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Developing a Novel, Multiplexed Immune Assay Platform to Screen Kinase Modulators of T Cell Activation

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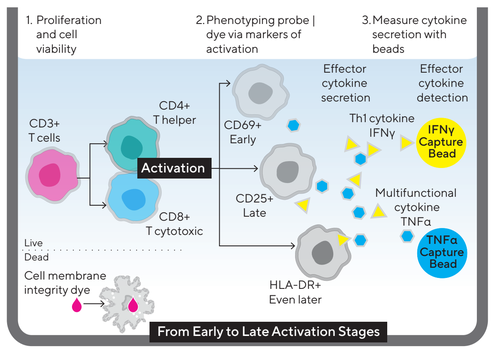
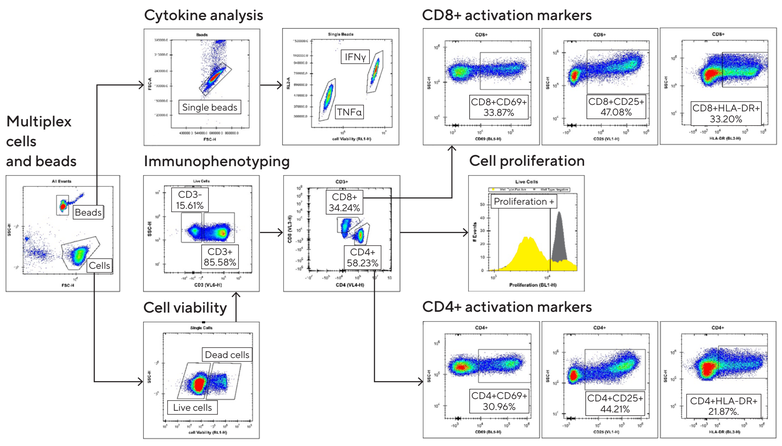
Abstract

T cell activation plays a central role in inflammation, autoimmune diseases, and cancer. Cancer immunotherapies, such as immune checkpoint inhibitor, bi-specific antibody, chimeric antigen receptor T (CAR-T) cell, and adoptive tumor-infiltrating lymphocyte (TIL) therapies require the characterization and monitoring of many aspects of T cell activation. Here we describe a novel, multiplexed immune assay platform based on high throughput flow cytometry technology and advanced computational algorithms for data analysis. From a mixture of cells and beads in microtiter plates, the assay simultaneously measures T cell phenotype, time-dependent expression of T cell activation markers, secreted effector cytokines, and T cell proliferation. We used this assay to screen a kinase chemogenomic library and identified 25 kinase inhibitors (KI) with distinct inhibition profiles on the early activation marker CD69, the late activation marker CD25, and the secreted cytokines IFNγ and TNFα. Interestingly, the screening revealed 3 kinase inhibitors for PKC, IKK2, and MEK1/2 respectively, all with a phenotypic signature similar to Ruxolitinib, a Jak1/2 inhibitor used to treat myelofibrosis. These results suggest that this multiplexed immune assay platform can be used as a primary screen for chemogenomic libraries, phenotypic or target-based drug discovery, target identification, and potential drug repositioning.

Multiplexed Assay Design and Gating Strategy





Materials and Methods

Cell and Reagents

Cryopreserved PBMCs (Astarte Biologics) from healthy donors were recovered overnight and cultured in media (RMPI-1640, Corning) containing 10% fetal bovine serum (Gibco) and supplemented with 10 ng/mL IL-2 and other media supplements. Cells were stimulated | activated with each of 3 different activators: CD3/CD28 Dynabeads® (Thermo Fisher Scientific), phytohemagglutinin (PHA, Sigma), or Staphylococcal Enterotoxin B (SEB, List Biological Laboratories). The kinase inhibitor library (Cayman) used for the screening assay has 152 known kinase inhibitory compounds at 10 mM stock concentration in DMSO.

