Customer Protocol

Use of Cyto-Cellect® Mouse assay kit with Viability Probe in Cyto-Mine® Chroma



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1. Introduction

This protocol describes how to perform a Cyto-Cellect® Mouse assay in combination with a Viability Probe (Fluidic Sciences) for rapid detection and selection of viable antibody secreting mouse cells using Cyto-Mine® Chroma.

The Cyto-Cellect® Mouse assay enables rapid detection of secreted mouse IgG1, IgG2a and IgG2b antibodies by utilising a Förster Resonance Energy Transfer (FRET) approach[1] enabling the identification of encapsulated IgG secreting cells. At the same time, the Viability Probe binds in a Ca^{2+}-dependent manner to any cells undergoing apoptosis or necrosis. This allows for exclusion of these cells from the selection during sorting, thereby improving the quality of sorted cells, by eliminating non-viable secreting cells from the sorted cell pool. This protocol describes the use of both exclusion and inclusion gates in Cyto-Mine® Chroma, to exclude cells stained with the viability probe (dead/dying), but include those positive for antibody secretion ($FRET^+$; **Figure 1**).

By multiplexing IgG-specific FRET detection with a Viability Probe and performing the assay using the Cyto-Mine® Chroma high-throughput, automated single-cell screening platform, this method provides a powerful and efficient technique for isolating high-quality secreting cells beneficial for Antibody Discovery (AbD) campaigns.

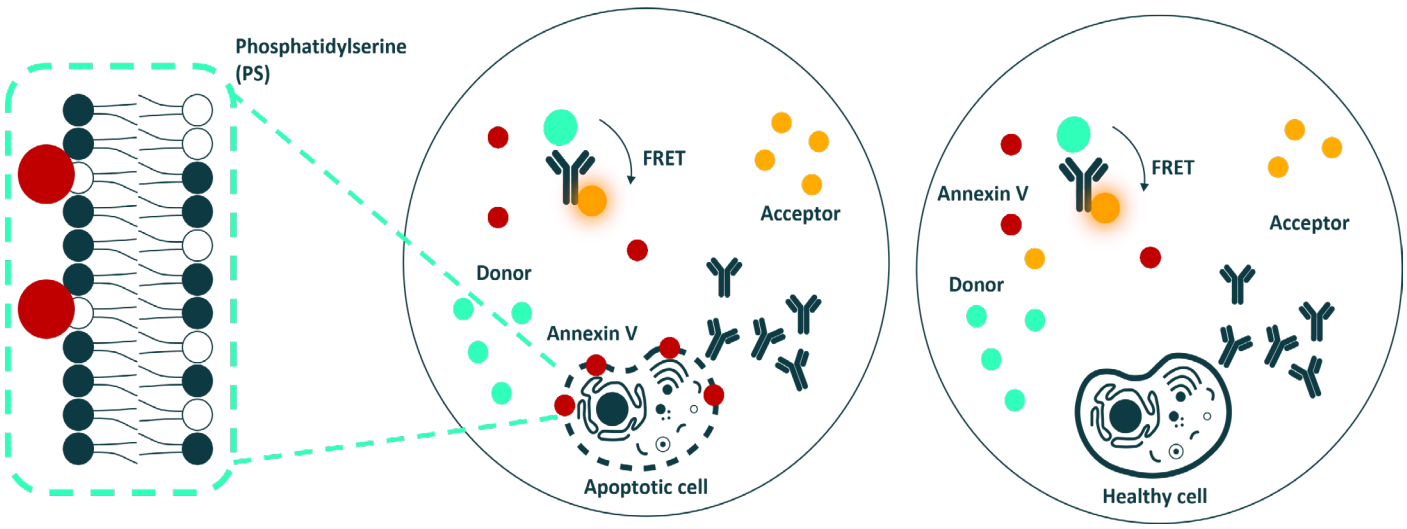


Figure 1. Schematic diagram for Cyto-Cellect® Mouse and Viability Probe assay on Cyto-Mine® Chroma. This method directly evaluates secretion by using FRET, whereby the FRET donor is paired with a FRET acceptor. The two FRET probes bind and form a three-molecule complex only in the presence of mouse IgG, providing sensitive and highly specific detection of secretion at single cell level. Simultaneous detection of apoptotic or dying cells by binding of the viability probe to the membrane-integrity compromised cells provides a readout for cell viability.

