Simplifying Progress

# Development of a Platform T Cell Exhaustion Assay Using an iQue® Screener PLUS

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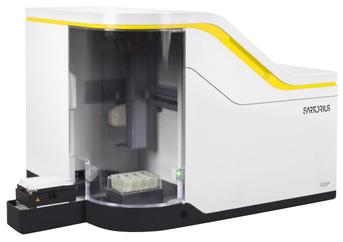
## Introduction

Sartorius has developed a platform T cell exhaustion assay using the Sartorius iQue® Screener PLUS combined with a Sartorius T cell exhaustion kit designed for iQue® flow cytometers. Reversal of T cell exhaustion is a crucial mechanism of action (MoA) of immuno-oncology therapeutic monoclonal antibodies (mAbs). The iQue® Screener PLUS achieves a faster assay throughput than traditional flow cytometers by sampling only microliters from each well and delivering an air-gap-delimited flow of samples to the detectors. This technology transforms a low throughput, time-consuming flow cytometry approach into a high throughput process.

T cell exhaustion occurs during prolonged exposure of the immune system to an antigen such as a virus or cancer cell. Persistent antigen exposure results in several physiological changes in the T cell population such as an increased expression of inhibitory receptors (IRs) and alterations in cytokine production including a decrease in IFNɣ secretion. Overall, these changes inhibit the T cell response to the persistent antigen. Immuno-oncology mAbs targeting IRs such as PD-1 are able to reverse T cell exhaustion, for example as measured by increasing IFNɣ secretion, thereby enhancing the immune response to promote the clearance of persistent antigens such a cancer cells. The platform format was tested with a number of immuno-oncology mAbs including nivolumab, pembrolizumab, atezolizumab and durvalumab all of which demonstrate the ability to reverse T cell exhaustion as measured by an increase in IFNɣ secretion of exhausted T cells.



## 1. Experimental approach

This in vitro primary cell-based platform assay approach utilizes exhausted T cells g enerated in house from fresh blood provided by a pool of on site donors. PBMCs isolated from fresh blood are treated with multiple rounds of stimulation with a CEF peptide pool. The CEF peptide pool is a group of 32 peptides, 8 – 12 amino acids in length, with sequences derived from the human Cytomegalovirus, Epstein-Barr virus and Influenza virus. The peptides bind directly to HLA-class I molecules on the surface of all Class I positive cells in the PBMCs. They will be recognized by peptide-specific CD8 cells without the need for additional antigen processing. PBMCs are put through multiple rounds of stimulation with the CEF peptide pool to generate exhausted cells that are frozen into single-use vials. The resulting vials are assessed in a 96 well assay plate with the molecule of interest and suitable controls followed by staining using a T cell exhaustion kit. The overall workflow is summarized in figure 1.

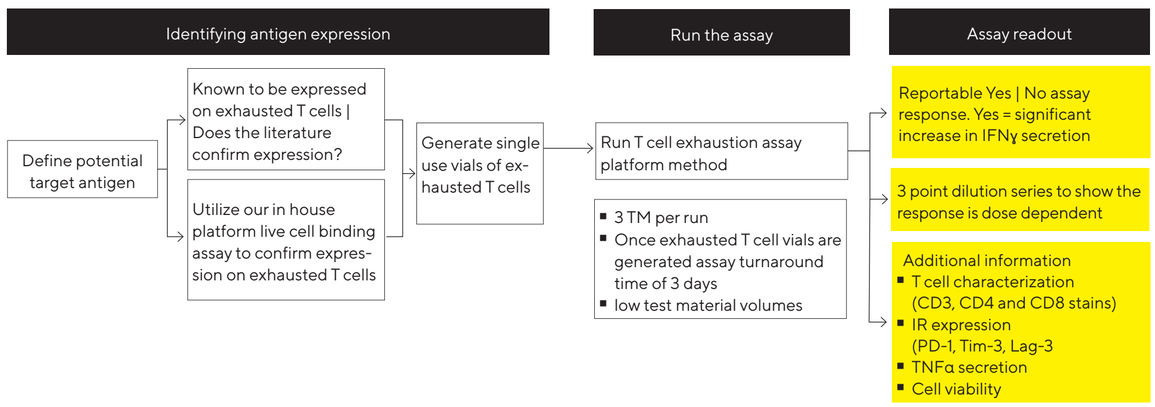
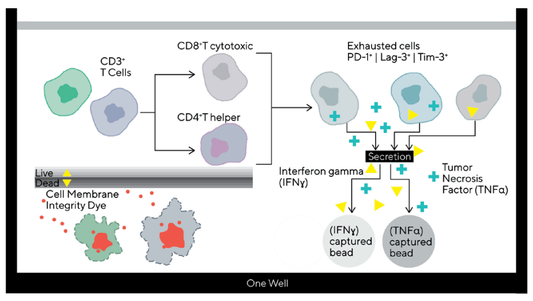



**Figure 1:** Schematic diagram of the workflow to assess the ability of a therapeutic antibody/NBE molecule to reverse T cell exhaustion.

