, Part 11 applies, with some specific exceptions, to drug producers, medical device manufacturers, biotech companies, developers of biologics, CROs and other industries controlled by the FDA. Regulated institutions are required to carry out controls, audits, system validations, audit trails, electronic signatures, and documentation for software and systems involved in generating, processing, and storing electronic data.

The 21 CFR Part 11 consists of 3 subparts, General Provisions, Electronic Records, and Electronic Signature. Because the General Provisions subpart includes explanations related to scope, implementation, and definitions; we will focus mainly on the details of subpart B and in this document.

| **SUBPART A**<br>**General Provisions** | **SUBPART B**<br>**Electronic Records** | **SUBPART C**<br>**Electronic Signatures** |
|---|---|---|
| The scope of the regulations, where and how the regulations should be applied, and provides definitions for key terms used in the regulations. | Requirements for controls of closed and open electronic record-keeping systems, signature manifestations and requirements for signatures and records to be linked. | Three parts:<br>1) general requirements for electronic signatures,<br>2) electronic signature components and controls, and 3) controls for identification codes/passwords. |
| 11.1 Scope<br>11.2 Implementation<br>11.3 Definitions | 11.10 Controls for closed systems<br>11.30 Controls for open systems<br>11.50 Signature Manifestations<br>11.70 Signature record/linking | 11.100 General Requirements<br>11.200 Electronic signature components and controls<br>11.300 Controls for Identification codes/passwords |

# CountWire™ System: 21 CFR Part 11 Ready

*CountWire™ System Components: Control – Store - Create*


CountWire™ Client in PC

CountWire™ Data Storage

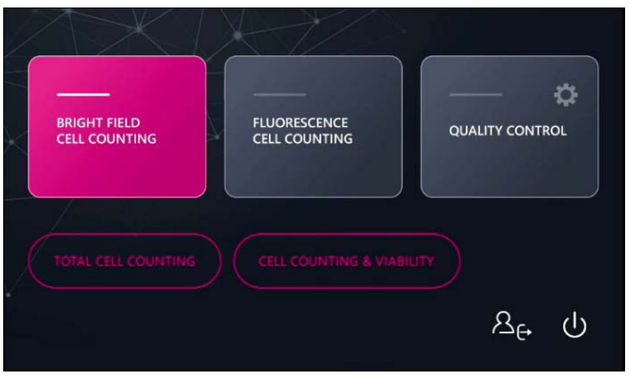
LUNA-FX7™

The CountWire™ System consists of the CountWire™ Client software installed in PCs, the CountWire™ Data Storage, and the LUNA-FX7™ Automated Cell Counter, connected and controlled under the same network.
The CountWire™ System allows multiple users to remotely access and manage data from multiple LUNA-FX7™ units connected to the network. Working through a PC connected to a network, authorized users may access data from one or more devices, no matter where they may be located. Further, users may approve counting reports on the LUNA-FX7™, without having to move to a PC.
The CountWire™ System completely supports 21 CFR Part 11 compliance, enabling the LUNA-FX7™ Automated Cell Counter to be used in a regulated process. This document provides guidelines on how the CountWire™ System is compliant with 21 CFR Part 11 regulations when it is used as a closed system.

## User Management & Access Control

The CountWire™ System establishes two user groups, Administrators and Users with different levels of access and privilege. Within Users, there are further access levels: Creator, Reviewer, and Approver. Users may be organized into groups based upon accessibility and approval lines.
The CountWire™ System's user management provides a high degree of flexibility in establishing user roles.
Only authorized administrators and users may access the system with a user ID and password. Administrators, among other roles, establish and manage password related settings and functions such as minimum length, valid period, account locks and invalid login attempt lockouts. Administrators also are responsible for establishing user and group levels that control accessibility to data.
Data may only be created after an authorized user logs into the system. Upon successful login, the LUNA-FX7™ switches to Security mode. When operating under Security mode all actions are recorded within the audit trail, time stamped, tagged to user, identified with the instrument name and serial number. Further, only data and counting reports created under Security mode may be forwarded for report approval.





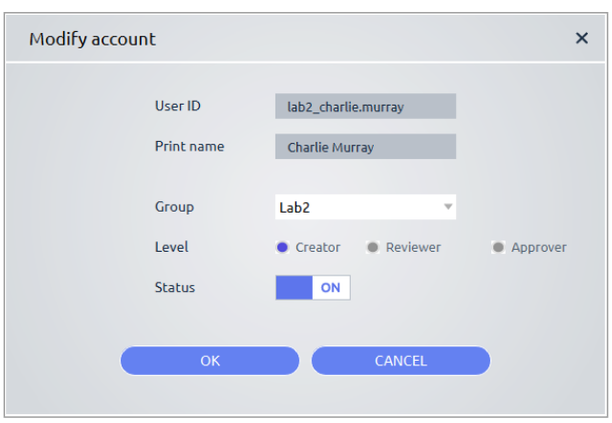

**Administrator** | **Users**

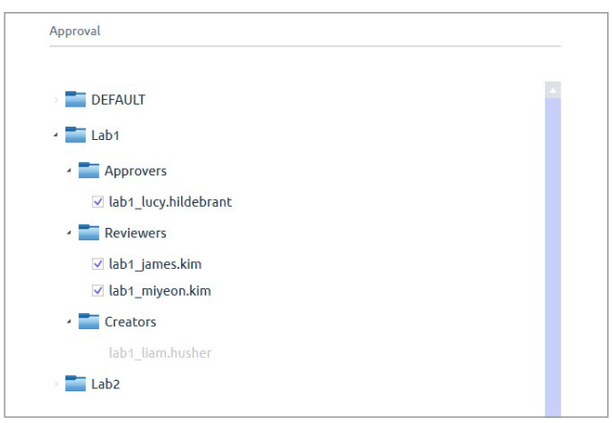
Approver

Reviewer

Creator

| Role/Level | | What they do |
|---|---|---|
| Administrator | | Responsible for system settings, management, and accessibility. |
| User | Approver | Responsible for final approval of counting reports. Allowed to perform reviewer and creator roles. |
| | Reviewer | Responsible for reviewing and approving counting reports. Allowed to perform creator role. |
| | Creator | Responsible for creating counting reports and approving the reports they create. |



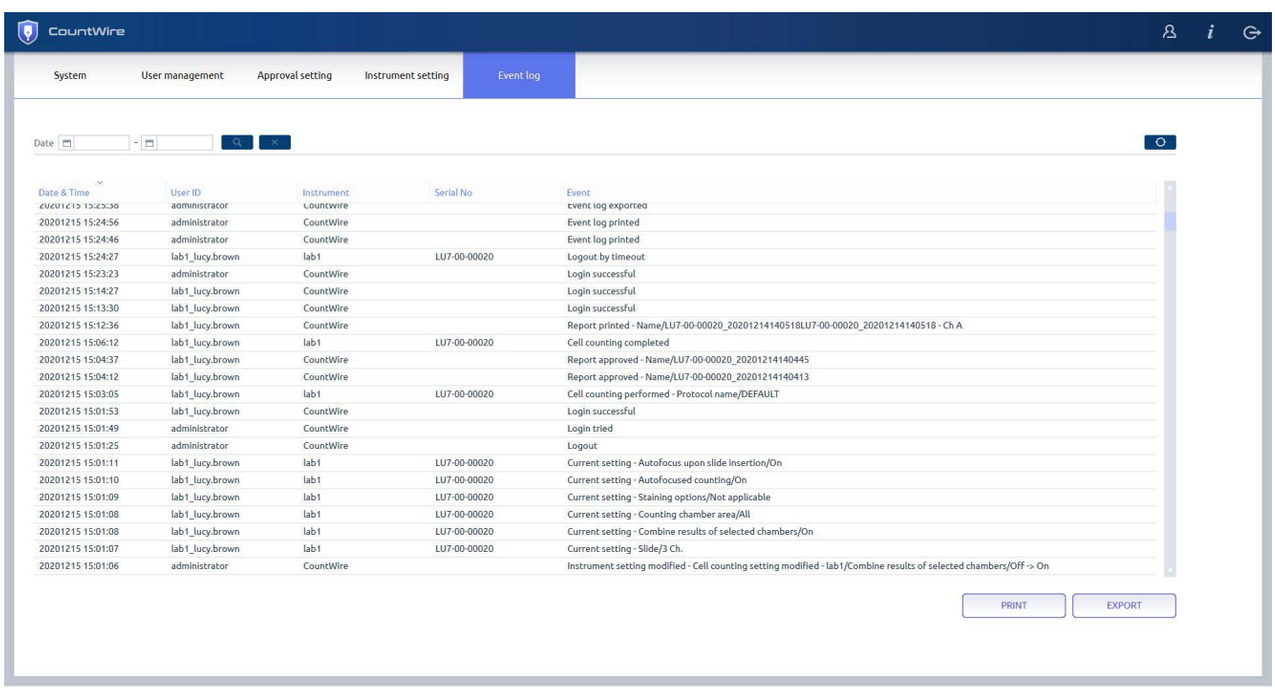



## Electronic Signature

All reports may be digitally signed. The electronic signature information includes the name and User ID of the signer, the date and time of signature execution and meaning (e.g. review or approval) of the signature.
Saves and approvals of the counting data require User ID and password. Each user ID and password must be unique and only one active user ID may be assigned to each person. All signatures are linked to the related electronic records and cannot be removed any record.


