Simplifying Progress

Advanced Cytometry Techniques for Immune-Based Therapy Analysis

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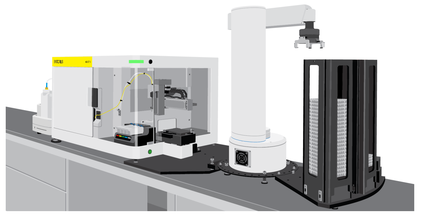
Introduction

- As interest in CAR-T cells and immune-based oncology therapies rises, efficient phenotyping of cell therapy products becomes essential. This involves analyzing surface markers associated with cell type characteristics.
- To address this need, tools for panel design and instruments for cell interrogation must be compatible.
- This study highlights the combined use of an advanced panel builder to simplify assay creation with quantification using the iQue® HTS Cytometer Platform.
- To showcase this workflow, data was generated for a T cell phenotyping panel and tested in PBMCs.
- In summary, the panel design tool, and the iQue® HTS Platform streamlines the creation of flexible panels for profiling immune cells.

High-Throughput Screening by Cytometry

- Scientists use flow cytometry to investigate complex biological processes as part of routine cell analysis workflows.
- To keep pace with market demands, scientists need a faster way to generate biologically relevant data from high-throughput research workflows.
- The iQue® Platform combines the ability to run complex panels using both cells and beads in the same well with the speed and throughput required for HTS workflows.
- The iQue® 5 instrument is an evolution, building on the established features to enhance the customer experience and support extra flexibility.



- Equipped with adaptable automation capabilities, it facilitates seamless integration with automation solutions, enhancing high-throughput screening and minimizing the need for manual intervention.
- Accelerated market-leading speed with the use of the patented air gap technology to deliver 384-well sampling in <20 min, 96-well <5 min.
- The platform is available in both a 3 laser (Violet, Blue, Red) and 4 laser (Violet, Yellow, Blue, Red) format for improved flexibility and easier panel design to enable greater workflow support.
- VBR format with up to 19 channel capacity.
- VYBR format with up to 27 channel capacity.
- Improved Clog detection and management with air-gap sensor for simplified monitoring.
- Easily set individual channel gains with SiPM.
- Improved usability, fluidics and data analytics including higher volume acquired in less time with reduced carry over between samples.

Emission Filter	Laser							
	405 nm		488 nm		561 nm *		640 nm	
445/45 nm	✓	Pacific Blue						
525/45 nm	✓	BV510	✓	FITC				
586/20 nm	✓	BV605	✓	EYFP	✓	PE		
615/20 nm	✓	Qdot 605	✓	PI	✓	PE-Dazzel594		
667/30 nm	✓	BV650	✓	PerCP	✓	PE-Cy5	✓	APC
695/40 nm	✓	BV711	✓	PerCP-Cy5.5	✓	PE-Cy5.5	✓	AF680
725/40 nm	✓	BV750	✓	PerCP-eFluor7	✓	PE-AF700	✓	AF700
780/60 nm	✓	BV786	✓	PE-Cy7	✓	PE-Cy7	✓	APC-Cy7

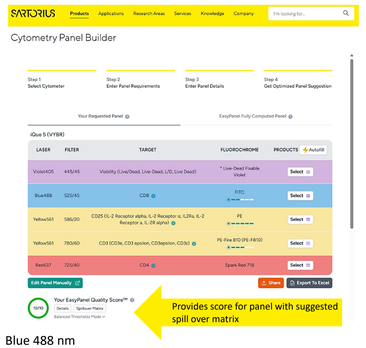
Laser and channel details for iQue® 5 Platform * 561 nm not on 3 laser (VBR) instrument

T Cell Panel Design

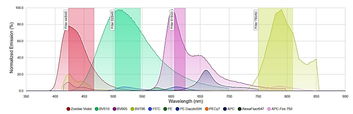
A comprehensive T cell panel was developed using the iQue® Cytometry Panel Builder (link can be found on our website).

