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# iQue® Human T Cell Activation Kit Protocol Optimization for Automated Liquid Handling Systems

## Introduction

The iQue® Human T Cell Activation Kit (TCA kit) was developed to rapidly analyze activation status of T cells providing information about their health and their role in cytokine secretion in a high throughput format. Using only 10 µL of sample, the TCA kit provides a readout of cell phenotype, T cell activation markers, cell proliferation, cell viability and the secretion of cytokines all in one well. Although the TCA kit protocol has been optimized for manual assay plate set up, we sought to adapt the protocol for use with an automated liquid handler. Using the Opentrons OT-2 liquid handler as an example platform, our results show that the TCA kit can be successfully carried out using an automated platform.

The TCA kit detection antibody cocktail includes antibodies directed against human CD3, CD4, CD8, CD25, CD69 and HLA-DR. The CD3, CD4 and CD8 markers are used to identify these cell subtypes while CD25, CD69 and HLA-DR are known markers of T cell activation. CD69 is an early T cell activation marker and its expression is elevated within hours up to two days.[1] CD25 is prominently upregulated within one day of stimulation and remains elevated for a few days.[1,2] HLA-DR is a very late T cell activation marker. We and others have noted that HLA-DR expression is poorly induced by CD3/CD28 Dynabeads.[1,2] It can be a late T cell activation marker when using strong stimulation.[3]

# Materials and Methods

### Instruments

The Opentrons OT-2 liquid handling robot used for these experiments was equipped with dual 8 channel pipettes, a GEN1 P300 8-channel pipette and a GEN2 P20 8-channel pipette (https://opentrons.com). Disposable 300 µL and 20 µL 96-tip racks (Opentrons) and 22 mL 12-well reservoirs (USA Scientific) were supplied. The automated protocol for running OT-2 to set up TCA plates was developed using Opentrons protocol designer. The OT-2 worktable layout is shown in Figure 1. Both manual and auto plates were centrifuged in Eppendorf® Centrifuge 5810. Manual plates were aspirated in a BioTek ELx405 plate washer.
The iQue® high-throughput flow cytometer configured with Violet-Blue-Red (VBR) lasers (405nm, 488nm, 640nm) was used for TCA plate flow cytometry analysis.

### Reagents

Frozen normal human peripheral blood mononuclear cells (PBMC) were purchased from ZenBio (Zen-Bio, Inc.). Cells were freshly thawed and cultured in RPMI 1640 media containing 10% FBS and 10 ng/mL of recombinant human IL-2 (PeproTech) at 2x10$^6$ cells/mL in six wells of a 24-well plate. Immediately prior to treatment, PBMCs were activated using Human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific) that were pre-washed with cell culture media and resuspended at 2x10$^6$ beads/mL. To obtain a bead-to-cell ratio of 1:1, an equal volume of pre-washed Dynabeads and PBMC were mixed.

Veri-Cells™ PBMC and Veri-Cells™ Activated (Surface) PBMC were purchased from BioLegend. Each 5-test size

