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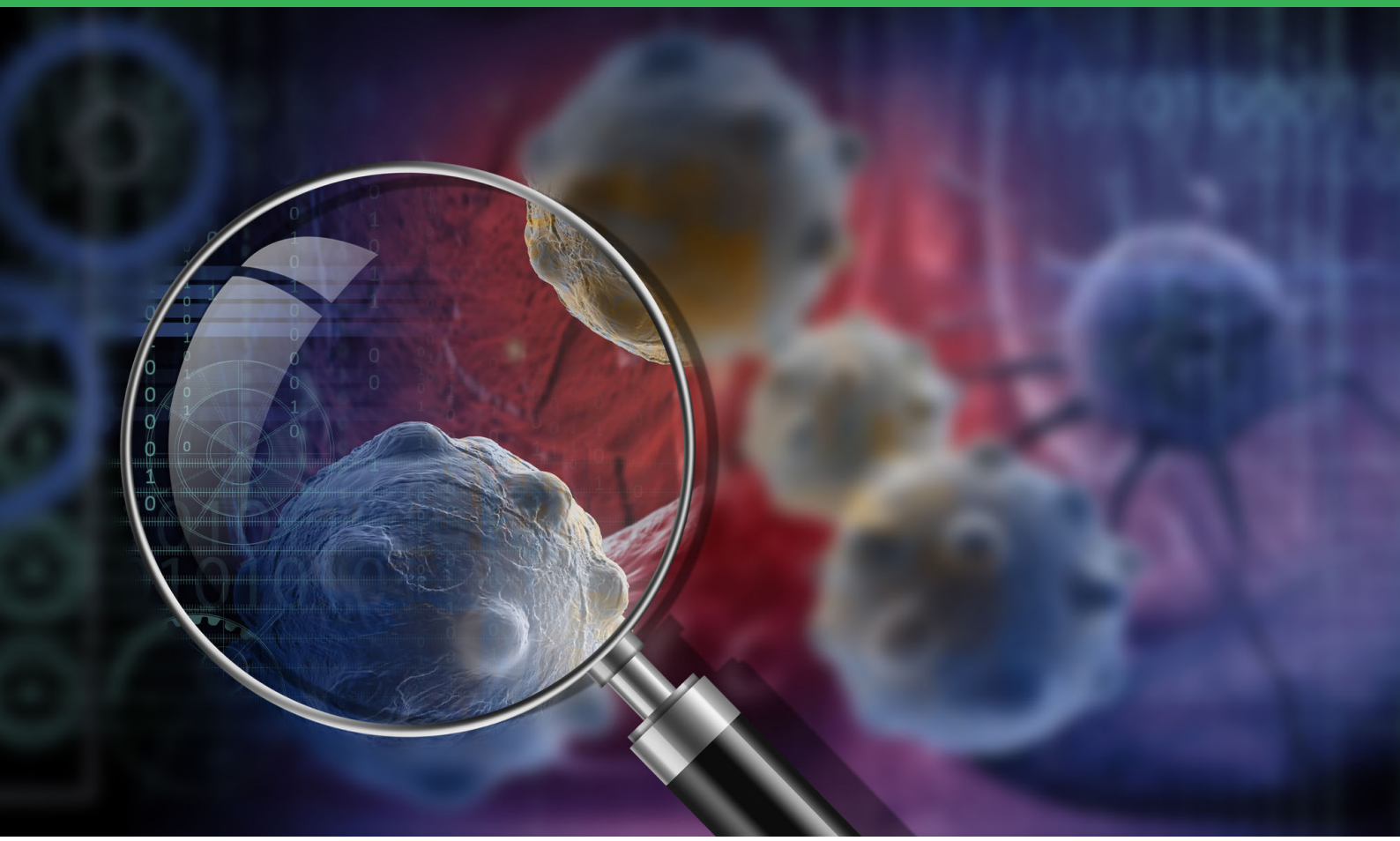

Introduction

Quantifying the number and type of cells in a sample is a fundamental part of life sciences research and has been for over 60 years. From humble beginnings counting whole cells in 2D in a Petri dish, a wide range of counting methods have been developed to help researchers manage the competing requirements for efficiency (speed) and accuracy, whatever the cell type. Whether for human, animal, or plant cells, modern counting technologies have brought automation, versatility, and high throughput to an activity that is crucial for research reproducibility and the success of many downstream workflows.

Failing to count cells accurately or distinguish reliably between viable and non-viable cells can compromise research integrity, waste resources and delay progress. Whether you are a researcher, lab professional, or a student counting cells for single-cell sequencing or dosing determination for a cell therapy, the stakes could hardly be higher when it comes to your choice of cell counting technology.

In this eBook, we take a look at Aligned Genetics' LUNA-FX7™ automated cell counter to help provide insight and practical solutions to optimize your cell counting workflows. Through a series of application case studies covering diverse and complex human, animal

Contents

and plant cell counting, understand how this versatile, automated technology can enhance your research capacity and laboratory efficiency for all cell types.

Human cells

Whole blood is a notoriously challenging medium for traditional counting techniques aimed at enumerating a single component such as leukocytes. Platelets and mature red blood cells in particular complicate reliable counting. Similarly, CAR-T cell therapies require a complex biomanufacturing process entailing strict adherence to regulatory and QA/QC guidelines. Cell health and viability must be evaluated and monitored throughout bioprocessing workflows to ensure the safety, quality, and efficacy of the final clinical product. From two case studies, gain insights into:

• How the dual fluorescent LUNA-FX7™ meets diverse counting needs through its use in first counting leukocytes in whole blood and then peripheral blood mononuclear cells (PBMCs) isolated from whole blood samples. Discover how this was achieved using LUNA 2-channel and 8-channel slides for high throughput, replicated analyses.

• How using the LUNA-FX7™ in fluorescent counting mode with the nucleic acid stains Acridine Orange and Propidium Iodide enables the monitoring and evaluation of CAR-T cell health at critical stages in the biomanufacturing process. Understand how this was achieved and met rigorous QC guidelines through the use of pre-set validation slides, internal QC software and optional 21 CFR Part 11-compliant software.

Animal cells

Monitoring the concentration and viability of animal cells is integral to a whole host of workflows covering areas such as livestock health, fertility assessment and cell biotechnology for protein production. From somatic cell counts in milk, to sperm cell assessment in cattle and insect cell measurement in high-quality protein production, the LUNA-FX7™ offers numerous advantages over traditional flow cytometry-based cell counters through reduced costs and enumeration of smaller-sized samples. Discover these benefits through three case studies:

• Learn how somatic cell count (SSC) determination in milk is challenging due to fat and protein debris, and how it can be overcome by using the LUNA-FX7™ in conjunction with the *Somatic Cell Staining Solution*.

• Discover the importance of determining SF9 insect cell line viability for robust protein production and how the LUNA-FX7™ was used to select the appropriate dyes and optimal machine exposure levels for monitoring SF9 cell quality.

• Understand the importance of semen analysis for determining cattle fertility and how a comparative analysis of different fluorescent dyes using the LUNA-FX7™ enabled the optimization of sample dilution and imaging conditions for convenient and rapid sperm cell assessment.

Plant cells

Traditional methods for assessing the viability of plant protoplasts – plant cells with removed cell walls – require culturing until the protoplasts have developed into complete plants. However, the LUNA-FX7™ enables immediate viability determination through double-staining with distinct-colored fluorescent dyes. Find out how the LUNA-FX7™ was used to identify the optimal dye combinations and parameters for reliable assessment of plant protoplast viability.

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A p p l i c a t i o n N o t e

Peripheral Blood Mononuclear Cells (PBMC), rapid evaluation, concentration and viability
AO/PI staining, dual fluorescence, 1, 2, 3, 8-channel slide options

Rapid evaluation of PBMC counting and viability

INTRODUCTION

Peripheral Blood Mononuclear Cells (PBMCs) are a vital source material used in myriad research applications, including single-cell sequencing to vaccine development and toxicological studies. Furthermore, PBMC derivatives such as T cells, B cells, NK cells, and stem cells are based on cell therapies, including CAR-T cell therapies and regenerative medicines. Therefore, accurately measuring numbers and viability of PBMCs after collection, isolation, or expansion are essential to making experimental or manufacturing decisions about downstream processes. Yet, directly obtaining leukocyte counts in whole blood using traditional counting techniques is complicated by the presence of mature, anucleated RBCs and platelets. Advantageously, the use of nucleic acid stains like Acridine Orange and Propidium Iodide (AO/PI) allow the nucleated leukocytes to be differentiated and accurately counted within a whole blood sample (Figure 1). Here, we exhibit the ability of the new dual fluorescent LUNA-FX7™ Automated Cell Counter to meet diverse cell counting needs by demonstrating its use in counting leukocytes in whole blood and the PBMCs isolated from whole blood samples.

LUNA-FX7™ Automated Cell Counter

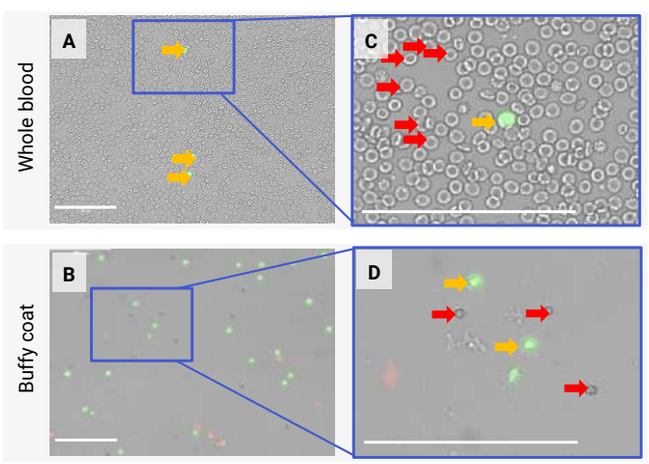


Figure 1. Diluted whole blood and PBMC-enriched buffy coat stained using AO/PI fluorescent dye. *The microscopic overlay images of the whole blood stained with AO/PI (A,C), and PBMCs from enriched buffy coat stained with AO/PI (B,D) were acquired using the CELENA® X High Content Imaging System with a 20X fluorite objective (www.logosbio.com). The yellow arrows indicate nucleated cells in AO/PI positive stained cells, while the red arrows indicate RBCs. The scale bar is 100 µm.*

MATERIAL AND METHODS

One milliliter of the human peripheral blood sample was prepared, and the PBMC sample was obtained by standard density gradient centrifugation technique using Histopaque-1083 (Sigma, #10831)[1]. After final washing, the PBMCs enriched in the buffy coat were resuspended in 100 µl PBS or RPMI +10% FBS media. Cell counts were performed in

the LUNA-FX7™ with either the 2-channel PhotonSlide™ (Cat# L12005) or LUNA™ 8-Channel Slides (Cat# F72001) and used a modified default protocol in the Fluorescence Cell Counting mode (Table 1). Before loading the cells, cells were stained at the standard ratio of the AO/PI reagent (Cat# F23001); 18 µl of cells + 2 µl of AO/PI, and then 10 µl of the mix was loaded into a slide chamber.

