A novel qPCR Method to Count CD4+ T helper Cells Using Dried Blood

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Introduction

The CD4+ T-helper cell quantification in people living with HIV (PLWH) is important to identify those suffering from advanced disease (<200 CD4 cells/µl) at antiretroviral therapy initiation and during monitoring[1]. Flow cytometric quantification, the gold standard technology for immune cell quantification, requires fresh blood which is challenging especially in resource-limited settings. In those areas, dried blood spots (DBS) of capillary blood have been implemented for HIV viral load testing, but not for CD4 counting yet[2]. Here, epigenetic quantification of CD4+ T-helper cells from dried blood spots or venous whole blood is demonstrated to overcome this obstacle.

Method: Epigenetic Real-Time PCR for the Quantification of CD4+ T-helper cells

To overcome current technological and diagnostic limitations, the principle of DNA unmethylation-based, quantitative real-time PCR assays of immune cells (epigenetic qPCR) is used.[3] This method provides relative and absolute counts of CD4+T-helper cells applicable to fresh or frozen EDTA whole blood or dried blood spots.

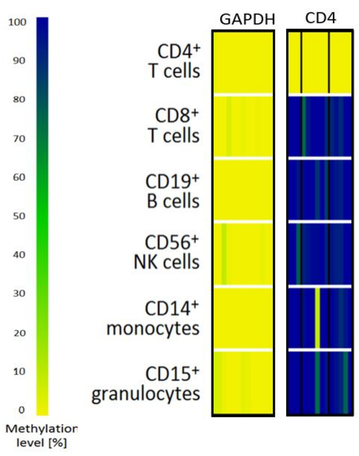
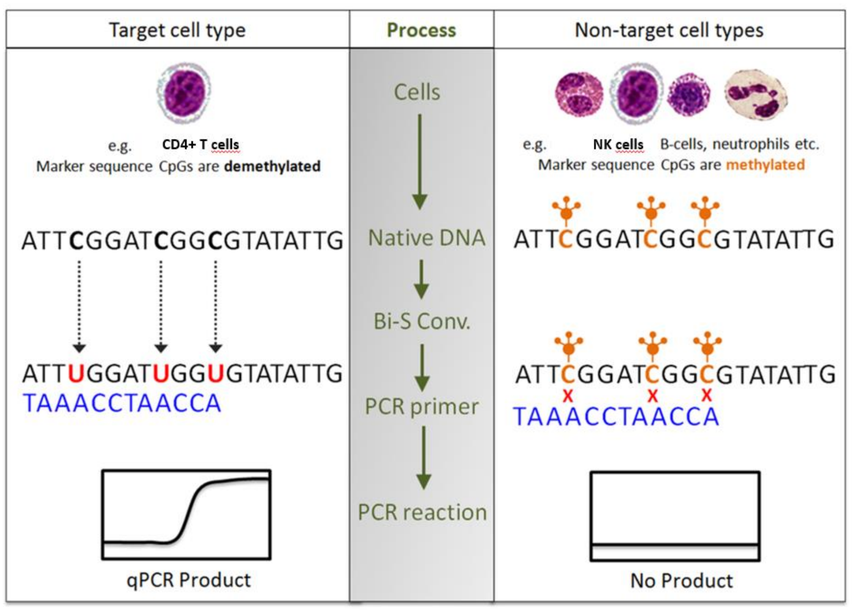


Figure 1: Identification of unmethylated genomic regions specific for CD4+ T-helper cells (CD4 locus) and leukocytes (GAPDH locus) in purified immune cell types. Gene loci of interest are arranged in columns and immune cell types, isolated from healthy donors, are arranged in rows. Each individual line of each loci represents a single CpG site. Methylation rate of each CpG is color-coded according to the scale.[3]

Figure 2: Development of unmethylation-specific real-time PCRs for the quantification of immune cells. DNA-methylation pattern is transferred into the DNA sequence by bisulfite conversion. Primers and probes match only converted, unmethylated target sequence. Methylation of CpGs in non-target cell types prevents conversion and, therefore, non-target cells are not detected. In parallel, the housekeeping gene GAPDH is analyzed. [3,4]

