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# Specifications of qPCR based epigenetic immune cell quantification

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**Abstract**

**Objectives:** Immune monitoring is an important aspect in diagnostics and clinical trials for patients with compromised immune systems. Flow cytometry is the standard method for immune cell counting but faces limitations. Best practice guidelines are available, but lack of standardization complicates compliance with e.g., *in vitro* diagnostic regulations. Limited sample availability forces immune monitoring to predominantly use population-based reference intervals. Epigenetic qPCR has evolved as alternative with broad applicability and low logistical demands. Analytical performance specifications (APS) have been defined for qPCR in several regulated fields including testing of genetically modified organisms or vector-shedding.

**Methods:** APS were characterized using five epigenetic qPCR-based assays quantifying $CD3^+$, $CD4^+$, $CD8^+$ T, B and NK cells in light of regulatory requirements.

**Results:** Epigenetic qPCR meets all specifications including bias, variability, linearity, ruggedness and sample stability as suggested by pertinent guidelines and regulations. The assays were subsequently applied to capillary blood from 25 normal donors over a 28-day period. Index of individuality (IoI) and reference change values were determined to evaluate potential diagnostic gains of individual reference intervals. Analysis of the IoI suggests benefits for individual over population-based references. Reference change values (RCVs) show that changes of approx. Fifty percent from prior measurement are suggestive for clinically relevant changes in any of the 5 cell types.

**Conclusions:** The demonstrated precision, long-term stability and obtained RCVs render epigenetic cell counting a promising tool for immune monitoring in clinical trials and diagnosis.

**Keywords:** epigenetics; immune monitoring; qPCR validation

## Introduction

The cellular composition of the human immune system ranges within defined physiological boundaries. Deviations of immune cell frequencies are an indicator for diseases, including immune deficiencies and cancer. Secondary immune disorders are caused by exogeneous influences, including infections with human immunodeficiency virus (HIV). Hematologic cancers lead to uncontrolled growth of myeloid or lymphoid cells. In addition to permanent deviations from physiological immune cell frequencies, reversible changes mark the physiological response to infections. Due to the general role of immune cells and their wide physiological range [1], their counting is rarely a stand-alone diagnostic tool, but rather supports diagnostics and monitoring.

In addition to biological significance, the value of biomarker data depends on accessibility of samples, analytical performance specifications (APS) and quality of data, including acceptable precision (coefficients of variation, CV) and bias (deviation), lower limit of quantification (LLoQ), assay ranges, reproducibility and ruggedness as well as availability of meaningful references [2]. While industry standards vary between assay types, they typically require that CVs are below 15 % for intra- and 20 % for inter-assay CV. Replicate CVs are considered acceptable if below 25 %. Reproducibility and ruggedness need to be fit for purpose which differs between various fields, such as between testing vector shedding [3] or genetically modified organisms [4].

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Flow cytometry is the gold standard for immune monitoring. However, it requires cumbersome sample management and logistically demanding procedures, since only intact suspension cells are feasible for analysis. This and a continuing lack of method standardization across laboratories with respect to protocols, reagents and gating strategies lead to an absence of normative reference data [5]. Moreover, technical requirements pose limitations for diagnostic procedures: Newborn screening is performed from dried blood spots (DBS) [6], and not compatible with flow cytometry. Similarly, DBS are suitable for viral load measurements, e.g., in rural areas [7], but not for flow cytometric cell counting. Hematological cancers are rare and flow cytometric screening is economically not feasible [8]. Moreover, for solid tumors or autoimmune diseases, quantification of tissue-infiltrating immune cells is diagnostically relevant and cannot be assessed by flow cytometry.

DNA methylation analysis has shown promise as alternative tool for immune cell quantification [9, 10]. Its mode of function is illustrated in Figure 1.

The underlying qPCR is based on amplification of bisulfite converted, single-stranded DNA, where unmethylated cytosines are changed to uracil. During amplification, uracil basepairs with adenine and is replaced by thymine in daughter strands. This process retains previously methylated CpG-sites but converts unmethylated CpG to TpG, translating methylation information into primary sequences. Standard conditions for epigenetic qPCR allow bisulfite-treatment directly on lysed cells. Differentially methylated CpG-sites serve as immune cell type-specific biomarkers. For example, a regulatory element of the CD4 gene is specifically unmethylated in $CD3^+CD4^+$ T cells, whereas methylated in other peripheral blood cells. Similar observations were reported for regulatory elements in CD3D/CD3G (CD3) or CD8B (CD8) genes in total $CD3^+$ T cells

