Customer Protocol

An antigen-specific cell-binding assay for Cyto-Mine® Chroma



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1. Introduction

Antigen-specific binding assays are widely used in antibody discovery (AbD) to identify antibody-secreting cells (ASC) that produce antibodies capable of recognizing a target antigen. Early identification of antigen-binding clones allows researchers to prioritize target-specific candidates, improving the efficiency of downstream characterization and reducing the time and resources required to identify high-quality antibody leads.

This protocol describes how to design, optimize, and perform an antigen-specific cell binding assay using Cyto-Mine® Chroma, in which the antigen is expressed on the surface of a target cell.

A simplified schematic of the assay principle is shown in **Figure 1**. Here both an ASC as well as target cells expressing the antigen of interest, are present in the same picodroplet. As antibodies are secreted, those that recognize the antigen bind to the surface of the target cell within the droplet (**Figure 1**). Detection is performed using a fluorescently labeled detection antibody that binds to the secreted antibody. In the absence of antigen-specific antibody, the detection antibody and the associated fluorescent signal is diffusely distributed throughout the droplet (**Figure 1A & B**).

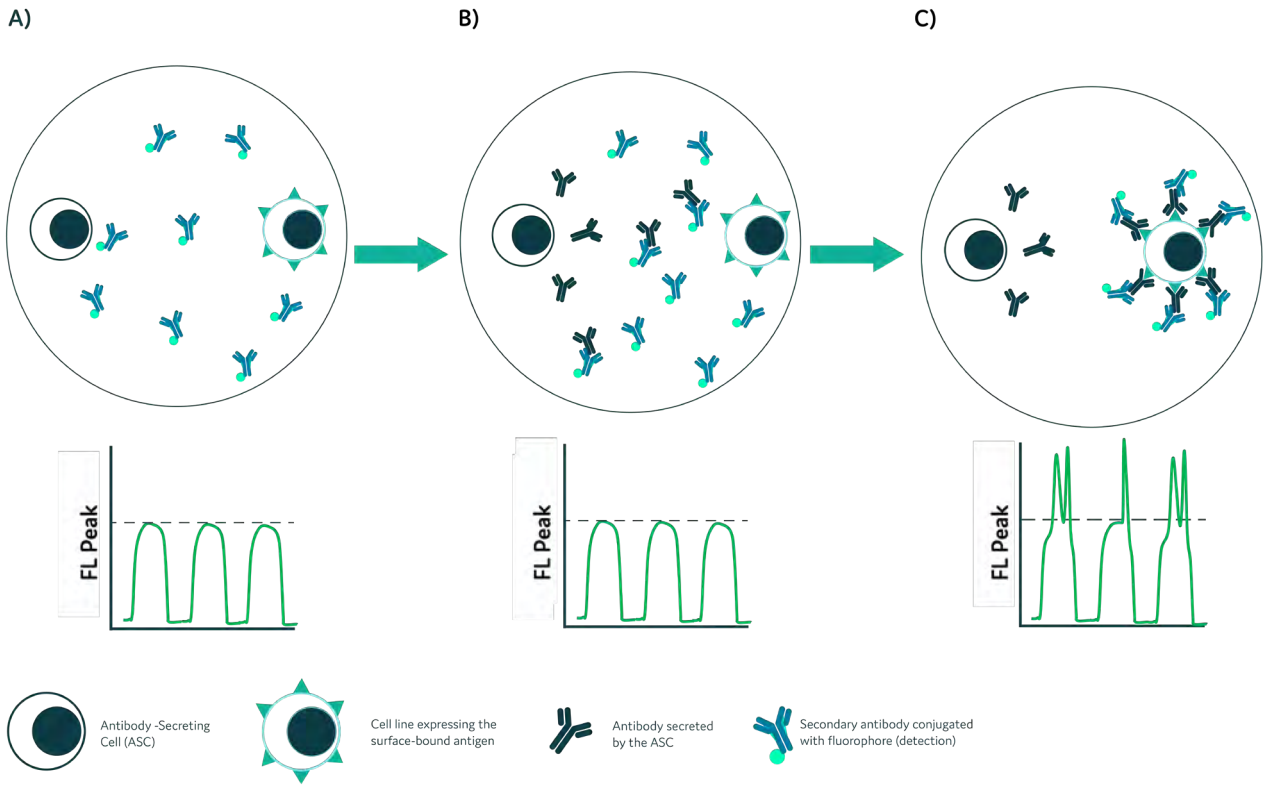


Figure 1. Schematic representation of antigen-specific binding assay, for secreted antibodies: A) Antibody-secreting cells are encapsulated together with target cells expressing antigen on the surface, and fluorescently-labeled detection antibody. **B)** Upon secretion, the primary antibody is recognized by the detection antibody and binds to the antigen on the surface of the cell. **C)** Binding of the antibody complex on the surface of the target cell results in translocation of some of the detection antibody and fluor from homogenously dispersed to localized on the cell surface, which is exhibited as an increase in peak green fluorescence signal. The fluorescence peak data (FL. Peak) for each scenario is shown for three sequential droplets passing the detection point. The dotted line shows the background peak signal, with peaks appearing above this line indicating binding event.

In contrast, when the antigen-specific antibody is present in the droplet, the detection antibody binds to the secreted antibody and the resulting complex accumulates on the surface of the target cell, generating a localized fluorescence signal (**Figure 1C**). In Cyto-Mine® Chroma, this signal can be distinguished from the diffuse fluorescence generated by unbound detection antibody distributed throughout the droplet (**Figure 1A & B**).

This protocol builds on the principles outlined above and takes advantage of the multiplexing capabilities of Cyto-Mine® Chroma to enable highly sensitive detection of antigen-specific ASC (**Figure 2**). In addition to the use of the fluorescent detection antibody described in **Figure 1**, both ASC as well as target cells are fluorescently labelled to improve signal discrimination and overall assay performance.

This protocol can be adapted to exclude non-viable cells, by incorporating a viability probe (using Fluidic Sciences' Viability Probe Kit, C315) or alternative cell-labelling strategies as appropriate for the experimental system. Please refer to **Appendix I** for more information about alternative experimental designs

Alternative protocols for detecting antibody binding to soluble antigen (FRET assay) or antigen-coated beads are available on spherebio.com.

