康维讯生物
KANWHISH

官方微信公众号
康维讯生物官网
www.kanwhish.com

# Establishment of qPCR Assay for Detection of CAR-T Cell Products in Human Serum

Lun Pan[1], Yu Liang[1], Hefen Zhang[1], Linglong Zou[1, 2]
([1] Kanwhish Biotechnology Co., Ltd., [2] Corresponding author)

## Introduction

Chimeric antigen receptor (CAR) T-cell technology reprograms T cells to engage and eliminate cancer cells. In this technology, patients' T cells are transduced in vitro using lentiviral or retroviral vectors containing a CAR transgene. Following infusion, CAR-T cells expand in vivo and may persist in the peripheral blood and bone marrow for years. Therefore, monitoring copies of the CAR transgene in vivo requires highly sensitive and reliable analytical methods validated for their intended use. Herein, we describe the validation of a qPCR assay to detect CAR transgene.

## Method Design

When using PCR method to analyze the pharmacokinetic characteristics of CAR-T cell therapy products, it is necessary to know the nucleic acid sequence of the CAR-T gene and design specific primers and probe for this sequence. However, the nucleic acid sequence information in the scFv domain is usually not available to a CRO's method developer due to sponsor's IP concern. In this case, a universal method can be established using the sequence of CAR's signal transduction domain for primer and probe design. To increase method specificity, Taqman probe approach is used to establish qPCR assay method. In addition, forward and reverse primers are designed from sequences encoding two adjacent domains, while probes are designed using the sequence at the junction of these two domains (Figure 1).

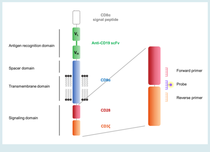



Figure 1 Assay design

## Assay Results

### Linearity and Dynamic Range

Conformational differences of plasmid DNA can cause a significant difference in standard curves resulting in inaccurate quantitation. Therefore, we tested two different conformational plasmid. First, the linearity and dynamic range of the CAR transgene qPCR assay were evaluated.

For the linearized plasmid, representative standard curves for the CAR qPCR assays are shown in Figure 2. The mean $R^2$ value (0.9998 ± 0.0002) from three independent CAR transgene qPCR assay runs indicated a high degree of linearity, defining a dynamic range of 10–10,000,000 transgene DNA copies/200 ng human gDNA. The mean slope of –3.4166 ± 0.0499 resulted in a mean amplification efficiency of 96.23% ± 1.90%.

For the plasmid, representative standard curves for the CAR qPCR assays are shown in Figure 3. The mean $R^2$ value (0.9998 ± 0.0001) from three independent CAR transgene qPCR assay runs indicated a high degree of linearity, defining a dynamic range of 20–10,000,000 transgene DNA copies/400 ng human gDNA. The mean slope of –3.4396 ± 0.0238 resulted in a mean amplification efficiency of 95.32% ± 0.90%.

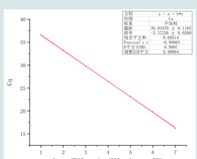



Figure 2. Standard curves of linearized plasmid qPCR assay

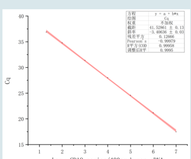



Figure 3. Standard curves of intact plasmid qPCR assay

### Accuracy & Precision

Five levels of QCs (ULOQ, H/M/LQCs, LLOQ) were used to assess assay precision and accuracy. These five QCs are prepared by spiking the linearized plasmid or plasmid containing the transgene into human gDNA isolated from healthy donors.