## 2. Gating strategy

The platform assay uses a Sartorius developed T cell exhaustion kit (cat# 97069 for 1 x 96 well plate). The multiplexed assay generates readouts for cell viability, T cell characterization, expression of IRs and cytokine release of IFNɣ and TNFα. The assay allows a small sample from an assay plate to be analyzed, the readout and methodology are summarized in figure 2. The gating strategy shown in figure 3, the assay design incorporates positive and negative controls to fine-tune gate placement.




| Detector | Spectrum | Violet Laser (405 nm) | | Blue Laser (488 nm) | | Red Laser (640 nm) | |
|---|---|---|---|---|---|---|---|
| 445/45 nm | | VL1 | CD3 | N/A | | | |
| 530/30 nm | | VL2 | – | BL1 | – | N/A | |
| 572/28 nm | | VL3 | – | BL2 | QBeads Cyt Det | | |
| 615/24 nm | | VL4 | – | BL3 | – | | |
| 675/30 nm | | VL5 | Tim-3 | BL4 | Lag-3 | RL1 | Cell Viability |
| 780/60nm | | VL6 | CD8 | BL5 | PD-1 | RL2 | CD4 |

**Figure 2:** Overview of the Sartorius T cell exhaustion kit for high throughput flow cytometry. Left shows the readout parameters and right shows the required flow cytometer lasers and channels, the kit was optimized on the Sartorius iQue® Screener PLUS and iQue3®.

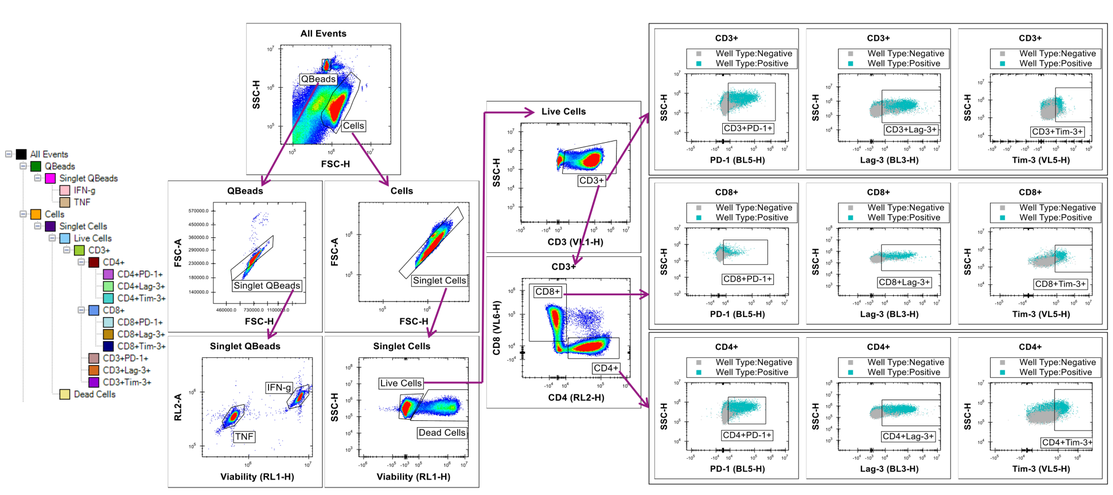



**Figure 3:** Overview of the gating strategy used in the Sartorius T cell exhaustion kit. Data shown is from an assay run using the platform T cell exhaustion assay at Sartorius Stedim BioOutsource. The overall gate hierarchy is shown on the left. The dot plots in the figure shows an example data set from an individual assay run. The positive well type contains exhausted T cells (Teal) and the negative well type contains non-treated PBMCs from the same donor (grey). The positive and negative conditions are used to set the IR gates

## 3. Results – CEF peptide pool treatment exhausts T cells

To demonstrate T cell exhaustion had occurred, exhausted T cells (no molecule) were compared to fresh PBMCs (NC) from the same blood donation and exhausted T cells + IL-2 (PC). IL-2 has been reported to reverse T cell exhaustion and acts as a positive control in the assay. Using the multiplexed T cell exhaustion kit readout, we confirmed that exhausted cells are enriched for T cells vs the control PBMC preparation (Figure 3A) and that IRs are upregulated on the various T cell populations (Figure 3B). As expected, we show that exhausted T cells secrete more IFNɣ than control PBMCs and that the secretion can be increased when rescuing the T cells from exhaustion by treatment with IL-2 (Figure 3C). Together the data shows T cells have been exhausted and that IFNɣ can be used as a measure of exhaustion.

