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Optimized Assessment of qPCR-Based Vector Copy Numbers as a Safety Parameter for GMP-Grade CAR T Cells and Monitoring of Frequency in Patients

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Chimeric antigen receptor (CAR) T cells are considered genetically modified organisms (GMOs) and constitute gene therapy medicinal products. Thus, CAR T cell manufacturing for clinical application is strictly regulated. Appropriate methods to assess vector copy numbers (VCNs) in CAR T cell products and monitoring of CAR T cell frequencies in patients are required. Quantitative polymerase chain reaction (qPCR) is the preferred method for VCN assessment. However, no standardized procedure with high reproducibility has been described yet. Here, we report on a single copy gene (SCG)-based duplex (DP)-qPCR assay (SCG-DP-PCR) to determine VCN in CAR T cell products. SCG-DP-PCR was validated and compared to the absolute standard curve method (ACM) within the framework of a clinical trial treating patients with good manufacturing practice (GMP)-grade CAR T cells at the University Hospital Heidelberg. Methodologically, SCG-DP-PCR displayed technical advantages over ACM and minimized mathematical analysis. SCG-DP-PCR, as a highly reproducible approach, can be used for clinical follow-up of patients treated with CAR T cells or other GMOs and might replace established methods for VCN quantification. This work will enable clinicians to assess VCN, as well as CAR T cell frequencies, in patients as a basis for decisions on subsequent therapies, including repeated CAR T cell administration.

INTRODUCTION

Chimeric antigen receptor (CAR) T cells constitute a highly promising adoptive immunotherapy for cancer. CAR T cells directed against CD19 have shown remarkable clinical results in heavily pretreated patients with relapsed or refractory lymphoid malignancies, including pediatric[1,2] and adult[3,4] acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL),[5,6] and other non-Hodgkin's lymphoma (NHL).[7–10] Development of CARs for treatment of other hematologic malignancies or solid tumors is ongoing.[11] Anti-CD19 CAR T cells have been approved by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The University Hospital Heidelberg initiated the first investigator-initiated trial (IIT) for CAR T cell therapy in Germany. The Heidelberg Chimeric Antigen Receptor T Cell Trial Number 1 (HD-CAR-1; European Union Drug Regulating Authorities Clinical Trials Database [EudraCT] no. 2016-004808-60; ClinicalTrials.gov: NCT03676504) is a monocentric, open-label, prospective phase I/II trial initiated in September 2018 with in-house leukapheresis as well as CAR T cell manufacturing for treating adult and pediatric ALL, adult CLL, and NHL patients with autologous T lymphocytes transduced with a third-generation CAR vector (RV-SFG.CD19.CD28.4-1BBzeta) targeting CD19.[12] Due to the fact that CAR T cells are considered genetically modified organisms (GMOs), they constitute gene therapy medicinal products (GTMPs). Hence, CAR T cells are manufactured according to good manufacturing practice (GMP) standards. Regulatory authorities require extensive safety evaluation of advanced therapy medicinal products (ATMPs), i.e., cellular immune therapies, such as CAR T cells. To warrant safety, transgene copies within a CAR T cell product, i.e., vector copy numbers (VCNs), have to be assessed prior to patient administration. Additionally, response to CAR T cell treatment is associated with expansion,[6] as well as persistence of CAR T cells in treated patients.[10,13] Therefore, the assessment of CAR T cell levels in patients at different time points after CAR T cell administration is crucial for deciding on further patient therapies. CAR VCN assessment in CAR T cell products and monitoring CAR T cells in the peripheral blood (PB) of treated patients are usually performed via quantitative PCR (qPCR).[14–17]. Here, we propose a duplex (DP) qPCR strategy based on a single-copy gene (SCG; SCG-DP-PCR), i.e. ribonuclease (RNase)P RNA component H1 gene (*RPPH1*; RNaseP in the following) (single copy per haploid human genome), for accurate and robust determination of VCN in CAR T cell products and in PB samples of treated patients. SCG-DP-PCR was compared to the absolute standard curve singleplex (SP)

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Table 1. Comparison of Relevant Parameters of qPCR Reactions (Efficiency, Linearity) in the Singleplex Setup (SP-CAR) of ACM and the SCG-DP-PCR Duplex Setup (DP-CAR, DP-RNaseP)

	PCR Reaction			Efficiency			Correlation Coefficient (R^2)		
ACM	SP-CAR			103.5% ± 7.1%			0.99		
SCG-DP-PCR	DP-CAR			104.2% ± 2.1%			0.99		
	DP-RNaseP			99.3% ± 1.6%			0.99		
Copies/Std.	Ct (SP-CAR)			Ct (DP-CAR)			Ct (DP-RNaseP)		
	Val. 1	Val. 2	Val. 3	Val. 1	Val. 2	Val. 3	Val. 1	Val. 2	Val. 3
3×10^5	20.9 ± 0.1	21.4 ± 0.1	21.5 ± 0.1	21.9 ± 0.1	21.9 ± 0.0	21.9 ± 0.1	21.5 ± 0.0	21.3 ± 0.0	21.5 ± 0.1
3×10^4	24.2 ± 0.1	24.2 ± 0.1	24.9 ± 0.2	25.1 ± 0.0	25.1 ± 0.0	25.1 ± 0.1	24.8 ± 0.0	24.6 ± 0.0	24.9 ± 0.0
3×10^3	27.5 ± 0.1	27.6 ± 0.1	27.6 ± 0.0	28.4 ± 0.0	28.4 ± 0.0	28.4 ± 0.0	28.1 ± 0.1	28.0 ± 0.0	28.2 ± 0.1
3×10^2	30.9 ± 0.0	31.1 ± 0.1	31.6 ± 0.3	31.8 ± 0.2	31.9 ± 0.2	31.8 ± 0.1	31.5 ± 0.1	31.3 ± 0.1	31.5 ± 0.1
30	33.1 ± 0.1	34.0 ± 0.3	35.3 ± 0.6	34.4 ± 0.6	34.7 ± 0.2	34.8 ± 0.2	34.9 ± 0.6	34.5 ± 0.0	35.1 ± 0.5

qPCR data from standards of three independent experiments were analyzed by linear regression (validation [Val.] 1, 2, and 3). Efficiencies of three experiments are represented as mean ± SD. Reactions were performed in triplicates. Ct values are represented as mean ± SD. Std., standard.

qPCR approach (absolute standard curve method [ACM]) and validated within the framework of HD-CAR-1.