## Audit Trail

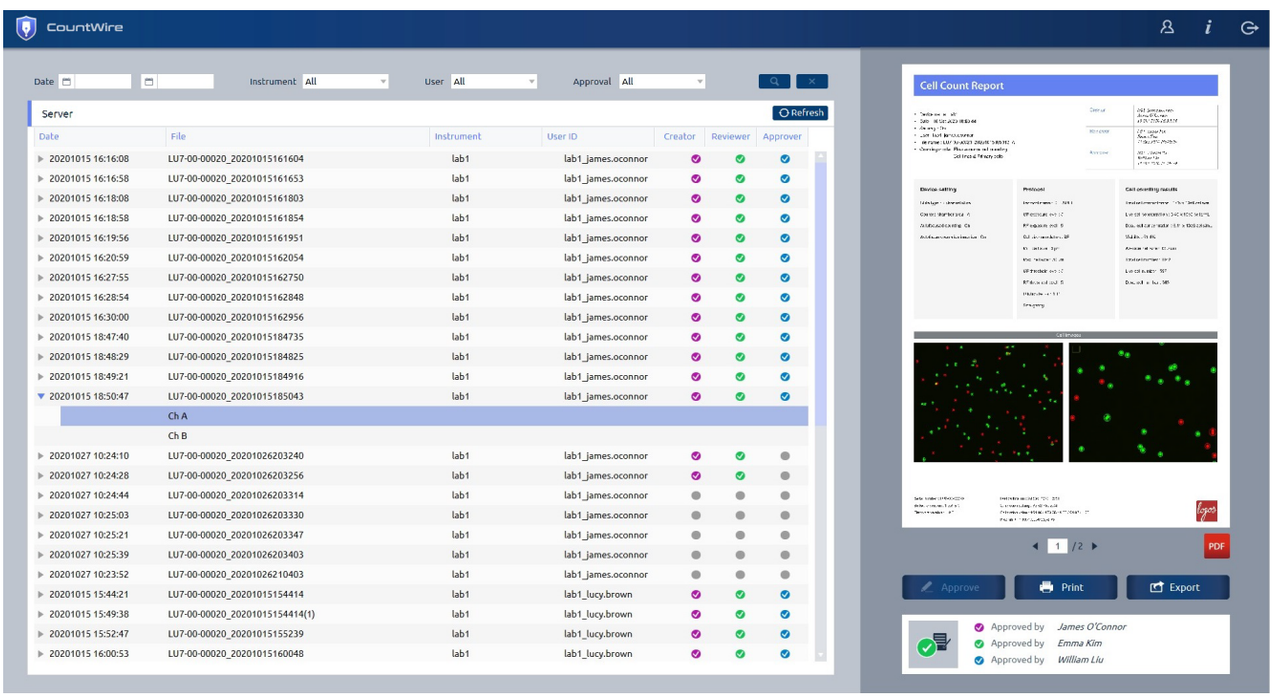
All actions and changes are retained in the event log. The event log includes, the Date & Time, User ID, Instrument name, Instrument serial number, and actions taken while logged in. New audit trail values are recorded in addition to old values; and audit trails may not be modified, deleted, or deactivated. Audit trails may be exported or printed for the review.



## Data Safety and Security

The CountWire™ System reliably protects all from deletion, overwriting, alteration, and/or possible accidents.
The CountWire™ Data Storage capacity is 4 TB with a mirrored 4 TB of storage to protect the data in the event of physical damage to the system.
Deletion or overwriting of data is not allowed in the CountWire™ System. The authenticity of the approved reports is confirmed and displayed by icons.
The authenticity of a report is represented by color coded icons.

| | |
|---|---|
|  | The original report is in good condition. |
|  | The original report may be damaged or forged. |




# Meeting the Regulatory Requirements of 21 CFR Part 11 with the CountWire™ System

The table below explains how the features and functions of the CountWire™ System connected with the LUNA-FX7™ satisfies the requirements of 21 CFR Part 11.

*Important ! Please note that the descriptions and explanations we provide represent our interpretations of the 21 CFR Part 11 regulations as the product provider, not representing any government agency.*

| Part and Description | Comment |
|---|---|
| **Subpart B - Electronic Records** | |
| *Controls for Closed Systems* | |
| System Validation | |
| 11.10 (a)<br>Validation of systems to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records. | Invalid or altered records are recognized by the system by comparing them with the encrypted original records.<br>CountWire™ Client displays the authenticity of the report.<br>All records are read-only and cannot be modified or removed by any user or administrator.<br>A validation guideline for the CountWire™ System is available. |
| Human Readable Records | |
| 11.10 (b)<br>The ability to generate accurate and complete copies of records in both human readable and electronic form suitable for inspection, review, and copying by the agency. Persons should contact the agency if there are any questions regarding the ability of the agency to perform such review and copying of the electronic records. | The CountWire™ Client software generates human-readable PDF reports that may be printed for inspection, off-line reviews, and duplication by the FDA.<br>Counting reports and audit trails may be exported in PDF format through the CountWire™ Client to a user's PC.<br>Reports may be printed from the PC. From the user's PC, the data may be transferred to USB flash drives or through the network. However, after any data has been exported, Logos Biosystems is not responsible for further data security. |
| Protection of Records | |
| 11.10 (c)<br>Protection of records to enable their accurate and ready retrieval throughout the records retention period. | All electronic records are backed up in the mirroring storage so that data may be recovered after a possible computer system failure.The CountWire™ Data Storage is up to 4 TB with an additional, mirrored 4 TBs storage.Data cannot be over written.<br>When a user attempts to save data under a previously used file name, the system automatically adds a suffix to the file's name to generate a unique name. |
| Limiting System Access | |
| 11.10 (d)<br>Limiting system access to authorized individuals. | The CountWire™ limits system access to authorized individuals. Users may access the system only with their own ID and password. The CountWire™ supports assigning 3 levels of users or user groups. A different level of privilege may be assigned to each user or group (Creators, Reviewers, or Approvers).<br>The administrator, at any time, may revoke or reassign the privilege of users and groups.The system automatically logs out users after a period of inactivity. An administrator is able to set the inactivity period (5-60 min). |

| | |
|---|---|
| | The CountWire™ allows password requirements to be established by an administrator that include minimum length (8-20 characters), password change cycle (1-12 months) and lockout after invalid log-in attempts (3-10 times/day). Previously used passwords are not allowed to be used by the same user.Only the administrator may unlock an account. All login attempts, successful or not, are recorded in the system event log. |
| **Audit Trails** | |
| 11.10 (e)<br>Use of secure, computer-generated, time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records.<br>Record changes shall not obscure previously recorded information.<br>Such audit trail documentation shall be retained for a period at least as long as that required for the subject electronic records and shall be available for agency review and copying. | The system automatically records and generates audit trails of all user actions that include the date & time, user ID, instrument name, and the instrument's serial number.<br>New audit trail values are recorded in addition to old values.<br>Audit trails are backed up to the mirror storage and not allowed to be deleted. Audit trails may be exported or printed for review.<br>Audit Trails cannot be modified, deleted, or deactivated. |
| **Operational System Checks** | |
| 11.10 (f)<br>Use of operational system checks to enforce permitted sequencing of steps and events, as appropriate. | The system enforces the sequence of data generation.<br>The CountWire™ restricts user access to specific protocols and counting settings (e.g. slide selection). Users are only allowed to perform the steps their level of privilege grants.All events within the system are ordered and time-stamped within the audit trail. |
| **Authority Checks** | |
| 11.10 (g)<br>Use of authority checks to ensure that only authorized individuals can use the system, electronically sign a record, access the operation or computer system input or output device, alter a record, or perform the operation at hand. | Accessing the CountWire™ software requires a unique user ID and PW in addition to the login information required to access the computer system. The system allows different access control level for the different user levels: creator, reviewer, and approver.<br>A user may only access the system with a valid user ID and password. Users are required to log-in after every inactivity or user-initiated logout. Any changes and modifications to the system by a user are recorded and assigned to their user ID. |
| **Device Checks** | |
| 11.10 (h)<br>Use of device (e.g., terminal) checks to determine, as appropriate, the validity of the source of data input or operational instruction. | The system checks the validity of the data source. By recognizing instrument names, serial numbers, and IP addresses through proprietary binary communications. |
| **Training** | |
| 11.10 (i)<br>Determination that persons who develop, maintain, or use electronic record/electronic signature systems have the education, training, and experience to perform their assigned tasks. | The organization using the system is responsible for ensuring that users and administrators have the education, training and experience required to perform their tasks.<br>Documents and training are provided only by Logos Biosystems.<br>External maintenance services may only be provided by Logos Biosystems. |

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| Policies for Signatures | |
| 11.10 (j)<br>The establishment of, and adherence to, written policies that hold individuals accountable and responsible for actions initiated under their electronic signatures, in order to deter record and signature falsification. | The organization is responsible for developing written policies that ensure the individuals are accountable and responsible for actions initiated under their electronic signatures. |
| System Documentation | |
| 11.10 (k) (1)<br>Adequate controls over the distribution of, access to, and use of documentation for system operation and maintenance. | Documentation such as the user manual and software update notices are available for the users and administrators. However, controls over the storage and distribution of any documentation are the organization's responsibility. |
| 11.10 (k) (2)<br>Revision and change control procedures to maintain an audit trail that documents time-sequenced development and modification of systems documentation. | Logos Biosystems follows revision and change control procedures and life cycle management procedures for document control. The CountWire™ Client software may be updated by the administrator or users. Only the administrator may update the CountWire™ Data Storage program. All data will remain intact with no loss of data security or traceability after any update.<br>Only creation of reports is allowed. Reports cannot be modified or deleted. All report creations are time-stamped and logged as part of the audit trail. |
| *Controls for Open Systems* | |
| 11.30<br>Persons who use open systems to create, modify, maintain, or transmit electronic records shall employ procedures and controls designed to ensure the authenticity, integrity, and, as appropriate, the confidentiality of electronic records from the point of their creation to the point of their receipt.<br>Such procedures and controls shall include those identified in 11.10, as appropriate, and additional measures such as document encryption and use of appropriate digital signature standards to ensure, as necessary under the circumstances, record authenticity, integrity, and confidentiality. | Logos Biosystems encourages the use of the system as a closed system. If used as an open system, the organization is responsible for ensuring the authenticity, integrity, and, if appropriate, the confidentiality of the system's electronic records. |
| *Signature Manifestations* | |
| Content of a Digital Signature | |
| 11.50 (a)<br>Signed electronic records shall contain information associated with the signing that clearly indicates all of the following<br>(1) The printed name of the signer;<br>(2) The date and time when the signature was executed;<br>(3) The meaning (such as review, approval, responsibility, or authorship) associated with the signature. | The CountWire™'s digital signatures contain<br>1) the printed name of the signer;<br>2) the date and time when the signature was executed;<br>and 3) the meaning (such as creator, reviewer, approver, responsibility, or authorship) of the signature.<br>The CountWire™ allows two roles: an administrator and users. For users there three levels: creator, reviewer, and approver. Only the system administrator can enable and modify the level of the user. |

