Comparison of Allglo and TaqMan Probes for Quantitative RT-PCR Detection of Simian Immunodeficiency Virus: Postprint

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Abstract

Objective To compare the lower limit of detection and repeatability of Allglo probe and TaqMan probe fluorescent quantitative RT-PCR for SIV detection. Methods The SIV standard was gradient diluted into six concentrations. For intra-batch variation analysis, 12 specimens were extracted simultaneously at each concentration; for inter-batch variation analysis, each specimen was extracted 12 times, followed by reverse transcription and quantitative PCR detection using both TaqMan and Allglo probes. For intra-batch analysis, reverse transcription and detection were performed simultaneously for each specimen; for inter-batch analysis, reverse transcription and detection were conducted in 12 separate runs. The PCR results from both probes were analyzed using the software accompanying the ABI7300 quantitative PCR instrument and relevant statistical methods. Results The lower limit of detection for both TaqMan and Allglo probe methods for SIV standards was 50 copies/mL. Repeatability results showed: intra-batch variation analysis revealed that the Allglo probe method had a maximum coefficient of variation (CV) of 0.63% and a minimum CV of 0.33%, while the TaqMan probe method had a maximum CV of 1.33% and a minimum CV of 0.2%; inter-batch variation analysis showed that the Allglo probe method had a maximum CV of 1.77% and a minimum CV of 0.95%, while the TaqMan probe method had a maximum CV of 1.86% and a minimum CV of 1.03%. Conclusion In the detection of simian immunodeficiency virus by fluorescent quantitative RT-PCR, the Allglo probe method may be superior to the TaqMan probe method.

Full Text

Preamble

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Abstract

Objective: To compare the sensitivity and reproducibility of Allglo and TaqMan probes in detecting simian immunodeficiency virus (SIV) using fluorescence quantitative RT-PCR (qRT-PCR). **Methods**: The SIV reference standard was diluted into six gradient concentrations. At each concentration, 12 samples were simultaneously extracted for intra-batch variation analysis, while each sample was extracted 12 times for inter-batch variation analysis. Following RNA extraction, reverse transcription was performed and quantitative PCR was conducted using both TaqMan and Allglo probes. For intra-batch analysis, all specimens underwent reverse transcription and qPCR simultaneously; for inter-batch analysis, these procedures were performed separately across 12 runs. The qPCR results from both probe systems were analyzed using the software integrated with the ABI 7300 quantitative PCR system and relevant statistical methods. **Results**: Both TaqMan and Allglo probe methods achieved a lower limit of sensitivity at 50 copies/mL for SIV detection. Reproducibility assessment revealed that for intra-batch analysis, the Allglo probe method exhibited a maximum coefficient of variation (CV) of 0.63% and minimum CV of 0.33%, compared to 1.33% and 0.2% for the TaqMan probe method, respectively. For inter-batch analysis, the Allglo probe method showed a maximum CV of 1.77% and minimum CV of 0.95%, versus 1.86% and 1.03% for the TaqMan probe method. **Conclusion**: In fluorescence quantitative RT-PCR detection of SIV, the Allglo probe method demonstrates superior performance compared to the TaqMan probe method.

Keywords: Allglo probe; TaqMan probe; simian immunodeficiency virus; fluorescence quantitative reverse transcription-polymerase chain reaction

Introduction

Simian immunodeficiency virus (SIV) exhibits high homology with human immunodeficiency virus (HIV) [1] and can infect rhesus macaques and other non-human primates to cause simian acquired immunodeficiency syndrome (SAIDS) [2-3]. Consequently, the SIV infection model has been widely employed as a gold standard animal model for HIV research in recent years, facilitating investigations into HIV pathogenesis, vaccine development, and drug screening [4-9]. The TaqMan probe method represents the most classical real-time quantitative PCR

technology and remains extensively used for rapid pathogen screening and viral load detection [10]. Allglo probes, developed by Allelogic Biosciences Corporation, represent a new generation of fluorescent dye probes offering advantages including lower cost, high sensitivity, strong specificity, and good reproducibility [12-13]. However, compared to TaqMan probes, the application of Allglo probe-based fluorescence quantitative RT-PCR for SIV/HIV viral load detection remains limited in China. This study aims to comparatively evaluate the performance of Allglo and TaqMan probe methods for detecting SIV viral load in standard samples.

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Materials and Methods

1.1.1 SIV Standard

SIVmac251 was cultured using the human chronic myeloid leukemia cell line (CEMx174 cells). The viral titer was quantified using the SIVmac251 viral standard (1×10 copies/mL, catalog number: 1338) obtained from the NIH AIDS Reagents Programme, yielding a final concentration of 4.5×10 copies/mL. The stock was diluted with fetal bovine serum (GIBCO) to the required concentrations.

1.1.2 Reagents for Reverse Transcription and Quantitative PCR

Reverse transcription was performed using a reverse transcription kit from MBI Fermentas. Quantitative PCR was conducted using a quantitative PCR kit from ABI.