2. Equipment and Materials

Equipment

Item	Supplier	Catalogue Number
Class II Microbiology Safety Cabinet	Scanlaf Mars 1200 or equivalent	91200-LAB
NanoDrop™ 2000/2000c Spectrophotometers	Thermo Fisher Scientific	ND-2000
NucleoCounter® NC-3000™	ChemoMetec	970-300
Centrifuge with microtiter plate rotor- 5804R	Eppendorf or equivalent	022623508
Multipette M4	Eppendorf or equivalent	4982000012
Pipettes (10 µL , 20 µL, 200 µL, 1,000 µL)	Gilson or equivalent	N/A
Cyto-Mine® Chroma	Fluidic Sciences	S008

Software

Item	Supplier	Catalogue Number
Cyto-Mine® Studio Suite – Instrument Control	Fluidic Sciences	N/A
Cyto-Mine® Data Analysis Software (v2.6.20)	Fluidic Sciences	N/A
FlowJo™ (updated version)	Becton, Dickinson and Company	N/A

Consumables

Product Name	Supplier	Catalogue Number
Micro centrifuge tubes 1.5 mL or 2 mL	Eppendorf or equivalent	N/A
Pipette tips (10 µL , 20 µL, 200 µL, 1,000 µL)	Various suppliers	N/A
10 mL Stripettes	Costar or equivalent	4488
NC Slide A8	ChemoMetec	942-0003

Cell strainer (30 - 70 µm mesh)	Sysmex	04-004-2326
25 mL reservoir	Integra	4382
50 mL tubes	SLS	352070
Cyto-Surf® A	Fluidic Sciences	C303
Cyto-Surf® B	Fluidic Sciences	C304
Cyto-Cartridge®	Fluidic Sciences	C302

Reagents

Product Name	Supplier	Catalogue Number
Cyto-Cellect® Mouse IgG Detection kit	Fluidic Sciences	C312
Viability Probe	Fluidic Sciences	C315
RPMI-1640	ATCC	30-2001
Hybridoma 357-101-4	ECCAC	92030603
Glutamine	Merck	G7513-100ML
Solution 13 (AO-DAPI)	Chemometec	910-3013
Foetal Bovine Serum (FBS)	Gibco	A5670701
OptiPrep™ Density Gradient agent	Sigma-Aldrich	D1556
Poloxamer 188 Non-ionic Surfactant	Thermo Fisher Scientific	24040032

3. Method

3.1 Important notes before the start of the experimental work

- Make sure all pipettes used have a valid calibration.
- Use best practice when pipetting, to ensure precision and accuracy.
- All steps involving handling any stock solution should be carried out in a Class II Microbiology Safety Cabinet (MBSC), except centrifugation and shaking operations (if relevant). During centrifugation and shaking all material must be contained within sealed containers to maintain sterility.
- Make sure to use the best aseptic practice possible throughout the operations.

3.2 Assay performance on Cyto-Mine® Chroma

This protocol provides information regarding experimental conditions and methods to perform sample preparation and run Cyto-Mine® Chroma for users that are familiar with operating the Cyto-Mine® Chroma. For more information, please refer to the Cyto-Mine® Chroma User Manual. Please note that the experiment outlined in this protocol requires the 4-laser 4-detector Cyto-Mine® Chroma and cannot be run using the 1- laser 2-detector Cyto-Mine®.

Experimental Procedure

Step	Operation	Details
1	Prepare the Cyto-Cellect® Mouse and Viability Probes	• Thaw one vial of Cyto-Cellect® Mouse containing the FRET probes and one vial of Viability Probe • Centrifuge both vials briefly in a microcentrifuge to collect the reagents at the bottom of the tube • Keep both vials cold until ready to use
2	Cyto-Mine® Chroma Set Up **System Warm Up**	• Start Cyto-Mine® GUI software and start System Warm Up step (Hands Off Step) ***Note:*** *To save time, run the System Warm Up before preparing Antibody Secreting Cells (ASC)*

