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A real-time quantitative PCR targeting the viral vector for the monitoring of patients treated with axicabtagene ciloleucel

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Short running head: Molecular monitoring of CAR T-cells therapy

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Abstract

Axicabtagene ciloleucel or axi-cel (CD19-CAR T-cells) has been recently approved for refractory/ relapsed diffuse large B cell lymphoma and primary mediastinal B-cell lymphoma. Proliferation of CAR T-cells after infusion and their persistence have been reported as important factors. Laboratory tools are needed for the monitoring of patients.

We developed a vector-based, simple and accurate real-time qPCR to measure axi-cel vector copy number (VCN) in peripheral blood samples.

Primers and probe targeting the *5'LTR* region of the gammaretroviral vector (mouse stem cell virus or MSCV) were designed for amplification. To generate standard curves MSCV plasmid was subcultured and quantified using droplet digital PCR (ddPCR). The method was applied to quantify VCN in blood samples from patients treated with axi-cel.

The limit of quantification of the qPCR assay was established at 2.2 copies/µL in DNA eluate. The qPCR method was well correlated with flow cytometry (FC) findings; however, the assay appeared to be more sensitive than FC. The kinetics observed in blood samples from treated patients was in agreement with previously reported findings.

In conclusion, we developed a sensitive and accurate qPCR assay for the quantification of transgenic CAR T-cells, which can be a useful additional tool for the monitoring of patients treated with of axi-cel.

Keywords: CAR T-cells, axi-cel, monitoring, qPCR

Introduction

Chimeric antigen receptor (CAR) -T cells are engineered-T cells to express CAR by gene transfer technology. CAR T cells have the targeting specificity of a monoclonal antibody combined with the effector functions of a cytotoxic T cell, and are able of specifically recognizing and killing their target cells. CAR T-cells therapy has revolutionized the immunotherapeutic field in cancer therapy, providing a potential therapeutic option for patients who are unresponsive to standard treatments [1,2].

CAR T-cells have yielded unprecedented efficacy in B-lineage malignancies, most remarkably in anti-CD19 CAR T-cells [3,4], and extensive research is being done to extend this treatment to solid tumors, autoimmune and chronic infectious diseases [5–7].

Recently, the US Food and Drug Administration and the European Medicine Agency have approved the first CAR T-cell products – tisagenlecleucel (Kymriah, Novartis) and axicabtagene ciloleucel (axi-cel) (Yescarta, Gilead) – for the treatment of B-cell precursor acute lymphoblastic leukemia and diffuse large B-cell lymphoma[8].

Proliferation of CAR T-cells after infusion and their persistence are important factors for anti-tumor activity [9–11]. The monitoring of CAR T-cells could be useful to better assess the association between the kinetics of CAR T-cells and clinical outcomes, including disease response and adverse events. Methods evaluating the integrated CD19 CAR transgene by quantitative PCR, or its phenotypic expression by flow cytometry have been used in clinical trials [12,13].

Targeting the retroviral vector can be an alternative approach. Tisagenlecleucel use a self-inactivating lentiviral vector system in which the transgene is encoded on a transfer plasmid between viral long terminal repeats (*LTR*) [14], while a gammaretroviral vector encoding the anti-CD19 CAR was used for axi-cel [15]. Routine HIV assays targeting the *LTR* region [16] can be used to monitor tisagenlecleucel, but not axicabtagene ciloleucel.

In this study, we developed a vector-based assay for the monitoring of patients treated with axi-cel.

Materials and methods

Primers and probe targeting the *5'LTR* region of mouse stem cell virus (MSCV)

The Mouse stem cell virus-based splice-gag vector is the retroviral backbone used in the axi-cel CAR construct [15]. Primers and probe to target MSCV *5' LTR* were designed using the Primer Express Software version 3.0 (Thermofisher Scientific, Les Ulis, France). The sequences of the primers and the internal probe are listed in Table 1.

MSCV plasmid subcloning

The MSCV plasmid previously published by Olive V et al [17], was obtained from Addgene (plasmid # 24828, Addgene, Watertown, MA). The 7656 bp-size plasmid was sent in bacterial culture as Agar stab. After culture on Luria Broth (LB) Agar plate containing ampicillin (200 µg/mL), a single bacterial colony was picked and inoculated in 3 ml of liquid LB containing ampicillin (200 µg/mL). The plasmid was purified from the overnight bacterial culture using the NucleoSpin Plasmid kit (Macherey Nagel, Hœrdt, France) and resuspended in Elution Buffer (5 mM Tris/HCl, pH 8.5) according to the manufacturer's guidelines. The plasmid purity analysis was then carried out by gel electrophoresis on 1.5% agarose gel. The plasmid stock solution (S1) was aliquoted and stored at -80 °C.

