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Application Note

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An Optimized, Multiplexed Assay for Screening *Ex Vivo* Conditions which Increase Memory T Cell Frequency

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Abstract

The *ex vivo* expansion of T cells is a critical process in the bio-manufacturing of adoptive cell therapies. Recent clinical studies show that a correlation between the persistence of subsets of functional memory T cells, including T memory stem cells (T_{SCM}), central memory T cells (T_{CM}) and other less differentiated T cell subsets, is responsible for long term anti-tumor responses in patient outcomes. This suggests *ex vivo* T cell expansion protocols generating higher percentages of T_{SCM} and T_{CM} in the T cell product are critical to significant clinical improvements in adoptive cell therapies.

To address this opportunity, we developed a robust T cell memory cell and cytokine profiling assay optimized for 96- or 384-well microtiter plates that runs on the iQue® advanced flow cytometry system. This assay includes antibody markers to identify T cells and identifies naive, T_{SCM}, T_{CM} and T_{EFF}. In addition, the functional analysis of the cells is assessed by quantitating the levels of secreted IFN and IL-10 simultaneously with phenotypic measurements.

As proof of concept, we tested various media components during *ex vivo* expansion of human PMBCs. We showed that media supplements had profound effects on the final T memory subset cellular composition and cytokine release.

Introduction

T cells are generated and released from the thymus as naive T cells (T_N). After infection or antigen encounter, the T_N cells proliferate and differentiate into effector T cells (T_E) which travel to the site of infected target cells for destruction, hence clearing out the infection. After the infection is cleared, the majority of the T_E die, but a small percentage develop into long lived resting state memory T cells (T_M). These T_M cells accumulate throughout life and have the capability to provide life-long protection against a number of pathogens.[1]

Additionally, the positive or negative expression of certain surface markers such as CD45RA, CD45RO, CD27, CD62L and CD95 identify multiple T cell subsets: T naive cells (T_N), T stem cell-like memory cells (T_{SCM}), T central memory cells (T_{CM}), T transitional memory cells (T_{TM}), T effector memory cells (T_{EM}), T effector memory cell re-expressing CD45RA (T_{EMRA}) and T terminal effector cells (T_{TE}). As T cells differentiate from T_N to memory and effectors cells, their effector function increases while their self-renewal (proliferation) ability decreases.[2]

Adoptive therapy with *ex vivo* expanded antigen-specific T cells, with or without genetic modification, can induce remissions in patients with relapsed | refractory cancer. The success of this therapy is dependent on the efficient expansion of T cells *ex vivo* and their homing, persistence and cytotoxicity following administration to the patient.[3] T cells from cancer patients are generally more differentiated and more challenging to expand than T cells from healthy donors. T_{SCM} and T_{CM} bio-distribution and long term persistence represent appealing targets to overcome the current limitation of cancer adoptive therapy. Studies of various *ex vivo* expansion protocols are geared toward generation and maintenance of the less differentiated subset phenotypes in hopes of improving the clinical efficacy of genetically modified cells for use in adoptive therapy.[4,5,6] One major challenge is that disease and/or prior treatment may have a permanent, detrimental effect on a patient's T cells. Not all patients with disease are suitable for adoptive cell therapy due to the lack of *ex vivo* expansion potential. This suggests that individualized T cell subset profiling and personalized immunotherapy are needed to effectively treat cancers.[7]

Ex vivo expansion of T cells continues to be a significant step in adoptive T cell therapy including CAR-T, TCR and TIL cell therapies. We have developed the iQue® Human T Cell Memory Kit based on high throughput flow cytometry to characterize and profile *ex vivo* expanded T cells. The miniaturized format of this assay conserves both reagents and precious sample. Multiplexing cells and beads in each assay well enables the simultaneous measurement of T cell phenotypes and secreted functional cytokines, workflows traditionally achieved independently on two separate instruments. The assay is optimized for use with the iQue® platform, which not only provides fast sample acquisition speed (5 min per 96-well plate), but also uses advanced algorithms tailored for immune screening and profiling, such as profiling maps and multi-plate analysis. Together, these tools enable the rapid translation of data into actionable results.

