Establishment of a general qPCR method for detecting nonclinical biodistribution of CGT products

Ling Zhao[1], Tong Ge[1], Haiwen Ruan[1], Yuanguo Cheng[1], Xinping Fang[1]
[1] TriApex Laboratories Co., Ltd., No.9, Xinglong Rd., Jiangbei New Area, Nanjing 211800, China

TRIAPEX

Introduction

Gene-modified cell therapy (GCT) products require comprehensive biodistribution assessment to monitor cell migration, homing patterns, and persistence across different administration stages for nonclinical safety evaluation. Quantitative real-time polymerase chain reaction (qPCR) has emerged as a preferred analytical method for such studies due to its high sensitivity, specificity, and reliability in detecting human-specific genomic sequences within complex biological matrices. However, current approaches lack standardized protocols for universal application in animal models, particularly when targeting conserved genomic regions such as the human *st6gal1* gene. To address this gap, we aimed to develop and validate a robust qPCR-based assay for nonclinical biodistribution analysis of GCT products through targeted amplification of the human-specific *st6gal1* sequence.

Methods

Experimental Materials:

- Human-specific gene fragment: The *st6gal1* gene fragment (709 bp) was identified and obtained from the human genome using the UCSC Table Browser and BLAST/BLAT tools. This fragment was selected for its human-specific sequence, which enables reliable detection of human-derived cells in animal models.
- Plasmid standard: The *st6gal1* fragment was cloned into the pUCm-T vector, linearized with restriction enzymes (*EcoR I* and *Hind III*), and quantified to prepare a plasmid standard with a concentration of 2.90×10^{11} copies/μL. This plasmid served as reference material for constructing calibration curves and quality control samples.
- Cynomolgus monkey genomic DNA: Genomic DNA was isolated from cynomolgus monkey liver tissue. Its concentration and purity were measured using a spectrophotometer, followed by dilution to 200 ng/μL for use as a blank matrix during method validation.

Experimental Methods

- Primer and probe design: Sequence-specific primers and a TaqMan probe were designed for the human *st6gal1* gene. The probe was conjugated with a FAM fluorescent reporter at the 5′-end and a minor groove binder (MGB) non-fluorescent quencher at the 3′-end.
- Standard curve and quality control samples: The plasmid standard was diluted to create a standard curve with concentrations ranging from 5.00×10^{1} to 5.00×10^{7} copies/5 μL. Quality control samples were spiked at five concentration levels (ULOQ, HQC, MQC, LQC, and LLOQ), to evaluate method performance.
- DNA extraction: Genomic DNA was extracted from samples using the DNeasy® Blood & Tissue Kit, and its concentration and purity were measured by spectrophotometric quantification, with acceptance criteria set as concentration ≥20.00 ng/μL and purity ratios of 1.75–2.14.
- PCR reaction setup: The PCR reaction mixture contained the following components: AceQ® Universal U+ Probe Master Mix V2, primers, probe, DEPC-Treated Water, and template DNA. Thermal cycling was performed for 40 amplification cycles, with real-time fluorescence acquisition during the annealing/extension phase of each cycle.

Table 1. PCR system

Component	Volume (μL)
2×AceQ Universal U+ Probe Master Mix V2	10
st6gal1-F (10 μM)	0.4
st6gal1-R (10 μM)	0.4
st6gal1-P (10 μM)	0.2
DEPC-Treated Water	4
Template DNA	5

Table 2. PCR amplified procedure

Cycles	Temperature	Time
1×	37 °C	2 min
	95 °C	5 min
40×	95 °C	10 s
	62 °C	30 s

Result

Accuracy and Precision:

The method demonstrated good accuracy and precision across different concentration levels. Intra-run precision ranged from 1.12% to 28.49%, and inter-run precision ranged from 7.41% to 17.93%. Accuracy ranged from -15.45% to 17.15% for intra-run and -4.17% to 1.05% for inter-run, meeting the predefined acceptance criteria.