Clinical samples

Whole-blood samples were collected from 3 patients treated with axi-cel. The samples were collected for routine care at serial time points after infusion of a single dose of axi-cel and stored at -80°C until DNA extraction. The characteristics of patients are summarized in Table 2. Blood samples from these 3 patients before axi-cell infusion, 5 patients treated with tisagenlecleucel and 5 HIV-1 patients were also collected for assay specificity testing. This observational study was based on medical records, in compliance with the French reference methodology MR-004, established by French National Commission on Informatics and Liberties (CNIL), and approved by the Institutional data protection authority of CHU Lille. All patients gave consent for data recovery.

Total DNA extraction

Total DNA extraction was performed on 400 µL of whole blood samples with the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, La Rochelle, France) on the MagNA Pure LC 2.0 Instrument according to the manufacturer's instructions. Total DNA was eluted in 100µL, and was stored at -20°C until PCR experiments.

Droplet digital polymerase chain reaction (ddPCR)

Digital PCR was carried out with the QX200™ Automated Droplet Generator (Bio-Rad, Marnes-la-Coquette, France) in accordance with the manufacturer's recommendations. For PCR amplification, 3 µL of DNA were mixed with primers and probe (final concentrations: 250nM for forward and reverse primers and 500 nM for probe), 2X ddPCR Supermix for Probes (Bio-Rad) and water in a 20 µL reaction volume. PCR reaction mixes were transferred to QX200™ Automated Droplet Generator (Bio-Rad) which generates approximately 20,000 nanoliter-sized droplets. Droplet-containing water-in-oil emulsion (40 µL) was transferred to a 96-well PCR plate (Bio-rad). The amplification was performed on a T100TM Thermal Cycler (Bio-Rad), using the following protocol: enzyme activation (95° for 10 min), amplification cycles (denaturation at 94°C for 30 sec, annealing/extension at 56°C for 1 min; 39 cycles), a ramp rate of 2.5°C/s, and a final 10 min enzyme inactivation step at 98°C. After cycling, the 96-well PCR plate was transferred to QX200™ Droplet Reader (Bio-Rad). Data analysis was performed using QuantaSoft™ software (version 1.6.6.0320; Bio-Rad). The software measures the number of positive and negative droplets for the fluorophore in each well, and then fits the fraction of positive droplets to a Poisson algorithm to determine the concentration of the target DNA molecule in copies/µL. QuantaSoft™ software provides the concentration in the PCR mix (X copies/µL). The concentration in the starting DNA material was obtained using 20X/3 formula.

Real-time Taqman PCR

Real time PCR was performed using the TaqMan Universal PCR Master Mix (Thermofischer Scientific). Reaction mix with a total volume of 25 µl was prepared as follows: 12.5 µl of 2X Master Mix, 0.5 µl of each primer (final concentration 250 nM), 1 µl of probe (final concentration 500 nM), 5.5 µl of nuclease-free water and 5 µl of DNA. Thermal cycling was

performed on an ABI 7500 Real-Time PCR System (Thermofischer Scientific) with the following protocol: AMPerase activation (50°C for 2 min), enzyme activation (95° for 10 min), amplification cycles (denaturation at 94°C for 15 sec, annealing/extension at 60°C for 1 min; 50 cycles).

Human albumin gene was used as internal control. The sequences (5' → 3') of oligonucleotides were as follows: forward primer (GCTGTCATCTCTTGTGGGCTGT), reverse primer (AAACTCATGGGAGCTGCTGGTTC); probe (VIC-CCTGTCATGCCCACACAAATCTCTCC-TAMRA). Primers and probe were at a final concentration of 200nM, and the reaction conditions were the same described for axi-cel vector.

Data analysis was performed using SDS software version 2.0.6 (Thermofischer Scientific).

Flow cytometry

Flow cytometry was performed using an in-house assay developed by Demaret et al [18]. The method is based on labeled CD19 protein, and is used for routine monitoring measurement of CAR T-cells in treated patients [18].

Statistical analysis

Linear regression was used for linearity analysis and the r Pearson coefficient to study correlation.

Results

Design of a real-time quantitative PCR targeting the MSCV *5'LTR*

We designed primers and probe that specifically target the MSCV *5'LTR* region. The 70 bp-size target product was detected in samples collected from patients treated with axi-cel. The target product was not detected in samples obtained before the infusion. In addition, no signal was found in samples from patients treated with tisagenlecleucel, nor in samples from HIV-1 patients.

In order to establish a standard curve for a quantitative PCR, the MSCV plasmid was quantified using ddPCR. For the set-up of ddPCR experiments, several temperatures were

tested for the annealing/extension step, and 56°C was found to provide optimal proportions of positive and negative droplets (see Figure 1).

Ten-fold dilutions of the MSCV plasmid were performed from the S1 stock solution and run by ddPCR in four replicates (see Figure 2). The standard deviation (SD) and the coefficient of variation (CV) were calculated for each dilution (see Table 3). The ddPCR method was found to be linear between S3 (mean concentration at 4.74 Log copies/µL) and S8 (0 Log copies/µL) (R^2= 0.998), as shown in Figure 3. Dilutions S4, S5 and S6 were the last three ones with a precise quantification (SD < 0.19 Log copies/µL, and a CV < 35% on absolute values [19]), and were used to calculate the average value of the S1 stock solution. The theorical concentration of the S1 stock solution was then established at 6.94 Log copies/µL.

