Application Note 19

Cyto-Cellect® Fc Fusion: Detection of Human Fc Fusion Proteins with Cyto-Mine®

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Background

The Cyto-Cellect® Fc Fusion Detection Kit from Sphere Fluidics is designed to work in conjunction with the Cyto-Mine® Chroma Single Cell Analysis Platform. It detects Fc fusion proteins with a human Fc region, secreted from cells encapsulated within picodroplets. Fc fusion molecules secreted from encapsulated cells disrupt the interaction of a pre-formed pair of FRET (Förster Resonance Energy Transfer) probes, resulting in a decreased FRET signal (Figure 1). Using the detection kit with Cyto-Mine® provides an assay for the measurement of single cell productivity by detecting secreted Fc fusion molecules, enabling the identification and selection of cells with the highest productivity.

The Cyto-Cellect® Fc Fusion Detection Kit is certified animal origin free (AOF) and is tested for isolation of Fc fusion protein secreting CHO cells (containing the Fc region of hIgG1) using Cyto-Mine®.

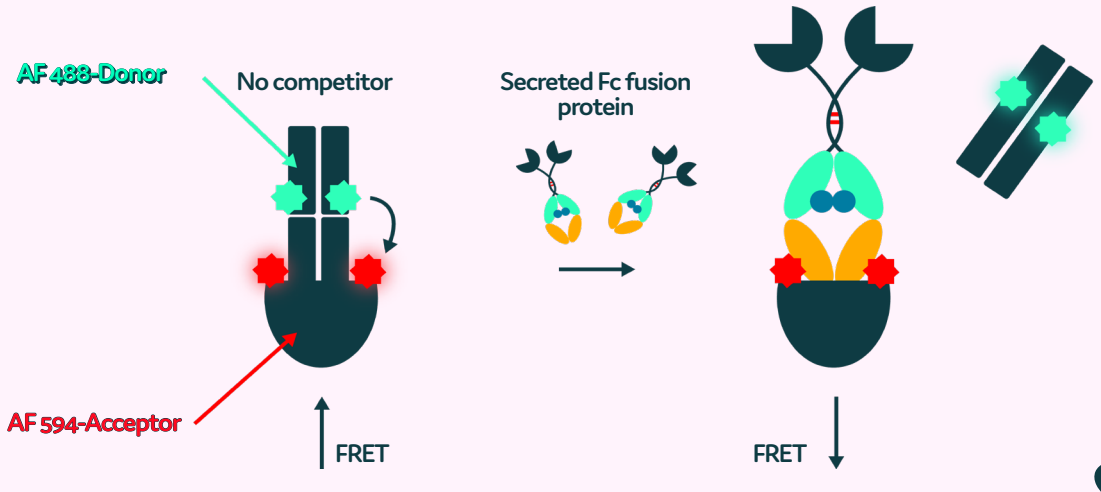

Figure 1. Schematic diagram of the Cyto-Cellect® Fc Fusion detection kit for Fc fusion protein secreting cells.

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Aims and Objectives

Here we demonstrate the suitability of Cyto-Cellect® Fc Fusion kit to detect Fc fusion proteins containing human IgG Fc domains. We show that the kit can be used in a plate reader, to reliably detect human Fc fusion proteins as well as human IgG 1-4. In Cyto-Mine®, the kit was successfully used to isolate human Fc fusion proteins as well as IgG4κ secreting cells.

Methods

Detection of Fc fusion proteins using Cyto-Cellect® Fc Fusion assay, in plate reader

Commercially available Fc fusion proteins (Abatacept, Abatacept (Fc silent), Etanercept) containing the Fc region of human IgG1 were titrated (150 nM – 2.34 nM, 2-fold dilution, 6-point titration) in the presence of Cyto-Cellect® Fc Fusion FRET probes. FRET response was determined in a plate reader, at excitation 488 nm; emission 520 nm (Green Channel) and 620 nm (Red Channel). FRET response was calculated by dividing the Red Channel Intensity by Green Channel Intensity. The FRET response was normalized to the response of 0 nM Fc fusion protein (control).

