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Brief report

Characterization of a multiplex digital PCR assay to quantify total T cells relative to chimeric antigen receptor-positive T cells

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Chimeric antigen receptor-T cells (CAR-T) have become a widely utilized therapy for B cell malignancies and are under investigation in early-phase clinical trials for a host of other hematologic and solid malignancies. Monitoring of CAR-T persistence has largely relied on quantitative PCR, flow cytometry, or a combination of these methodologies. Digital PCR (dPCR) has gained favor as a sensitive and user-friendly method for monitoring CAR-T persistence in patients after infusion and can be adapted to any CAR-T construct. Historically, CAR-T quantitation has been expressed in copies per microliter (copies/μL) or as a percentage of total nucleated cells, both of which fail to provide information on the broader immunologic context for the patient. We have developed a T cell-specific dPCR assay that can be multiplexed with CAR-T and control gene assays to provide quantitation of total T cells as well as CAR-T and total nucleated cells. This assay eliminates the need for redundant quantitation of T cells by flow cytometry and in combination with ultra-sensitive CAR-T assays can allow a greater depth of CAR-T quantitation relative to total T cells with minimal source sample needs.

INTRODUCTION

Chimeric antigen receptor-T cells (CAR-T) have revolutionized the treatment of B cell malignancies in pediatric and adult patients.[1–3] Prospective CAR-T target antigens in other hematologic and solid tumors are being evaluated in an ever-growing number of early-phase clinical trials.[4–6] CAR-T persistence is a crucial, albeit not exclusive, predictor of short-term efficacy and long-term survival in these patients.[7,8] Digital PCR (dPCR) is a sensitive and user-friendly monitoring method for CAR-T persistence in patients after infusion and can be adapted to any CAR-T construct.[9–14] Historically, dPCR CAR-T quantitation has been expressed in copies per microliter (copies/μL) or as a percentage of total nucleated cells, neither of which provides information on CAR-T cells as a percentage of total T cells. Flow cytometry can bridge this gap but requires a high level of expertise given the low sensitivity and high background of many CAR detection reagents.[10,12] We have designed a dPCR assay to quantify T cells relative to total nucleated cells, which can be multiplexed with CAR-specific dPCR to minimize sample volume, time, and resource needs while providing valuable clinical information for CAR-T patients.

RESULTS

The T cell receptor (TCR) dPCR assay was developed to be multiplexed with customized CAR-specific dPCR assays and with an albumin (ALB, HGNC:399) reference gene assay on a Qiacuity dPCR instrument (QIAGEN). It amplifies non-T cells with germline TCR alpha (TRA, HGNC:12027) loci (Figure 1 and methods); percentage of T cells can be calculated based on copies per microliter (copies/μL) of amplified TCR versus copies/μL of total nucleated cells (albumin reference gene). To characterize our assay, we generated serial dilutions of Jurkat cells, a T cell line, into MOLM 13 cells, an AML cell line (Jurkat:MOLM13), and vice versa (MOLM13:Jurkat). DNA from these dilutions was analyzed with the TCR and albumin assays to quantify percentage of T cells and compare with the calculated values (Figures 2A and 2B). The Pearson correlation coefficient (r) for Jurkat:MOLM13 dilutions was 0.995 (95% confidence interval [CI] = 0.9963–0.9999, p value < 0.0001). For MOLM13:Jurkat dilutions, r = 0.9965 (95% CI = 0.9753–0.9995, $p < 0.0001$).

To further characterize the TCR assay, peripheral blood mononuclear cells (PBMCs) from 8 healthy donors, as well as T cell-enriched fractions from the same donors, were tested. The percentage of T cells was quantified by flow cytometry, used as the "gold standard" against which the TCR assay was compared (Figures 2C and 2D). For bulk PBMCs, r = 0.9938 (95% CI = 0.9650–0.9989, $p < 0.0001$). For enriched T cell fractions, r = 0.8610 (95% CI = 0.3975–0.9744, $p = 0.006$). For both cell line serial dilutions and for healthy donor samples, we performed parallel experiments with the TCR assay via standard quantitative real-time PCR