1.2 Primers and Probes

Primers and probes were designed using ABI Primer Express 3.0 software. The Allglo probe design principle involved shortening the probe sequence to fewer than 20 bases while maintaining a Tm value around 60°C, based on the TaqMan probe design. The upstream primer sequence was AACCTTCGGTTCCAATTCCGGAAC; the downstream primer sequence was CTTCAACGGTTCATTCCCGGGACC. The TaqMan probe sequence was FAM-CACTTGCTGAAATGGCCTTAAAGCCAGTTC-TAMRA. The Allglo probe sequence was FAM-CACTTGCTGAAATGGCC-Allglo.

1.3.1 Establishment of Allglo and TaqMan Probe-based Fluorescence Quantitative RT-PCR Methods

SIV RNA was extracted using an in-house one-tube viral nucleic acid extraction reagent (patent number: CN200910039350.8). The reverse transcription reaction system (8 L total) consisted of: 5× Buffer (1.6 L), reverse primer (0.4 L), dNTP (10 mmol/L, 0.8 L), RNase inhibitor (40 U/ L, 0.25 L), water (4.55 L), and reverse transcriptase (0.4 L). After preparation on ice, 8 L of the reverse transcription mixture was added to each RNA precipitate, mixed by pipetting, and incubated at 42°C for 1 hour. The resulting cDNA was stored at -20°C until analysis.

For quantitative PCR, the frozen SIV standard cDNA was thawed at room temperature. The 25 L PCR reaction mixture contained: forward and reverse primers (0.25 L each), probe (0.2 L), water (9.8 L), denatured SIV cDNA (2 L), and PCR Mix (12.5 L). The thermal cycling conditions were: initial denaturation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

1.3.2 Sensitivity Testing

The SIV viral standard was diluted with fetal bovine serum to six concentrations: 5×10 , 5×10 , 5×10 , 5×10^{3}, 5×10^{2}, and 5×10^{1} copies/mL, with fetal bovine serum alone serving as negative control. RNA was extracted and reverse transcribed from each dilution, followed by quantitative PCR detection using both TaqMan and Allglo probes to determine the minimum detectable concentration.

1.3.3 Reproducibility Comparison

SIV viral standards at different concentrations (5×10 to 5×10^{1} copies/mL) were subjected to SIV RNA extraction. For intra-batch variation analysis, 12 specimens were simultaneously extracted at each concentration; for inter-batch variation analysis, each specimen was extracted 12 times independently. The extracted RNA was reverse transcribed, with intra-batch samples processed simultaneously and inter-batch samples processed across 12 separate runs. Quantitative PCR was performed using both TaqMan and Allglo probes, with intra-batch samples analyzed in parallel and inter-batch samples analyzed as single wells across 12 separate runs. Data were analyzed using the software integrated with the ABI 7300 quantitative PCR system.

1.3.4 Analysis of CT Values and SIV Standard Copy Numbers

The CT value represents the cycle number at which the fluorescent signal in each reaction tube reaches a predetermined threshold, and is inversely proportional to the initial copy number of template DNA (dRn) [14]. In this study, a standard curve was generated using standards with known initial copy numbers, with the

vertical axis representing CT values and the horizontal axis representing the logarithm of initial copy numbers (as shown in [Figure 2: see original paper] and [Figure 4: see original paper]). The correlation coefficient (R^2) value from the standard curve indicates the degree of correlation between CT values and fluorescence accumulation.

1.3.5 Statistical Analysis Methods

Quantitative PCR data files were analyzed using the software integrated with the ABI 7300 system. An Excel database was established to calculate mean CT values, standard deviations (SD), and coefficients of variation (CV%).

Results

2.1 Sensitivity Experimental Results Analysis

Both TaqMan and Allglo probe methods achieved a lower limit of detection at 50 copies/mL for SIV standards. However, the amplification curves from the TaqMan probe method showed that 2 out of 12 replicate wells at the 50 copies/mL concentration exhibited significant deviation. Inclusion of these 2 deviant amplification curves would reduce the R^2 value below 0.99; therefore, they were excluded from analysis, yielding an R^2 of 0.993 and demonstrating good correlation between CT values and fluorescence accumulation ([Figure 1: see original paper], [Figure 2: see original paper]). The Allglo probe amplification curves and standard curve for SIV standard cDNA showed no significantly deviant wells across all 12 replicates at each concentration, with an R^2 value of 0.993, indicating good correlation between CT values and fluorescence accumulation ([Figure 3: see original paper], [Figure 4: see original paper]).

2.2.1 Intra-batch Variation Analysis of SIV cDNA Using TaqMan Probe

The TaqMan probe method, after excluding the 2 deviant wells at 50 copies/mL, showed the maximum coefficient of variation (1.33%) at the 50 copies/mL concentration (10 replicates) and minimum CV (0.2%) at 5×10 copies/mL, indicating low variation and good reproducibility (, [Figure 1: see original paper], [Figure 2: see original paper]).

