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TECHNICAL ADVANCE

Accurate Quantification of T Cells by Measuring Loss of Germline T-Cell Receptor Loci with Generic Single Duplex Droplet Digital PCR Assays

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Quantifying T cells accurately in a variety of tissues of benign, inflammatory, or malignant origin can be of great importance in a variety of clinical applications. Flow cytometry and immunohistochemistry are considered to be gold-standard methods for T-cell quantification. However, these methods require fresh, frozen, or fixated cells and tissue of a certain quality. In addition, conventional and droplet digital PCR (ddPCR), whether followed by deep sequencing techniques, have been used to elucidate T-cell content by focusing on rearranged T-cell receptor (TCR) genes. These approaches typically target the whole TCR repertoire, thereby supplying additional information about TCR use. We alternatively developed and validated two novel generic single duplex ddPCR assays to quantify T cells accurately by measuring loss of specific germline *TCR* loci and compared them with flow cytometry–based quantification. These assays target sequences between the *Dδ2* and *Dδ3* genes (*TRD* locus) and *Dβ1* and *Jβ1.1* genes (*TRB* locus) that become deleted systematically early during lymphoid differentiation. Because these ddPCR assays require small amounts of DNA instead of freshly isolated, frozen, or fixated material, initially unanalyzable (scarce) specimens can be assayed from now on, supplying valuable information about T-cell content. Our ddPCR method provides a novel and sensitive way for quantifying T cells relatively fast, accurate, and independent of the cellular context. *(J Mol Diagn 2017, 19: 236–243; http://dx.doi.org/10.1016/j.jmoldx.2016.10.006)*

Quantification of (malignant) T cells in benign, inflammatory, and malignant tissue or body fluids can be of great value with respect to diagnostics, prognostics, and even therapeutic approaches. T-cell infiltration of tumors and (chronic) inflammatory lesions is a common phenomenon and has clinical consequences. Migration toward tumors and the magnitude of infiltration have been correlated positively and/or negatively to tumor growth and clinical prognosis.[1,2] Moreover, (intratumor) T cells are triggered and guided in an anti-tumor response by treatment with monoclonal antibodies that are increasingly being used therapeutically in clinical studies.[3,4] Hence, the interest in monitoring (infiltrating) T cells in the context of malignancies has increased vastly over time.

To determine T-cell proportions in body fluids or solid tissue, flow cytometry and immunohistochemistry are commonly used. However, cell counting is dependent on membrane epitopes that may be lost during cell preparation, depending on the preparation method. In addition, for a decent quantification, both flow cytometry and immunohistochemistry require advanced standardization and are dependent on sample type and quality of input material.[5,6] Therefore, T-cell receptor (TCR) sequencing was introduced as an unbiased approach to elucidate the extent of T-cell infiltrate and composition of the TCR repertoire in particular.[7]

Mature T cells differ genetically from other cell types as a result of *TCR* gene rearrangements. During early lymphoid

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differentiation, many distinct variable, diversity, and joining *TCR* genes are rearranged to generate a highly diverse TCR repertoire.[8] Four gene complexes are responsible for the variety of expressed TCRs and rearrange sequentially in a highly ordered manner, starting with *TRD*, followed by *TRG*, *TRB*, and finally *TRA*. Depending on the productivity of the rearranged *TCR* genes, the order of rearrangements continues from *TRD* to *TRA* until a functional recombined TCR sequence is obtained. However, some parts of the four *TCR* genes rearrange biallelically regardless of this order of recombinations and become deleted early in the T-cell maturation.[9]

Digital droplet PCR (ddPCR) represents the third generation of PCR and provides new possibilities for molecular quantification. ddPCR allows absolute quantification of nucleic acids by combining sample partitioning (limiting dilution) with Poisson statistical data analysis.[10]

In contrast to other ways of quantifying T cells by (multiplex) PCR techniques, we took advantage of the genetic dissimilarity between T cells and cells of other origin by measuring loss of germline *TCR* loci. Thus, instead of counting a whole repertoire of rearranged TCRs, we used an indirect counting approach.[7,11]

This article describes in detail how T cells can be quantified by measuring loss of germline *TRB* and/or *TRD* genomic loci using ddPCR. By focusing on the most frequently deleted loci of rearranged *TCR* genes, we were able to determine the contribution of T cells in between other cell types (Figure 1A). As most suitable target sequences to be used, we choose *Dδ2-Dδ3* intergenic sequences (from now on called ΔD) (Figure 1B) and *Dβ1-Jβ1.1* intergenic sequences (ΔB) (Figure 1C). Because *Dδ2-Dδ3* rearrangements occur on both alleles at the most immature stage of human T-cell development, virtually all mature T cells are lacking ΔD.[9] *Dβ1-Jβ1.1* rearrangements also occur early during T-cell maturation, and thus ΔB sequences are biallelically lost in practically all peripheral T cells.[9] By measuring loss of these specific *TCR* loci and normalization against a copy number stable reference gene, T-cell quantity can be effectively determined (Figure 1D). Finally, we performed a thorough validation of this newly developed technique relative to T-cell quantification by flow cytometry.