- Panel builder has simple steps to guide you through building the panel, powered by Easy Panel.
- Two panels were designed for use on either iQue® 3 or 5 using a base panel covering CD3, CD4, CD8 and a viability marker.
- Further markers for activation (CD69, CD25), exhaustion (LAG-3, TIM-3, PD-1) or memory (CCR7, CD62L), were added for further characterization.
- Using the iQue® 5 HTS platform a further 4 markers were incorporated to enable the capture of 11 data channels, along with forward and side scatter, from a 96-well plate.
- The addition of a fourth laser increases the flexibility for panel design and ability to capture more data.

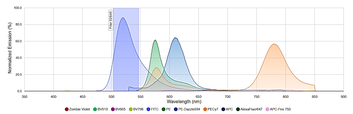
Laser	Channel	Target	Fluorochrome	iQue®3 panel
Violet 405	V445/45	Viability	Zombie Violet	VL1 – 445/45
Violet 405	V525/45	CD8	BV510	VL2 – 530/30
Violet 405	V615/20	CD4	BV605	VL4 – 615/24
Violet 405	V780/60	CD69	BV786	
Blue 488	B525/45	CD62L	FITC	
Yellow 561	Y586/20	CD25	PE	BL2 – 572/28
Yellow 561	Y615/20	CCR7	PD-Dazzle594	
Yellow 561	Y780/60	PD-1	PECy7	BL5 – 780/60
Red 640	R667/30	LAG-3	AF647	RL1 – 675/30
Red 640	R695/30	TIM-3	APC	
Red 640	R780/60	CD3	APC-Fire750	RL2 - 780/60



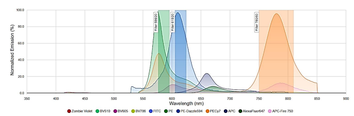
Violet 405 nm



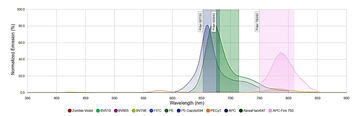
Blue 488 nm



Yellow 561 nm



Red 640nm



Compensation Assessment

Initially, compensation particles were tested with the selected antibodies to automatically calculate compensation spillover values using iQue Forecyt® software, before applying the panel to immune cells.

- In iQue® 5, PE conjugated antibodies can use the yellow laser excitation for improved resolution.
- Spillover matrix highlights potential issue with TIM-3 and LAG-3 due to overlapping spectra.
- iQue Forecyt® compensation allows overlapping fluorophores to be isolated and successfully analyzed.

Spillover Matrix

Primary Channel \ Spillover Channel	CD62L (BV525-H)	TIM-3 (R667-H)	LAG-3 (R695-H)	CD3 (R780-H)	V/Blue MI (V445-H)	CD8 (V525-H)	CD4 (V615-H)	CD69 (V780-H)	CD25 (Y586-H)	CCR7 (Y615-H)	Y667-H	PD-1 (Y780-H)
CD62L (BV525-H)		100.00	0.00	0.02	0.18	3.99	0.36	0.04	0.11	0.23	0.00	0.16
TIM-3 (R667-H)	0.02		13.50	11.39	0.09	0.13	0.15	0.75	0.23	0.88	0.00	5.00
LAG-3 (R695-H)	0.09	399.11		61.97	0.60	0.42	0.19	0.32	0.45	0.97	0.00	12.77
CD3 (R780-H)	0.03	3.01	0.44		0.11	0.10	0.06	6.43	0.13	0.37	0.00	35.94
V/Blue MI (V445-H)	0.21	0.05	0.02	0.02		19.84	0.70	0.04	0.06	0.06	0.00	0.04
CD8 (V525-H)	0.48	0.00	0.00	0.01	13.13		20.70	1.57	0.29	0.73	0.00	0.08
CD4 (V615-H)	0.18	0.42	0.08	0.05	19.58	1.75		8.39	49.72	72.33	0.00	7.71
CD69 (V780-H)	0.11	0.13	0.06	16.87	17.59	1.61	0.32		0.18	0.33	0.00	5.42
CD25 (Y586-H)	1.58	0.01	0.00	0.01	0.09	0.28	4.99	0.13		24.52	0.00	0.93
CCR7 (Y615-H)	0.67	0.41	0.10	0.24	0.11	0.16	14.73	0.69	34.80		0.00	5.94
Y667-H	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
PD-1 (Y780-H)	0.24	0.01	0.01	9.88	0.05	0.06	0.05	6.22	2.07	0.69	0.00	