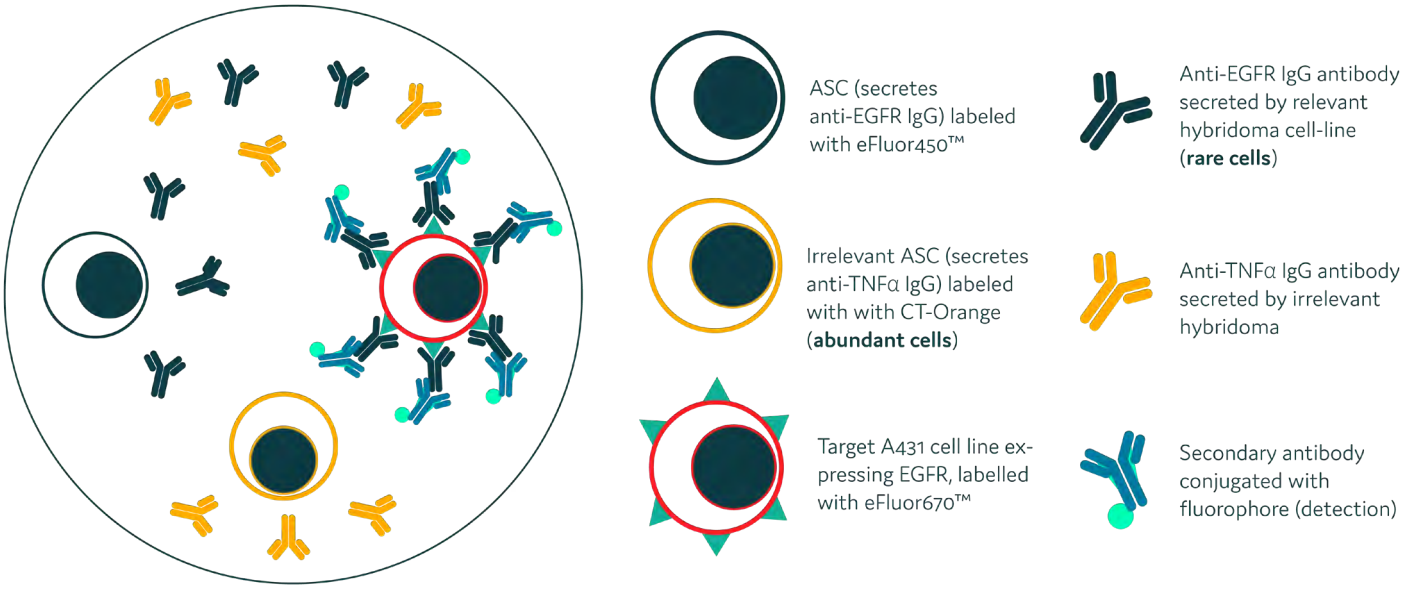


Figure 2. Schematic representation of the antigen-specific assay used in the protocol: Three types of cells were stained and prepared for co-encapsulation in picodroplets: target A431 cells (over-expressing Epidermal growth factor receptor, EGFR) were stained with eBioscience™ Cell Proliferation Dye eFluor™670, hybridoma cells (secreting anti-human EGFR antibodies) were stained with eBioscience™ Cell Proliferation Dye eFluor™ 450, and a different hybridoma cell-line (secreting irrelevant anti-human TNF-α antibodies) was stained with Cell Tracker Orange CMRA. For detection of antigen-specific binding of anti-EGFR Ab to the surface of target cells, anti-mouse AF488-conjugated antibody was added to the reaction.

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™	BD Biosciences (Becton, Dickinson and Company)	N/A

Equipment

Item	Supplier	Catalogue Number
Class II Microbiology Safety Cabinet	Various	Various
NanoDrop™ 2000/2000c Spectrophotometers	Thermo Fisher Scientific	ND-2000
NucleoCounter® NC-3000™	ChemoMetec	970-300
Centrifuge	Eppendorf or equivalent	022623508
Multipette	Eppendorf or equivalent	4982000012
Pipettes (10 µL, 20 µL, 200 µL, 1,000 µL)	Gilson or equivalent	N/A
SYNENTEC NYONE Plate Scanner	SYNENTEC GmbH	N/A
Cyto-Mine® Chroma	Fluidic Sciences	S008

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
Millex™ PVDF syringe filter: pore size 0.22 µm, diam. 4 mm, sterile, hydrophilic	Millipore	SLGV004SL
Nunc Lab-Tek II 8-Well (or other multi-well chamber compatible with imaging)	Thermo Fisher Scientific	154534
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

Category	Material	Supplier	Catalogue Number
Cells	A431 cells	ECCAC	85090402
	Hybridoma 455 cells	ECCAC	92090408
	Hybridoma 357-101-4 cells	ECCAC	92030603
Cell handling	StemPro™ Accutase™ Cell Dissociation Reagent	Thermo Fisher Scientific	A1110501
	DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190094
Media	455 Complete Media (RPMI, 10% Horse Serum, L-Glutamine)	N/A	
	RPMI1640	Gibco	16050-122
	Horse Serum	Gibco	A10491-01
	L-Glutamine (200 mM)	Gibco	25030081
	A431 Complete Medium (DMEM, 10% Foetal Bovine Serum (FBS), Glutamine)	N/A	
	DMEM	ATCC	30-2002
	FBS	Gibco	A5256701
	L-Glutamine (200mM)	Gibco	25030081

Counting	Solution 13 (AO-DAPI)	ChemoMetec	910-3013
Encapsulation media	OptiPrep™ Density Gradient agent	Sigma-Aldrich	D1556
	Poloxamer 188 Non-ionic Surfactant	Thermo Fisher Scientific	24040032
Detection Molecule	Alexa Fluor® 488 AffiniPure® Goat Anti-Mouse IgG, Fcγ fragment specific	Jackson ImmunoResearch	115-545-008
Positive control	Goat Anti-Human IgG Fc (DyLight® 488)	Abcam	AB97003
	Cetuximab (anti-EGFR recombinant Ab)	Absolute Antibody	C225
Blocking	Purified Rat Anti-Mouse CD16/ CD32 (Mouse BD Fc Block™)	BD Biosciences	553141
	AffiniPure® Goat Anti-Mouse IgG, Fcγ fragment specific	Jackson ImmunoResearch	115-005-008
Cell Labelling	eBioscience™ Cell Proliferation Dye eFluor™ 450	Thermo Fisher Scientific	65-0842-85
	CellTracker™ Orange CMRA	Thermo Fisher Scientific	C34551
	eBioscience™ Cell Proliferation Dye eFluor™ 670	Thermo Fisher Scientific	65-0840-85

3. Method

The antigen-specific binding assay is developed in a stepwise workflow to ensure optimal performance on the Cyto-Mine® Chroma platform (**Figure 3**). Initial optimization is performed **off Cyto-Mine® Chroma** using microscopy to confirm assay configuration, controls, and detection molecule concentration. The assay is then evaluated **on Cyto-Mine® Chroma** to assess background signal and maximal signal response, before performing the **complete assay** to assess overall performance and enable detection of ASC.

Please note that this protocol is intended for users that are familiar with operating the Cyto-Mine® Chroma platform. For more information, please refer to the Cyto-Mine® Chroma User Manuals.