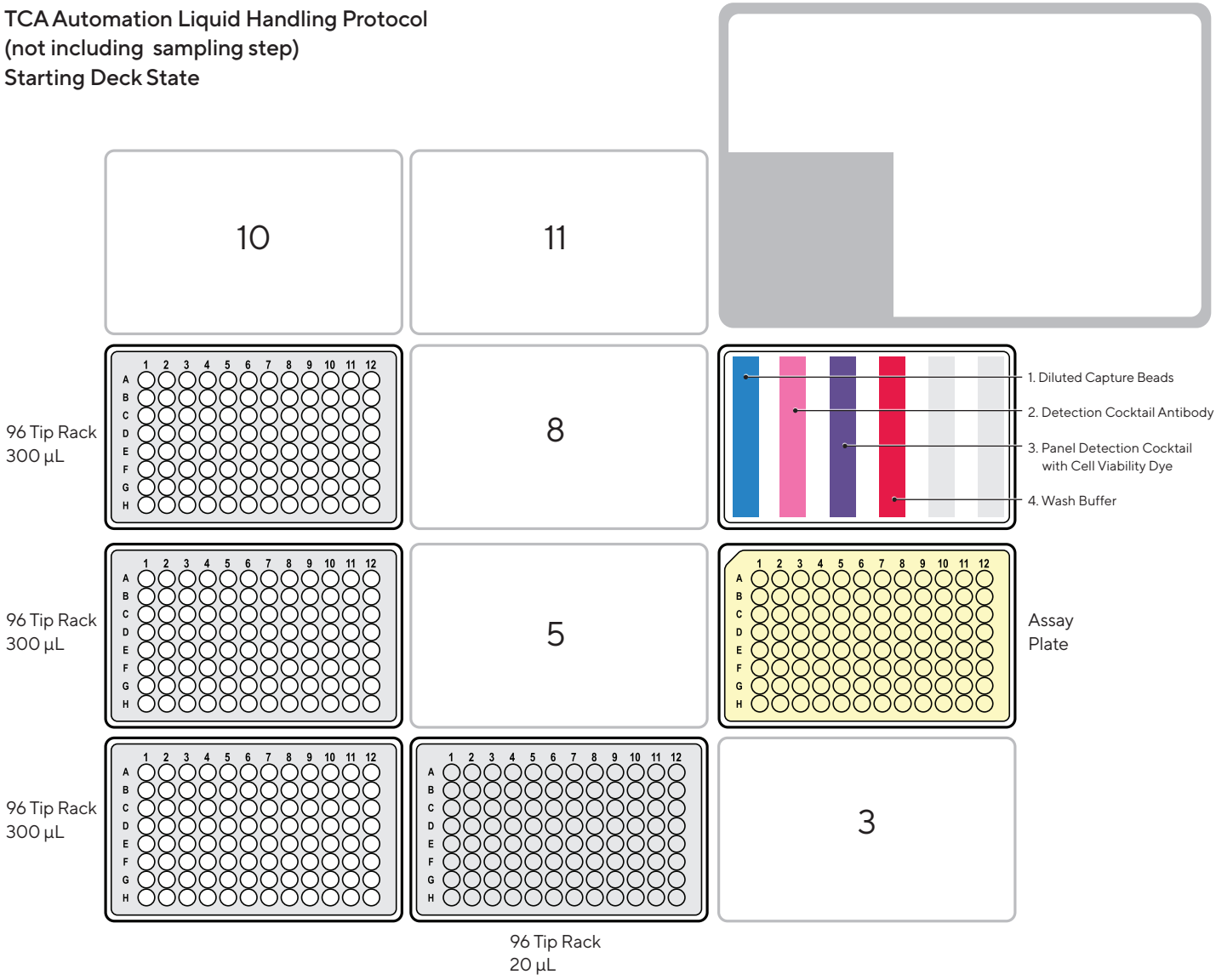


Figure 1: The Opentrons OT-2 deck slots layout. The reagent reservoir plate has four wells containing the following four reagents: 1) Diluted capture beads; 2) Detection Cocktail Antibody; 3) Panel Detection Cocktail with Cell Viability Dye; 4) Wash Buffer.

vial was reconstituted with 325 µl of Veri-Cells™ Buffer A, and 10 µL of reconstituted cells were loaded in each assay plate well (96 or 384-well plate).

iQue® Human T Cell Activation Kit (Sartorius PN 90560, 90561, 90562, 90563) was used to detect T cell activation following the kit manual and the automated workflow detailed herein.

### Workflow

The OT-2 operating protocol was designed using the Opentrons Protocol Designer BETA online free software. Standards, samples and reagents were prepared following TCA kit manual. In a 12-well v-bottom reservoir, load the reagents into the designated wells: 20 mL of pre-diluted cytokine capture beads, mix well before loading; 2 mL of Cytokine Detection Cocktail; 2 mL of Antibody Panel Detection Cocktail; 15 mL of Wash Buffer. Cover the reservoir with a black lid during the incubation periods. The workflow for setting up a TCA plate in OT-2 liquid handler is shown in Figure 2.

