applied biosystems

HIGHER ACCURACY DETERMINATION OF IMMUNE CELL IDENTITY AND PURITY USING THE PUREQUANT REAL-TIME PCR ASSAY

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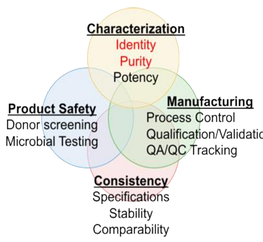
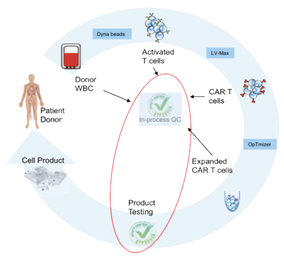
ABSTRACT

In the past several years significant improvement has been made in treating certain kinds of cancers by harnessing the power of immune system, in particular, T cells. One of the key challenges in developing immune cells as therapeutic agents is the accurate estimation of their identity and purity. Current methods used for characterization of immune cell types rely on flow cytometry. Flow cytometry can accurately estimate CD8+ T lymphocytes and other surface markers. However, this method is challenging to implement in a GMP manufacturing environment posing logistical challenges such as requirement for live cells, variability leading to difficult in standardizing and high throughput. In addition, cytometric methods are not accurate for specific intracellular targets that positively identify Regulatory T (Treg) cells and T Helper 17 (Th17) cells. Therefore, there is an emerging need for alternative assay methods. Epigenetic DNA methylation is known to be unique for specific cell types and can thus be used as an identifier in heterogeneous population of cells. Exploiting differences in cell type-specific methylation signatures, we developed assay kits that quantify the percentage of Treg and Th17 by detecting methylation status of FoxP3 and IL17A via qPCR of bisulfite converted genomic DNA. In contrast to flow analysis, sample requirement is minimal and the assay works well with fresh/frozen cells or genomic DNA. This assay has been implemented to accurately identify and estimate different T cell population in Chimeric Antigen Receptor (CAR)-modified T cells. The combination of accuracy, low sample requirement and flexibility provides an ideal measurement system for confirmation of identify and purity of T cell types specifically critical for therapeutic applications.

INTRODUCTION

Characterization of a biological product (includes determination of physiochemical properties, biological activity, immunochemical properties, purity and impurities) is necessary to allow relevant specifications to be established. The **identity** of the final biologic product must be verified by assays that will identify the product for proper labeling and will distinguish the product from other products being manufactured in the same facility (21 CFR 610.14). Examples: Cell surface markers , Gene expression, Secreted molecules, Peptide sequences.





While surface markers are useful, in cases where they are not definitive, epigenetic assays are useful. PureQuant Methylation Assays utilizes cell type specific methylation patterns to identify cell types of interest in heterogeneous populations. Ammonium bisulfite converts unmethylated cytosine to uracil indiscriminately through out the entire genome. Primer pairs are specifically designed to pair within target genes and the newly converted uracil bases. The sequence with un-methylated cytosine will not bind these primers and consequently will not have amplification during the qPCR reaction.

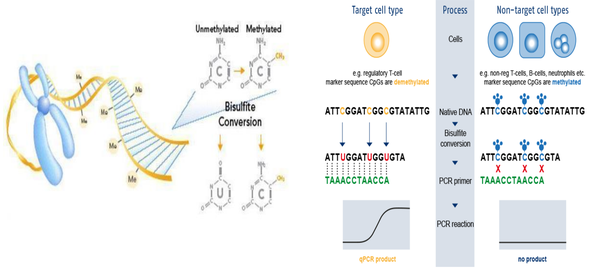
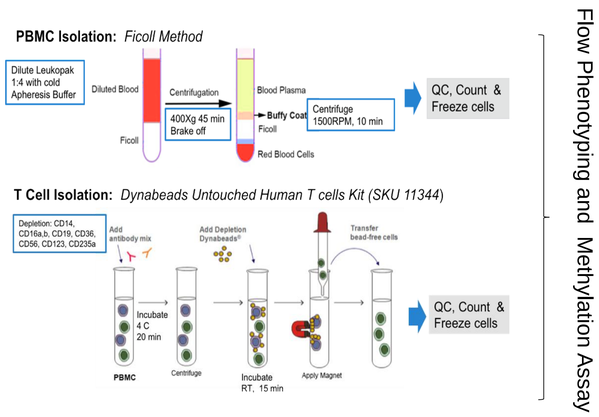