Proof-of-concept (POC) screening studies were performed by using human peripheral blood mononuclear cells (PBMCs) from three donors cultured in a single, 96-well plate over 3 days using 14 different culture conditions. Results demonstrated that the iQue® Human T Cell Memory Kit could be used to quickly identify T cell culture conditions, such as media supplemented with cytokines IFNß or IL-21, which may increase the frequency of less differentiated T memory cells such as T_{CM} and T_{SCM}. More interestingly, the PBMCs with secreted IL-10 showed a significant correlation with T_{CM} frequency, which suggest the secreted IL-10 may be used as an early indicator of increased T_{CM} frequency in *ex vivo* T cell expansion culture. These results suggest that the iQue® Human T Cell Memory Kit, when used with the iQue® platform and integrated Forecyt® software, may provide a robust solution for quick characterization of T memory cells and for *ex vivo* expanded T cell product intended for adoptive T cell therapy.

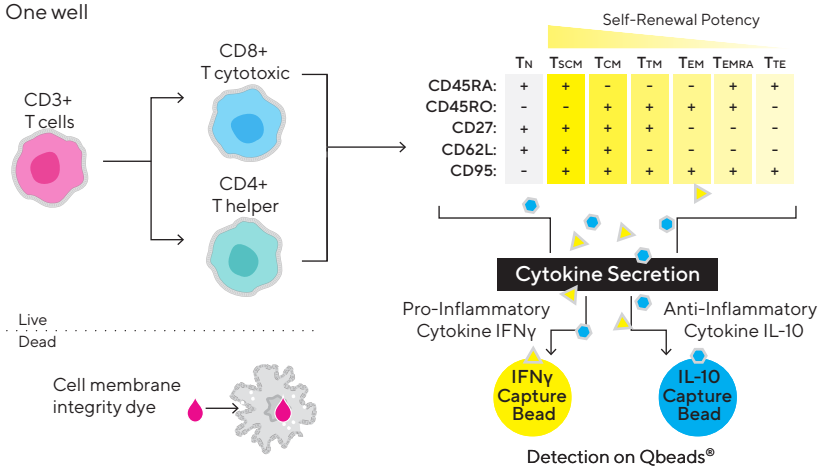


Figure 1: Multiplex assay design

Assay Principles

In each assay well, live immune cells are distinguished from dead cells by staining with a fluorescent membrane integrity dye. The dye enters only dead cells or those with a compromised membrane, staining the nucleic DNA by intercalation. Live cells are immunophenotyped by staining with a fluorescent antibody panel to separate CD3+ (T cells), CD3- (non-T cells), CD4+ (T helper cells) and CD8+ (T cytotoxic cells). The panel also includes 5 different T cell surface markers for T naive | memory | effector cell phenotyping: CD45RA, CD45RO, CD27, CD62L and CD95.

Effector cytokines secreted by *ex vivo* expanded T cells, including pro-inflammatory cytokine IFNγ and anti-inflammatory cytokine IL-10, are measured in a "sandwich" immune assay format by two different iQue® Qbeads® included in the same well (Figure 1). If necessary, up to six additional cytokines may also be quantified in a multiplexed assay format by combining the iQue® Human T Cell Memory Kit with the iQue® Human T Cell Companion Kits of user's choice (IL-2, IL-6, IL-13, IL-17A, T_{NF}, or GM-CSF).

Materials

Cells and Reagents

Cryopreserved human PBMCs (Astarte Biologicals) were cultured in X-VIVO™ 15 media (Lonza), supplemented with 5% human AB sera (Sigma). CD3 | CD28 Dynabeads® (ThermoFisher Scientific), and cytokines IL-4, IL-6, IL-7, IL-15, IL-21 and IFNß (PeproTech) were used for *ex vivo* T cell activation and expansion. The iQue® Human T Cell Memory Kit was used to assess cell count, viability, and secreted cytokines (INFγ and IL-10), as well as to identify memory T cell subsets by their cell surface phenotypic markers (CD27, CD45RA, CD45RO, CD62L and CD95).