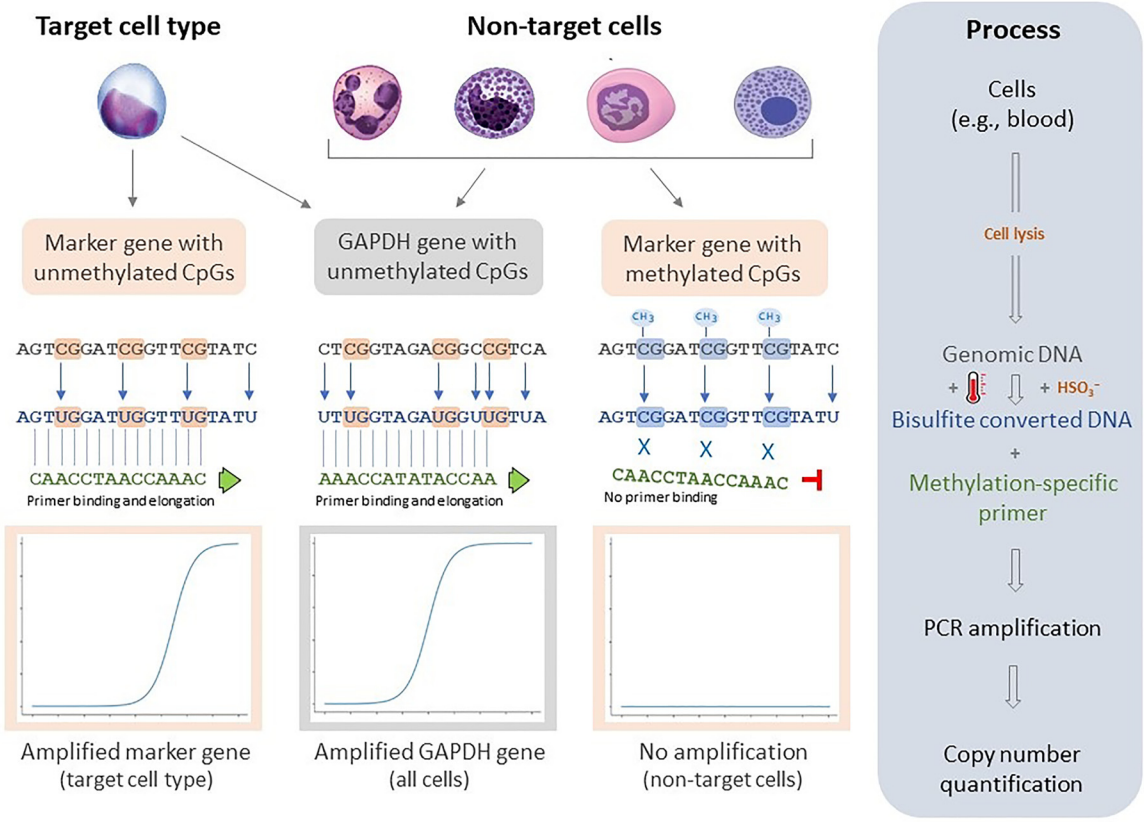


**Figure 1:** Principle of cell counting by epigenetic qPCR. Gene loci with cell type specifically unmethylated CpGs are used for analysis. Upon cell lysis, genomic DNA is chemically treated with bisulfite. In this reaction, unmethylated cytosine is converted to uracil (and then, in PCR reactions, replaced by thymine (T)), whereas methylated cytosine (C) remains unchanged. Methylated cytosines occur exclusively in the dinucleotide context of CpG. By means of the described chemical treatment, the differential methylation is translated into primary sequence information. Using oligonucleotides that base-pair with converted UpG, only the marker gene in the target cell type is PCR amplified. The corresponding sequence in non-target cells (i.e., with unconverted CpGs) is not amplified. In order to amplify DNA from all cell, a locus in the GAPDH gene is used, which is constitutively unmethylated. This latter epigenetic marker serves as reference to determine the copy number of all DNAs and thus to quantify all cells in a sample.

and $CD3^+CD8^+$ T cells, respectively [11, 12]. Cell type specific methylation imprints have also been identified for $CD19^+$ B and $CD56^+$ NK cells [10] in genes encoding for the lipoprotein receptor–related protein 5 (LRP5), a Wnt co-receptor for bone mass regulation [13], and mevalonate diphosphate decarboxylase (MVD) converting mevalonate pyrophosphate into isopentenyl pyrophosphate playing a role in the isoprenoid and vitamin K synthesis, respectively [14]. With these markers, epigenetic immune cell counting shows substantial equivalence with corresponding data from flow cytometry. While cell type specificity for each marker has been validated extensively in blood, the method is in principle applicable to all body fluids [15] and tissues [16]. However, cell type specificity has to be verified for each tissue.

DNA as substrate for qPCR-based methods is undemanding in terms of storage and transportation. Short PCR products (90–120 base pairs) render the process stable and make cell quantification feasible from various matrices including dried blood spots [10], even when DNA is fragmented. However, to monitor stable performance, qPCR systems require negative (no-template) controls, positive controls and biological references. Complexity is increased by additional controls for bisulfite-conversion and normalization between single-stranded sample DNA and double-stranded plasmid DNA used as quantification standard. Beyond those technical aspects, result interpretation depends on the use of optimal references, i.e., population-reference intervals (RI) or a donor's previous result [17]. Often, population-based RIs are not sensitive enough to identify significant changes in individuals [18]. The index of individuality (IoI), i.e., the ratio between intra- and inter-subject variation, assesses the usefulness of a population-based RI. If inter-subject variations become too large, intra-subject variations are more applicable and the reference change value (RCV) assesses whether observed changes are unusual, i.e., are significantly different from previous measurements in a patient [19]. For standard immune monitoring, population-based RIs are used, despite wide normal ranges [1], and immune monitoring may gain resolution when using intra-individual changes.

Here, we assessed technical parameters of epigenetic qPCR and its suitability for diagnostic and clinical analysis. We determined qPCR precision upon analysis of intra- and inter-assay CVs and evaluated bias using deviation between observed and expected copies. A range of artificially generated experimental conditions with different biological samples was used to show robustness of the technical approach. EDTA-anticoagulated human blood was analyzed under deliberately varied reaction conditions. Then, measurement stability was investigated in human blood in various collection tubes containing different anti-coagulants or from dried blood spots (DBS). Finally, in a longitudinal study, fingerprick blood was sampled daily from normal subjects for 28 days followed by counting unmethylated CD3, CD4, CD8, LRP5 and MVD loci to assess value of population-based or individual-based reference values.

## Materials and methods

Peripheral blood, DBS and urine were obtained from healthy volunteers with written consent and approval (Ruhr-Universität Bochum (20–6886) and Charité-Universitätsmedizin Berlin: (EA2/028/13, EA2/029/13)). $K_3$EDTA-, citrate-dextrose or heparin-anticoagulated blood showed mean deviations below 7.4 % compared to $K_2$EDTA-anticoagulated tubes indicating minor, non-significant matrix-dependent differences (Supplementary Material). Blood is the primary target of immune cell analyses, but other organs and tissues are also of interest due to the role of tissue infiltrating lymphocytes (TiLs) [20]. Urine, in contrast, may serve as substrate for non-invasive analysis of kidney damage or, in transplant settings, as marker for rejection [15, 21].

### qPCR

Biological specificity of each epigenetic qPCR assay was verified during assay validation in blood and was described previously [9, 10, 12]. The assays are highly specific for the detection of each target cell population and show no or negligible cross-reactivity with other leukocyte populations. For other tissues, specificity of the markers must be verified individually. Oligonucleotides were purchased from Eurofins GmbH (Supplementary Material). Sequences of *in silico*-converted markers (standard) and genomic marker genes (calibrator) were inserted into plasmid pUC57 (GenScript), quantified, linearized and diluted in λ-phage DNA (10 ng/mL; New England Biolabs) obtaining 31250, 6250, 1250, 250 and 50 copies in the final reaction. Calibrator was bisulfite-converted parallel with samples. Copy number of locus-specific unmethylated amplicons from calibrator plasmid was divided by the copy number of unmethylated GAPDH copies. These quotients served to equalize amplification efficiencies between GAPDH and specific assays [10]. 75 µL sample was lysed using 67 µL Lysis solution (Dynabeads SILANE Genomic DNA Kit, Invitrogen). For urine, this step was performed after centrifugation at 16,000 g for 3 min and resuspension in 75 µL PBS. For DBS, three 3.2 mm punches were given to 68.75 µL lysis buffer. All samples were incubated at 56 °C for 60 min. DNA conversion was performed at 80 °C for 55 min with 270 µL bisulfite solution and 90 µl THFA (Merck, Germany). DNA purification was performed following Dynabeads manual. Thermal cycling was as follows: 1 × 95 °C for 35 min, followed by 50 × 95 °C for 15 s, 61 °C for 1 min in 5 µl using appropriate oligonucleotides (Supplementary Material) and Roche 2xLightCycler480 Probes Master and cycler. Samples were analyzed in triplicates. Crossing points (Cps) were computed by the second-derivative maximum method and converted to unmethylated copies (plasmid units) from the 5-point standard regression curve. The regression slope and coefficient of determination ($R^2$) are used to assess quality of the fit. To calculate the percentage of cell type-specific loci relative to unmethylated GAPDH DNA copies, a resampling scheme

was used. All copies of cell type specific marker-replicates were paired with all copy numbers of GAPDH replicates. In each combination the ratio of cell type-specific to GAPDH values are averaged and corrected by the resampled calibrator.