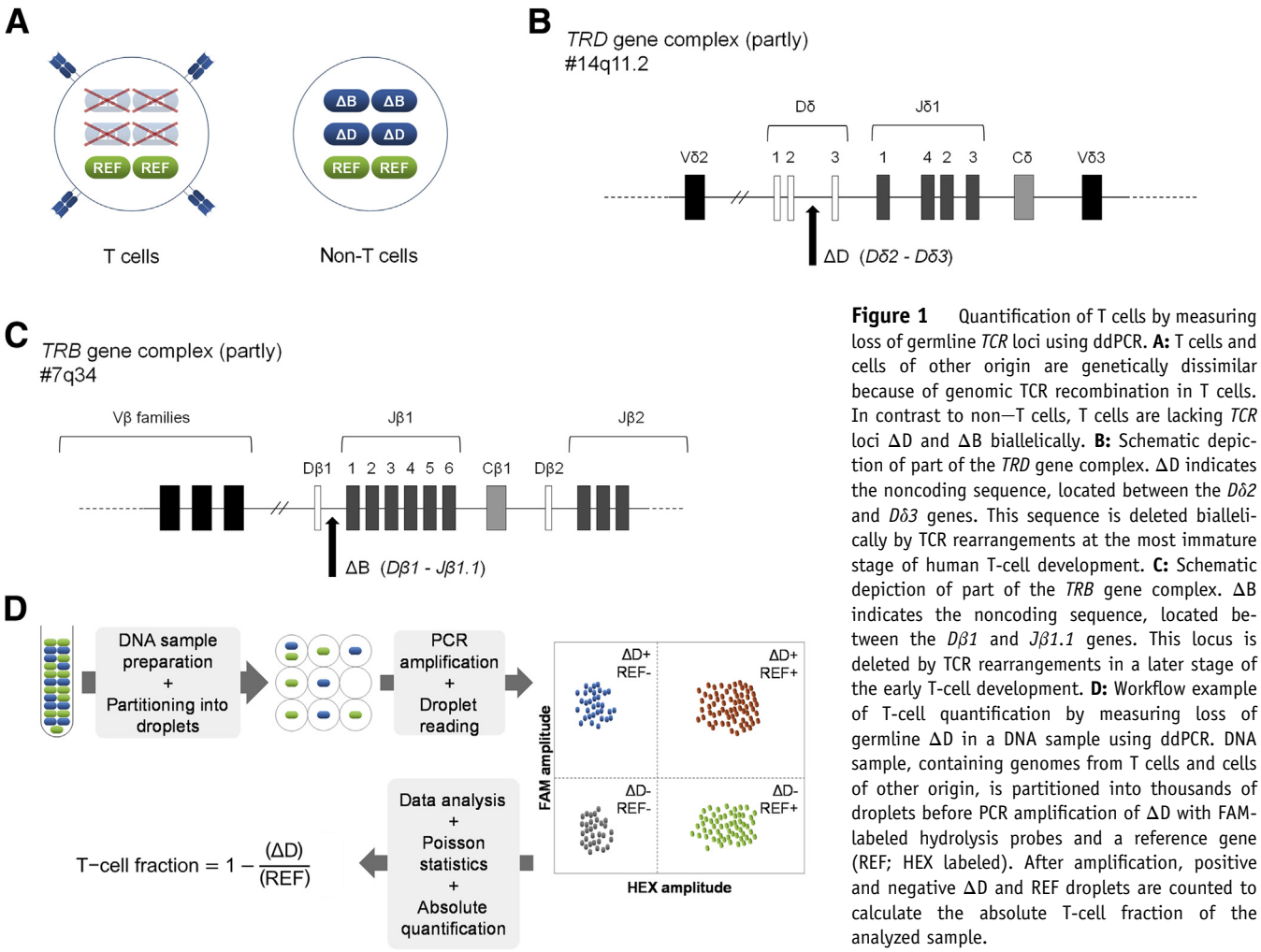

Figure 1 Quantification of T cells by measuring loss of germline *TCR* loci using ddPCR. **A:** T cells and cells of other origin are genetically dissimilar because of genomic TCR recombination in T cells. In contrast to non—T cells, T cells are lacking *TCR* loci ΔD and ΔB biallelically. **B:** Schematic depiction of part of the *TRD* gene complex. ΔD indicates the noncoding sequence, located between the *Dδ2* and *Dδ3* genes. This sequence is deleted biallelically by TCR rearrangements at the most immature stage of human T-cell development. **C:** Schematic depiction of part of the *TRB* gene complex. ΔB indicates the noncoding sequence, located between the *Dβ1* and *Jβ1.1* genes. This locus is deleted by TCR rearrangements in a later stage of the early T-cell development. **D:** Workflow example of T-cell quantification by measuring loss of germline ΔD in a DNA sample using ddPCR. DNA sample, containing genomes from T cells and cells of other origin, is partitioned into thousands of droplets before PCR amplification of ΔD with FAM-labeled hydrolysis probes and a reference gene (REF; HEX labeled). After amplification, positive and negative ΔD and REF droplets are counted to calculate the absolute T-cell fraction of the analyzed sample.

Materials and Methods

Blood Donors and Cell Isolation

Approval for this study was obtained from the institutional review board of Leiden University Medical Center (Leiden, the Netherlands). All participants provided written informed consent according to the Declaration of Helsinki.

Peripheral heparinized blood was drawn from healthy donors and Sézary syndrome patients. Sézary syndrome (SS) is an aggressive type of cutaneous T-cell lymphoma characterized clinically by erythroderma, generalized lymphadenopathy, and the presence of malignant T cells in the skin, lymph nodes, and peripheral blood.[12] SS is a malignant clonal proliferation of mature, $CD4^+$ skin-homing central memory T cells.[13]

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. For standard curve preparation, a freshly isolated PBMC sample from a healthy donor was enriched for $CD4^+$ T cells by depletion of non-$CD4^+$ T cells, using the $CD4^+$ T Cell Isolation Kit I, according to the protocol supplied by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Furthermore, passage 5 cultured fibroblasts from a healthy donor were harvested. These cells were cultured from donor skin, as obtained during mamma reduction surgery.[14] PBMCs, $CD4^+$ enriched T cells, and fibroblasts were aliquoted in nuclease-free microcentrifuge tubes in quantities of 5×10^6 cells and stored as dry pellets at $-80°C$ until DNA extraction. For flow cytometry, PBMC samples were cryopreserved in ice-cold fetal calf serum supplemented with 10% (v/v) dimethyl sulfoxide at a final concentration of 10×10^6 cells/mL until analysis.[15]