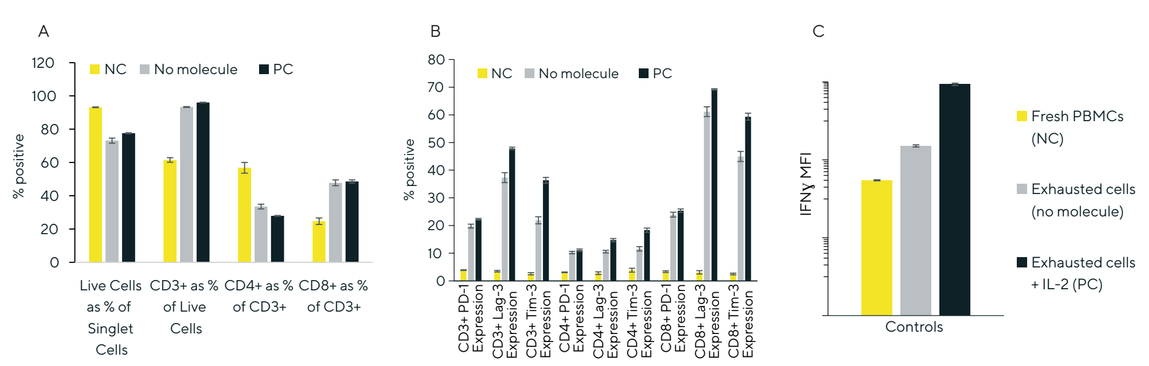



**Figure 4:** Data generated from a T cell exhaustion kit to assess exhausted T cells (no molecule) vs exhausted T cells + IL-2 supplement (PC) vs untreated PBMCs from the same blood donation (NC). Exhausted T cells were generated by treating PBMCs with multiple rounds of CEF peptide pool treatment. (A) Viability and T cell characterization. (B) Expression of IRs on the various T cell subtypes. (C) Comparison of IFNɣ secretion.

## 4. Results – anti-PD-L1 T cell exhaustion assay

A therapeutic mAb against PD-L1 can enhance T cell activation by disrupting the inhibitory signals from PD-1:PD-L1 binding. There are many PD-L1 inhibitors on the market including atezolizumab (Tecentriq®), avelumab (Bavencio®) and durvalumab (Imfinzi®). We tested two of these molecules in 14 sample positions across seven assay runs using our platform T cell exhaustion method. Example data for the reportable IFNɣ MFI response is in Figure 5 and a summary of responses across the runs is available in Table 1.

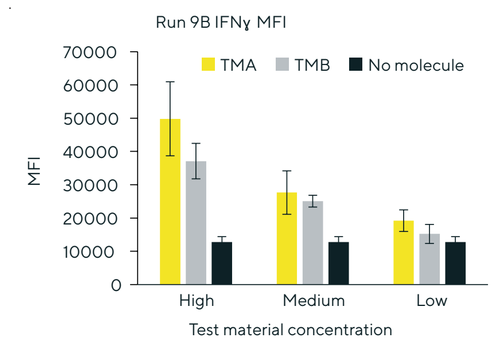



**Figure 5:** Example IFNɣ MFI data generated from a T cell exhaustion assay run testing durvalumab (TMA) and atezolizumab (TMB) in the assay format

| Donor | Run | Molecule | Test material position | Yes \| No IFNɣ MFI assay response | IFNɣ MFI fold increase |
|---|---|---|---|---|---|
| A | 9B | durvalumab | TMA | Yes | 3.9 |
| A | 9B | tecentriq | TMB | Yes | 2.9 |
| A | 9C | durvalumab | TMA | Yes | 1.5 |
| A | 9C | durvalumab | TMB | Yes | 1.6 |
| A | 9C | durvalumab | TMC | Yes | 1.4 |
| B | 10C | atezolizumab | TMA | Yes | 1.9 |
| B | 10C | atezolizumab | TMB | Yes | 2.0 |
| C | 11B | durvalumab | TMA | Yes | 1.4 |
| C | S11C | durvalumab | TMA | Yes | 1.4 |
| D | 12B | durvalumab | TMA | Yes | 2.6 |
| D | 12B | durvalumab | TMC | Yes | 2.9 |
| D | 12C | durvalumab | TMA | Yes | 2.3 |
| D | 12C | durvalumab | TMB | Yes | 3.0 |
| D | 12C | durvalumab | TMC | Yes | 2.4 |

**Table 1:** Assessment of anti-PD-L1 molecule responses in the platform T cell exhaustion assay. 2 way ANOVA and Bonferroni post test used to test for a significant increase IFNɣ vs no molecule control = yes assay response. Fold increase calculated from mean test material response at the high concentration/ mean no molecule response.