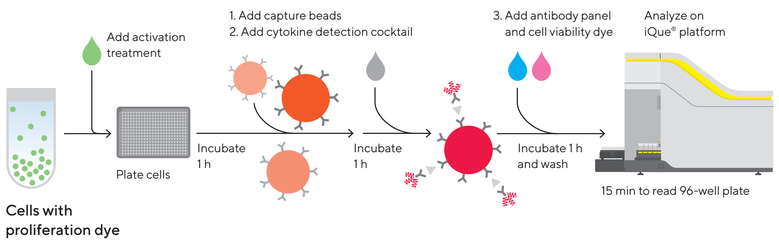
Profiling T Cell Activation

PBMCs, stained with the iQue® Cell Proliferation and Encoding (B/Green) Dye (part of the iQue® Human T Cell Activation Kit) and stimulated using an 11-point, 2-fold serial dilution series. Media without activation compounds was used as a negative control. The total culture volume was 100 µL per well, with a final cell density of 1 million/mL. On days 1, 3, and 6 after stimulation, 10 µL of sample containing cells and supernatant were transferred to an assay plate (Costar). The iQue® Human T Cell Activation Kit (Sartorius) was used to assess cell count, viability, proliferation, and secreted cytokines as well as to identify activated T cells. To quantitate cytokine secretion, a standard curve was used with a top concentration of 50,000 pg/mL, with a 1:3 serial dilution series (including a zero-concentration negative control) and duplicate wells. Data were acquired on the iQue® platform (VBR) with integrated iQue Forecyt® software in approximately 15 minutes per 96-well plate.

Kinase Inhibitor Screen

PBMCs from a single donor were recovered for 24 hours as above. Cells were pre-treated with the kinase inhibitor library (10 µM), Cyclosporine A as the positive control, or media as the negative control for 1 hour. Cells were activated using Dynabeads® and incubated for 24 hours. 10 µL of sample containing cells and supernatant were transferred to an assay plate and analyzed using the iQue® Human T Cell Activation Kit as above.

Assay Workflow



Endpoints

	T Cell ID			Cell Surface Activation Markers			Secreted Cytokines	
	CD3	CD4	CD8	CD69 (early)	CD 25 (late)	HLA-DR (even later)	TNFα	IFNγ
T cytotoxic cells	+	-	+	+|-	+|-	+|-	+	+|-
T helper cells	+	+	-	+|-	+|-	+|-	+|-	+ (Th1)

Activation Profiles of Different Modulators

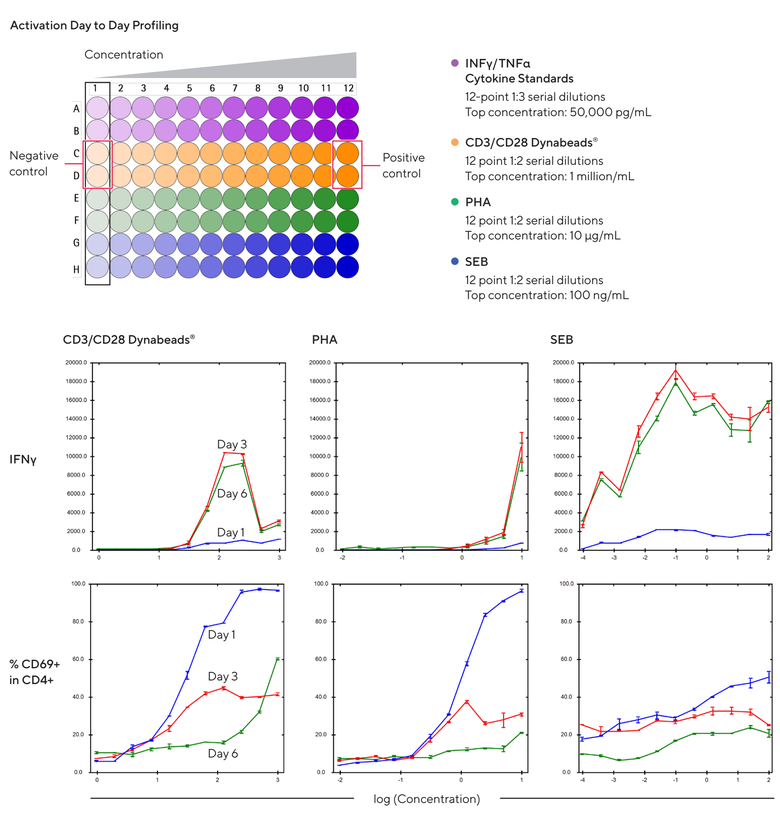


Figure 1: Day to day profiling of Dynabeads®, PHA and SEB demonstrate assay robustness. The 3 different modulators show distinct profiles in IFNγ secretion and in the expression of the early activation surface marker CD69 as well as a day-to-day effect. CD3/CD28 Dynabeads® treatment on day 1 shows a dose-dependent IFNγ secretion, and days 3 and 6 show similar IFNγ secretion and saturation at a relatively high concentration. The top 2 doses on days 3 and 6 show reduced IFNγ secretion ("hook" effect), which is consistent with the T cell exhaustion phenomenon characteristic of T cell activation.