### 3-Level quality control (QC)

First, replicate CV for standard dilutions, references and calibrators must be below 25 %. For standards, Cp must be within a 3× standard deviation (SD) and deviation below 12 % from the expected value. No-template controls must measure above Cp 33 or 50 (see below). Calibrators must not deviate more than 21 % from mean control chart count. Second, the standard curve requires 3 of 5 valid standard points. Linear regression must result in amplification efficiency >1.98 and $R^2$>0.980. 2/3 of 6 no-template controls must measure Cp>50, the remaining 1/3 may measure between 33 and 50. 1/2 of references and all calibrators must pass QC. Third, standards, references, calibrators and no-template controls must be valid according to level 2. If level 3 is passed, the assay on the analysed plate is valid. Samples with replicate CV<25 % and above LLoQ are valid. Replicate measurements with 25 % <CV<40 % are considered non-compliant.

### Statistics

Deviation is expressed as relative percent difference to the expected value calculated as (x^ – x_0)/x_0*100[%]. x_0 represents expected and x^ observed value. CV is defined as SD to mean ratio. Intra-assay CV was obtained from averaged replicates of each input level in one experiment. Inter-assay CV was obtained from different runs of averaged observations per run. Amplification efficiency and slope are extracted from LightCycler480II software. LLoQ is expressed as Cp and determined based on five measurements of four blood dilutions. Their intra- and inter-assay CVs and deviation were used to fit an exponential curve (y=c*xb) where c and b are parameters estimated by curve fit. The observed Cp is considered x and y is the observed assay CV. The fitted curve was used to calculate a Cp of 15 and 20 % for intra- and inter-assay CVs and 15 % deviation. The corresponding Cps were considered LLoQ candidates with the largest being the final LLoQ. Based on back-calculation with the standard curve, LLoQ is given as copy number. Statistical analyses were performed using R (4.2.2). Kwiatkowski–Phillips–Schmidt–Shin and Augmented–Dickey–Fuller tests were used for time series analysis.

## Results

A plasmid containing in silico converted sequences for the CD3, CD4, CD8, LRP5, MVD and GAPDH loci (standard) was measured in a fivefold dilution series ranging from 31,250 to 50 plasmid copies. The mean amplification slopes were between −3.32 (LRP5) and −3.41 (MVD). Mean amplification efficiency was between 1.97 (MVD) and 2.00 (LRP5) and mean $R^2$ was above 0.997 for all assays. A second plasmid, containing native genomic sequences of all measured loci (calibrator) was bisulfite-converted in parallel to samples and accounted for differences between *in silico*-converted standard and *in vitro*-converted biological DNA. It served both as control for bisulfite-conversion and to normalize copy numbers between the different cell type-specific unmethylated loci and constitutively unmethylated GAPDH (Table 1). EDTA-anticoagulated blood was diluted to characterize qPCRs by determining CV values and linearity of measurements (bias). The undiluted sample showed mean relative counts of unmethylated CD3, CD4, CD8, LRP5 and MVD of 27.2, 11.8, 3.2, 3.0 and 6.0 % relative to unmethylated GAPDH. Then, the sample was diluted with PBS at a stable total volume followed by qPCR. Compared to standard APS, CD3 and LRP5 assays passed criteria for precision (CV) and linearity (deviation) for all dilutions. For measurements of diluted blood with other markers, APS were met down to 1:9 dilutions. The lowest estimated concentration determined by regression analysis with all APS fulfilled, reflects the LLoQ. Crossing points (Cp) of qPCR analysis for LLoQs were 34.35 for CD3, 33.66 for CD4, 32.15 for CD8, 33.87 for LRP5 and 30.40 cycles for MVD. Back-calculation from Cp into copy numbers was based on estimated diluted standard values and yielded LLoQs of 12, 9, 16, 12 and 87 copies for the respective markers (Table 2).

To test robustness of bisulfite conversion, protocol conditions were deliberately varied. Bisulfite concentration, incubation time and temperature were changed in independent experiments. EDTA-anticoagulated whole blood was analysed by qPCRs targeting unmethylated GAPDH and CD3. For GAPDH, Cp was stable between 3.8 mol/L (Cp=23.78) and 3.3 mol/L (Cp=23.79) but shifted at lower concentrations (2.7 mol/L; Cp=23.91) and (2.2 mol/L; Cp=23.96). For CD3, stable Cps (26.52 and 26.50) were observed for the two higher concentrations, but shifted at lower concentrations to 26.56 (2.7 mol/L) and 26.69 (2.2 mol/L). Calculated percentages of CD3 copies from incubations with 3.8 mol/L and 3.3 mol/L measured within range of normal intra- and inter-assay CVs. For lower bisulfite concentrations, CV and deviation exceeded acceptable levels. Cytosine deamination temperature was changed from 80 to 85 °C, 75 °C or 65 °C keeping other conditions stable. Amplification showed Cps between 23.7 and 23.9 for GAPDH and 26.5 and 26.7 for CD3 and CVs and deviations within range of repeat measurements. Only incubation at 95 °C led to shifting Cp for GAPDH (1.1) and CD3 (0.8). Deamination between 35 and 55 min resulted in small effects on amplification and relative CD3 counts. Incubation for 25 and 65 min led to changing CD3 counts indicating loss of stability. Thermal desulfonation yielded stable results between 25 and 60 min, but Cps for GAPDH and CD3 were higher at 15 min (Table 3). Performance