Methods

Cell Treatment

PBMCs from three different healthy donors were recovered overnight in X-VIVO15 media with 5% human AB sera (hereafter referred to as media). On Day 0, cells from each donor were counted and cell density was adjusted to 4 million/mL (4X). Cells were plated at 25 µL/well into corresponding wells of a 96-well v-bottom plate (Cat. No. 10149). CD3 | CD28 Dynabeads® were washed once with media prior to use. The beads and wash media were then placed on a Dynamag™ magnet (ThermoFisher Scientific) for a minimum of 1 min. The wash media was then removed and the beads were resuspended in fresh media at a final density of 0.8 million/mL (4X). Dynabeads® were added to the designated wells in the culture plate at 25 µL/well. Fourteen different cytokine cocktail combinations were prepared at (2X) in media. These combinations were added to designated wells at 50 µL/well. The total culture volume was 100 µL per well, with a final cell density of 1 million/mL and a final CD3 | CD28 Dynabeads® density of 0.2 million/mL (final cell/bead ratio: 4:1). The final cytokine concentrations in culture wells were: IL-7 and IL-15, 10 ng/mL; IL-4, IL-6, IL-21, and IFNß, 100 ng/mL. The 14 cytokine combinations are shown in Figure 2 (Treatments 3–16). Treatment 1 is a negative control (cells and media only) and Treatment 2 is cells activated only with Dynabeads® (no supplemented cytokines). The prepared culture plate was placed in a high humidity chamber and kept in a 37° C incubator with 5% CO_2 for up to 8 d to allow for expansion of the T cell populations with minimum edge effect.

Treatment	Cocktail
1	Negative Control
2	CD3 | CD28 Dynabeads® (DB)
3	IL-4, Il-7, DB (Core)
4	IL-6
5	IL-15
6	IL-21
7	IFNβ
8	IL-15, IL-6
9	IL-21, IL-6
10	IFNβ, IL-6
11	IL-21, IL-15
12	IFNβ, IL-15
13	IFNβ, IL-2
14	IL-21, Il-15, IL-6
15	IFNβ, IL-15, IL-6
16	IFNβ, IL-21, IL-15

	1	2	3	4	5	6	7	8	9	10	11	12
A	1		9		1		9		1		9	
B	2		10		2		10		2		10	
C	3		11		3		11		3		11	
D	4		12		4		12		4		12	
E	5		13		5		13		5		13	
F	6		14		6		14		6		14	
G	7		15		7		15		7		15	
H	8		16		8		16		8		16	
	Donor 1				Donor 2				Donor 3			

	Possible Effect
CD3 | CD28 Dynabeads®	T cell stimulation | expansion
IL-4 | IL-7	Help *in vitro* expansion | maintain memory T cells
IL-6	Promote T folicular helper cells
IL-15	Increase survival of memory T cells
IL-21	Increase Th17 cells and CD8 effector function
IFNβ	Increase CD8 effector function and survival

Figure 2: Plate design and screening conditions

Assay Setup

On Days 2, 5 and 8 post-treatment, culture samples were manually mixed by up-and-down pipetting to ensure cell resuspension, and 10 µL of sample containing cells and supernatant were transferred from the culture plate to an assay plate (Cat. No. 10149). Samples were analyzed using the iQue® Human T Cell Memory Kit with workflow shown in Figure 3. Total assay time was approximately 3 h, with hands-on time of 30 min each day. Data was acquired on the iQue®, with a total acquisition time of approximately 25 min per 96-well plate. Event gates and all experimental metrics including the T cell identifiers (CD3 | CD4 | CD8), T cell memory subsets (naive | memory | effector), cell viability and quantitation of secreted cytokines were auto generated using the integrated Forecyt® software. To measure the effect of the cytokine treatment, data from each donor were normalized against samples from the same donortreated with only CD3 | CD28 Dynabeads® and expressed as the fold increase using the advanced metric tool in Forecyt®. On each assay day, a separate plate with 2 rows of cytokine standards were run using the iQue® Human T Cell Memory Kit to generate standard curves for cross-plate quantitation of IFN and IL-10 in the screening plate using Forecyt®.

