Customer Protocol

Antigen Specific anti-mouse IgG (Fc) Bead-Based Assay For Use With Cyto-Mine® Chroma



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1. Introduction

Antigen-specific assays are essential for identifying target-specific antibodies early in the therapeutic discovery process, as they enable early validation of antibody binding and help streamline the workflow. Combining these assays with Cyto-Mine® Chroma, an automated high-throughput picodroplet platform, provides a powerful approach for isolating rare, high-value antigen-specific cells at an early stage of discovery.

Here, we describe the development and implementation of anti-mouse IgG (Fc) bead-based assays for detection of antigen-specific antibodies for use in Cyto-Mine® Chroma. We assess the sensitivity of the bead-based format for detecting rare events and compare this method to the previously described Förster Resonance Energy Transfer (FRET) - based antigen specific assay (**Figure 1**).

In the bead-based assay, the anti-mouse IgG coated beads accumulate secreted antibody on their surface (**Figure 1A**), allowing capture of fluorescently labelled antigen by target specific antibodies. The resultant complex formation on the bead results in a localized fluorescent signal detectable by Cyto-Mine® Chroma.

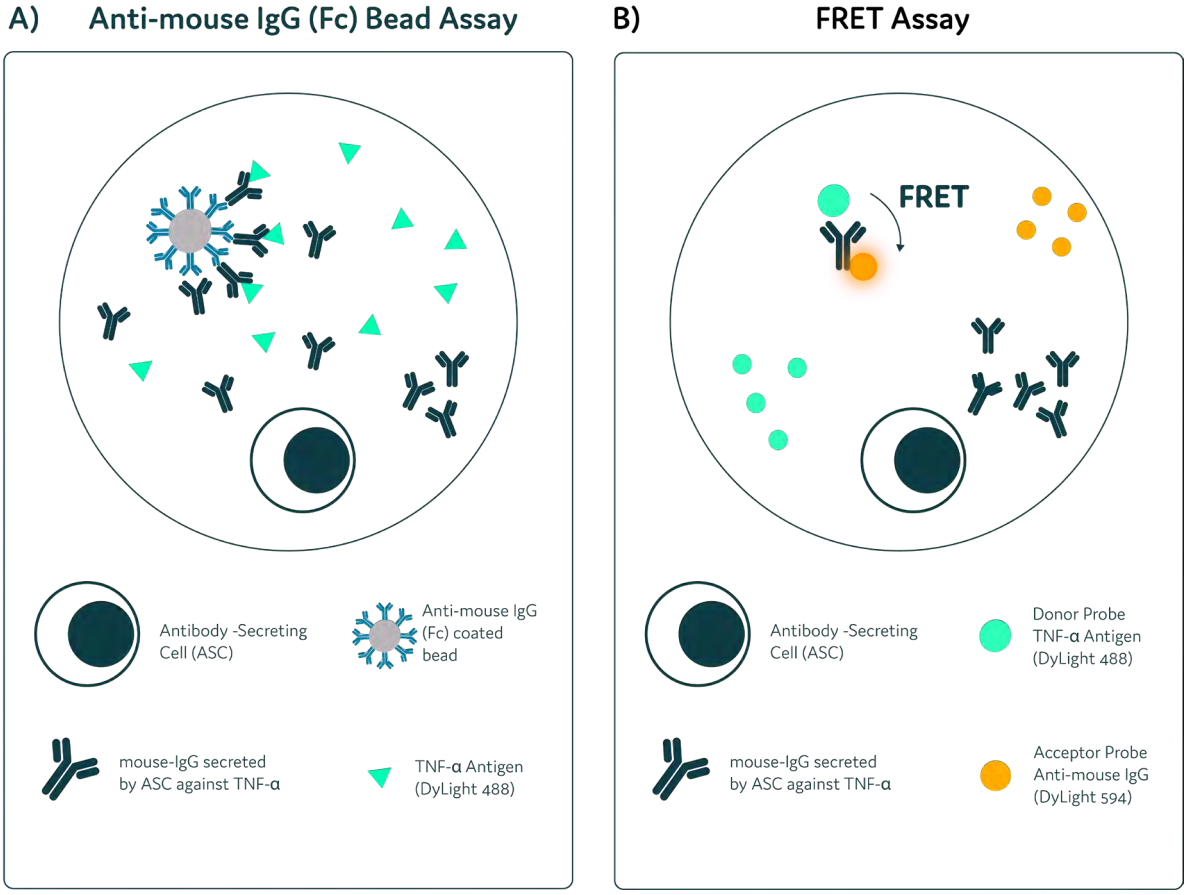


Figure 1. Schematic Diagram for Antigen-Specific Bead and FRET Assays: A) Anti-mouse IgG bead-based assay: Antibody-secreting cells (ASC) are co-encapsulated with anti-mouse IgG -coated beads and fluorescently- labelled TNF-α antigen. Upon secretion, anti–TNF-α antibodies bind to the bead and simultaneously interact with the labelled antigen, resulting in the formation of a localized fluorescent complex on the bead surface **B)** Antigen-Specific FRET assay: ASC are co-encapsulated with fluorescently-labelled donor (TNF-α antigen) and acceptor (anti-mouse IgG). Upon binding of the antigen-specific antibody to the donor and acceptor, these are brought into proximity, resulting in a FRET signal. This interaction generates a measurable dispersed fluorescence signal, indicating the presence of antigen-specific antibody in the picodroplet.

In this protocol, TNF-α is used as the model antigen to validate the performance of the bead-based screening workflow. The assay capability to isolate rare hits is demonstrated by screening a population of anti-TNF-α specific antibody-secreting hybridoma cells (357-101-4 cells) spiked into a background of irrelevant A20 cells at 1:100, respectively. A20 is a B lymphocyte cell, which secretes and expresses surface IgG[1], thus the spike-in technique simulates a culture containing 1% antigen-specific ASCs in the total population of ASCs. Picodroplets containing the relevant ASCs that generated a localized, bead-bound fluorescent complex are identified by the Cyto-Mine® Chroma's integrated optics and the positive droplets are sorted. We demonstrate the bead-based antigen-specific assay can detect rare antigen specific ASC with similar performance to the FRET assay.

While TNF-α is used as the model system, the methodology is inherently adaptable, providing a framework for customizing the assay to other antigens relevant to specific discovery campaigns.

The Cyto-Mine® Chroma platform can exploit bead-based formats, reporter cell-based formats, and FRET-based formats to provide exceptional flexibility across various antigen types and labelling requirements. Alternative protocols for detecting antibody binding to soluble antigen (FRET assay)[2] or antigen-specific cell-binding assay[3] are available on spherebio.com.

2. Equipment and Materials

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™	Becton, Dickinson and Company	N/A

Equipment

Item	Supplier	Catalogue Number
Class II Microbiology Safety Cabinet	Scanlaf Mars 1200 or equivalent	N/A
Plate Reader	BMG Labtech CLARIOstar®plus or equivalent	N/A Note: The plate reader must support absorbance at 500 nm.
EVOS M7000 Imaging System	Thermo Fisher Scientific	AMF7000

Light Microscope	Carl Zeiss Micoscopy GmbH	Primovert
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
Centrifuge 5424R	Eppendorf	N/A
Multi-channel Pipette 1250 µL	Integra	4624
Multi-channel Pipette 300 µL	Integra	4623
SYNENTEC NYONE Plate Scanner	SYNENTEC GmbH	NyOne V 2.0
Eppendorf Tube Rotator	Stuart	SB3
Pipettes (2 µL, 10 µL, 20 µL, 200 µL, 1,000 µL)	Various suppliers	N/A
Cyto-Mine® Chroma	Fluidic Sciences	S008

Consumables

Product Name	Supplier	Catalogue Number
Corning® 384 Well Plates, Black (Low volume)	Corning	4511
96 Well flat-bottom microtiter plates	Corning	3595
Micro centrifuge tubes 1.5 mL or 2 mL	Eppendorf or equivalent	N/A
Protein LoBind® Eppendorf Tube (1.5 mL)	Eppendorf	EP0030108116
Protein LoBind® Eppendorf Tube (2 mL)	Eppendorf	EP0030108132
Pipette tips (2 µL, 10 µL , 20 µL, 200 µL, 1,000 µL)	Various suppliers	N/A
Integra Pipette tips (1250 µL)	Integra	3745
Integra Pipette tips (300 µL)	Integra	3735

10 mL Stripettes	Costar or equivalent	N/A
NC Slide A8	ChemoMetec	942-0003
CellTrics 10 µm Disposable Filter	Sysmex	04-004-2324
Millex™ PVDF syringe 0.22 µm Filter	Merck Life Science	SLGV004SL
Single Use Syringe Injekt-F, 1ml, sterile	Braun Petzold	PZ9166017V
25 mL Reservoir	Integra	4382
50 mL Falcon Tubes	SLS	352070
15 mL Falcon Tubes	SLS	352095
Cyto-Surf® A	Fluidic Sciences	C303
Cyto-Surf® B	Fluidic Sciences	C304
Cyto-Cartridge®	Fluidic Sciences	C302

Reagents

Product Name	Supplier	Catalogue Number
Human TNF-alpha Recombinant Protein	Thermo Fisher Scientific	300-01A-500UG
DyLight® 488 NHS Ester	Thermo Fisher Scientific	46402
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190094
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	10977035
Goat anti-Mouse IgG (Fc) Coated Polystyrene Particles (5.0% w/v; 6.0 µm)	Spherotech	MPFc5-60-5
C-CHIP Disposable Hemocytometer	NanoEntex Inc.	DHC-N01
357-101-4 Cells Complete Media RPMI1640 9.4 % FBS 1% L-Glutamine	 Gibco Gibco Gibco	 Cat. A1049101 Cat. A5670701 Cat. 25030-81
Goat Anti-Mouse IgG Fc (DyLight® 594)	Abcam	Ab97017

Anti-TNF-α antibody [52B83]	Abcam	Ab1793
FcR Block – Purified Rat Anti-Mouse CD16/CD32	BD Biosciences	553141
Viability Probe	Fluidic Sciences	C315
DMSO	Merck	67-68-5
Hybridoma 357-101-4	ECCAC	92030603
A20 Cells	ATCC	TIB-208
Solution 13 (AO-DAPI)	Chemometec	910-3013
OptiPrep™ Density Gradient agent	Sigma-Aldrich	D1556
Poloxamer 188 Non-ionic Surfactant (10%)	Thermo Fisher Scientific	24040032

3. Method

The bead-based assay is developed in a stepwise workflow to ensure optimal performance on the Cyto-Mine® Chroma (**Figure 2**).