Performance of the real-time PCR

To determine the limit of detection (LOD) and the limit of quantification (LOQ) of the real-time qPCR assay, serial dilutions (S6 to S9) of the plasmid were run in 10 replicates. Data for each dilution are summarized in Table 4. The LOD 95% was determined by probit regression analysis at 1.6 copies/µL. The LOQ was defined as the lowest concentration of target that can be detected with a CV< 35% on absolute values, and was established at 2.2 copies/ µL.

A PCR run was also performed on dilutions S3 to LOQ (three replicates), and the real-time PCR was found to be linear up to S3 (4.94 Log copies/µL), as shown in Figure 4.

Plasmid dilutions S3 to S7 were used as standard points to perform the quantification of axi-cel vector copies. The obtained eluate concentration (Ce in copies/µL) and extraction patterns were used to calculate the initial concentration in the patient specimen. Given that DNA was extracted from 400 µL of whole blood and eluted in 100 µL, the concentration in the starding sample (Cs in copies/mL) was calcutated with the formula: Cs = Ce*100*1000/400. The corresponding values of LOD and LOQ in a blood sample were then estimated to 400 copies/mL (2.60 Log copies/mL) and 550 copies/mL (2.74 Log copies/mL) respectively.

Quantification of axi-cel Vector copies in clinical samples

The quantification was performed on available blood samples, collected for routine care. All the samples collected before infusion, were tested negative. The kinetics for each patient is

shown in Figure 5. For Patient 1, the peak of axi-cel vector copies (5.41 Log copies/mL) was observed on day 7 post infusion (pi), followed by a slight decrease and stabilization around 3 Log copies/mL from 4 months pi. In patient 2, the quantity peaked at 4.44 Log copies/mL on day 14 pi (no sample available between 1 and 14 days), with a decrease to reach 3 Log copies/mL at 1 month pi, followed by a fluctuation around the limit of quantification. Then, a re-increase was observed after administration of nivolumab, and another peak at 4.26 Log copies/mL was reached more than 8 months after infusion. The kinetics in Patient 3 was quite similar to Patient 1. The highest value (4.51 Log copies/mL) was recorded on day 20 pi (no sample available between day 3 and 20), and then a slight decrease and a stabilization around 3 Log copies/mL, after 3 months pi.

Correlation between Taqman qPCR results, and ddPCR and flow cytometry findings

Samples from one patient were run both by ddPCR and Taqman qPCR. A very good correlation was found between both methods, as shown in Figure 6. The Pearson r coefficient was 0.995 ($p< 0.0001$).

Furthermore, qPCR results were compared to findings obtained with FC. The comparison between qPCR and FC for the 3 patients is shown in Figure 7. Overall, the kinetics was similar for both assays. In Patient 2, the re-increase with qPCR seemed delayed, as compared to FC. In Patients 1 and 2, when signal in FC was undetectable, axi-cel vector was still quantifiable in qPCR.

Discussion

Axicabtagene ciloleucel is one of the first anti-CD19 CAR T-cells therapy approved for the treatment of refractory large B-cell lymphoma in adults, and showed an impressive efficacy with a complete response (CR) rate of 58% at a median follow-up of 27.1 months[20]. *In vivo*, anti-CD19 CAR T-cells persistence has been reported as a marker of long-term clinical efficacy of this therapy, but there are currently no standard guidelines for the monitoring of CAR T-cells kinetics in patients' peripheral blood. Therefore, routine laboratory tools need to

be developed to fill this gap and to meet the current needs [21,22]. Using the CAR engineering of axi-cel, we designed a set of primers and probe in order to set-up a quantitative polymerase chain reaction (qPCR) to measure expansion and persistence of CAR T-cells in vivo. Recent reports described the use of a ddPCR approach for CD-19 CAR T-cells quantification[21,22]. In the present work, we chose to develop a qPCR method which is more suitable to routine, and widely available in most clinical laboratories. Our method targets MSCV *5' LTRs*, a region of the viral vector integrated into the genome of the transduced cells[15]. A quantified MSCV plasmid was used to generate standard curves for theTaqMan-based real-time PCR method. We used ddPCR for MSCV plasmid quantification, since this method has already been reported to be precise and accurate for plasmid copy number quantification[23]. The limit of quantification was set at 2.2 copies/µL (2.74 Log copies/mL in patients' blood samples). The reliability of the qPCR assay was also assessed by evaluation of the analytical specificity on blood samples from patients prior to the axi-cel infusion, as well as patients treated with tisagenleceucel, and HIV-1 patients. Furthermore, the method was shown to be accurate and correlated with ddPCR.