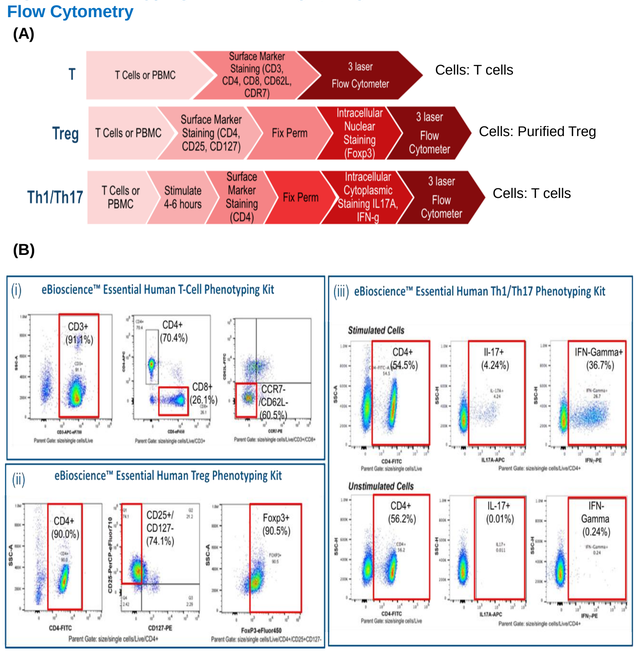
Image adapted from: www.epiontis.com (www.precisionmedicinegrp.com)

MATERIALS AND METHODS



RESULTS

Figure 1. Phenotyping of Cells Using T, Treg and Th1/Th17 Panels with Flow Cytometry

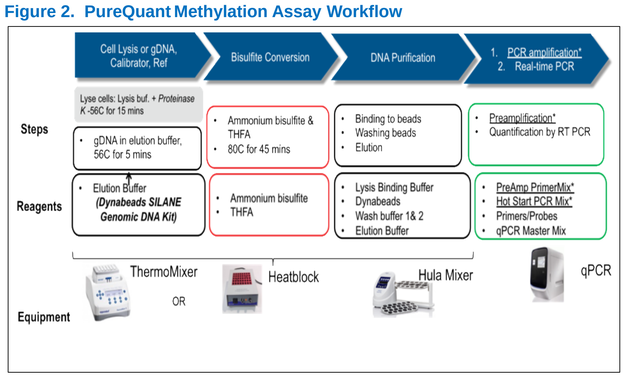


Flow cytometry is the predominant method used to determine identify and purity of immune cells.

(A) Workflow to determine the percent of CD8, Treg and Th1/Th17 positive cells in human T cells

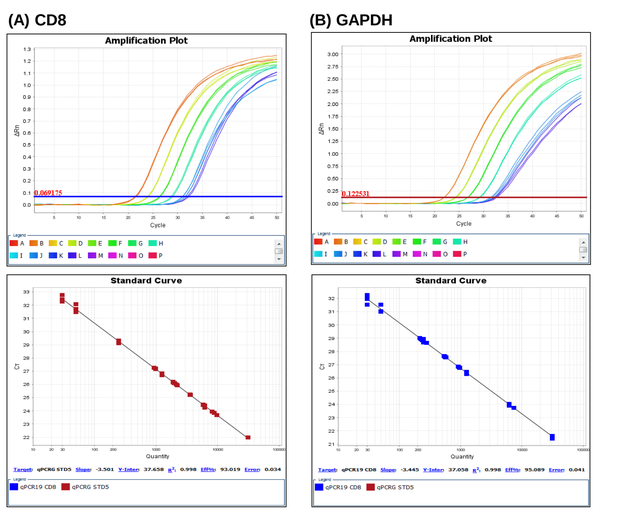
(B) Representative phenotyping data of human T cells stained with validated antibody panels and gated for live and FMO/isotype controls,