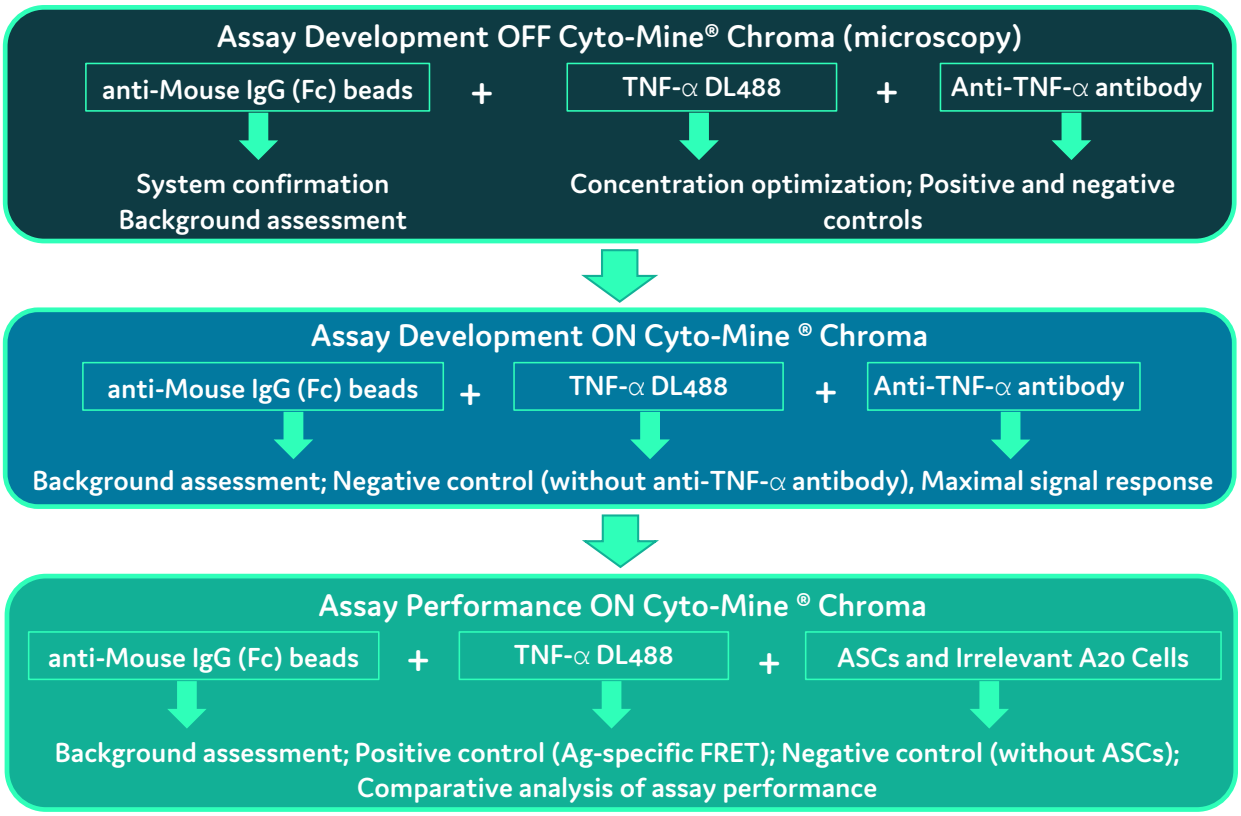


Figure 2. Workflow for the development and optimization of an antigen-specific bead-based assay. The chart outlines the key steps involved in the development and optimization of the antigen-specific bead-based assay for Cyto-Mine® Chroma.

Antigen labelling is extensively described in our antigen-specific FRET protocol[2] and will not be detailed in this protocol. Firstly, assay optimization is performed off Cyto-Mine® Chroma using microscopy to confirm assay configuration, background signal, positive and negative controls, and detection molecule concentration. The optimal condition is then evaluated on Cyto-Mine® Chroma to assess on-system background signal and maximal signal response. Finally, the complete assay is performed with ASCs to assess overall performance of the assay comparative to FRET.

3.1 Assay Optimization and Development OFF Cyto-Mine® Chroma (microscopy)

Aim

This optimization step aims to:

- Determine autofluorescence signal associated with the beads.
- Determine the extent of non-specific binding of the antigen and the beads.
- Determine the optimal concentration of antigen.

Experimental Design

The optimization conditions evaluated in this step are summarized in the table below. All conditions were tested in duplicates. Recombinant anti-TNF-α was assessed at 0.5 and 4 µg/mL; 0.5 µg/mL has been reported to approximate the IgG secreted by a single ASC over a 2-hour incubation[4,5]. Although only two antigen concentrations were evaluated here, assessment of a broader concentration range may further support optimization.

Condition	1	2 & 3	4	5 & 6
Beads	1.0 x 10^5	1.0 x 10^5	1.0 x 10^5	1.0 x 10^5
TNF-α DL488	-	1 µg/mL	2 µg/mL	2 µg/mL
anti-TNF-α IgG	-	0.5 or 4.0 µg/mL	-	0.5 or 4.0 µg/mL
Purpose	**Negative control** · Background from beads	**Positive control** · Testing sensitivity of the assay · Condition with 4.0 µg/mL anti-TNF-α IgG	**Negative control** · Assess non-specific binding of TNF-α DL488 to beads	**Positive control** · Testing sensitivity of the assay · Condition with 4.0 µg/mL anti-TNF-α IgG

Table 1. Experimental design conditions. This table presents the conditions set up for the OFF Cyto-Mine® Chroma assay development.

Step	Operation	Details
1	Prepare beads	• Mix the bead stock thoroughly. • Make 1 mL bead suspension at 1/100 dilution in DPBS. • Count beads using a C-CHIP hemocytometer under a light microscope. • Calculate the beads concentration (beads/mL) using the formula below: $\frac{beads}{mL} = \frac{beads\ \ in\ \ 4\ \ corner\ \ squares}{4} \times dilution\ \ factor \times 10^4\ \ \left(volume\ \ factor\right)$ • Transfer the volume of beads required for 2.0 x10^6 beads into a LoBind Eppendorf tube and add up to 1 mL of complete media. Divide the final volume equally into two 1.5 mL LoBind Eppendorf tubes, each with 500 µL. • Centrifuge the tubes at 5,000 g for 5 minutes. • Carefully remove the supernatant without disturbing the pellets. • Resuspend both pellets in a total of 1 mL of complete media to achieve a final 2.0 x10^6 beads/mL. ***Note:*** • *Use 1.5 mL Protein LoBind Eppendorf tube for any steps involving beads.* • *To minimize beads sticking to the tube walls, keep the beads suspension to a maximum of 500 µL per 1.5 mL LoBind Eppendorf tube during centrifugation.*
2	Prepare the final reaction	• Label 6 x 1.5 mL LoBind Eppendorf tubes for all the conditions (**Table 1**). • Mix the beads suspension and add 50 µL of it to each of the Eppendorf tubes. • Add the respective concentration of TNF-α DL488 to the conditions mentioned in **Table 1**. • Add the respective concentration of anti-TNF-α antibody to the relevant tubes (**Table 1**). • Make up the final reaction volume to 100 µL using complete media. • Incubate for 1 hour at room temperature in the dark.

3	Fluorescence Microscopy Imaging	• Label C-CHIP hemocytometer slide (or any other slide compatible with imaging) according to **Table 1**, allowing duplicate wells per condition. • Following incubation, load 10 µL of each reaction onto a microscope slide and examine the samples using a fluorescence microscope. • Make sure to use the same imaging setting for exposure time, light intensity, and gain for the entire experiment.
4	Data Analysis	**Determine the background signal:** Conditions 1 and 4: • These serve as negative controls. Beads should not exhibit any detectable fluorescent signal. • Detection of a fluorescent signal in condition 1 indicates bead autofluorescence. • Detection of a fluorescence signal in condition 4 indicates non-specific binding of TNF-α DL488 to the bead surface. • If a fluorescence signal is observed, optimize the concentration of TNF-α DL488 **Assess the suitability of the assay for the detection of anti-TNF-α antibodies:** Conditions 2& 3 and 5&6: • The presence of green fluorescent beads when recombinant anti-TNF-α antibody is used at 0.5 µg/mL indicates that the assay conditions are sufficently sensitive to detect low concentrations of secreted antibodies. • Conditions where recombinant antibody has been tested at 4 µg/mL may be used as positive controls.