RESULTS

Evaluating the PBMC concentration and viability with the LUNA-FX7™

The separated PBMCs were analyzed by counting a series of 2-fold dilutions using both the 8-channel and 2-channel slides (Figure 2). The counts using both slide types showed linearity with an R-square value of 0.99 or above on the logarithmic scale of concentrations over the serial dilutions. Not surprisingly, with a greater volume of analysis, the 2-channel slide showed slightly better consistency than the 8-channel slide.

Table 1. *The optimized parameter settings for PBMC or leukocytes counting of the LUNA-FX7™ on Fluorescence Cell Counting mode*

Basic

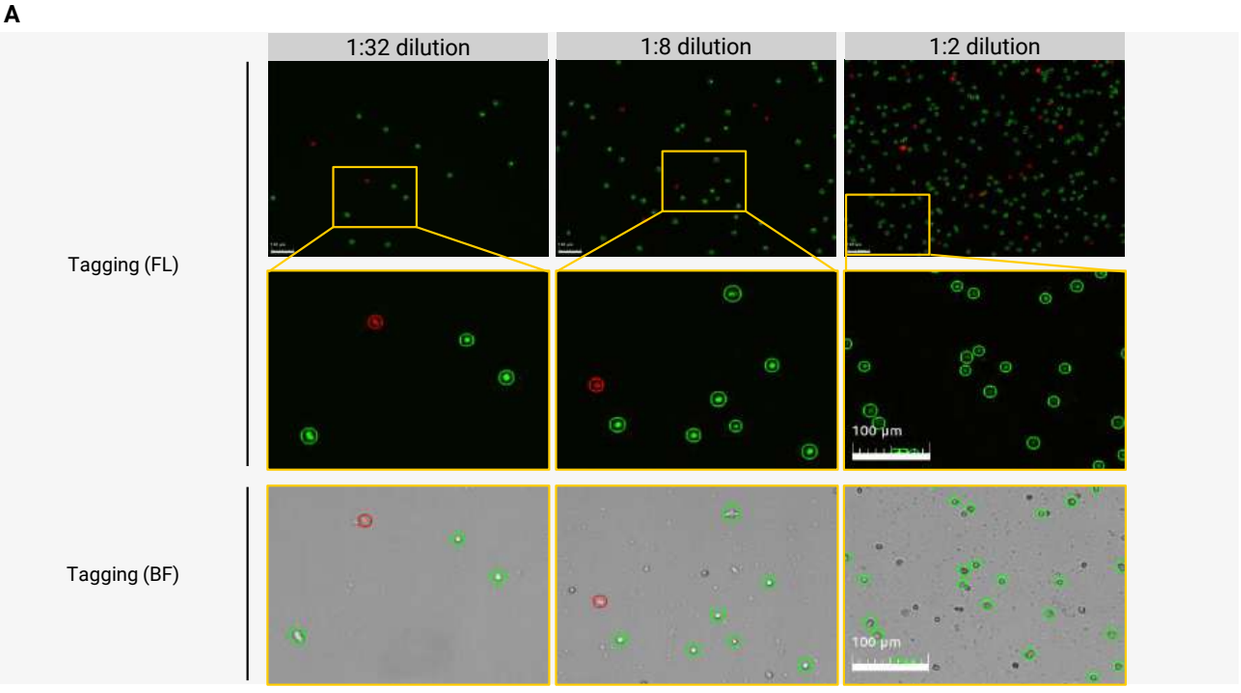
Counting mode	Fluorescence Cell Counting
GF exposure level	5
RF exposure level	5
Cell size calculation	BF
Min. cell size	3 µm
Max. cell size*	30 µm
GF threshold level	5
RF threshold level	5
Dilution factor	1.11

Advanced

Counting mode	Fluorescence Cell Counting
GF exposure level	5
RF exposure level	5
Min. cell size*	3 µm
Max. cell size*	30 µm
Declumping sensitivity	5
Min. FL intensity	0
Min. roundness	3
Dilution factor	1.11

** Changed from the DEFAULT values.*

A



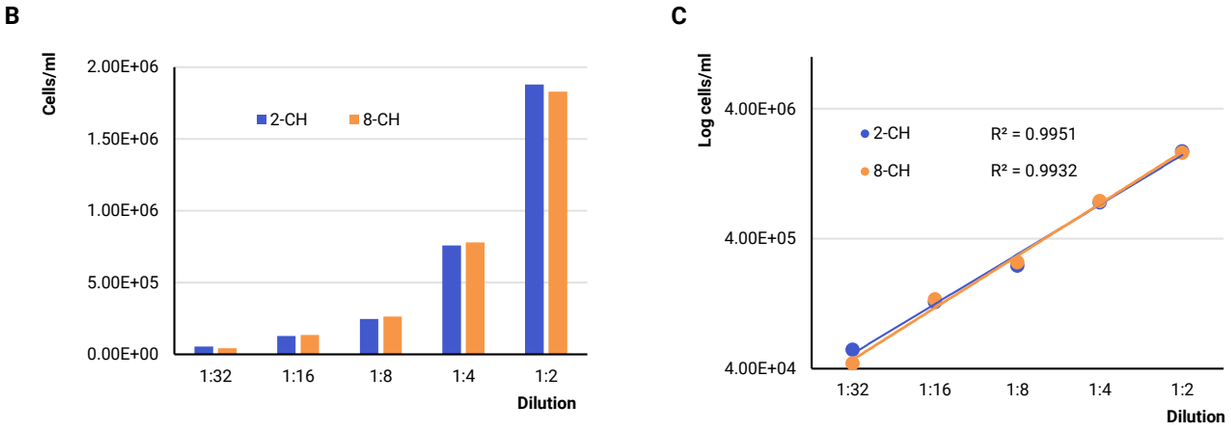
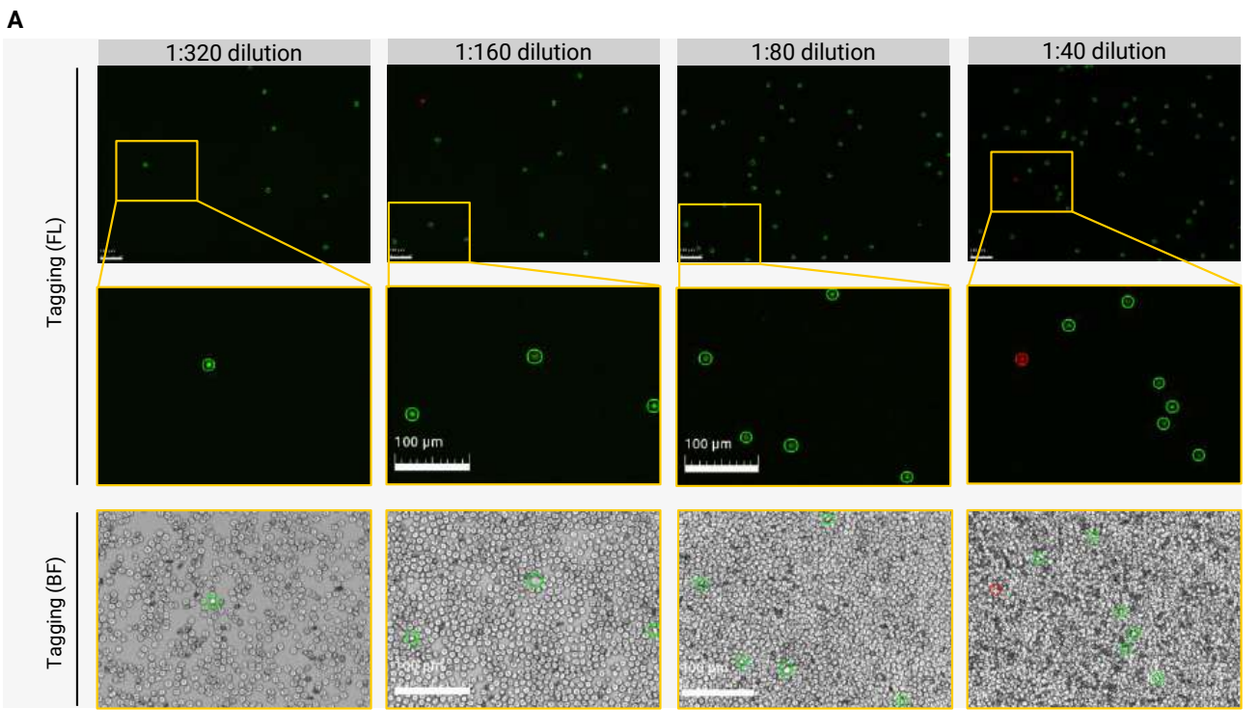


Figure 2. The linearity of counting in serial dilutions of PBMCs. *(A) Tagged (live or dead) fluorescent and brightfield overlay of several dilutions. (B) Bar graph showing the results of 5 serial dilutions. (C) The logarithmic scale of the counts over the dilutions using both 2-channel and 8-channel slides down to concentrations less than 4.00E+04 cells/ml. The scale bar represents 100 µm.*

The easy counting of leukocytes in whole blood

The leukocytes in whole blood were visualized by fluorescence and also accurately enumerated with the LUNA-FX7™. Among countless mature RBCs, the fluorescence leukocytes were distinctively counted from diluted whole blood (Figure 3).