RESULTS

Method validation and evaluation of SCG-DP-PCR were performed as follows: efficiencies and correlation coefficients (R^2) of PCR reactions within the duplex PCR (targeting the CAR transgene and SCG) were compared before similar efficiencies over the relevant transgene copy number range were confirmed. A final proof of similar efficiencies was achieved by the use of an efficiency control (EC) sample (description in Materials and Methods) as a direct testing method. Following this method validation, VCNs in healthy donor samples were assessed via ACM and SCG-DP-PCR, and both methods were directly compared. Subsequently, SCG-DP-PCR was applied on follow-up samples of patients treated with HD-CAR-1 CAR T cells. Differences and influencing factors of ACM and SCG-DP-PCR were determined.

Efficiencies and Linearity of PCR Reactions (ACM and SCG-DP-PCR)

For method validation, efficiency and linearity (correlation coefficient) of PCR reactions of ACM (SP-CAR) and SCG-DP-PCR (DP-CAR, DP-RNaseP) were assessed by linear regression analysis of standards. Standard curves were only considered valid if a R^2 of above 0.98 and mean efficiencies of 100% ± 10% were achieved. SP-CAR PCR reaction displayed an efficiency of 103.5 ± 7.1%; efficiencies of 104.2 ± 2.1% and 99.3 ± 1.6% were achieved for DP-CAR and DP-RNaseP PCR reactions, respectively (Table 1).

Standard curves were generated for SP-CAR, DP-CAR, and DP-RNaseP in three validation experiments. Figure 1 illustrates the results obtained in one of three validation experiments.

Relative Efficiency Plot of SCG-DP-PCR

The relative efficiency plot compared simultaneous PCR reactions over the tested transgene copy number range by calculation of comparative threshold cycle (ΔCt; DP-CAR − DP-RNaseP) and graphical analysis (semi-logarithmic display; transgene copies [log10]: x axis; ΔCt: y axis). Figure 2 displays generated relative efficiency plots. ΔCt (DP-CAR − DP-RNaseP) in the DP-PCR standards was similar for the 4 higher copy standards ($3 \times 10^2 - 3 \times 10^5$ copies/reaction). The smallest standard (30 copies/reaction) was excluded from analysis due to high standard deviation in replicates. Consequently, diagnostic measuring range for this method setup was defined within $3 \times 10^2 - 3 \times 10^5$ copies/reaction. In this range, the slope of the regression curve was 0.0168.

Efficiency Control Sample

The EC sample was added to every duplex PCR experiment. The calculated ΔCt values (DP-CAR − DP-RNaseP) in this control sample ranged between $Ct \geq -0.31$ and $Ct \leq 0.17$. The application of $2^{-\Delta Ct\ (DP\text{-}CAR\ -\ DP\text{-}RNaseP)}$, acceptable copy numbers of the CAR transgene relative to RNaseP gene of 0.89, 1.06, and 1.24 (1.06 ± 0.18) was achieved (Table 2). The amplification plot of the EC sample from one validation experiment (Figure S1; Supplemental Materials and Methods) illustrates similarity of the CAR transgene and RNaseP gene amplifications in the SCG-DP-PCR setup.

Vector Copy Numbers

VCNs were obtained by mathematical extrapolation of regression curves to sample signals via ACM and by relative SCG-DP-PCR applied. The application of SCG-DP-PCR on CAR T cell samples generated from healthy donors, an increase of 0.8 ± 0.2 VCN/cell, was observed when compared to ACM (Table 3).

CAR T Cell Monitoring in Patients Using a Validated SCG-DP-PCR

Following validation on healthy donor-derived CAR T cells, SCG-DP-PCR was also used to assess CAR T cell expansion in HD-CAR-1 patient samples. Figure 3 displays CAR T cell expansion

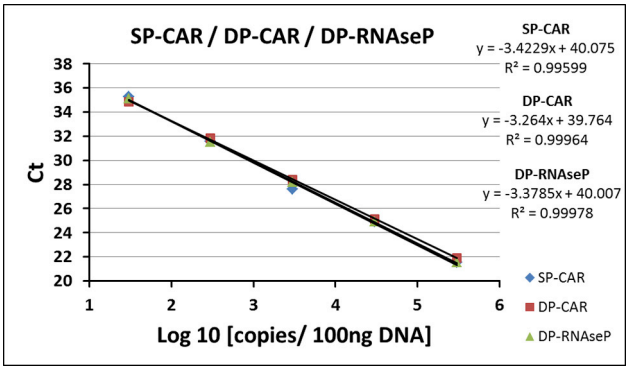

Figure 1. Efficiencies and Linearity of PCR Reactions (ACM and SCG-DP-PCR)

Standard curves of qPCR reactions. Exemplary data from one validation experiment are shown. Mean Ct values from qPCR were used for linear regression. Reactions were performed in triplicates. Ct, threshold cycle.

