Flow Cytometric Analysis of Urine Cell 18-Color Immunophenotyping Panel

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Flow Cytometry for NMIBC

Context

ZH9 is a novel live attenuated bacterial immunotherapy currently in clinical development for **Non-Muscle Invasive Bladder Cancer (NMIBC)**, aiming to redesign the treatment paradigm with a single induction dose therapy.

For biomarker analysis of treatment response and we set out to develop a suitable assay for exploratory analyses of urine samples from the clinical trial.

Approach

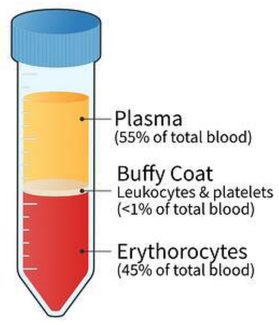
Buffy coats were isolated from human whole blood in **sodium heparin** tubes. To approximate patient samples, freshly isolated buffy coats were spiked into healthy donor urine. **Urine sample** were spiked in and stored at 2-8°C for 24 hours prior to staining.

Samples processed and stained with a **Full (18 color) and FMX (12 color panel).**

1. Establish Qualification Plan and an Immunophenotyping Panel

A. Assay Evaluation

Study needs	Solutions
• Develop a panel to evaluate NMIBC urine samples	• Evaluate markers on healthy donor buffy coat sample to assess rare populations • Spike urine in to evaluate healthy urine background



Assay precision: Three replicates each of sample of buffy coat spiked with urine from three donors were assayed.
Analyst Precision: Two analysts and one replicate donor for three healthy donor samples using human urine from one healthy donor spiked with fresh buffy coats isolated from human whole blood in sodium heparin tubes.
Fixed sample stability: Three replicates of spiked and treated whole blood from each of 3 donors were assayed. One set of replicates as acquired at each of the following time points following fixation: within 3 hours, 24±3 hours, and 24±3 hours.
LLOQ: Spiked buffy coat cells were serially diluted in 1x DPBS for an eight-point, 2-fold serial dilution.

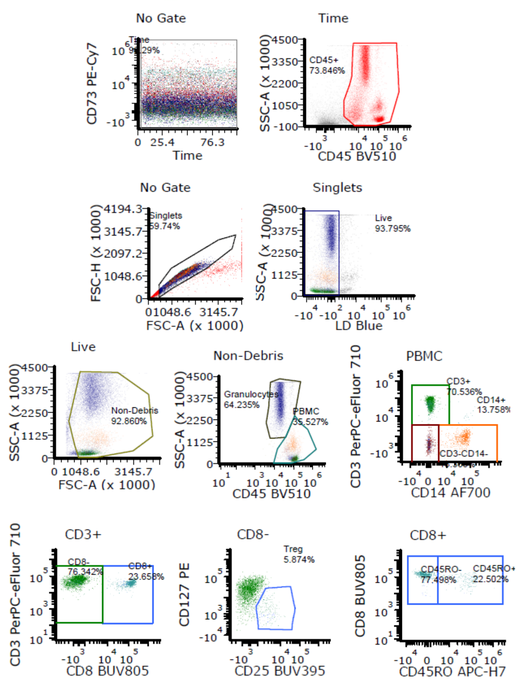
B. Full Immunophenotype Panel

Target	Fluorophore	Marker for	Location
CD25	BUV395	T reg	Surface
Live/Dead	Blue	Dead cells	Surface
CD15	BUV737	Granulocytes	Surface
CD8	BUV805	T cell subset	Surface
CD1c	BV421	DC (cDC type 1)	Surface
CD45	BV510	Total leukocytes	Surface
HLA-DR	BV605	Activation	Surface
CD141	BV650	DC (cDC type 1)	Surface
CD39	BV711	Exhaustion	Surface
CD193	BV786	Eosinophils	Surface
CD56	AF488	NK Cells	Surface
CD3	PerCP-eFluor710	T cells	Surface
CD127	PE	T reg	Surface
CD279 (PD-1)	PE-CF594	Exhaustion	Surface
CD73	PE-Cy7	Suppression	Surface
CD274 (PD-L1)	APC	Suppression	Surface
CD14	AF700	Monocytes	Surface
CD45RO	APC-H7	Memory	Surface

2. Gating Strategy

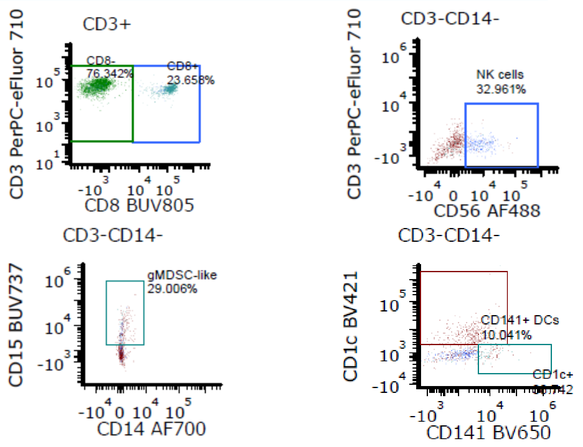
Preliminary Gating

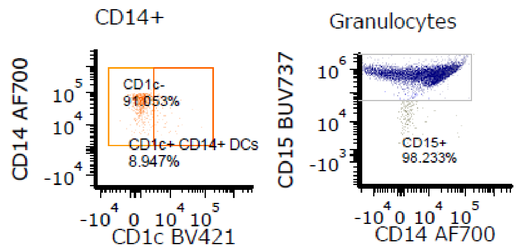
Preliminary Gating: Fluidic instability were excluded using a Time gate (CD73 versus Time). Downstream white blood cells were identified based on CD45 expression and singlets were identified from FSC-A and FSC-H. From singlets, live cells were identified as negative for live/dead blue. Debris was excluded and, PBMCs and Granulocytes were identified based on FSC-A and SSC-A profile. The PBMC compartment was gated into CD3 single-positive, CD14 single-positive, and CD3/CD14 double negative subsets. The CD3+ cells were then gated into CD8+ and CD8- subsets. Within the CD8- subset, Tregs were identified as CD25+ and CD127-. The CD8+ subset was gated into CD45RO+ activated cells and CD45RO- quiescent cells.