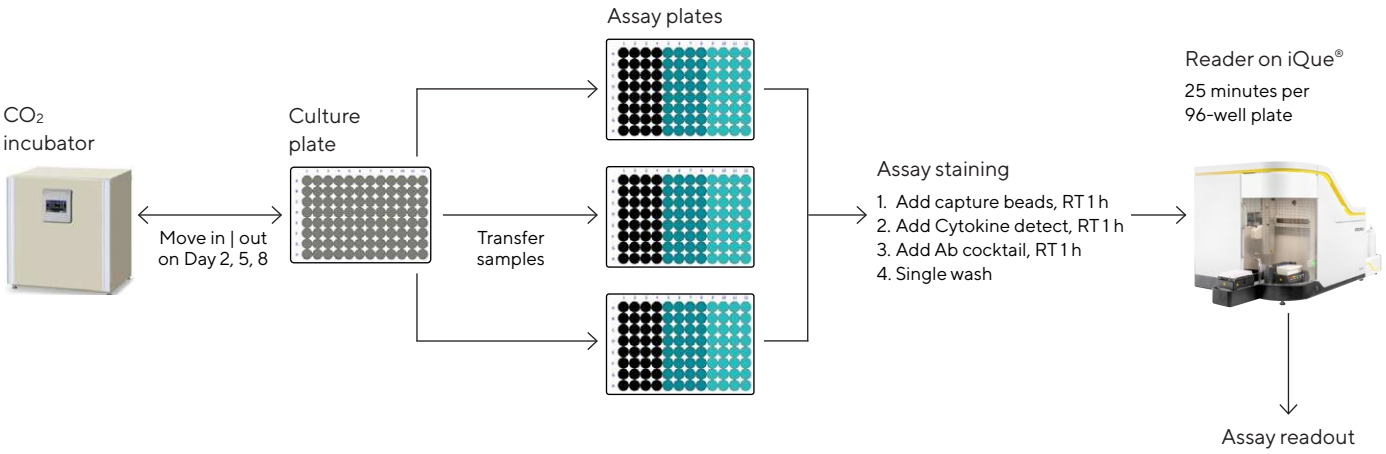


Figure 3: Screening workflow

Basic T Cell ID		T Naive | Memory | Effector ID							Secreted Cytokines		Cell Count	Viability
CD3+	CD4+	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{TEM}	T_{EMRA}	T_{TE}	IFNγ	IL-10	+	+ | -
	CD8+	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{TEM}	T_{EMRA}	T_{TE}				