## 5. Results – anti-PD-1 T cell exhaustion assay

A therapeutic mAb against PD-1 can enhance T cell activation by disrupting the inhibitory signals from PD-1:PD-L1 binding. There are several PD-1 inhibitors on the market including Opdivo® (nivolumab) and Keytruda® (prembrolizumab). Here we test these molecules in 11 sample positions across six assay runs using our platform T cell exhaustion method. Example data for the reportable IFNɣ MFI response is shown in Figure 6 and a summary of responses across the runs is available in Table 2.

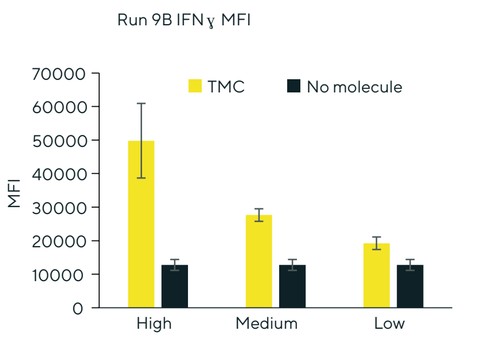



**Figure 6:** Example IFNɣ MFI data generated from a T cell exhaustion assay run testing durvalumab (TMA) and atezolizumab (TMB) in the assay format

| Donor | Run | Molecule | Test material position | Yes \| No IFNɣ MFI assay response | IFNɣ MFI fold increase |
|---|---|---|---|---|---|
| A | 9B | pembrolizumab | TMC | Yes | 3.6 |
| B | 10B | pembrolizumab | TMA | Yes | 2.0 |
| B | 10B | pembrolizumab | TMB | Yes | 2.1 |
| B | 10B | pembrolizumab | TMC | Yes | 2.0 |
| C | 11B | pembrolizumab | TMB | Yes | 1.4 |
| C | 11B | nivolumab | TMC | Yes | 1.3 |
| D | 12D | nivolumab | TMA | Yes | 1.5 |
| D | 12D | nivolumab | TMB | Yes | 1.6 |
| D | 12D | nivolumab | TMC | Yes | 1.5 |

**Table 2:** Assessment of anti-PD-1 molecule responses in the platform T cell exhaustion assay. 2 way ANOVA and Bonferroni post test used to test for a significant increase IFNɣ vs no molecule control = yes assay response. Fold increase calculated from mean test material response at the high concentration/ mean no molecule response.

## 6. Conclusion

The data presented demonstrates that the platform T cell exhaustion method will exhaust T cells in fresh PBMC preparations via stimulation with multiple rounds of CEF peptide pool. The exhausted cells can be frozen into single-use vials to allow ease of downstream testing. Using the Sartorius T cell exhaustion kit, a yes/no assay reportable value is generated for the reversal of T cell exhaustion. The readout was shown to be robust across different molecules, donors and assay runs. A workflow of how we can support your molecule testing is available in Figure 7.

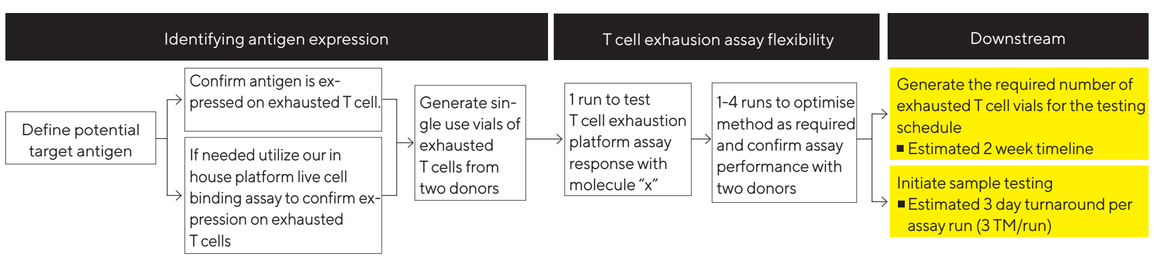



**Figure 7:** Schematic diagram of the workflow to use our platform T cell exhaustion assay