<table>
<tr><th>Step</th><th>Operation</th><th>Details</th></tr>
<tr><td>3</td><td>Prepare Encapsulation Medium</td><td>
Prepare the Encapsulation Medium as follows (2 mL total):
<table>
<tr><th>Component</th><th>Stock Conc.</th><th>Final Conc.</th><th>Volume (µL)</th></tr>
<tr><td>Culture medium</td><td>N/A</td><td>74% v/v</td><td>1480</td></tr>
<tr><td>Opti-Prep</td><td>60% w/v</td><td>16% v/v</td><td>320</td></tr>
<tr><td>Pluronic™ F-68</td><td>10%</td><td>10% v/v</td><td>200</td></tr>
</table>
Note: Protocol is shown for 2 mL of Encapsulation Medium to accommodate for sample loss during the preparation. Only 1 mL of Encapsulation Medium will be required for a single Cyto-Mine® experiment
Do not include any media that is expected to contain secreted antibodies (i.e. "conditioned" media) as the presence of antibody in media will affect the FRET assay
</td></tr>
<tr><td>4</td><td>Prepare Antibody Secreting Cells (ASC)</td><td>

Resuspend ASC (357-101-4) in a tissue culture flask
To count cells using NucleoCounter® NC300™:
- Take out 19 µL of cell suspension
- Add 1 µL Solution 13 (AO-DAPI)
- Add 10 µL of this mix into a glass slide of the NucleoCounter® NC300™
- Measure cell number and viability
Calculate the volume of cells corresponding to 0.3 x 10^6 cells (for final cell concentration of 3 x 10^5 cells in 1.5 mL of sample)
Transfer the calculated volume (+10% to allow for cell loss) into a clean 1.5 mL Eppendorf tube
Spin cells down at 300 x g for 5 minutes at room temperature
Gently remove medium from the tube without disturbing the pellet
Gently resuspend the pellet in 1 mL of fresh culture medium
Spin cells down at 300 x g for 5 minutes at room temperature
Gently remove medium from the tube without disturbing the pellet
Gently resuspend the pellet in 100 µL of Encapsulation Medium
Add the cell suspension to the Viability Probe vial (Step 1) and mix
Incubate for 15 minutes at room temperature in the dark

</td></tr>
</table>

		• Add 900 µL of the Encapsulation Medium (**Step 3**) to the sample • Take the resulting 1 mL of cell sample and add to the vial containing Cyto-Cellect® Mouse Probes • Gently mix the sample by pipetting up and down • Filter the mixture through a 30 µm cell strainer to eliminate any cell clumps or aggregates and collect into a sterile 1.5 mL Eppendorf tube. • Determine the cell concentration (cells/mL) and viability (%) in the final sample using the NucleoCounter® NC300™ ***Note:*** *Cells are kept at room temperature during the washes to avoid 'cold capture'.*
5	**Load** cells into Cyto-Cartridge®	• Load 1 mL of prepared sample into the Cyto-Cartridge®, then place the Cyto-Cartridge® into the Cyto-Mine® Chroma • Follow the instructions shown on screen ***Note:*** *Load the Cyto-Cartridge® aseptically in a Biosafety Cabinet.* ***Cyto-Cartridge® is single-use*** *and disposable to prevent any cross-contamination.* *There will be certain sample loss due to sample filtering, but the filtered sample will have sufficient volume for the generation of 1.5 x 10^6 droplets.*
6	Cyto-Mine® Chroma Set Up & Run	<u>Experimental Parameters</u> • Number of picodroplets to generate: 1.5 x 10^6 • Time and temperature for incubation: 120 minutes at 37°C • Type of microtiter plates (MTPs) for dispensing: 96-Well Plate • Input cell density loaded based on the information obtained from **Step 4** • Set up cameras focus and other parameters (please refer to Cyto-Mine® Chroma User Manual for further information) <u>Cyto-Cartridge® priming</u> • Hands-off step • Time: 30 minutes <u>Picodroplet generation/cell encapsulation</u> • Hands-off step • Picodroplet size: 450 pL

- Time: 36 minutes

Picodroplet incubation/antibody secretion

- Hands-off step
- Incubation temperature: 37 °C
- Incubation time: 2 hours

Note: *based on expected cell productivity, a shorter or longer incubation time can be applied*

Flow Stabilization before Sorting

- Wait until this step is automatically finished (5 minutes)