### Sample Acquisition and Data Analysis

Following staining, the assay plates were analyzed on the iQue® advanced high-throughput flow cytometer. Cells and cytokine beads were gated and analyzed using the template as a guide. The cytokine standard curves, concentration in samples and cell phenotypes were reported using the metrics provided in the template. Intraplate signal dynamic and variance were estimated with Z' factor. Z'-factor is calculated using $1-\{3(\sigma_p + \sigma_n)/|\mu_p-\mu_n|\}$, where $\mu_p$ and $\sigma_p$ are the mean and standard deviation values of the positive control (the treated samples) and $\mu_n$ and $\sigma_n$ are those of the negative control (the untreated samples).[4,5]

## Results and Discussion

### Adapting of the TCA Kit Protocol for Use in an Automated Liquid Handling System

We ran a series of experiments to determine the feasibility of using the iQue® Human T Cell Activation Kit in an automated liquid handling system. For these experiments, we selected the OpenTrons OT-2 liquid handler as an inexpensive entry level system for widest applicability. Critical features in our selection were the ability to be outfitted with multichannel pipets capable of delivering between 10 to 195 µL. Although we used 8-channel pipets, we anticipate that systems with 4 or 16 channels would also work. Systems with fixed 96- or 384-well pipets will not work due to their greatly increased reagent requirements.

After several rounds of automated method optimization, we arrived at the workflow shown in Figure 2. Our considerations included a) keeping the automated

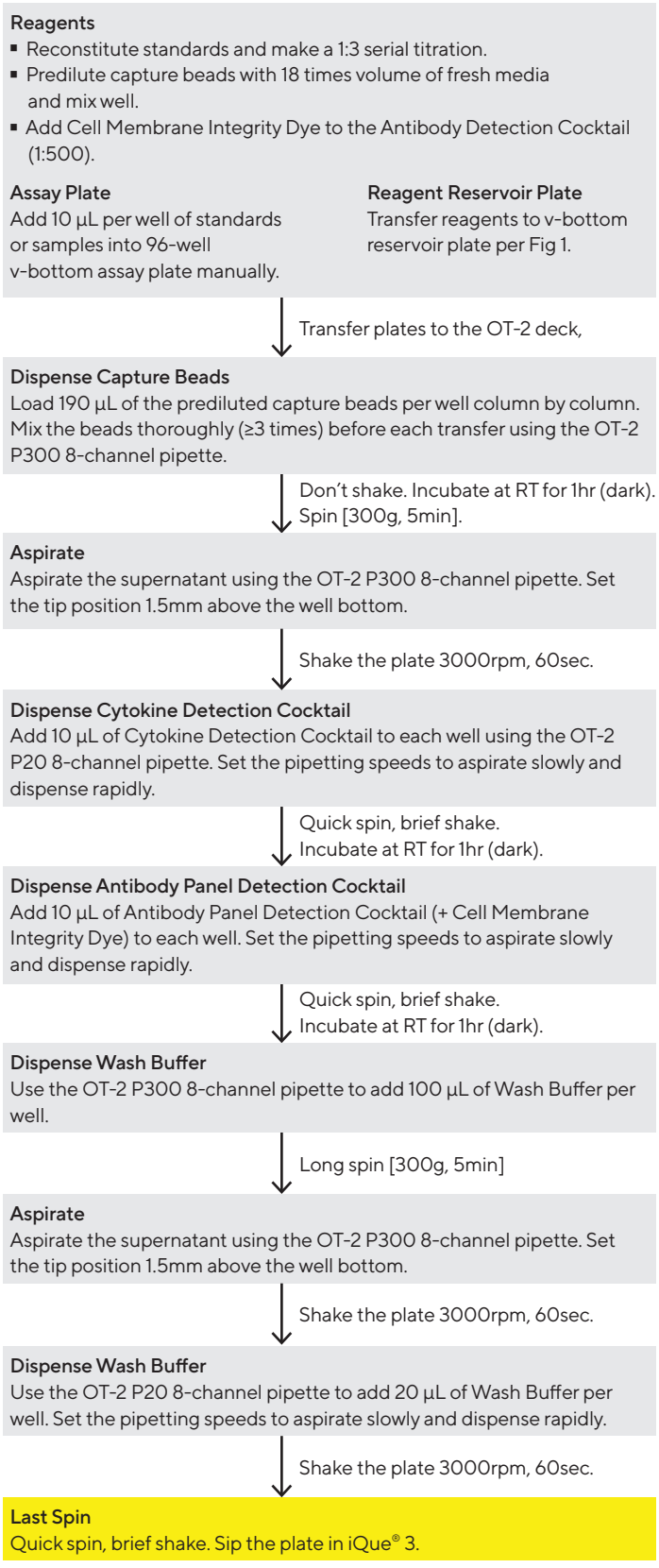


Figure 2. Workflow for setting up TCA plate in OT-2.