DNA Isolation

Stored pellets of PBMCs, $CD4^+$ enriched T cells, and fibroblasts were subjected to DNA isolation using the QIAamp DNA Blood Mini Kit, according to the protocol supplied by the manufacturer (Qiagen, Hilden, Germany). Purity of the isolated DNA was evaluated by measuring A260/280 and A260/230 ratios by UV spectrophotometry using a NanoDrop system (NanoDrop Technologies, Wilmington, CA). Integrity was evaluated by gel electrophoresis on 0.7% agarose (ethidium bromide) gels. DNA samples with A260/280 ratios between 1.7 and 1.9, A260/230 ratios >1.8, and high molecular weight, showing no degradation, were included for subsequent ddPCR analysis.

Standard Curve Preparation

To evaluate the technical validity of these assays, a standard curve of T-cell DNA was prepared and analyzed to determine the dynamic range and accuracy of T-cell quantification by ddPCR. DNA extracted from a $CD4^+$ T-cell enriched PBMC sample was diluted in a background of germline (nonrearranged) TCR DNA, originating from fibroblasts. Prepared standard curve contained 15 dilution points consisting of 0%, 0.5%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, 90%, 95%, 98%, 99%, 99.5%, and 100% (w/w) T-cell DNA. ddPCR of the standard curve was performed in duplicate as independently executed experiments, resulting in a cumulative partitioning of approximately 40,000 droplets.

ddPCR

To quantify T cells accurately, duplex ddPCR assays with primers and FAM- or HEX-labeled hydrolysis probes were developed. Probes directed at target loci ΔD and ΔB were labeled with FAM, whereas probes directed at the reference gene *DNM3* were labeled with HEX (Table 1). For a proper normalization and calculation of ΔD and ΔB loss, it is essential that the used reference gene is located in a genomically stable locus. Especially, when the T-cell content must be calculated in a tumorous environment or when T cells themselves are malignantly transformed, copy number alterations of reference (and target) loci will have a disruptive effect on the calculations. In particular, when calculating ΔD and ΔB loss in SS, *DNM3* is most suitable to use because this gene is generally copy number stable in patients experiencing this disease.[15]

ddPCR was performed using ddPCR Supermix for probes (Bio-Rad Laboratories, Hercules, CA) in 20 μL with 50 ng of DNA, resulting in 0.75 copies per droplet of haploid genomes after partitioning into 20,000 droplets.