To examine its analytical performance the qPCR method was applied for axi-cel vector quantification in frozen samples collected from 3 patients treated with axi-cel. The kinetics in peripheral blood was as expected for 2 patients with a peak within 7-14 days post CAR-T infusion followed by a progressive decline[24,25]. Axi-cel vector was still detectable up to 3 months post-infusion. A different pattern was observed in the third patient who developed a progressive disease 3 months after axi-cel injection and a rapid decline on both CF and qPCR after the expansion phase. The patient was then treated with Nivolumab, a PD-1 inhibitor. A second axi-cel peak at a level, similar to the first one, followed the first Nivolumab injections. As reported in a precedent study, these results suggest that Nivolumab may enhance the expansion of anti-CD19 CAR T cells but not necessarily over time[26].

The correlation between qPCR and FC was good. Nevertheless, our approach offers the advantage of detecting CAR T-cells at lower concentrations in peripheral blood. Indeed, for

one patient who showed a complete response, anti-CD19 CAR T cells remained detectable 6 months after the infusion using qPCR assay, while FC was undetectable. Thus, axi-cel vector quantification could be used as a complementary tool to FC for the long-term monitoring of patients.

In conclusion, we report an accurate, sensitive and easy to perform qPCR assay for the quantification of axi-cel vector, which can be used for the monitoring of patients treated with axi-cell. The clinical relevance of the levels of axi-cel vector, and thus the potential of a quantification routine method require further investigation in large cohort studies.

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Figure Legends

Figure 1. Annealing/extension temperature set-up for ddPCR experiments. The S3 dilution of MSCV plasmid was run by ddPCR using several temperatures for annealing/extension step. The positive droplets are represented in blue, whereas negative droplets are in black. Yellow lines indicate the separation between the different conditions tested.

Figure 2. Plasmid quantification by ddPCR. Ten-fold dilutions (S2 to S8) of MSCV plasmid were run by ddPCR. The positive droplets are represented in blue, whereas negative droplets are in black. Yellow lines indicate the separation between the different samples, and the purple line represent the set fluorescence threshold to distinguish positive and negative droplets.

Figure 3. Linearity in ddPCR. Ten-fold dilutions (S3 to S8) of MSCV plasmid were run in 4 replicates. Data were presented as mean+/-SD. The regression line was shown with 95% confidence band.

Figure 4. Linearity in real time Taqman PCR. Serial dilutions (from S3 to LOQ) of MSCV plasmid were run in 3 replicates. The concentration was plotted against Ct values. The regression line was shown with 95% confidence band.

Figure 5. Quantification of axi-cel vector in clinical samples. Real-time qPCR targeting axi-cel vector was performed on whole blood samples collected at various time points for 3 patients. The result is presented as Log copies/mL of blood. The dotted line represents the LOQ.

Figure 6. Correlation between ddPCR and qPCR. Axi-cel vector was quantified in blood samples from one patient. Concentration obtained with ddPCR were plotted against values obtained with qPCR. Data are presented as Log copies/mL. The regression line was shown with 95% confidence band.

Figure 7. Correlation between qPCR and flow cytometry. Axi-cel vector was quantified in blood samples from 3 patients using qPCR. CART cell number in samples collected the same day were evaluated with a flow cytometry method using labeled CD19 protein. qPCR data are presented as Log copies/mL, and the LOQ is 2.74 Log copies/mL. The flow cytometry findings are presented as Log cell number/mL, and the LOQ is 3 Log cell number/mL.

Table 1. Primers and probe targeting MSCV 5'LTR

Oligonucleotide	Sequence (5′–3′)	Nucleotide Position
Forward-primer	5'-TTGCAAGGCATGGAAAATACAT-3'	50-71
Reverse-primer	5'-GCTGTCTCTCTGTTCCTAACCTTGA-3'	95-119
Probe	5'-6FAM-ACTGAGAATAGAGAAGTTC-MGB-3'	73-91

Table 2. Characteristics of patients

Characteristics	Patient 1	Patient 2	Patient 3
Age (years)	77	69	48
Sexe	Male	Female	Female
Diagnosis	Transformed MZL	DLBCL	DLBCL
Previous chemotherapy lines	4	3	3
CRS	Grade 2 (day 1)	Grade 2 (day 1)	Grade 2 (day 3)
ICANS	Grade 2 (day 5)	Grade 4 (day 5)	Grade 4 (day 5)
Disease status			
Before CAR T-cell	Progressive disease	Progressive disease	Progressive disease
1 month after CAR T-cell	Complete response	Partial response	Partial response
3 months after CAR T-cell	Complete response	Progressive disease	Complete response
Last follow-up	OS: 11 months (death from MDS)	OS: 17 months (alive)	PFS: 13 months (alive)

CRS: cytokine release syndrome; DLBCL: diffuse large B-cell lymphoma; ICANS: immune effector cell-associated neurotoxicity syndrome; MDS: myelodysplastic syndrome; MZL: marginal zone lymphoma; OS: overall survival; PFS: progression-free survival