Note: *Flow of picodroplets normally stabilizes following 3-4 minutes of run.*

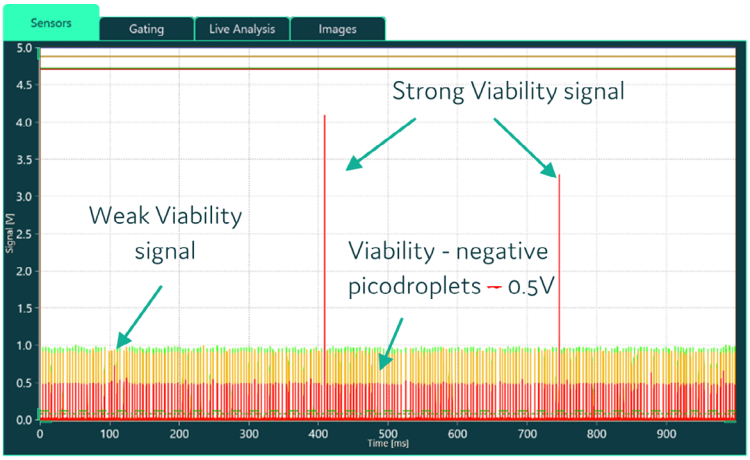
Sorting

Lasers and Detectors Required:

Probe	Laser	Detector
Cyto-Cellect® Mouse Donor	Blue : Exc.488 nm	Green: Em.520/18nm
Cyto-Cellect® Mouse Acceptor	N/A	Orange: Em. 605/20 nm
Viability	Red: Exc. 638 nm	Far-Red: Em. 678/30 nm

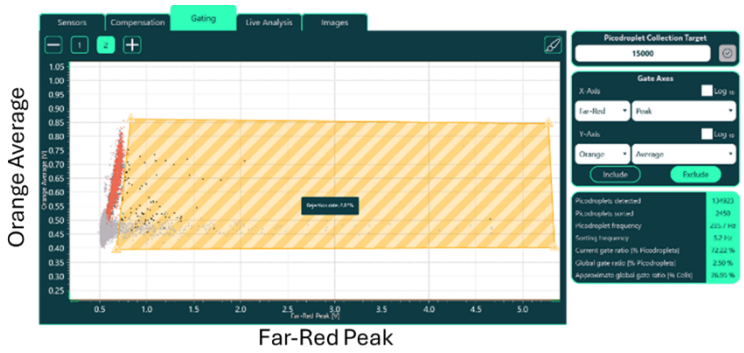
PMT Gains Set up:

- Set up PMT Gains to achieve the readout peak (Y- axis=Signal) at the height of 1-1.5 V for Orange, Green & Far-Red. Example of background, low and high Viability signal is shown in the figure below. **High Viability signal= Dying/dead cells**
- Detection trigger: Green above Threshold
- Auto-Thresholds for detection of fluorescence
- Max Picodroplets to collect: 15,000



Gate 1: Excluding non-viable cells

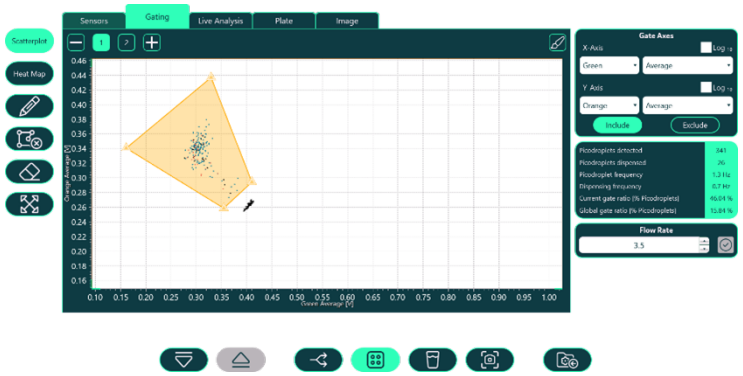
- Non-viable cells are Viability$^+$ (Far-Red Peak$^+$) and will be detected as localized fluorescence in the Far-Red PMT
  - X-Axis: Far Red Peak
  - Y-Axis: Orange Average
- Define gate logic as **"Exclude"**



Gate 2: Including secreting cells

- Secretion is detected by FRET probes
  - X-Axis: Green Average (Donor)
  - Y-Axis: Orange Average (Acceptor)
- Define gate logic as **"Include"**