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| Human Readable Form | |
| 11.50 (b)<br>The items identified in paragraphs (a)(1), (a)(2), and (a)(3) of this section shall be subject to the same controls as for electronic records and shall be included as part of any human readable form of the electronic record (such as electronic display or printout). | All items in the signatures, user ID, printed name, date and time, and meaning are included in the human-readable form, PDF files. |
| *Signature/Record Linking* | |
| 11.70<br>Electronic signatures and handwritten signatures executed to electronic records shall be linked to their respective electronic records to ensure that the signatures cannot be excised, copied, or otherwise transferred to falsify an electronic record by ordinary means. | Signatures and approvals require user ID and password.<br>Signatures cannot be excised, copied, or transferred.<br>All electronic signatures are linked to the respective electronic records. Electronic signatures are embedded in the document and encrypted. |
| **Subpart C - Electronic Signatures** | |
| *General Requirements* | |
| Uniqueness | |
| 11.100 (a)<br>Each electronic signature shall be unique to one individual and shall not be reused by, or reassigned to, anyone else. | Electronic signatures cannot be reused and should be executed for each new electronic record. The organization is responsible for verifying the identity of the individual executing the signature. |
| Verification of Identity | |
| 11.100 (b)<br>Before an organization establishes, assigns, certifies, or otherwise sanctions an individual's electronic signature, or any element of such electronic signature, the organization shall verify the identity of the individual. | Electronic signatures cannot be reused and should be executed for each new electronic record. The organization is responsible for verifying the identity of the individual executing the signature. |
| Certification of Equivalence | |
| 11.100 (c)<br>Persons using electronic signatures shall, prior to or at the time of such use, certify to the agency that the electronic signatures in their system, used on or after August 20, 1997, are intended to be the legally binding equivalent of traditional handwritten signatures. | The organization is responsible for certifying that digital signatures are intended to be legally finding equivalent of handwritten signatures. |
| 11.100 (c) (1)<br>The certification shall be submitted in paper form and signed with a traditional handwritten signature, to the Office of Regional Operations (HFC-100), 5600 Fishers Lane, Rockville, MD 20857. | The organization is responsible for certifying that digital signatures are intended to be legally finding equivalent of handwritten signatures. |
| 11.100 (c) (2)<br>Persons using electronic signatures shall, upon agency request, provide additional certification or testimony that a specific electronic signature is the legally binding equivalent of the signer's handwritten signature. | The organization is responsible for certifying that digital signatures are intended to be legally finding equivalent of handwritten signatures. |

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| *Electronic Signature Components and Controls* | |
| Signature with Biometrics or Code and Password | |
| 11.200 (a) (1)<br>Employ at least two distinct identification components such as an identification code and password. | CountWire™ employs two distinct identification components: a user ID and password. |
| 11.200 (a) (1) (i)<br>When an individual executes a series of signings during a single, continuous period of controlled system access, the first signing shall be executed using all electronic signature components; subsequent signings shall be executed using at least one electronic signature component that is only executable by, and designed to be used only by, the individual. | When an individual executes one or more signings during a continuous period of controlled system access, all electronic signature components must be executed at each signing. |
| 11.200 (a) (1) (ii)<br>When an individual executes one or more signings not performed during a single, continuous period of controlled system access, each signing shall be executed using all of the electronic signature components. | Each signature not performed during a single, continuous period of controlled system access requires all signature components. |
| 11.200 (a) (2)<br>Be used only by their genuine owners. | No two users may have the same user ID/password combination. The organization is responsible for ensuring that proper rules and documentation for executing an electronic signature are in place. |
| 11.200 (a) (3)<br>Be administered and executed to ensure that attempted use of an individual's electronic signature by anyone other than its genuine owner requires collaboration of two or more individuals. | The CountWire™ does not provide a proxy signature function. Only the account owner may modify the password and only the administrator can reset the password after proper verification of the user. The enforcement of this policy is the responsibility of the organization that operates the system. |
| Biometrics Ensure Genuine Owners | |
| 11.200 (b)<br>Electronic signatures based upon biometrics shall be designed to ensure that they cannot be used by anyone other than their genuine owners. | CountWire™ does not support biometric signatures. |
| *Controls for Identification Codes/Passwords* | |
| Uniqueness of Code/Password | |
| 11.300 (a)<br>Maintaining the uniqueness of each combined identification code and password, such that no two individuals have the same combination of identification code and password. | No two individuals may have the same combination of ID and password.<br>User IDs and passwords are required to be unique. |
| Periodical Check of Issuance (e.g. Password Aging) | |
| 11.300 (b)<br>Ensuring that identification code and password issuances are periodically checked, recalled, or revised. | Passwords will expire and need to be rest after an expiration period (1-12 months) that is set by the administrator. |

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| **Loss Management** | |
| 11.300 (c)<br>Following loss management procedures to electronically deauthorize lost, stolen, missing, or otherwise potentially compromised tokens, cards, and other devices that bear or generate identification code or password information, and to issue temporary or permanent replacements using suitable, rigorous controls. | Only the administrator may reset a password after proper verification of the individual if a user has lost or forgotten a password.<br>In the case of a forgotten user ID is forgotten, a new account should be generated. User IDs are not allowed to be removed or deleted. |
| **Safeguards and Detection of Unauthorized Attempts** | |
| 11.300 (d)<br>Use of transaction safeguards to prevent unauthorized use of passwords and/or identification codes, and to detect and report in an immediate and urgent manner any attempts at their unauthorized use to the system security unit, and, as appropriate, to organizational management. | Both components of the electronic signature, ID and password, are executed with each signing. All passwords are stored encrypted and cannot be accessed by any user or administrator. After an administrator-set number (3–10 times/day) consecutive unsuccessful login attempts, a user's account is locked, preventing access. The account remains locked until the administrator resets the password. |
| **Testing of Devices, Cards, etc.** | |
| 11.300 (e)<br>Initial and periodic testing of devices, such as tokens or cards, that bear or generate identification code or password information to ensure that they function properly and have not been altered in an unauthorized manner. | The CountWire™ does not support devices that bear or generate identification code or password information, such as tokens or cards. |
| **Additional Features and Capabilities which Increase Data Integrity** | |
| The system's administrative software, installed on a PC, may be connected to the network, allowing multiple devices to provide remote access and data management. | The system allows for CountWire™ Client to be installed on a computer, which is physically separated from the location of the LUNA-FX7™ connected over Ethernet or WiFi. This allows for multiple LUNA-FX7™ devices and the software installed PCs to be connected and managed. |
| After approvals are completed, the data is permanently locked to be read-only to prevent editing. | The system allows for counting reports to be permanently locked to be read-only after results have been approved and signed off. |
| Data backup and restore procedures to prevent data loss. | The system allows administrative users to perform a backup and restore data. The organization is responsible for establishing and implementing operating procedures to prevent data loss. |
| Data migration after the system version-up. | All data will remain intact with no loss of data security or traceability after system version updates. |
| The system must be programmed to automatically log out after an inactivity time set. | The system has an inactive period Auto logout function that is set by an administrator (5–60 min). |

logos biosystems

Application Note

Single-cell omics sequencing, single-cell isolation, accurate cell concentration, viability measurement, cell cluster graph, cell size histogram, analyzing intact nuclei

# Automated Cell Evaluation for Single-Cell RNA-seq Analysis

## INTRODUCTION

Single-cell sequencing allows researchers to characterize abnormal cell populations, discover and analyze rare cells, cellular map networks, and discover subtle, yet important, heterogeneities. Given the incredible potential, it is not a surprise that single-cell sequencing technology is seeing explosive growth and demand[1, 2]. Harnessing the power of single-cell sequencing, however, requires significant investment in cost and time. To ensure that the investment in time and resources is rewarded with quality data, the quality of the sample, prior to processing, is critical. Further, because all single-cell sequencing protocols rely on accurately quantifying cells before processing, accurate cell counts are a vital first step.

Therefore, an automated cell counter used to quantify single-cell sequencing samples must accurately and reliably count cells of all sample types, including dissociated tissues, separated nuclei, whole blood, and cultured cell lines. Here, we demonstrate how the LUNA-FX7™ Automated Cell Counter provides not only accurate counts for many diverse sample types but also just as importantly, verification of sample quality.

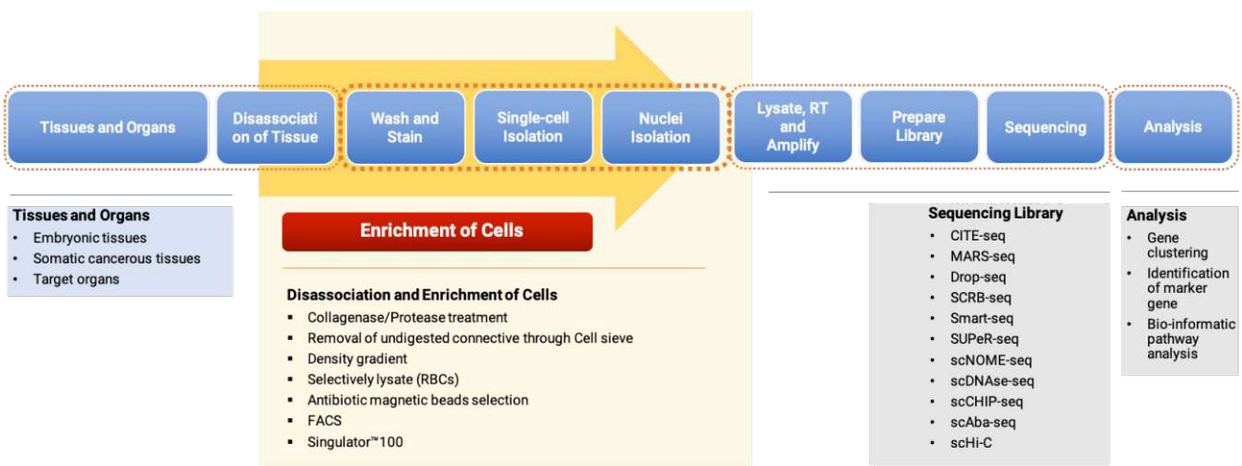



***Figure 1.*** Singe-cell sequencing workflow: 1) Tissue Collection, 2) Isolated Cell Preparation, 3) Sequencing Library Preparation, 4) Sequencing and 5) Analysis.

***IMPORTANT***

Generating isolated cells from whole tissues suitable for both counting and single-cell sequencing requires complete dissociation. Cell clusters can not only yield inaccurate counts but negatively influence sequencing results. The composition of the extracellular matrix (ECM) of each tissue type can vary; thus, the enzymatic and chemical composition of the dissociation methods must be optimized accordingly to ensure dissociated tissue to obtain isolated cells.

## Cell Sample Quality Assessment

Initially, two cell samples were collected for single-cell RNA-seq analysis. Both samples were counted using standard cell viability protocol with the Acridian Orange/ Propidium Iodide cell staining dye ( Cat# F23001). The first sample, B cells isolated from human peripheral blood, a high degree of viability and single-cell separation (Figure 2) suitable for downstream library preparation. By contrast, the second sample, adherent AsPC-1 cells, provides an example of how an accurate image-based counter is vital to assessing the quantity and quality of the sample preparation.

After harvesting, the AsPC-1 cells were subject to an incomplete dissociation. The resulting image clearly shows that a significant portion of the cell content is still incorporated into bound clusters as much as 9%, and the AsPC-1 viability was only 56%. Subject to the viability and cell separation, this sample is not suitable for downstream processing (Figure 3). In many cases, the sample cells for single cells are limited and require low cell concentrations and replications of count for the best and rapid cell counting. In the accurate count of low cell concentration, we use the LUNA™ 1-Channel Slide (Cat# L72011), which holds a larger cell volume to count even the cell concentration is low to 1.00E+04 cells/mL (Table 1).