Using Cyto-Cellect® Fc Fusion for detection and dispensing of CHO cells in Cyto-Mine®

CHO cells, secreting Fc fusion proteins or human IgG4κ, were resuspended in Encapsulation Medium (83% growth medium, 16% Opti-prep, 1% Poloxamer 188) and Cyto-Cellect® Fc Fusion FRET probes. Cells were then loaded onto the Cyto-Cartridge and secretion was detected using Cyto-Mine®. The integrated stages of the Cyto-Mine® process are summarized in Figure 2. Briefly, cells and FRET probes are encapsulated into picodroplets. These are incubated within the incubation chamber at 37°C to allow optimum conditions for target protein secretion. The secreted target protein accumulates inside the picodroplet, which is captured and detected by FRET probes. Cells secreting the desired protein are detected in Cyto-Mine® during sorting and dispensed into tissue culture plates.

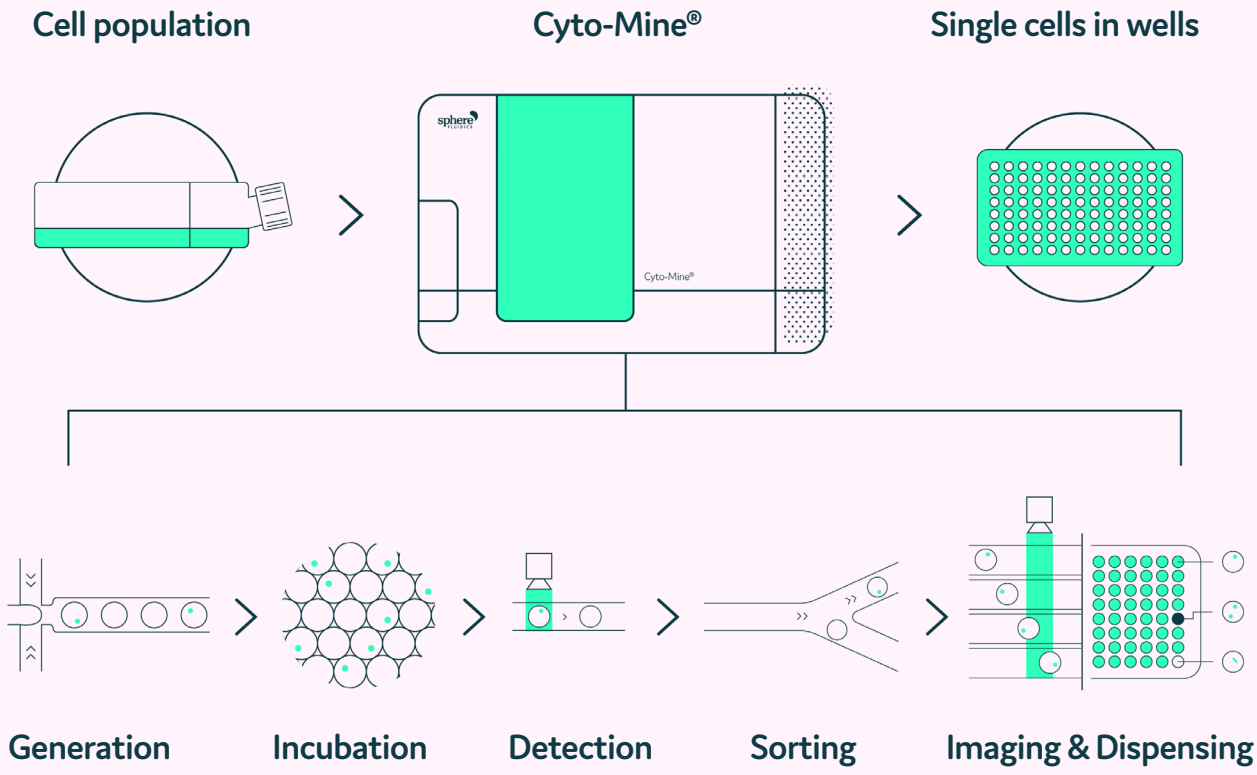


Figure 2. A Cyto-Mine® workflow. Integration of single cell screening, sorting, isolation, and verification using a fully integrated microfluidic process.

Detection of human IgG1-4 antibodies using Cyto-Cellect® Fc Fusion assay, in plate reader

Commercially available human IgG1-4 κ or λ antibodies were titrated (133 nM – 2.1 nM, 2-fold dilution, 6-point titration) in the presence of Cyto-Cellect® Fc Fusion FRET probes. Following a 2 hour incubation period, FRET response was determined in a plate reader, at excitation 488 nm; emission 520 nm (Green Channel) and 620 nm (Red Channel). FRET response was calculated by dividing the Red Channel Intensity by Green Channel Intensity. The FRET response was normalized to the response of 0 nM Fc fusion protein (control).