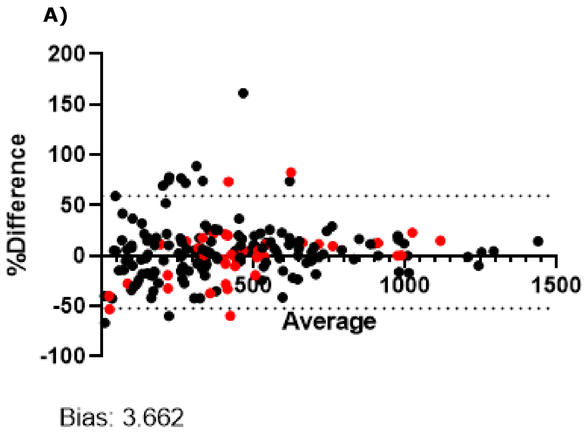


Correction Equation and Bland-Altman Analysis

For each PCR instrument and sample material, a correction equation was defined to reduce the bias between epigenetic and flow cytometric results. The bias between the two technologies was reduced to <5% under all conditions.

Figure 4: A) Exemplary presentation of the Bland-Altman analysis for DBS cards using Roche LC 480. Data points in red are from HIV patients. Black dots are data points from patients with divers immunological deficiency. B) Summary of Bias and Standard deviation (SD) per PCR instrument using liquid whole blood and C) DBS samples.

A)



B)

Liquid whole blood	Leukocytes		CD4/µl		CD4%	
	Bias	SD of Bias	Bias	SD of Bias	Bias	SD of Bias
LightCycler 480	1.577	18.24	4.011	29.52	3.017	24.39
LightCycler Pro	1.494	17.9	4.435	31.01	3.248	25.89
QuantStudio6	1.8	19.54	5.6	34.59	4.279	29.4

C)

DBS	Leukocytes		CD4/µl		CD4%	
	Bias	SD of Bias	Bias	SD of Bias	Bias	SD of Bias
LightCycler 480	0.8026	12.8	3.662	28.4	3.337	26.35
LightCycler Pro	0.9149	13.56	4.475	31.8	4.176	29.75
QuantStudio6	0.8393	13.16	3.944	29.82	3.55	26.65

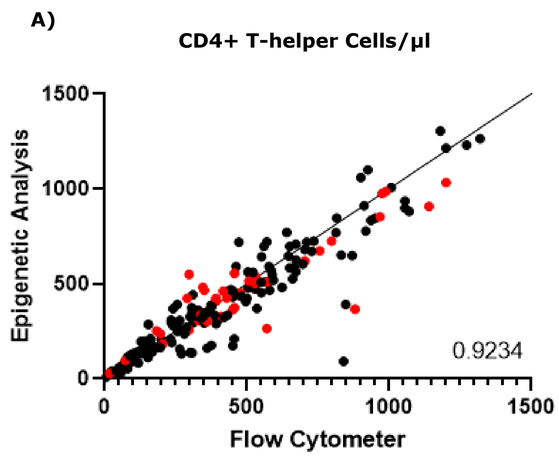
Results:

Correlation between Epigenetic PCR and Flow Cytometry

EDTA whole blood samples from 200 patients with diverse hematological diseases and conditions (e.g. HIV, PID or stem cell transplanted patients) were collected at Uniklinikum Leipzig. For each patient, 100 µl liquid EDTA whole blood and one DBS spot (~75µl) on Whatman paper were collected (left over material of routine blood collection). All samples were analysed on the LightCycler (LC) 480, LightCycler (LC) Pro and QuantStudio 6 Flex and results were compared to flow cytometric data. For both substrates, a significant Spearman's correlation factor of >0.9 was determined when comparing flow cytometric and epigenetic CD4+ T cell data on all three devices.

Figure 3: A) Exemplary presentation of the Spearman's correlation comparing epigenetic results of DBS cards using Roche LC 480 with flow cytometric data. Data points in red are from samples from HIV patients. Black dots are data points from patients with divers immunological deficiencies. **B)** Summary of Spearman's rho per PCR instrument using liquid whole blood and **C)** DBS samples.