Table 3. ddPCR results for plasmid dilutions

Plasmid dilutions	Mean concentration (Log copies/µL)	Standard deviation (Log copies/µL)	Coefficient of variation (%)
S2	>5.82	NA	NA
S3	4.74	0.12	26.20
S4	3.88	0.04	8.53
S5	2.94	0.02	3.70
S6	2.01	0.03	7.82
S7	0.94	0.16	36.77
S8	0	0.32	82.1

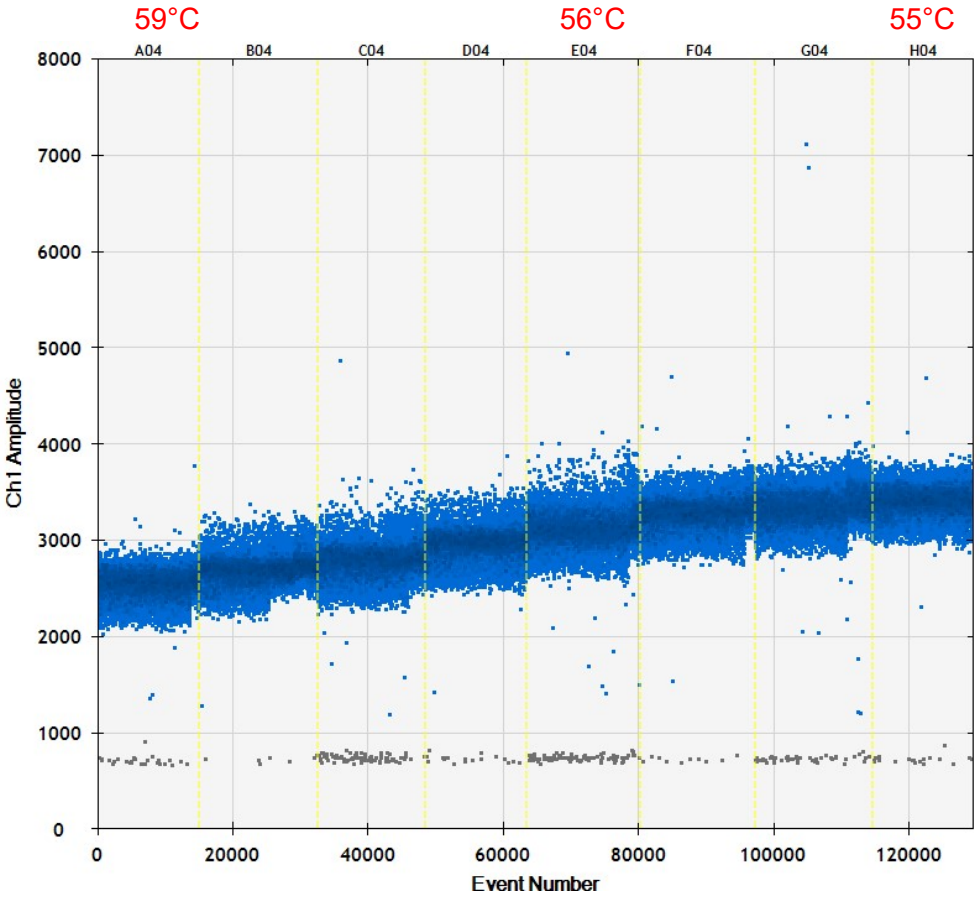
NA : non applicable

Table 4. Determination of LOD and LOQ for real-time qPCR

Plasmid dilutions	Theorical value (copies/µL)	Number of positive results (Total = 10)	Mean concentration (copies/µL)	Coefficient of variation (%)
S6	88	10	100.8	21.5
S7	8.8	10	10.36	28.2
S7/2	4.4	10	4.54	30.1
S7/4	2.2	10	2.02	33.2
S7/8	1.1	9	1.02	80.3
S8	0.88	4	NA	NA
S9	0.088	0	NA	NA