Figure 2. PureQuant Methylation Assay Workflow



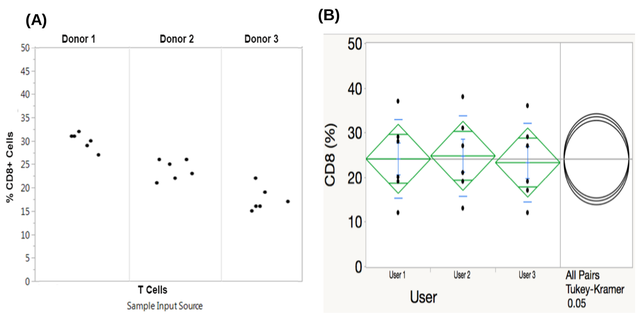
PureQuant methylation assay comprises of 4 key steps starting from cells or isolated genomic DNA. All necessary controls such as calibrator and reference are provided to create standard curves to extract copy number which is normalized to total copy number (GAPDH) to determine percentage of cell type of interest.

Figure 3. Representative Standard Curve Used To Estimate Copy Number



Linear plot amplification curve produced by the standards for CD8 (A) and GAPDH (B). A linear regression is produced using the QuantStudio software. This curve is then used to estimate the copy numbers present within the sample based on the average Ct of three technical replicates. The standard curve is further qualified under strict parameters which access the slope, R2 and primer efficiency.

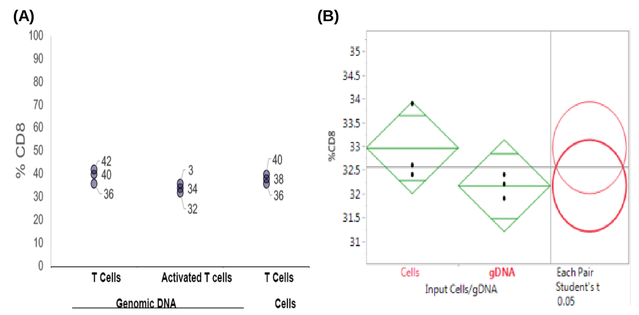
Figure 4. Determination of CD8+ Cells in Multiple Donor T Cells



(A) PureQuant CD8 Methylation assay was carried out with genomic DNA isolated from T cells from three different donors. Multiple technical and biological replicates was carried out for each donors. The results were expressed as % CD8+ cells.

(B) To determine the robustness of the assay, three independent users carried out the assay and the results were consistent as determined by statistical analysis using JMP

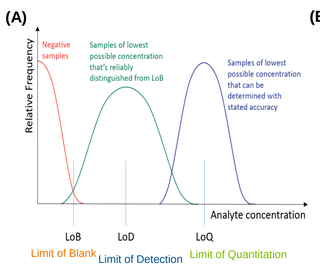
Figure 5. Cells or Isolated Genomic DNA as Sample Source



(A) Cells in the range of 1-2 million or isolated genomic DNA (400-1200 ng) can be used in the Methylation assay workflow as shown with CD8 assay

(B) The input sample of genomic DNA or cells in the recommended range does not have statistically significant impact on assay performance

Figure 6. Analytical Performance of PureQuant Methylation Assays



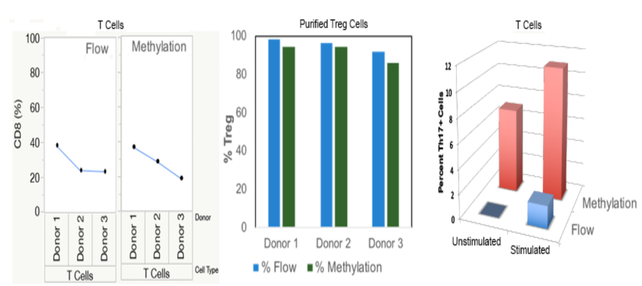
(B)

Assay	LoB	LoD	LoQ
CD8	0	19	52
GAPDH	0	7	21
FoxP3	0	3	12
Th17			
TpG	0	35	73
CpG	0	38	73

(A) It is important to fully characterize the analytical performance of tests to understand their capability and limitations, and to ensure that they are "fit for purpose." The terms LoB, LoD, and LoQ describe the smallest concentration of a measurand that can be reliably measured by an analytical procedure

(B) Standard DNA was prepared as per protocol. DNA serially diluted (40, 20, 10, 5 copies and blank) using diluent (TE + 10 ng/uL Lambda DNA). 10 replicates was included for each data point and LoB, LoD , LoQ calculated according to the standard equations