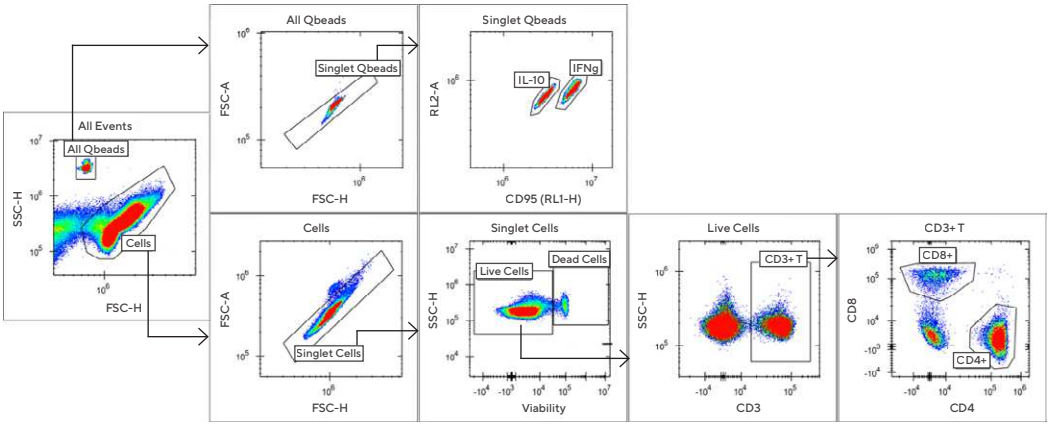


Figure 4: Cell and bead gating strategy at full plate level

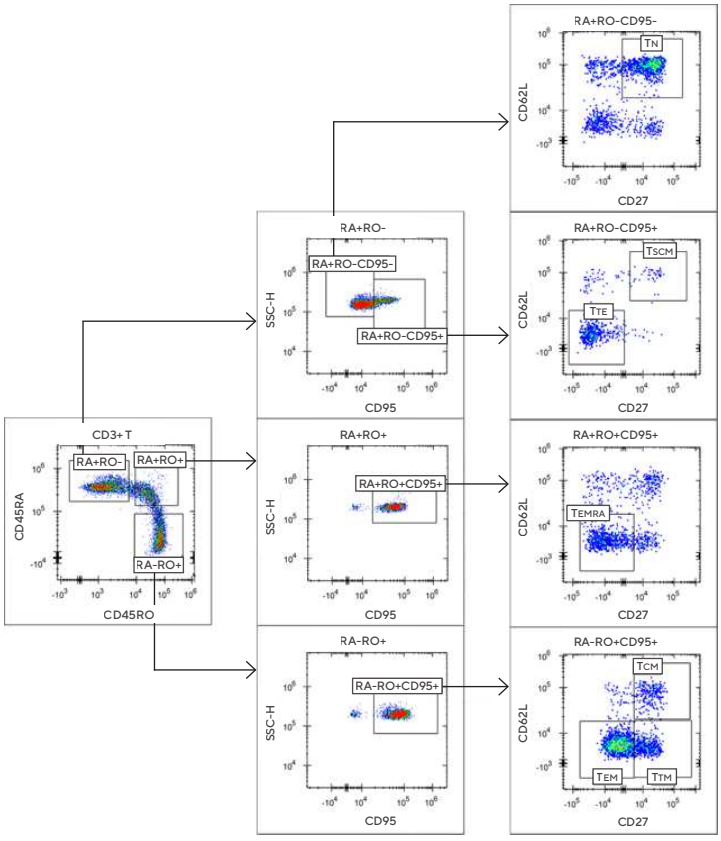


Figure 5: Gating of T memory cells at plate level

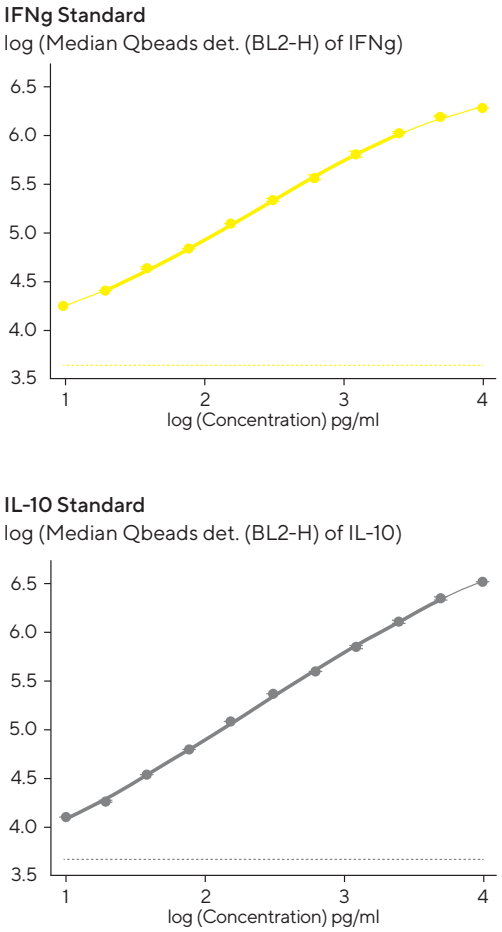
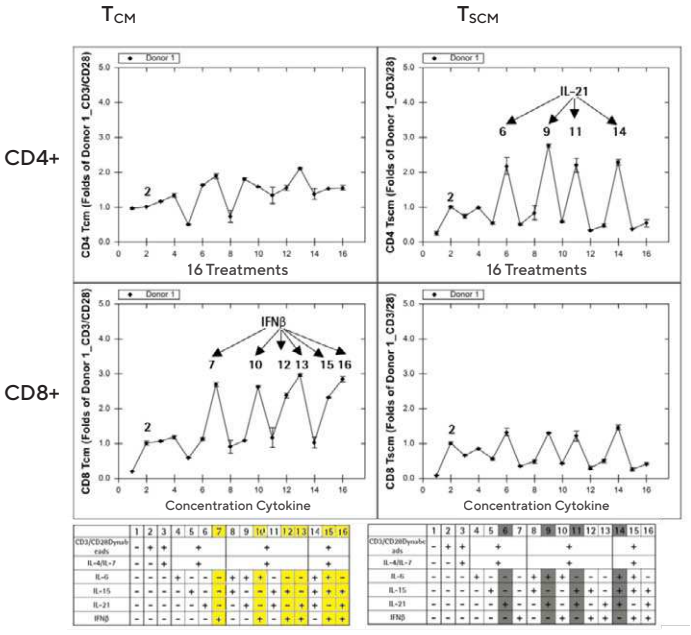