Data

At the physiologically relevant recombinant antibody concentration of 0.5 µg/mL, 2 µg/mL antigen gave a stronger fluorescence signal than 1 µg/mL, with no background signal observed. This condition was therefore selected for subsequent optimization.

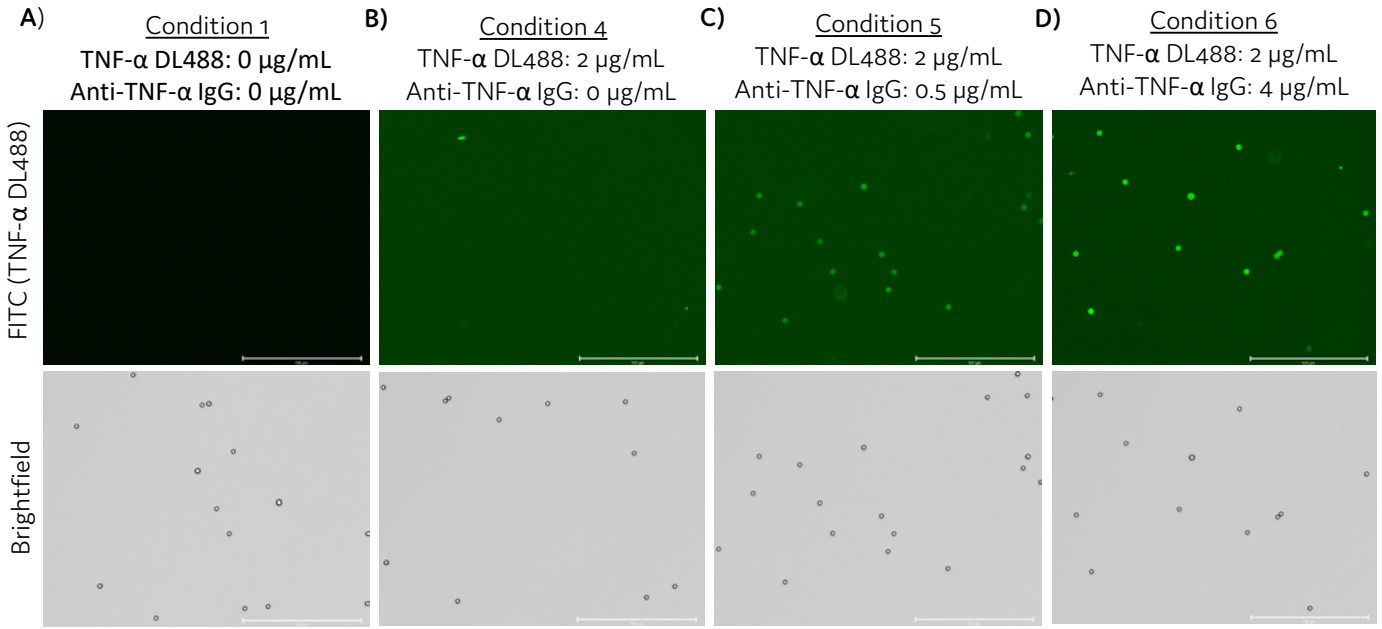


Figure 3. Representative fluorescence microscopy images for assay optimization. A) Beads incubated in the absence of TNF-α DL488 and anti–TNF-α IgG. **B)** Beads incubated with TNF-α DL488 (2 µg/mL) in the absence of anti–TNF-α IgG. **C)** Beads incubated with TNF-α DL488 (2 µg/mL) and anti–TNF-α IgG (0.5 µg/mL). **D)** Beads incubated with TNF-α DL488 (2 µg/mL) and a higher concentration of anti–TNF-α IgG (4 µg/mL).

3.2 Assay Development ON Cyto-Mine® Chroma

Aim

The aim of this development step is to:

- Determine the background signal associated with the beads and TNF-α antigen on Cyto-Mine® Chroma.
- Determine whether the optimal concentration of the TNF-α antigen derived from microscopy experiments is detectable on Cyto-Mine® Chroma.

Experimental Design

Two Cyto-Mine® Chroma experiments are required for this section:

Run 1 (Experimental Condition): A bead-based assay to test the sensitivity of the assay in the presence of 0.5 µg/mL recombinant anti-TNF-α antibody.

Run 2 (Background Control): A control bead-based assay to evaluate non-specific background signal in the absence of recombinant anti-TNF-α antibody. This control condition will allow assessment of bead- and TNF-α associated background fluorescence.

In this step, the performance of the bead-based assay is evaluated using recombinant anti-TNF-α antibody as a model to validate signal detection

Encapsulation of entities (beads or cells) within droplets in Cyto-Mine® Chroma is a stochastic process that can be modelled using the Poisson distribution. This model can be used to predict picodroplet occupancy by single or multiple entities based on the input concentration. The protocol below uses a typical operating point expected to give approximately 41.7% picodroplets containing one or more beads.

Input entity concentration can be used to control occupancy and is a useful experimental parameter. Please contact Technical Support for more information.

Experimental design should include the following conditions:

	Condition	1	2
Beads	**anti-Mouse IgG (Fc)**	1.2 × 10^6/mL	1.2 × 10^6/mL
Antigen	**TNF-α DL488**	2 µg/mL	2 µg/mL
Recombinant Antibody	**Anti-TNF-α IgG**	0.5 µg/mL	-
Purpose		Main experimental condition	Negative Control

Table 2. Experimental design conditions. Table shows the conditions set up for assay development on Cyto-Mine® Chroma.

Experimental Procedure

This protocol is intended for users already proficient in Cyto-Mine® Chroma operation. For comprehensive instructions on general system operation, please refer to the Cyto-Mine® Chroma User Manual.

<table>
<tr><th>Step</th><th>Operation</th><th>Details</th></tr>
<tr><td>1</td><td>Cyto-Mine® Set Up System Warm Up</td><td>• 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 sample.</td></tr>
<tr><td>2</td><td>Prepare beads</td><td>• Prepare beads as outlined in Section 3.1, Experimental Procedure Step 1

• Beads required: 2.0 x10^6

• Following centrifugation, discard supernatant without disturbing the pellets and store the pellets at 4°C until use.

Note: Use 1.5 mL Protein LoBind Eppendorf tube for any steps involving beads.</td></tr>
<tr><td>3</td><td>Prepare Encapsulation Medium (EM)</td><td>• Prepare the Encapsulation Medium (EM) as follows (3 mL total):

<table>
<tr><th>Component</th><th>Stock Conc.</th><th>Final Conc.</th><th>Volume (µL)</th></tr>
<tr><td>Complete Culture Media</td><td>N/A</td><td>74% v/v</td><td>2,220</td></tr>
<tr><td>Opti-Prep</td><td>60% w/v</td><td>16% v/v</td><td>480</td></tr>
<tr><td>Poloxamer-188</td><td>10%</td><td>10% v/v</td><td>300</td></tr>
</table>
</td></tr>
</table>

		• Filter the 3 mL EM through a 10 µm filter. ***Note:*** • *Protocol is shown for 3 mL of EM to accommodate for sample loss during the preparation. Only 2 mL of sample mix is required for this Cyto-Mine® Chroma experiment.* • *Filtration of EM through a 10 µm filter helps to remove the particles/debris.* • *Culture media containing supplements required for cell growth may be used to make up the EM, however, please do not use more than 10% foetal bovine serum (FBS) and any detergents as they may affect droplet integrity.*
4	Prepare TNF-α DL 488	• Prepare 2.5 mL of 2 µg/mL TNF-α DL 488 in filtered EM and mix. • Filter the mixture through 0.22 µm filter using a 1 mL syringe. ***Note:*** • *Use one 0.22 µm filter per 1 mL of suspension. Passing more than 1 mL through a single filter may clog the membrane.* • *Filtration of TNF-α DL 488 through a 0.22 µm filter significantly reduces non-specific green-fluorescent aggregates/debris.*
5	Prepare the sample	• Take the volume of bead suspension required for 2.0 x10^6 beads into a 1.5 mL LoBind Eppendorf tubes and add up to 500 µL of 1x DPBS. • Centrifuge the tube at 5,000g for 5 minutes. • Carefully remove the supernatant without disturbing the pellet. • Resuspend the bead pellet in 1 mL EM+TNF-α DL488 (prepared in **Step 4**) and mix well by pipetting 10-20 times. • Transfer the bead suspension into a 2 mL LoBind Eppendorf tube and top up to a final 2 mL using the EM+TNF-α DL488. • Filter the mixture through a 10 µm filter to remove any bead clumps. • Take 10 µL of sample, make a 1/50 dilution, and perform bead counting using a C-CHIP hemocytometer. • Divide the beads into two LoBind 1.5 mL Eppendorf tube and make up the beads to 1.0 x10^6 beads/mL using EM+TNF-α DL488. • Only to one tube, add recombinant anti-TNF- α antibody to achieve a working concentration of 0.5 µg/mL. • The tube without the recombinant antibody will be the control condition to check for non-specific background signal.