NA: non applicable

FIGURE 1


59°C
56°C
55°C
A04
B04
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D04
E04
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G04
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8000
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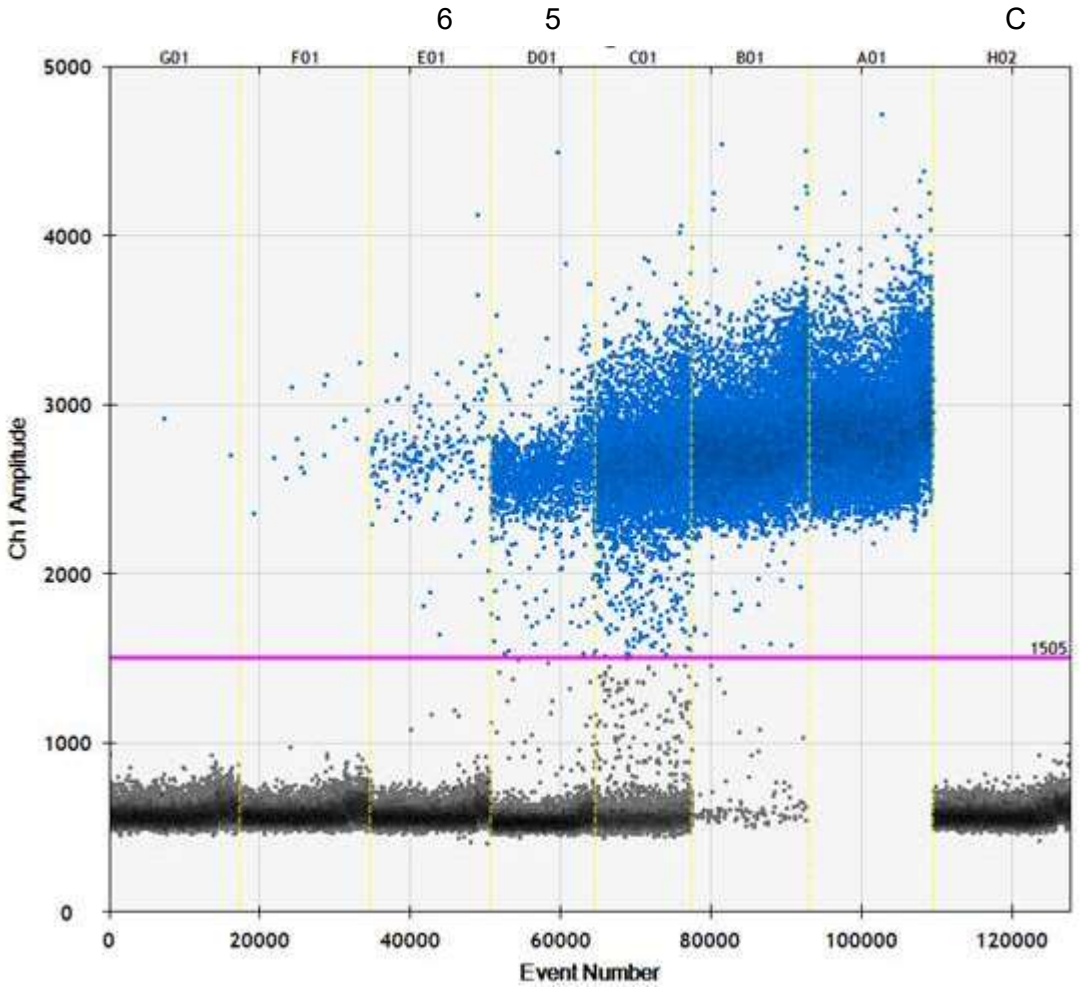
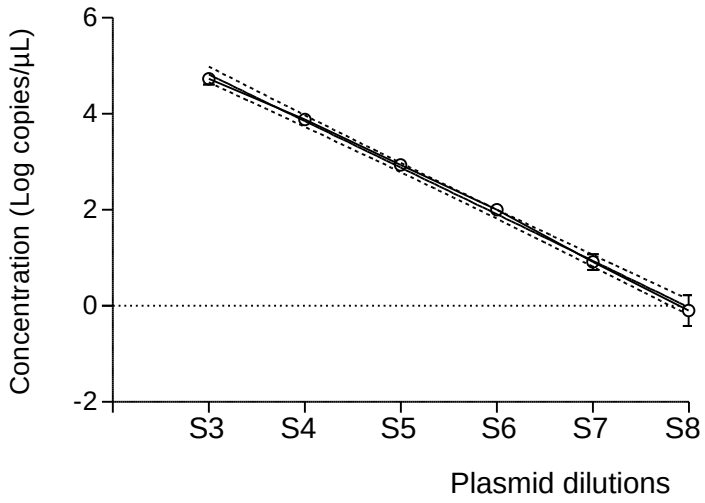
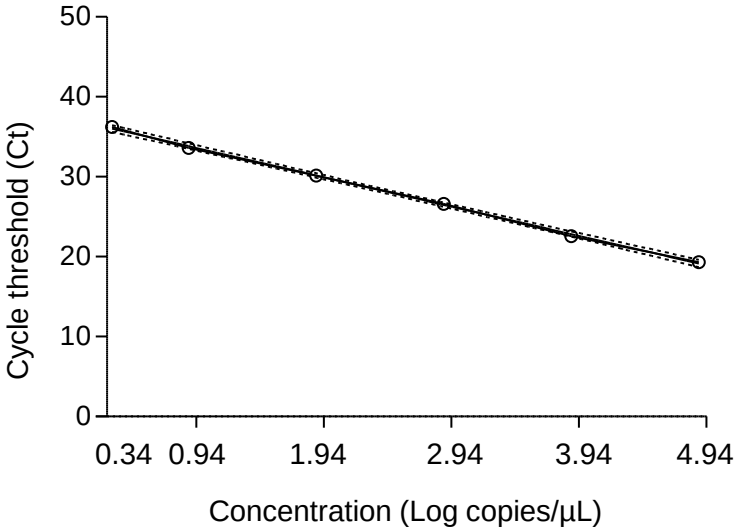
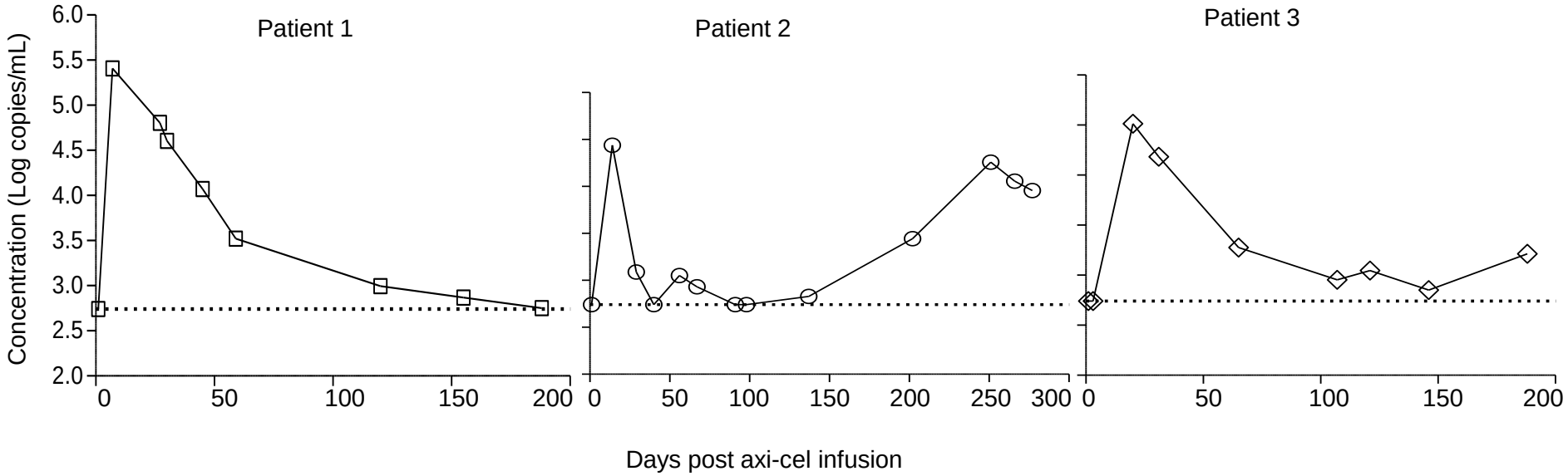
2000
1000
0
Ch1 Amplitude
0
20000
40000
60000
80000
100000
120000
Event Number

