A sensitive epigenetic immune cell counting assay for identity and purity testing of cellular therapies

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ABSTRACT

Manufacturing cell therapy products often rely on laborious cell isolation techniques that result in contaminating byproducts. In order to ensure product quality and consistency, thorough tests are performed throughout the manufacturing process and for product release. Characterization of a biological product includes determination of biological activity, identity, purity and impurities. Identity and purity assays commonly rely on cell surface markers or secreted molecules that are measured using flow cytometry. A critical challenge with flow cytometry is the need for live samples and lack of standardization due to biological, technical and operational variability. An appealing alternative that overcomes these constraints is the PureQuant assay, a cell type-specific, epigenetic qPCR based method that is sensitive, consistent and standardized.

PureQuant assays were evaluated in CAR-T cells using assays specific for T-cell specific CD3, CD4, CD8, and contaminating B cells and monocytes. The individual assays each comprise of a reference to check assay performance, calibrator that corrects for bisulfite conversion efficiencies and standards to deduce the exact copy number. The panel of five assays was used to determine the purity of multiple healthy donor-derived CAR-T cells generated using a second generation (CD3ζ and 4-1BB) anti-CD19 CAR lentivirus. Sensitivity to detect contaminating cells was further established by spike-in experiments with addition of purified B-cell and monocytes to CAR-T cells. These studies demonstrate that the combination of accuracy, low sample requirement and flexibility provides an ideal measurement system for confirmation of identity and purity critical for therapeutic applications.

INTRODUCTION

Current methods used for the characterization of T cell types and other immune cell products rely on flow cytometry. Additionally, characterization methods that utilize live cells pose a challenge in large scale GMP manufacturing environment due to complicated logistics, limited throughput and difficulties standardizing. These challenges highlight the need for alternative assay methods. The PureQuant methylation assays offers a standardized solution in determining the identity and purity of immune cell therapies. PureQuant utilizes unique methylation patterns which can be interrogated to determine cell types of interest.

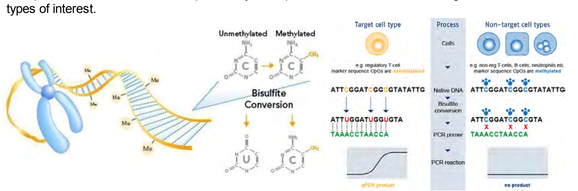


Image adapted from: www.epiontis.com (www.precisionmedicinegrp.com)

MATERIALS AND METHODS

PBMCs are isolated using the Ficoll method are a typical starting material for immune cell workflows. From the PBMCs, T cells, or other cell types can be enriched and manipulated or expanded. Following the PureQuant methylation assay user manual, gDNA extracted from cell samples is used as the input material, while live cells are used for flow cytometric analysis.

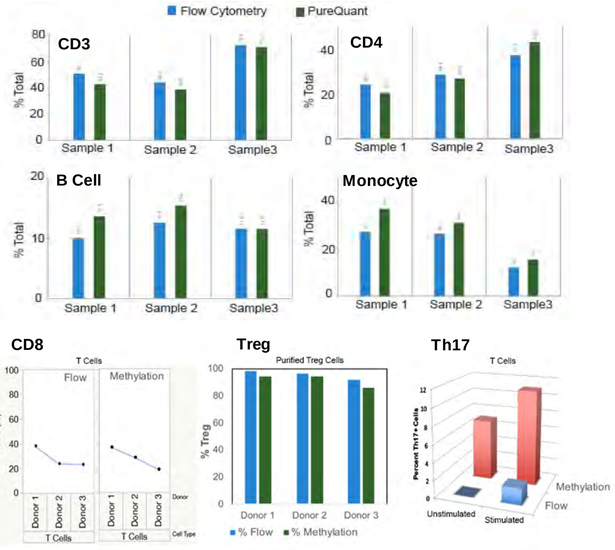
Calibrator: Corrects for qPCR assay specific performance differences - Plasmid-based calibrator harboring the genomic target regions of all qPCRs, including GAPDH as universal denominator, is subjected to bisulfite conversion followed by qPCR. This allows calculation of efficiency factor by dividing the TpG copy numbers for each cell type–specific assay by the GAPDH copy numbers measured from the same plasmid.

Reference: Checks for assay performance - A valid measurement of at least 2,000 GAPDH copies is needed, and the calibrator and reference should show values above the LoQ.

Standards: Helps determine the copy number - Standards diluted over 6 different concentrations are used to generate a standard curve, from which the slope & intercept are used to determine the copy number.

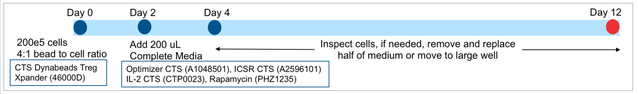
RESULTS

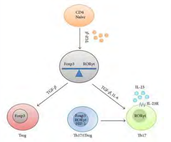
Figure 1. Comparison between Flow Cytometry and PureQuant



Three PBMC donors were used to assay for various sub cell types shown, except for Treg assay where purified Treg cells were used. Enriched T cells were used for CD8 and Th17. Live samples were used for flow cytometry analysis while cell pellets fresh or frozen was used for gDNA isolation and further analysis using PureQuant. Results for all assays with three different donors indicate comparable results. In the case of T17, Flow analysis requires upfront stimulation of cells which is not required for PureQuant assays,.