**Table 1:** qPCR performance of *in silico* converted plasmid standard dilutions and *in vitro* converted calibrator.

| | | *In silico* converted plasmid-based standard | | | | | | | | | | Calibrator plasmid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean crossing points (standard deviation) | | | | | Slope of calibration curve | | Amplification efficiency | | Coefficient of determination | | Ratio of cell type-specific locus copies to GAPDH copies (standard deviation) |
| Marker gene | No. of experiments | 31,250 copies | 6,250 copies | 1,250 copies | 250 copies | 50 copies | Mean | Range | Mean | Range | Mean | Range | |
| Unmethylated GAPDH | 5 | 22.9 (0.3) | 25.3 (0.3) | 27.6 (0.3) | 30.0 (0.3) | 32.2 (0.1) | −3.34 | −3.50 to −3.15 | 1.99 | 1.93 to 2.06 | 0.997 | 0.992 to 0.999 | n.a. |
| Unmethylated CD3D/G | 3 | 23.7 (0.5) | 26.0 (0.3) | 28.8 (0.3) | 30.8 (0.3) | 33.1 (0.3) | −3.35 | −3.44 to −3.25 | 1.98 | 1.92 to 2.06 | 0.999 | 0.998 to 0.999 | 1.20 (0.09) |
| Unmethylated CD4 | 3 | 22.3 (0.1) | 24.8 (0.2) | 26.9 (0.2) | 29.3 (0.2) | 31.3 (0.2) | −3.34 | −3.44 to −3.16 | 1.99 | 1.95 to 2.08 | 0.998 | 0.995 to 1.000 | 0.63 (0.05) |
| Unmethylated CD8B | 3 | 22.6 (0.3) | 24.5 (0.2) | 26.8 (0.2) | 29.2 (0.2) | 31.6 (0.4) | −3.37 | −3.57 to −3.14 | 1.98 | 1.91 to 2.08 | 0.998 | 0.994 to 1.000 | 0.92 (0.07) |
| Unmethylated LRP5 | 3 | 23.1 (0.2) | 25.4 (0.1) | 27.8 (0.2) | 30.1 (0.2) | 32.8 (0.2) | −3.32 | −3.34 to −3.31 | 2.00 | 1.96 to 2.00 | 0.997 | 0.994 to 0.999 | 0.73 (0.05) |
| Unmethylated MVD | 5 | 22.0 (0.2) | 24.4 (0.3) | 26.7 (0.3) | 29.2 (0.3) | 31.6 (0.4) | −3.41 | −3.31 to −3.52 | 1.97 | 1.92 to 2.01 | 0.999 | 0.999 to 1.000 | 1.12 (0.09) |

equivalence was confirmed in different laboratories (data not shown).

To monitor long-term precision, biological references were included in all qPCR runs. Results were used to assess potential longitudinal systematic deviations over approx. 2 years (05/2021 to 05/2023). Mean frequency of unmethylated CD3 of 21.6 % was found with a 2-fold standard deviation (SD) range of ±3.5 %. For CD4, CD8, LRP5 and MVD, mean values were 16.3 % (2-fold SD ±3.3 %), 6.5 % (±1.7 %), 3.2 % (±0.7 %) and 5.6 % (±1.2 %) (Figure 2A). From plotting respective frequencies over time, a moving average for intervals of 25 measurements was calculated. For moving averages, the 1 and 99 % quantiles on the distribution of median deviations from the total means for CD3, CD4, CD8, LRP5 and MVD were at −0.979 and 1.088 % for CD3, −1.260 and 1.187 % for CD4, −0.484 and 0.537 % for CD8, −0.208 and 0.219 % for LRP5, and −0.203 and 0.202 % for MVD (Figure 2). The Augmented–Dickey–Fuller (ADF) test became significant ($p \leq 0.012$) for all markers suggesting absence of systematic shifts or drifts. Kwiatkowski–Phillips–Schmidt–Shin testing did not suggest a unit root for CD3, CD8, LRP5 and MVD ($p > 0.1$), but for CD4 ($p = 0.017$). In conjunction with ADF testing, heteroscedasticity for CD4 cannot be excluded.

Substrate-dependent data quality was assessed for epigenetic qPCR quantification in 100 whole blood, 183 PBMC, 151 FFPE and 57 urine sediment samples. The 384-well plate set-up allowed measurement of up to 17 samples in duplicates for CD3, CD4, CD8, LRP5, MVD and GAPDH assays. Each epigenetic qPCR system and plate run undergoes a defined QC process. To measure all 491 samples with valid batch QC results (methods section), 58 (CD3, CD4, CD8) and 59 plates were needed (instead of 54) suggesting a plate QC-failure of 5.6 and 7.2 %, respectively. The number of failed measurements depended on substrate and assay. With exception of LRP5, all assays and matrices passed QC in above 95 % of measurements. Overall, from measurement of 491 samples 99.4 , 98.8, 98.8, 96.3 and 97.7 % passed QC as valid or non-compliant for CD3, CD4, CD8, LRP5 and MVD, respectively (Table 4).

Ambient EDTA-anticoagulated blood from 9 donors was analyzed for unmethylated CD3, LRP5 and GAPDH verifying dependency of measurement stability on storage times. The mean relative content of the cell type-specific unmethylated loci for freshly drawn samples was at 25.3 % with donor-specific variations ranging from 16.7 to 33.3 % for CD3 and 3.9 % ranging from 1.5 to 6.1 % for LRP5. After ambient storage for 24, 48 and 72 h, mean deviations from freshly collected samples were between 5.0 and 8.1 % (CD3) and 5.3 and 10.7 % (LRP5), with no significant deviation from fresh

**Table 2:** Dilution series of whole blood or DNA to determine bias (deviation) and variability (c.v.) to derive LLoQs of the 5 epigenetic qPCR assays.