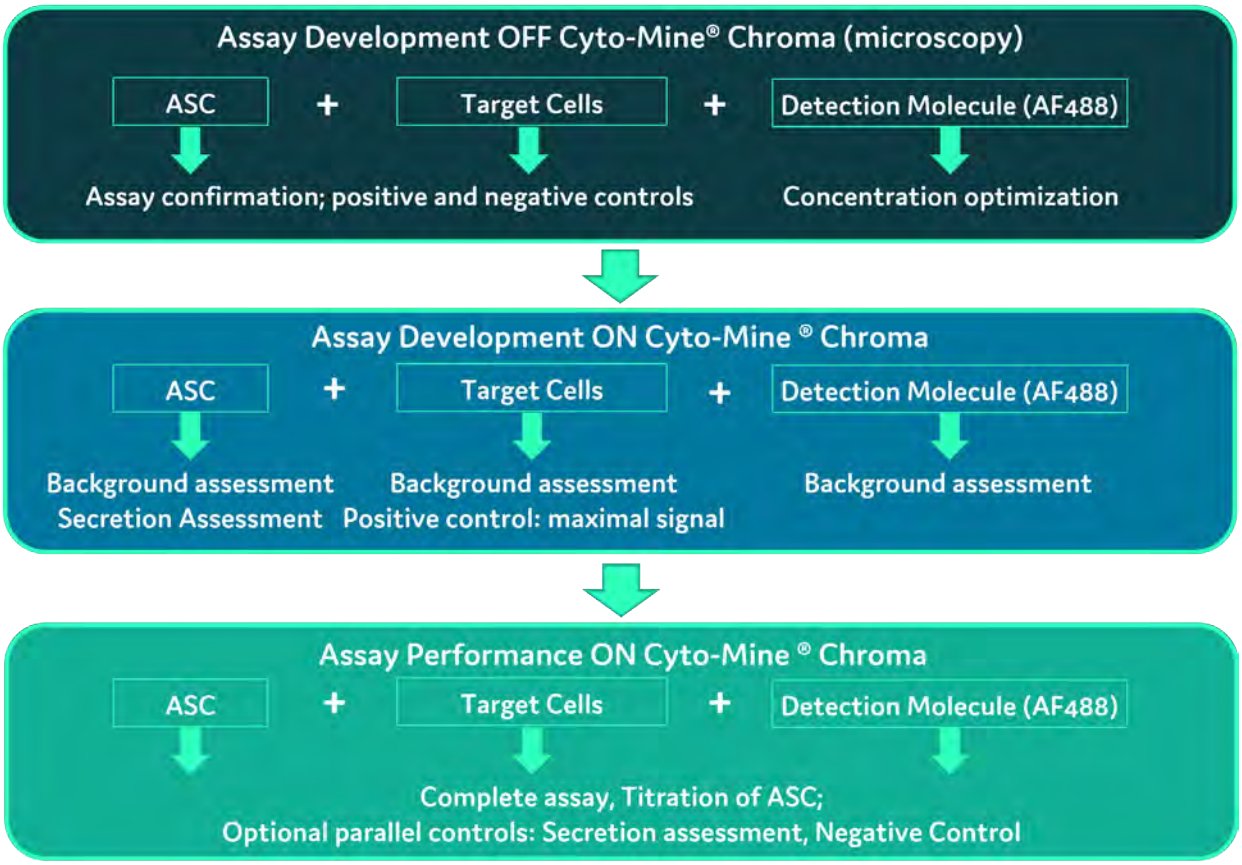


Figure 3. Workflow for the development and optimization of an antigen-specific binding assay. The chart outlines the key steps involved in the development and optimization of the antigen-specific binding assay for Cyto-Mine® Chroma.

3.1 Assay Optimization and Development OFF Cyto-Mine® Chroma

Aim

This optimization step aims to:

- Determine the background signal
- Assess the suitability of the assay for detection of the secreted antibody

It is essential to evaluate the specificity of the assay designed to detect the secreted antibody. While positive controls (such as commercially available recombinant antibodies) should be included, hybridoma culture supernatant or purified secreted antibody should also be tested wherever available.

Although not detailed extensively in this protocol, determining the optimal concentration of the detection antibody would be essential in developing a robust assay. The procedure below describes the workflow using a single concentration of detection antibody. To optimize the detection antibody concentration, repeat the procedure while titrating this antibody over an appropriate range (recommended: 0.5–5 µg/mL).

Experimental Design

For this optimization assay, cells are not pre-seeded, and no wash steps are included after antibody incubation. Instead, cells and antibodies are incubated together to replicate the assay conditions that will be present in Cyto-Mine® Chroma.

Experimental design should include the following samples in duplicates:

	Cells		Primary Ab		Secondary Ab		
Condition	**ASC**	**Target Cells**	**Secreted Ab**	**Control IgG**	**α-Mouse Ab**	**α-Human Ab**	**Purpose**
1	0.4×10^5 cells	-	-	-	2.0 µg/mL	-	Background from secreting cells
2	-	0.4×10^5 cells	-	-	2.0 µg/mL	-	Background from target cells
3	-	0.4×10^5 cells	-	1.0 µg/mL	-	2.0 µg/mL	Positive control (commercially verified primary Ab)
4	-	0.4×10^5 cells	1.0 µg/mL or 0.2 mL	-	2.0 µg/mL	-	Positive control (verification of in-house system)
5 ***Optional***	-	0.4×10^5 cells	-	1.0 µg/mL (isotype control with no specificity to EGFR)	2.0 µg/mL	-	Negative control

Table 1. Experimental design conditions. This table presents the conditions set up for the off- Cyto-Mine® Chroma assay development. Details of material and cells used:

- ASC: 455 Hybridoma cells, secreting anti-epidermal growth factor receptor (EGFR) antibody
- Target cells: A431 cell line, expressing EGFR
- Secreted antibody: Supernatant from 455 hybridoma cells or purified antibody derived from 455 hybridoma supernatant
- Control IgG: Cetuximab
- Detection antibody:
  - α-Mouse Ab: Anti-Mouse IgG- DyLight® 488
  - α-Human Ab: Anti-Human IgG- DyLight® 488
- An antibody with no specificity to EGFR may be used as negative control.

Experimental Procedure

Step	Operation	Details
1	Collect Cell Culture Supernatant	• Carefully collect 1 mL from ASC culture into an Eppendorf tube. • Filter through 0.22 µm filter to remove cells and protein aggregates/debris. • Keep the supernatant at 4°C until use. ***Note:*** • *The ASC should be the same as that used in the main assay.* • *It is recommended to use 48 – 72 hours old culture (to allow accumulation of secreted antibody).* • *It is recommended to purify anti-EGFR Ab from cell culture supernatant.*
2	Prepare Primary and Secondary (detection) Antibody	• Label 4 x1.5 mL Eppendorf tubes for Ab mixes as per Experimental Design (**Table 1**). • Remove 455 Complete Media for ASC (455 hybridoma) from the fridge. • Add 0.5 mL Culture Medium into the 4 x 1.5 mL Eppendorf tubes. • Add relevant primary and/or detection antibody to achieve **2-fold final concentratio**n (2 µg/mL or 4 µg/mL, respectively): - Condition 1: 0.5 mL medium + 4.0 µg/mL Detection Ab - Condition 2: 0.5 mL medium + 4.0 µg/mL Detection Ab - Condition 3: 0.5 mL medium + 2.0 µg/mL Primary Ab (Cetuximab) + 4.0 µg/mL Detection Ab - Condition 4: 0.5 mL medium + 2.0 µg/mL Purified IgG (or 0.5 mL Supernatant if purified IgG is not available) + 4.0 µg/mL Detection Ab *- Condition 5 (Optional): 0.5 mL medium + 2.0 µg/mL isotype Ab + 4.0 µg/mL Detection Ab* ***Note:*** • *Eppendorf tubes for Conditions 3, 4 & 5 will contain both primary and detection antibodies.* • *Condition 4 will either contain IgG purified from cell culture supernatant or unpurified supernatant. Where 0.5 mL supernatant is added, no medium should be added to the Eppendorf tube.*

		• *Antibody concentrations are prepared at twice the final assay concentration, as they will be mixed 1:1 with cell-containing culture media to achieve the desired final concentration.*
3	Prepare Target Cells and ASC	• Harvest the target cells and ASC following supplier recommendations. • Count cells and calculate the volumes corresponding to the following: - Target cells: 4 x10^5 cells - ASC: 1 x10^5 cells • Take the calculated volumes (+10% to allow for cell loss) into separate 2 mL Eppendorf tubes • Spin cells down at room temperature. • Gently discard the supernatant from the Eppendorf tube without disturbing the pellet. • Add 2 mL of 455 Complete Medium to target cells and 0.5 mL to ASC
4	Imaging	• Label Nunc Lab-Tek II 8-Well (or other multi-well chamber compatible with imaging) according to **Table 1**, allowing duplicate wells per condition. • Add 0.2 mL of the relevant antibody containing sample (**Step 2**) and 0.2 mL of ASC (Condition 1) or target cells (Conditions 2 – 5; **Step 3**). • Incubate for 30-60 minutes at 37°C • Image wells under fluorescence microscope. Use Conditions 1 and 2 to determine minimal starting exposure. ***Note:*** *Nunc Lab-Tek II 8-Well working volume is 0.2-0.5 mL. Current experiment is designed with final 0.4 mL/well.*
5	Data Analysis	**Determine the background signal:** Conditions 1 and 2 (and optional Condition 5): • These serve as negative controls. Cells should not exhibit any detectable fluorescent signal. • Detection of fluorescent signal indicates non-specific binding of the detection antibody to the cell surface. • If fluorescent signal is observed, check cell viability, adequacy of the wash steps and optimize the detection antibody concentration.