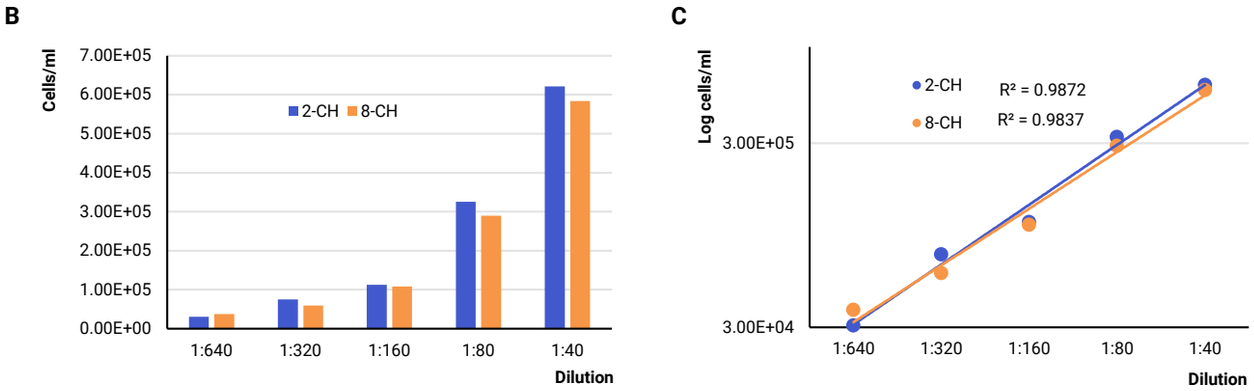


Figure 3. The Leukocyte count of whole blood on Fluorescent Cell Counting mode in the LUNA-FX7™. *(A) Tagged leukocyte images of fluorescent and brightfield overlay in serial dilutions of whole blood samples. (B) Bar graph displaying counting of 5 serial dilutions from 1:40 to 1:640 of whole blood cells. (C) The linearity of counts appears across concentration on both 2-channel and 8-channel slides. The scale bar represents 100 µm.*

CONCLUSION

The enumeration of PBMCs was evaluated adequately on the LUNA-FX7™ Automatic Cell Counter with the 2-channel PhotonSlide™ and LUNA™ 8-Channel Slides. With more slide options for the LUNA-FX7™, LUNA™ 1-Channel Slides (Cat# L72011) may be applied for a more comprehensive concentration range by analyzing 47 image fields, and LUNA™ 3-Channel Slides (Cat# L72021) are beneficial for preset of triplicate analysis as counting options. Indeed, leukocyte counting is much more straightforward by loading the diluted blood after AO/PI fluorescence staining. So, you can use the LUNA-FX7™ to respect the archived records of PBMCs along with leukocyte counts without intensive labor.

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[1] S Parasuraman, R Raveendran, and R Kesavan Blood sample collection in small laboratory animals J Pharmacol Pharmacother. 2010 Jul-Dec; 1(2): 87–93. doi: 10.4103/0976-500X.72350

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Application Note

CAR-T cell therapy production, initial WBC, leukapheresis, T cells, CARs expressed T cells, final dosing of CAR-T cells, concentration and viability, AO/PI staining

Monitoring CAR-T Cell Therapy Production Using the LUNA-FX7™

INTRODUCTION

Chimeric Antigen Receptor (CAR) T cell therapy is a combination of personalized Immunotherapy and gene[1]. CAR-T cell therapy is a 'living' drug established from a patient's own isolated and engineered T cells. Since the first FDA approval in 2018, CAR-T therapies have achieved 80% in remission rates for hematologic malignancy patients[2]. As with all adoptive cell therapies, the production of autologous or allogeneic CAR-T therapies is a complicated biomanufacturing process that requires strict adherence to regulatory and QA/QC guidelines. To be ensured the safety, quality, and efficacy of the final product, cell health and viability must be evaluated and monitored throughout the process of development and bioprocessing workflows.

Here, we demonstrate how the LUNA-FX7™ may be used to monitor and evaluate cell health at critical stages in the CAR-T process: 1) Collection of the primary source material, 2) Post-Leukapheresis, 3) Isolation / Activation, 4) Expansion, and 5) Production. Curocell Inc., a leading company developing innovative anti-cancer immune therapy in Korea, kindly provided cell count data of two samples randomly selected from each stage of the CAR-T cell production (Figure 1). Cell counts were performed with the LUNA-FX7™ in fluorescent cell counting mode using the nucleic acid stains Acridine Orange and Propidium Iodide (AO/PI, Cat# F23001, Logos Biosystems). In brief, for each count, 2 µl of pre-mixed AO/PI solution was combined with 18 µl of cells, loaded into a 2-, 3-, or 8-channel slide, and counted utilizing the autofocus feature. For the final dosing determinations, the LUNA™ 1-Channel Slides (Cat# L72011) with an analysis volume of 5.1 µl (>10X volume of most automated volumes) were used.

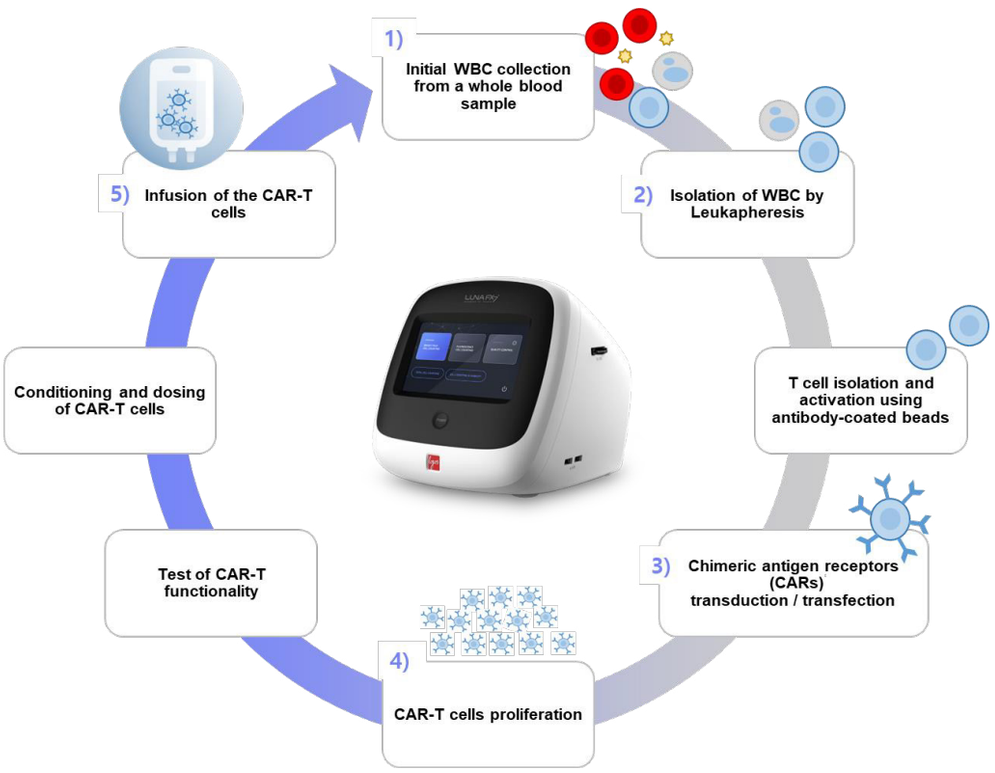


Figure 1. *Each step of the CAR-T cell production for clinical drugs needs to be characterized by purposely designed cell formulations and defined cell concentration and viability by LUNA-FX7™ Automated Cell Counter.*

APPLICATION

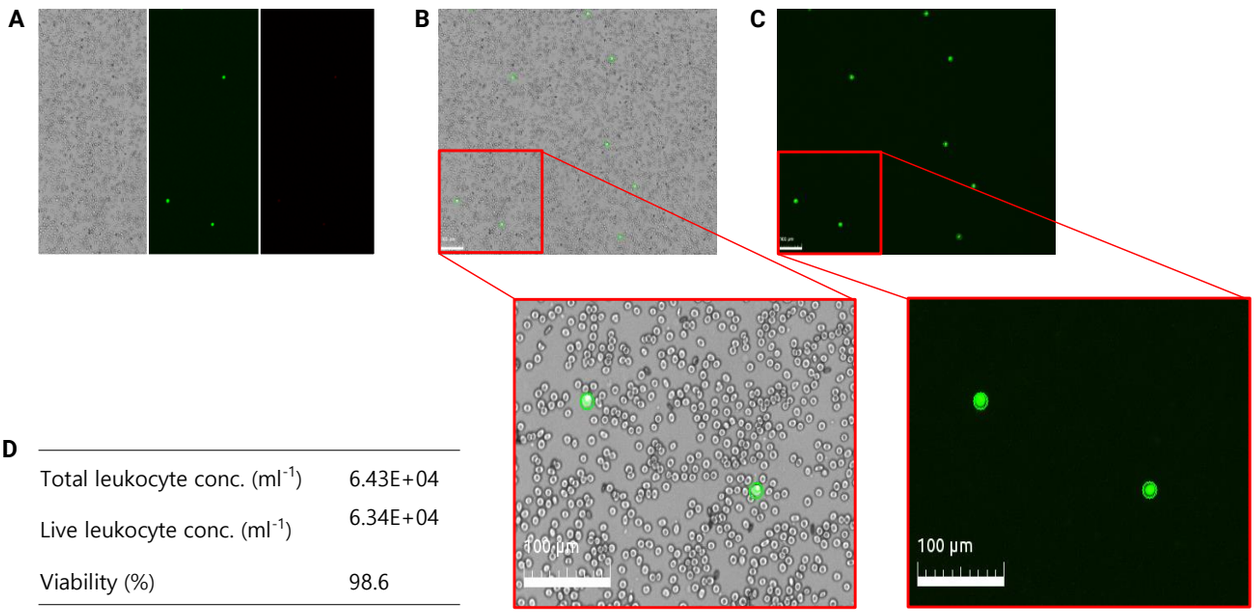
Table 1. *Protocol parameter settings for CAR-T cells in Fluorescence Cell Counting mode*

Protocol Parameter	Value
GF exposure level (0.1-10)	5
RF exposure level (0.1-10)	5
Cell size calculation (BF/FL)	FL*
Min. cell size (1-89 µm)	4*
Max. cell size (2-90 µm)	70
GF threshold level (1-10)	5
RF threshold level (1-10)	5

** Changed from the DEFAULT values.*

1. Initial WBC concentration and viability from a whole blood sample

After initial blood collection and prior to leukapheresis, cells were diluted 1:100 with 1X PBS for counting in LUNA-FX7™. Despite the fact that white blood cells (WBC) comprise only 2% of a whole blood population, Figure 2 shows how the LUNA-FX7™ can easily differentiate nucleated cells from anucleated cells (i.e. RBC) in a heterogeneous population.