Kinase Inhibitors Decrease Expression of Early Activation Marker CD69

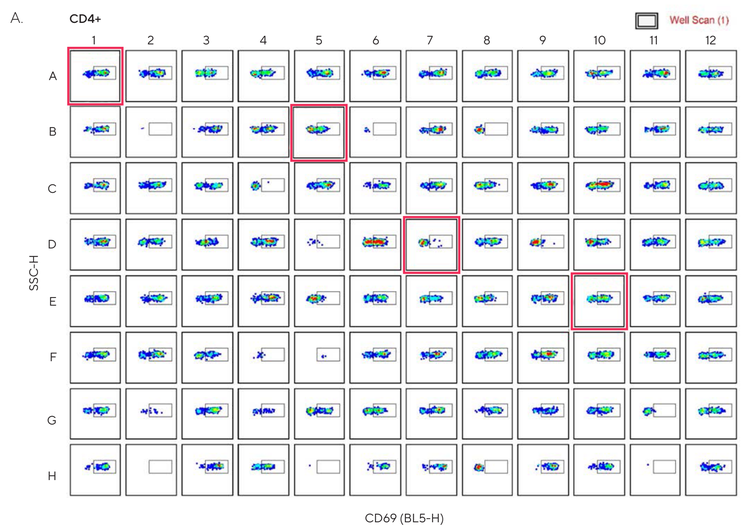


Figure 2: Kinase inhibitors decrease the expression of early activation marker CD69. A representative result of CD4+ T helper cells. (A) Plate view of 2D plots (CD69 vs. SSC) show the expression of CD69 in each well. Five screening wells with decreased CD69 expression are highlighted with brown boxes. (B) The well-based 2D plots (CD69 vs. SSC) from the five highlighted wells and a negative control well (A1 well) display the varying effects on the expression of the early activation marker CD69 caused by different kinase inhibitors.

Kinase Inhibitor Hit Finding and Ranking

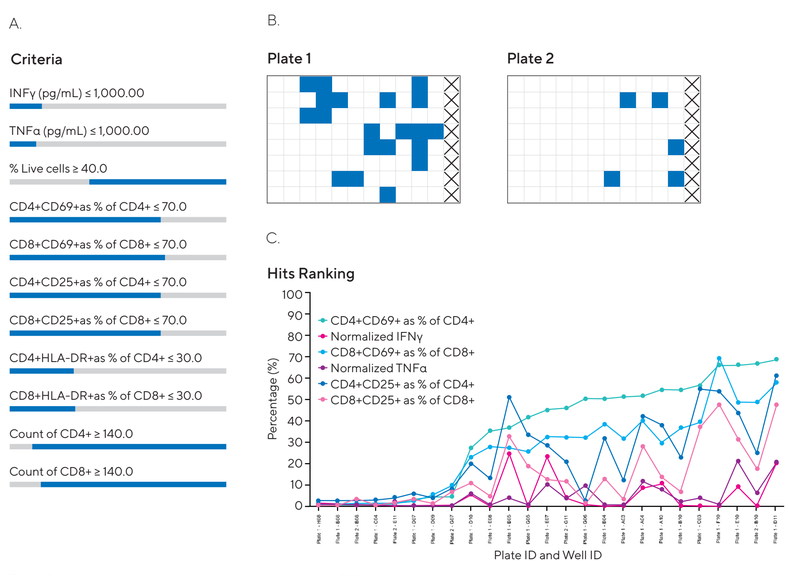


Figure 3: Identification and ranking of hits that broadly inhibit T Cell Activation. (A) 11 criteria applied simultaneously and utilized to identify the screening hits. (B) The profile maps of two kinase inhibitor screening plates with a total of 25 hits (blue boxes). (C) 25 screening hits out of 152 kinase inhibitors from the entire library were ranked, based on the decrease of CD69 expression on CD4+ T cells (low to high, red curve) by using line graph feature in iQue Forecyt®. Other parameters are also shown for the 25 hits, including CD69 expression in CD8+ T cells, CD25 expression in CD4+ and in CD8+ T cells, and the secretion of IFNγ and TNFα (normalized to the mean value of the negative wells, expressed as a percentage).