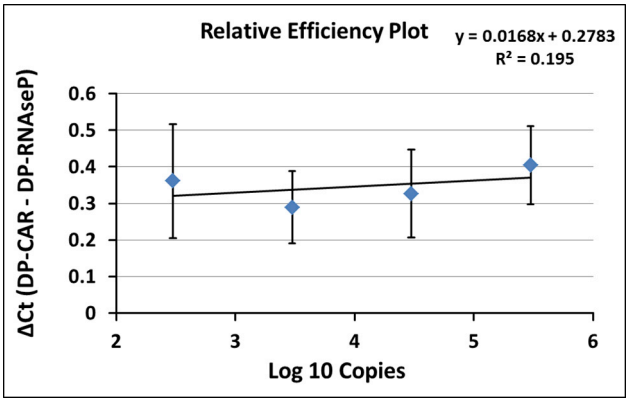

Figure 2. Relative Efficiency Plot of SCG-DP-PCR

Graphical analysis of relative efficiencies from 4 higher duplex standards (3×10^2 – 3×10^5 copies) of three independent experiments (validation 1, 2, and 3). Results are represented as mean ± standard deviation (SD). Reactions were performed in triplicates. Smallest duplex standard, i.e., 30 copies, was excluded from analysis due to high SD.

within PB samples of 3 HD-CAR-1 patients at different time points after CAR T cell administration.

DISCUSSION

CAR-expressing T cells targeting CD19 are currently revolutionizing the treatment of patients with hematologic malignancies and are becoming an integral part of the clinical hematological practice.[18] CAR-transduced T cells in clinical practice are mostly generated by the use of viral vectors. These vectors originate from the *Orthoretrovirinae* subfamily of *Retroviridae*, i.e., γ-retrovirus and lentivirus. They convert their RNA genome into cDNA and integrate this genetic information into the genome of the target cell, thus enabling long-term transgene expression. However, the use of viral vectors for CAR T cell manufacturing for therapeutic purposes requires strict biosafety testing. The exclusion of the presence of replication-competent retrovirus (RCR) within CAR T cell samples, as well as evaluation of the average number of integrated vector copies per transduced T cell, is mandatory. A variety of different strategies for VCN determination have been used, relying mainly on ACM,[14] as well as relative quantification approaches.[15–17]. ACM is associated with potential inaccuracies, due to the need of standard curves. Relative quantification of VCN displays practical and technical issues. The $2^{-\Delta\Delta Ct}$ method for relative quantification was described by Livak and Schmittgen[19] and was originally used for qPCR analysis of fold changes in gene expression. It has been also applied to quantify transgene VCNs in CAR T cell samples.[16,17] In addition to the general preconditions of approximately similar and optimal efficiencies of 100% for the target and the reference qPCR reactions, VCN quantification by the use of the $2^{-\Delta\Delta Ct}$ method requires special conditions, i.e., a SCG as a reference gene and a special calibrator sample. Especially, the generation of a calibrator sample consisting of a CAR T cell clone with a defined and stable VCN of the CAR transgene constitutes a major issue in terms of practicability and feasibility. Hence, a quantitative approach that operates independently of impractical factors, such as standard curves or calibrator samples, additionally economizing material and time, is a highly desirable tool for clinical CAR T cell research. We developed and validated an adapted SCG-based quantification approach (SCG-DP-PCR) to address these issues. SCG-DP-PCR VCN assessment applies the $2^{-\Delta Ct}$ method that is derived after implementation of the following assumption to the $2^{-\Delta\Delta Ct}$ method:[19] similar and optimal efficiencies of 100% for the target and the reference qPCR reactions result in similar Ct values of the EC sample (for mathematical deduction of the $2^{-\Delta Ct}$ method see Supplemental Materials and Methods A2). Figure 4, left, summarizes ACM, SCG-DP-PCR ($2^{-\Delta Ct}$), and the established $2^{-\Delta\Delta Ct}$[19] methods. Corresponding required samples for VCN determination are depicted in Figure 4, right.

The validation of SCG-DP-PCR was performed by testing the following: (1) the efficiency and linearity of PCR-reactions, (2) the constancy of PCR efficiencies within the relevant transgene copy range, and (3) the similarity of PCR efficiencies, as well as RNaseP SCG status, by using an EC sample and applying the $2^{-\Delta Ct}$ method.

SCG-DP-PCR reactions displayed similar efficiencies close to 100% and almost optimal linearities ($R^2 > 0.98$) (Table 1; Figure 1). Constant PCR efficiencies over the relevant concentration range, i.e., similar ΔCt (DP-CAR − DP-RNaseP) in every duplex standard sample, were confirmed by a relative efficiency plot. Here, log10 copies (x axis) were plotted against ΔCt (DP-CAR − DP-RNaseP) (y axis). Via linear regression, a slope of <0.1 (optimal 0) was obtained, proving constancy (Figure 2). Efficiency validation was completed, verifying similar amplification efficiencies of DP-CAR and DP-RNaseP PCR reactions and confirming the SCG status of RNaseP using the EC sample. CAR transgene copies relative to the RNaseP gene of 0.89, 1.06, and 1.24 (1.06 ± 0.18) for the EC sample were achieved, lying within our accepted range (Table 2; Figure S1). Consequently, we established a relative qPCR approach that is independent from a calibrator sample. RNaseP could be detected in all samples we assessed.

Table 2. Analysis of EC Samples from Three Validation Experiments (Validation 1, 2, and 3)

	Validation 1	Validation 2	Validation 3
Ct (DP-CAR)	24.74 ± 0.08	24.25 ± 0.01	25.19 ± 0.04
Ct (DP-RNaseP)	24.83 ± 0.04	24.56 ± 0.04	25.02 ± 0.09
ΔCt	−0.09	−0.31	0.17
SCG-DP-PCR $2^{-\Delta Ct}$ method	1.06	1.24	0.89

Reactions were performed in triplicates. Ct values are represented as mean ± SD. Mean Ct values from qPCR were used for ΔCt calculation.