		Note: • *To minimize beads sticking to the tube walls, keep the beads suspension to a maximum of 500 µL per 1.5 mL LoBind Eppendorf tube during centrifugation.* • *As beads are in EM and will remain floating in the C-CHIP, make a 1/50 dilution of the beads sample for counting.* • *Target a bead concentration of ~1.2 × 10^6 beads/mL in the final sample to achieve ~41.7% of picodroplets containing one or more beads.*
6	Load the sample into Cyto-Cartridge®	• Load 0.9 mL of each final 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.*
7	Cyto-Mine® Chroma Set Up & Run	<u>Experimental Parameters</u> • Number of picodroplets to generate: **1.0 x 10^6** • Time and temperature for incubation: **0 minutes** • Type of microtiter plates (MTPs) for dispensing: **96-Well Plate** • Input final bead density loaded based on the information obtained from **Step 5**. • 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: **30 minutes** <u>Picodroplet incubation/antibody secretion</u> • Hands-off step. • Incubation temperature: **37 °C**

- Incubation time: **0 minutes**

Note: *No incubation is required for this assay as no cells are used.*

Flow Stabilization before Sorting

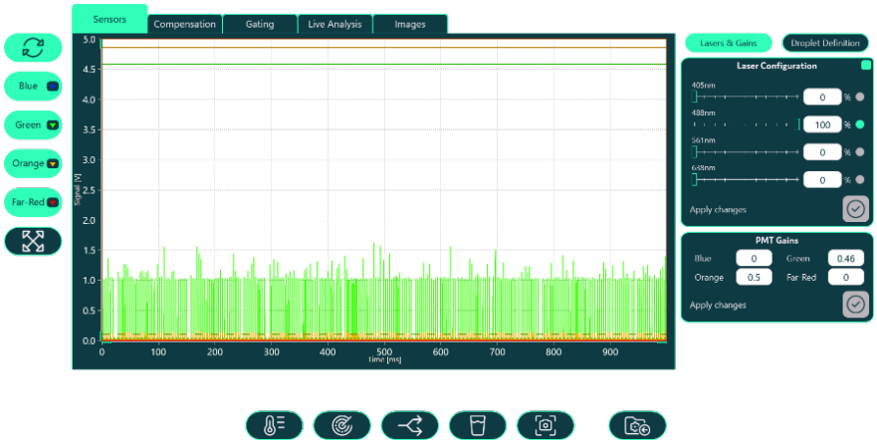
- Wait until this step is automatically finished

Note: *Flow of picodroplets stabilizes takes approximately 3-4 minutes.*

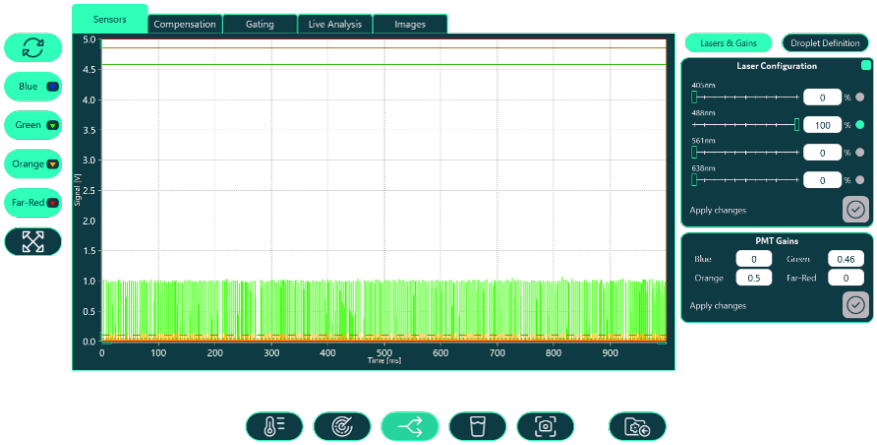
Sorting

- Turn "ON" the 488 laser only.
- Set up PMT Gains for Green to achieve the background signal peak (y axis=Signal) at the height of 1.0 voltage (for representative example see below).
- Droplet Definition: **Green**; Mode: **Default**.
- Auto-Thresholds for detection of fluorescence.
- Apply changes

Condition 1 (with recombinant anti-TNF-α antibody)



Condition 2 (without recombinant anti-TNF-α antibody)

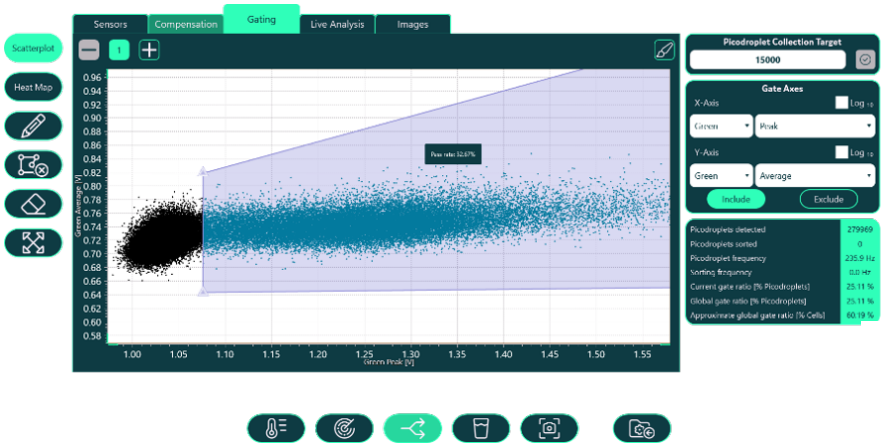


Gating

- Max. Picodroplets to Collect: **15,000**
- Y-Axis: **Green Average**
- X-Axis: **Green Peak**
- Draw a polygon on the scatter plot to gate the positive events (for representative example see below).

Note: *The Sorting Picodroplet Collection Target is set to 15,000 as a default, but sorting does not need to be activated for this experiment*

Condition 1 (with recombinant anti-TNF-α antibody)



Condition 2 (without recombinant anti-TNF-α antibody)



Acquire data

- To analyze positive rate within the bead population without dispensing, acquire 5 x10^5 total picodroplets.

		• The data acquired from this step will be used for further analysis (see **Analysis of Sorting Data Step 9**)
8	End of experiment	• Dispensing of droplets is not required if the aim is solely to evaluate the positive rate. • Skip dispensing by clicking End Run. Follow instructions as prompted by Cyto-Mine® Chroma
9	Analysis of Sorting Data	• The expected results from the two experiments are shown below (**Figure 4**). ***Note:*** *The green peak positive population represents picodroplets where the bead has captured the anti-TNF-α antibody, which in turn has bound specifically to the TNF-α DL488. This interaction results in localized green fluorescence within the picodroplet, which is identified by the Cyto-Mine® Chroma as a green peak positive population.* The percentage of green peak positive population can be used to determine the percentage of positive picodroplets that exhibit bead-based antigen-specific antibody binding. • Poisson Distribution: % Picodroplets containing one or more beads • Dot Plot analysis by FlowJo: % Green Peak⁺ $\%\ positive\ \ picodroplets^{+} = \frac{\%\ \ green\ \ peak^{+}\ \ from\ \ FlowJo}{\%\ \ picodroplets\ \ containing\ \ beads} \times 100$ At the physiologically relevant recombinant antibody concentration of 0.5 µg/mL, green peak positive picodroplets were detected on Cyto-Mine® Chroma, with no background signal observed for condition without anti-TNF-α antibody (**Figure 4**).

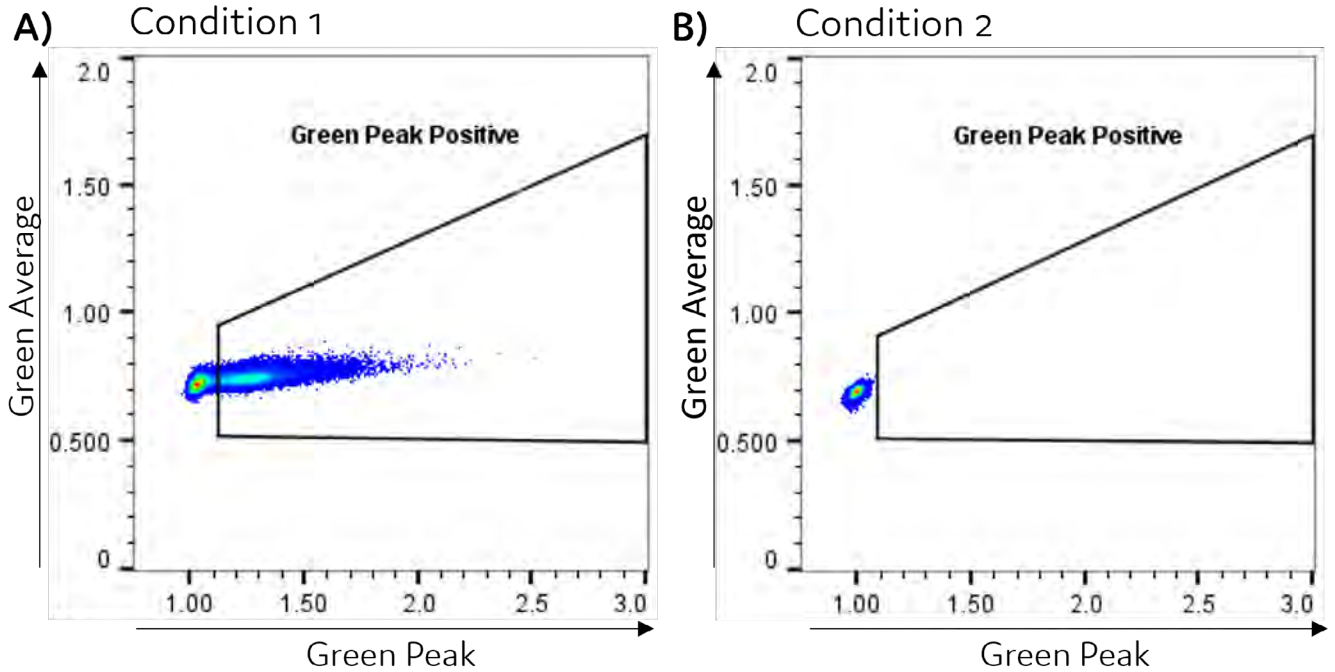


Figure 4. Sorting scatter plot data for bead-based assay performance on Cyto-Mine® Chroma. Beads were mixed with antigen in the presence **(A)** or absence **(B)** of anti-TNF-α antibody at 0.5 µg/mL. Data shows sorting data analyzed using FlowJo™ software to generate scatter plots.