protocol as close as possible to the familiar manual protocol, b) ensuring that the reagent volumes provided in the kit are sufficient and c) that the cytokine detection beads are sufficiently mixed to ensure even distribution. This workflow should be adaptable to other liquid handlers with a minimum of 6 free deck positions and one or more 4-, 8- or 16-channel pipet(s) capable of accurately delivering between 10 to 195 µL. To keep the system requirements at a minimum, our workflow consists of both on and off instrument steps. Steps involving shaking and centrifugation are performed off instrument. If your liquid handler has an integrated shaker and centrifuge it may be possible to further adapt this protocol to remove the need for performing any of the steps off instrument.

We performed a series of experiments to verify that assay plates prepared using automation have equivalent performance to those prepared using the standard manual protocol. Figure 3 shows representative results comparing assay plates prepared using the automated or manual protocol. In brief, duplicate sample plates were prepared with human PBMCs that had been activated with anti-CD3/CD28 Dynabeads for periods of 0-5 days. One plate was stained using the standard manual protocol while the other was stained in parallel using the OT-2 liquid handler. The plates were sipped in an iQue® 3 and the results analyzed using iQue Forecyt® with the provided gating template. Cytokine standard curves and the time-dependent cytokine secretion in manual plate and auto plate were compared to see their consistency. As shown in Figure 3A and 3B, the IFNγ and TNFα standard curves are nearly identical when overlaid. Figure 3C and 3D show the comparison of the concentration of IFNγ and TNFα in the activated PBMC samples. The difference of the IFNγ concentration in manual and auto plates was in a range of -1.5% to 6.1%. The concentration of TNFα in manual and auto plates was closer. The difference was between -0.1% to 2.3%. These data show that equivalent results are obtained for cytokines quantified using either the automated or manual staining protocol.