		• *If fluorescent signal is observed in Condition 5, determine the FcR expression on target cells.* **Assess the suitability of the assay for detection of the secreted antibody:** Condition 3: • Serves as a positive control. Recombinant antibody, Cetuximab, will bind its specific antigen expressed on target cells. • Target cells coated with Cetuximab will subsequently bind the fluorescently labeled detection antibody and appear fluorescent under microscope (**Figure 4**). • If required, optimize fluorescent signal by titrating the concentrations of Cetuximab and the detection antibody. Condition 4: • The presence of fluorescently labeled target cells indicates that the assay conditions are suitable for detecting secreted antibody binding to its antigen on the target cells. • The fluorescent signal should be bright, and most target cells should be labelled. • If no or low signal is detected, determine the concentration of secreted antibody in the supernatant and consider collecting the supernatant at a later time point.

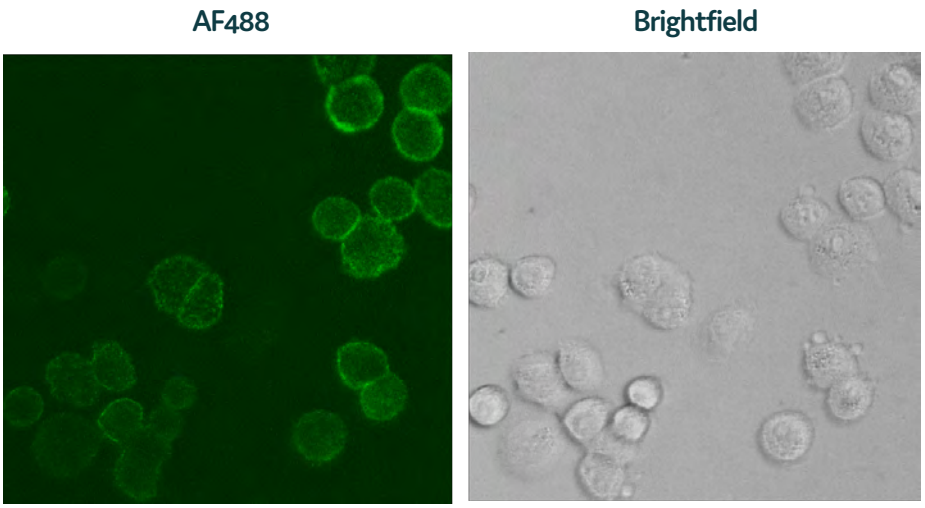


Figure 4. A431 cells were stained with anti-EGFR IgG (Cetuximab) and anti-human IgG AF488 antibodies (as in Condition 3, Positive control).

3.2 Assay Development ON Cyto-Mine® Chroma

Aim

The aim of this development step is to:

- Determine the background signal on Cyto-Mine® Chroma.
- Test the assay format on Cyto-Mine® Chroma.
- *Determine the proportion of secreting cells within the culture**

** Instructions for how to do this can be found in the Use of Cyto-Cellect® Mouse Assay Kit with Viability Probe in Cyto-Mine® Chroma protocol and will not be discussed in this protocol.*

Experimental Design

Control experiments should be designed as follows:

1. Negative Control and Background assessment: Target cells with anti-mouse detection antibody and blocking reagents.

2. Positive Control and maximum signal assessment: Target cells with the antigen-specific primary antibody (Cetuximab), anti-human detection antibody and blocking reagents.

	Cells		Primary Ab	Detection Ab		Blocking		
Condition	**ASC**	**Target Cells**	**Control IgG**	**α-Mouse Ab**	**α-Human Ab**	**α-Mouse Ab (not fluorescent)**	**Fc Block**	**Purpose**
1	-	2×10^6 cells	None or 1.0 µg/mL of irrelevant IgG	2.0 µg/mL	-	2.0 – 5.0 µg/mL (Remove before run)	1 µg/ million cells	Negative control
2		2×10^6 cells	1.0 µg/mL of anti-EGFR IgG	-	2.0 µg/mL	2.0 – 5.0 µg/mL (Remove before run)	1 µg/ million cells	Positive control and maximum signal assessment

Table 2. Experimental design conditions. Table shows the conditions set up for the on Cyto-Mine® Chroma assay development. Details of material and cells used:

- Target cells: A431 cell line, expressing EGFR
- Control IgG:
  - Anti-EGFR: Cetuximab
  - Irrelevant IgG: isotype control
- Detection antibody:
  - α-Mouse Ab: Anti-Mouse IgG- Alexa-Fluor® 488
  - α-Human Ab: Anti-Human IgG- Alexa-Fluor® 488
- Blocking:
  - α-Mouse Ab: Anti-Mouse IgG and Fc Block: Anti-Mouse CD16/ CD32
  - α-Human Ab: Anti-Human IgG and Fc Block: Anti-Mouse CD16/ CD32

Each control condition requires a **separate Cyto-Mine® Chroma** run.

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® Chroma 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 target cells.</td></tr>
<tr><td>2</td><td>Prepare Encapsulation Medium (EM)</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>455 Complete Medium</td><td>N/A</td><td>74% v/v</td><td>1,452</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>
<tr><td>Rat Anti-Mouse CD16/ CD32 (Mouse BD Fc Block™)</td><td>0.5 mg / mL</td><td>1 µg / million cells</td><td>20</td></tr>
<tr><td>Alexa Fluor® 488 Anti-Mouse IgG</td><td>0.5 mg / mL</td><td>2 µg / mL</td><td>8</td></tr>
</table>
· Filter through 0.22 µm filter to remove protein aggregates and debris associated with detection molecule

· Keep Encapsulation Medium at 4°C until use

Note:

· Make Encapsulation Medium fresh, on the day of assay.

· Current protocol is shown for 2 mL of sample mix to accommodate for sample loss during the preparation. Only 1 mL of sample mix is required for a single Cyto-Mine® Chroma experiment.

· Do not include any media that is expected to contain secreted antibodies (i.e. “conditioned” media) as the presence of pre-existing antibodies in media will result in high background/false positive events.</td></tr>
<tr><td>3</td><td>Prepare Target Cells</td><td>· Harvest and count target cells.

· Calculate the volume of cells required corresponding to 2 x 10^6 of target cells.

· Transfer the required volume (+10% to allow for cell loss) into a clean 1.5 mL Eppendorf tube.</td></tr>
</table>

		• Spin cells down at room temperature. • Gently remove medium from the tube without disturbing the pellet. ***Note:*** *Target cells are deliberately used at a relatively low concentration (2×10^6 cells/mL) to ensure that a proportion of droplets remain cell-free, providing a baseline population for setting sorting thresholds.*
4	Blocking and Staining of Target Cells	• Resuspend the pellet in 500 µL of basal culture media (without serum). • Add 2.0 – 5.0 µg / mL of the unconjugated form of the anti-mouse detection antibody to block surface-associated IgG on ASCs • Add Mouse BD Fc Block™ (or any analogous product) according to manufacturer's instructions (≤ 1 µg / 1 x10^6 cells) • Add eBioscience™ Cell Proliferation Dye eFluor™ 670 (2 µM) to label target cells (for protocol follow manufacturer's instructions). • Incubate at 4°C for 30 minutes. • Spin cells down. • Gently discard supernatant from the tube without disturbing the pellet. • Resuspend the pellet in 1 mL of complete medium. • Spin cells down at room temperature. • Gently remove supernatant from the Eppendorf tube without disturbing the pellet. ***Note:*** • *Other Cell Dye or Markers can be introduced at this step. For more information see* ***Appendix I****.*
5	Load cells into Cyto-Cartridge®	• Resuspend the pellet in 1 mL of Encapsulation Medium **Positive Control Experiment:** Add the positive control Ab (Cetuximab) to a final concentration of 1 µg/mL. **Negative Control Experiment:** Add negative control Ab (unrelated mouse Ab) to a final concentration of 1 µg/mL. • Filter the cell suspension through a 30 µm cell strainer.