In the unlikely case of a missing RNaseP signal, a repetition of the PCR reaction is highly advised.

After validation, SCG-DP-PCR was compared to ACM by measuring VCN of CAR T cells generated from healthy donors (Table 3). For SCG-DP-PCR, a higher VCN 0.8 ± 0.2/cell was assessed when compared to ACM. We assume that via ACM VCN might be under-represented due to the influence of differing reaction conditions within an experimental setup with standard samples (standard curve; no genomic DNA) and GMO samples (target sample; genomic DNA). Additional factors, such as well-to-well variations or errors in DNA concentration measurement, influence ACM results. Moreover, sequential dilution of standards, as well as mathematical extrapolation, affects ACM and might contribute to observed VCN variations. However, underlying reasons for VCN discrepancy of ACM and SCG-DP-PCR were not analyzed any further. In SCG-DP-PCR, reactions are performed within one well. Hence, a main technical requirement for SCG-DP-PCR is the use of highly efficient and compatible primer and probe sets targeting the CAR transgene and the SCG. Methodical differences and influencing factors on ACM and SCG-DP-PCR are summarized in Table 4. SCG-DP-PCR was subsequently applied on HD-CAR-1 patient samples achieving clinical relevant

Table 3. Validation and Comparison of the VCN Determination by Different Strategies ACM and SCG-DP-PCR

	Validation 1	Validation 2	Validation 3
Ct (DP-CAR)	24.77 ± 0.03	24.28 ± 0.03	25.31 ± 0.02
Ct (DP-RNaseP)	24.93 ± 0.05	24.80 ± 0.01	25.50 ± 0.02
ΔCt	−0.16	−0.52	−0.19
VCN/cell (SCG-DP-PCR)	2.2	2.9	2.3
VCN/cell (ACM)	1.3	2.3	1.5
Copies/Std. (ACM)	Ct (Validation 1)	Ct (Validation 2)	Ct (Validation 3)
3×10^5	20.9 ± 0.1	21.4 ± 0.1	21.5 ± 0.1
3×10^4	24.2 ± 0.1	24.2 ± 0.1	24.9 ± 0.2
3×10^3	27.5 ± 0.1	27.6 ± 0.1	27.6 ± 0.0
3×10^2	30.9 ± 0.0	31.1 ± 0.1	31.6 ± 0.3
30	33.1 ± 0.1	34.0 ± 0.3	35.3 ± 0.6

Three independent experiments were performed (validation 1, 2, and 3). Mean Ct values from qPCR were used for linear regression (ACM) and ΔCt calculation (SCG-DP-PCR). Reactions were performed in triplicates. Ct values are represented as mean ± SD.

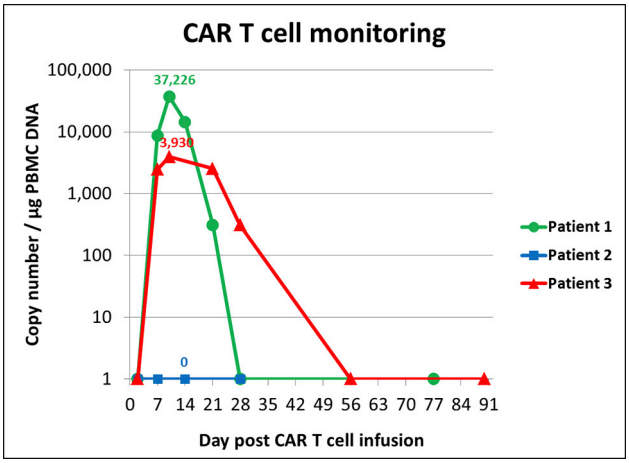

Figure 3. CAR T Cell Monitoring in Patients Using a Validated SCG-DP-PCR
CAR T cell monitoring in peripheral blood (PB) samples of three different patients, assessed by validated SCG-DP-PCR. Patient 1 was assessed by absolute standard curve method (ACM) before SCG-DP-PCR was established in our GMP facility for all further quantification experiments. Patients were treated with a dose of 1×10^6 $CD19^+CAR^+$-transduced T cells per square meter body surface at day 0. Different kinetics of CAR T cells were observed. Determined peak copy numbers are included into the graph above peak data points. No CAR T cells were detected in samples of patient 2. The samples were not measured by other validated methods.

data on CAR T cell expansion (Figure 3). No CAR T cells could be detected in samples of peripheral blood in patient 2. Given that the patient displayed a partial response to treatment, CAR T cells might have accumulated at the cancer cell site without circulating into the peripheral blood.

Overall, validated SCG-DP-PCR represents a less error-prone method to address the regulatory safety release criterion VCN in CAR T cell products compared to ACM. It is highly suitable to follow up CAR T cells in the peripheral blood of patients. Additionally, RNaseP represents an internal control for every PCR reaction of patient samples. Our main aim, to develop a suitable approach for standardization of VCN assessment in the field of clinical CAR therapy, was achieved. Importantly, the lack of standardized detection methods for the monitoring of CAR T cells or other GMOs in patients post-treatment could be overcome by SCG-DP-PCR. Moreover, given the lack of a calibrator sample, this relative quantification approach can be easily transferred and established in other laboratories. Subsequently, the important ability to monitor the expansion of CAR T cells or other GMOs in patients could be extended to many hospitals. This might improve the assessment of the course of diseases of patients in the field of gene therapy, particularly CAR T cell therapy.