Table 3. Data of accuracy and precision

	Sample	Precision	Accuracy
Intra-assay	ULOQ	1.12 to 11.00	-5.16 to 8.05
	HQC	2.14 to 10.08	-5.44 to 5.00
	MQC	2.95 to 13.16	-6.16 to 14.07
	LQC	2.40 to 14.30	-15.45 to 17.15
	LLOQ	8.24 to 28.49	-13.29 to 14.97
Inter-assay	ULOQ	7.70	1.05
	HQC	7.41	-0.71
	MQC	10.00	-1.76
	LQC	13.28	-4.17
	LLOQ	17.93	-1.62

Standard Curve, Linear Range, and Sensitivity:

The method showed a linear range from 5.00×10^{1} to 5.00×10^{7} copies/5 μL, with an R^2 value of 0.999, indicating high linearity and PCR amplification efficiency. The sensitivity was confirmed at 5.00×10^{1} copies/5 μL, which is suitable for detecting low levels of human cells in animal tissues.

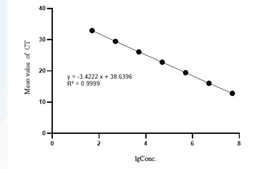

Figure 1. Standard curve for detection of *st6gal1* in cynomolgus monkeys by qPCR

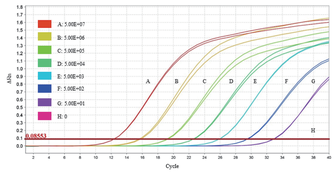

Figure 2. Amplified curve of standards (unit: copies/5 μL)

Specificity:

The assay specifically detected the *st6gal1* gene in cynomolgus monkey liver tissue spiked with human genomic DNA, exhibiting no observable cross-reactivity with the background genomic matrix. This validates the method's specificity in differentiating human-derived DNA from nonhuman sources.

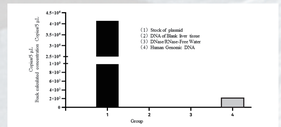

Figure 3. Result of specificity

Selectivity:

The method showed good selectivity across different tissues (liver, heart, spleen, lung, kidney, brain, muscle, stomach, testis/ovary, epididymis/uterus) and blood samples from both male and female cynomolgus monkeys. This indicates that the method can be applied to various tissue types without significant matrix effects.

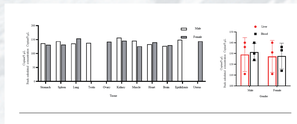

Figure 4. Data of selectivity

Extraction Recovery:

The extraction recovery rates were 56.49% for liver tissue homogenate and 54.60% for whole blood, with %CV values of 13.90% and 17.24%, respectively. These recovery rates meet the acceptance criteria, indicating efficient DNA extraction from different matrices.

Table 4. Data of extraction recovery

Matrix	Concentration (copies/5μL)	CV (%)	Extraction recovery (%)	Mean of extraction recovery (%)	Precision (%)
Liver tissue homogenate of cynomolgus monkeys	5.87E+05	3.12	65.28	56.49	13.90
	4.51E+05	6.02	50.17		
	4.86E+05	2.97	54.03		
Blood from cynomolgus monkeys	2.50E+05	2.30	49.98	54.60	17.24
	3.27E+05	6.24	65.43		
	2.42E+05	1.91	48.39		

Stability:

The plasmid standard was stable under various conditions, including long-term storage at -65°C to -90°C for 66 days, room temperature for 24 hours, 2°C to 8°C for 24 hours, and after five freeze-thaw cycles. This demonstrates the robustness of the method under different storage and handling conditions.

Bridging Assay for Matrices with Different Genomic Content:

The method performed well with different concentrations of cynomolgus monkey liver DNA (10 ng/μL and 500 ng/μL), meeting the acceptance criteria for precision and accuracy. This indicates that the method is robust across different DNA concentrations.