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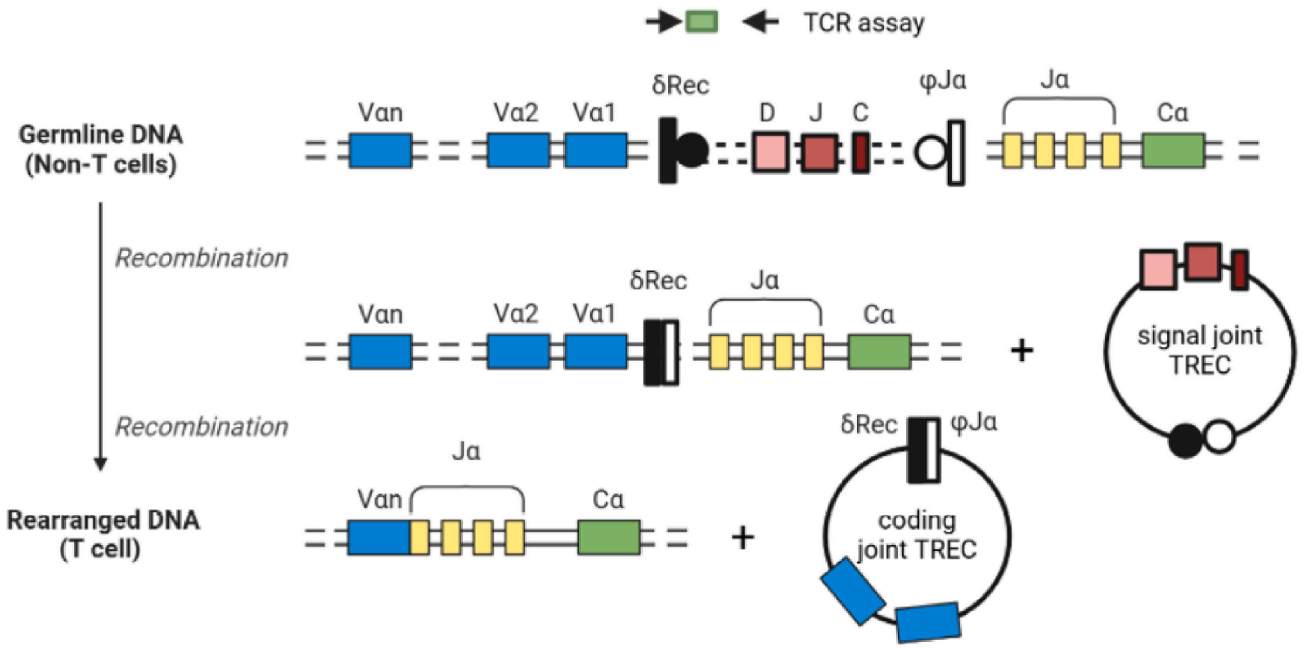

Figure 1. Location of TCR assay within TCRα/δ locus

Shown is a schematic of the rearrangement of the TCRα receptor (TRA, HGNC:12027) from germline state (present in non-T cells) to full rearrangement (mature T cells). The T cell receptor rearrangement excision circles (TRECs) produced by the rearrangement are shown (figure adapted from ref.[15]). The forward primer (right-pointing arrow) and probe (green rectangle) of the TCR assay are located in the δRec region and the reverse primer (left-facing arrow) is past the TREC cut site. Created in BioRender. Winters, A. (2024) BioRender.com/o75y339.

(real-time qPCR) and confirmed strong correlation persisted in all experiments (Figure S1).