3. Subset Analysis

CD8+/CD8-: HLA-DR, PD-L1, PD-1, CD39, CD73, and CD127 assessed as functional markers.
NK cells : HLA-DR, PD-L1, PD-1, CD39, CD73, and CD127 assessed as functional markers.
CD3-CD14- : CD39, CD73, and CD127 assessed as functional markers.
CD1c+CD14-/CD141+/ gMDSC : HLA-DR, and PD-L1 assessed as functional markers.





CD14+ : HLA-DR, CD39, CD73, and CD127 assessed as functional markers.

CD1c+CD14+ : HLA-DR,PD-L1, assessed as functional markers.

CD15+ Granulocytes : CD39, CD73, CD127 assessed as functional markers

Eosinophils and Neutrophils: PD-L1, HLA-DR assessed as functional markers.

4. Statistics

Assay Precision

CD8+/-:

Sample	Replicate	CD8+CD39+ Freq. of Parent	CD8+CD39+ Median	CD8+CD127 + Freq. of Parent	CD8+CD127 + Median	CD8+CD73+ Freq. of Parent	CD8+CD73+ Median	CD8+HLA-DR+ Freq. of Parent	CD8+HLA-DR+ Median	CD8+PD-L1+ Freq. of Parent	CD8+PD-L1+ Median	CD8+PD-1+ Freq. of Parent	CD8+PD-1+ Median
Mean of All Donors	Mean	1.17	4538.65	88.96	25009.45	73.06	33911.18	6.91	11023.03	1.19	3179.84	4.09	7754.96
	SD	0.08	1275.91	0.62	417.16	0.55	671.61	0.57	1262.47	0.20	631.65	0.76	701.18
	%CV	10.22	29.69	0.69	1.63	0.74	1.89	11.58	10.67	17.43	17.87	18.68	9.70

*Green highlight: CV< 30 percent

Granulocytes:

Sample	Replicate	Eosinophils/CD39+ Freq. of Parent	Eosinophils/CD39+ Median	Eosinophils/CD127+ Freq. of Parent	Eosinophils/CD127+ Median	Eosinophils/CD73+ Freq. of Parent	Eosinophils/CD73+ Median	Eosinophils/HLA-DR+ Freq. of Parent	Eosinophils/HLA-DR+ Median	PD-L1+ Freq. of Parent	Eosinophils/PD-L1+ Median
Mean of All Donors	Mean	20.89	35835.59	35.39	9152.33	54.22	14053.55	12.18	16270.57	10.69	31486.40
	SD	1.97	1186.87	2.84	657.68	3.13	894.71	2.07	3141.53	1.53	6677.64
	%CV	9.24	3.34	7.61	6.28	5.63	5.99	16.04	20.31	14.66	21.36

*Green highlight: CV< 30 percent

CD14+

Sample	Replicate	CD1c-/Monos/CD73+ Freq of Parent	CD1c-/Monos/CD73+ Median	CD1c-/Monos/CD127+ Freq. of Parent	CD1c-/Monos/CD127+ Median	CD1c-/Monos/CD39+ Freq. of Parent	CD1c-/Monos/CD39+ Median	CD1c-/Monos/PD-L1+ Freq. of Parent	CD1c-/Monos/PD-L1+ Median	CD1c-/Monos/HLA-DR+ Freq. of Parent	CD1c-/Monos/HLA-DR+ Median
Mean of All Donors	Mean	2.15	5718.67	1.57	3134.15	99.85	27331.89	2.05	3354.24	100.00	64287.49
	SD	0.57	874.23	0.39	154.38	0.06	320.99	0.27	575.31	0.00	1483.00
	%CV	29.54	16.70	22.59	4.90	0.06	1.16	12.10	14.24	0.00	2.41

*Green highlight: CV< 30 percent

5. Conclusions

- **Assay Precision:** 138 reportables were assessed with out pass /fail criteria. Due to rare population assessment most of the reportable CV percent is under 30 percent.
- **Analyst Precision:** 138 reportables were assessed with out pass /fail criteria. Due to rare population assessment most of the reportable CV percent is under 30 percent.
- **Fixed Stability:** 138 reportables were assessed with out pass /fail criteria. Due to rare population assessment most of the reportable CV percent is under 30 percent.
- **LLOQ:** Populations assessed for LLOQ were, CD45+, CD3+, CD14+, and CD15+. The lower limit of quantitation for CD45+ was 1155 gated events. The lower limit of quantitation for CD3+ was 84 gated events. The lower limit of quantitation for CD14+ was 33 gated events. The lower limit of quantitation for CD15+ was 2107.33 gated events
- **Conclusions:** The Cytek Aurora was able to provide high resolution data with the use of a combination of bead reference controls and cell reference controls, with a percent CV consistently below 30 percent for all major populations. This assay holds applicability for monitoring cellular phenotypes in human urine, providing relevant data on the mechanisms of action of novel therapies and facilitating the longitudinal evaluation of phenotypic changes in cellular composition and phenotype during therapeutic intervention

Reach out today to start a conversation in developing a complex and specialized flow cytometry panel.