		• Determine cell concentration (cells/mL) and viability (%) in the final sample using NucleoCounter® NC-3000™ or equivalent cell counter. • 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:*** • *There will be certain sample loss due to sample filtering, but the filtered sample will have sufficient volume for the generation of up to 1.5 x 10^6 droplets.* • *Load the Cyto-Cartridge® aseptically in a Biosafety Cabinet.* • ***Cyto-Cartridge® is single-use** and disposable to prevent any cross-contamination.*
6	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: **120 minutes @ 37°C** • Type of microtiter plates (MTPs) for dispensing: **96-Well Plate** • Input cell density loaded based on the information obtained from **Step 5**. • Set up camera 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** • Number of picodroplets generated: **1 million** • Time: **24 minutes** <u>Picodroplet incubation/antibody secretion</u> • Hands-off step. • Incubation temperature: **37 °C** • Incubation time: **120 minutes**

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 stabilization may take up to 4 minutes.*

Sorting

- Lasers and Detectors required:

Probe	Laser	Detector
Alexa Fluor® 488 Anti-Mouse IgG	Blue Exc. 488 nm	Green Em. 520/18nm
Far-Red Cell dye	Red Exc. 638 nm	Far-Red Em. 678/30

- PMT Gains set up:

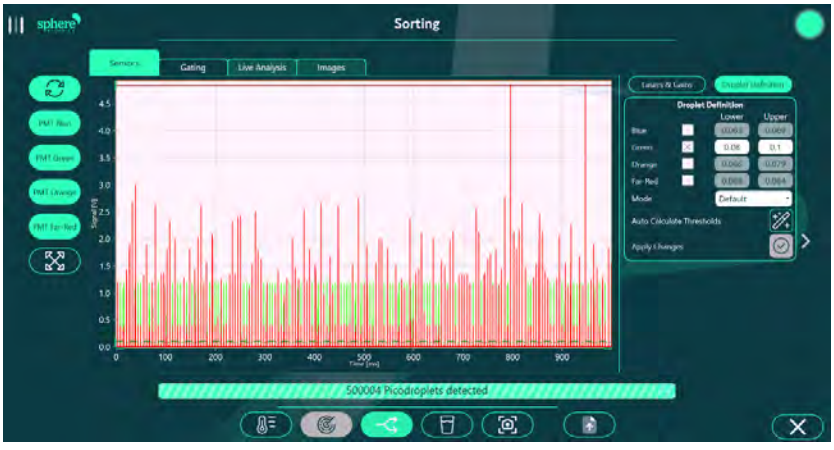
  - Set up PMT Gains to achieve the readout peak (Y- axis=Signal) at the height of 0.5-1.5 V for Green & Far-Red.

  - Detection trigger: Green above Threshold.

  - Auto-Thresholds for detection of fluorescence.

  - Max. picodroplets to collect: 15,000 if needed.

Note: *Ensure that the PMT settings used for these controls are the same as those to be used in the main assay to allow direct comparison of population distributions on the scatter plots.*



Gating Strategy

Gate 1: Exclude fused droplets

- Fused droplets can be excluded based on their increased width
- X-Axis: Inter Droplet Width
- Y-Axis: Intra Droplet Width

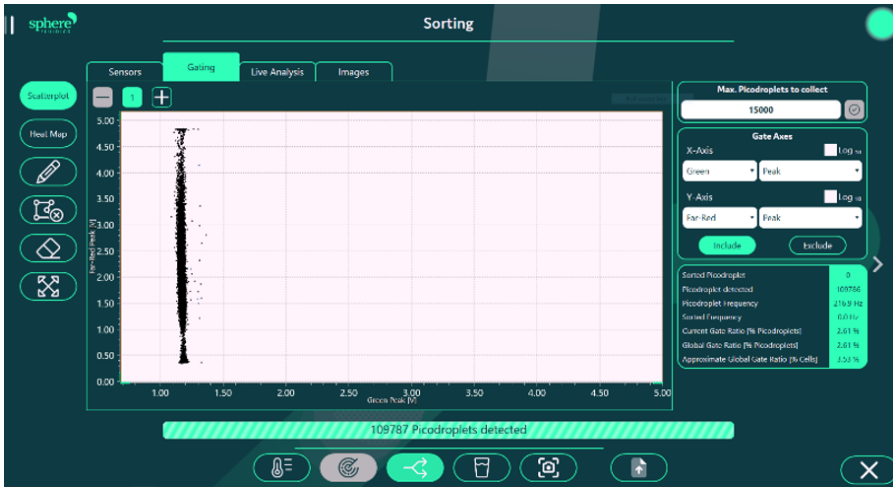


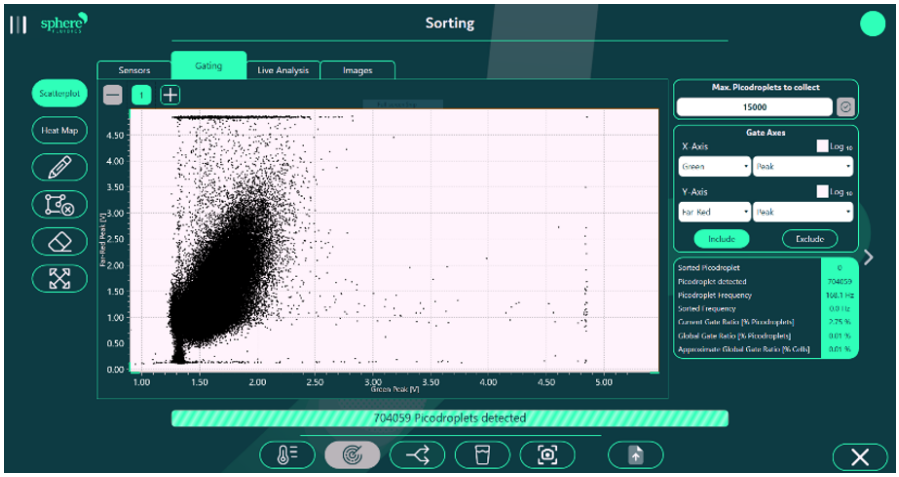
Gate 2: Include positive events (Ab binding on target cells)

- X-Axis: Green Peak Voltage
- Y-Axis: Far-Red Peak Voltage

Note: *For a typical experiment the expected proportion of fused droplets is < 1%, however this may increase in the presence of destabilising factors such as detergents and high serum content (>10%).*

Negative Control Scatterplot (no binding events detected)



		Positive Control Scatterplot (all input Target cells exhibit positive binding)  Acquire data · Sorting does not have to be activated, detection of 250k-500k droplets is sufficient for obtaining the necessary information. · The data acquired during sorting will be used to determine the optimal conditions for the main assay.
7	Data analysis	Determine background noise on Cyto-Mine® (negative control assay): · In the absence of ASC or Control Antibody, no positive Green fluorescent peak signal should be detected. · The presence of high peak signal indicates non-specific binding. Test the assay format on Cyto-Mine® (positive control assay): · If the percentage of droplets identified as containing target cells (Far-Red signal) is lower than expected, assess the efficiency of target cell labeling (for calculation of expected positive rate based on Poisson distribution see Antigen-Specific FRET assay protocol). · Ensure that the labeling conditions are optimized to achieve uniform staining without compromising cell viability. Labeling should be sufficient to detect all target cells, but not excessive to the point of causing cell stress or death. · Droplets containing target cells (Far-Red positive) should also show a Green Peak signal, indicating detection of recombinant antibody binding to target cells.