D

Total leukocyte conc. (ml^{-1})	6.43E+04
Live leukocyte conc. (ml^{-1})	6.34E+04
Viability (%)	98.6

Figure 2. ***Cell concentration and viability of WBC in whole blood samples.*** *Prior to leukapheresis, a whole blood sample was dilute 1:100 with PBS, stained with AO/PI, and counted. Tiled images of Brightfield (BF), Green channel (GF), and Red channel (RF) (A). Overlay images of tagged (live/dead nucleated cells) images (B, C). Total WBC cell numbers and viability of the 1/100 diluted blood (D). The protocol settings are shown in Table 1.*

2. Isolation of WBC by Leukapheresis

Leukapheresis is the process of separating WBCs from the whole blood. Figures 3 shows the viability of 2 different samples. RBCs were not seen in either sample, but some cell debris may still be observed. Here again, the LUNA-FX7™ can easily distinguish between cells and cellular or non-cellular debris, minimizing the possibility of counting false positives.

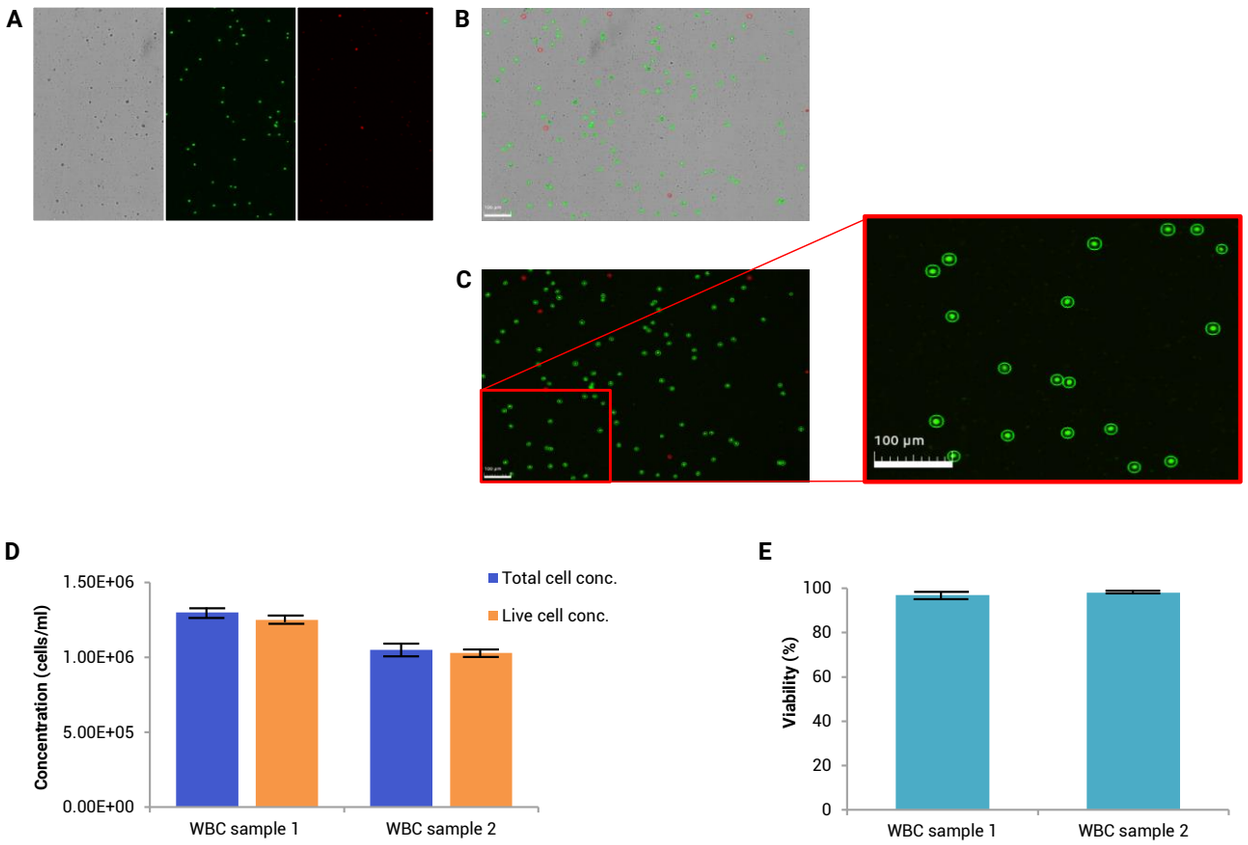
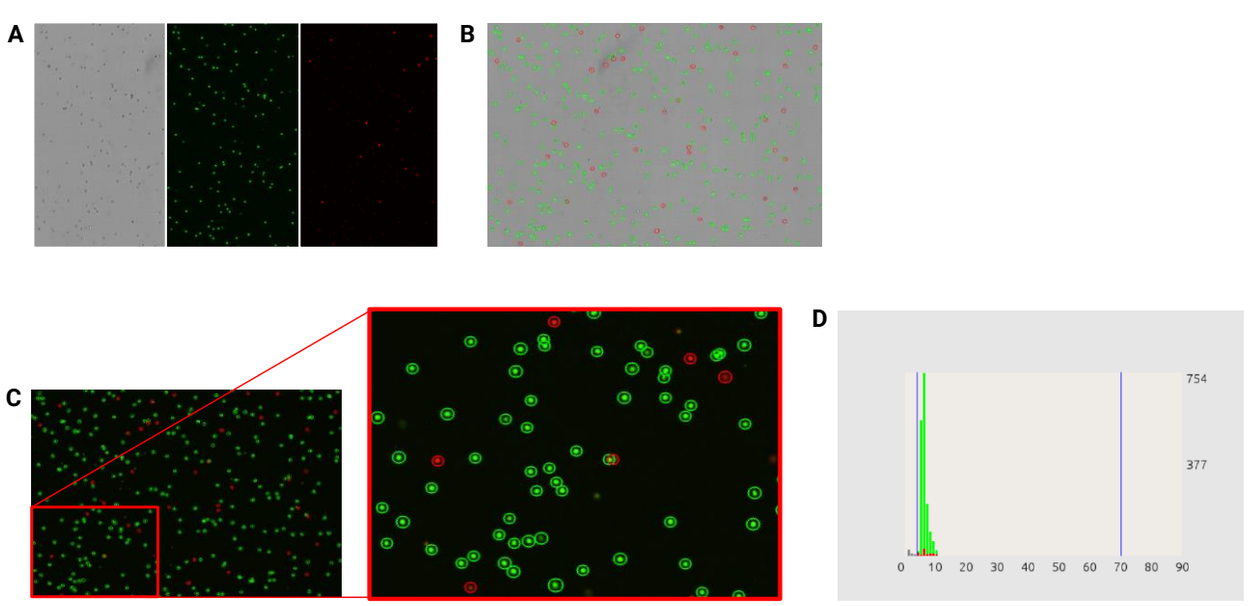


Figure 3. *Cell concentration and viability post-leukapheresis. WBC samples counted post-leukapheresis and BF, GF, and RF raw images (A), FL tagged image, and the magnified image (B, C). Two samples were evaluated and the cell concentration and viability were plotted. (D, E). The protocol settings are shown in Table 1.*

3. T cell isolation and activation

After leukapheresis, T cells are further isolated and then activated, yielding a homogenous T cell population. At this stage, there is a marked contrast in the quality of samples (Figure 4).



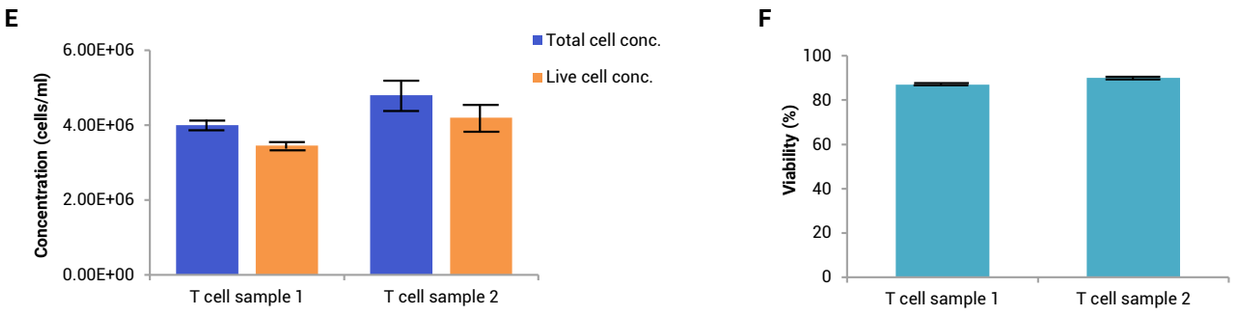


Figure 4. *Cell concentration and viability post isolation and activation. T cells were counted post isolation and activation, and BF, GF, and RF raw images (A), and FL tagged image, and enlarged image (B, C). The false-positive debris can be eliminated by a minimum cell size setting (D). Two samples were evaluated and the cell concentration and viability were plotted (E, F). The protocol settings are shown in Table 1.*

4. CARs expressed T cells

The isolated and activated T cells are transduced through retroviruses to produce the chimeric antigen receptors (CARs). After transduction, the newly engineered CAR-T cells are expanded *ex vivo*. Figure 5 shows cell viability and numbers of expanded CAR-T cells.