Results

Cyto-Cellect® Fc Fusion probes detect human Fc fusion proteins in a concentration-dependent manner

The ability of the Cyto-Cellect® Fc Fusion probes to detect human Fc fusion proteins was examined by a plate reader-based FRET assay. For this purpose, we titrated commercially sourced Abatacept (CTLA-4 Fc fusion) and Etanercept (TNF receptor 2 Fc fusion), in the presence of Cyto-Cellect® Fc Fusion assay probes. In addition, we assayed an Fc silent version of Abatacept to determine whether mutations in the Fc region affect assay performance.

The data in Figure 3A show that increasing concentrations of Abatacept and Etanercept result in a decreased FRET response, consistent with these molecules displacing the FRET Donor from the acceptor probe. Importantly, an Fc silent mutation in Abatacept did not impact the FRET response.

Figure 3B depicts the FRET response observed over time for Abatacept as target molecule. A short incubation of 30 minutes was sufficient to induce a 50% change in FRET signal compared to control at the highest concentration of Abatacept tested.

Further incubation for up to 3 hours only minimally reduced this response (41% reduction in signal).

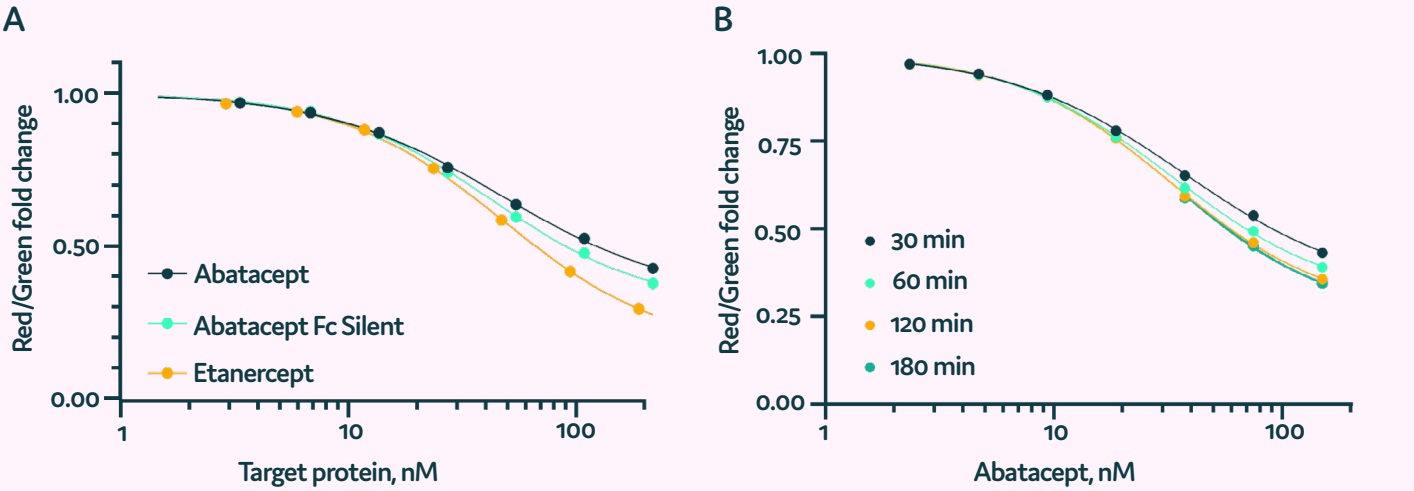
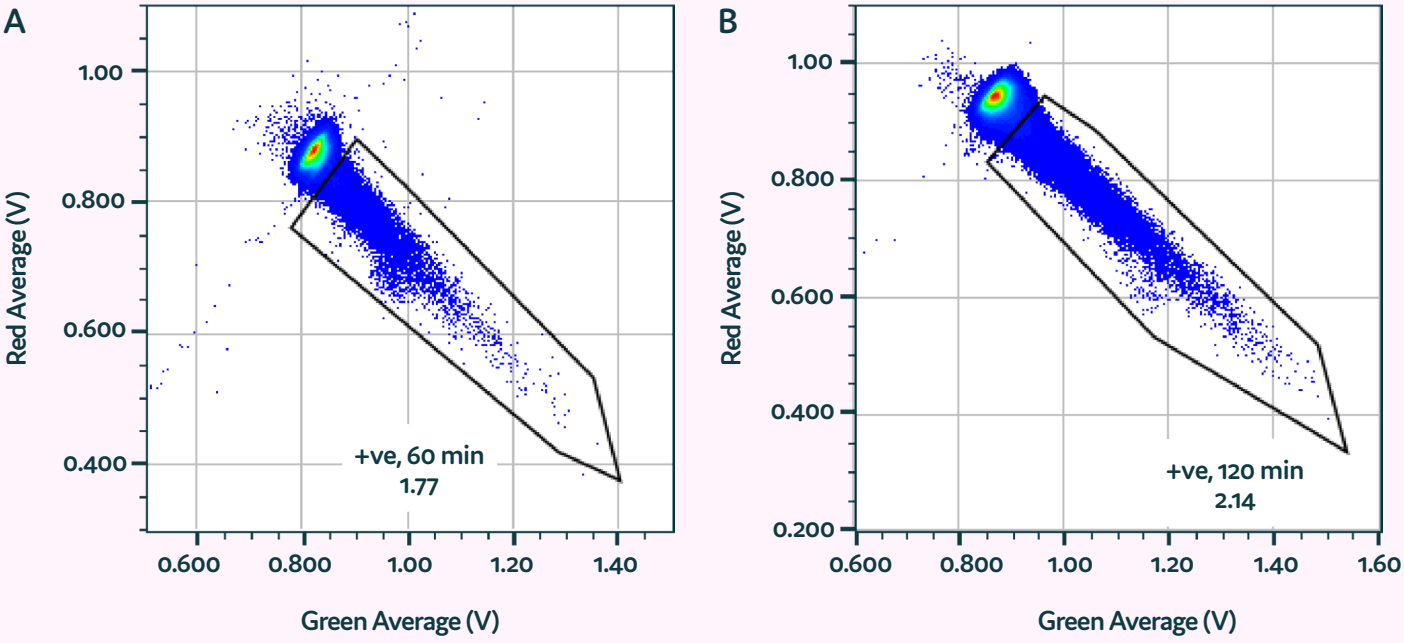