Lastly, banked peripheral blood samples from subjects treated with the CAR-T construct UCD19 on a clinical trial (NCT05535855) were analyzed via multiplexed dPCR for quantitation of the UCD19 CAR with a CAR-specific assay, the TCR assay to quantify percentage of T cells, and albumin for total nucleated cells. We confirmed both strong correlation between TCR-calculated percentage of T cells and clinical flow cytometry data (Figure 2E) and correlation between clinical flow cytometry for UCD19 and our experimental multiplex assay (Figure 2F). For T cell comparisons, r = 0.9486 (95% CI = 0.7358–0.9909, $p = 0.0003$). The correlation was strongest with live cells, but was still high with total events as well (r = 0.9126, 95% CI = 0.5101–0.9872, $p = 0.0041$) (Figure S2). For UCD19 quantitation, r = 0.9947 (95% CI = 0.9696–0.9991, $p < 0.0001$) when comparing the multiplex dPCR to flow-based CAR quantitation (Figure 2F) and r = 0.9991 (95% CI = 0.9950–0.9998, $p < 0.0001$) when comparing dPCR in the clinical setting versus laboratory/multiplex setting (Figure S3). Visualization of the multiplexed dPCR data is shown in Figure S4. UCD19 CAR-T assay sensitivity and specificity are demonstrated in Figures S5 and S6, respectively.

DISCUSSION

We have developed and characterized a T cell-specific dPCR assay that can be multiplexed with CAR-T and control gene dPCR assays to provide quantitation of CAR-T cells, T cells, and nucleated cells in a single reaction. This assay eliminates the need for redundant quantitation of T cells by flow cytometry and in combination with ultrasensitive CAR-T dPCR assays can allow a greater depth of CAR-T quantitation relative to T cells with minimal source sample. The assay is built around the TREC locus within the TCRα gene (TRA, HGNC:12027),[15] such that the dPCR amplicon is only present in non-T cells with non-rearranged TCRα. The percentage of T cells can then be calculated relative to total nucleated cells in the sample. We confirm a strong correlation between flow cytometric and dPCR-based T cell quantitation in both healthy donor and patient-derived PBMC samples. We further demonstrate proof-of-concept for CAR- and T cell dPCR multiplexing experiments.

We faced some limitations with characterization of these assays including the need to validate copy numbers within cell lines that are known to develop aneuploidy when cultured over time. Albumin and TCR assays confirmed anticipated copy numbers in all cell lines when compared to published chromosome numbers.[16,17] Additionally, in calculating CAR cells/μL from average VCN, we were unable to correct for non-integrated viral copies due to the proprietary nature of the full vector sequence. Correlation coefficients at higher T cell concentrations, such as in the T cell-enriched donor samples, were generally weaker than for other samples. However, T cells at 80%–100% of a peripheral blood sample are not typical of patients receiving CAR-T cells, particularly after lymphodepleting chemotherapy. Given that