		Acquire data: • Sort about 15,000 positive droplets (Viability^{-} and FRET^{+}) • The data acquired from this will be used for further analysis (**see Data Analysis and Interpretation**)
7	Dispensing flow stabilization	• Start Flow Stabilization. It will take 4-10 minutes for droplets to appear • Slowly reduce flow rate from 20 → 15 → 10 → 5 µL/hr with 30 seconds delay between changing each flow rate
8	Prepare 96-Well plate for dispensing	Label 96-Well plates with the following information: • Cell Type • Date • Instrument ID • Plate Number For subsequent cloning: Use a multi-channel pipette to pipet 30 µL of fresh culture media into each well ***Note:*** *For dispensing into 384-well plates pre-fill the plates with 6 µL of fresh Culture Media into each well.* *For dispensing into PCR plates please refer to the Cyto-Mine® Chroma User Manual or contact Fluidic Sciences' customer support.*
9	Dispensing	• Adjust flow rate so Picodroplet frequency is ≤ 1Hz. • Set up Gains and Thresholds for detection of fluorescence signal, similar to **Step 6** for Sorting • On the 'Gating' tab ensure the following is set up: X-Axis: Green Average Y-Axis: Orange Average • Draw a gate on scatterplot for the FRET^{+} population • Dispense the FRET^{+} population into 96 Well plate for subsequent culturing or PCR analysis

		
10	Post-Dispensing top-up of plates	• Following dispensing, incubate the plates in a 37°C incubator, 5% CO_2 for 15 minutes • After 15 minutes incubation, use multi-channel pipette to top-up 70 µL of fresh complete media to all wells (to a final volume of 100 µL) • Culture cells for 2-weeks to allow single cells grow into colonies ***Note:*** *This step ensures that the oil from the dispensed droplets evaporate from wells.* *For 384-well plates, top up with 44 µL of fresh Culture Media.to a final volume of 50 µL.* *For scanning the plates, centrifuge the plates for ~10 seconds at 250 x g prior to imaging.*
11	End of run	*When the plates are dispensed, click "End run" and follow instructions as prompted by Cyto-Mine® Chroma*

4. Data Analysis and Interpretation

This section explains how to use FlowJo™ to analyse Cyto-Mine® Chroma data, assess sample quality, and determine the number of viable IgG-secreting cells. For viewing in-run scatter plots or analyzing droplet images, use the Cyto-Mine® Data Analysis software. For more details on the use of the Cyto-Mine® Data Analysis software, please refer to the Cyto-Mine® Chroma User Manual

4.1 Using FlowJo™ to Identify the Dead/Dying Population

Using FlowJo™ to Identify the Dead/Dying Population

- Open the experiment file using Cyto-Mine® Data Analysis software
- Export the sorting data as .csv file
- Import the sorting .csv file into FlowJo™
- To determine the viability of cell sample sorted and whether these did show FRET response:

  Plot Far-Red Peak (X-axis) vs Orange Average (Y-axis; **Figure 2A**)

  Gate the Far-Red positive population to identify dead/dying cells (Viability$^+$; **Figure 2A**)

  Plot this gated population as Green Average (X-axis) vs Orange Average (Y-axis; **Figure 2B**)

  Identify and gate FRET positive population as shown in **Figure 2B**

The data in **Figure 2A** indicates that 1.97% of droplets contained cells stained with the Viability Probe, indicating apoptotic or necrotic cells. Of these, approximately 9% displayed a FRET signal (**Figure 2B**).

Calculating the Percentage of Dead/Dying IgG-Secreting Cells

To determine the proportion of loaded cells that were dead/dying, use the following equation:

$$\%\ Dead/dying\ cells\ =\ \frac{\%\ Gated\ \ Population}{\%\ \ Occupied\ \ Droplets\ \ (Poisson)} \times 100$$

Example calculation:

- % Gated population: 1.97% (**Figure 2**)
- % Occupied Droplets (Poisson): 14.6% of droplets contain ≥1 cell (example based on 0.35 × 10^6 loaded cells and 450 pL droplets)

$$\%\ Dead/dying\ cells\ =\ \frac{1.97}{14.6} \times 100 =\ \ 13.49\%$$

The analyzed sample showed 86.5% viability at sorting. Approximately 9% of the Viability Probe-positive population showed a FRET signal, consistent with a population in early to mid-apoptosis