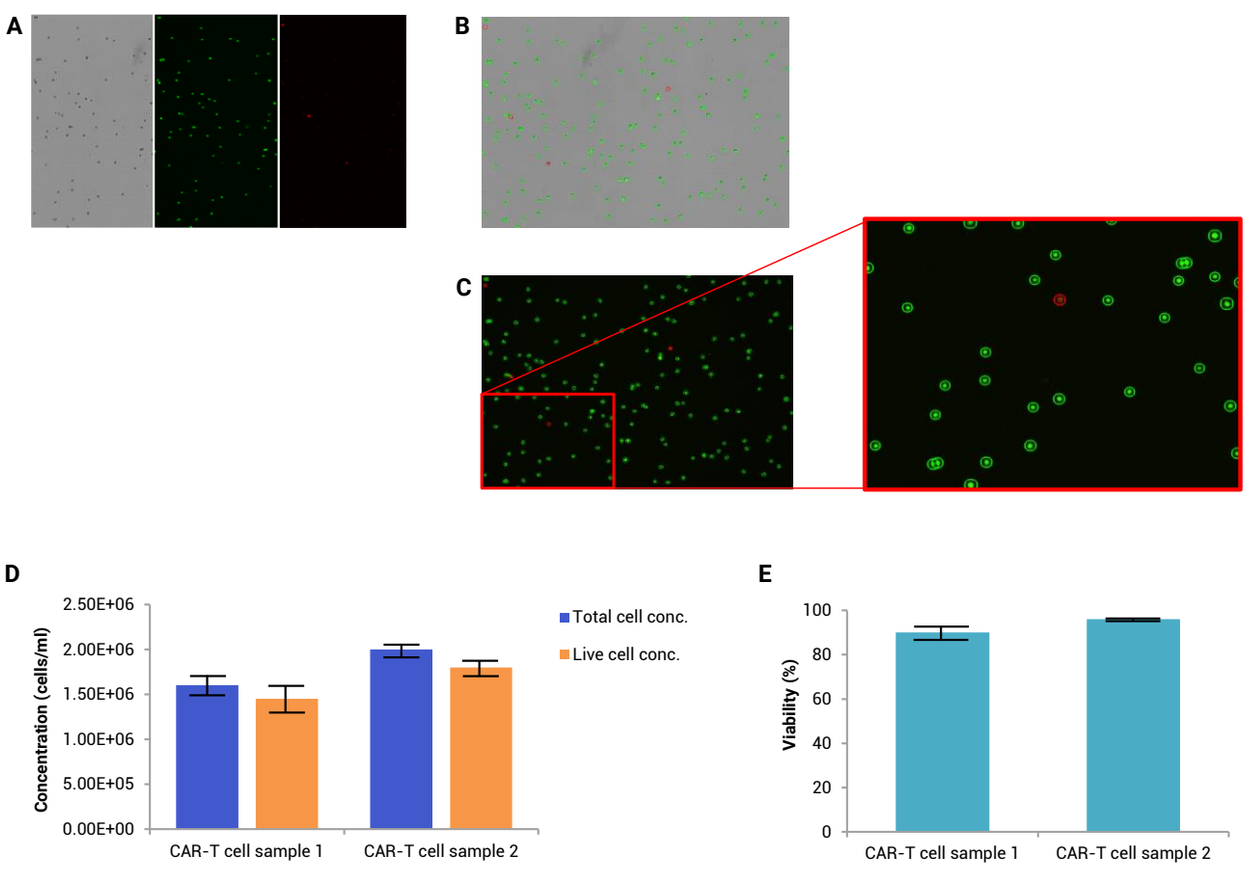


Figure 5. *Cell concentration and viability after expansion of CAR-T cells. The raw and tagged images (A, B, C) and the cell concentration and viability (D, E) of CAR-T cell samples. The protocol settings are shown in Table 1.*

5. CAR-T cell product

Expanded CAR-T cells are cryopreserved and evaluated for appropriate dosing. Figure 6 shows the viability and concentration of 2 samples with different cell viability.

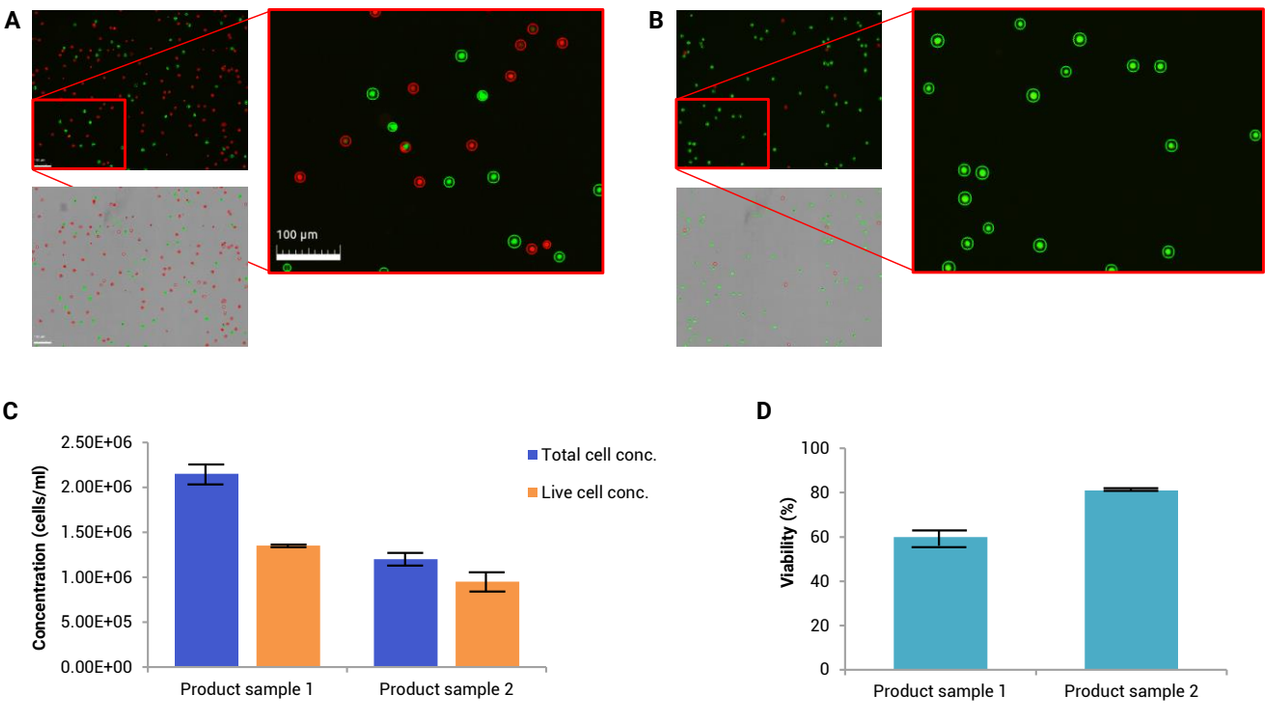


Figure 6. *Cell concentration and viability of CAR-T cell final products. Tagged images of the low viability sample (A) and the high viability sample (B) and the corresponding cell concentration and viability (C, D). The protocol settings are shown in Table 1.*

CONCLUSION

From the initial collection of primary source material to final dosing determinations, the LUNA-FX7™ provides the required accuracy and flexibility for monitoring cell health and viability through the entire CAR-T process. Furthermore, in conjunction with pre-set validation slides, internal QC software, and optional 21 CFR Part 11 compliant software ensures that the rigorous QC and regulatory guidelines are met and maintained.

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ACKNOWLEDGEMENTS

We, Aligned Genetics, Inc. would like to express our sincere appreciation to Curocell Inc. for sharing the count data produced with the LUNA-FX7™, enabling us to write this application note.

Somatic cell counting using the LUNA-FX7™ Automated Cell Counter

INTRODUCTION

Somatic cell count (SCC) is a well-established parameter used in the dairy industry to manage udder health. An increase in SCC indicates intramammary gland infection (IMI) leading to mastitis. This condition can result in poor pregnancy rates and impact milk production. Hence, dairy farms use flow cytometry-based cell counters such as the FossoMatic. Although this approach is efficient for high-throughput analysis, there is a demand for inexpensive methods of enumerating smaller sized samples in the research field.

However, SCC determination in milk is challenging due to fat and protein debris. To overcome this issue, the *Somatic Cell Staining Solution* has been developed for uncomplicated and straightforward SCC determination with the LUNA-FX7™. Here, we show the ability of the LUNA-FX7™ Automated Cell Counter to efficiently count somatic cells in milk using *Somatic Cell Staining Solution*.

MATERIALS AND METHODS

Raw milk samples were obtained from POSTBIO, Inc. in high, medium, and low concentrations. Only low concentration had SCC below 2 x 10^5 cells/mL. Each milk sample was mixed with the *Somatic Cell Staining Solution* (Cat# F23101) at a 1:4 ratio. Cell counting was performed with the LUNA-FX7™ using 1-channel slides (Cat# L72011). A modified default protocol was used in the Fluorescence Cell Counting mode (Table 1).

STAINING PRINCIPLE

SCC determination in brightfield (BF) channel is not effective due to the fat and protein debris in milk (Figure 1A). Since these debris in milk have little to none fluorescence signal at the wavelengths that can be detected by the LUNA-FX7™, SCC determination was conducted using *Somatic Cell Staining Solution* contains propidium iodide (PI). It also contains a detergent to induce cell lysis as PI is not able to permeate intact cells. Once cells are permeabilized, PI binds to DNA by intercalating between base pairs and its fluorescence is increased 20- to 30-fold upon binding. These allow for the accurate distinction of somatic cells from debris in milk. Accordingly, SCC can be determined without being interrupted by debris in milk.

RESULTS

The results from the LUNA-FX7™ were closely analyzed and inspected to confirm whether the PI labeled and identified somatic cells are accurately. The LUNA-FX7™ successfully distinguished somatic cells from debris in milk which can be seen in tagged images (Figure 1A). Moreover, SCC determination results from the LUNA-FX7™ were compared with the known concentrations from the flow-cytometry based cell counter. The results showed strong correlation between two different platforms (with the low concentration being below 2 x 10^5 cells/mL) (Figure 1B).