MATERIALS AND METHODS

Manufacturing of CAR T Cells

Clinical-grade CAR T cells were produced in the GMP Core Facility of our institution from healthy donors, as well as from patients enrolled in the HD-CAR-1 trial. Standardized CAR T cell

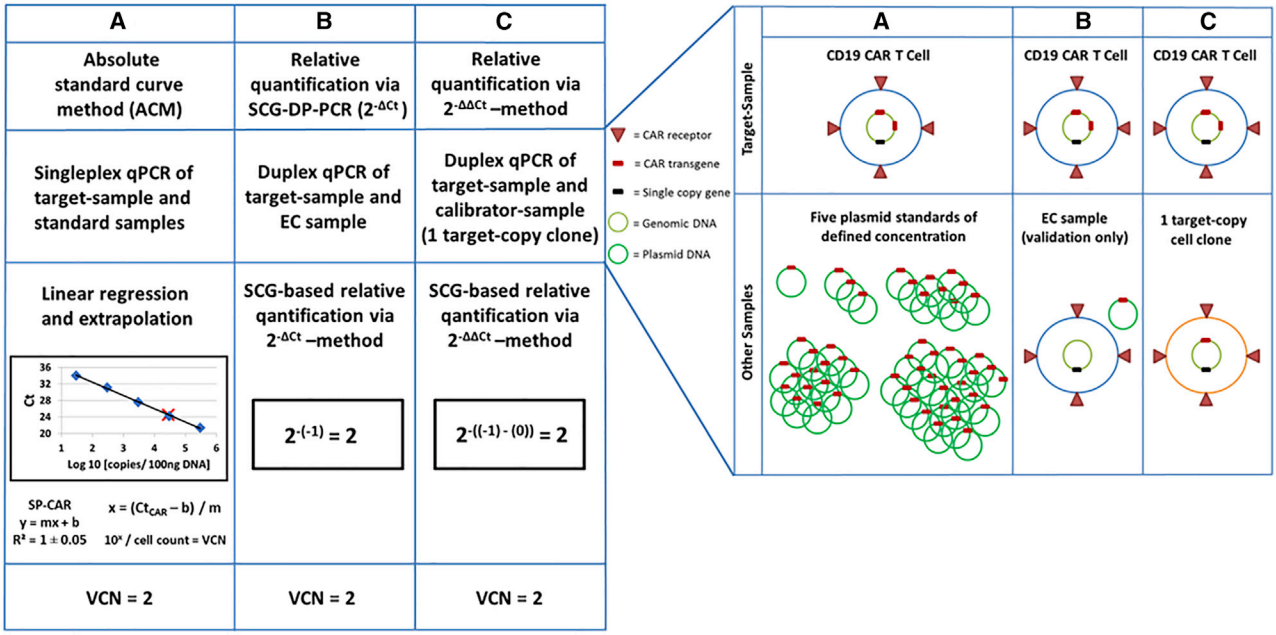

Figure 4. Summary of ACM, SCG-DP-PCR ($2^{-\Delta Ct}$), and the Established $2^{-\Delta\Delta Ct}$[19] Methods

(Left) Schematic illustration of VCN determination with anti-CD19 CAR T cells harboring 2 CAR transgene copies under assumption of optimal PCR conditions. The figures and calculations refer to a haploid human genome to decrease complexity. Different qPCR strategies are illustrated. (A) Absolute standard curve method (ACM). (B) Relative quantification via the $2^{-\Delta Ct}$ method (SCG-DP-PCR). (C) Relative quantification via the $2^{-\Delta\Delta Ct}$ method. SCG, single copy gene; VCN, vector copy number; qPCR, quantitative polymerase chain reaction; Ct, threshold cycle; DP, duplex; EC, efficiency control. (Right) Corresponding required samples for VCN determination by different qPCR methods. Schematic illustration of samples required for different qPCR-strategies. (A) Absolute quantification via the standard curve method. (B) Relative quantification via the $2^{-\Delta Ct}$ method. (C) Relative quantification via the $2^{-\Delta\Delta Ct}$ method.

manufacturing was established and validated before initiating HD-CAR-1.[12] Informed consent was obtained from all healthy donors and HD-CAR-1 patients, according to the Declaration of Helsinki. Approval from the Ethics Committee of the University Heidelberg (AFmu-405/2017), the Paul-Ehrlich-Institut (PEI) competent authority (EudraCT no. 2016-004808-60), and the responsible regional authority (federal authority no. 3148/02) was granted in October 2017, September 2018, and August 2018, respectively. The first HD-CAR-1 patient was included in September 2018 and dosed in October 2018. Currently, 14 patients have been screened for treatment, and 12 patients have been enclosed.

In brief, healthy donors and patients following enrollment underwent unstimulated leukapheresis for collection of peripheral blood mononuclear cells (PBMCs). PBMCs were transduced with the RV-SFG.CD19.CD28.4-1BBzeta retroviral vector (kindly provided by Professor Malcolm Brenner, Baylor College of Medicine, Houston, Texas) at our GMP Core Facility after activation with anti-CD3 and anti-CD28 antibodies (MACS GMP Pure; Miltenyi Biotec, Bergisch Gladbach, Germany) and culturing with interleukin (IL)-7 (10 ng/mL) (R&D System, Minneapolis, USA) and IL-15 (5 ng/mL) (Cellgenix, Freiburg, Germany). RV-SFG.CD19.CD28.4-1BBzeta carries an anti-CD19 single-chain variable fragment (scFv) derived from the FMC63 antibody inserted within the SFG retroviral backbone. The transmembrane domain is derived from CD28, the hinge domain from the human immunoglobulin G_1 (IgG_1)-CH_2CH_3 domain. 4-1BB is inserted between the CD28 and the CD3ζ domain. CAR T cells derived from healthy donors were collected and directly analyzed for VCN validation experiments. HD-CAR-1 patients were treated with escalating CAR T cell doses (1–20 × 10^6-transduced cells/m^2 body surface area [BSA]) after lymphodepletion with fludarabine (30 mg/ m^2 BSA) and cyclophosphamide (500 mg/m^2 BSA) for 3 days. PB samples from HD-CAR-1 patients were collected for analysis on days 1, 2, 3, 7, 14, 21, 28, 56, and 90 after CAR T cell administration.