## Analyzing Intact Nuclei

For several single-cell sequencing applications, isolated cells need to be further processed to generate separated and intact nuclei. We next evaluated the utility of the LUNA-FX7™ to assess the overall quality and quantity of the fully separated nuclei. For this, we used 3 cell lines - HL60, HeLa cells, and HEK cells. Figure 4 shows 3-panel images containing intact, healthy HL60 cells (left), HL60 cells with the nuclei partially isolated (middle), and the nucleic completely separated (right), and the live cells with intact cell membranes appear green fluorescence by Acridian Orange staining. In contrast, both dead cells and isolated nuclei turn red fluorescence by Propidium Iodide uptaken through broken or absent cell membranes. However, the LUNA-FX7™ allows you to distinguish intact dead cells vs. intact nuclei using the cell size information (Figure 4, Figure 6). For example, average cell size of intact live HL-60 was measured as 13.4 um, and the average size of the completely isolated nuclei was measured as 8.3 um, indicating cell membrane and plasma part was successfully removed. Incompletely isolated nuclei size was measured as 10.2 um in average indicating the quality of isolated nuclei is not good enough for downstream processing (Figure 4).

Also, high resolution brightfield images of the LUNA-FX7™ can be used to visually verify the quality of isolated nuclei. The isolated nuclei, for instance, can be easily distinguished from intact cells because the borders of nuclei are thinner and not evident due to the lack of the cell membrane. (Figure 5, and Figure 6).

In summary, the LUNA-FX7's capabilities and sensitivity in three-channel imaging (BF, GF, and RF), along with a sophisticated tagging algorithm, allow you to establish quality control criteria for sample pass or fail before committing expensive downstream processing.

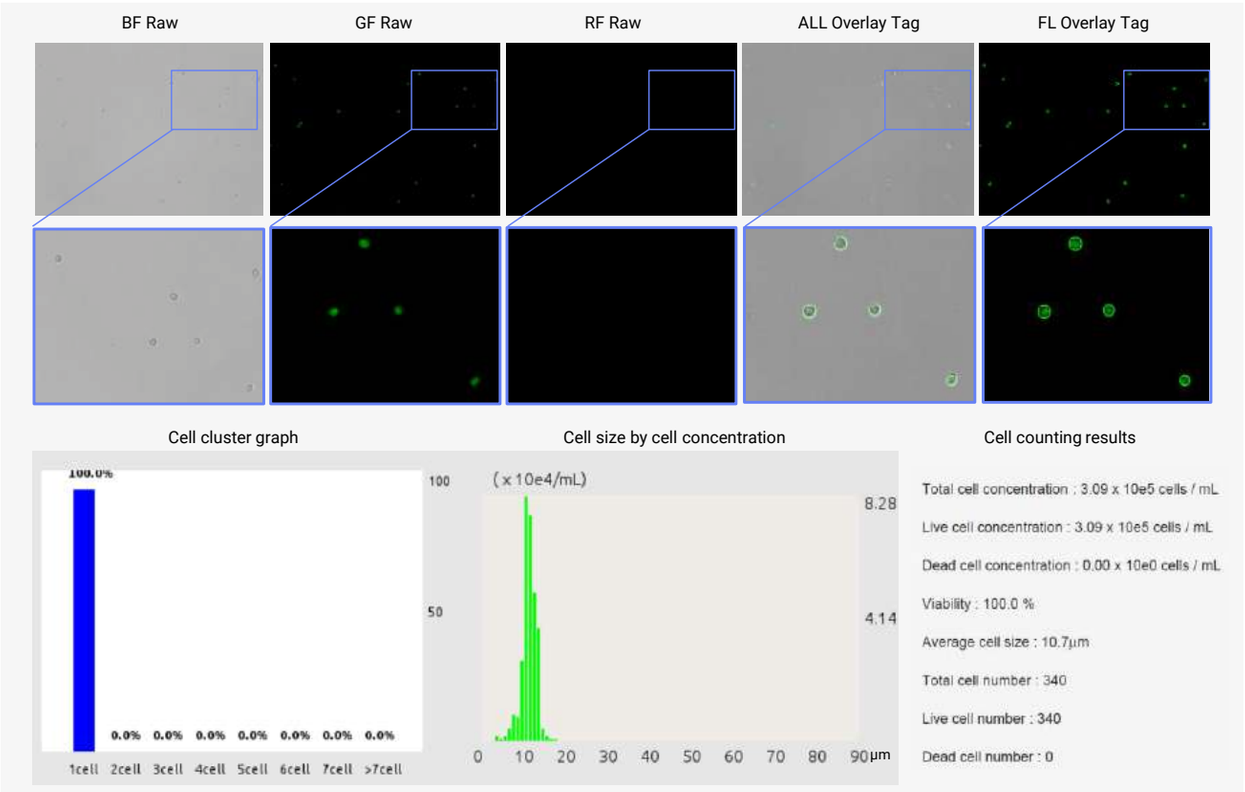



***Figure 2.*** *The B cells appeared well prepared for the single-cell library, including all positive green cells without dead cells (RF) or debris of particles (BF) with an excellent tag of 100% viability. These healthy cells demonstrated an average cell size of 10.7 µm, 100% single-cell separation in the cell cluster plot at a concentration of 3.00E+05 cells/mL as cell counting*

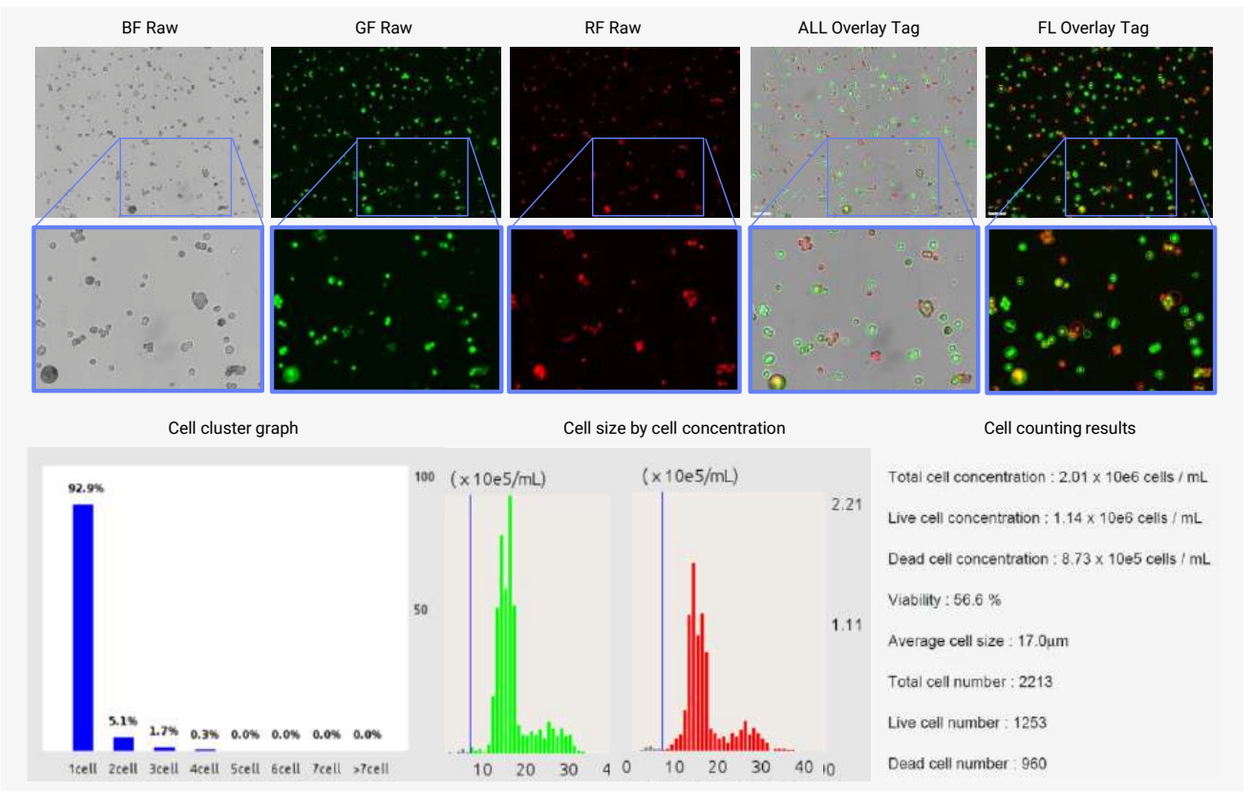
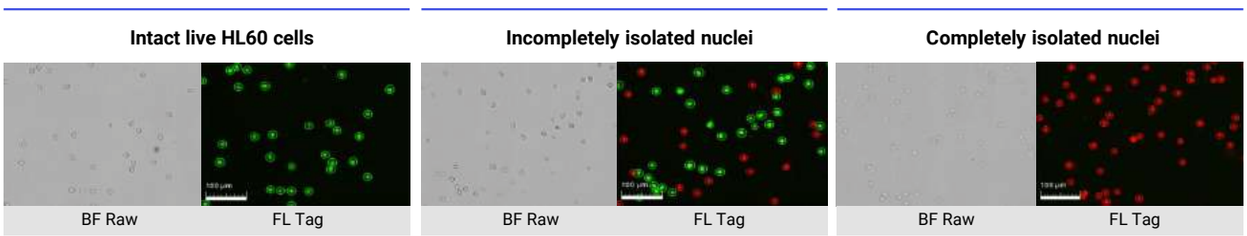



***Figure 3.*** *Example of inadequately prepped cell sample for single-cell sequencing. The AsPC-1 cells of inadequate cell profiles for single-cell sequences have been described. 'Failing' cell preps show both a high content of dead cells and contain incompletely dissociated clusters of cells. The cluster histogram feature of the LUNA-FX7™ allows the accurate assessment of cell cluster percentages vs. isolated cells.*




| Cell count | Intact live cells | Incompletely isolated | Completely isolated nuclei | Nuclei count |
|---|---|---|---|---|
| Total cells | 1.27 x $10^6$/mL | 2.81 x $10^6$/mL | 1.96 x $10^6$/mL | Total cells |
| Live cells | 1.16 x $10^6$/mL | 1.56 x $10^6$/mL | 0.00 x $10^0$/mL | Intact cells |
| Dead cells | 1.10 x $10^5$/mL | 1.24 x $10^5$/mL | 1.96 x 106/mL | Isolated nuclei |
| Viability | 91.3% | 55.07% | 0.00% | cell quota |
| Ave. cell size | 13.4 µm | 10.2 µm | 8.3 µm | Ave. object size |