Figure 3. FRET response of Cyto-Cellect® Fc Fusion in the presence of commercial Fc fusion proteins. (A) Commercially sourced Fc fusion proteins were titrated in the presence of Cyto-Cellect® Fc Fusion probes and fluorescence signals measured on a Clariostar® Plus plate reader. Data show normalized FRET response, calculated by dividing Red intensity/Green intensity. (B) Abatacept was titrated in the presence of Cyto-Cellect® Fc Fusion probes. FRET response was determined as in (A) after the indicated time points.

Using Cyto-Cellect® Fc Fusion for detection and isolation of Fc fusion protein- secreting CHO cells in Cyto-Mine®

Cyto-Cellect® Fc Fusion was used to detect Fc fusion protein-secreting CHO cells in Cyto-Mine®. Figure 5A shows the decreasing FRET signal from CHO cells secreting a Fc fusion protein, following a 1-hour incubation of cells with the Cyto-Cellect® Fc Fusion probes. Incubating cells for a longer period can lead to increased detection of the gated population as shown in Figure 5B. Sorted picodroplets were dispensed into microtiter plates and cell outgrowth monitored for 2 weeks. Data show that the dispensed cells remain viable and grow into colonies. Subsequent screening of >600 colonies showed 89.8% of clones secreting the desired Fc fusion protein.