3.3 Assay Performance ON Cyto-Mine® Chroma

Aim

The aim of this final step is to:

- Determine the background signal associated with beads and TNF-α DL488 in the absence of relevant ASCs.
- Assess the suitability of the bead-based workflow for identifying, sorting, and isolating rare antigen-specific ASCs on Cyto-Mine® Chroma.
- Compare bead-based assay performance with the antigen-specific FRET assay

Experimental Design

To evaluate the performance of the bead-based assay for detecting rare TNF-α-specific ASCs, the 357-101-4 cells were spiked into irrelevant IgG-secreting A20 cells in a final ratio of 1:100, respectively. Four Cyto-Mine® Chroma runs are required for this section, as follows:

1. Cell Characterization: The TNF-α-specific ASC (357-101-4) cells used in this assay were initially tested for IgG secretion using the Cyto-Cellect® Mouse FRET assay kit (Fluidic Sciences; data not shown); this characterization and procedure is not discussed further. It was determined that approximately 50% of these cells secreted detectable levels of IgG following 2 hours incubation on Cyto-Mine® Chroma. Therefore, based on this secretion rate, in Cyto-Mine® Chroma assays, a total of 2×10^4 cells were loaded to ensure the presence of 1×10^4 actively antibody-secreting cells; henceforth, this will be referred to as 1×10^4 antibody secreting cells, except in Data Analysis and Interpretation section.

2 . FRET Control: A TNF-α antigen-specific FRET secretion assay with approximately 1.0×10^4 of 357-101-4 cells spiked into 1.0×10^6 A20 cells in a final sample volume of 1mL to provide baseline performance.

3. Negative Control: A bead-based assay with approximately 13.0×10^6 anti-Mouse IgG (Fc) beads and 1.0×10^6 A20 cells in a final sample volume of 1mL to assess background on Cyto-Mine® Chroma.

4. Main Experimental Condition: bead-based assay format with approximately 13.0×10^6 anti-Mouse IgG (Fc) beads, and 1.0×10^4 of 357-101-4 cells spiked into 1.0×10^6 A20 cells in a final sample volume of 1mL, representing a rare-event scenario often found in antibody discovery workflows. The bead concentration is set at 13.0×10^6/mL to achieve ≥ 99% picodroplet occupancy with one or more beads, minimizing the probability of missing relevant ASCs.

Experiments two to four include a viability probe (Fluidic Sciences Viability Probe Kit, C315). This is used to identify apoptotic (non-viable) cells within the population. Experiments 2-4 are summarized in the table below (**Table 4**).

Condition	2 (FRET Control)	3 (Negative Control)	4 (Main)
357-101-4 antibody secreting cells (Relevant)	1.0 $\times 10^4$/mL	-	1.0 $\times 10^4$/mL
A20 cells (Irrelevant)	1.0 $\times 10^6$/mL	1.0 $\times 10^6$/mL	1.0 $\times 10^6$/mL
Beads	-	13.0 $\times 10^6$/mL	13.0 $\times 10^6$/mL
TNF-α DL488	1 µg/mL	2 µg/mL	2 µg/mL
anti-Mouse IgG antibody-DL594	20 µg/mL	-	-
BD Fc Block	1 µg / 1.0 $\times 10^6$ cells	1 µg / 1.0 $\times 10^6$ cells	1 µg / 1.0 $\times 10^6$ cells
Viability Probe	1 vial/1.0 $\times 10^6$ cells	1 vial/1.0 $\times 10^6$ cells	1 vial /1.0 $\times 10^6$ cells
Purpose	FRET positive control	• Negtive Control • Background signal assessment	Main experimental condition

Table 4. Experimental design conditions. Table shows the conditions set up for the ON Cyto-Mine® Chroma assay development.

Experimental Procedure

The procedures outlined below describe the three Cyto-Mine® Chroma experiments.

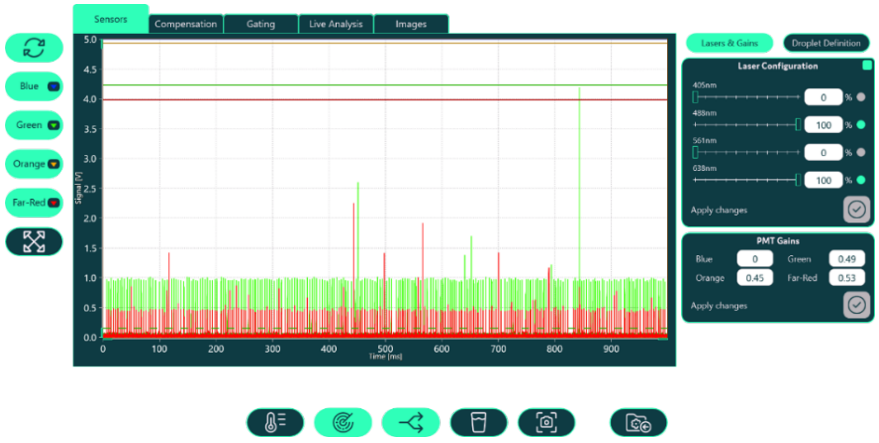
Step	Operation	Details
1	Cyto-Mine® Chroma Set Up System Warm Up	• Refer to **Section 3.2 Experimental Procedure, Step 1**
2	Block FcR and BCR on 357-101-4 and A20 cells	• Resuspend the 357-101-4 and A20 cells in tissue culture flasks. • Filter ~5 mL of each culture separately through a 10 µm mesh filter. Collect the filtrates in separate 5 mL falcon tubes. • Measure cell density and viability of the filtered cells using a NucleoCounter® or similar instruments. • Calculate the required volumes to obtain ~5.0 $\times 10^6$ A20 cells and ~2.0 $\times 10^6$ 357-101-4 cells and transfer these into separate 15 mL Falcon tubes. • Centrifuge cells at room temperature. • Carefully remove the medium without disturbing the cell pellet. • Resuspend the pellets in 5 mL RPMI 1640.

		• Centrifuge and discard the supernatant. • Resuspend the 357-101-4 pellet (~2.0 × 10^6 cells) in 200 µL RPMI 1640 and add 2 µg BD Fc Block to achieve a final working concentration of 1 µg/100 µL per 1.0 × 10^6 cells. • Resuspend the A20 pellet (~5.0 × 10^6 cells) in 500 µL RPMI 1640 and add 5 µg BD Fc Block. • Gently mix and incubate for 30 min at 4°C. ***Note:*** *Before filtering the cells through a 10 µm mesh filter, ensure that the cells can pass through and are not retained. Filtration is crucial to remove any non-specific debris that could result in false positive signal during the Cyto-Mine® Chroma run.*
3	Prepare Beads	Refer to **Section 3.2 Experimental Procedure, Step 2**, except for the specific modifications listed below: • Calculate the number of beads required for two Cyto-Mine® *Chroma* sample preparations (i.e., ~1.0 ×10^7 beads per sample to achieve ≥99.0% bead occupancy). • To count beads, dilute these 1/100 in DPBS. ***Note:*** *It is recommended to use 4-5 x10^6 excess beads to account for potential bead loss during sample preparation.*
4	Prepare EM	• Prepare 5 mL of EM and filter it through a 10 µm mesh filter. For the rest of the instruction, refer to **Section 3.2 Experimental Procedure, Step 3**.
5	Prepare TNF-α DL488	• Prepare 2 mL of 2.22 µg/mL (127.6 nM) of TNF-α DL488 in EM and mix. • For the rest of the instruction, refer to **Section 3.2 Experimental Procedure, Step 4** ***Note:*** *The final concentration of TNF-α DL488 antigen will be 2 µg/mL (115 nM), as 900 µL of this suspension is added to 100 µL of Viability Probe-stained cell suspension in EM..*
6	Stain cells with Viability Probe	• Keep three vials of Viability Probe at room temperature for 5 min and spin them briefly in a Quick-Spin centrifuge. • Upon completion of blocking, add 2 mL of media to a 15 mL Falcon tube and transfer the 500 µL of blocked A20 cells to it and mix gently. • Add 800 µL of media to the blocked 357-101-4 cells tube and mix gently. • Centrifuge the cells at room temperature. • Resuspend the A20 cells pellet in 400 µL of EM and filter through a 10 µm mesh filter.