***Figure 4.*** *Possible results from nuclei isolation processes of HL 60 cells. Brightfield (BF) images and overlaid fluorescence images with tag allow you to distinguish the different cell 'states easily'. Intact cells appear noticeably more prominent and darker than the isolated nuclei in BF images. At the same time, the fluorescent images and counts allow accurate quantitative assessment.*

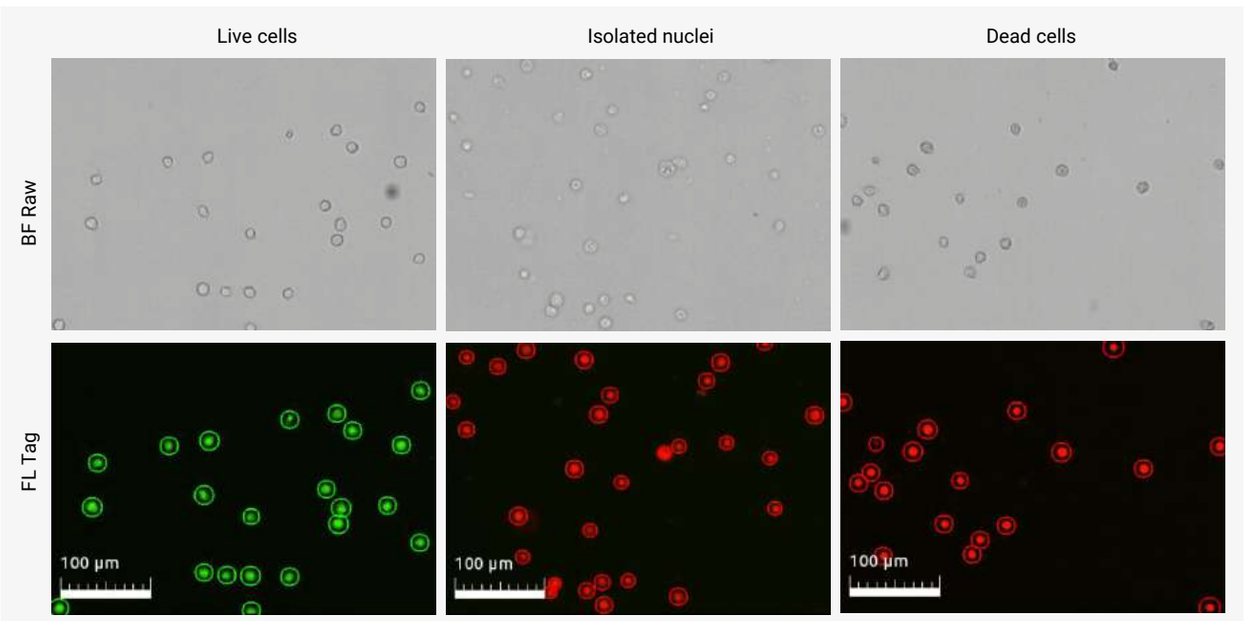
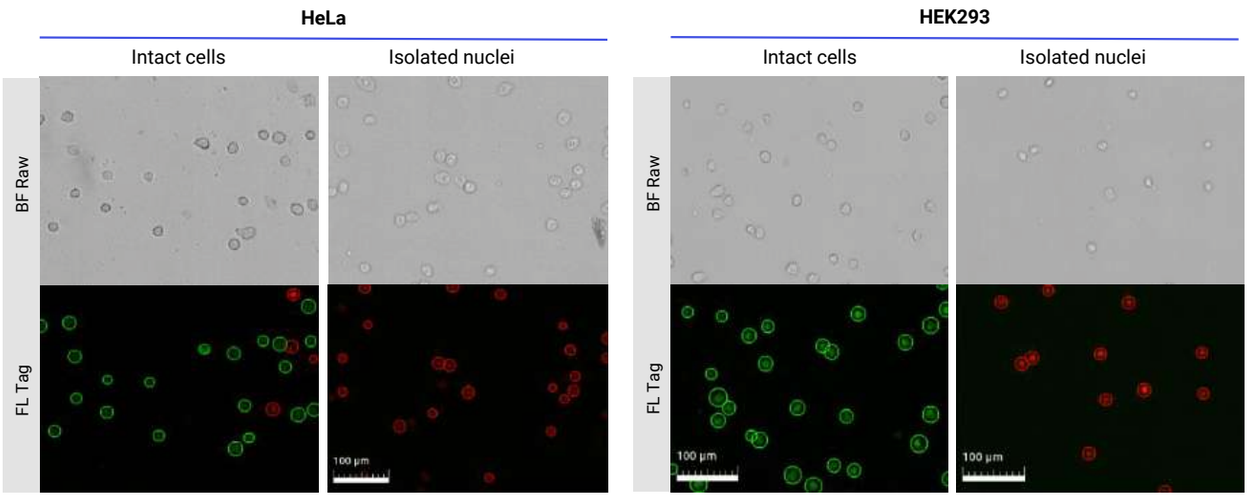


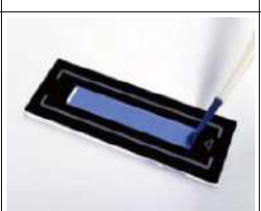

***Figure 5.*** *Differentiation between live, dead, and intact nuclei. Because both dead cells and intact nuclei lack an intact cell membrane, the red fluorescent would be perceived from propidium iodide staining. However, the BF images and size measurements generated by the LUNA-FX7™ enable the routinely distinguish between dead cells and intact, isolated nuclei.*




| | HeLa | | HEK293 | |
|---|---|---|---|---|
| | Intact cell | Isolated nuclei | Intact cell | Isolated nuclei |
| Total cells | 1.27 x $10^6$/mL | 1.80 x $10^6$/mL | 2.66 x $10^6$/mL | 3.91 x $10^5$/mL |
| Intact live cells | 1.16 x $10^6$/mL | 0.00 x $10^0$/mL | 2.56 x $10^6$/mL | 9.05 x $10^3$/mL |
| Isolated nuclei | 1.10 x $10^5$/mL | 1.80 x $10^6$/mL | 9.59 x $10^4$/mL | 3.82 x $10^5$/mL |
| Intact cell | 91.3% | 0.00% | 96.4% | 2.3% |
| Ave. object size | 13.4 µm | 6.4 µm | 13.3 µm | 11.7 µm |

***Figure 6.*** *Evaluation of isolated nuclei and possible results. Brightfield (BF) images and overlaid fluorescence images allow you to distinguish the different cell 'states easily'. Intact cells appear noticeably more prominent and darker borders than the isolated nuclei in BF images. At the same time, the fluorescent images and counts allow accurate quantitative assessment. Live intact cells are stained with acridine orange (GF), and separated nuclei are stained red. The LUNA-FX7™ can accurately discriminate from samples with large cytoplasm and small nuclei (HeLa) and cell types with large nuclei relative to the cytoplasm (HEK293).*

**Table 1. The counting slide options and their specifications.**

| Compatible slides | LUNA™ 1-Channel Slides | PhotonSlide™ | LUNA™ 3-Channel Slides | LUNA™ 8-Channel Slides |
|---|---|---|---|---|
| Sample throughput | 1 sample | Up to 2 samples | Up to 3 samples | Up to 8 samples |
| Sample loading volume | 50 µL | 10 µL/chamber | 10 µL/chamber | 10 µL/chamber |
| Analysis volume | 5.1 µL | 1.3 µL/chamber | 1.3 µL/chamber | 0.5 µL/chamber |
| Number of image fields | 47 fields | 12 fields/chamber | 12 fields/chamber | 5 fields/chamber |
| Counting concentration | 1.00E+04 - 1.00E+07/mL | 5.00E+04 - 1.00E+07/mL | 5.00E+04 - 1.00E+07/mL | 1.00E+05 - 1.00E+07/mL |
| Image |  |  |  |  |
| Specialty | Larger volume to effectively enumerate in the extreme cases of the low concentration of cells | Comfortable and familiar to use for cell counting, just like using a hemocytometer | Additional statistical report available from triple replicates of mean, SD and CV | Eight samples at once in a high-throughput manner Compatible with multichannel pipettes |

## CONCLUSION

For single-cell sequencing, the LUNA-FX7™ provides insight for researchers exploring to improve cell sample accuracy and reliability to assess both the quantity and quality of cells and isolated nuclei samples in a wide range of sample types.

## REFERENCES

[1] L., Xiong, F., Wang, Y., Zhang, S., Gong, Z., Li, X., . . . Guo, C. (2021). What are the applications of single-cell RNA sequencing in cancer research: a systematic review. *J Exp Clin Cancer Res*, 40(1), 163. doi:10.1186/s13046-021-01955-1

[2] Dong, X., Liu, C., & Dozmorov, M. (2021). Review of multi-omics data resources and integrative analysis for human brain disorders. *Brief Funct Genomics*. doi:10.1093/bfgp/elab024

Application Note

Automated cell counter, nuclei isolation, nuclei quality assessment, fluorescent nuclear staining, nuclei fixation

# Fluorescent Dyes and Automated Cell Counters: Towards Reliable Nuclei Quality in Single Cell Genomics

## Introduction

The accurate evaluation of nuclei quality is pivotal for single cell genomics research. Combining fluorescent dyes with imaging tools such as automated cell counters, provides an effective method to evaluate nuclei quality. With a myriad of fluorescent dyes available, choosing the optimal combination to assess nuclei quality is paramount to ensuring the reliability of results. Furthermore, given the advanced capabilities of modern automated cell counters like the LUNA-FX7™ and LUNA-FL™, there is an increasing necessity to understand how these systems perform when integrated with different fluorescent dyes. This study aims to identify the most effective dye combinations for nuclei quality assessment using different cell types, different systems, and nuclei fixation. Through our comprehensive analysis, we seek to offer insights that streamline and enhance the accuracy of nuclei assessment procedures in single cell genomics research settings.

## Materials and Methods

Four different fluorescent dyes, each with distinct final concentrations, were utilized in the study: Acridine Orange (AO) at 28 µM, Calcein AM at 12.5 µM, Propidium Iodide (PI) at 15 µM, and Ethidium Homodimer-1 (EthD-1) at 20 µM. These dyes were incorporated into different dye combinations, including AO/PI, AO/EthD-1, Calcein AM/PI, and Calcein AM/EthD-1. Each combination was prepared by adding 1 µL of each dye to create a total of 2 µL, which was then mixed with 18 µL of the cell sample.

## Optimal Fluorescent Dye Combinations for Nuclei Assessment

Experiments were conducted to identify optimal dye combinations for assessing the nuclei quality using different fluorescent dyes on U937 cells and 3T3 cells. Theoretically, an intact cell should be selectively stained only by membrane-permeable dyes like AO and Calcein AM. On the other hand, nuclei isolated after cell membrane removal should be stained by membrane-impermeable dyes such as PI and EthD-1, displaying viability similar to that of dead cells.