| Marker gene (target cell type) | Blood volume used, µL | DNA amount used, ng | Dilution factor | Measured CP value, cycles | Cell type-spec. TpG copies, PU | Unmethylated GAPDH copies, PU | Target cells, % | Coefficient of variation, % | | Deviation, % (limit: 15 %) | Lower limit of quantification | | R-squared linear regression (cell type-spec. TpG copies over blood volume) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Intra assay (limit: 15 %) | Inter assay (limit: 20 %) | | CP value, cycles | Copy number | |
| Unmethylated CD3D/CD3G ($CD3^+$ T cells) | 75 | n.a. | n.a. | 27.8 | 1696.3 | 5,241.3 | 27.2 | 10.6 | 4.8 | n.a. | 34.35 | 12 | 0.97 |
| | 25 | n.a. | 3 | 29.3 | 665.1 | 1957.5 | 28.4 | 9.5 | 4.6 | 7.8 | | | |
| | 8.3 | n.a. | 9 | 30.8 | 238.5 | 654.4 | 30.4 | 2.9 | 5.0 | 11.7 | | | |
| | 2.8 | n.a. | 27 | 32.5 | 79.0 | 210.5 | 31.2 | 7.0 | 11.4 | 14.9 | | | |
| Unmethylated CD4 ($CD4^+$ T cells) | 75 | n.a. | n.a. | 28.0 | 492.1 | 7,596.3 | 11.8 | 3.9 | 4.7 | n.a. | 33.66 | 9 | 0.93 |
| | 25 | n.a. | 3 | 29.3 | 196.1 | 3,347.2 | 10.7 | 5.0 | 10.5 | 9.5 | | | |
| | 8.3 | n.a. | 9 | 31.0 | 60.5 | 885.7 | 12.5 | 9.6 | 12.4 | 5.7 | | | |
| | 2.8 | n.a. | 27 | 32.6 | 21.0 | 295.3 | 13.2 | 12.4 | 4.5 | 11.8 | | | |
| Unmethylated CD8B ($CD8^+$ T cells) | 75 | n.a. | n.a. | 28.8 | 195.3 | 7,064.2 | 3.2 | 5.9 | 3.1 | n.a. | 32.15 | 16 | 0.94 |
| | 25 | n.a. | 3 | 30.0 | 84.8 | 3,490.0 | 2.8 | 5.7 | 9.2 | 11.3 | | | |
| | 8.3 | n.a. | 9 | 31.7 | 25.7 | 911.8 | 3.3 | 13.3 | 14.4 | 0.9 | | | |
| | 2.8 | n.a. | 27 | 33.1 | 12.1 | 303.4 | 5.0 | 38.9 | 53.6 | 48.4 | | | |
| Unmethylated LRP5 (B cells) | 75 | n.a. | n.a. | 30.5 | 146.2 | 7,596.3 | 3.0 | 7.9 | 8.1 | n.a. | 33.87 | 12 | 0.92 |
| | 25 | n.a. | 3 | 31.8 | 60.4 | 3,347.2 | 2.8 | 10.3 | 19 | 6.1 | | | |
| | 8.3 | n.a. | 9 | 33.5 | 18.7 | 885.7 | 3.3 | 13.3 | 19 | 9.7 | | | |
| | 2.8 | n.a. | 27 | 35.1 | 6.6 | 295.3 | 3.5 | 23.4 | 20.9 | 24.8 | | | |
| Unmethylated MVD (NK cells) | n.a | 4,000 | n.a. | 26.9 | 978.0 | 15,233.0 | 6.0 | 4.5 | 13.3 | n.a. | 30.40 | 87 | 0.96 |
| | n.a | 1,000 | 4 | 28.9 | 259.0 | 3,671.0 | 6.6 | 4.6 | 9.4 | 9.3 | | | |
| | n.a | 250 | 16 | 30.9 | 68.0 | 888.0 | 7.1 | 5.9 | 9.2 | 18.3 | | | |
| | n.a | 50 | 80 | 33.3 | 15.0 | 172.0 | 7.9 | 13.8 | 12.3 | 30.8 | | | |

PU, plasma units.

**Table 3:** Assay robustness against deliberate process variations during bisulfite conversion.

| Modified parameter | Bisulfite con., mol/L | Incubation temp., °C | Incubation time, min | | GAPDH-specific qPCR | | CD3D/G-specific qPCR | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Deamination | Desulfonation | Mean Cp, cycles | C.V. of Cp value, % | Mean, Cp, cycles | CV of Cp value, % | Mean CD3, % | Assay CV, % | Deviation, % (limit: 15 %) |
| Standard condition (S.C.) | 3.8 | 80 | 45 | 35 | 23.7 | NA | 26.5 | NA | 18.5 | NA | NA |
| Bisulfite concentration | 3.3 | S.C. | S.C. | S.C. | 23.8 | 0.5 | 26.5 | 0.4 | 21.1 | 7.5 | 14.8 |
| | 2.7 | | | | 23.9 | 0.6 | 26.6 | 0.3 | 19.1 | 13.7 | 4.2 |
| | 2.2 | | | | 24.0 | 0.6 | 26.7 | 0.7 | 15.0 | *15.0* | *18.0* |
| Temperature | S.C. | 65 | S.C. | S.C. | 23.9 | 0.6 | 26.7 | 0.4 | 18.8 | 4.8 | 4.4 |
| | | 75 | | | 23.7 | 0.4 | 26.5 | 0.3 | 19.6 | 4.3 | 9.1 |
| | | 85 | | | 23.8 | 0.4 | 26.7 | 0.4 | 18.0 | 2.8 | 0.1 |
| | | 95 | | | 24.8 | 0.3 | 27.3 | 0.2 | 19.2 | 3.8 | 6.8 |
| Deamination time | S.C. | S.C. | 25 | S.C. | 23.7 | 0.2 | 26.6 | 0.2 | 19.7 | 2.7 | 10.9 |
| | | | 35 | | 23.7 | 0.2 | 26.7 | 0.3 | 18.7 | 3.4 | 4.9 |
| | | | 55 | | 23.8 | 0.5 | 26.7 | 0.4 | 17.9 | 4.7 | 0.7 |
| | | | 65 | | 23.8 | 0.4 | 26.7 | 0.4 | 14.4 | 1.9 | *19.1* |
| Desulfonation time | S.C. | S.C. | S.C. | 15 | 24.0 | 0.3 | 26.6 | 0.2 | 18.7 | 2.1 | 4.9 |
| | | | | 25 | 23.8 | 0.3 | 26.6 | 0.2 | 19.0 | 4.3 | 3.4 |
| | | | | 45 | 23.7 | 0.3 | 26.5 | 0.2 | 18.1 | 2.9 | 8.2 |
| | | | | 60 | 23.7 | 0.6 | 26.2 | 0.5 | 17.9 | 7.6 | 9.1 |

Numbers in italics show those values that are above the acceptance criteria. Such values render the analysis failed and show when the measurement gets to unprecise due to the changed conditions.

blood, while storage for 96 h resulted in 34.5 % deviation in LRP5. For longer storage, blood was spotted as DBS or EDTA-anticoagulated blood was frozen. Storage for up to 12 months showed mean deviations between 1.8 and 12.0 % for DBS and 12.3 and 18.1 % for frozen samples for CD3. LRP5 was only analyzed for frozen EDTA-anticoagulated blood indicating only small deviations between 13.2 and 4.2 % (Table 5).