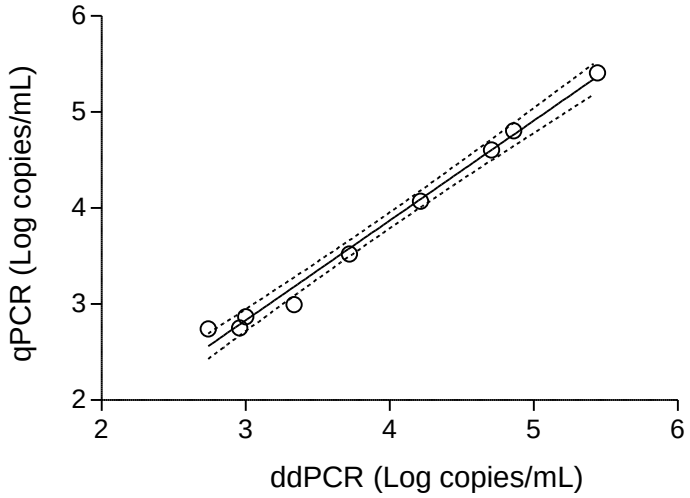
FIGURE

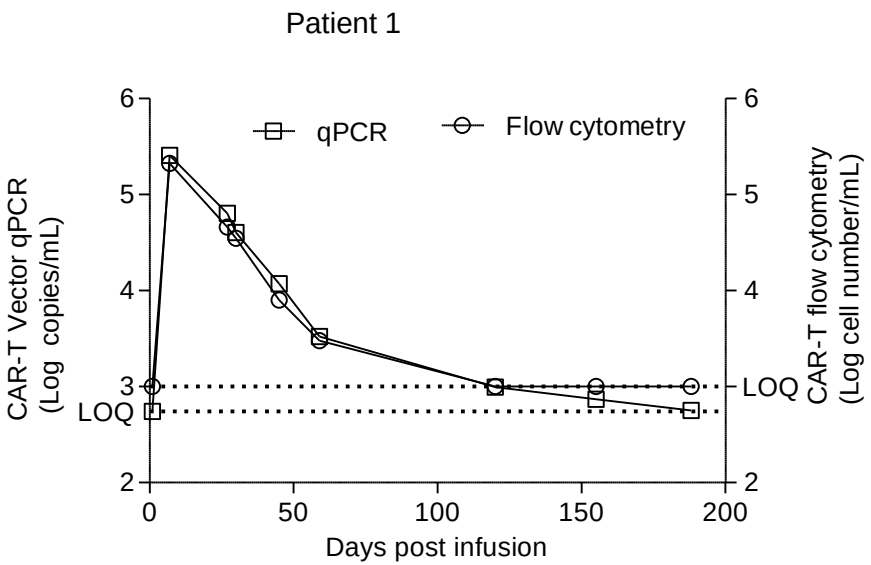

6
5
C
G01
F01
E01
D01
C01
B01
A01
H02
5000
4000
3000
2000
1000
0
Ch1 Amplitude
1505
0
20000
40000
60000
80000
100000
120000
Event Number