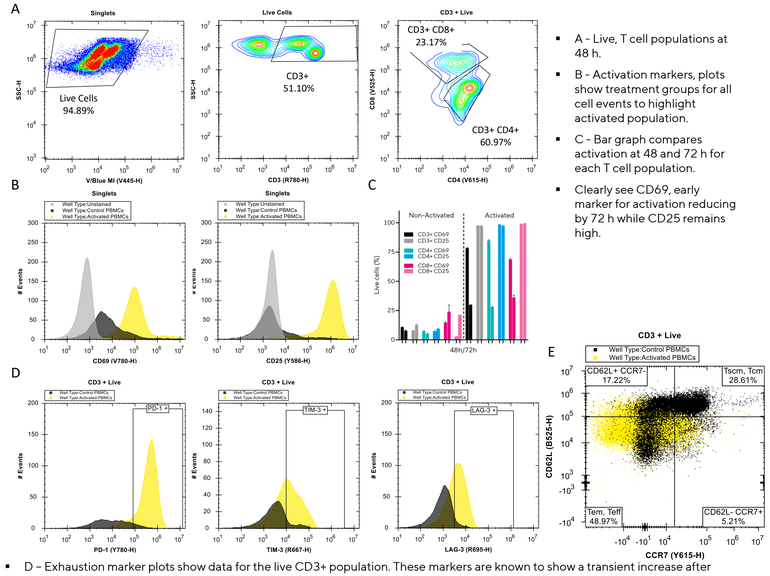
Phenotype Profiling in PBMCs

Human PBMCs were cultured in RPMI 1640 media with 10% serum and IL-2, with or without CD3/CD28 T cell activation beads (1 bead per cell) to activate the T cell population. Over the following 72 hours, the cells were assessed using the panel.

Cells were assessed using the following strategy:

- All events to detect cells (FSC Vs SSC) and singlets (not shown)
- Viability marker for live cells
- CD3+ cells
- CD4+ and CD8+ populations

→ Activation – CD69, CD25
→ Exhaustion – PD-1, LAG-3, TIM-3
→ Memory – CCR7, CD62L



- A - Live, T cell populations at 48 h.
- B - Activation markers, plots show treatment groups for all cell events to highlight activated population.
- C - Bar graph compares activation at 48 and 72 h for each T cell population.
- Clearly see CD69, early marker for activation reducing by 72 h while CD25 remains high.

- D – Exhaustion marker plots show data for the live CD3+ population. These markers are known to show a transient increase after activation and at 48 h all three show some increase but unlikely to be true exhaustion.
- E - Displays potential memory phenotypes. Using CCR7 and CD62L it is possible to define two groups Tscm/Tcm (less differentiated) and Tem/Teff (more differentiated) populations. Post activation the CD3+ T cells have moved to more differentiated state (control 16%, activation 76%).

Conclusion

- In summary, the iQue® 5 HTS platform support users with increased flexibility for designing complex panels, suitable for cell therapy characterization.
- The integration of the panel builder with the iQue® Platform offers a streamlined approach for creating complex panels to profile immune cell populations.
- Panels can be further enhanced by incorporating cytokine profiling using the iQue Qbead® portfolio, leveraging the iQue® Platform's capacity to independently track both cell and bead populations within the same sample.