Fingerprick-capillary blood from 24 normal donors (16 female/8 male) was collected over 28 days, yielding 546 samples and approx. 98 % results passed QC. The relative count of unmethylated CD3, CD4, CD8, LRP5 and MVD were at means (SD) of 23.2 % (±6.3), 16.3 % (±5.0), 7.6 % (±2.6), 5.1 % (±2.5) and 8.1 % (±3.0), respectively. 95 % of values were between 12.7 and 36.3 % unmethylated CD3, 7.6–26.2 % unmethylated CD4, 4.3–14.8 % unmethylated CD8, 1.8–12.4 % unmethylated LRP5 and 3.4–15.3 % unmethylated MVD. This confirms a rather wide population interval for immune cells. Samples from individual donors measured over time had means as low as 14.8 % and as high as 32.9 % for CD3, 9.0 % up to 22.8 % for CD4 and from 5.2 % up to 9.4 % for CD8. For LRP5, individual medians were as low as 2.0 % and reached 6.6 % and for MVD 4.4–12.7 % (Figure 3). From this, mean individual CV (CVI) was determined to be at 17.2 % vs. a population CV (CVP) of 27.0 % for CD3. Also, CVI is significantly lower than CVP for all markers. Therefore, index of individuality (IoI), i.e., the ratio of within-subject variation to between-subject variation, was determined. If IoI is above 1.4, a population-based reference interval identifies most abnormal measurements, whereas a subject-based analysis is required for most abnormal counts when IoI is below 0.6. Here, IoI ranges between 0.758 and 0.472 indicating significant benefits for subject-based assessment of immune cell counts. The reference change value (RCV) from the individual median value is at approx. Fifty percent ranging from 44.2 to 53.6 % for these markers (Table 6).

## Discussion

Limitations of flow cytometry-based immune cell monitoring include challenging sample logistics, a partial lack of guidelines and standardization and acceptably narrow reference values [22]. Contrary to this, guidelines have been developed for qPCRs [23] and such assays can be standardized. DNA, the substrate for epigenetic immune cell counting by qPCR, is chemically stable and logistics are not as challenging [24]. As the copy number of any given gene is directly linked to the cell count, cell type-specifically unmethylated gene copies translate directly into the number of cells with the associated phenotype. Despite this simplistic theory, DNA methylation analysis, like other analytical techniques,

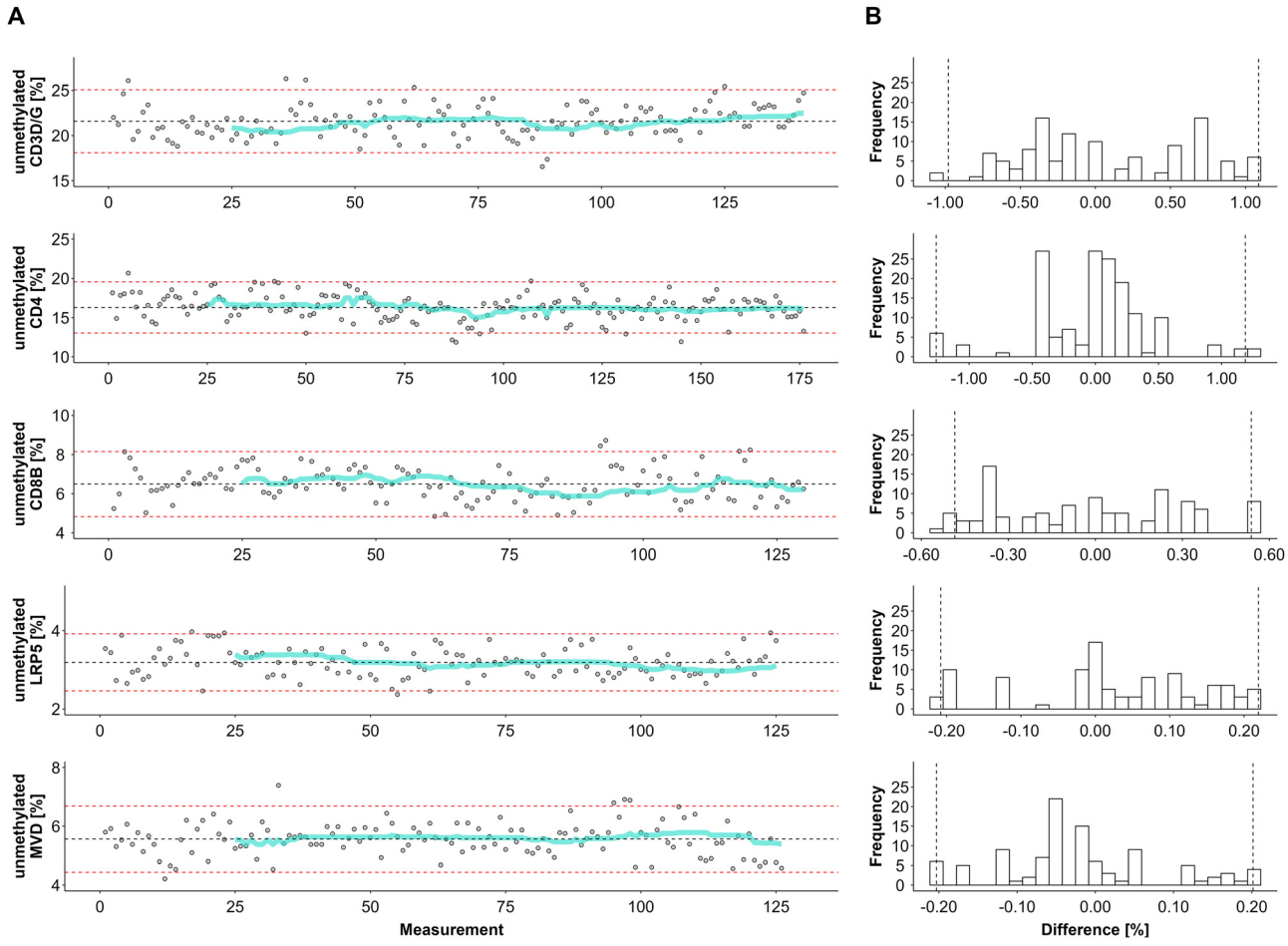


**Figure 2:** Longitudinal immune cell quantification to monitor qPCR stability. (A) Measured relative frequencies of epigenetic markers (in % unmethylated, Y-axis) is plotted against the number of measurements (CD3: n=141; CD4: n=176; CD8: n=130; LRP5: n=125 and MVD: n=126). The black dashed line depicts the calculated mean. The red dashed lines indicate 2-fold SD. The light blue curve shows the calculated moving average with an interval set to 25. (B) Histograms show distribution of differences between overall median and each 25-sample median interval. 1 and 99 % quantiles are indicated by dashed vertical lines.