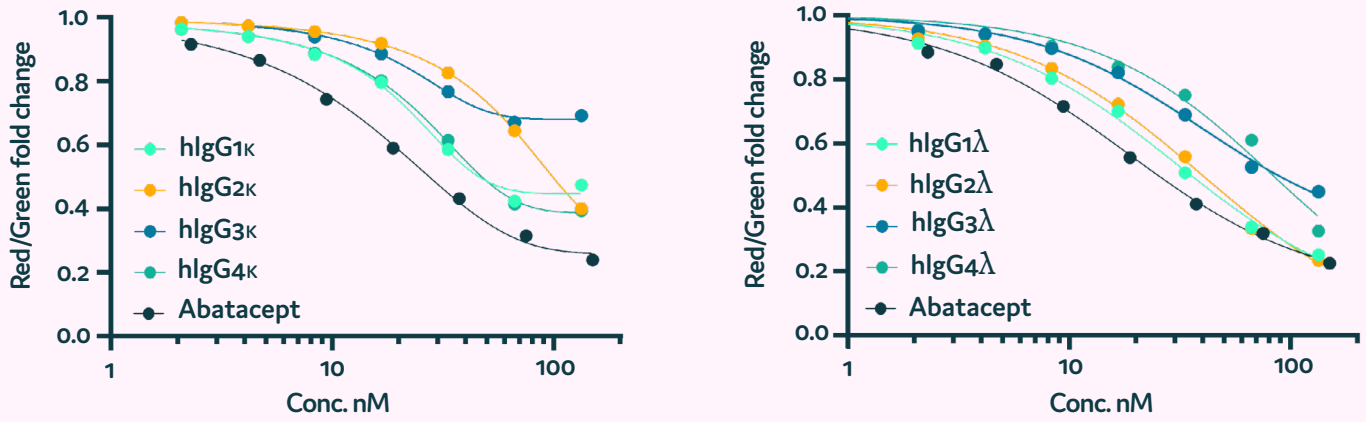
Monoclonal droplets	87.5%
Cell outgrowth	54%

Monoclonal droplets	90.52%
Cell outgrowth	43%

Figure 4. Cyto-Cellect® Fc Fusion used for enrichment and single cell dispensing of Fc fusion secreting CHO cells in Cyto-Mine®. CHO cells, secreting Fc fusion protein were resuspended in Encapsulation Medium ($1.1x10^5$) containing Cyto-Cellect® Fc Fusion FRET probes and loaded onto Cyto-Mine®. Cells were incubated for 1 (A) or 2 hours (B) and dispensed as single cells into microtiter plates. Dot plots show sorting data. Tables show clonality data and cell outgrowth for dispensed cells.

Detection of human IgG1-4 using Cyto-Cellect® Fc Fusion kit, in plate reader

As the commercially available Fc fusion proteins tested contained a human IgG1 Fc domain, we tested the ability of Cyto-Cellect® Fc fusion kit to detect other IgG Fc domains using commercially available human IgG1-4 antibodies. Cyto-Cellect® Fc Fusion probes showed dose-dependent responses to commercial IgG1-4κ (Figure 5A) and IgG1-4λ (Figure5), with higher EC_{50} values compared to Abatacept, suggesting that Fc fusion proteins can compete more efficiently with the Donor probe than parent IgG molecules. Interestingly, differences between responses to human IgG κ and λ molecules were observed, most notably in the case of IgG1, 3 and 4κ for which the response plateaued at ~66 nM. This was not observed for the corresponding IgG λ subclasses.



Target	EC_{50}
Abatacept	15 nM
hIgG1κ	18 nM
hIgG2κ	43 nM
hIgG3κ	19 nM
hIgG4κ	29 nM

Target	EC_{50}
Abatacept	18 nM
hIgG1λ	31 nM
hIgG2λ	40 nM
hIgG3λ	40 nM
hIgG4λ	83 nM

Figure 5. FRET response of Cyto-Cellect® Fc Fusion in the presence of commercial hIgGκ and λ subclasses. Commercially sourced IgGκ (A) or IgGλ (B) antibodies were titrated in the presence of Cyto-Cellect® Fc Fusion probes. Data show normalized FRET response, calculated by dividing Red intensity/Green intensity. Commercially available Abatacept was used as a control. EC_{50} values were determined with GraphPad Prism 10.3 using the built-in [Inhibitor] vs. response - Variable slope (four parameters) non-linear fit.

Detection of human IgG4κ antibody using Cyto-Cellect® Fc Fusion kit, in Cyto-Mine®

Cyto-Cellect® Fc Fusion was used to detect antibody- secreting cells in Cyto-Mine®. Figure 6 shows FRET response of CHO cells secreting human IgG4κ, following a 1- and 2- hour incubation of cells with the Cyto-Cellect® Fc Fusion probes. The Fc Fusion probes were able to detect human IgG4κ secreting cells in Cyto-Mine® following a 1- hour incubation (Figure 6A). Although a more prolonged incubation time increased signal intensity, the percentage gated population remained the same (Figure 6B).