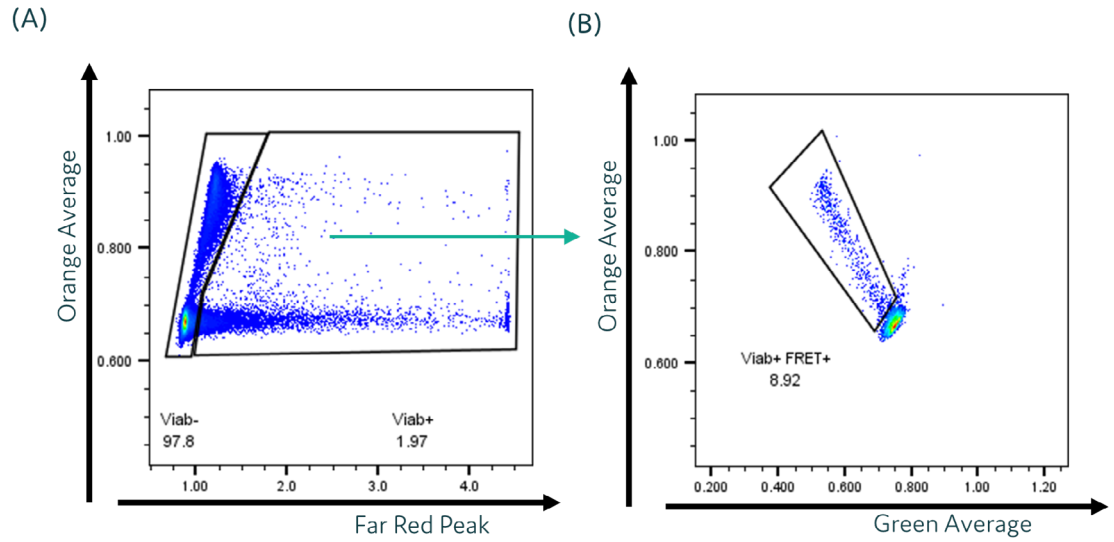


Figure 2. Gating strategy to determine IgG secretion in unhealthy population. The analysis of gating strategy to determine the percentage IgG secreting population in unhealthy population is shown. (**A**) Droplets sorted were analyzed for staining with Viability Probe. Data shows gating of droplets containing dead/dying cells (Viab$^+$) and droplets that are negative for staining with the Viability Probe (Viab$^-$). (**B**) IgG secretion (FRET$^+$ signal) was analyzed in Viab$^+$.

4.2 Determine Number of Viable, IgG Secreting Cells

The sorting data can also be used to quantify the proportion of viable cells exhibiting detectable FRET signal.

Using FlowJo™ to Identify the FRET-Positive Viable Population

- Open the experiment file using Cyto-Mine® Data Analysis software
- Export the sorting data as .csv file
- Import the sorting .csv file into FlowJo™
- To determine the percentage FRET-positive population within the viable cell gate:

  Plot Far-Red Peak (X-axis) vs Orange Average (Y-axis; **Figure 3A**)

  Gate the Far-Red low/negative population to exclude dead/dying cells (Viab$^-$; **Figure 3A**)

  Plot this gated population as Green Average (X-axis) vs Orange Average (Y-axis; **Figure 3B**)

  Identify and gate FRET positive population as shown in **Figure 3B**

Calculating the Number of Viable IgG-Secreting Cells

To determine number of viable IgG secreting cells that the 4.78% represents, use the following equations:

$$\%\ gated\ Viable\ ASC\ =\ \frac{\%\ Gated\ Population}{\%\ Occupied\ Droplets\ (Poisson)} \times 100$$

$$No.\ Viable\ ASC\ =\ \%\ Viable\ ASC \times\ Loaded\ Cell\ \ No.$$

Example calculation:

• % gated population: 4.78% of 97.8% (**Figure 3**)

• % Occupied Droplets (Poisson): 14.6% of droplets contain ≥1 cell (example based on 0.35 × 10^6 loaded cells and 450 pL droplets)

• Loaded cell number: Cell number loaded onto Cyto-Cartridge® for data presented was 0.35 x 10^6

$$\%\ Gated\ Viable\ ASC\ =\ \frac{(4.78\ x\ 0.978)}{14.6} \times 100 =\ 32.02\%$$

$$No.\ Viable\ ASC\ =\ 0.32\ \times\ \left(0.35\ x\ 10^6\right) =\ 0.11\ x\ 10^6$$

Based on the observed 32% FRET-positive rate, the sample contained an estimated 0.11 × 10^6 IgG-secreting cells out of the 0.35 × 10^6 cells initially loaded.