is subjected to various sources of imprecision. For example, bisulfite conversion and amplification of DNA may vary between different experimental conditions. Analytical performance depends on stability over time, which may be compromised by slow changes to a reagent or when using different reagent lots [25]. Here, analytical performance specifications (APS) for epigenetic qPCRs specific to total, helper and cytotoxic T cells as well as B and NK cells were investigated. APS for qPCR-based quantification systems were defined previously in various areas [26]. Slopes of standard curves, amplification efficiency and $R^2$ are primary performance indicators for qPCR [3, 27]. All of them meet the required values with target slopes between −3.1 and −3.6, amplification efficiencies above 1.98 and $R^2$ above 0.980 [28]. Moreover, measurements are stable in a dynamic range between 50 and 31,250 copies covering the 5,000–30,000 total and 50–9,000 locus-specifically unmethylated DNA copies empirically observed when eluting with 50–70 µL buffer from 75 µl whole blood, 3×3 mm dried blood spots or 1–5 FFPE slides. In gene therapy applications, LLoQs of 50 copies and an intra-assay CV of <25 % are recommended detection limits [3]. With exception to MVD, which meets all requirements down to 87 copies, all assays presented have a back-calculated LLoQ lower than 16 copies and measured at acceptable CV values down to 1:9 or 1:27 whole blood dilutions. The use of the calibrator plasmid served to backtrace the double-stranded *in silico* converted quantification plasmid (standard) to the single-stranded *in vitro* converted endogenous biomolecule in the sample to allow for definitive quantification [29]. The calibration factor is the single most influential parameter for the results and faces the strictest quality criteria. To pass QC, two independent calibrators per

**Table 4:** Assay quality and performance statistics in various biological matrices.

| Specimen | QC | qPCR assay specific for | | | | |
|---|---|---|---|---|---|---|
| | | $CD3^+$ T cells | $CD4^+$ T cells | $CD8^+$ T cells | B cells | NK cells |
| PBMC (n=183) | Passed, n (%) | 182 (99.5) | 181 (98.9) | 179 (97.8) | 179 (97.8) | 181 (98.9) |
| | Failed, n (%) | 0 (0) | 2 (1.1) | 2 (1.1) | 2 (1.1) | 2 (1.1) |
| | Non-compliant, n (%) | 1 (0.5) | 0 (0) | 2 (1.1) | 2 (1.1) | 0 (0) |
| | Non-failed, % | 100 | 98.9 | 98.9 | 98.9 | 98.9 |
| Whole blood (n=100) | Passed, n (%) | 100 (100) | 100 (100) | 91 (91) | 68 (68) | 88 (88) |
| | Failed, n (%) | 0 (0) | 0 (0) | 1 (1) | 9 (9) | 4 (4) |
| | Non-compliant, n (%) | 0 (0) | 0 (0) | 8 (8) | 23 (23) | 8 (8) |
| | Non-failed, % | 100 | 100 | 99 | 91 | 96 |
| FFPE (n=151) | Passed, n (%) | 149 (98.7) | 146 (96.7) | 144 (95.4) | 147 (97.3) | 134 (88.7) |
| | Failed, n (%) | 2 (1.3) | 3 (2) | 3 (2) | 3 (2) | 4 (2.7) |
| | Non-compliant, n (%) | 0 (0) | 2 (1.3) | 4 (2.6) | 1 (0.7) | 13 (8.6) |
| | Non-failed, % | 98.7 | 98 | 98 | 98 | 97.3 |
| Urine sediment (n=57) | Passed, n (%) | 52 (91.2) | 53 (93.0) | 53 (93.0) | 39 (68.4) | N.D. |
| | Failed, n (%) | 1 (1.8) | 1 (1.7) | 0 (0) | 4 (7.0) | N.D. |
| | Non-compliant, n (%) | 4 (7.0) | 3 (5.3) | 4 (7.0) | 14 (24.6) | N.D. |
| | Non-failed, % | 98.2 | 98.3 | 100 | 93.0 | N.D. |
| Total (n=491) | Valid and non-compliant, n (%) | 488 (99.4) | 485 (98.8) | 485 (98.8) | 473 (96.3) | 424 (97.7) |

**Table 5:** Stability of epigenetic qPCR results from ambient and frozen blood as well as dried blood spots.

| | | Epigenetic qPCR assay specific for | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Unmethylated CD3 locus | | | | Unmethylated LRP5 locus | | | |
| | | [%] of unmethylated GAPDH locus | | | | | | | |
| Time point | Donors or measurements [no.] | Mean | Min. | Max. | Mean deviation from $t_1$, % | Mean | Min. | Max. | Mean deviation from $t_1$, % |
| $t_1$ambient (at blood draw) | 9 | 25.3 | 16.7 | 33.3 | NA | 3.9 | 1.5 | 6.1 | NA |
| $t_2$amb. (24 h after $t_1$amb.) | 9 | 25.2 | 16.3 | 34.6 | 5.0 | 3.9 | 1.4 | 6.3 | 5.3 |
| $t_3$amb. (48 h after $t_1$amb.) | 9 | 24.7 | 16.7 | 30.6 | 5.1 | 4.1 | 1.7 | 6.3 | 10.7 |
| $t_4$amb. (72 h after $t_1$amb.) | 9 | 26.9 | 17.6 | 34.1 | 8.1 | 4.2 | 1.5 | 6.8 | 9.2 |
| $t_5$amb. (96 h after $t_1$amb.) | 9 | 26.5 | 17.5 | 35.4 | 9.9 | 5.1 | 2.1 | 7.5 | 34.2 |
| $t_1$frozen (direct after freezing) | 8 | 15.7 | 13.5 | 17.1 | NA | 3.1 | 2.6 | 3.9 | NA |
| $t_2$froz. (1–3 m after $t_1$froz.) | 3 | 17.6 | 15.12 | 19.5 | 12.3 | 3.5 | 3.2 | 3.9 | 13.2 |
| $t_3$froz. (5–6 months after $t_1$froz.) | 8 | 18.5 | 15.8 | 21.9 | 18.1 | 3.2 | 2.6 | 3.5 | 1.8 |
| $t_4$froz. (10–12 months after $t_1$froz.) | 8 | 17.8 | 16.3 | 20.8 | 13.7 | 3.2 | 2.9 | 3.8 | 4.2 |
| $t_1$dbs (at spotting) | 3 | 16.6 | 15.3 | 17.5 | NA | | | | |
| $t_2$dbs (1–3 months after $t_1$dbs) | 3 | 16.3 | 14.0 | 19.7 | −1.8 | Not determined | | | |
| $t_3$dbs (5–6 months after $t_1$dbs) | 3 | 18.6 | 17.5 | 19.9 | 12.0 | | | | |
| $t_4$dbs (12 months after $t_1$dbs) | 3 | 17.5 | 16.5 | 18.9 | 5.4 | | | | |