Isolation of PBMCs

PBMCs containing CAR T cells from healthy donors and HD-CAR-1 patient PB samples were isolated by Ficoll density gradient (Linaris, Dossenheim, Germany) and genomic DNA extracted (cat. #51104, QIAamp DNA Blood Mini; QIAGEN). DNA concentration was measured by UV spectroscopy (NanoDrop OneC; Thermo Fisher Scientific, Applied Biosystems). Samples were diluted to a final concentration of 20 ng/μL in nuclease-free H_2O.

qPCR Methods

SCG-DP-PCR and ACM qPCR were performed on genomic DNA derived from PBMCs. To determine the number of integrated CD19.CD28.4-1BBzeta copies in CAR T cells, i.e., VCN, 100 ng genomic DNA isolated from patients and healthy-donor PBMCs

Table 4. Summary of Differences/Influencing Factors of Experimentally Compared Two Strategies ACM and SCG-DP-PCR

Factors	Absolute Standard Curve Method (ACM)	SCG-DP-PCR
Experiment	multiple wells (single PCR reaction/well)	single well (duplex PCR reactions)
	standard curve	no standard curve required
	no genomic DNA within PCR reaction	genomic DNA within PCR reactions
	no internal control	RNaseP signal as internal control
	extensive experimental setup	fast experimental setup
VCN analysis	extensive: linear regression	fast: relative calculation
	mathematical extrapolation	$2^{-\Delta Ct\ (DP\text{-}Car\ -\ DP\text{-}RNaseP)}$
Method validation	standard curve validation	extensive efficiency validation

was amplified using the StepOnePlus real-time PCR system (Applied Biosystems). Whereas ACM was performed as the SP PCR targeting only the CAR transgene (SP-CAR), SCG-DP-PCR amplifies the CAR transgene (DP-CAR) and the SCG RNaseP (DP-RNaseP) simultaneously.

Thermal cycling for all PCR experiments was performed using the following amplification conditions: 2 min for 50°C, 10 min for 95°C, followed by 40 cycles of 15 s 95°C and 1 min 60°C. Primers, probes, and TaqMan Gene Expression Master Mix were purchased from Applied Biosystems. Detailed parameters of the qPCR experiments are comprised within Supplemental Materials and Methods B). Nontemplate control (NTC), biological negative control (nontransduced donor cells), and the RV-SFG.CD19.CD28.4-1BBzeta plasmid, as positive control, were included within all experiments.

Absolute Standard Curve Method

ACM was performed using conventional plasmid standard curves generated via serial dilution of RV-SFG.CD19.CD28.4-1BBzeta plasmid DNA in nuclease-free H_2O (30, 3×10^2, 3×10^3, 3×10^4, 3×10^5 plasmid copies per PCR reaction). The following primers and probe were used for the SP-CAR qPCR: CAR transgene forward primer (FP): 5′-AGCTGCCGATTTCCAGAAGA-3′, reverse primer (RP): 5′-GCGCTCCTGCTGAACTTCA-3′, and probe: FAM-5′-AAGGAGGATGTGAACTGAGA-3′-MGB/NFQ. FP binds within the 4-1BB sequence, RP within the CD3ζ sequence, and the probe in the transition between 4-1BB and CD3ζ.

SCG-DP-PCR

SCG-DP-PCR was established and validated on CAR T cells generated from 3 healthy donors within the framework of the approval of the HD-CAR-1 trial for the regulatory authorities.

For SCG-DP-PCR validation, genomic DNA was added to RV-SFG.CD19.CD28.4-1BBzeta plasmid DNA in a 1:1 ratio of copies. Via serial dilution, a duplex standard curve (30, 3×10^2, 3×10^3, 3×10^4, 3×10^5 copies per PCR reaction) was generated to target the CAR transgene, as well as the RNaseP gene.

For SCG-DP-PCR VCN calculation, the $2^{-\Delta Ct}$ method, based on the previously described $2^{-\Delta\Delta Ct}$ method,[19] was used. The mathematical evaluation of experimentally generated qPCR data applying SCG-DP-PCR using the $2^{-\Delta Ct}$ method or via ACM is described in detail within the Supplemental Materials and Methods A.

The following primer sets were used for SCG-DP-PCR reactions:

(1) Sequences of forward, reverse primer, and probe targeting the CAR transgene were used, as described before for the SP-CAR qPCR (ACM).
(2) RNaseP: Copy Number Reference Assay, RNaseP (cat. #4403326, TaqMan; Applied Biosystems), containing RNaseP gene-specific forward primer, reverse primer, and probe (VIC/TAMRA) within a reaction mix.

Besides NTC, a biological negative control, as well as the RV-SFG.CD19.CD28.4-1BBzeta plasmid as a positive control, an EC sample was included within each experiment for SCG-DP-PCR validation to verify similar amplification efficiencies of CAR transgene and RNaseP. The EC sample consisted of genomic DNA from a nontransduced cell sample (comprising RNaseP) combined with the RV-SFG.CD19.CD28.4-1BBzeta plasmid in a 1:1 ratio. Calculations for EC sample generation and preparation are described within the Supplemental Materials and Methods. Besides testing similar PCR efficiencies, the EC sample verifies the SCG status of RNaseP in genomic DNA when a VCN of 1 is achieved.

