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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:

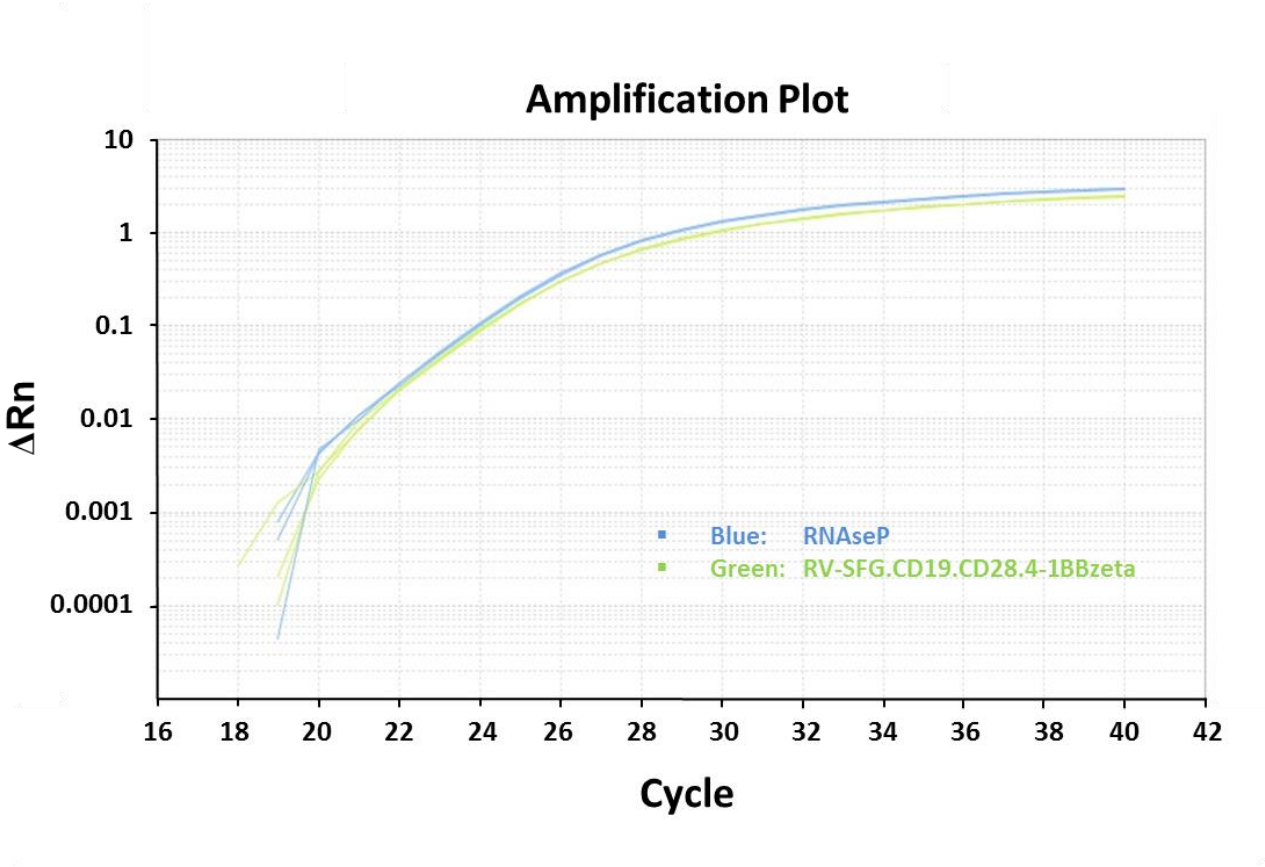

Figure S1: Logarithmic amplification curves for the duplex-qPCR reaction in the efficiency control (EC) sample. Exemplary data from one validation experiment are depicted. Reactions were performed in triplicates.