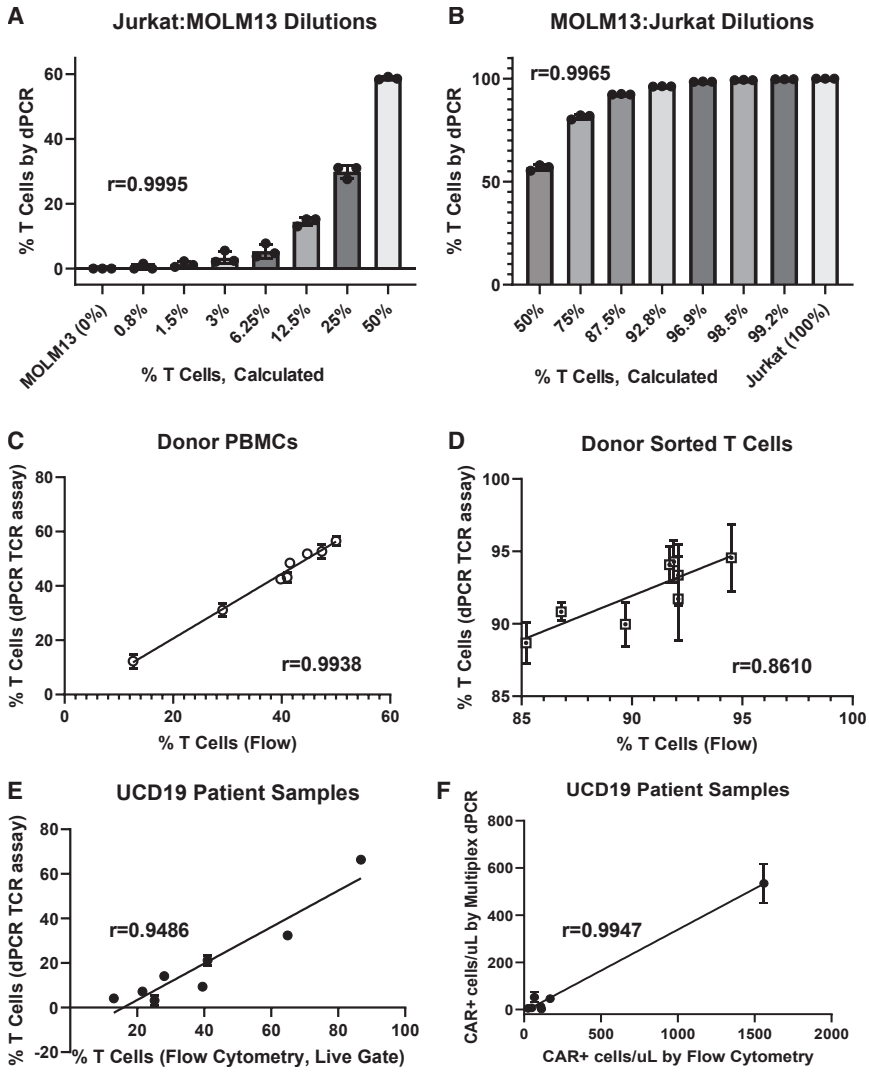

Figure 2. Correlation between T cell quantitation as measured by the TCR dPCR assay and theoretical or flow cytometry quantified T cells

(A and B) Jurkat and MOLM13 serial dilutions. *p*-values for correlation < 0.0001. (C and D) Healthy donor peripheral blood mononuclear cells (PBMCs) and enriched T cells. *p*-values for correlation < 0.0001 (C) and 0.006 (D). (E) Peripheral blood from UCD19 subjects at study time points ($p = 0.0003$). (F) Correlation between UCD19 CAR quantitation by clinical flow cytometry versus our multiplexing assay ($p < 0.0001$). dPCR CAR quantitation (copies/uL provided as output obtained from QIAcuity) was normalized to known VCN of the product (copies/cell) by dividing CAR copies/uL by CAR copies/cell to calculate CAR cells/μL.

dPCR is DNA-based and flow cytometry is cell-based, there may be inherent discrepancies related to live vs. dead cells—both of which can yield DNA. For UCD19 subjects, we compared dPCR quantitation to flow cytometry quantitation gated on both live cells and total events. Similarly, although the sample source for flow cytometry was enriched PBMCs whereas the source for dPCR was whole blood, the correlations were still strong. Therefore, the TCR dPCR assay serves as a valuable surrogate to flow cytometry in the context of immunologic monitoring of CAR-T patients. Its application to other clinical scenarios is likely to broaden as it is utilized in clinical laboratories.

MATERIALS AND METHODS

Assay design

The TCR assay was built across the TCR rearrangement excision circle (TREC) sequence of the TCRα/δ locus (TRA, HGNC:12027) on chromosome 14,[15] for direct detection of non-T cells with germline TRA (Figure 1; Table 1). To calculate percentage of T cells relative to total nucleated cells, this assay was paired with a control gene, albumin (ALB, HGNC:399), and the percentage of T cells was calculated as ([ALB copies/μL – TCR copies/μL]/ALB copies/μL × 100). To demonstrate multiplexing capability with CAR dPCR assays, the TCR and ALB assays were combined with a CAR assay specific to the UCD19 construct (Table 1). Primers for TCR and UCD19 assays were ordered from Integrated DNA Technologies, Inc.; custom minor groove binder (MGB) Taqman probes were obtained from Thermo Fisher Scientific. The ALB dPCR assay was purchased from Bio-Rad (assay ID dHsaCNS381279675).

Sample acquisition and processing

Jurkat and MOLM13 cell lines were propagated in RPMI 1640 medium + 10% fetal bovine serum. For serial dilutions, live cells were counted via Trypan blue exclusion; Jurkat and MOLM13 cells were mixed at the described ratios. Mixtures of Jurkat and MOLM13 cells were pelleted, and DNA was extracted using a QIAmp DNA Mini kit (QIAGEN). DNA quantity and purity were assessed via Nanodrop.