The accepted range for ΔCt (DP-CAR − DP-RNaseP) in the EC sample was defined per calculation between $\Delta Ct \geq -0.4$ and $\Delta Ct \leq 0.56$. Application of $2^{-\Delta Ct\ (DP\text{-}CAR\ -\ DP\text{-}RNaseP)}$ results in an accepted variance for the copy number of 1 ± 0.32 for Ct values of DP-CAR relative to DP-RNaseP.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.02.003.

AUTHOR CONTRIBUTIONS

A.K., M.L.S., and M.S. conceived the qPCR validation study and wrote the manuscript. U.G., B.N., L.W., and A.H.K. supported study conception. A.K. designed, performed and analyzed qPCR experiments. U.G. and B.M. processed patient samples. M.S., A.S., S.H., C.M.T., and P.D. treated patients and provided clinical data. All authors reviewed and edited the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

Optimized Assessment of qPCR-Based Vector Copy Numbers as a Safety Parameter for GMP-Grade CAR T Cells and Monitoring of Frequency in Patients

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Supplementary data:

a) Mathematical evaluation of qPCR data:

1. Copy number determination for standards:

For standard curves, obtained data were analyzed by linear regression prior to mathematical extrapolation. Linear regression for standard curve generation was performed using Microsoft Excel Software. 3.3 mega base pairs (Mbp) were assumed as haploid human genome with 650 Da weight for each base pair. The RV-SFG.CD19.CD28.4-1BBzeta plasmid comprises 8581 bp. The following equation was applied to calculate the human genome weight per copy as well as RV-SFG.CD19.CD28.4-1BBzeta plasmid weight per copy:

$$DNA\ weight\ per\ copy\ (\frac{g}{copy}) = \frac{DNA\ length\ (bp)\ x\ 650\ (\frac{g}{mol})}{6.0221\ x\ 10\text{^}23\ mol\text{^} - 1}$$

Obtained DNA weight per copy was used to determine RNAseP (1 copy per haploid genome) and RV-SFG.CD19.CD28.4-1BBzeta plasmid copy numbers per µl:

$$Copies/\mu l = \frac{DNA\ concentration\ (\frac{g}{\mu l})}{DNA\ weight\ per\ copy\ (\frac{g}{copy})}$$

For standard curves, the copies / µl were calculated and standards diluted accordingly.

2. Derivation of the $2^{-\Delta Ct}$ –method:

VCN using SCG-DP-PCR was calculated by relating CD19.CD28.4-1BBzeta transgene to RNAseP gene copies applying the equation $2^{-\Delta Ct\ (DP\text{-}CAR\ -\ DP\text{-}RNAseP)}$. This equation was obtained via conversion of the $2^{-\Delta\Delta Ct}$ –method and is independent from the calculations mentioned before.

$2^{-\Delta\Delta Ct}$ –method is based on the following equation:

$$\Delta\Delta Ct = (Ct_{Target} - Ct_{Reference})_{CAR\ sample} - (Ct_{Target} - Ct_{Reference})_{Calibrator\ sample}$$

A calibrator sample, i.e. the EC sample, contains similar copy numbers of the CAR transgene and RNAseP gene. Thus, under optimal and similar PCR conditions for both reactions, the last part of the equation can be deleted:

$$\Delta Ct = (Ct_{Target} - Ct_{Reference})_{CAR\ sample} \underbrace{- (Ct_{Target} - Ct_{Reference})_{Calibrator\ sample}}_{=0}$$

$2^{-\Delta Ct}$ –method (SCG-DP-PCR) was used for VCN determination. The calculated copy number corresponds to the copy number of haploid human genomes, hence multiplication by the factor 2 results in vector copy number per cell (VCN / cell).

3. Copy number determination for patient samples:

For the calculation of CAR transgene copies per ng or µg PBMC DNA, two sequential equations were applied. First, the genomic copies corresponding to 100ng genomic DNA were calculated by the following equation:

$$CN\ human\ genome = \frac{DNA\ weight\ per\ qPCR\ reaction\ (ng)}{DNA\ weight\ per\ copy\ of\ diploid\ human\ genome\ (\frac{ng}{copy})}$$

This resulted in 100ng genomic DNA corresponding to 14,037 PBMCs. CAR transgene copies per ng or µg PBMC DNA were calculated by multiplying the VCN per cell assessed via SCG-DP-PCR with the corresponding cell number used within the PCR reaction by one of the following equations:

$$CN\ per\ 100ng = VCN\ per\ cell\ x\ 14{,}037$$

$$CN\ per\ 1\mu g = VCN\ per\ cell\ x\ 140{,}370$$

b) Technical details of SP- and DP- qPCR experiments:

The final concentration of two unlabeled primer pairs was 900nM per primer and 250nM for probes in all PCR reactions. The 2x TaqMan™ Universal PCR Master Mix (cat # 4304437) and each 20x primer probe mastermix (Custom TaqMan™ Gene Expression Assay, FAM, cat # 4331348 for the CAR transgene PCR and RNAseP Copy Number Reference Assay, cat # 4403326 for the RNAseP PCR) were purchased from Applied Biosystems™. The final reaction volume per PCR reaction in the test samples was 25µl containing 1.25µl per 20x primer probe mastermix (no RNAseP mastermix in SP-CAR PCR reactions),12.5µl of 2x TaqMan™ Universal PCR Master Mix, 5µl of sample (20ng / µl) and nucleasefree water (6.25µl for SP-PCR and 5µl for DP-PCR). Differing preparations and sample components for standards and the EC sample are described in the following.