6	Stain cells with Viability Probe	• Measure cell density and viability and calculate the volume required for ~1.0 × 10^6 live cells. • Resuspend the 357-101-4 cells pellet in 500 µL of EM and filter through a 10 µm mesh filter. Measure cell density and viability and calculate the volume required for ~1 x10^4 IgG secreting live cells. • Remove the three vials of Viability Probe and label them as Main, Negative Control, and FRET Positive Control. • Add the following to Main and FRET Positive Control vial: - A20 cells: 1 x10^6 cells - 357-101-4 cells: 1 x10^4 antibody secreting cells • To the Negative Control vial, add: - A20 cells: 1 x10^6 cells only • Make up the total volume to 100 µL with EM. • Mix the vials gently and incubate at room temperature in dark, for 15 min.
7	Prepare the bead sample	• Resuspend the bead pellets in ~2.0 mL of filtered EM + TNF-α DL488 • Transfer the bead suspension into a 2 mL LoBind Eppendorf tube and mix thoroughly. • Filter the suspension through a 10 µm mesh filter and collect the filtrate in a 2 mL LoBind Eppendorf tube.
8	Prepare the final Cyto-Mine® Chroma cell sample	After the completion of the Viability Probe staining, prepare the final sample as follows: <u>Main</u> • To the stained cells, add 0.9 mL of the EM + TNF-α DL488 from **Step 7**. • Add 1 µg/mL of BD Fc Block and mix gently. • Check cell density and viability. • Take 10 µL of sample, make a 1/50 dilution, and perform bead counting using a C-CHIP hemocytometer. <u>Negative Control</u> • To the stained cells, add 0.9 mL of the EM + TNF-α DL488 from **Step 7**.

		• Add 1 µg/mL of BD Fc Block and mix gently. • Check cell density and viability. <u>FRET Positive Control</u> • To the stained cells, add 0.9 mL of the EM only. • Add 1 µg/mL of the TNF-α DL488 and 20 µg/mL Anti- mouse IgG DL594 (Ab97017). • Add 1 µg/mL of BD Fc Block and mix gently. • Check cell density and viability.
9	Load the samples into Cyto-Cartridge®	• Load ~1.0 mL of each sample into their respective Cyto-Cartridge®, then place the Cyto-Cartridge® into the Cyto-Mine® Chroma. • For the rest of the instruction, refer to **Section 3.2 Experimental Procedure, Step 6.**
Note: *The below section outlines three Cyto-Mine® Chroma runs, including a benchmark antigen-specific FRET control and a negative control to assess non-specific background. While the FRET procedure follows previously published protocols, the bead-based experiments detailed here focus on sorting and data collection, with the main experimental sample also including full dispensing procedures.*		
10	Cyto-Mine® Chroma Set Up & Run	Follow the instruction in **Section 3.2 Experimental Procedure, Step 7**, except for the specific modifications listed below: <u>Experimental Parameters</u> • Number of picodroplets to generate: **1.5 x 10^6** • Time and temperature for incubation: **120 minutes at 37 °C** ***Note:*** • *According to predicted cell secretion rate, a shorter or longer incubation can be applied.* • *For workflows such as antibody discovery (AbD) where a small number of positive cells are expected ("rare events"), it is recommended to generate 2.0 × 10^6 picodroplets and implement the "Bulking-Up Sorting Strategy" to enable efficient dispensing after sorting. For more information on bulking-up contact Fluidic Sciences Technical Support.* **<u>Cyto-Mine® Chroma – Main</u>** <u>Sorting</u> Set up detection: • Turn "ON" the 488 nm and 638 nm lasers.

- Set up PMT Gains for Green, Orange, and Far-Red to achieve the background signal peak (y axis=Signal) as follows: Green ~1.0 V, Orange ~0.1–0.25 V, and Far-Red ~0.5 V (see image below).
- Droplet Definition: **Green**; Mode: **Default**.
- Auto-Thresholds for detection of fluorescence.
- Apply changes

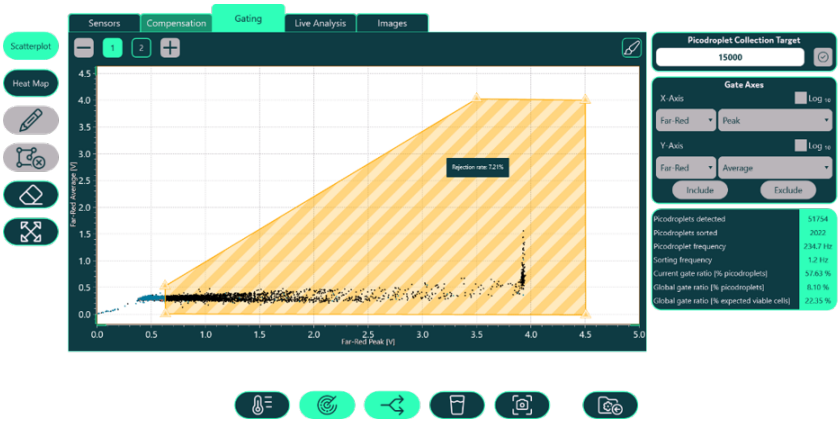


Gating 1: Exclude apoptotic cells (Far-Red Viability Probe±)

- Picodroplet Collection Target: **15,000**
- Y-Axis: **Far-Red Average**
- X-Axis: **Far-Red Peak**
- Draw a polygon on the scatter plot to gate the Far-Red Viability Probe+ (Apoptotic) population (see image below).
- Press "Exclude" to exclude the Far-Red Viability Probe+ (or the "Dead).

Note:

- *It is recommended to apply a less strict gating to provide a buffer zone between the empty (negative) population and the start of the excluding gate, rather than placing the gate immediately after the empty population. This approach ensures that small differences in staining brightness or background fluorescence do not lead in excluding the relevant population.*
- *For a typical experiment the expected proportion of fused droplets is < 1%, however this may increase in the presence of destabilizing factors such as detergents and high serum content (>10%) and requires exclusion of the fused droplet during gating (for explanation see "An Antigen-Specific Cell-Binding Assay for Cyto-Mine® Platforms",* **Section 3.2. Experimental Procedure, Step 6)**[3].



Gating 2: (to sort positive population)

Picodroplet Collection Target: **15,000**

- Y-Axis: **Green Average**
- X-Axis: **Green Peak**

In this gating, we will be using the Bulking-Up Sorting Strategy.

- Draw a Polygon 1 on the scatter plot to gate the Green Peak (Live) events (see image below).
- Draw another Polygon 2 on the left side of the empty population.
- Start sorting and sort up to ~2,000 picodroplets, then Pause sorting.
- Move the Polygon 2 away from the empty population and continue sorting the Green Peak (Live) population for an additional ~2,000 picodroplets (total ~4000 picodroplets sorted), then Pause sorting.
- Return Polygon 2 to the empty population and sort an additional ~3,000 picodroplets.
- Once total ~7000 picodroplets are sorted, move to Dispensing.

Note: *Green peak positive population represent picodroplets where the bead binds to the anti-TNF-α antibody secreted by the 357-101-4 cells, which in turn binds specifically to the TNF-α DL488, therefore exhibiting localised green fluorescence on the bead within the picodroplets, which is detected on Cyto-Mine® Chroma as green peak positive population.*

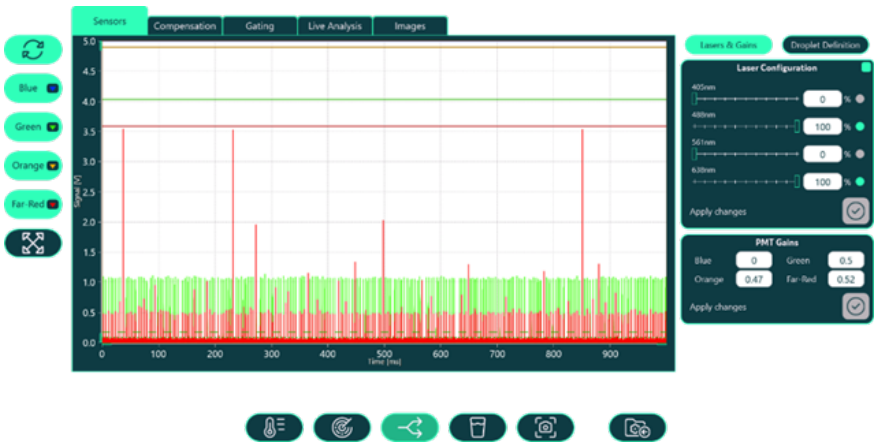


Cyto-Mine® Chroma – Negative control

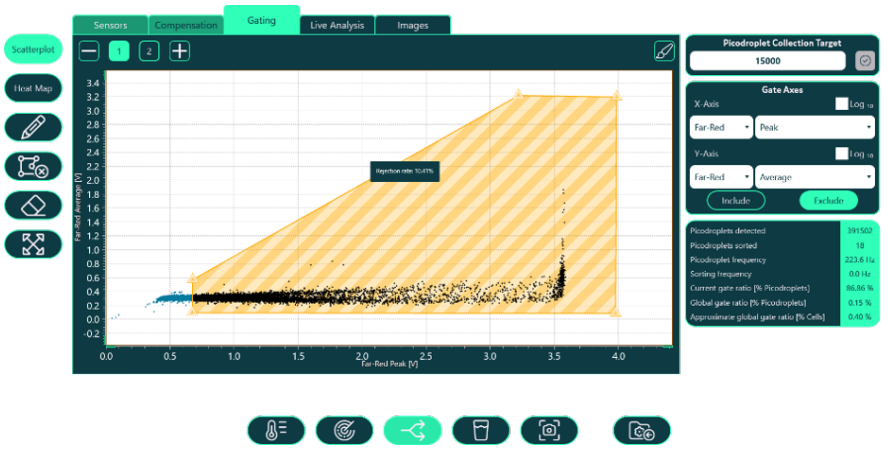
For Sorting and Gating strategy, refer to the 'Main' assay, with the exception that no bulking-up and Sorting is required for the Negative Control experiment.