Table 1. *The optimize parameter settings for SCC in Fluorescence Cell Counting mode of the LUNA-FX7™*

Counting Mode	Fluorescence Cell Counting
GF exposure level	5
RF exposure level	5
Min. cell size	5
Max. cell size	30
Declumping sensitivity	5
Min. FL intensity	0
Min. roundness	3
Dilution factor	5

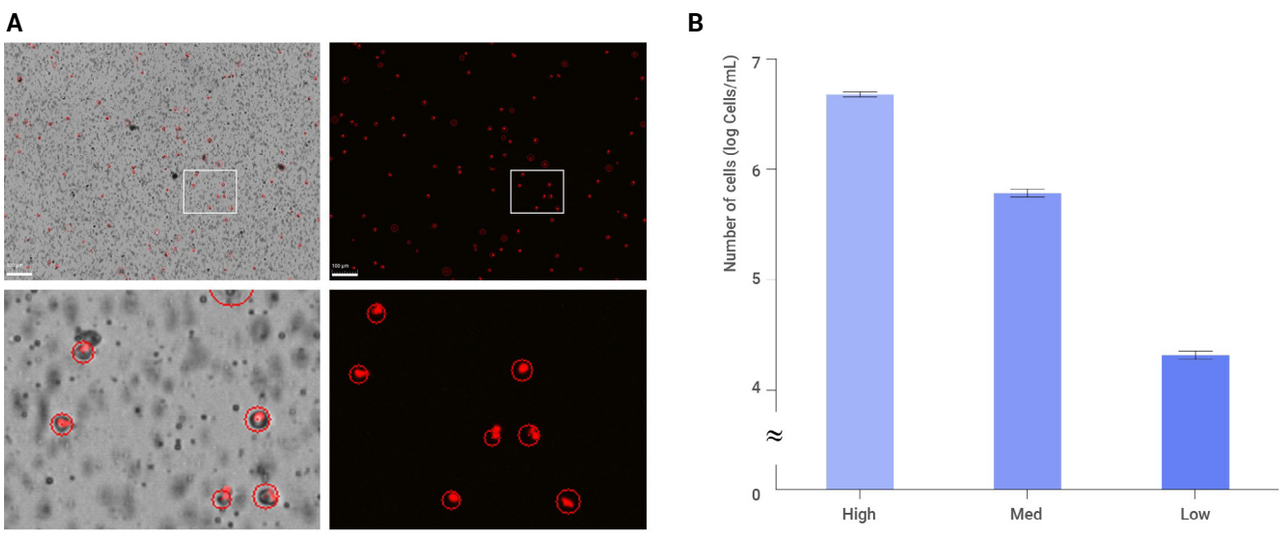

Figure 1. *(A) The image shows successfully stained and tagged somatic cells in milk samples using the LUNA-FX7™. (B) The bar graph shows strong correlation of SCC data obtained from the LUNA-FX7™ and the flow-cytometry based cell counter.*

CONCLUSION

We showed that LUNA-FX7™ automated cell counter could effectively count somatic cells using Somatic Cell Staining Solution. Moreover, the SCC results comparison between the LUNA-FX7™ and the flow cytometry-based cell counter showed a strong correlation to each other. Therefore, the LUNA-FX7™ automated cell counter is versatile enough to be used for many applications including SCC determination.

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Application Note

Automated cell counter, protoplast isolation, protoplast viability assessment, dual staining, dye assessment

Counting Protoplast with the LUNA-FX7™ Automated Cell Counter: Optimal Fluorescent Dye Combinations

Introduction

Protoplasts are plant cells with removed cell walls through enzymatic or mechanical removal of the cell wall. These spherical, cell-wall-free plant cells exhibit unique characteristics, including totipotency, making them versatile tools in plant science. They serve as an experimental system that allows to explore the structure, chemistry, and function of plant cells.

Traditionally, viability assessment involved culturing protoplasts until they developed into complete plants, but this method could not provide immediate viability assessment. The LUNA-FX7™ Automated Cell Counter now enables rapid viability determination through double staining with distinct-colored fluorescent dyes. However, it is essential to understand how the LUNA-FX7™ Automated Cell Counter performs with different dyes and to adjust analysis parameters accordingly. This study aims to identify optimal dye combinations and parameters for assessing protoplast viability using the LUNA-FX7™ Automated Cell Counter.

Protoplast Isolation and Staining

Protoplast Isolation

1. Prepare 5 g of seedlings and wash them once with ethanol and twice with distilled water (DW).
2. Gently pat dry the seedlings with a paper towel and place them in a Petri dish. Use scissors to finely chop them.
3. Add 25 ml of digestion buffer to the finely chopped seedlings, mix well, and transfer to a 50 ml tube. Cover with foil to protect from light.
4. Incubate on a rotator at 20 rpm for 6 hours.
5. After incubation, add 20 ml of wash buffer to the tube, gently mix, and filter through a 100 µm strainer.
6. Centrifuge at 100 g for 3 minutes.
7. Remove the supernatant, add 20 ml of wash buffer to the tube, gently mix, and filter through a 40 µm strainer.
8. Centrifuge at 100 g for 3 minutes.
9. Remove the supernatant and resuspend the pellet in 3 ml of wash buffer.

Fluorescence Staining

1. Mix:
   - 18 µL of protoplast cells
   - 2 µL of dyes prepared by mixing green and red fluorescent dyes
2. Load 10 µL of stained cells.
3. Perform analysis using the LUNA-FX7™.

*Note: Protoplasts have diverse sizes and may exhibit varying fluorescent intensities. Please make adjustments as needed.

Table 1. *The suggested parameter settings for protoplast counting of the LUNA-FX7™ on Fluorescence Cell Counting mode*

Basic

Counting Mode	Fluorescence Cell Counting
GF exposure level	5
RF exposure level	9
Cell size calculation	BF
Min. cell size	3 µm
Max. cell size	70 µm
GF threshold level	5
RF threshold level	5
Dilution factor	1.11

Advanced

Counting Mode	Fluorescence Cell Counting
GF exposure level	5
RF exposure level	9
Min. cell size	3 µm
Max. cell size	70 µm
Declumping sensitivity	5
Min. FL intensity	0
Min. roundness	3
Dilution factor	1.11

Fluorescent Dyes for Viability Measurement

We have selected five commonly used fluorescent dyes for viability assessment:

Dye	Properties	Colors
Calcein AM	Membrane-permeable Esterase-activated dyes	Green
Fluorescein diacetate (FDA)		
Acridine Orange (AO)	Membrane-permeable nuclear dye	
Propidium Iodide (PI)	Membrane-impermeable nuclear dye	Red
Ethidium Homodimer-1 (EthD-1)		

In principle, live cells should emit a green fluorescence when stained with membrane-permeable dyes while dead cells are expected to produce a red signal when stained with membrane-impermeable dyes. These dyes function through distinct mechanisms. For instance, nuclear dyes like AO, PI, and EthD-1 become significantly brighter when bound to DNA. Additionally, both Calcein AM and FDA require esterase activity to generate a green fluorescent signal which serves as an indicator of cell vitality for assessing cell health. In our experiments, we paired these green and red dyes to determine the viability of protoplasts, thus providing a comprehensive assessment of cell viability.

Optimal Fluorescent Dye Combinations for Protoplast Viability Assessment

FDA/PI or FDA/EthD-1 is the most effective choice for protoplast viability assessment (Figure 1). Both PI and EthD-1 exhibited the ability to stain protoplast nuclei; however, one may need to adjust the RF exposure level to 9 to detect sufficient signals (Table 1).

Among the green dyes we tested, only FDA yielded a bright and reliable signal with no need of adjusting GF exposure level. Both FDA and Calcein AM rely on esterase activity to produce signals, but Calcein AM exhibited almost no signal. However, it's important to be aware that FDA can generate high background noise during prolonged incubation periods. We recommend performing cell counting immediately after staining to achieve optimal results in viability assessment. If the signal is excessively high, adjusting the GF exposure level can be applied. Moreover, we found a significant decrease in AO intensity although AO is generally expected to stain all cells.

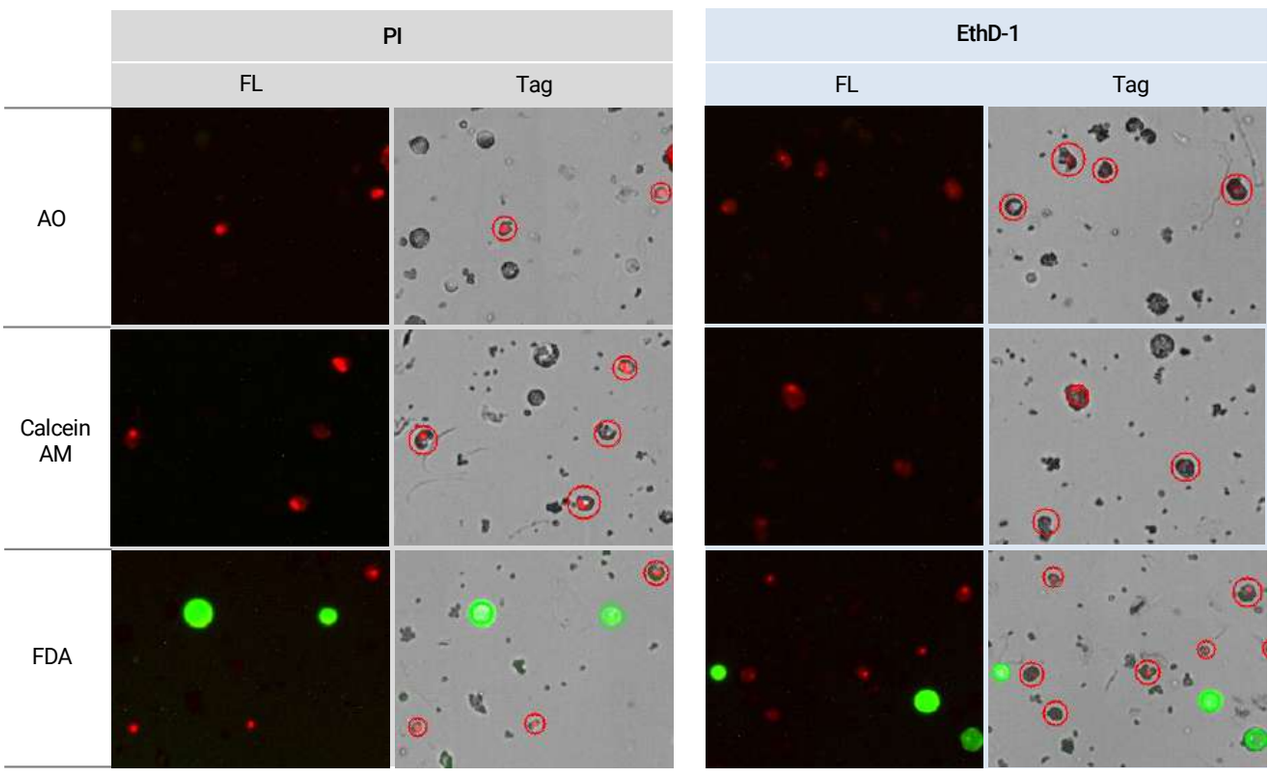

Figure 1. *The staining results of isolated protoplast. Protoplasts were effectively stained with FDA/PI or FDA/EthD-1, whereas AO/PI or EthD-1 and Calcein AM/PI or EthD-1 exhibited no green signals.*
FL: Images merged from both green and red fluorescence channels.
Tag: Composite images of all channels, fluorescent and brightfield, with identified objects marked using red and green circles. Red circles indicate dead cells, while green circles represent live cells.