Figure 6: IFN and IL-10 standard curves

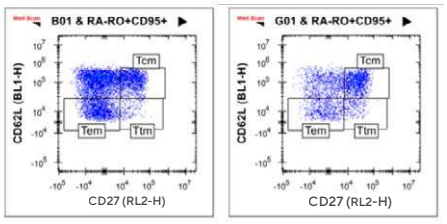
A.



B. T_{CM} Comparison

Treatment 2 (CD3 | CD28 Dynabeads only)

Treatment 7(CD3 | CD3 | (CD28 Dynabeads + IL4 | IL7 |IFNβ)



C. CD3+ T CellCount

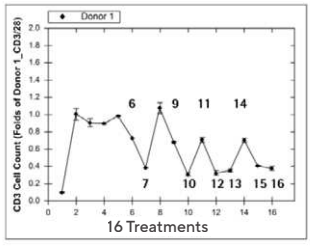


Figure 7: Increased T memory cell frequency after *ex vivo* expansion in cytokine supplemented media

Data Acquisition and Analysis

Samples were acquired on the iQue® using the assay template provided with the iQue® Human T Cell Memory Kit. The violet, blue and red (VBR) laser configuration is required due to the antibody panel design of the kit. The acquisition protocol and data analysis including event gates and gating strategy (Figures 4 and 5), data metrics, heat maps and standard curves were auto-generated using the iQue® Human T Cell Memory Kit template and Forecyt® software. The template includes validated compensation metrics which automatically apply to the data analysis, without the need for single stain compensation. For the gating strategy, all populations were separated at the plate level, not at the well level. The cells and beads were separated using the scatter plot due to size and granularity difference. The 2 different cytokine capture beads (IFNγ and IL-10) were separated based on bead intrinsic fluorescence in the RL1 and RL2 channels of the iQue®. RL1 is also shared with CD95 detection of cells, but will not interfere with bead classification. Cells were separated using 2D plots with various CD markers | viability staining signal. Different T naive | memory | effector cell subsets were identified in CD3+ T cell populations (Figures 4 and 5). To identify each corresponding CD4+ and CD8+ T naive | memory | effector cell subsets, Boolean logic gates were used (data not shown).

Standard curves to quantitate the levels of secreted IFNγ and IL-10 were generated using a 4-parameter curve fit with 1/Y2 weighting, and the linear range (indicated by bold lines in Figure 6) for each standard curve was calculated using Forecyt®. Standard curves were prepared with 1:2 serial titration with a top concentration of 10,000 pg/mL per standard protein using standards provided in the kit. The linear ranges for standards were as follows: IFN , 10–2, 100 pg/mL; IL-10, 10–6, 400 pg/mL. The detection ranges were wider than the linear range, and the dash line represents the fluorescent background when the standard concentration was zero.

Results and Discussion

The T cell expansion time course data was analyzed and series graphs from the POC studies were generated for each donor using Forecyt®. Culture media supplemented with cytokine cocktails containing IFNß or IL-21 cytokine increased the T_{CM} or T_{SCM} frequency, respectively. Data were presented as fold increase against the control wells with CD3 | CD28 Dynabeads® treatment. Figure 7 shows the results from Donor 1 on Day 5. T_{CM} (especially cytotoxic CD8+ T_{CM}) show frequency increased in six cocktails containing IFNß, and T_{SCM} (particularly helper CD4+ T_{SCM}) showed frequency increased in four cocktails containing IL-21 (Figure 7A). The full mechanism of selective effect on CD8+ T_{CM} by IFNß and on CD4+ T_{SCM} by IL-21 is unknown, though it is reported type I interferons, including IFNß, act directly on CD8+ T cells to allow clonal expansion and memory formation.[8] In Figure 7B, the 2D plots (CD27 vs. CD62L) show T_{CM} cell frequency increased in the wells treated with a cocktail containing with IFNß compared to the well treated with CD3 | CD28 Dynabeads® alone.