Sorting

Sensors and Detection (see image below)



Gating 1: Exclude apoptotic cells (Far-Red Viability Probe+, see image below)

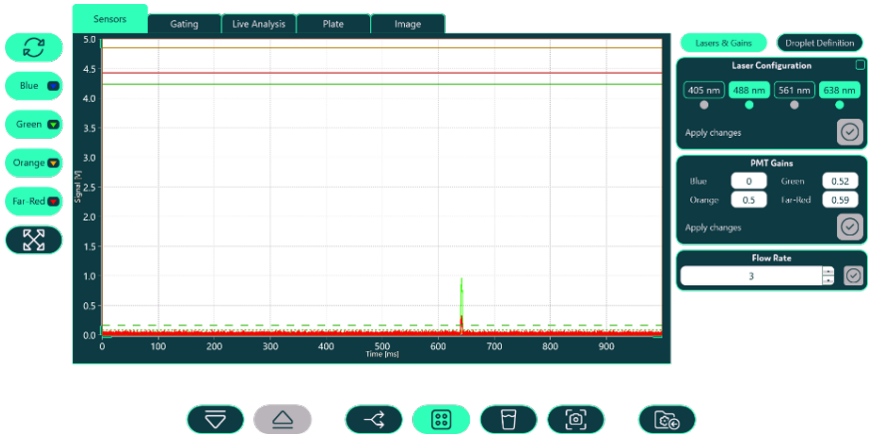


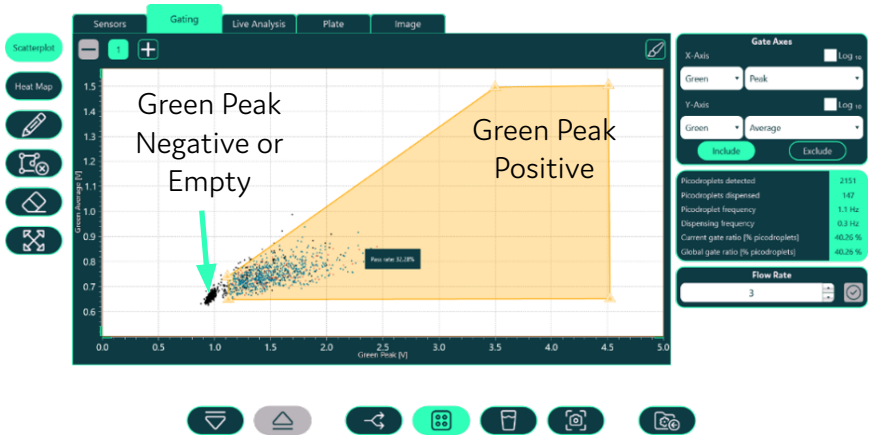
Gating 2: (only detection; see image below)



Cyto-Mine® Chroma – Antigen-specific FRET Positive control

- Follow the gating strategy and the experimental procedure outlined in "Antigen-Specific FRET Assay For Use With Cyto-Mine® Platforms" protocol[2].
- Acquire sorting data for all the experiments
  - The data acquired from this step will be used for further analysis, see **Section 4**.
  - To analyze positive rate within the bead population with/without dispensing, acquire 5 x10^5 total picodroplets.
- Dispensing of droplets is not required in this Cyto-Mine® Chroma runs, as the aim is to estimate percentage of Antigen-Specific ASC using FRET assay.
- Label 96-Well plates with following information:

11	Pre-Dispense: Prepare the 96-Well Plate	- Cell Type; Date; Plate no. - Cyto-Mine® Chroma Unit no. · For subsequent cloning: Use a multi-channel pipette to pipet 30 µL of complete media into each well. ***Note:*** · *For dispensing into 384-well plates pre-fill the plates with 6 µL of Complete Media into each well.* · *For dispensing into PCR plates please refer to the Cyto-Mine® Chroma User Manual or contact Fluidic customer support.*
13	Dispensing Flow Stabilization	· Once 7,000 droplets are sorted, start Flow Stabilization. This will take 4-10 minutes to appear then slowly reduce flow rate from 20 -> 15 -> 10 -> 5 µL/hr with 30 seconds wait between changing each flow rate. · Load the pre-filled 96-Well plate.
14	Picodroplet Dispensing	**<u>Cyto-Mine® Chroma – Main</u>** <u>Dispensing</u> Set up detection: · Turn "ON" the 488 and 638 lasers. · Set up PMT Gains for Green, Orange, and Far-Red to achieve the background signal peak (y axis=Signal) as follows: Green ~1.0 V, Orange ~0.1–0.25 V, and Far-Red ~0.25 V (see image below). · Droplet Definition: **Green**; Mode: **Default**. · Auto-Thresholds for detection of fluorescence. · Apply changes 

		Gating • Adjust Flow Rate so Picodroplets frequency is ≤ 1.5Hz • Y-Axis: **Green Average** • X-Axis: **Green Peak** • Draw a Polygon on the scatter plot to gate the Green Peak Positive events (see image below). • Start dispensing the Green Peak Positive population into the 96-Well plate. • Dispense more plates, if required.  ***Note:*** *The Green Peak Negative or Empty population will be the first to arrive during dispensing.*
14	Post-Dispensing top-up the plates	• Following dispensing, incubate the plates in the 37°C incubator, 5% CO_2 for 15 minutes. • After incubation, use multi-channel pipette to top-up 70 µL of 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:*** • *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 250g prior to imaging.* • *After dispensing into PCR plates follow your standard downstream processing protocol or contact Fluidic customer support for more information.*
15	End Run	• Finish the run, as per the Cyto-Mine® Chroma Manual.

4. Data Analysis

This section explains how to use FlowJo™ to analyze Cyto-Mine® Chroma data, include assessing sample quality, and comparing the specific IgG-secreting positive population across Antigen-Specific FRET and Bead-Based assay. For viewing in-run scatter plots or analyzing picodroplet 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 Determining the Antigen-Specific ASCs using FRET assay

To quantify the FRET-positive population for the '**FRET control**' condition (see **Section 3.3**):

- Open the experiment file using Cyto-Mine® Data Analysis software.
- Export .csv format files of the sorting data.
- Open the .csv files in FlowJo™ software.
- Plot the sorting graph using Green Average Fluorescence and Red Average Fluorescence as X and Y axes, respectively.
- Identify the FRET positive population (population of picodroplets that extend towards top left corner – reduced red signal and increased green signal; **Figure 5**).
- Draw a polygon around the FRET positive population to quantify the percentage of FRET positive picodroplets (**Figure 5**).
- This information can be used to determine the percentage of cells that show antigen-specific antibody secretion. Calculation shown below for **Figure 5**, where 2.0 x10^4 IgG secreting 357-101-4 cells were encapsulated in Cyto-Mine® Chroma:

  - Estimated proportion of ASC in total cell population (Poisson Distribution):
    - 99.10% of empty droplets
    - 0.89% of picodroplets containing ASC
  - % FRET positive picodroplets 0.55%
  - Calculate % ASC as follows:

$$\%\mathrm{ASC} = \frac{\%\ \mathrm{FRET+}}{\%\ \mathrm{expected\ \ droplets\ \ containing\ \ ASC}} \times 100$$

$$\%\text{ASC} = \frac{0.55}{0.89} \times 100 = \sim 61.8\%$$

The antigen-specific FRET assay yielded a ~61.8% specific IgG-secreting positive population. Although including a viability probe is beneficial it was found not to be necessary for this assay, because the 357-101-4 cells (total 2.0 x10^4) are significantly outnumbered by A20 cells (total 1.0 x10^6). Due to the high specificity of the antigen-specific FRET signal, any contribution from apoptotic 357-101-4 cells through non-specific binding would be negligible. In contrast, the bead-based assay is highly sensitive to non-specific binding to apoptotic cells of either type; failing to exclude the apoptotic population (both 357-101-4 and A20 cells) significantly increases the false positives.

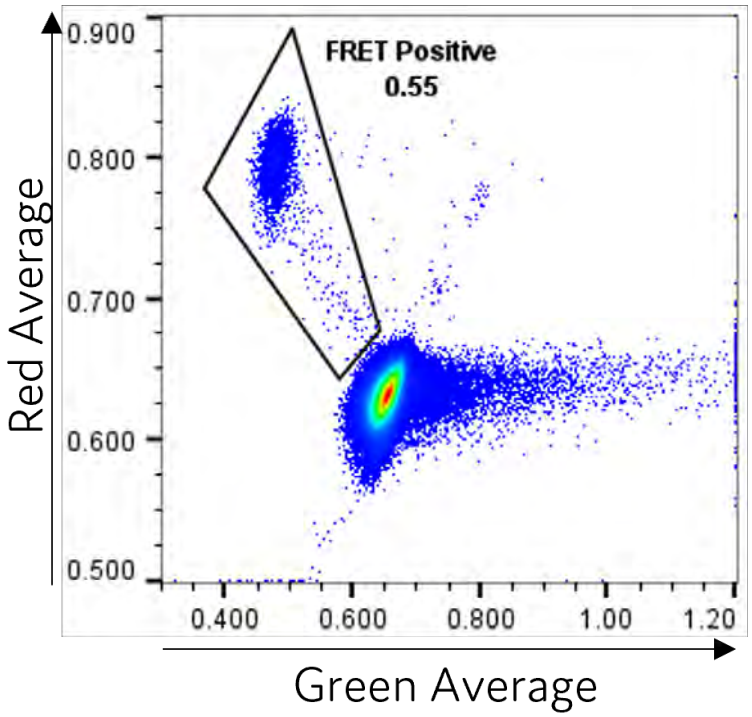


Figure 5. TNF-α antigen-specific FRET assay on Cyto-Mine® Chroma using 357-101-4 cells. Sorting scatter plot from the TNF-α antigen-specific FRET assay run on Cyto-Mine® Chroma. Sorting data for picodroplets were analyzed using FlowJo™ software.