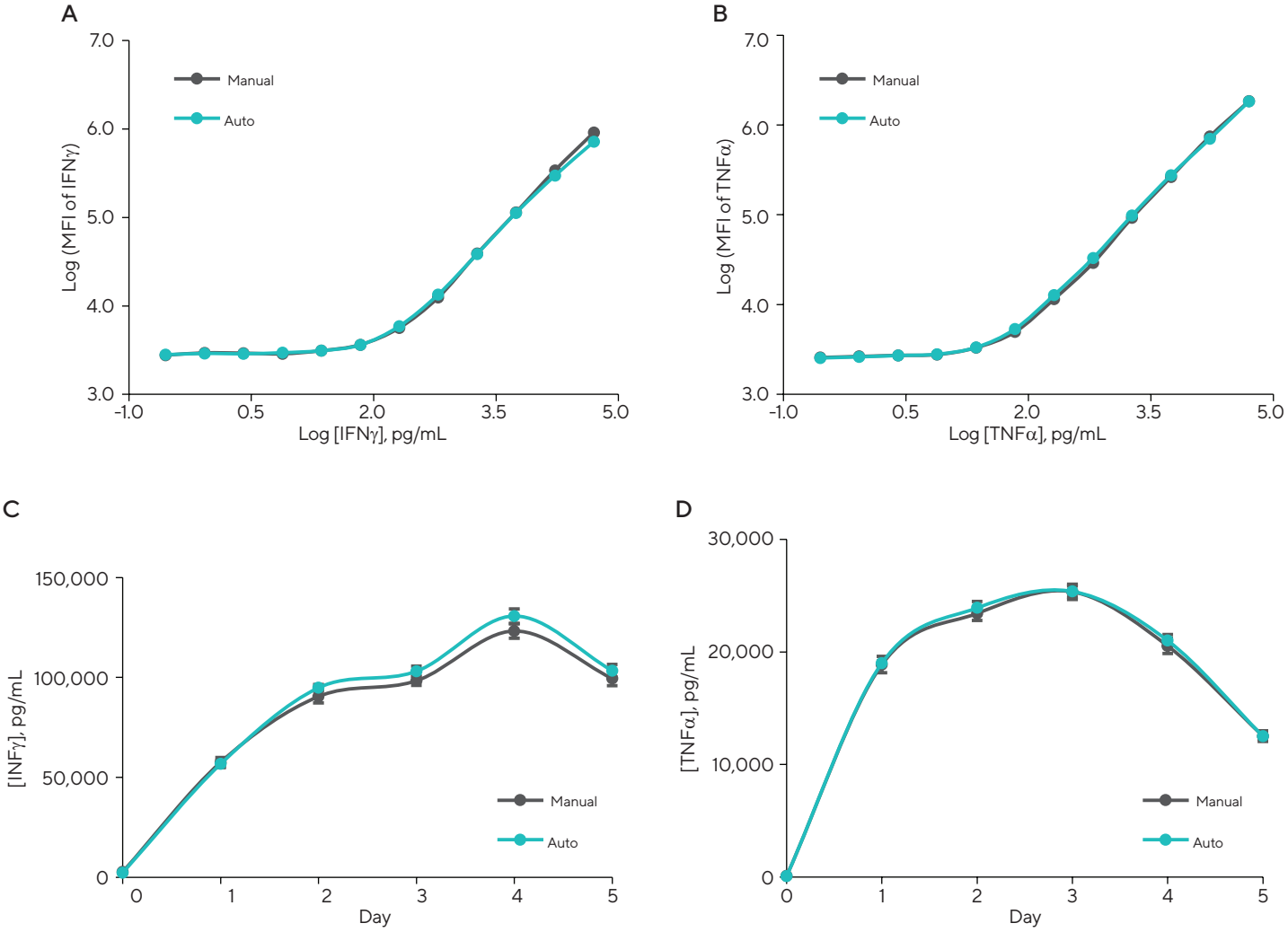


Figure 3. Comparison of cytokine standard curves and cytokine measurements using manual or automated plate preparation. (A) IFNγ standard curves, (B) TNFα standard curves. Figures C and D show IFNγ and TNFα production, respectively, in human PBMCs activated with CD3/CD28 Dynabeads for 0-5 days prior to assay. Mean and standard deviation of six replicates are reported for each time point.

We also compared the T cell subsets and activation markers in the manual versus automated assay plates. Figure 4A and 4B show the percentage of CD69+, CD25+ and HLA-DR+ cells in CD4+ or CD8+ T cells. The percentage of each subpopulation closely coincided between the plates, indicating that the identification of cell populations in the auto plate showed no obvious difference from the manual plate.

To further understand the signal dynamic and data variation in each plate, we calculated Z' factor for each plate using the percentage of CD69 and CD25 positive cells in activated PBMCs as positive controls and their expression in non-activated PBMCs as negative controls. CD69 expression peaked after one day of anti-CD3/CD28 stimulation and declined quickly thereafter. CD25 was upregulated from day 1 to day 5 upon anti-CD3/CD28 stimulation. Therefore, we calculated Z' factors in CD69+ T cell subsets after 1-day stimulation and in CD25+ T cell subsets after 1 to 5 days of stimulation. The Z' factors calculated with CD69+CD4+ T cells were 0.96 vs 0.97, with CD69+CD8+ T cells were 0.79 vs 0.70 in manual and auto plates respectively. The difference of Z' factors in CD69+CD4+ vs CD69+CD8+ cells was caused by the higher background percentage in CD69+CD8+ subset. The mean of CD69+CD4+ percentage at day 0 was 2%, but it was 22% in CD69+CD8+ cells. At the peak time, though both reached 98-99%, the dynamic range in CD69+CD8+ was smaller leading to smaller Z' factors. In the CD25+CD4+ and CD25+CD8+ T cell subsets, the day 0 background was low, and the activation remained at peak level from day 1 to day 5. The Z' factors calculated with either population were > 0.9 in both manual and auto plates. The similarity of Z' factors suggests both the manual and automated workflows result in similarly consistent results.