DNA restriction digestion was performed using HaeIII directly in the ddPCR reaction solution, according to the

Table 1 Detailed Primers and Probes: Information Used for Quantification of T Cells by ddPCR

Gene	Genomic feature	Forward primer (900 nmol/L)	Reverse primer (900 nmol/L)	Probe (250 nmol/L)	Label
TRD	Dδ2-Dδ3 (ΔD)	5′-GCTGGCTGTAATGGG-AATGT-3′	5′-TAATGGCTTGATAAA-GATAAGTGATCAT-3′	5′-TGTGAAGATGTCTGT-AGCCATCTTAT-3′	FAM
TRB	Dβ1-Jβ1.1 (ΔB)	5′-GCCATGCACTTTCCC-TTTCG-3′	5′-ACAGAGTCCATCCAC-AGGG-3′	5′-TGGACCCTCACAGAG-GGAGCA-3′	FAM
DNM3	Exon 14	5′-CTAAACACCTCTGCT-GATTTCTGC-3′	5′-CCGCCTTTCATGATG-CCAATG-3′	5′-TGAGCCACCCCTTGC-GAATCACCT-3′	HEX

ddPCR, droplet digital PCR; FAM, carbaxyfluorescein; HEX, hexachlorofluorescein.

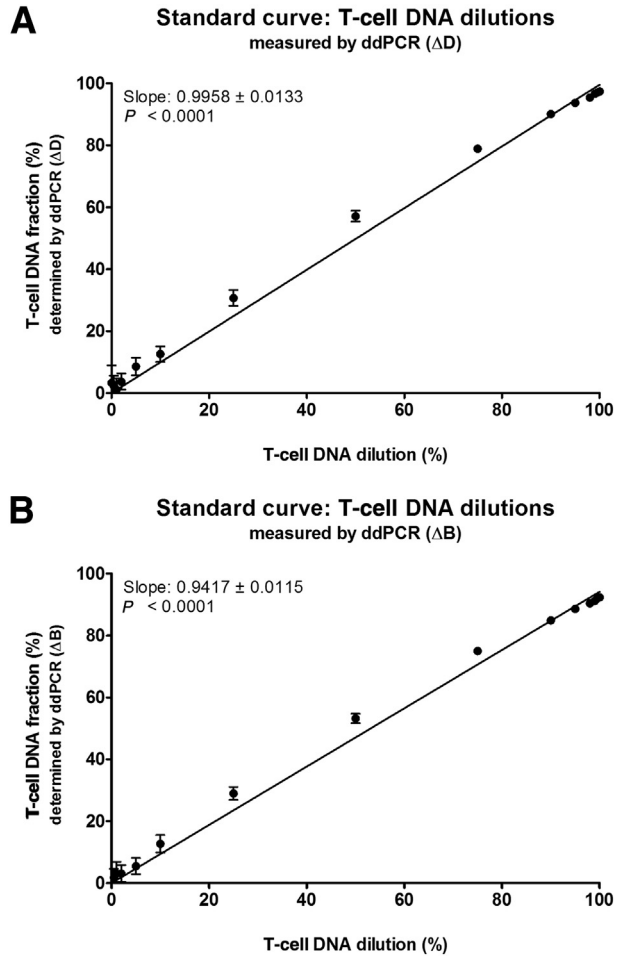

Figure 2 Scatter plots with linear regression for measuring T-cell DNA dilutions as determined by ddPCR. Scatter plots illustrating the relation between input percentage of enriched T-cell DNA sample (*x* axis) and T-cell DNA fraction as determined by ΔD ddPCR (**A**) and ΔB ddPCR assay (**B**). Error bars indicate 95% CIs, as calculated by QuantaSoft according to Poisson distribution on scored droplets.

protocol supplied by Bio-Rad. Droplets were generated using an AutoDG System (Bio-Rad), and droplet emulsion was transferred to a 96-well PCR plate for amplification in a T100 Thermal Cycler (Bio-Rad). Cycle parameters were as follows: enzyme activation for 10 minutes at 95°C; denaturation for 30 seconds at 94°C and annealing and extension for 1 minute at 60°C for 40 cycles; enzyme deactivation for 10 minutes at 98°C; and infinite cooling at 12°C. The ramp rate for all cycles was 2°C/second. Cycled droplets were stored at 4°C to 12°C until reading. Positive and negative droplets were measured as a CNV1 experiment using a QX200 Droplet Reader (Bio-Rad).

ddPCR Data Analysis

Quantified droplets were analyzed and normalized using QuantaSoft software version 1.7.4 (Bio-Rad). To calculate normalized haploid copy numbers for ΔD and ΔB, a genomic copy number stable reference (REF) gene was used (*DNM3*). Obtained normalized ΔD and ΔB values are ratios typically lying between 0 and 1. The T-cell fraction was calculated by subtracting this determined ratio from 1 according to the following formulas: T-cell fraction = 1−(ΔD/REF) and T-cell fraction = 1−(ΔB/REF).