Buffer-Dependent Performance of Green Fluorescent Dyes

Despite of AO's known ability to stain all cells regardless of viability, the unexpected low AO signal when used with protoplasts led to further investigation. We conducted experiments using green dyes on mammalian cells, including U937 cells, which are known to be stained by AO. The buffer was changed to either PBS or a wash medium to assess the impact on dye signals, and the default protocol was used for analysis. Indeed, there was a significant reduction in AO signal intensity in the wash medium compared to PBS (Figure 2A). Moreover, Calcein AM exhibited more consistent signals when the washing buffer was used rather than PBS, and FDA showed a minor decrease in overall intensity when the washing buffer was used (Figure 2B and 2C).

Although the precise factors contributing to the differences in dye performance remain unclear, the differences in osmolarity and pH between the wash medium and PBS may influence cells and dye performance. For example, it's important to note that AO can display varying colors at different pH levels. The difference in osmolarity made a reduction in cell size approximately 13 µm to 1-2 µm smaller. Considering these factors, these dyes can be influenced by diverse mechanisms based on the chemical conditions surrounding the cells.

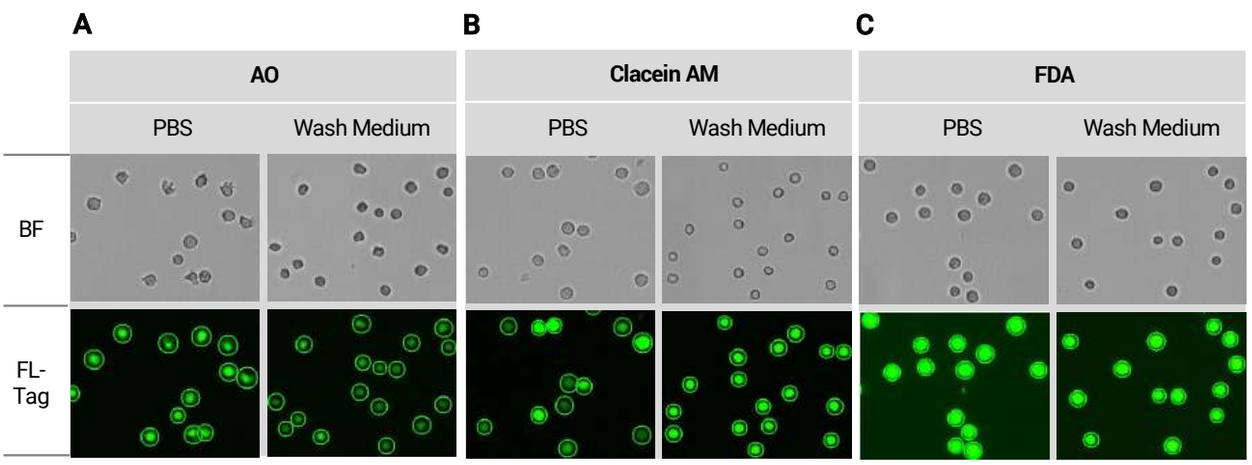

Figure 2. *The impact of different buffers on the performance of AO (A), Calcein AM (B), and FDA (C) on U937 cells. The AO signal decreased in wash medium, while Calcein AM exhibited improved performance in wash medium. The performance of FDA was not influenced by the type of buffer.*

BF: Images captured in brightfield.
FL-Tag: Composite fluorescent channel images with identified objects marked using red and green circles. Red circles indicate dead cells, while green circles represent live cells.

Conclusion

FDA/PI or FDA/EthD-1 dyes are the most effective dyes that effectively stain protoplasts. While FDA consistently produces a reliable signal, it is important to conduct cell counting immediately to minimize potential background noise during incubation. Calcein AM showed discrepancies in performance depending on the cell types and buffers. Interestingly, the AO signal exhibits a significant reduction when the wash medium is used. Additionally, both PI and EthD-1 are effective in staining protoplasts, but it requires an RF exposure level adjustment from 5 to 9. In summary, choosing the appropriate dye combinations and optimal analysis protocols of the LUNA-FX7™ Automated Cell Counter serve as a great method for protoplast viability assessment.

logos biosystems

Application Note

Automated cell counter, protoplast isolation, protoplast viability assessment, dual staining, dye assessment

Automated cell counting and viability measurement of Insect cells (SF9): Comparison of staining method

Introduction

Protein production systems such as mammalian cells, and insect cells are widely used across life sciences, biotechnology, and biomedical industry. Notably, insect cells like the SF9 cell line are extensively used in protein expression thanks to their following benefits: ease of culture, cost-effective in scale-up processes, and higher tolerance to osmolality compared to mammalian cells. However, ensuring high-quality protein production requires monitoring both cell concentration and viability to maintain healthy cell cultures and robust protein production. The LUNA-FX7™ automated cell counter can provide a convenient technique for this purpose when paired with appropriate staining methods. This study aims to recommend the optimal dyes for assessing the viability of SF9 cells after comparing different viability stains.

Staining Protocols

SF9 cells were stained with Trypan Blue Stain, 0.4% (T13001), Acridine Orange (AO)/Propidium Iodide (PI) Stain (F23001) and Fluorescein Diacetate (FDA) / Propidium Iodide (PI) Stain (F23214).

Trypan blue staining

1. Mix:
   - 10 µL TB, 0.4%
   - 10 µL cell sample
2. Load 10 µL of stained cells.
3. Perform analysis using the LUNA-FX7™.

*Default protocol

Fluorescence Staining

1. Mix:
   - 2 µL pre-mixed dyes or 1µL per individual dye
   - 18µL cell sample

2. Load 10 µL of stained cells.

3. Perform analysis using the LUNA-FX7™.

*Note: SF9 cells may express varying fluorescent intensities based on dyes used.

Please make adjustments as needed.

Dyes for Viability Measurement

We have selected four commonly used dyes for viability assessment:

Dye	Properties	Colors
Trypan Blue (TB)	Membrane-impermeable dye	Blue (BF)
Fluorescein diacetate (FDA)	Membrane-permeable esterase-activated dye	Green (FL)
Acridine Orange (AO)	Membrane-permeable nuclear dye	
Propidium Iodide (PI)	Membrane-impermeable nuclear dye	Red (FL)

Optimal dyes for SF9 cell assessment

Summary: Both TB and FDA/PI are viable for SF9 cell staining for viability measurement (Figure 1 A), but FDA/PI is the preferred option.

TB is commonly used to assess cell concentration and viability of SF9 cell. However, we recommend avoiding TB with adherent cultures as it requires applying physical manipulations during subculturing. SF9 cells are susceptible to mechanical force and agitation which can introduce cellular debris with adherent cultures. This can result in non-specific binding of TB to various particles in the sample. This issue may be less pronounced in suspension cultures.

In the evaluation of fluorescent dye combinations, U937 cells stained with the AO/PI dye combination used as a reference to validate dye performance. While AO/PI staining is generally effective for most mammalian cells, its efficacy may vary across various cell types. Indeed, SF9 cells stained with the AO/PI dye combination expressed low signal intensity. We speculate that this variation could be caused by the relatively small genome size of insect cells compared to mammalian cells. To overcome this issue, both GF and RF exposure levels were adjusted to 7 compared to the typical exposure level of 5 for mammalian cells. While PI expressed sufficient signal, the signal intensity from AO remained low after adjusting exposure levels. Although all cells were successfully labeled, weak AO signal can compromise overall performance (Figure 1B).

Considering the issues mentioned above, the most effective option for SF9 cell counting and viability assessment was FDA/PI among the options tested. FDA relies on cellular esterase activity, which is effective for various cell types such as yeast and insect cells regardless of genome sizes. FDA can express a high signal in the cell cytosol, which requires lowering GF exposure level to 1. With the appropriate LED levels – GF 1 / RF 7, the FDA/PI dye combination was the most recommended option for assessing SF9 cells.