Among four different dye combitions, AO/PI, AO/EthD-1, Calcein AM/PI, and Calcein AM/EthD-1, the combination of AO with PI or AO with EthD-1 accurately displayed the viability of both intact cells and isolated nuclei (Figure 1A). The presence of green and red signals allowed for measuring viability in both U937 cells and 3T3 cells. However, we observed nearly no Calcein AM signal in 3T3 cells, while approximately two-thirds of U937 cells exhibited a Calcein AM signal (Figure 1B). This discrepancy can be attributed to Calcein AM's reliance on esterase activity, which may vary among different cell types. Consequently, the use of Calcein AM resulted in the omission of certain intact cells, compromising the reliability of the viability assessment.

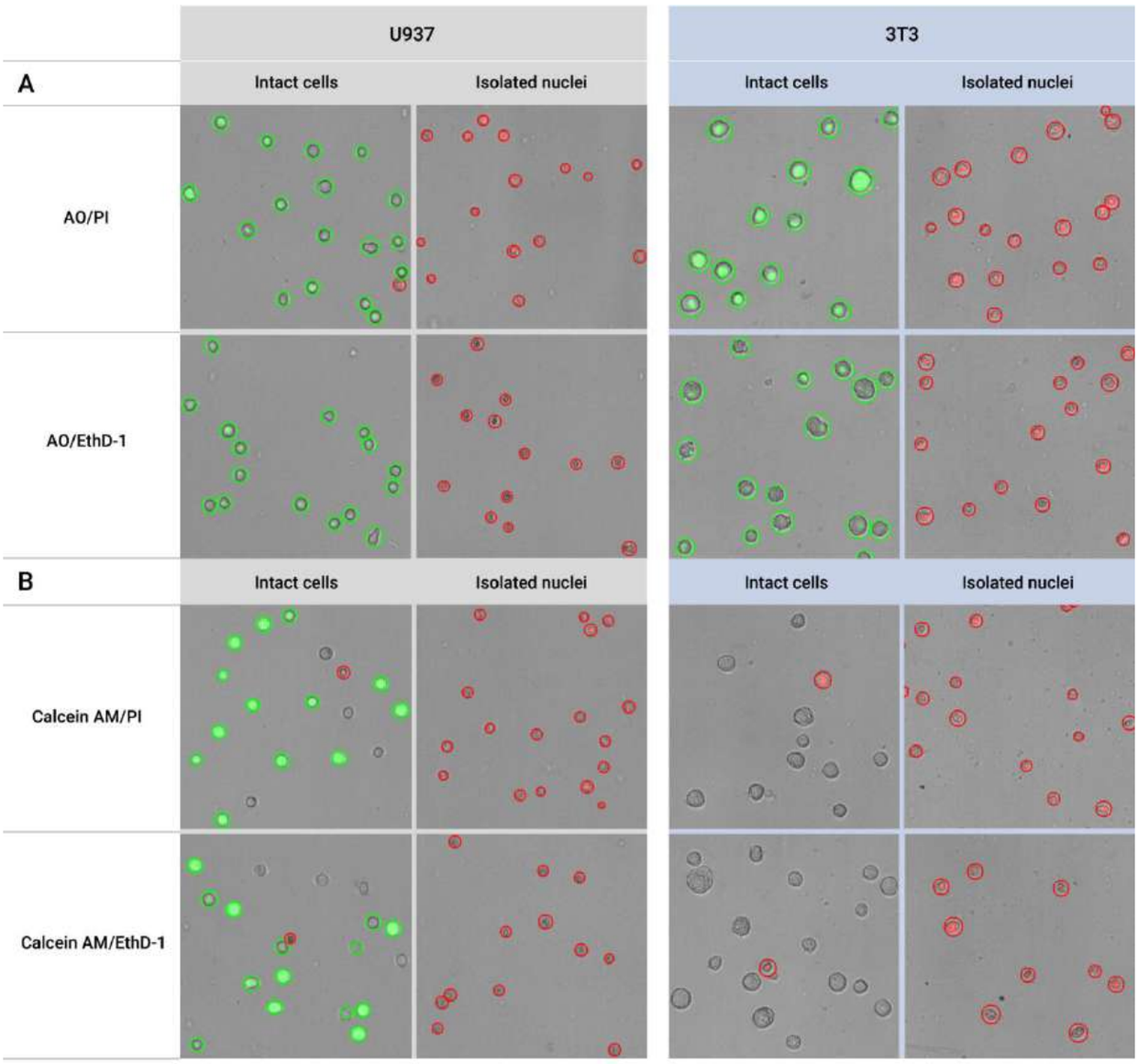



***Figure 1.*** *(A) Staining results of U937 cells and 3T3 cells using the combinations of AO with PI and AO with EthD-1. (B) Staining results of U937 cells and 3T3 cells using the combinations of Calcein AM with PI and Calcein AM with EthD-1.*

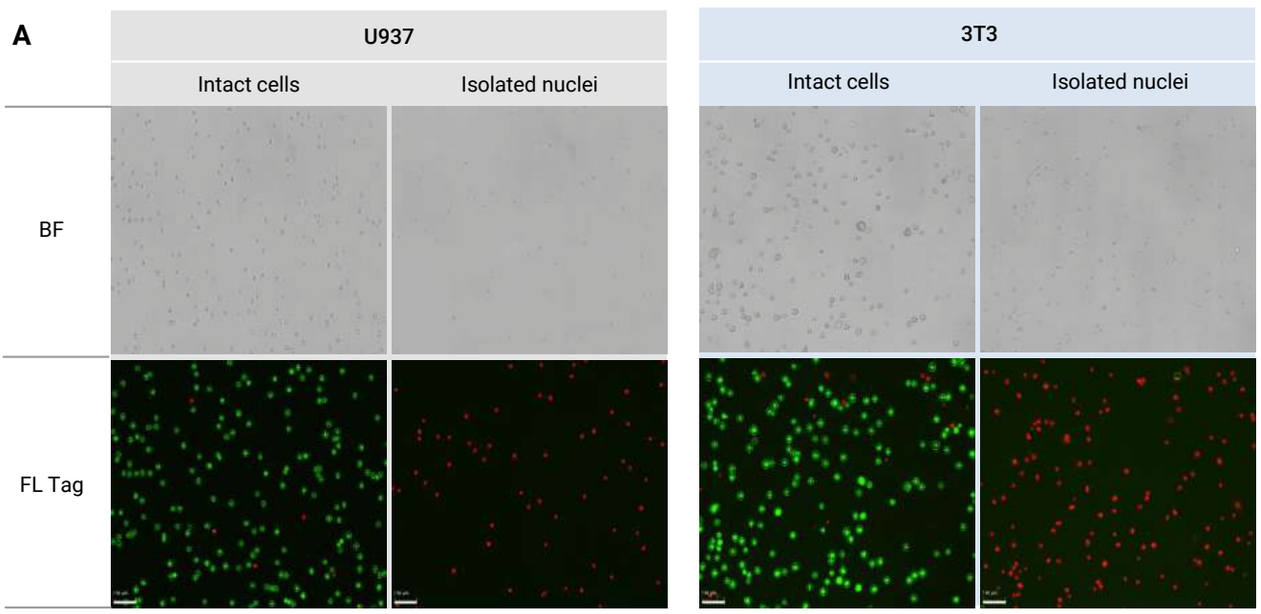
## Quantitative Nuclei Assessment

The LUNA-FX7™ and LUNA-FL™ systems are advanced platforms with robust capabilities for comprehensive analysis. These systems seamlessly capture both brightfield and fluorescent images that are then analyzed to obtain valuable data such as size, viability, and cell count using fluorescent signals.

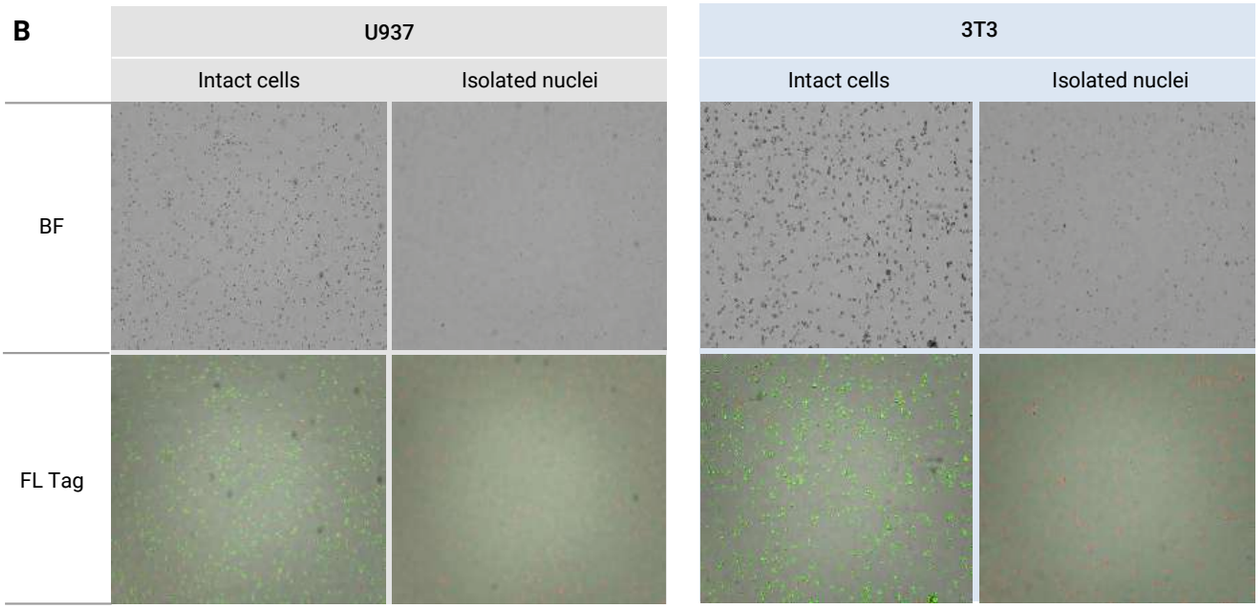
To evaluate the quality of isolated nuclei, one can utilize the average cell size data provided by these systems. For instance, nuclei from which the membrane has been removed are measured to be significantly smaller in size compared to intact cells. This is because the removal of the cell membrane and cytoplasm leads to a reduction in cell

size by several micrometers. In particular, the LUNA-FL™ and LUNA-FX7™ extract cell size information from the brightfield image. As a result, there's minimal distortion of cell size due to fluorescence intensity that might accompany calculations from fluorescent images. This ensures a more reliable cell size measurement when assessing the quality of isolated nuclei.