A)



B)

Liquid whole blood	Leukocytes	CD4/µl	CD4%
LightCycler 480	0.9197	0.9216	0.9654
LightCycler Pro	0.9231	0.9181	0.9614
QuantStudio6	0.9117	0.9063	0.9338

C)

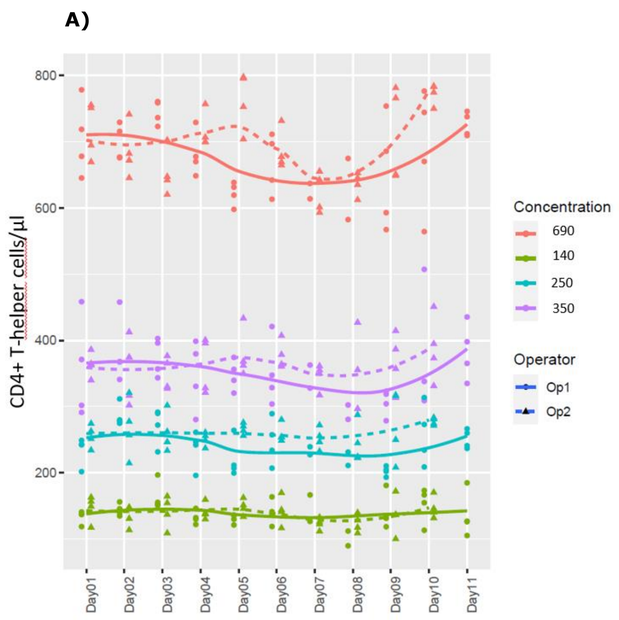
DBS	Leukocytes	CD4/µl	CD4%
LightCycler 480	0.9517	0.9235	0.9546
LightCycler Pro	0.9447	0.9109	0.9503
QuantStudio6	0.9484	0.9197	0.9497

Precision Measurement (CLSI EP05-A3)

To asses the repeatability and reproducibility of this test method, two operators performed two experiments on 10 days with two replicates of four samples with defined CD4+T-helper cell count for liquid whole blood and DBS. For liquid blood sample <200 CD4 cells/µl, CVs ≤ 15% were reached. The precision when using DBS cards is slightly lower but is less than 21% at about 100 CD4 cells/µl.

Figure 4: A) Exemplary presentation of the data points over the time from the single-site study for liquid blood samples with approx. 140 (green), 250 (turquoise), 350 (purple) and 690 (red) CD4+ T-helper cells/µl using Roche LC 480 performed by two operators. **B)** Summary of the coefficient of variations (CV) according to the cell counts and PCR devices using liquid whole blood and **C)** DBS.

A)



B)

	Liquid Whole Blood			
CV [%]	140 CD4/µl	250 CD4/µl	350 CD4/µl	690 CD4/µl
LightCycler 480	12%	11%	9%	10%
LightCycler Pro	12%	12%	10%	10%
QuantStudio6	15%	13%	13%	9%

C)

	DBS			
CV [%]	100 CD4/µl	220 CD4/µl	420 CD4/µl	610 CD4/µl
LightCycler 480	17%	15%	15%	12%
LightCycler Pro	17%	13%	14%	15%
QuantStudio6	21%	15%	16%	14%

Conclusion

We have demonstrated that our novel epigenetic qPCR assay accurately quantifies CD4+ T helper cells with minimal bias to the standard of care, flow cytometry. The ability to use dried blood may greatly simplify the determination and monitoring of CD4+ T cell counts in PLWH, especially when combined with viral load testing on dried blood. We are currently developing the assay under the in vitro diagnostic regulation (IVDR) and will complete analytical validation in the near future. Clinical validation of the assay is planned in South Africa.

References

[1]Boyd et al., Addressing advanced HIV disease and mortality i global HIV programming. AIDS Res Ther. 2020 Jul 10;17(1):40

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[3]Baron et al., Epigenetic immune cell counting in human blood samples for immunodiagnostics. Sci. Transl. Med. 2018 Aug; 10 –pp1-11

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