3.3 Assay Performance ON Cyto-Mine® Chroma

Experimental Design

Using the optimized conditions determined in **Sections 3.1** and **3.2**, the antigen specificity of the ASC can now be determined in Cyto-Mine® Chroma. The following conditions were used in this experimental setup:

1. ASC: Two hybridoma cell lines with different antigen specificities were used to assess the sensitivity of the assay for detecting antigen-specific ASCs at 2% of the total ASC population.

- Relevant ASC (secreting anti-EGFR antibody): 0.02×10^6 cells, labeled with eBioscience™ Cell Proliferation Dye eFluor™ 450.
- Irrelevant ASC (secreting non-relevant antibody): 1×10^6 cells, labeled with CellTracker™ Orange CMRA.

2. Target cells: Target cells expressing surface EGFR were used at 8×10^6– 10×10^6 cells; Cells were labelled with eBioscience™ Cell Proliferation Dye eFluor™ 670.

Note: *Target cells are used in excess to achieve ~100% droplet occupancy, ensuring that droplets containing an ASC also contain at least one target cell.*

Experimental Procedure

Step	Operation	Details
1	Prepare the reagents	· Thaw the relevant vials of the Cell labeling reagents. · Centrifuge the vial briefly in a microcentrifuge to collect the reagents at the bottom of the tube. · Keep the vial cold until ready to use
2	Cyto-Mine® Chroma Set Up System Warm Up	· Start Cyto-Mine® GUI software and start System Warming Up Step (Hands Off Step).
3	Prepare Encapsulation Media	· Prepare the Encapsulation Medium as in **Section 3.2, Experimental Procedure, Step 3**
4	Prepare ASC	· Harvest and count the ASC. · Calculate the volume of cells corresponding to 0.02×10^6 of anti-EGFR IgG secreting hybridoma cell line (relevant ASC) and 1×10^6 cells of irrelevant hybridoma cell line. ***Note:*** *For planning the desired number of secreting cells, take the number of ASC within the relevant ASC population into consideration. Our Cyto-Cellect® Mouse assay kit can be used with the relevant ASC prior to determine percentage secreting cells (see Antigen-specific FRET assay protocol for more information)*

		• Take the calculated volume of cells (+10% to allow for cell loss) into separate clean 1.5 mL Eppendorf tubes. • Spin cells down at room temperature. • Gently discard supernatant from the Eppendorf tubes without disturbing the pellet.
5	Blocking and staining ASC	• Follow **Section 3.2 Experimental Procedure Step 4** for blocking and staining of cells with the following exceptions: • Label cells: - Relevant ASC (anti-EGFR): eBioscience™ Cell Proliferation Dye eFluor™ 450 (follow manufacturer's instructions) - Irrelevant ASC: CellTracker™ Orange CMRA (follow manufacturer's instructions) • Following the wash, resuspend the pellet in 0.4 mL Encapsulation Medium • Filter the cell suspension through a 30 µm cell strainer. • Determine the cell concentration (cells/mL) and viability (%) in the final sample using NucleoCounter® NC-3000™ or equivalent cell counter. • Keep cells at 4°C until final Sample preparation ***Note:*** *Cells are kept at room temperature during initial washes to avoid 'cold capture' of the antibody on cell membrane.*
6	Prepare target cells	• Harvest and count target cells as described in **Section 3.2 Experimental Procedure Step 3-4.** • Calculate the volume of cells corresponding to 8 x 10^6 - 10 x 10^6 of target cells. • **Follow the protocol as in 3.2. Experimental Proecure Step 3-4.**
7	Final Sample Preparation	• Keep cells at 4°C throughout this section of sample preparation to reduce antibody secretion. • Combine the ASC and target cells in a fresh 1.5 mL Eppendorf tube • Make up the volume of sample to 1 mL by adding Encapsulation Medium.
8	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. • Follow the step-by-step instructions on the screen.

<table>
<tr><td></td><td></td><td>Note: 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. If generating 2 x 10^6 droplets, make sure to have 1 mL of sample volume.</td></tr>
<tr><td>9</td><td>Cyto-Mine® Chroma set up & run</td><td>
Follow the set-up information in Section 3.2 Experimental Procedure Step 6, with the following exceptions:
<u>Experimental Parameters</u>
Number of picodroplets to generate: 1.5 x 10^6 (up to 2 x10^6 droplets can be generated)
<u>Picodroplet generation/cell encapsulation</u>
Number of picodroplets generated: 2 x10^6
Time: 48 minutes
<u>Sorting</u>
Lasers and Detectors required:
<table>
<tr><th>Probe</th><th>Laser</th><th>Detector</th></tr>
<tr><td>Alexa Fluor® 488 Anti-Mouse IgG</td><td>Blue
Exc. 488 nm</td><td>Green
Em. 520/18nm</td></tr>
<tr><td>Orange Cell Dye (CellTracker™ Orange CMRA</td><td>Yellow
Exc.561 nm</td><td>Orange
Em. 605/20 nm</td></tr>
<tr><td>Violet Cell Dye (eBioscience™ Cell Proliferation Dye eFluor™ 450)</td><td>Violet
Exc. 405 nm</td><td>Blue
Em. 446/34 nm</td></tr>
<tr><td>Far-Red Cell dye (eBioscience™ Cell Proliferation Dye eFluor™ 670)</td><td>Red
Exc. 638 nm</td><td>Far-Red
Em. 678/30 nm</td></tr>
</table>
PMT Gains set up:
Set up PMT Gains to achieve the readout peak (Y- axis=Signal) at the height of 0.5-1.5 V for Blue, Orange, Green & Far-Red.
Detection trigger: Green above Threshold.
Auto-Thresholds for detection of fluorescence.
Max. picodroplets to collect: 15,000
</td></tr>
</table>



Gating Strategy

Set Up Three Gates

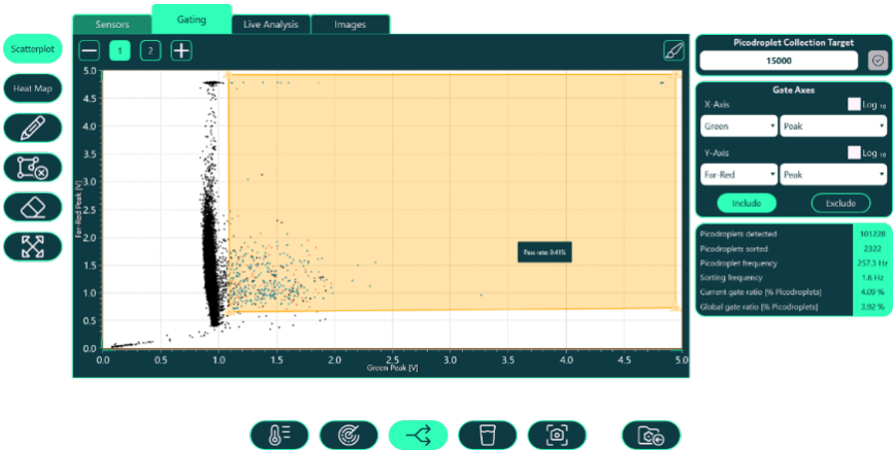
Gate #	X-Axis	Y-Axis	Gating Logic
1	Green Average (V)	Intra Droplet Width	Include
2	Green Peak (V)	Far-Red Peak (V)	Include
3	Blue Peak (V)	Orange Peak (V)	Include

Gate 1: Exclude fused droplets

- Please follow the information as in **Section 3.2 Experimental Procedure, Step 6**

Gate 2: Droplets including target cells (Far-Red) and binding (Green Peak)