Another useful data for nuclei quality assessment is cell viability using the fluorescent dyes. As indicated in prior results in Figure 1, when combined with AO/PI or AO/EthD-1, healthy intact cells exhibit cell viability close to 100%. In contrast, well-separated nuclei display cell viability close to 0%. Notably, automated cell counters like LUNA FL™ and LUNA FX7™ enable accurate quantification of isolated nuclei cell concentration when stained with these dyes (Figure 2). These results provide valuable data, confirming the quality of nuclei for subsequent applications.




| | U937 | | 3T3 | |
|---|---|---|---|---|
| | Intact cells | Isolated nuclei | Intact cells | Isolated nuclei |
| Total cell concentration | $1.97*10^6$ cells/mL | $7.41*10^5$ cells/mL | $2.61*10^6$ cells/mL | $1.74*10^6$ cells/mL |
| Live cell concentration | $1.89*10^6$ cells/mL | $9.05*10^3$ cells/mL | $2.52*10^6$ cells/mL | $2.54*10^4$ cells/mL |
| Dead cell concentration | $7.69*10^4$ cells/mL | $7.32*10^5$ cells/mL | $9.04*10^5$ cells/mL | $1.71*10^6$ cells/mL |
| Viability | 96.1% | 1.4% | 96.5% | 1.4% |
| Average cell size | 12.5 µm | 5.6 µm | 17.8 µm | 7.2 µm |




| | U937 | | 3T3 | |
|---|---|---|---|---|
| | Intact cells | Isolated nuclei | Intact cells | Isolated nuclei |
| Total cell concentration | $1.89*10^6$ cells/mL | $7.40*10^5$ cells/mL | $2.11*10^6$ cells/mL | $1.30*10^6$ cells/mL |
| Live cell concentration | $1.70*10^6$ cells/mL | $7.16*10^3$ cells/mL | $2.02*10^6$ cells/mL | $7.16*10^4$ cells/mL |
| Dead cell concentration | $1.81*10^5$ cells/mL | $7.33*10^5$ cells/mL | $8.83*10^5$ cells/mL | $1.29*10^6$ cells/mL |
| Viability | 90.4% | 1.0% | 95.8% | 0.6% |
| Average cell size | 13.4 µm | 7.5 µm | 20.6 µm | 11.4 µm |

***Figure 2.*** *Montages displaying the staining results of different combinations, AO/PI, and AO/EthD-1, for U937 cells and 3T3 cells. The quantitative analyses from LUNA-FX7™ (A) and LUNA-FL™ (B) are also shown.*

## Nuclei Staining After PFA Fixation

Nuclei fixation is a common practice for long-term storage, as it helps preserve cellular structures and enables subsequent analysis. Hence, to verify if the aforementioned nuclei quality assessment methods can be applied to fixed nuclei as well, we tested AO/PI and AO/EthD-1 on PFA-fixed nuclei. When using the AO/PI dye combination, a distinct and strong PI fluorescence signal was observed regardless of fixation status or dye incubation time. This indicates that the AO/PI dye can be used for both fresh isolated nuclei and fixed nuclei. In contrast, when using AO/EthD-1, there was a reduction in the red fluorescence of EthD-1 in fixed nuclei, and even with prolonged dye incubation, there was no increase in the fluorescence signal. To address this challenge, we adjusted the exposure value, increasing it from the default setting of 5 to 8. This adjustment led to stronger red signals, comparable to those achieved with the AO/PI combination. These findings suggest that when using the AO/EthD-1 dye, a reduction in fluorescence signal can be expected for fixed nuclei, as opposed to fresh isolated nuclei. Therefore, adjustments to the parameters of the automated cell counter may be necessary.

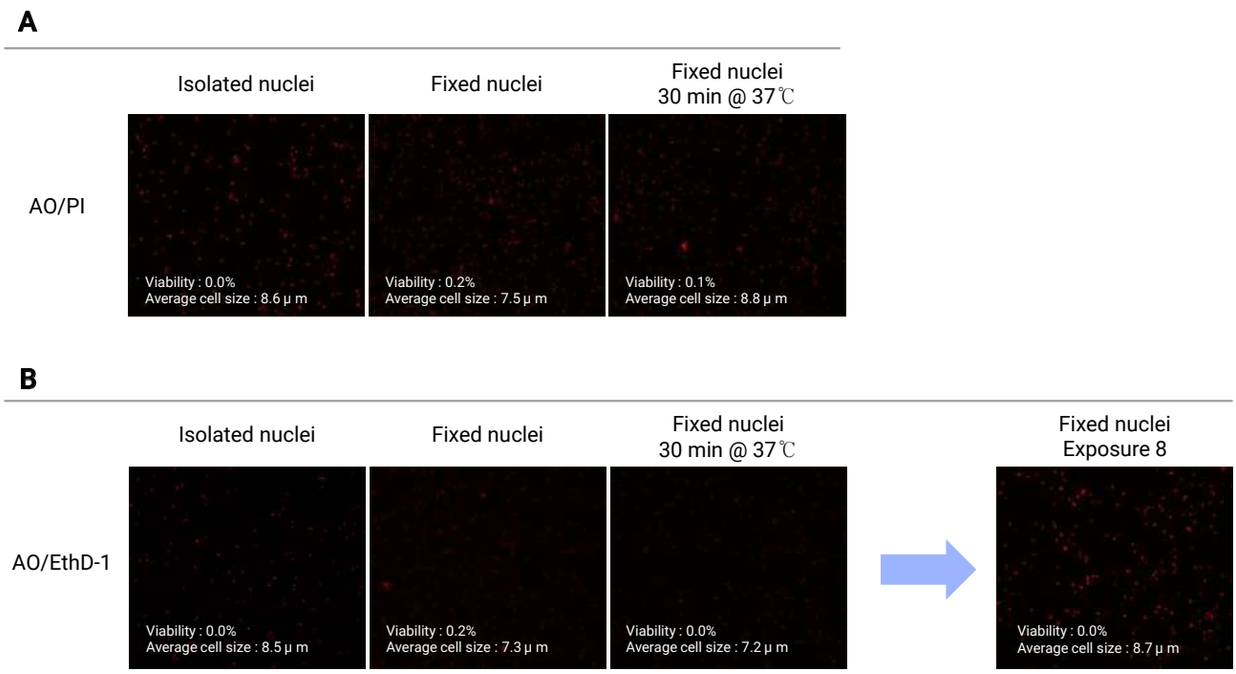



***Figure 3.*** *A montage showing the staining results of nuclei before and after PFA fixation using AO/PI and AO/EthD-1. The image obtained using an exposure setting of 8 is also displayed on the right.*

## Conclusion

In the rapidly evolving realm of single cell genomics research, the precise evaluation of nuclei quality stands as a cornerstone for dependable outcomes. Among the dye combinations assessed, AO/PI and AO/EthD-1 emerged as the most promising for distinguishing viability differences between intact cells and isolated nuclei. Notably, while AO/PI proved effective for both fresh and fixed nuclei, the AO/EthD-1 combination required optimization to achieve comparable results, especially for fixed nuclei. Our study has demonstrated a highly effective and reliable method by combining the appropriate dye compositions with the advanced LUNA-FX7™ and LUNA-FL™ systems to assess isolated nuclei quality. This well-established approach provides a valuable technique for assessing isolated nuclei, thereby contributing to the advancement of single cell genomics research.

logos biosystems

Application Note

Bioprocess monitoring, biomanufacturing, cell therapies, bioactive production
automatic calculation, elapsed time, growth rate, doubling time, growth curves

# Bioprocess analysis with the LUNA-FX7™

## INTRODUCTION

The biomanufacturing industry is experiencing rapid growth due to improvements in cell therapies, such as CAR-T cell therapy, and increasing bioactive production[1]. It is expected to grow at least 12 to 17% annually over the next decade[2]. In response to these demands, biomanufacturers have been expanding the production capacity while maintaining quality and regulatory compliance. Furthermore, it is even more critical to monitor cell growth and health accurately while managing multiple cell batches. To provide biomanufacturers with the flexibility, accuracy, and power to accommodate these analysis demands, we recently developed the LUNA-FX7™ Automated Cell Counter.

The LUNA-FX7™ Automated Cell Counter provides a new bioprocess monitoring feature as well as a Quality Control mode with unique validation slides that can be utilized for quality outcomes. The Bioprocess feature enables scientists to monitor, record, and analyze an individual batch of bioprocessing activities. Once counting is performed under the Bioprocess mode, the LUNA-FX7™ automatically generates growth rates, cell doubling time, and trend charts. Furthermore, entire cell counting data may easily be saved and transferred via a USB flash drive, Wi-Fi, or Ethernet. Notably, the 21 CFR Part 11 compliant "CountWire™" software packages allow more advanced remote access and data management for multiple LUNA-FX7™ devices connected to the same network. By providing a convenient means to trace individual batches and monitor various operations securely, the LUNA-FX7™ can facilitate a more efficient workflow. Here, we demonstrate how the LUNA-FX7™ monitors the cell growth of three suspension cell lines using the Bioprocess option. Each cell line was applied to the three counting modes, Fluorescence Cell Counting mode, Bright Field Total Cell Counting mode, and Bright Field Cell Counting & Viability mode.

## MATERIALS AND METHODS

### Cell preparation and counting of batch run simulation

Three suspension cell lines, HL60, K562, and U937, were used in this assessment. All cell lines were grown in RPMI 1640 culture media supplemented with 10% fetal bovine serum (100 units/mL). Batch runs were simulated by seeding 10 mL of initial concentrations at 6 x $10^4$ cells/mL in 100 mm cell culture dishes and grown for seven days. During batch runs, counts were performed twice a day for all seven days utilizing the Bioprocess feature. For each count, 100 µl of cells were sampled. Each sample was counted via three different cell counting modes: Fluorescence Cell Counting mode, Bright Field Total Cell Counting mode, and Bright Field Cell Counting & Viability mode. For brightfield viable cell counting, a 1:1 mix of 0.4% Trypan blue stain (Cat# B13101) was used. For fluorescent cell counting, a 2:18 mix of Acridine Orange/Propidium Iodide (AO/PI) (Cat# F23001) was used.