For example, a 100% loss of ΔD or ΔB (representing a 100% T-cell fraction) results, after normalization, in ratios of 0, resulting in a T-cell fraction of 1−0 = 1 (100%).

Flow Cytometry

Flow cytometry was performed on frozen PBMC samples. Cryopreserved cells were quickly thawed at 37°C, washed with phosphate-buffered saline supplemented with 5% (v/v) fetal calf serum, and washed again in phosphate-buffered saline without fetal calf serum.

On ice, cells were stained in phosphate-buffered saline supplemented with 0.2% (w/v) bovine serum albumin with CD3-allophycocyanin conjugated monoclonal antibodies (BD Biosciences, San Jose, CA; catalog number 345767). To determine background and non-specific signals each run, a relevant isotype-specific (IgG1-allophycocyanin) control was included (BD Biosciences; catalog number 555751).[15]

Using a FACSCalibur flow cytometer (BD Biosciences), 20,000 events per sample were measured and analyzed using FlowJo software version 7.2.5 (FlowJo, Ashland, OR). Lymphocytes were gated on the basis of forward and sideward scatter patterns. Second, T-cell content ($CD3^+$ cells) was quantified relative to all measured events of the forward and sideward scatter plots. Specific antigen expression was determined relative to autofluorescence and (non-) specific signals.[15]

Statistical Analysis

Statistical evaluation of all data was performed by using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). To evaluate the dynamic range and accuracy of both ddPCR assays, linear regression was performed on the standard curve data. T-cell quantification data from healthy donor and SS patient samples were depicted as dot plots separately for each method and tested for differences in median counts by Kruskal-Wallis testing. In addition to standard Pearson *r* correlation test, Bland-Altman plots were generated from flow cytometry and ddPCR data. Bland-Altman plots examine differences in ddPCR output relative to flow cytometry in more detail: Besides generating 95% limits of agreement, also the bias of differences can be computed, thus supplying insights in possible consistent underquantification or overquantification of T cells by ddPCR relative to the considered gold-standard method of flow cytometry.