4.2 Determining the Antigen-Specific ASCs using Bead-Based Assay

To quantify the antigen-specific positive population for the '**Main**' condition (see **Section 3.3**):

- Open the experiment file using Cyto-Mine® Data Analysis software.
- Export .csv format files of the sorting data.
- Open the .csv files in FlowJo™ software.

- Plot the sorting graph using Green Average Fluorescence and Far Red Peak Fluorescence as X and Y axes, respectively.
- Identify the **Live cell population** (Viability Probe negative), see Figure **6A & B (i)**.
- Gate on the Live cell population and analyze these for Green Average and Green Peak, see Figure **6A & B (ii)**.
- This percentage of Green Peak positive population can be used to determine the percentage of cells that exhibit antigen-specific antibody secretion. Calculation shown below for **Figure 6A** where 2.0 x10^4 IgG secreting 357-101-4 cells were encapsulated in Cyto-Mine® Chroma:
  - Estimated proportion of 357-101-4 cells in total picodroplet population (Poisson Distribution):
    - 99.10% of empty droplets
    - 0.89% of picodroplets containing ASC
  - % Green Peak$^+$: 0.53%

$$\%\text{ASC} = \frac{0.53}{0.89} \times 100 = \quad 59.5\%$$

The data shown above indicates that the bead-based assay (59.5%) and the antigen-specific FRET assay (61.8.0%) yield comparable results for identifying rare antibody-secreting cells (ASCs). Furthermore, this data confirms that both assays provide good sensitivity and specificity in detection and isolation of rare antigen-specific IgG secreting cells within a population as the proportion of positive cells recovered aligns with the ~50% secretion levels first measured for the 357-101-4 cells.

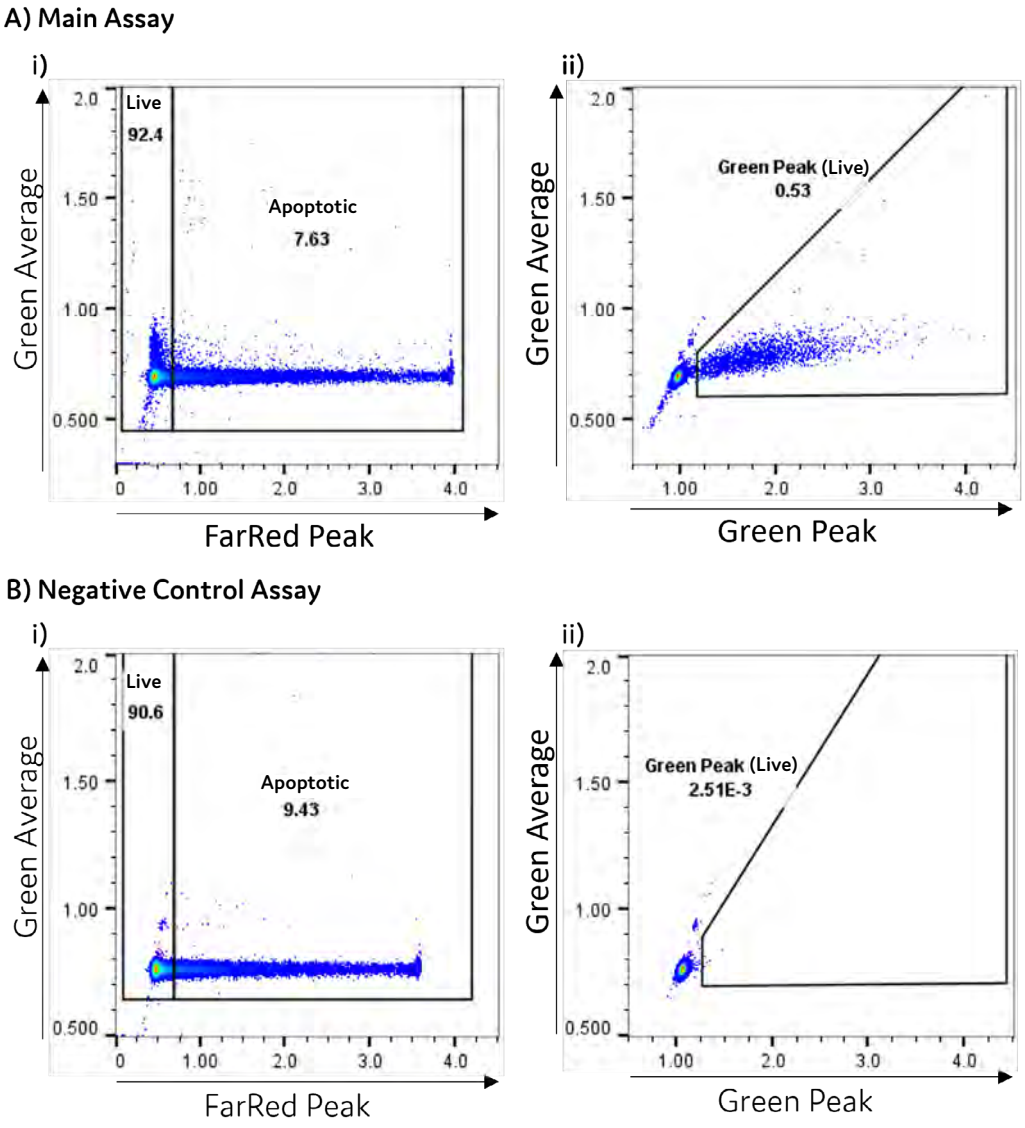


Figure 6. Data analysis for antigen-specific bead-based assay on Cyto-Mine® Chroma. A) Scatter plots for the bead-based assay with 357-101-4 cells spiked into irrelevant A20 cells (1:100), measured on Cyto-Mine® Chroma. As shown in (i), the "Apoptotic" gated population, representing FarRed positive (Viability Probe$^+$) picodroplets, was excluded. The "Live" gated population, representing FarRed negative picodroplets, was sequentially gated for sorting the "Green Peak (Live)" population, as shown in (ii). **B**) Scatter plots for the bead-based assay using irrelevant A20 cells, measured on Cyto-Mine® Chroma. As shown in (i), the "Apoptotic" gated population, representing FarRed positive (Viability Probe$^+$) picodroplets, was excluded. The "Live" gated population, representing Far-Red negative picodroplets, was sequentially gated to identify the "Green Peak (Live)" population, as shown in (ii). Sorting data for picodroplets were analyzed using FlowJo™ software to generate scatter plots.

5. Experiment Optimization and Troubleshooting

Experimental Step	Specific issue	Solution/Optimization
Sample preparation	False-positive signals on Cyto-Mine®Chroma	• Filter the fluorescent reagent mixture in Encapsulation Media through a 0.22 µm filter before resuspending cells and beads.
Preparation of bead suspension in Encapsulation Media (EM)	Beads sediment or float	• Beads sediment or float if their density is not matched to the EM. • Optimize the EM density by adjusting OptiPrep concentration (e.g., 12-18%). • Note that conditions close to 16% OptiPrep will be more suitable for maintaining homogeneous ASCs distribution.
Cell Sample Preparation for Cyto-Mine® Chroma	Cell loss following filtering	• If significant cell loss is observed, verify compatibility of cell size with the filter. Use a filter with larger pore size (e.g., 15 µm). Filtration should effectively remove debris while preserving cell recovery.
Gating Strategy during Cyto-Mine® Chroma Experiment	False positives or loss of relevant events.	• Apply less stringent gating by introducing a buffer zone between the negative population and the sorting/exclusion gate.
Bead-Based Assay in Cyto-Mine® Chroma	Low proportion of positive picodroplets	• Assure no cell loss in the final Cyto-Mine® Chroma sample. • Ensure all required additives (Opti-Prep, Poloxamer, and probes) have been added to Encapsulation medium. • Perform bead counting of the final sample prior to loading into the Cyto-Cartridge®.
Bead-Based Assay in Cyto-Mine® Chroma	High level of fused droplets	• Encapsulation Media should not contain detergents or more than 10% FBS.

6. References

1. Word, C.J. and Kuehl, W.M., 1981. Expression of surface and secreted IgG2a by a murine B-lymphoma before and after hybridization to myeloma cells. Molecular Immunology, 18(4), pp.311–322.

2. Antigen-Specific FRET Assay For Use With Cyto-Mine® Platforms; available on spherebio.com.

3. An Antigen-Specific Cell-Binding Assay for Cyto-Mine® Platforms; available on spherebio.com.

4. Rutkauskaitė, J., et al., 2022. High-throughput functional antibody screening using droplet microfluidics. iScience, 25(12), 105299.

5. Stojanović, I., et al., 2015. Droplet-based microfluidic platform for high-throughput analysis of antibody secretion. Analytical Biochemistry, 480, pp.1–8.

Notes



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