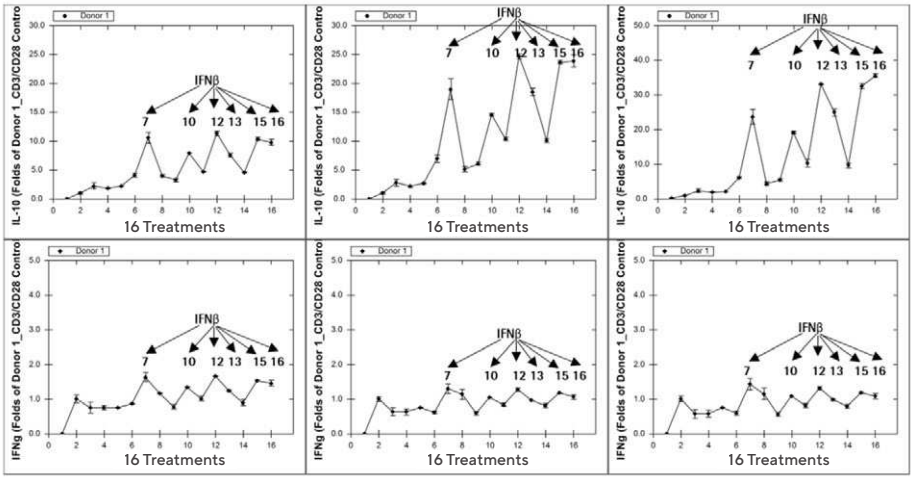


Figure 8: IL-10 secretion correlates with CD8+ T_{CM} cell frequency

CD3+ T cell counts in culture supplemented with cytokine cocktails containing IFNß also slowed down cell proliferation by approximately 50% (Figure 7C). Day 2 post treatment T_{CM} and T_{SCM} cell frequencies were only moderately effected by the treatments in the same direction as on Day 5, and Day 8 achieved similar results (data not shown).

Temporal analysis of IL-10 and IFN secretion was determined by using Forecyt® software. Figure 8 shows the results from Donor 1. Data were presented as fold increase against the control wells with CD3 | CD28 Dynabeads® treatment only. Six cytokine cocktails containing IFNß in the culture increased both IL-10 and IFN secretion from Day 2 to Day 8. Interestingly, the IL-10 secretion pattern was very similar to CD8 T_{CM} frequency pattern (Figure 7A). Even on Day 2, when there was no significant increase of T_{CM} in most cocktail treatments (data not shown), IL-10 secretion showed a similar pattern as that on Day 5. This suggests that the increase in IL-10 secretion by immune cells may be an indicator of increasing T_{CM} frequency in upcoming days.

Additional results from our POC screening studies show donor-to-donor variation in CD8+ T memory cell frequencies, which is not surprising as not all patients are expected to be suitable for adoptive T cell therapy. Day 5 results are shown as an example (Figure 9). Donors 1 and 3 had a higher frequency of CD8+ T_{CM} when cultured in media containing IFNß, increased frequencies of CD8+ T_{SCM} and CD8+ Ttm when cultured in media containing in IL-21, and elevated CD8+ T_{EM} frequency with IL-15 supplemented media. In contrast, all media combinations had only moderate or no effect on the frequency of T cell memory subsets from Donor 2, suggesting donor dependent sensitivities to various cytokine treatment in *ex vivo* T cell expansion. These results suggest it is necessary to screen culture conditions for every donor, as one optimal expansion condition for a specific donor may not apply to other donors.