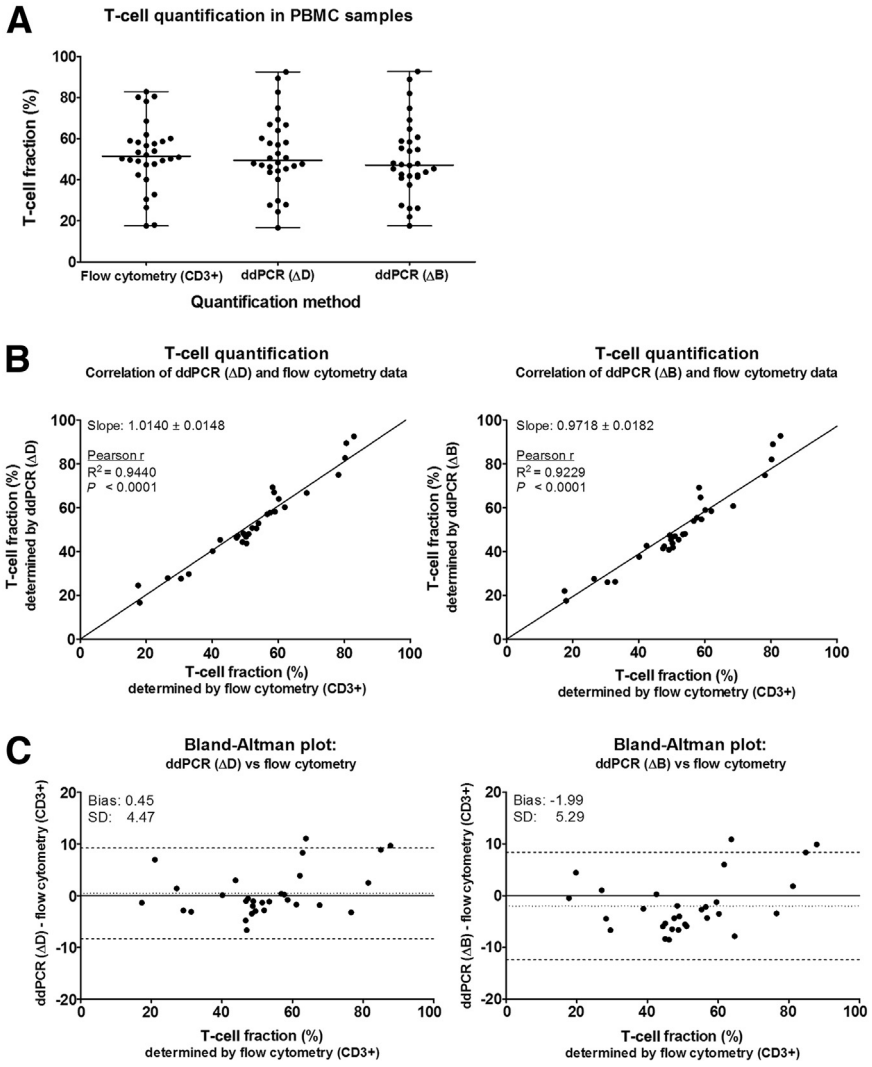

Figure 3 Comparison of T-cell quantification by ΔD and ΔB ddPCR to flow cytometry. **A:** Kruskal-Wallis testing of three T-cell quantification methods. Determined T-cell fractions of 30 analyzed peripheral blood mononuclear cell (PBMC) samples are plotted jointly for each applied quantification method: flow cytometry, ΔD ddPCR, and ΔB ddPCR assay. Bars indicate median, minimum, and maximum levels of quantification. **B:** Scatter plots with Pearson *r* test for correlation of T-cell quantification, as determined by flow cytometry and ddPCR assays. Linear association in T-cell quantification between ddPCR assays (ΔD and ΔB) and flow cytometry was statistically evaluated. **C:** Bland-Altman plots for assessment of quantification bias, as determined by ddPCR relative to flow cytometry. Individual T-cell measurement differences were plotted against the horizontal zero value (*y* axis), representing T-cell proportions as determined by gold-standard method of flow cytometry (*x* axis) for ΔD and ΔB ddPCR assay. The **dotted lines** represent the bias in quantification relative to flow cytometry. **Dashed lines** represent 95% limits of agreement.

Results

Accuracy and Dynamic Range of ΔD and ΔB ddPCR Are High

Accuracy and dynamic range of T-cell quantification by ddPCR was evaluated by analyzing standard curve data from 15 T-cell DNA dilution points (ie, 15 different fractions of T-cell DNA diluted in a background of germline TCR DNA). Linear regression revealed a high relationship between DNA input and the fraction of T cells as determined by ddPCR. Slopes for both ΔD and ΔB ddPCR assay were significantly ($P < 0.0001$) close to a perfect linear regression: 0.9958 and 0.9417, respectively (Figure 2). Dynamic range of quantification guarantees accuracy for detecting low to absolute proportions of T-cell DNA, as indicated by the small 95% CIs. Because $CD4^+$ T-cell enrichment by cell depletion ensures a T-cell purity of >95%, an undiluted sample should reflect a T-cell count between 95% and 100%.[15] The undiluted T-cell DNA sample gave a cell count of 97.4% for the ΔD assay and therefore met the criterion for purity as supplied by the manufacturer. However, by using the ΔB assay, the undiluted sample was calculated to have a T-cell count of 92.3%.

Comparison of T-Cell Quantification by ΔD and ΔB ddPCR to Flow Cytometry Shows a Good Correlation

The main aim of this study was to compare and assess the performance of both the ΔD and ΔB ddPCR assays relative to the standard way of T-cell quantification, as performed by flow cytometry. To ensure a panel of PBMC samples with a wide range of T-cell proportions, blood samples from healthy donors ($n = 20$) as well as blood samples from SS patients ($n = 10$) were selected. In an advanced disease state, SS patients characteristically harbor high numbers of clonally expanded T cells in the periphery, whereas patients in a more medically managed disease state can have relatively low numbers of T cells (lymphocytopenia) as compared to a typical distribution, as seen in PBMCs from healthy individuals

(40% to 60%).[16] By using this selection of samples, representing a wider range of T-cell proportions, the validity of ddPCR assays could be evaluated in a broader range.

First, we compared T-cell proportions as determined by flow cytometry and both ddPCR assays (Figure 3A). Because our set of 30 selected PBMC samples are not representing a normal distribution of T-cell proportions, Kruskal-Wallis testing was applied to compare all three methods of T-cell quantification. Although ΔD and ΔB ddPCR assays measure reduced median levels of T cells (2% and 4% reduction, respectively) and a broader range of T-cell quantities as compared to flow cytometry, statistical testing indicated that these differences were not significant ($P = 0.57$).