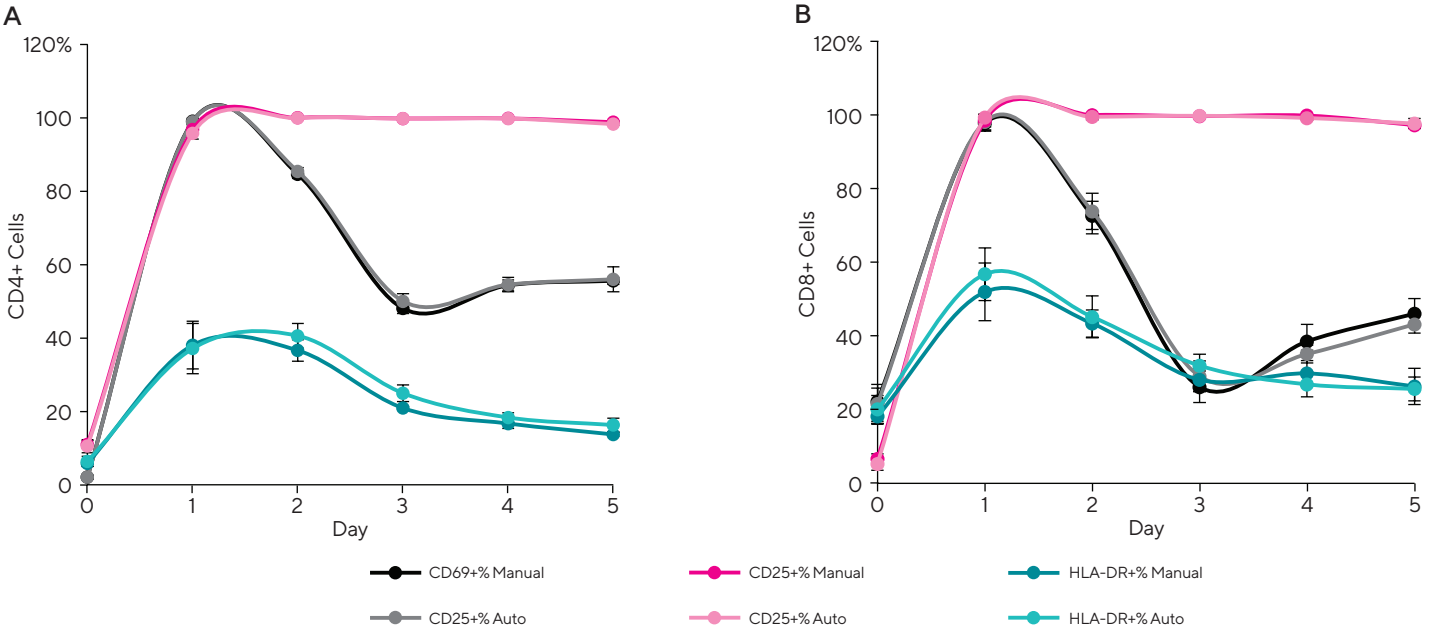


Figure 4. Comparison of activated T cell subpopulations using manual or automated plate preparation. Human PBMCs were activated with CD3/CD28 Dynabeads at 1:1 ratio for 0-5 days. Shown are the percentages of CD69+, CD25+ or HLA-DR+ cells in CD4+ (A) or CD8+ (B) T cells. The mean and standard deviation of six replicates are reported for each time point.

## Consistent Results Are Obtained Using an Automated Liquid Handling Platform

We next investigated the inter-experiment reproducibility of TCA results from plates prepared using the automated protocol. To avoid day-to-day variation of PBMC activation, we designed an experiment using IFNγ and TNFα cytokine standards and fixed PBMCs (VeriCells) to compare cytokine measurements and T cell subpopulations between plates prepared on separate days. Three plates were run in three days using the automated protocol. Each plate had two copies of the standard curve, and each sample of the standard curve was duplicate. A total of six replicate curves in three plates analyzed in three days are shown in Figures 5A and 5B. The six replicates of IFNγ (5A) and TNFα (5B) standard curves from three plates overlay each other.