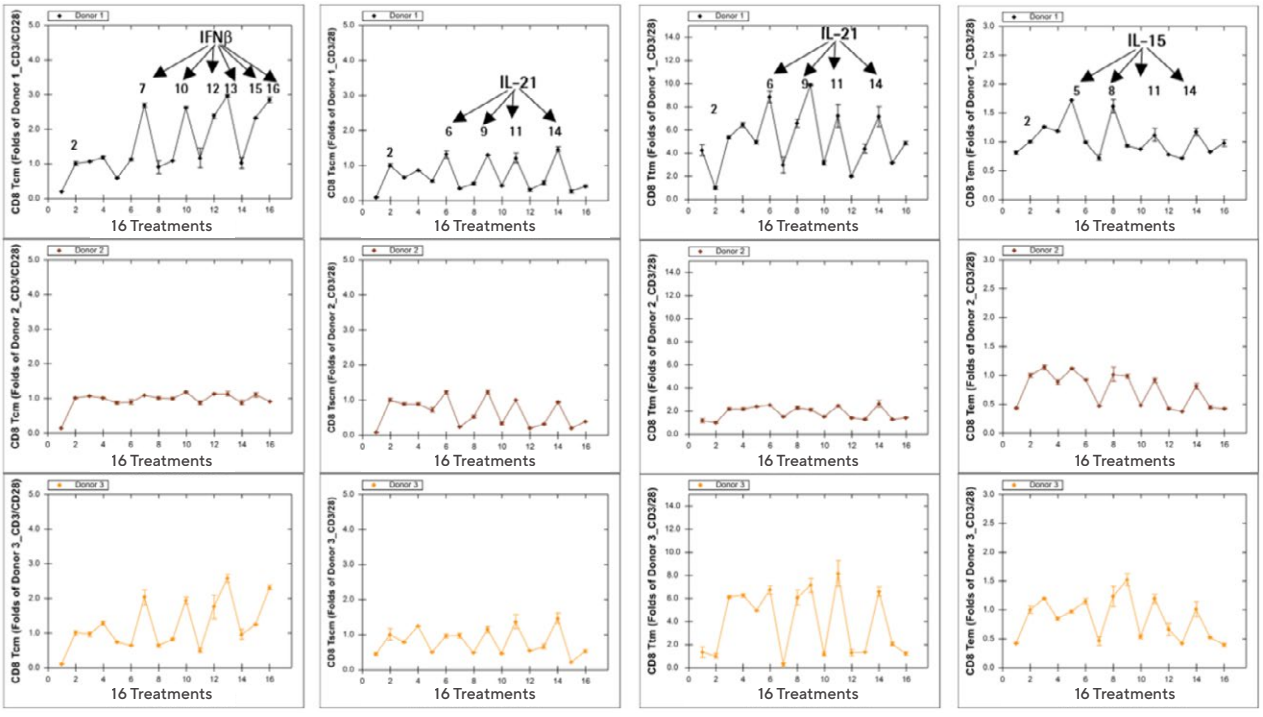


Figure 9: Donor-to-donor variation in *ex vivo* T cell expansion

Conclusion

This application note demonstrates the simple assay and analysis workflow of iQue® Human T Cell Memory Kit using a screening model that, despite the complexity of T cell biology, could be useful for adoptive T cell therapy workflows. The kit is designed for ease of use by combining cells and beads, multiplexing measurements in each assay well. It offers these unique advantages:

- Multiplexed cell and secreted cytokine measurement in a single assay, improvement over common immunology research workflows that generally require multiple assays run on different platforms.
- iQue® advanced flow cytometry platform with integrated Forecyt® software offers a single platform and data analysis package. Streamlines data acquisition and analysis workflow including multi-plate analysis, solving data synchronization issues. Quickly translate data into actionable results.
- Simplified Plug-and-Play assay workflow with no need of color compensation. Most of the reagents are pre-mixed for ease of use. The assay template includes validated compensation metrics that are automatically applied to your data analysis. Cytokine concentration is also automatically interpolated by referring to the corresponding standard curve.
- Spatial-temporal analysis of T memory cell phenotypes and functions at different stages in a single high-content miniaturized assay. This format saves precious samples, decreases reagent costs and also enhances data integrity.
- Flexibility to choose additional cytokine measurements from validated iQue® Human T Cell Companion Kits: IL-2, IL-6, IL-13, IL-17A, GM-CSF and TNF.

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