DEVELOPMENT OF A MULTIPLEXED QUANTITATIVE PCR ASSAY FOR CD19-DIRECTED CAR-T CELL PERSISTENCE MONITORING

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INTRODUCTION

Chimeric antigen receptor (CAR) T cell therapy has transformed hematological cancer treatment, with six therapies now approved by the FDA. Four of these therapies (tisa-cel, axi-cel, brexu-cel, and liso-cel) target CD19 using single chain variable fragments (scFvs) derived from the heavy and light chain variable (V_H and V_L) regions of the monoclonal antibody FMC63.

The primary objective of this study was to evaluate the ability of FMC63 scFv-specific real-time PCR (qPCR) to detect and quantify FMC63 DNA in whole blood specimens. This assay is intended for the quantitative detection of FMC63 DNA from CAR-T cells in whole blood specimens for the purpose of monitoring CAR-T cell engraftment, expansion, and persistence in individuals receiving CD19-directed CAR-T cell therapy.

Here we describe the development, validation and performance characteristics of a multiplexed, quantitative qPCR assay targeting FMC63 for the monitoring of CD19-directed CAR-T cell therapy.

MATERIALS AND METHODS

Multiple qPCR primers and probes were designed to target the V_H and V_L regions of FMC63 and screened to identify those with the highest amplification efficiency and sensitivity. The most promising candidates were multiplexed with an RNase P single-copy human reference gene assay, optimized, and further characterized using whole blood spiked with linearized plasmid containing the FMC63 target sequence.

The assay demonstrating the best preliminary efficiency, precision, and sensitivity was then validated in accordance with guidelines recommended by the New York State Department of Health, College of American Pathologists (CAP), and Clinical and Laboratory Standards Institute (CLSI) to establish the analytical specificity, linearity and dynamic range, analytical sensitivity (limit of detection and lower limit of quantification), intra- and inter-assay precision (reproducibility), and analytical accuracy of the test method[1-7].

DNA was extracted from EDTA whole blood using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit and KingFisher™ Flex system (Thermo Fisher). Amplification and detection were performed using TaqMan™ Fast Advanced Master Mix (Thermo Fisher) and the Applied Biosystems™ 7500 Fast instrument. Quantification was performed using FMC63 and RNase P linearized plasmid standards containing scFv FMC63 and RNase P qPCR target sequences, and quantitative results were evaluated in copies/mL and copies/µg of genomic DNA assuming one copy of RNase P per haploid cell.

RESULTS

- **Table 1.** Analytical specificity against human genomic DNA was demonstrated by testing DNA isolated from EDTA whole blood collected from 20 individuals. FMC63 signal was undetected in all specimens tested, with all samples positive for RNase P, demonstrating successful extraction and amplification.

References:

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RESULTS

- **Figure 1.** Linear regression of plasmid standard dilutions resulted in an R^2 value of 0.9998 and a slope of -3.3235 (99.93% efficiency) over seven orders of magnitude (5 x 10^0 to 5.000 x 10^7 copies per reaction or the pre-extraction equivalent of 6.250 x 10^1 to 6.250 x 10^8 copies per mL). Full-process sample dilutions produced an R^2 of 0.9982, a slope of 1.0041, and a y-intercept of -0.0474 when analyzed in $\log_{10}$ copies/mL and an R^2 of 0.9984, a slope of 0.9867, and a y-intercept of 0.0507 when analyzed in $\log_{10}$ copies/µg DNA.
- **Table 2.** Excellent precision was observed, with intra-assay copies/mL %CVs results ranging from 1.51 to 20.68% across all concentrations tested and inter-assay %CVs of 5.53%, 8.45%, and 20.97% observed for high, medium, and low analyte concentrations, respectively. When analyzing copies/µg DNA results, intra-assay %CVs ranged from 2.32 to 16.44% across all concentrations and inter-assay %CVs were 7.58%, 6.86%, and 15.80% for high, medium, and low concentrations, respectively.
- **Table 3.** The limit of detection (LOD_{95}) predicted by Probit analysis was 87.61 copies/mL (95% confidence interval of 51.99 to 197.8 copies/mL) and 1.816 copies/µg (95% confidence interval of 1.077 to 4.099 copies/µg). The lower limit of quantification (LLoQ) was determined to be 100.0 copies/mL and 2.072 copies/µg of DNA.
- **Table 4.** Analytical accuracy was demonstrated with FMC63 target detected in 100% (60/60) of blinded and randomized positive samples with all observed $\log_{10}$ copies/mL and $\log_{10}$ copies/µg results within ±0.5 $\log_{10}$ copies of expected values. 100% (20/20) of blinded negative samples were negative for the FMC63 target and positive for RNase P, as shown in **Table 1**.