To evaluate linear association in T-cell quantification between ddPCR and flow cytometry statistically, linear regression and Pearson r tests were executed. T-cell quantification either by ΔD or ΔB ddPCR assays and flow cytometry was highly correlated, resulting in significant coefficients of determination (R^2) of 0.9440 and 0.9229 (both $P < 0.0001$), respectively (Figure 3B). Linear regression was applied by forcing a regression line to start from zero x and y coordinates (0,0) and by extending the range to $x = 100$. Desirably, both quantification methods are not deviating much, resulting in linear regression slopes close to 1. Regarding the ΔD assay, the corresponding slope approached this perfect value, namely, 1.0140 ± 0.0148. The slope of regression of the ΔB assay was slightly lower (0.9718 ± 0.0182).

To examine the nature of the differences in quantification by ddPCR relative to the gold-standard method of flow cytometry, Bland-Altman plots were generated (Figure 3C). Individual T-cell measurement differences were plotted against the horizontal zero value (y axis), representing T-cell proportions as determined by flow cytometry (x axis). This analysis revealed that the biases of the ΔD and ΔB assays were small and close to zero: 0.45 and −1.99, respectively. For the ΔD assay, the 95% limits of agreement were almost symmetrically positioned around zero values, reaching from −8.3 to 9.2. As expected from the slightly higher and negatively charged bias for the ΔB assay, divergence for the ΔB assay is larger and more systematically displaced lower than the zero value of differences, suggesting some underestimation of T cells as compared to flow cytometry.

When comparing ddPCR assays with each other, the underestimation of T cells with the ΔB assay is evident in corresponding Bland-Altman analysis (in which the ΔD ddPCR assay was considered as gold-standard method), showing a bias of −2.43. However, 95% limits of agreement were tighter as compared to flow cytometry as gold standard, namely, −6.0 to 1.1 (Figure 4A). Even correlation was better as compared to flow cytometry, resulting in a R^2 of 0.9906. Regarding mutual linear association, in accordance with the Bland-Altman data, regression slope was less potent, but still strong, namely, 0.9597 ± 0.0182 ($P < 0.0001$) (Figure 4B).

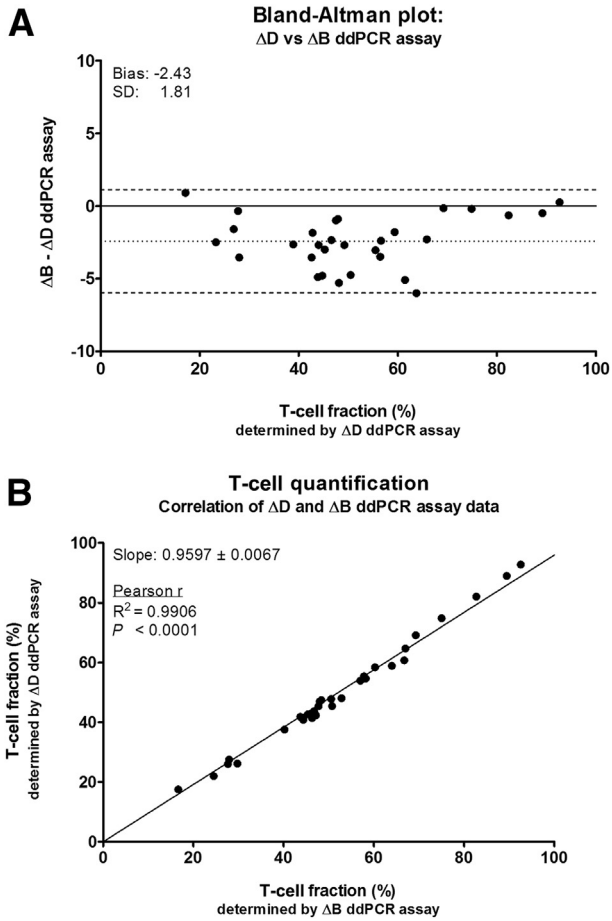

Figure 4 Bland-Altman plot and scatter plot with Pearson r test for correlation of T-cell quantification, as determined by ΔB ddPCR relative to ΔD ddPCR assay. **A:** Individual T-cell measurement differences were plotted against the horizontal zero value (y axis), representing T-cell proportions as determined by ΔD ddPCR (x axis) for ΔB ddPCR assay. The **dotted line** represents the bias in quantification relative to ΔD ddPCR assay. **Dashed lines** represent 95% limits of agreement. **B:** Linear association in T-cell quantification between ΔB ddPCR and ΔD ddPCR assay was statistically evaluated.