Table 5. Data of bridging assay for matrices

Concentration level of samples	Matrix concentration 10 ng/μL		Matrix concentration 500 ng/μL	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
ULOQ	1.13 to 2.48	-2.10 to 5.97	0.34 to 2.31	-5.22 to 2.22
HQC	0.71 to 4.18	-1.76 to 10.17	0.74 to 1.66	-2.83 to 7.62
MQC	0.84 to 1.05	-9.78 to -2.33	0.26 to 1.77	-17.77 to -0.97
LQC	0.98 to 5.45	-16.52 to 0.88	6.33 to 7.20	-26.27 to -6.15
LLOQ	6.38 to 12.25	-11.69 to -4.59	8.14 to 13.47	-25.04 to -4.44

Universality:

The method successfully detected *st6gal1* in 40 human peripheral blood DNA samples, demonstrating its universality for detecting human DNA in various contexts. This suggests that the method can be applied to different types of human cell therapy products.

Conclusions

Full method validation indicated this study established a general qPCR method for nonclinical biodistribution assay of cellular therapy products. Compared with the routine qPCR method targeting chimeric antigen receptor (CAR), this method eliminates the limitation on drug type and reduces the cycle and cost of method development and validation, thereby promoting cellular therapy products nonclinical research.

Novel Aspects:

- **1. Comparison with Alu-based qPCR Methods:**
  Existing Methods: Many existing qPCR methods rely on multi-copy Alu sequences in the human genome for detection. However, Alu sequences are also prevalent in primate genomes, leading to insufficient specificity, especially when detecting human cells in primate models.
  Novelty of This Method: This study selects the single-copy *st6gal1* gene as the detection target, which is highly specific to humans and does not cross-react with non-human primate genomes. This approach significantly improves specificity, particularly in non-human primate models such as cynomolgus monkeys.
- **2. Comparison with Gender-Specific Markers (e.g., SRY or TSPY):**
  Existing Methods: Some methods use gender-specific markers like SRY (for male cells) or TSPY (for male cells in female recipients). These methods are limited to detecting cells from male donors in female recipients and cannot be applied universally.
  Novelty of This Method: The *st6gal1*-based qPCR method is gender-independent and can detect human cells regardless of the donor or recipient's gender. This universality makes it applicable to a broader range of cell therapy products and non-clinical studies.
- **3. Comparison with Multi-Species qPCR Methods:**
  Existing Methods: Some qPCR methods have been developed to detect human cells in rodent models by simultaneously amplifying human and mouse genomic DNA. However, these methods often suffer from lower sensitivity and are not suitable for non-rodent models like primates.
  Novelty of This Method: This method is specifically designed to detect human cells in non-human primates, such as cynomolgus monkeys, with high sensitivity (50 copies/5 μL). It bridges the gap in existing methods that are primarily optimized for rodent models.
- **4. Comparison with Universality:**
  Existing Methods: Some qPCR methods are tailored for specific cell therapy products or limited to certain tissues, requiring separate method development and validation for each product or tissue type.
  Novelty of This Method: The *st6gal1*-based qPCR method is universally applicable across various tissues (e.g., liver, heart, spleen, blood) and can be used for different cell therapy products without the need for extensive re-validation. This universality reduces the time and cost associated with method development.

Conclusion on Novelty:

- The novelty of this study lies in the development of a universal, highly specific, and sensitive qPCR method targeting the human-specific *st6gal1* gene for non-clinical biodistribution studies. Unlike existing methods that rely on multi-copy sequences, gender-specific markers, or are limited to rodent models, this method offers a robust and versatile solution for detecting human cells in non-human primates and other animal models. Its comprehensive validation according to regulatory guidelines further enhances its applicability in the development and regulatory approval of cell therapy products.
- In summary, this method addresses several limitations of existing qPCR approaches, providing a novel and reliable tool for non-clinical biodistribution studies in cell therapy research. Its ability to detect human cells with high specificity and sensitivity, combined with its universality and regulatory compliance, confirms its novelty and potential impact on the field.

Acknowledgement: Thanks to TriApex Laboratories Co., Ltd. and all the technicians involved in the project.

www.tri-apex.com