Table 1. Analytical specificity and negative analytical accuracy sample data

Sample ID	FMC63 CAR Copies/mL	RNase P Copies/mL	Sample ID	FMC63 CAR Copies/mL	RNase P Copies/mL	Sample ID	FMC63 CAR Copies/mL	RNase P Copies/mL	Sample ID	FMC63 CAR Copies/mL	RNase P Copies/mL
Negative Donor 1	Not Detected	14,166,792	Negative Donor 6	Not Detected	8,144,858	Negative Donor 11	ND	9,936,369	Negative Donor 16	Not Detected	10,572,669
Negative Donor 2	Not Detected	9,849,029	Negative Donor 7	Not Detected	12,436,385	Negative Donor 12	ND	10,793,698	Negative Donor 17	Not Detected	23,401,263
Negative Donor 3	Not Detected	9,290,654	Negative Donor 8	Not Detected	7,743,921	Negative Donor 13	ND	9,226,385	Negative Donor 18	Not Detected	14,941,500
Negative Donor 4	Not Detected	14,334,866	Negative Donor 9	Not Detected	16,994,108	Negative Donor 14	ND	10,786,426	Negative Donor 19	Not Detected	19,012,166
Negative Donor 5	Not Detected	17,739,561	Negative Donor 10	Not Detected	18,815,863	Negative Donor 15	ND	18,806,989	Negative Donor 20	Not Detected	15,103,488

Figure 1. Linearity and dynamic range

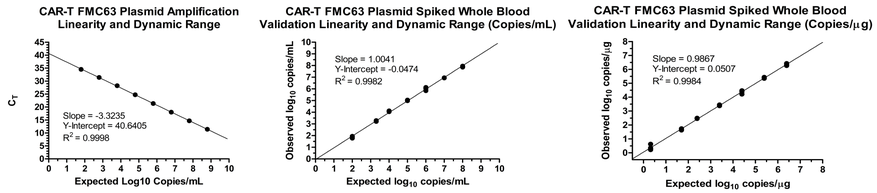

Table 2. Intra- and inter-assay precision

High Samples				Medium Samples				Low Samples			
Data Set		FMC63 CAR copies/mL	FMC63 CAR copies/µg DNA	Data Set		FMC63 CAR copies/mL	FMC63 CAR copies/µg DNA	Data Set		FMC63 CAR copies/mL	FMC63 CAR copies/µg DNA
Intra-assay precision day 1	Mean	9,124,534	228,215	Intra-assay precision day 1	Mean	100,999	2,727	Intra-assay precision day 1	Mean	10888	44.36
	SD	621,309	12,583		SD	3,232	150.2		SD	224.9	7.294
	%CV	6.81%	5.51%		%CV	3.20%	5.51%		%CV	11.92%	16.44%
Intra-assay precision day 2	Mean	8,810,215	240,034	Intra-assay precision day 2	Mean	118,127	2,973	Intra-assay precision day 2	Mean	1,734	46.63
	SD	132,788	18,718		SD	6,572	68.94		SD	124.2	4.375
	%CV	1.51%	7.80%		%CV	5.56%	2.32%		%CV	7.16%	9.38%
Intra-assay precision day 3	Mean	9,315,644	211,940	Intra-assay precision day 3	Mean	103,907	2,627	Intra-assay precision day 3	Mean	2,404	55.19
	SD	563,169	5,236		SD	5,088	142.2		SD	497.1	7.324
	%CV	6.05%	2.47%		%CV	4.90%	5.41%		%CV	20.68%	13.27%
INTER-ASSAY PRECISION	Mean	9,083,464	226,730	INTER-ASSAY PRECISION	Mean	107,678	2,776	INTER-ASSAY PRECISION	Mean	2,009	48.73
	SD	502,463	17,185		SD	9,096	190.5		SD	421.1	7.700
	%CV	5.53%	7.58%		%CV	8.45%	6.86%		%CV	20.97%	15.80%

RESULTS

Table 3. Analytical sensitivity (LOD and LLoQ)