Figure 7. Comparison of Methylation and Flow Assays for CD8, Treg and Th17

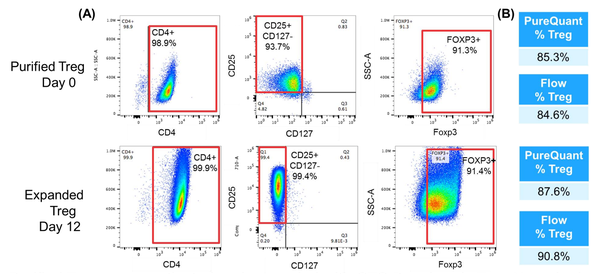


(A) CD8 assays are comparable for Flow panels and Methylation Assay

(B) Treg assay tends to shows higher degree of variability in results between Flow panels and methylation assay since Flow measures transient expression of FoxP3 upregulated during activation of T cells

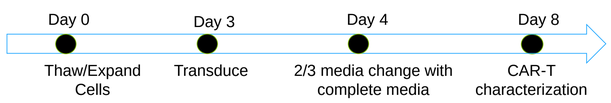
(C) Th17 assay is cumbersome with Flow panels requiring pre-stimulation while Methylation does not require this step

Figure 8. Flow phenotyping and PureQuant Methylation Assays performed on Tregs expanded using CTS Dynabeads Treg Xpander



Purified Tregs were activated and expanded with CTS Dynabeads Treg Xpander in CTS Optimizer supplemented with CTS Immune Cell Serum Replacement, and CTS IL-2. Cells were analyzed at Day 0 and Day 12. The Treg percentage was assessed using eBioscience Essential Treg Phenotyping Kit and CTS PureQuant Methylation Treg Assay. (A) Flow plots were generated using the eBioscience Essential Treg Phenotyping Kit (B) Treg percentage of the total population for both flow and Methylation. % Treg was calculated by multiplying the percentages of CD4+, CD25+/CD127- and FOXP3+ cells to compare to methylation.

Figure 9- PureQuant Methylation Assays performed on CAR-T Cells



Sample	% CD8+	% Treg	% Th17
Untransduced	63.4	0.1	0.4
CAR-T (ICSR)	59.4	0.3	5.5
CAR-T (HuS)	57.0	0.1	5.2

CAR-T cells were generated in house and PureQuant Methylation assay was performed alongside untransduced control cells. CD8, Treg and Th17 PureQuant Methylation Assays were successfully performed using reference DNA, matched Untransduced T-Cells, and CAR-T Cells

SUMMARY & CONCLUSIONS

- **Essential human flow phenotyping kits**
  - Validates assays to phenotype CD8+ T cells, Treg and Th1/Th17 cells was designed for easy and consistency across users and Flow instruments with standard 3 laser configuration.
  - Isotype controls for each kit can be used for more accurate analysis by using a combination of including FMO and isotype controls.
- **PureQuant Methylation Assay**
  - PCR based assay provides complete solution with controls and references that can be carried out on any standard qPCR instrument
  - Assay outcome measure precise copy number which can be normalized to total copy number to determine % total cell types of interest.
  - Requirement of cells or isolated genomic DNA as the starting material offers flexibility and also allows for easier logistics, smaller cell sample requirement and ability to scale up while maintaining standardized procedures and methods.
  - Analytical performance and consistency of the CD8+ T cells, Treg and Th17 assays further confirm the robust nature of assay components, workflows and methods
  - The assay was successfully implemented in reliably identifying and quantifying CD8+ T cells, regulator T cells and Th17 cells in CAR-T prep.
- **Comparison of Flow Phenotyping and Methylation Assays**
  - PureQuant Methylation assays provide a complementary orthogonal method to determine sample identify and purity of specific immune cells type in a heterogenous mixture of cells
  - While Flow cytometry offers a simple means to assess surface markers, this is more challenging with intracellular and low abundance markers such as Treg and Th17. The logistics of using live cells and in some cases fixed cells can introduce variability with extended testing times and higher sample requirement

In summary, PureQuant Methylation assay offers an appealing alternate for hard to phenotype cell types such as Treg and Th17 positive immune cell types. The can be adopted for both in-process and release testing for Research use as well as Cell and Gene Therapy Manufacturing applications.

TRADEMARKS/LICENSING

For Research Use Only. Not for use in diagnostic procedures.