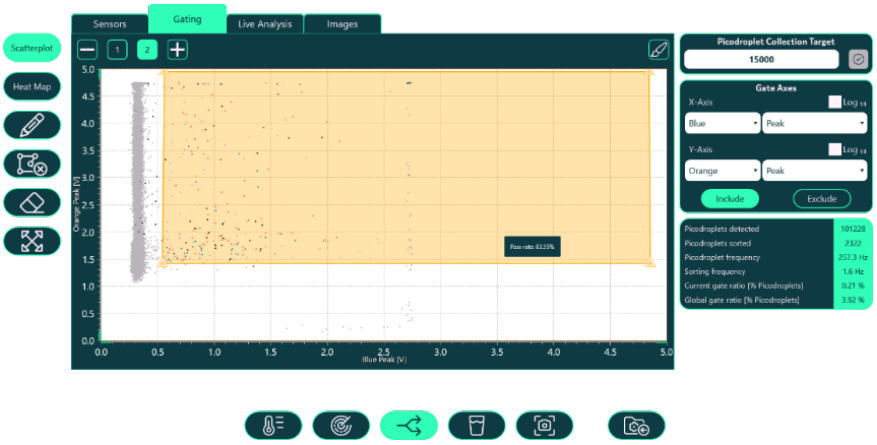
- Binding of anti-EGFR IgG to the target cells will be detected as increased Green Peak signal.
- X-Axis: Green Peak
- Y-Axis: Far-Red Peak
- Draw a polygon on the scatter plot to gate the (Green Peak+/Far-Red Peak+) events.
- Define gate logics as "Include"



Gate 3: Droplets containing relevant ASC but not the irrelevant ASC

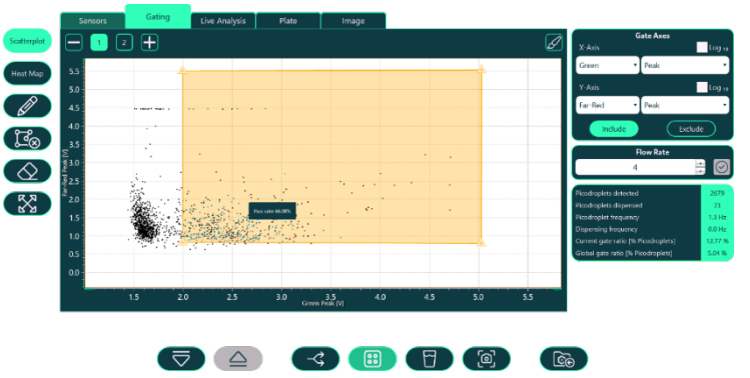
- X-Axis: Blue Peak
- Y-Axis: Orange Peak
- Draw a polygon on the scatter plot to gate the all the Blue Peak+ events
- Define gate logics as "Include"

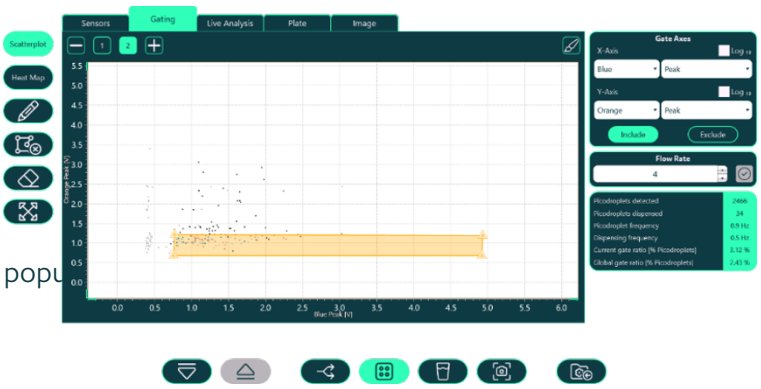
Note: *Based on the user's objective, polyclonal droplets (containing both types of secreting cells) can be included or excluded from the polygon area.*



Acquire Data

- Sort about 7,000- 15,000 positive droplets
- The data acquired from this will be used for further analysis (see below)

<table>
<tr><td>10</td><td>Dispensing Flow Stabilization</td><td>• Start Flow Stabilization. This will take 4-10 minutes to appear
• Slowly reduce flow rate from 20 -> 15 -> 10 -> 5 µL/hr with 30 seconds wait between changing each flow rate.</td></tr>
<tr><td>11</td><td>Prepare 96-Well plate for dispensing</td><td>• Label 96-Well plates with following information: Cell Type; Date; Instrument ID, Plate no.
• 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 customer support.</td></tr>
<tr><td>12</td><td>Dispensing</td><td>• Adjust flow rate so that Picodroplets frequency is ≤ 1Hz.
• Set up Gains and Thresholds for detection of fluorescence signal, similar to Step 9 for Sorting
• On the ‘Gating’ tab ensure the following is set up:
<table>
<tr><th>Gate #</th><th>X-Axis</th><th>Y-Axis</th><th>Gating logic</th></tr>
<tr><td>1</td><td>Green Peak (V)</td><td>Far-Red Peak (V)</td><td>Include</td></tr>
<tr><td>2</td><td>Blue Peak (V)</td><td>Orange Peak (V)</td><td>Include</td></tr>
</table>


<u>Gate 1:</u> Draw a gate on scatterplot for the Green Peak⁺/Far-Red Peak⁺


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

		Gate 2: Draw a gate on scatterplot for the Blue Peak^{+}/Orange Peak^{-} population (adjust polygon to include or exclude unwanted cell population). • Dispense the positive population into Multi-Well Plate (96 or 384 well) for subsequent culturing or PCR analysis ***Note:****Current gating is restricted to monoclonal droplets with relevant secreting cells only (Blue Peak^{+}/Orange Peak^{-}). If interested in any droplets containing positive signal, expand the gate 2 to Blue Peak^{+}/Orange Peak^{+} (polyclonal droplets containing positive cells along irrelevant secreting cells)*
13	Post-Dispensing top-up of plates	• Following dispensing, incubate the plates in the 37°C incubator, 5% CO_2 for 15 minutes. • After incubation, use a 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.
14	End of run	• When the plates are dispensed, Click "End run" and follow instruction as prompted by Cyto-Mine® Chroma

4. Data Analysis

This section explains how to use FlowJo™ to analyze Cyto-Mine® Chroma data, assess sample quality, and determine the number of viable IgG-secreting cells. For viewing in-run scatter plots or analysing 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 Determining the number of binding events

Droplets containing antigen-specific ASC can be identified using increased localized green fluorescence signal (Green Peak$^+$), indicating binding of the detection molecule to the target cell within the droplet. As the probe remains confined within droplets throughout the Cyto-Mine® Chroma run, binding of the secreted antibodies to their target antigen allows true single-cell assessment without cross-talk.

Follow the steps below to determine the number of surface-binding events in the cell sample. Additional labeling of the other components of this assay (labeling of target and secreting cells) can provide better evaluation of assay quality and the validity of measured positive events. Critically, in this assay format, true positive hits are expected to occur in the subpopulation of droplets containing both target cells and relevant secreting cells.

Determine the percentage of antigen-specific ASC binding events in subpopulation of droplets

- Open the experiment file using Cyto-Mine® Data Analysis software.
- Export .csv format files of the sorting data.
- Import the sorting .csv file into FlowJo™ software.
- Gate events:

    Gate 1: Contains relevant ASC and target cells: Far-Red$^+$/Blue$^+$ (**Figure 5A**).

    Gate 2: Using the population in Gate 1, create a daughter gate on droplets showing binding: Gate on droplets containing the two components of a functional assay that showing binding events: Far-Red$^+$/Green Peak$^+$ (**Figure 5B**).