Identify and Compare Compounds Similar to a Well-Known JAK1/2 Inhibitor—Ruxolitinib

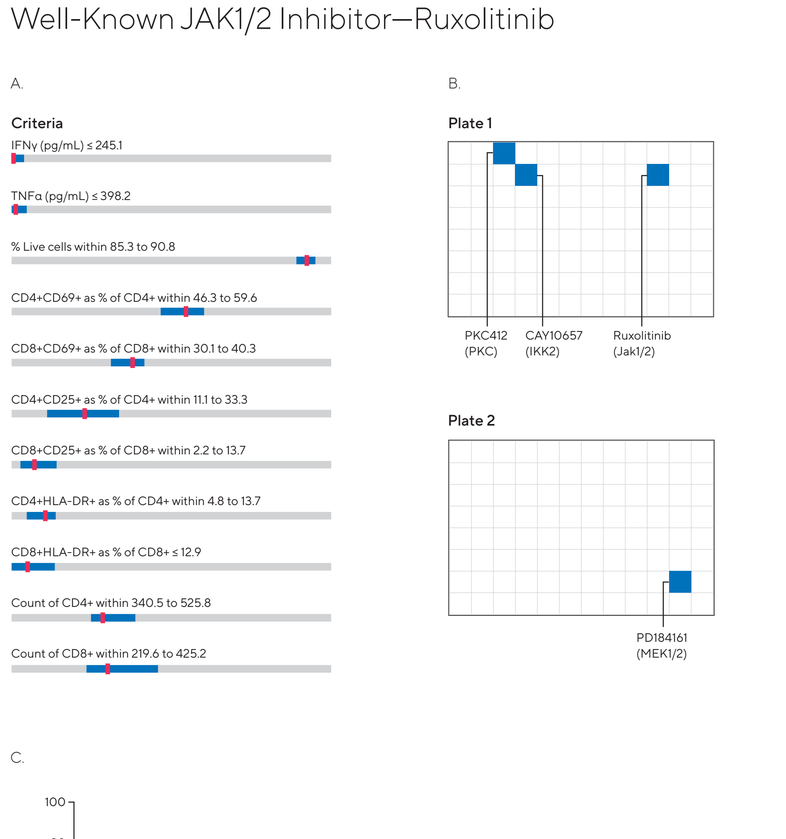


Figure 4: Identification of 3 kinase inhibitors with similar phenotypic signatures as a well-known myelofibrosis drug Ruxolitinib (Jak1/2 inhibitor). Use of Multi-Plate Analysis and Profile Maps Boolean logic algorithm in iQue Forecyt® enabled the identification of 3 distinct kinase inhibitors with functional profiles similar to Ruxolitinib. Selecting the Ruxolitinib well (B) triggers the algorithm to place a red tick mark in the criteria slider bars (A) to show the value for each endpoint of the Ruxolitinib treatment. iQue Forecyt® allows easy threshold adjustments (by click and drag) of criteria bars to bracket the Ruxolitinib endpoint values. (C, D) Further quantitative screening results suggest these 3 compounds inhibit the T cell activation markers including the expression of CD69 and CD25, and the secretion of cytokines IFNγ and TNF.

Summary

- The iQue® Human T Cell Activation Kit provides multiplexed cell and secreted cytokine measurements in a single assay
- Delivers spatial-temporal analysis of T cell phenotype and function at different stages in a single, high-content miniaturized assay
- The Sartorius iQue® platform with integrated iQue Forecyt® software offers a single platform and data analysis package—quickly translate data into actionable results
- Optimized assay format avoids the guesswork of sample dilution, saves precious samples, decreases reagent costs, and enhances data integrity
- Simplified plug-and-play assay workflow with pre-mixed reagents and no need of color compensation
- Flexibility to choose additional cytokine measurements from validated iQue® Human T Cell Companion Kits: IL-2, IL- 6, IL-10, IL-13, IL-17A and GM-CSF