Dilution sets for the standard curves were prepared independently for each experiment and each method. Standard samples for validation of the SP- and DP- PCR reactions were prepared as follows: For the SP-CAR PCR, a stock plasmid solution with a concentration of 100 ng / µl in TE buffer (cat # 93283, Sigma Aldrich) was used to generate the highest plasmid standard with a concentration of 6 x 10^4 copies / µl by sequential dilution in nucleasefree H_2O (cat # 129114, Qiagen). Calculations described in A) section 1 were applied. Starting from the highest plasmid standard four more standards were generated for the SP-CAR PCR by serial 1:10 dilution. The used DNA amount of every test sample was 5 µl of a prior prepared 20ng / µl dilution of human genomic DNA in nuclease-free H_2O resulted in 100 ng human genomic DNA per PCR reaction corresponding to 14,037 copies of diploid human genomes (see A) section 3). By multiplication with the factor 2 this results in 28,074 copies of RNAseP (single copy per haploid human genome) in 100 ng of human genomic DNA. For the duplex standards, a highly concentrated stock of an untransduced patient sample ($\geqslant$ 228ng / µl) to prepare 100µl of a sample with 6 x 10^4 copies / µl of RNAseP equivalent to the highest plasmid standard was used. To this sample, the same volume of the plasmid sample that was used for the generation of the highest plasmid standard (6 µl of 1 x 10^6 plasmid copies / µl to 6 x 10^6 RNAseP copies in an overall volume of 100 µl) was added. The prepared highest duplex standard with a copy concentration of 6 x 10^4 copies / µl for the CAR transgene and RNAseP gene was serially diluted four times in 1:10 steps to generate the duplex standards. For every standard a volume of 100 µl was prepared and aliquoted for further use. All standard PCR reactions contained 5µl of standard instead of test sample.

The EC sample was prepared directly in the reaction well by mixing 4.7 µl of the second plasmid standard (6 x 10^3 copies / µl) with 5µl of the non-transduced patient sample with a concentration of 20 ng / µl (5,615 copies RNAseP / µl). 0.3 µl nucleasefree H_2O, 12.5 µl of 2x TaqMan™ Universal PCR Master Mix and 1.25µl of each 20x primer probe master mix were added. As assessed via calculation, the EC sample contained a similar concentration of approx. 28,100 copies of the CAR transgene and RNAseP gene (1:1 ratio).

c) Supplementary Figure

Figure S1:

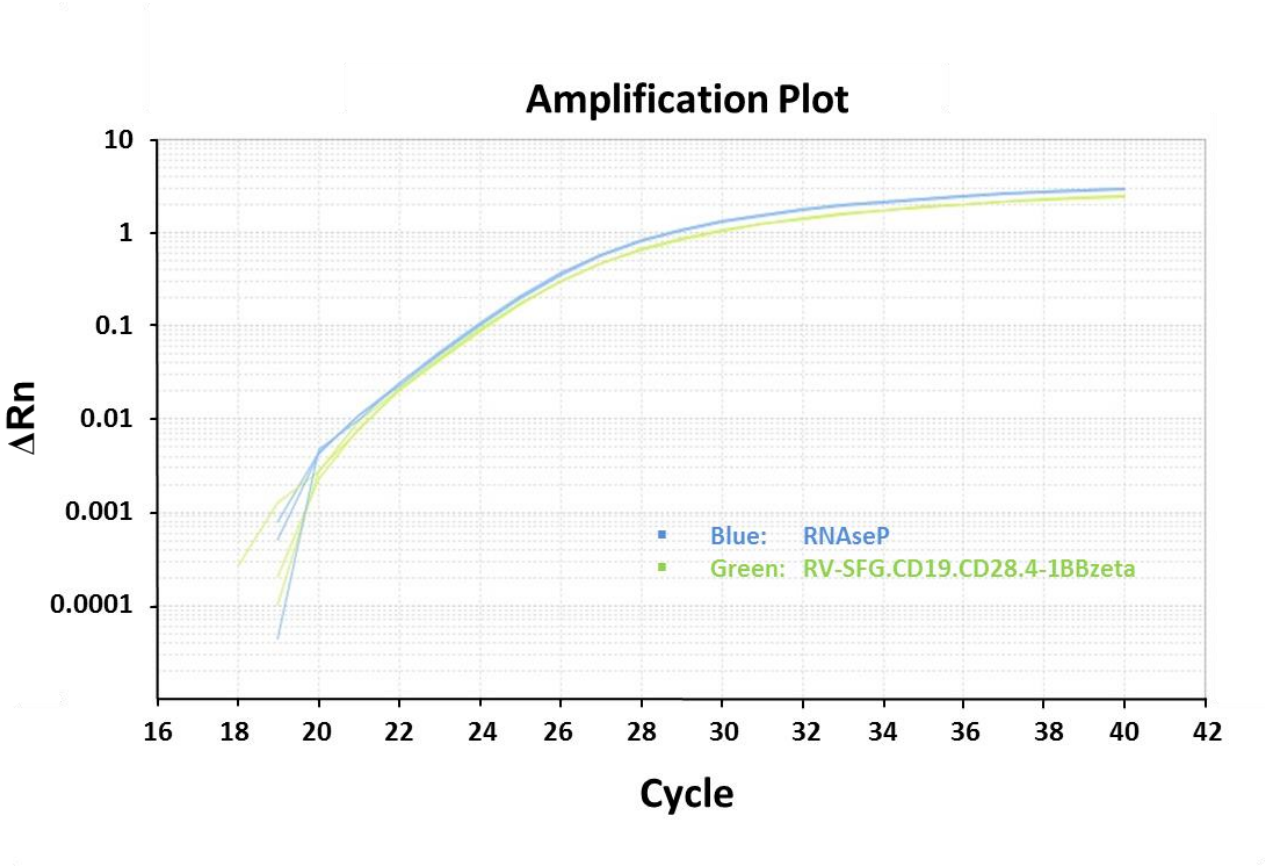

Figure S1: Logarithmic amplification curves for the duplex-qPCR reaction in the efficiency control (EC) sample. Exemplary data from one validation experiment are depicted. Reactions were performed in triplicates.