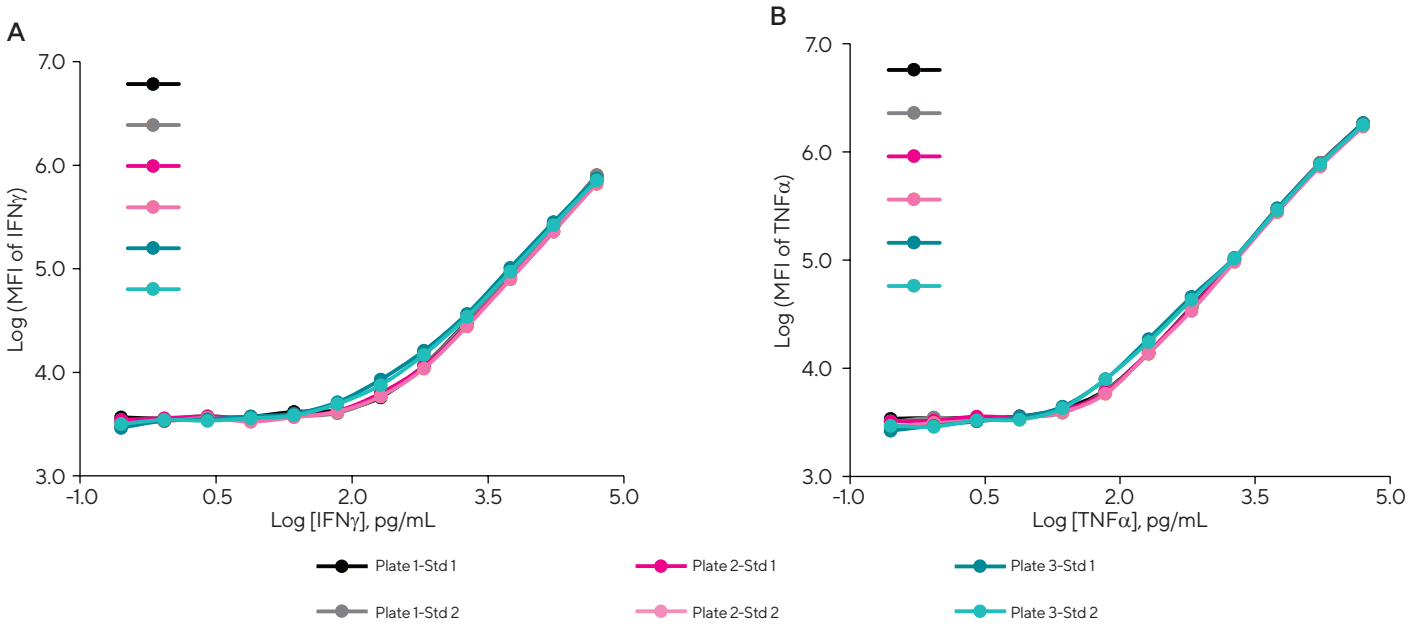


Figure 5. Inter-plate reproducibility of cytokine standard curves generated using the automated protocol on three separate days. There are two copies of the standard curve on each plate, and each sample of the standard curve is duplicate. Total six replicate curves in three plates analyzed in three days are reported. IFNγ (A) and TNFα (B) standard curves.

| Cell populations with CD markers | | Plate 1 Mean ± SD (N = 12) | Z′ factor | Plate 2 Mean ± SD (N = 12) | Z′ factor | Plate 3 Mean ± SD (N = 12) | Z′ factor | Mean Mean ± SD (N = 36) | CV, % |
|---|---|---|---|---|---|---|---|---|---|
| CD69+ % of CD4+ | VeriCells | 0.3 ± 0.1 | 0.820 | 0.2 ± 0.1 | 0.851 | 0.3 ± 0.2 | 0.824 | 0.3 ± 0.1 | 52.5 |
| | VeriCell-Act | 47.8 ± 2.7 | | 43.3 ± 2.1 | | 45.9 ± 2.5 | | 45.7 ± 3 | 6.6 |
| CD69+ % of CD8+ | VeriCells | 1.9 ± 0.4 | 0.771 | 1.3 ± 0.4 | 0.797 | 1.9 ± 0.6 | 0.741 | 1.7 ± 0.6 | 32.6 |
| | VeriCell-Act | 41.4 ± 2.6 | | 38.0 ± 2.1 | | 38.7 ± 2.6 | | 39.4 ± 2.8 | 7.1 |
| CD25+ % of CD4+ | VeriCells | 3.4 ± 0.4 | 0.949 | 2.0 ± 0.4 | 0.956 | 2.2 ± 0.3 | 0.925 | 2.6 ± 0.7 | 27.5 |
| | VeriCell-Act | 85.5 ± 1 | | 80.5 ± 0.8 | | 80.4 ± 1.6 | | 82.1 ± 2.7 | 3.2 |
| CD25+ % of CD8+ | VeriCells | 0.9 ± 0.3 | 0.885 | 1.2 ± 0.7 | 0.840 | 0.8 ± 0.5 | 0.850 | 1.0 ± 0.5 | 56.2 |
| | VeriCell-Act | 51.8 ± 1.7 | | 48.0 ± 1.8 | | 49.9 ± 1.9 | | 49.9 ± 2.3 | 4.7 |