### Bioprocess operations

The LUNA-FX7™, the most advanced model in the LUNA™ Family comes equipped with the Bioprocess software plus an expanded 1 TB hard drive. First, a unique protocol name for an individual batch is assigned to start bioprocess monitoring. In this manner, multiple batches are easily monitored and stored in the profiles of status. For this demonstration, the three protocols for bioprocess monitoring were named as the cell lines - HL60, K562, and U937* [1],

*[1] *As the three cell lines have shown almost the same trends, the detailed bioprocess data of HL60 is representatively selected and presented in this application note.*

respectively. Actual protocol parameters may be customized to specific cell lines or remain unchanged. Here, the default protocol parameters were used for all cell lines. Prior to counting, the assigned protocol for a specific batch is loaded. For each counting, load the target protocol for the bioprocess, count the cells, and save the results with a check on the Bioprocess saving option. The LUNA-FX7™ automatically calculates the growth rate and the doubling time, generating updated growth curves for individual batches based on the counting in the last two intervals (Figure 1). The on-screen chart is flexible; the y-axis can be toggled to total cell concentration, viable cell concentration, and viability; simultaneously, the x-axis is changeable to time, date, and month (More details in the User Manual).

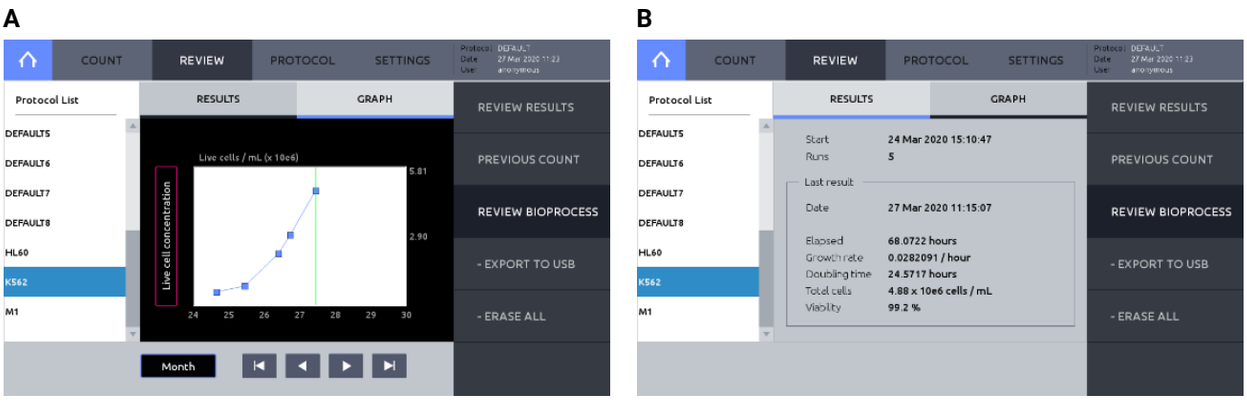



***Figure 1. On-screen growth chart and results of the K562 cell in the Bioprocess Review.*** *(A) The growth chart is showing the cumulative results of cell concentration over date. (B) Summarized results show the start date, the number of measurements, current growth rate, doubling time, current concentration, and viability.*

## Data visualization and extrapolation

The LUNA-FX7™ automatically records and calculates bioprocess indicators such as growth rate, viability, and doubling time. Thus, the report is generated including the trend charts of the data and tables of cell counting status (Figure 2). Further, the cell status also includes cumulative tracking at each time point, along with immediate or overall cell growth rates. The data can be exported as a CSV file for more compiled analysis if needed (Table 1).

***Table 1. The report in Fluorescence Cell Counting Mode****. The example CSV report from the HL60 batch run. The report contains date, time, concentration and viability. The data of escape time, growth rate, and doubling time are calculated from the last count (Blue shadows) and the run's total time (Green shadows).*

| Date & Time | Total cell conc. (/mL) | Live cell conc. (/mL) | Total cell # | Live cell # | Viability (%) | Elapsed time (last) (hrs) | Growth rate* (last) (/hr) | Doubling time** (last) (/hr) | Elapsed time (total) (hrs) | Growth rate* (total) (/hr) | Doubling time** (total) (hrs) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3/31/2020 5:34 | 6.75E+05 | 6.55E+05 | 742 | 720 | 97 | | | | | | |
| 3/31/2020 23:09 | 9.53E+05 | 9.28E+05 | 1048 | 1020 | 97.3 | 17.01 | 1.96 | 35.34 | 17.59 | 1.96 | 35.34 |
| 4/1/2020 7:41 | 1.24E+06 | 1.23E+06 | 1360 | 1350 | 99.3 | 8.01 | 3.08 | 22.47 | 26.12 | 2.33 | 29.77 |
| 4/1/2020 21:59 | 1.84E+06 | 1.82E+06 | 2028 | 2005 | 98.9 | 14.01 | 2.76 | 25.11 | 40.42 | 2.48 | 27.94 |
| 4/2/2020 5:39 | 2.23E+06 | 2.21E+06 | 2447 | 2433 | 99.4 | 7.01 | 2.51 | 27.64 | 48.09 | 2.49 | 27.89 |
| 4/2/2020 22:55 | 3.61E+06 | 3.59E+06 | 3972 | 3951 | 99.5 | 17.01 | 2.79 | 24.84 | 65.35 | 2.57 | 27.02 |
| 4/3/2020 5:58 | 3.80E+06 | 3.79E+06 | 4176 | 4168 | 99.8 | 7.00 | 0.73 | 95.28 | 72.40 | 2.39 | 29.04 |
| 4/3/2020 23:46 | 5.33E+06 | 5.29E+06 | 5863 | 5820 | 99.3 | 17.01 | 1.90 | 36.46 | 90.20 | 2.29 | 30.26 |
| 4/4/2020 6:45 | 5.43E+06 | 5.37E+06 | 5967 | 5905 | 99 | 6.00 | 0.27 | 260.40 | 97.19 | 2.15 | 32.31 |
| 4/5/2020 1:16 | 5.43E+06 | 5.18E+06 | 5970 | 5690 | 95.3 | 18.01 | 0.00 | 0.00 | 115.71 | 1.80 | 38.47 |

*Growth rate per hour = (LN V2 - LN V1) / (t2 - t1)
**Doubling time in hours = LN (2) / Growth rate per hour
LN = Natural log,
t1, t2 = Unit of hour,
V1 = Cell concentration in cells/mL at elapsed time t1 in hours,
V2 = Cell concentration in cells/mL at elapsed time t2 in hours

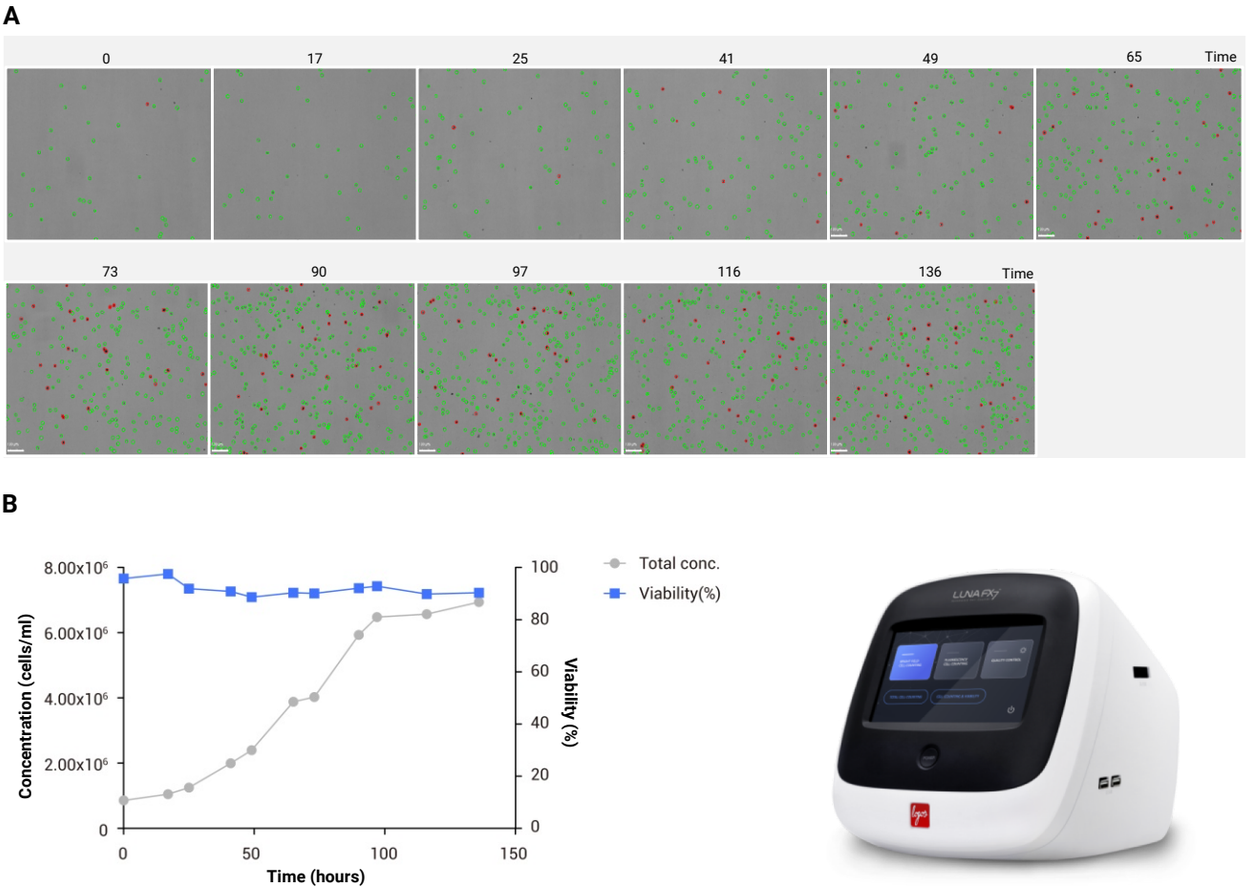



***Figure 2. Bioprocessing data output.*** *Images and charts from fluorescent counting of HL60 cells during a 136-hour batch run. (A) Tagged images at each sampling of the batch run. (B) The plots of cell concentration and viability curves over date were accordingly generated in the LUNA-FX7™.*



*LUNA-FX7™ Automated Cell Counter*

## CONCLUSION

The Bioprocess option in the LUNA-FX7™ reduces unnecessary effort by automating the recording and analyzing status indicators such as cell growth and viability, simultaneously performed three cell culture batches of 3 different counting modes. Remarkably, the automated calculations of doubling times, growth curves, and viability status provide near-real information to monitor and forecast bioprocess production timelines reliably and accurately. When combined with the CountWire™ software package, the Bioprocess feature allows team members to monitor multiple culture batches in real-time across multiple facilities.

## REFERENCES

[1] Joubert S, Dodelet V, Béliard R, Durocher Y. La bioproduction des anticorps monoclonaux [Biomanufacturing of monoclonal antibodies]. Med Sci (Paris). 2019;35(12):1153-1159. doi:10.1051/medsci/2019219

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