Figure 2. PureQuant as a reliable indicator of Treg Cells

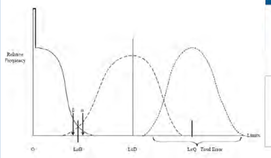




	PureQuant Assay			Flow
Sample	% CD8	% Treg	% Th17	% CD25+CD127-FoxP3+
Purified Treg		85.3		81.4
Expanded Treg	1.3	87.6	4.8	82.8

Purified Treg cells were expanded for 12 days using the CTS Dynabead Treg Xpander. Cells before and after expansion was analyzed for Treg positive cells via flow cytometry by measuring percentage of CD25+. CD1270 and FoxP3+ cells. PureQuant Treg assay was carried out in parallel and Th17 also measures to determine contaminating Th17 cells with expansion.

Figure 3. Analytical Performance of PureQuant Assays

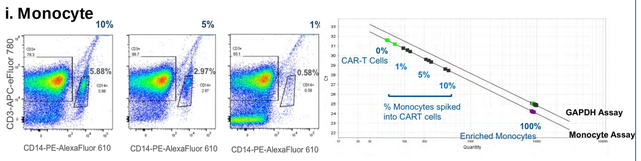


Control	Assay	LoB	LoD	LoQ
Calibrator A	GAPDH	0	7	21
	CD8	0	19	52
	FoxP3	0	3	12
	TpG	0	35	73
	CpG	0	38	73
Calibrator B	GAPDH*	0	21	55
	CD3*	0	6	17
	CD4	0	11	37
	B cell	0	7	22
	Monocyte	0	17	28

Limit of Blank (LoB) determines the lowest possible amount of analyte detected with 95% confidence there is no analyte present.. Limit of Detection (LoD) determines the lowest possible analyte to be a true detection with 95% confidence. The Limit of Quantification (LoQ) is the lowest amount of analyte which can be accurately quantified. LoQ is determined by the limit of detection.

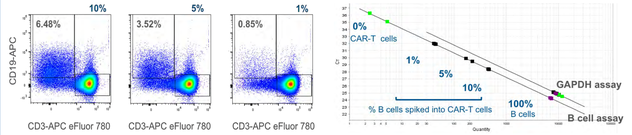
Figure 4. Sensitivity of Monocyte and B Cell contamination detection

i. Monocyte



Spike-in of Monocytes into 1M CAR-T		PureQuant		Flow cytometry
# Monocytes	% Monocytes	# copies	% Monocytes	% CD14+
100,000	10	404	3.09	5.88
50,000	5	232	1.78	2.97
10,000	1	110	0.85	0.58
CAR-T	0	60	0.49	2.80
Monocyte	>80	8758	74.28	83.00
Monocytes: LOB-0; LOD-17; LOQ-28				

ii. B cell



Spike-in of B cells into 1M CAR-T		PureQuant		Flow cytometry
# B cells	% B cells	# copies	% B cells	% CD19+
100,000	10	467	6.3	6.48
50,000	5	207	3.1	3.52
10,000	1	43	0.7	0.85
CAR-T	0	4	0.1	0.13
B Cells	>99	7,232	99	90
B Cell: LOB-0; LOD-7; LOQ-22				

Monocyte (i) or B cells (ii) were spiked into CAR-T cells and samples analyzed using flow cytometry or PureQuant assay. Flow analysis is represented as dot plots and PureQuant assay results are shown relative to the standard curve for that assay. Table summarizes the observation of spike-in experiments from Flow cytometry and PureQuant. Results indicate detection of as low as 1% cells by both methods

Figure 5. PureQuant assays for purity assessment of CAR-T cells

	Sample	Reference	Donor 1 CAR-T	Donor 6 CAR-T	Donor 7 CAR-T
T cell Sub sets	CD3	23.81	90.6	82.7	88.1
	CD4	16.16	54.6	51.9	49.9
	CD8	7.08	37.3	32.9	41.3

	Sample	Reference	Donor 1 CAR-T	Donor 6 CAR-T	Donor 7 CAR-T
Contaminating cells	B cell	4.73	0	0.1	0.1
	Monocyte	8.15	0.6	0.3	0.4

Three different CAR-T donors were prepared and analyzed via PureQuant Methylation assay for each target cell type. Low quantities of contaminating cell types (B-cell and monocytes) are present, while observing high pan T-cell and T-cell subsets.

SUMMARY & CONCLUSIONS

- PureQuant PCR based assay provides a complete solution with controls and references that can be carried out on any standard qPCR instrument.
- Assay outcome measures precise copy number which can be normalized to total copy number to determine % total cell type of interest.
- Results for all assays tested showed comparability between flow-based method and PureQuant assay
- Analytical performance and consistency confirms the robust nature of assay components, workflows and methods
- Contaminating cell types can be accurately detected ~1% without the need of gating strategies when taking a sample of immune cell prep
- Assays can be successfully performed on newly generated CAR-T cells and Tregs expanded using CTS Dynabeads Treg Xpander
- PureQuant assays provide a complementary orthogonal method to determine sample identify and purity of specific immune cell types in a heterogenous cell

TRADEMARKS/LICENSING