Table 1. Standard curve performance of linearized plasmid

| Unit: copies/200 ng human gDNA | STD1 1E7 | STD2 1E6 | STD3 1E5 | STD4 1E4 | STD5 1E3 | STD6 100 | STD7 10 |
|---|---|---|---|---|---|---|---|
| Back-calculated | 10664490.7 | 951161.2 | 97583.7 | 9774.2 | 1032.0 | 99.7 | 10.2 |
| Inter-run %CV | 6.7 | 2.8 | 7.6 | 4.4 | 5.2 | 6.4 | 9.1 |
| Inter-run %RE | 6.6 | -4.9 | -2.4 | -2.3 | 3.2 | -0.3 | 1.7 |
| n | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

Table 2. Standard curve performance of plasmid

| Unit: copies/400 ng human gDNA | STD1 1E7 | STD2 1E6 | STD3 1E5 | STD4 1E4 | STD5 1E3 | STD6 100 | STD7 20 |
|---|---|---|---|---|---|---|---|
| Back-calculated | 10679875.7 | 957733.9 | 98239.9 | 9587.1 | 1017.9 | 98.5 | 20.9 |
| Inter-run %CV | 4.4 | 1.5 | 3.5 | 2.7 | 5.1 | 8.7 | 7.6 |
| Inter-run %RE | 6.8 | -4.2 | -1.8 | -4.1 | 1.8 | -1.5 | 4.4 |
| n | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

Table 3 Accuracy and precision of QC samples (with linearized plasmid standards)

| Unit: copies/200 ng human gDNA | LLOQ (10) | LQC (30) | MQC (3E4) | HQC (7.5E6) | ULOQ (1E7) |
|---|---|---|---|---|---|
| Back-calculated | 11.0 | 34.1 | 22030.0 | 7342474.8 | 10059604.8 |
| Inter-run %CV | 15.9 | 12.1 | 9.5 | 8.2 | 9.7 |
| Inter-run %RE | 10.0 | 13.6 | 10.2 | -2.1 | 0.6 |
| n | 18 | 18 | 18 | 18 | 18 |

Table 4 Accuracy and precision of QC samples (with intact plasmid standards)

| Unit: copies/400 ng human gDNA | LLOQ (20) | LQC (60) | MQC (3E4) | HQC (7.5E6) | ULOQ (1E7) |
|---|---|---|---|---|---|
| Back-calculated | 23.2 | 69.8 | 32114.0 | 7293447.9 | 9883775.3 |
| Inter-run %CV | 12.1 | 11.8 | 12.6 | 7.9 | 7.6 |
| Inter-run %RE | 16.2 | 16.4 | 7.0 | -2.8 | -1.2 |
| n | 18 | 18 | 18 | 18 | 18 |

As summarized in Table 1-4, the intra- and inter-assay precision & accuracy of standard calibrators and QC (ULOQ, H/M/LQCs, LLOQ) were all within the acceptable range, which indicates that the method is both accurate and precise.

### Limit of Detection

Gradient dilutions were made with intact versus linearized plasmid standards and analyzed for detection at each dilution level. Six independent assay batches were performed with a total of 18 replicates at each dilution level. Copy number were used to quantify gradient dilution standards with a 8-point standard curve while calculating the precision. By Probit analysis of the test results of the standard, the LOD was 6.18 copies/200 ng human gDNA with a 95% confident detection rate for linearized plasmid standards, and 14.52 copies/400 ng human gDNA with a 95% confident detection rate for plasmid standards.

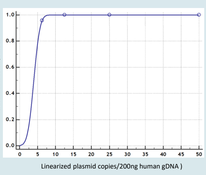



Figure 4. limit of detection of the linearized plasmid qPCR assay

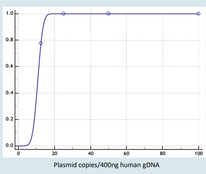



Figure 5. limit of detection of the plasmid qPCR assay

### Assay Specificity

In order to verify the specificity of this assay, the genome DNA of human and E.coli were added, respectively, to perform qPCR amplification reaction, and the specificity of this method was verified by amplification curve. The results showed that the qPCR assay had no amplification, which indicates that this assay tolerated the 400 ng human and E. coli genome DNA well.

## Conclusion/Discussion

We have developed a qPCR analysis to detect CAR transgene. The validation results demonstrated adequate assay sensitivity, accuracy, precision, specificity. In comparison, linearized plasmids have better performance than plasmids, due to the advantages in conformation.

© 2023 CBF China