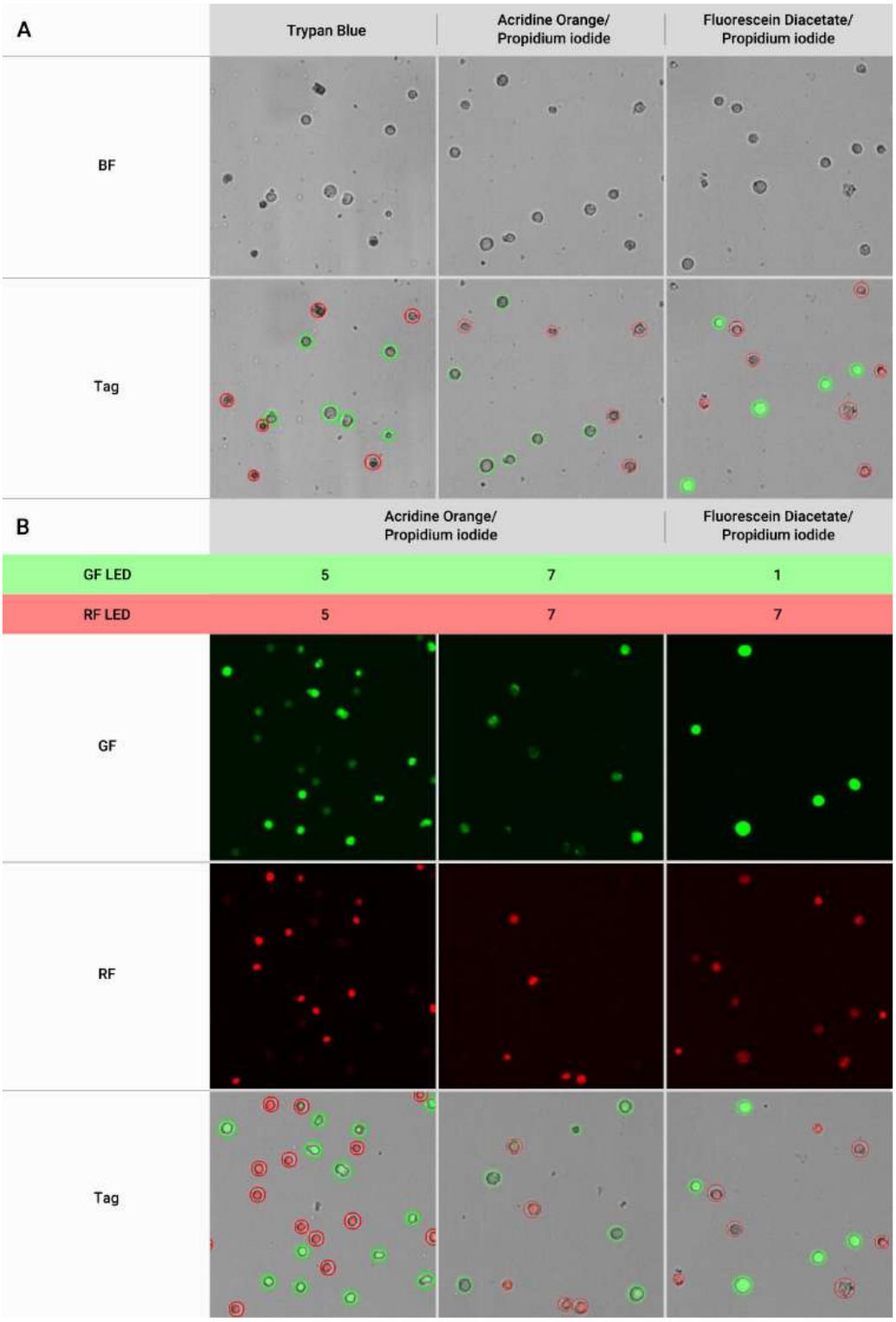

Figure 1. *(A) The staining results of SF9 using TB, AO/PI and FDA/PI. SF9 cells were effectively stained with TB and FDA/PI, while AO/PI expressed low green signals.*
(B) Comparison of staining results between SF9 cells stained with AO/PI and FDA/PI and U937 cells stained with AO/PI. Different LED levels were applied for each sample.
Tag: Composite images of all channels, fluorescent and brightfield, with identified objects marked using red and green circles. Red circles indicate dead cells, while green circles represent live cells.

Conclusion

The most recommended choice for SF9 cell viability assessment is FDA/PI due to its consistent performance and compatibility. The LED levels are required to be adjusted to GF 1 / RF 7 when using FDA/PI with SF9 cells, as FDA can generate a high signal and PI signal may be lower compared to when applied to mammalian cells. TB staining is not recommended for SF9 subculturing with adherent cultures, though this issue may be mitigated when using suspension cultures. Moreover, increasing LED exposure levels may partially address the low signals from AO after staining SF9 cells with AO/PI. However, this approach could compromise the overall performance of the LUNA-FX7™ Automated Cell Counter. In summary, choosing the appropriate dyes and optimal exposure level of the LUNA-FX7™ Automated Cell Counter can offer a great method for monitoring SF9 cell quality.

logos biosystems

Application Note

Automated cell counter, Image-based sperm cell assessment, cell counting, viability assessment, dual staining

Optimizing Sperm Cell Assessment with the LUNA-FX7™ Automated Cell Counter

Introduction

Sperm cell assessment, also known as a semen analysis, plays an important role in determining male pregnancy potential. While not a direct measure of fertility, male pregnancy significantly influences both human fertility and livestock breeding. Hence, there is high interest in sperm cell assessment.

There are methods available for sperm cell assessment using flow cytometry and image-based analysis tools. Automated cell counters like the LUNA-FX7™ automated cell counter can offer a rapid and convenient methods for assessing sperm samples using images. However, optimized analysis parameters and suitable dyes are required due to the unique morphology and properties of sperm cells compared to mammalian cells.

We conducted a comparative analysis of different fluorescent dyes such as SYBR14, acridine orange (AO), and propidium iodide (PI) to optimize sperm cell analysis using the LUNA-FX7™. This study aims to assess the efficacy of dye combinations and optimize parameters for sperm cell assessment.

Method and Material

Sample Preparation:

The cryopreserved semen samples from Korean cattle were thawed, and the buffer was replaced with PBS. The semen samples were diluted in PBS at a ratio of approximately 1:20 to 1:40, depending on the semen samples.

Dyes:

Dye	Properties	Colors	Company (Cat#)
SYBR14 (1 μM)	Membrane-permeable nuclear dye	Green	AAT Bioquest (17563)
Acridine Orange (AO)			Logos Biosystems (F23002)
Propidium Iodide (PI)	Membrane-impermeable nuclear dye	Red	Logos Biosystems (F23003)

Fluorescence Staining

1. Mix:
   - 18 µL sperm cells in PBS
   - 2 µL of dyes prepared by mixing green and red fluorescent dyes
2. Incubate for 10 min at 37℃
3. Load samples on a desirable slide.
4. Perform analysis with LUNA-FX7™.

Table 1. *The optimized parameter settings for sperm cell assessment in advanced mode of the LUNA-FX7™*

Protocol : Sperm cell counting	
Fluorescence cell counting : Advanced mode	
GF Exposure level	6
RF Exposure level	9
Min. search size	7 µm
Max. search size	30 µm
Declumping sensitivity	7
Min. FL intensity	0
Min. roundness	3
Dilution fator	1.11

Optimizing Cell Counting Protocol and Dye Combinations for Sperm Cell Assessment

Summary: Both SYBR14/PI and AO/PI staining methods are viable options for assessing sperm cell viability with optimized cell counting protocol. However, SYBR14/PI staining has shown better performance compared to AO/PI.

The default protocol for LUNA-FX7™ is already optimized for commonly used mammalian cells. However, adjustments were required for assessing sperm cells including exposure levels, cell size, and declumping sensitivity. The min search size was increased to 7 µm from 3 µm, while declumping sensitivity was adjusted to 7 from 5 to improve overall detection performance (Table 1). Additionally, exposure levels were set to GF 6 and RF 9 for both SYBR14/PI and AO/PI dye combinations.

Following these adjustments, we conducted a comparative analysis between the SYBR14/PI and AO/PI staining methods. Considering the known efficacy of the SYBR14/PI staining method for sperm cell assessment, our interest was the performance of AO/PI staining method to assess sperm cells. Our findings showed that both staining methods are viable options for sperm cell assessment (Figure 2). However, the results suggested that SYBR14 is better suited for staining sperm cells compared to AO, suggesting that the chemical properties of SYBR14 may be more suitable for this purpose.

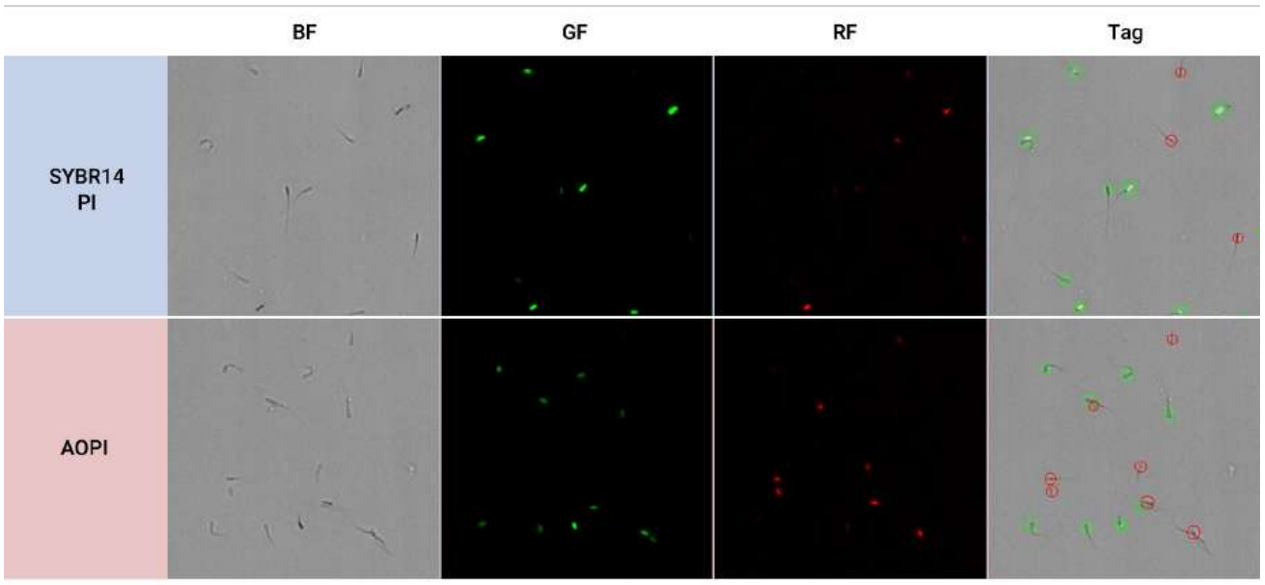

Figure 1. *Fluorescence imaging of sperm cells stained with two different dyes: SYBR14/PI and AO/PI. The montage displays BF, GF, RF, and Tag images after analysis.*
**Tag : Composite fluorescent channel images with identified objects marked using red and green circles. Red circles indicate dead cells, while green circles represent live cells.*

Conclusion

We found that both SYBR14/PI and AO/PI staining methods are viable options for assessing sperm cell viability, with SYBR14/PI exhibiting superior performance compared to AO/PI. Adjustments to exposure levels and other parameters were crucial for overall performance. Despite the LUNA-FX7™ not being specifically optimized for sperm sample analysis, it still offers a convenient and efficient solution for quick sperm assessment. Through recommended procedures and adjustments, such as sample dilution and optimization of imaging conditions, the LUNA-FX7™ can offer a simple solution for sperm cell assessment.

Featured products

LUNA-FX7™ Automated Cell Counter
by Logos Biosystems

★★★★★

"A good investment in cell counting standardization."

Jorge Ferreira, Stemmatters

★★★★★

"The cell counter is very quick, easy to use and user friendly."

Edma Loku, ECCPs

★★★★★

"Great instrument for accurate cell counting at an affordable price."

Nalini Kolar Srinivas, Uniklinikum Essen

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Additional resources

App Note

- **Yeast Cell Counting using the LUNA-FX7™Automated Cell Counter - Download app note »**
- **The Counting Accuracy of the LUNA-FX7™ Automated Cell Counter - Download app note »**
- **Automated Cell Evaluation for Single-Cell RNA-seq Analysis - Download app note »**

White Paper

- **Complete Compliance with 21 CFR Part 11 CountWire™ System for the LUNA-FX7™ - Download white paper »**

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- **Introducing the LUNA-FX7 Automated Cell Counter - Watch video »**

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