2.2.2 Intra-batch Variation Analysis of SIV cDNA Using Allglo Probe

The Allglo probe method exhibited the maximum CV (0.63%) at the 50 copies/mL concentration (12 replicates) and minimum CV (0.33%) at 500 copies/mL, demonstrating low variation and good reproducibility (, [Figure 3: see original paper], [Figure 4: see original paper]).

2.2.3 Inter-batch Variation Analysis of SIV cDNA Using TaqMan Probe

The TaqMan probe method showed the maximum inter-batch CV (1.86%) at the 5×10 copies/mL concentration and minimum CV (1.03%) at 500 copies/mL, indicating low variation and good reproducibility ().

2.2.4 Inter-batch Variation Analysis of SIV cDNA Using Allglo Probe

The Allglo probe method demonstrated the maximum inter-batch CV (1.77%) at the 5×10 copies/mL concentration and minimum CV (0.95%) at 5×10^3 copies/mL, showing low variation and good reproducibility ().

Discussion

Simian immunodeficiency virus is a retrovirus [15] that, upon infecting host cells, produces reverse transcriptase to convert viral RNA into DNA, which then integrates into the host genome. Mature virions are packaged and released into the bloodstream or tissue fluid [16]. During different stages of SAIDS in infected macaques, plasma viral loads exhibit corresponding changes [17], making plasma viral load a primary indicator for studying disease progression, monitoring antiviral treatment efficacy, and predicting prognosis [18-19]. Studies have shown that SIV viral load in macaque plasma during the plateau phase typically ranges from 1×10^3 to 1×10 copies/mL [20], and may decrease to below 0.5×10^2 copies/mL or become undetectable following effective highly active antiretroviral therapy (HAART) [21]. Current methods for detecting macaque plasma viral load include branched DNA technology, nucleic acid sequence-based amplification, and fluorescence quantitative PCR, with the latter being widely adopted due to its high specificity and sensitivity [23].

Optimal probe selection is critical for real-time fluorescence quantitative PCR detection of SIV viral load. Major probe types include TaqMan probes, modified TaqMan MGB probes, dual hybridization probes, molecular beacon probes, Simple probes, Allglo probes, and dual-labeled probes. Among these, TaqMan probe technology remains the most classical and widely used real-time quantitative PCR method in laboratories [11, 24]. The new-generation Allglo probe incorporates all advantages of conventional TaqMan, TaqMan MGB, and molecular beacon probes [25], prompting our comparison of TaqMan and Allglo probe-based qPCR methods for SIV viral load detection. TaqMan probes contain a fluorescent reporter at the 5' end and a quencher at the 3' end, with sequences fully complementary to the DNA template between the two primers. Fluorescence signal intensity correlates positively with PCR product quantity, enabling quantification through standard curves generated from known template standards. However, TaqMan probes have limitations including high background fluorescence and design challenges [26]. Allglo probes utilize specially labeled nucleotides that serve as both reporter and quencher groups, with chemical modifications that significantly increase Tm values. These probes feature single-dye

labeling without background fluorescence, achieving quenching through structural changes. After probe hydrolysis, the terminal fluorescent groups revert to reporter status, resulting in higher fluorescence accumulation values.

This study compared the sensitivity and reproducibility of both probe systems for SIV viral load detection. Sensitivity analysis revealed that both TaqMan and Allglo probes achieved a detection limit of 50 copies/mL. However, TaqMan probe amplification curves showed 2 out of 12 replicates with significant deviation at 50 copies/mL, suggesting that Allglo probes provide greater stability at this concentration. Reproducibility analysis demonstrated that although the TaqMan method (after excluding deviant wells) showed a lower minimum intra-batch CV than Allglo, the Allglo probe method exhibited smaller maximum and minimum CVs for both intra-batch and inter-batch analyses, indicating superior overall reproducibility. These findings suggest that Allglo probe-based fluorescence quantitative RT-PCR may be superior to TaqMan probe methods for SIV detection.

While TaqMan probes are widely used for pathogen detection and quantification in fungi, bacteria, and viruses [27-32], comparative studies on Allglo and TaqMan probes for detecting knockdown resistance gene mutations in mosquitoes, H7N9 influenza virus, and GI/GII noroviruses have reported that Allglo probes offer advantages in specificity, sensitivity, reproducibility, and cost-effectiveness [11-12, 33], consistent with our findings. These results indicate that Allglo probe technology holds promise for rapid screening of diverse pathogens, providing more effective detection methods for clinical diagnosis and related research applications.

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Figure Captions

[Figure 1: see original paper] Amplification plot of reference SIV cDNA by qPCR using TaqMan probe.

[Figure 2: see original paper] Standard curve of SIV detection corresponding to Fig.1 (the two wells with obvious deviation from the concentration of 50 copies/mL were removed).

[Figure 3: see original paper] Amplification plot of reference SIV cDNA by qPCR using Allglo probe.

[Figure 4: see original paper] Standard curve of SIV detection corresponding to Fig.3.

Note: Figure translations are in progress. See original paper for figures.