Probit LOD Prediction:			87.61 copies/mL		LLoQ:	100.0 copies/mL	
Expected copies/mL	Observed Copies/mL	Expected $\log_{10}$ Copies/mL	Observed $\log_{10}$ Copies/mL	Standard deviation	Bias	% Detection	Total analytical error
800.0	660.5	2.9031	2.8114	0.0883	0.0916	100%	0.3
400.0	269.1	2.6021	2.4106	0.1323	0.1915	100%	0.5
200.0	145.6	2.3010	2.1434	0.1398	0.1576	100%	0.4
100.0	67.11	2.0000	1.7937	0.1808	0.2063	100%	0.6
50.00	26.32	1.6990	1.3104	0.3444	0.3885	80%	1.1
12.50	10.14	1.0969	0.9180	0.2539	0.1789	65%	0.7
6.250	6.706	0.7959	0.7918	0.1835	0.0041	40%	0.4
3.125	4.623	0.4949	0.6395	0.1660	0.1447	32%	0.5
1.563	4.430	0.1938	0.6460	0.0260	0.4522	10%	0.5

Probit LOD Prediction:			1.816 copies/µg		LLoQ:	2.072 copies/µg	
Expected copies/µg	Observed Copies/µg	Expected $\log_{10}$ Copies/µg	Observed $\log_{10}$ Copies/µg	Standard deviation	Bias	% Detection	Total analytical error
16.58	13.52	1.2196	1.1226	0.0887	0.0970	100%	0.3
8.289	5.287	0.9185	0.7059	0.1233	0.2126	100%	0.5
4.145	3.061	0.6175	0.4639	0.1490	0.1536	100%	0.5
2.072	1.659	0.3165	0.1670	0.2269	0.1495	100%	0.6
1.036	0.5762	0.0154	-0.3429	0.3409	0.3584	80%	1.0
0.5181	0.3079	-0.2856	-0.5856	0.2654	0.3000	75%	0.8
0.2590	0.2343	-0.5866	-0.7505	0.2907	0.1638	65%	0.7
0.1295	0.1344	-0.8877	-0.9103	0.1848	0.0226	40%	0.4
0.06476	0.08893	-1.1887	-1.0691	0.1429	0.1196	32%	0.4
0.03238	0.08737	-1.4897	-1.0647	0.1032	0.4250	10%	0.6

Table 4. Analytical accuracy positive sample data

Expected copies/mL	Expected $\log_{10}$ copies/mL	Minimum observed $\log_{10}$ copies/mL	Minimum observed vs. expected $\Delta\log_{10}$ copies/mL	Maximum observed $\log_{10}$ copies/mL	Maximum observed vs. expected $\Delta\log_{10}$ copies/mL
100,000,000	8.0000	7.8663	-0.1337	7.9528	-0.0472
10,000,000	7.0000	6.9245	-0.0755	7.0050	0.0050
1,000,000	6.0000	5.8460	-0.1540	6.1331	0.1331
100,000	5.0000	4.9882	-0.0118	5.1051	0.1051
10,000	4.0000	4.0447	0.0447	4.1172	0.1172
2,000	3.3010	3.2071	-0.0939	3.4796	0.1785

Expected copies/µg	Expected $\log_{10}$ copies/µg	Minimum observed $\log_{10}$ copies/µg	Minimum observed vs. expected $\Delta\log_{10}$ copies/µg	Maximum observed $\log_{10}$ copies/µg	Maximum observed vs. expected $\Delta\log_{10}$ copies/µg
2,469,131	6.3925	6.2698	-0.1228	6.4234	0.0308
246,913	5.3925	5.3159	-0.0767	5.4302	0.0376
24,691	4.3925	4.2172	-0.1754	4.4659	0.0734
2,469	3.3925	3.3815	-0.0110	3.4881	0.0956
246.9	2.3925	2.4551	0.0626	2.4906	0.0980
49.38	1.6936	1.5616	-0.1320	1.8235	0.1299

CONCLUSIONS

Monitoring of CAR-T cell expansion and persistence can provide important insight into therapeutic efficacy, durability of response, potential for relapse, and uncontrolled proliferation. Currently, there are no direct CAR-T cell monitoring options commercially available, with B cell aplasia typically used as a surrogate marker for CD19-directed CAR-T cell efficacy. The validated test method described here demonstrates excellent specificity, sensitivity, linearity, precision, and accuracy, providing a reliable means of monitoring anti-CD19 CAR-T cell persistence in patients receiving any of the currently approved therapies, as well as others under investigation using the FMC63 scFv.