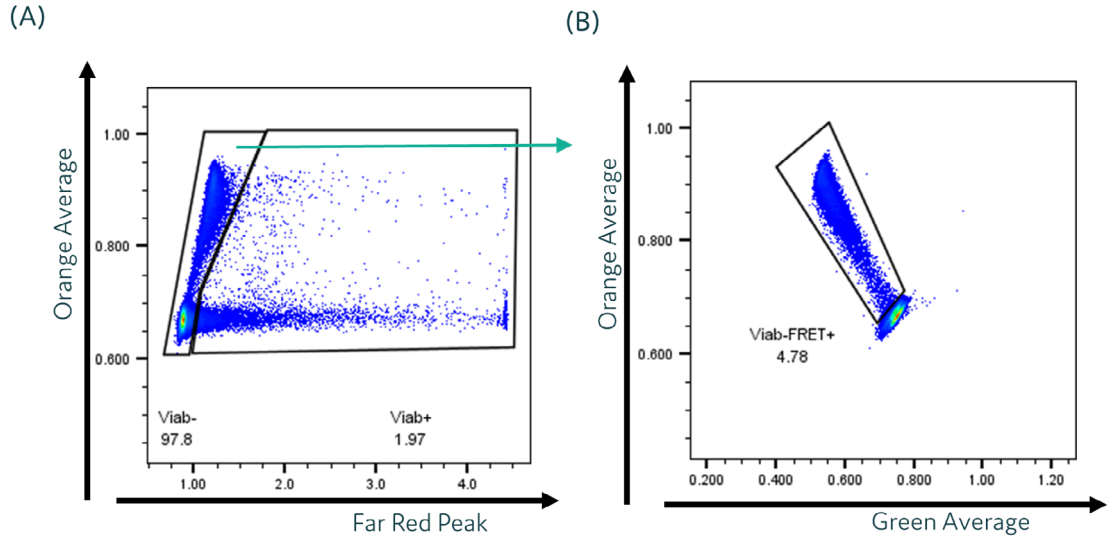


Figure 3. Analysis of sorting data for antibody secretion in viable population. The figure shows the gating strategy to determine the percentage of IgG secreting population in the live population. (**A**) Droplets sorted were analyzed for staining with Viability Probe. Data shows gating of droplets containing dead/dying cells (Viab$^+$) and droplets that are negative for staining with the Viability Probe(Viab$^-$). (B) IgG secretion (FRET$^+$ signal) was analyzed in the healthy population (Viab$^-$). Data shows percentage FRET$^+$ population in droplets containing viable and heathy cells.

5. Experiment Optimization and Troubleshooting

Step	Specific Issue	Solution/Optimization
Viability Probe detection in Cyto-Mine®	No Viability Probe stained ($Viab^+$) population	• The binding of the viability probe to cells is dependent on the presence of Ca^{2+} in the medium • We have verified probe binding in several commonly used media, including RPMI, DMEM and CD-CHO • If there is any uncertainty, we recommend testing probe binding in the medium of interest by staining heat-shocked cells with the Viability Probe and assessing the signal using fluorescence microscopy or flow cytometry
FRET assay in Cyto-Mine®	Low proportion of positive picodroplets / no positive population	• Prior to the Cyto-Mine® run assess the compatibility of the Cyto-Cellect Mouse® assay kit with the secreted antibody by testing the cell culture supernating using FRET assay in a plate reader • Make sure to avoid any Ab contamination in the encapsulation media (i.e. conditioned media) and to remove any secreted antibody from your cell sample by washing cells during sample preparation • Ensure no cell loss in the final Cyto-Mine® sample • Ensure all required additives (Opti-Prep, PF-68 and FRET probes) have been added to Encapsulation medium • Test cell productivity of cells using a different method (ELISA, flow cytometry).
FRET assay in Cyto-Mine®	High level of fused / destabilized droplets	• Make sure your Encapsulation Media does not contain any detergents or more than 10% FBS.

Notes

Notes

Notes



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Cyto-Mine® and Cyto-Mine® Chroma: CLASS 1 LASER Product – BS EN / IEC 60825-1:2014

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