A very small proportion (0.6%) of total droplets contain both ASC and target cells, which is in line with Poisson-based estimation of expected positive rate for 0.02×10^6 cells of relevant ASC. About 70% of these droplets exhibit positive binding events, in correlation to the previously determined secretion rate (50%-70%) of this hybridoma cell-line.

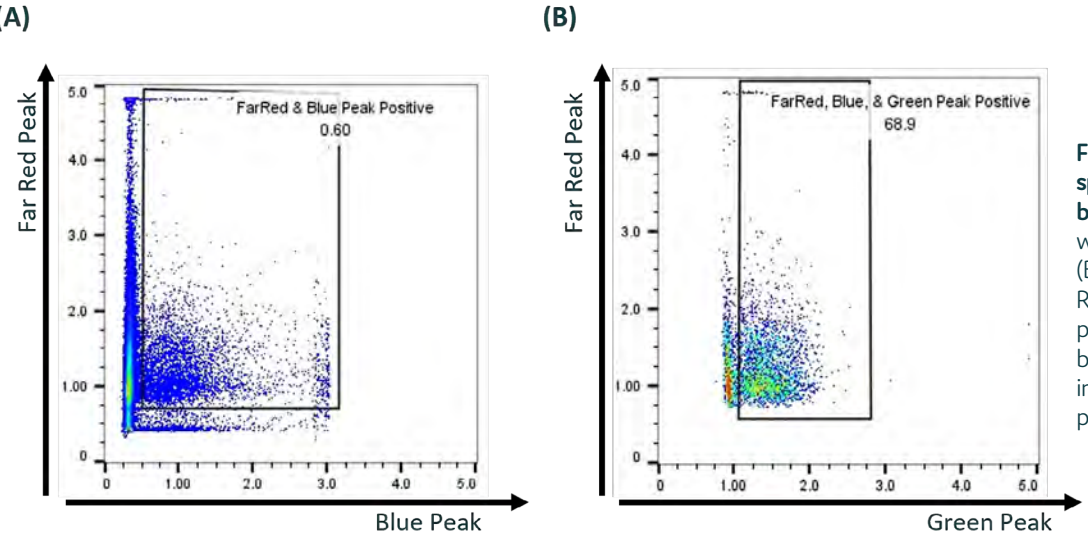


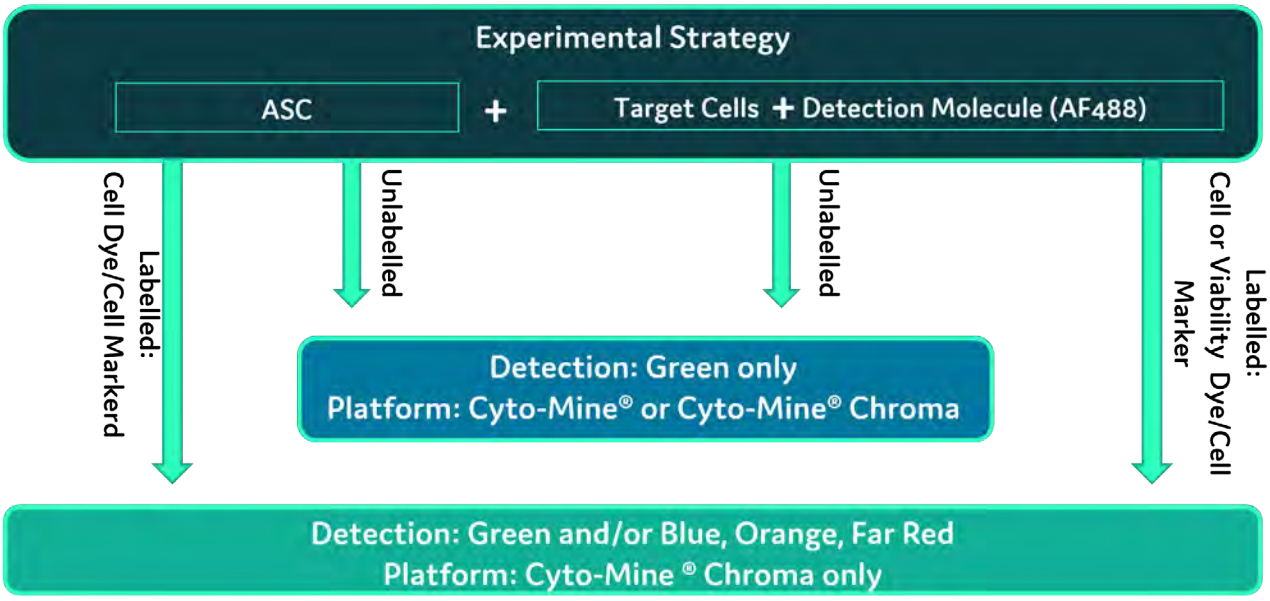
Figure 5. Detecting antigen specific ASC using Cell surface binding assay. (**A**) Droplets were gated for relevant ASC (Blue Peak$^+$) and target cells (Far Red Peak+). (B) Double positive population was gated for positive binding events (Green Peak$^+$ in Blue Peak$^+$ /Far-Red Peak$^+$ population).

5. Experiment Optimization and Troubleshooting

Experimental Step	Specific issue	Solution/Optimization
Assay development off-Cyto-Mine® Chroma	No/Lower than expected proportion of surface-bound signal	· Use other methods (microscopy imaging or flow cytometry) to develop and optimize the assay by including positive and negative controls, titrating the amount and the type of detection molecule, testing antigen expression on the surface of target cells.
Detection of Ag-specific surface binding assay on Cyto-Mine® Chroma	Low proportion of binding events/No positive population	· Include Positive control with recombinant /purified control IgG to validate robust assay performance · Prior to the Cyto-Mine® Chroma run assess the compatibility of the Detection Molecule (i.e. fluorescent antibody) with secreted molecule by testing the cell culture supernatant with target cells and detection molecule using microscopy. · Make sure to avoid any Ab contamination in the encapsulation media (i.e. conditioned media) and to remove any secreted molecules from cell sample by washing cells during sample preparation. · Ensure no cell loss in the final Cyto-Mine® Chroma sample by counting both secreting and target cells in the final sample · Ensure all required additives (Opti-Prep, PF-68 and Detection Molecule) have been added to Encapsulation medium. · Test productivity of cells using different method (ELISA, flow cytometry).

Detection of Ag-specific surface binding assay in Cyto-Mine® Chroma	High level of fused / destabilized droplets	• Make sure Encapsulation Media does not contain any detergents and does not contain more than 10% FBS. • During the preparation ensure the sample is filtered and does not contain and clumps or fibres that can destabilise droplets thought different steps of Cyto-Mine® Chroma run.
Detection of Ag-specific surface binding assay in Cyto-Mine® Chroma	High level of background/higher than expected positive population	• Ensure blocking approach is efficient to reduce/block background (use other methods i.e. microscopy, flow cytometry to validate materials). • Filter all the buffers to remove any debris and protein aggregates. • Due to tendency of dead cells for unspecific binding reduce the number of dead/dying cells using Dead Cells Removal Kit (130-090-101, Miltenyi Biotec). • Label dead/dying cells to exclude from downstream analysis. For example, the Viability Probe Kit by Fluidic Sciences (C315) can be used to exclude dying/dead cells during sorting and dispensing of cells in Cyto-Mine® Chroma. As the Viability probe is encapsulated together with the cells, any cells starting to undergo apoptosis during incubation in Cyto-Mine, will be labelled and can be excluded from dispensing. For the use for the Viability Probe Kit please contact the Fluidic Sciences Sales team. Alternatively, Propidium Iodide (or other fluorescent DNA binding reporter dyes) can be added to help exclude dead cells from positive population.

6. Appendix I- Alternative Experimental designs



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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