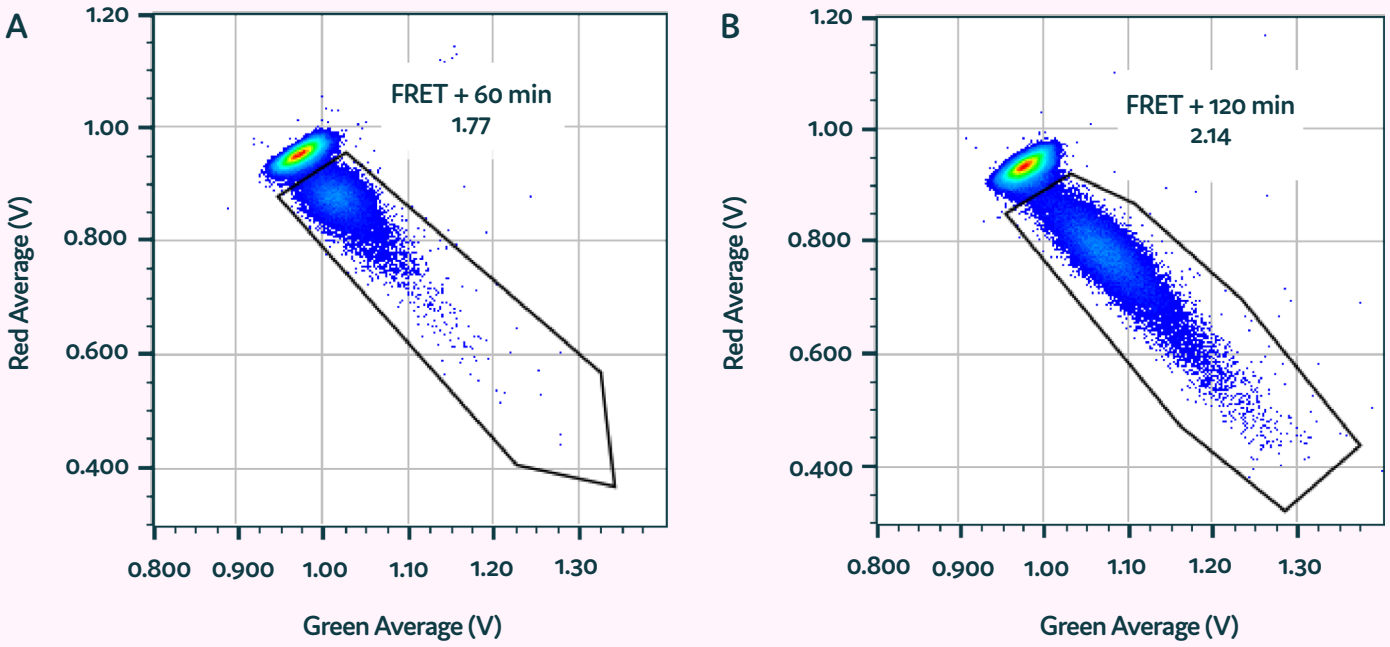


Figure 6. Cyto-Cellect® Fc Fusion kit used for enrichment of human IgG secreting CHO cells in Cyto-Mine®. CHO cells, secreting human IgG4**K**, were resuspended in Encapsulation Medium (3.4 x105 cells/mL) containing Cyto-Cellect® Fc Fusion FRET probes and loaded onto Cyto-Mine®. Cells were incubated for 1 (A) or 2 hours (B) and gated during sorting based on fluorescence signal. Dot plots show sorting data.

Conclusions

The data presented here shows that the Cyto-Cellect® Fc Fusion kit provides a sensitive FRET response to secreted Fc fusion proteins and is compatible with both plate reader and Cyto-Mine®. In addition, human IgG 1-4 (κ and λ) as well as Fc silent Abatacept were detectable using Cyto-Cellect® Fc Fusion probes in plate reader. In Cyto-Mine®, the kit enables the enrichment and single cell dispensing of CHO cells secreting Fc fusion molecules and human IgG (IgG4κ tested). Dispensed cells are viable and demonstrate successful proliferation and cell outgrowth in culture.

Compatibility with Cyto-Mine®:

The Cyto-Cellect® Fc Fusion kit can be used in combination with Cyto-Mine® to allow sorting and dispensing of single cells, based on their productivity.

Detection of Fc fusion proteins:

The kit detects Fc domains from all four IgG subclasses, as well as Fc- silent mutated Fc domains.

Animal Origin Free (AOF):

The Cyto-Cellect® Fc Fusion kit is AOF and can be used as part of a therapeutic Cell Line Development (CLD) workflow along with Cyto-Mine®.

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