Collectively, these data demonstrate that ddPCR, by using either ΔD or ΔB assay, correlates highly with the gold-standard approach of T-cell quantification as performed by flow cytometry.

Discussion

Herein, we described how genetic dissimilarity between T cells and cells of other origin can be exploited for quantification of T cells. Measuring loss of germline TCR sequences ΔD and ΔB allowed us to measure T-cell proportions as accurate as the current gold-standard method of quantification as performed by flow cytometry. Testing of the dynamic range of T-cell quantification by ddPCR ensured sensitive and accurate detection of low to absolute proportions of T cells.

In contrast to other techniques, ddPCR has a digital dimension. Although flow cytometry, immunohistochemistry, and real-time quantitative PCR are more subject to

arbitrary aspects because of instrument settings, dependency on replicates and standards, and even personal factors, ddPCR offers absolute and direct quantification by its digital design.[5,6,10,11,17]

Exploiting the genetic dissimilarity between T cells and cells of other origin in terms of detection, quantification, and characterization is not a novel approach. With the introduction of conventional multiplex PCR assays (also used in a ddPCR format) that recognize a whole repertoire of rearranged TCRs, T cells (and T-cell clones) can be determined in body fluids or solid tissue.[11,18] However, by focusing at the most frequent deleted sequences in rearranging *TCR* genes [ie, the intergenic *Dδ2-Dδ3* (ΔD) and *Dβ1-Jβ1.1* (ΔB) regions], we were able to quantify T cells accurately by using generic single duplex ddPCR assays.

When comparing ΔD and ΔB assays statistically, we concluded that both ddPCR assays were similar in accuracy and in perfect line with flow cytometric quantification of T cells in particular. However, Bland-Altman analysis as well as correlation and regression tests revealed that the ΔB assay slightly underestimates T cells in comparison with the ΔD assay. This observed difference in quantification can be linked to the stage in which both ΔD and ΔB loci become deleted because of TCR rearrangements in the early lymphoid differentiation. As demonstrated before, *Dδ2-Dδ3* rearrangements occur biallelically at the most immature stage of human T-cell development, whereas *Dβ1-Jβ1.1* rearrangements follow later. Furthermore, it is not clear whether this second particular rearrangement occurs biallelically in all T cells during maturation.[9] On the basis of our current data, biallelic deletion of ΔB seems almost ubiquitous. Nevertheless, despite small differences in performance, both ddPCR assays are evidently useful and accurate for T-cell quantification. In some situations, use of the ΔB assay might be preferred (eg, when copy number alterations in malignancies could disrupt the calculation of T-cell infiltrate). Therefore, knowledge about the copy number alteration status of 14q11.2 (*TRD*) and 7q34 (*TRB*) in the malignancy of interest is advisable. Whenever gains or losses of one of these loci are common or expected, switching assays would be recommended.

Counting T cells in body fluids by measuring loss of germline *TCR* loci (using either ΔD or ΔB assay) can be of great value, but can additionally be used in quantifying tumor infiltrating lymphocytes accordingly. A major advantage of using ddPCR instead of standard methods like flow cytometry and immunohistochemistry is the type and quality of required input material. Although conventional methods of T-cell quantification require fresh, frozen, or fixated cells and tissue, for this technique DNA specimens are sufficient.[5,6,10] Another benefit of using DNA instead of tissue is the presence of intratumor heterogeneity and possible corresponding heterogeneous T-cell infiltration in malignancies. Local differences in T-cell counts, as determined by immunohistochemistry, are equaled out in DNA samples of the same specimen.[19] Besides the type of material, also the required quantity varies for each technique. In this study, ddPCR experiments were accurately performed on as little as 50 ng of DNA. This represents, on average, 0.75 copies of haploid genomes in every PCR droplet and is regarded as optimal, but this quantity can be lowered when necessary and still provide significant data. However, with respect to the statistics of Poisson distribution, ddPCR allows copies per droplet values not <0.11 (representing 7 ng of human genomic DNA in a 20-μL reaction mixture) to ensure CVs $<2.5\%$ [bulletin 6407 RevA, applications guide (p97); Bio-Rad, Herculus, CA]. Because ddPCR requires small amounts of DNA instead of freshly isolated, frozen, or fixated cells and tissue, the sample size and variety of (scarce) material for quantifying (infiltrating) T cells can be expanded significantly.

In conclusion, these validated generic single duplex ΔD and ΔB ddPCR assays provide a novel and sensitive way for quantifying T cells relatively fast, accurate, and independent of the cellular context.

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