PCR plate and assay require replicate CVs below 25 % and deviation below 20 % compared to data derived from the previous 25 measurements. The lack of guidelines for such calibrators, however, leaves room for discussion regarding quality criteria. Introducing deliberate changes to reaction parameters of bisulfite-conversion included

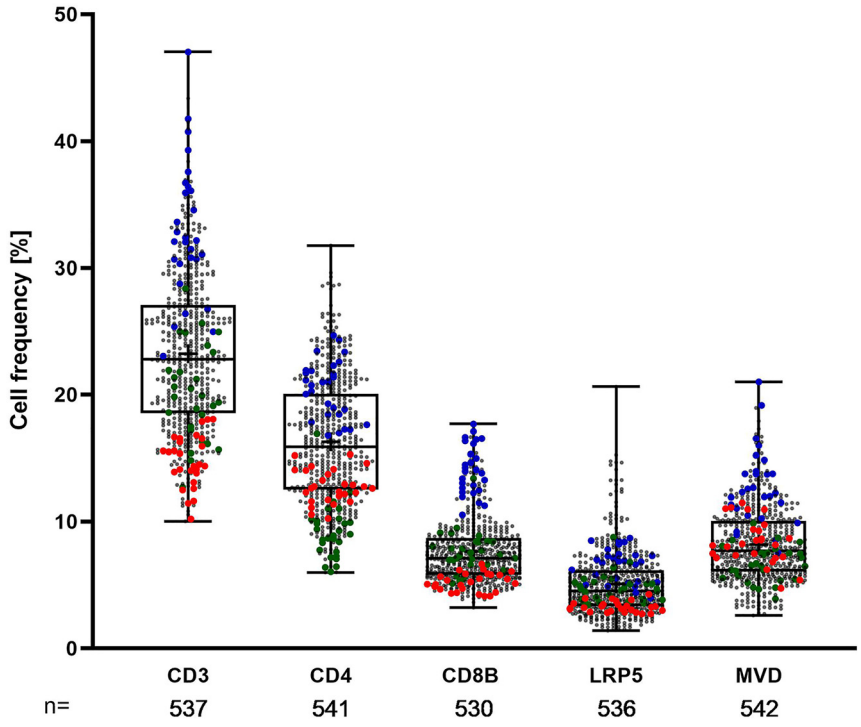


**Figure 3:** Epigenetic analysis of immune cell frequency. Boxplots illustrate counts of CD3, CD4, CD8B, LRP5 and MVD relative to GAPDH in capillary blood samples. Boxes represent interquartile ranges (IQR) of the median with whiskers extending maximally 1.5 times from the upper/lower box. Colored dots represent data from 3 individuals with high (blue), intermediate (green) and low (red) counts.

**Table 6:** Performance and variation characteristics of the epigenetic qPCRs in capillary blood from healthy donors over 28 days.

| | Cell type-specifically unmethylated locus | | | | |
|---|---|---|---|---|---|
| | **CD3D/G** | **CD4** | **CD8B** | **LRP5** | **MVD** |
| Number of donors (at up to 28 days) | 24 | 24 | 24 | 24 | 24 |
| Valid measurements, % (of 546 total samples) | 98.5 | 99.1 | 97.1 | 98.2 | 99.3 |
| Mean, % | 23.2 | 16.3 | 7.6 | 5.1 | 8.1 |
| (standard deviation) | (6.3) | (5.0) | (2.6) | (2.5) | (3.0) |
| Individual C.V., % | 17.2 | 16.8 | 17.7 | 20.1 | 19.6 |
| Population C.V., % | 27.1 | 30.5 | 34.1 | 49.0 | 36.1 |
| Analyte C.V., % (derived from | 11.2 | 9.0 | 10.1 | 11.4 | 10.1 |
| number of measurements) | (158) | (330) | (119) | (181) | (126) |
| Index of individuality | 0.758 | 0.627 | 0.598 | 0.472 | 0.612 |
| Reference change value | 47.7 | 44.2 | 47.3 | 53.6 | 52.0 |

chemical components and incubation temperatures and served as an assessment of assay ruggedness. Potentially coincidental incubation differences of up to 10 °C or 10 min have no measurable impact beyond expected unsystematic variations compared to standard conditions. In longitudinal studies, changes over time in materials and equipment may be disguised by noise, but potentially affect results [25]. Changes in a reference blood sample were analyzed over approx. 2 years in more than 150 experiments. Ideally, assays are stationary with homoscedastic errors over the entire period. All five assays were stationary, while CD4 showed possible heteroscedasticity, which appeared uncritical with lower variation for a short interval. Overall, these data indicate stable long-term performance. No significant differences between supportive matrices were observed, although this poses a potential concern for qPCR reliability [30]. Fresh anticoagulated blood was stable for up to 3 days at ambient temperature and for up to one year after freezing supporting previous observations [24]. 491 whole blood, PBMCs, embedded tissue and urine sediment samples demonstrated high robustness against different biological matrices. Technically, this robustness is enabled by the use of small DNA fragments with relatively high CpG density leading to stable amplification even if DNA suffers from fragmentation and deamination, both known to impair PCRs [31]. The value of population- or subject-based reference intervals was determined by the IoI on a 1-month time-course of 24 clinically normal donors suggesting a benefit of the latter [32]. This is at odds with current clinical practice, which usually tests lymphocytes against population-based references [33]. The reference change value (RCV) suggests that an observed change of more than 50 % between measurements indicates a biologically significant alteration [34]. The use of epigenetic immune monitoring from fingerprick-capillary blood may allow regular immune cell counting with high diagnostic resolution and has potential applications in screening, monitoring and risk assessment [35].

Epigenetic immune monitoring performs with technical precision, lack of bias and ruggedness as required by regulations and is a simple but precise way to complement flow cytometric immune monitoring. The approach allows the analysis of samples from the same donors and patients at any interval without compromising life quality and improves diagnostic precision. Overall, epigenetic qPCR analysis may turn out as tool for individualized immune monitoring.

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**Research ethics:** The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

**Informed consent:** Peripheral blood in different tubes, self-collected capillary DBS, tissue and urine sediment were obtained from volunteers with written consent and approval (Ruhr-Universität Bochum (20-6886) and Charité-Universitätsmedizin Berlin: (EA2/028/13 and EA2/029/13)).

**Author contributions:** The authors have accepted responsibility for the entire content of this manuscript and approved its submission. KS performed statistical analysis. BS, AR and IJ performed experiments. ER, NB and KG contributed to study design and UB contributed to manuscript writing. SO designed study and wrote manuscript.

**Competing interests:** KS, BS, JG, UB, ER, JO, AR, IJ and SO are employed at Precision for Medicine, a company offering molecular biology services.

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**Data availability:** The raw data can be obtained on request from the corresponding author.

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