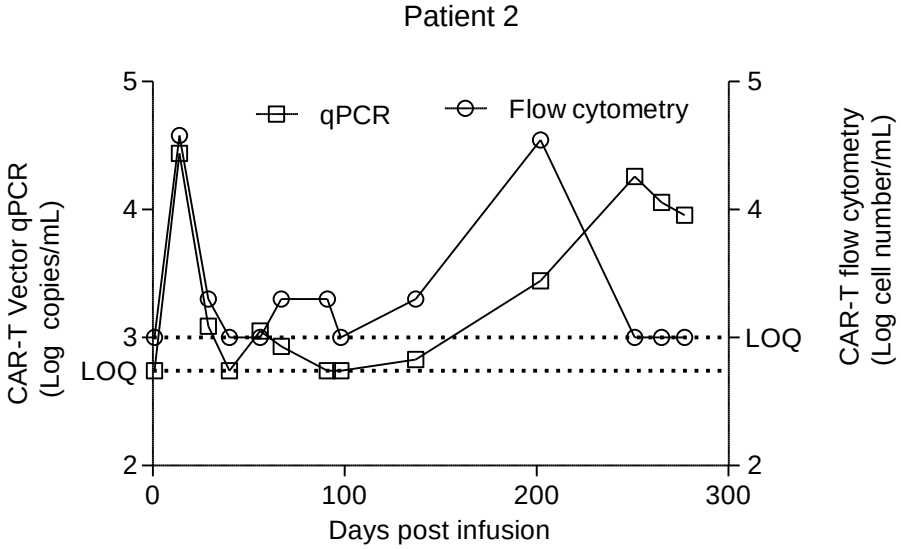

6
4
2
0
-2
Concentration (Log copies/µL)
S3
S4
S5
S6
S7
S8
Plasmid dilutions

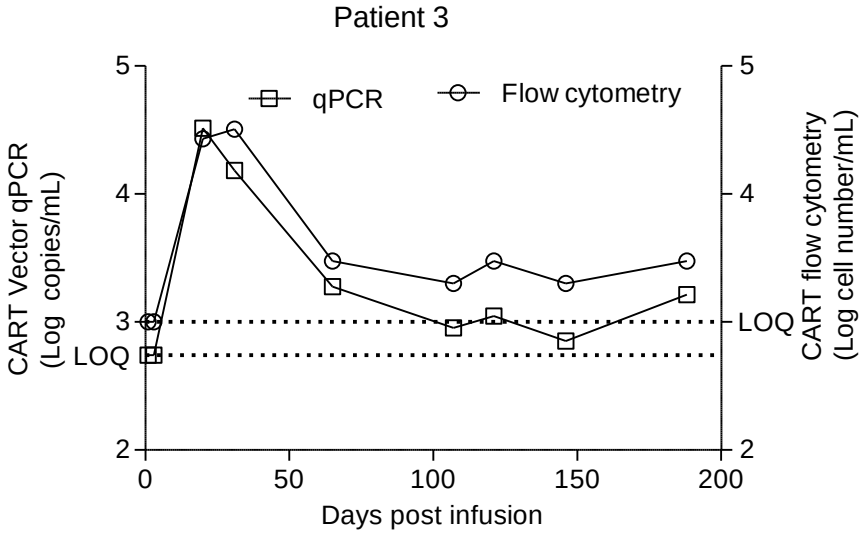

50
40
30
20
10
0
Cycle threshold (Ct)
0.34
0.94
1.94
2.94
3.94
4.94
Concentration (Log copies/µL)


Patient 1
Patient 2
Patient 3
Concentration (Log copies/mL)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
50
100
150
200
250
300
Days post axi-cel infusion


qPCR (Log copies/mL)
ddPCR (Log copies/mL)
2
3
4
5
6


Patient 1
qPCR
Flow cytometry
CAR-T Vector qPCR
(Log copies/mL)
CAR-T flow cytometry
(Log cell number/mL)
LOQ
LOQ
Days post infusion
0
50
100
150
200
2
3
4
5
6


Patient 2
qPCR
Flow cytometry
CAR-T Vector qPCR
(Log copies/mL)
CAR-T flow cytometry
(Log cell number/mL)
LOQ
LOQ
Days post infusion
0
100
200
300
2
3
4
5


Patient 3
qPCR
Flow cytometry
CART Vector qPCR
(Log copies/mL)
CART flow cytometry
(Log cell number/mL)
LOQ
LOQ
Days post infusion
0
50
100
150
200
2
3
4
5