Table 1. Inter-plate reproducibility of T cell subpopulations were analyzed and evaluated with Z′ factor and % CV.

Each plate also included 12 replicates of VeriCells and 12 replicates of activated VeriCells (Surface). Activated VeriCells have high levels of CD69 and CD25 staining. We used the percentage of CD69+ and CD25+ in CD4+ or CD8+ cells as positive controls and the relevant populations in VeriCells as negative controls. The Z′ factors for each subpopulation were calculated with the mean and standard deviation of their expression percentage and the results were summarized in Table 1.

Within each plate, according to the Z′ factor, the detection of CD69+ and CD25+ T cells was evident and consistent. The variance among three plates was evaluated with %CV. As shown in Table 1, the %CV of each T cell subsets in activated VeriCells was < 10%. In VeriCells, the %CV was high because the detection was at the limit end. These data clearly showed the application of TCA kit in automated liquid handler could obtain reliable and repeatable results.

### Tips for Adapting the TCA Kit to Your Automated Liquid Handling Platform

Due to the diverse configurations and capabilities of liquid handling systems available, this protocol will almost certainly need to be adapted for your instrument. The minimal liquid handling system requirements are at least 6 free deck positions for the reagent plate, the sample and assay plates as well as positions for the tips. For the transfer and mixing steps, the workflow requires one or more multichannel pipets (4-, 8- or 16-channels) capable of accurately delivering volumes between 10 to 195 µL for the 96 well assay format or 5 to 195 µL for the 384 well assay format. Systems with fixed 96- or 384-well pipets will not work due to their greatly increased reagent requirements.

One concern is uneven distribution of the capture beads across the assay plate due to settling in the reservoir. We found it necessary to incorporate a bead reservoir mixing step prior to each transfer (3 x 190 µL). We recommend using SPHERO AccuCount Particles (Spherotech, Cat# ACBP-50-10) or similar to verify the effectiveness of the mixing steps in your system.

For transferring reagents in the 10-20 µL range, we recommend using slow aspiration (eg. 4 µL/s) and rapid dispense (eg. 20 µL/s) speeds with a final blowout step. The tip position should be set at half the well height and a touch-tip step incorporated at the top of the well to avoid cross-well contamination. For small volumes, a blowout operation should be incorporated into the protocol. For aspiration steps involving bead suspensions, it's important to leave sufficient space between the tip and the bottom of the well. We found a minimum distance of ~1.5 mm from the bottom of the well was sufficient. Users can use SPHERO AccuCount Particles to test the aspiration tip position and verify no loss of beads. We strongly recommend including a final assay plate spin step prior to loading in the iQue® to ensure everything is brought to the bottom of the well.

## Conclusions

Our study demonstrates high assay quality obtained using the iQue® Human T Cell Activation Kit in an automated liquid handling system. Data generated when employing a relatively inexpensive liquid handling system like the OpenTrons OT-2, displayed results consistent with manually prepared plates, indicating it's compatibility for automated high-throughput screening with excellent day-to-day reproducibility. The automation friendly iQue® Human T Cell Activation Kit provides miniaturized sample volume in a high throughput format for efficient time to answer using the iQue® advanced flow cytometer.

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