Leukopaks were obtained as non-regulated medical waste from Children's Hospital Colorado Blood Donation Center. PBMCs were isolated from 8 healthy donors using lymphocyte separation medium (Corning) following a density gradient centrifugation protocol. Half of these PBMC samples were enriched with EasySep Human T cell Isolation Kit (StemCell). Flow cytometry was performed on bulk PBMCs or enriched T cell fractions using a Fortessa X-20 (T cell population defined as $CD3^+$, $CD56^-$, $CD4^+$, or $CD8^+$), followed by DNA extraction as described previously.

Whole blood samples were collected at clinically relevant time points from subjects on a UCD19 CAR-T cell clinical trial (NCT05535855)

Table 1. Digital PCR assay details

Assay Name	Forward primer	Reverse primer	MGB probe	Fluorophore
TCR	TTTGTAAAGGTGCCCACTCC	ACACTCATCTTTTGTTCCTGTTTG	TGGGTACCGGGTTAA	FAM or VIC[a]
UCD19	GGAACCGTGAAACTGCTGAT	CGAAGGTGTAGGGAAGGGTA	ACTGACTACTCACTGAC	FAM
ALB	MIQE context sequence (hg19|chr4:74269957-74270079:+) ATTATTGGTTAAAGAAGTATATTAGTGCTAATTTCCCTCCGTTTGTCCT AGCTTTTCTCTTCTGTCAACCCCACACGCCTTTGGCACAATGAAGTGGG TAACCTTTATTTCCCTTCTTTTTCT			Cy5

[a]FAM fluorophore used for experiments without UCD19 assay; VIC fluorophore used for multiplexing experiments.

and used for trial-specified correlative studies. DNA aliquots from 3 subjects at 2–3 time points apiece (8 samples) were obtained after study approval from the Gates Institute BioBank Advisory Committee and utilized for multiplexing experiments of TCR, UCD19, and ALB assays. Samples were utilized under IRB secondary use protocol no. 21–3730 (approved October 21, 2022). dPCR CAR quantitation was normalized to average vector copy number (VCN) of the product to calculate CAR cells/μL.

PCR experiments

Nanoplate-based dPCR was performed using the Qiacuity platform (QIAGEN) per manufacturer protocols, as described previously.[18] A 55°C annealing temperature was used, with 200 nanograms/sample of DNA template. Samples were analyzed in duplicate in 4 separate experiments, except for UCD19 samples, which were analyzed in triplicate in 3 separate experiments. Cell line and PBMC/T cell experiments were performed utilizing both FAM- and VIC-labeled TCR probe in combination with Cy5-labeled ALB probe. Real-time qPCR was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) as described previously,[18] utilizing FAM-labeled TCR and Cy5-labeled ALB probes, and consisted of 2 separate experiments with samples analyzed in triplicate. Additional details regarding PCR experiments to determine sensitivity and specificity of the CAR-T specific assay can be found in the supplemental material.

Statistical analyses

Correlation between calculated percent T cells (for cell line dilutions) or percent T cells quantified by flow cytometry (PBMC and T cell fractions, UCD19 subject samples) was quantified by Pearson correlation coefficient (r). r was also utilized to compare quantitation of the UCD19 CAR gene between our research calculations and the UCD19 dPCR quantitation performed as part of the clinical trial.

DATA AVAILABILITY

Raw data will be made available upon reasonable request to the corresponding author. Assay details are listed in Table 1.

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AUTHOR CONTRIBUTIONS

L.A.M., T.J.F., and A.C.W. conceptualized the study. L.A.M., T.J.F., M.E.K., and J.E.S. designed the TCR assay. L.A.M. designed the UCD19 assay. L.A.M., L.S., M.Y., and A.C.W. performed all experiments and data analysis. K.R.J. and A.O. generated flow cytometry and clinical dPCR data for UCD19 subject samples. A.C.W. was the lead author on the manuscript, but all authors contributed to the preparation and editing of the manuscript.

DECLARATION OF INTERESTS

T.J.F. discloses employment with Sana Biotechnology, Inc.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2025.200981.

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