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Design and Optimization of a CD4 Primer for Quantitative Real-Time PCR (qRT-PCR)

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Abstract: Quantitative real-time polymerase chain reaction (qRT-PCR) is being explored as an affordable alternative to flow cytometry for monitoring CD4+ T lymphocytes in HIV-infected patients, but detailed design and validation of CD4-specific primers are rarely reported. To design a CD4-specific primer-probe set for qRT-PCR, assess its in silico specificity, and determine the optimal annealing temperature. A forward primer, reverse primer, and hydrolysis probe targeting CD4 transcript were designed from published CD4 gene sequences. Specificity was evaluated using NCBI Primer-BLAST against the Human mRNA RefSeq database. Optimal annealing temperature was determined using an 8-point thermal gradient (54.0-66.0°C) on a Bio-Rad CFX96 system with SensiFAST™ Probe No-ROX chemistry, comparing Cq values across the gradient. Results: Primer-BLAST confirmed specificity of the forward (20nt, Tm 57.42°C, GC 50.00%) and reverse (24nt, Tm 57.69°C, GC 41.67%) primers, with low self-complementarity (3.00 and 2.00) and no 3' self-complementarity. Cq values were tightly clustered (23.25-23.76) across the gradient; with the lowest Cq (23.25) at 54.8°C. The CD4-specific primer-probe set showed confirmed specificity and robust tolerance across a wide annealing temperature range, with an optimum of 54.8°C, supporting its use as a foundational tool for CD4+ T lymphocyte monitoring where flow cytometry is inaccessible.

Keywords: Annealing temperature; CD4; Primer-BLAST; Primer design; qRT-PCR.

Introduction

CD4+ T lymphocyte enumeration remains the principal biomarker for assessing immune status and guiding clinical decisions in individuals infected with Human Immunodeficiency Virus (HIV), informing decisions on antiretroviral therapy (ART) initiation, monitoring of treatment response, and detecting first-line treatment failure (WHO, 2024). Flow cytometry is the recognized gold standard for CD4+ T lymphocyte enumeration; however, its dependence on costly equipment, consumables, and trained personnel limits its availability in many resource-constrained healthcare settings, and reliance on clinical or immunological criteria alone in such settings has demonstrated limited sensitivity for detecting treatment failure (Glencross et al, 2008; Lynen et al, 2010). This has motivated growing interest in molecular alternatives, particularly quantitative real-time polymerase chain reaction (qRT-PCR)-

based approaches that quantify CD4 gene expression at the mRNA level as a surrogate for CD4+ T lymphocyte count (Hu et al., 2013; Coelho et al., 2021; Lee et al, 2004; Tessema et al, 2022).

The analytical validity of any qRT-PCR-based assay depends on the quality of the primer-probe set. According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, appropriate primer design, verified specificity, and empirically optimized reaction conditions - particularly annealing temperature - are prerequisites for generating reliable, reproducible Cycle Threshold (Ct) data (Bustin et al, 2009, Taylor et al, 2019). Primers with insufficient specificity or unoptimized thermal conditions risk non-specific amplification, primer-dimer formation, or inconsistent amplification efficiency, all of which can compromise downstream quantitative interpretation (Bustin et al, 2009, Taylor et al, 2019). Despite this, published reports describing CD4 qRT-PCR assays frequently report only the final primer sequences used, with limited documentation of the specificity assessment and thermal optimization process that preceded their application (Hu et al, 2013 ; Coelho et al,, 2021 ; Lee et al, 2004 ; Tessema et al, 2022).

This study reports the design, in silico specificity assessment, and annealing temperature optimization of a CD4-specific primer-probe set intended for use in qRT-PCR-based CD4+ T lymphocyte quantification. This primer-probe set was subsequently used to develop a CD4+ T lymphocyte count estimation model based on qRT-PCR Ct values, the clinical performance of which is described in a separate manuscript currently in preparation (Oktavia et al., unpublished data). The present report focuses specifically on the molecular assay development process: primer and probe design, confirmation of target specificity, and determination of the optimal annealing temperature.

Materials and Methods

This study was conducted at the Center for Diagnostic and Research on Infectious Diseases (PDRPI), Faculty of Medicine, Universitas Andalas, Padang, Indonesia, as part of a broader study developing a qRT-PCR-based CD4+ T lymphocyte count estimation model (Oktavia P, et al., unpublished data). Ethical approval was obtained from RS M. Djamil Padang, Indonesia, number DP.04.03/D.XVI.10.1/281/2025.

Primer and Probe Design

A forward primer, reverse primer, and hydrolysis probe targeting the CD4 transcript were designed by the research team based on the published CD4 gene sequences. The primer and probe sequences used in this study are listed in Table 1.

Table 1. CD4 Primer and Probe Sequences

Component	Sequence (5'→3')	Length (nt)
Forward primer (CD4 F)	CCATTCAGGACACAGGGAAA	20
Reverse primer (CD4 R)	AAAGACAGGAGGAGAAGGAAATAG	24
Probe	TGATGTGGGCAGGAGGGGTGGTGAA	25

Notes: *nt, nucleotides.*

In Silico Specificity Assessment

The specificity of the forward and reverse primers was evaluated using NCBI Primer-BLAST, with the target template set to the NCBI Messenger RNA Reference Sequences database restricted to Homo sapiens.[10] This tool screens candidate primer pairs against the selected reference database to identify potential amplification products at both the intended target site and any unintended genomic or transcript locations. It also reports primer length, melting temperature (Tm), GC content, and self-complementarity scores, which are indicators of primer dimer risk.

Annealing Temperature Optimization

The optimal annealing temperature for the CD4 primer-probe set was determined empirically using a temperature gradient qRT-PCR run on a Bio-Rad CFX96 Real-Time PCR System, which permits simultaneous amplification across a programmed temperature gradient distributed over eight wells (columns A-H). A pooled sample (designated 1MF) was

amplified in each of the eight gradient wells, spanning an annealing temperature range of 54.0–66.0°C, using the SOLIScript® 1-step Multiplex Probe Kit with FAM-labeled CD4-specific probe detection. The Cycle Quantification (Cq) value obtained at each temperature point was recorded and compared to identify the annealing temperature that yielded the lowest and most consistent Cq value.

Results and Discussion

Primer and Probe Specificity

Primer-BLAST analysis of the NCBI Human mRNA RefSeq database confirmed that both the forward and reverse primers amplified only the intended CD4 target template, with no unintended amplification products identified in the database. Table 2a summarizes the design and specificity parameters for each primer.

Table 2a. Design Parameters of the CD4 Primer Pair

Parameter	Forward Primer	Reverse Primeya
Sequence (5'→3')	CCATTCAGGACAC AGGGAAA	AAAGACAGGAGGAG AAGGAAATAG
Length (nt)	20	24
Melting temperature (Tm, °C)	57.42	57.69
GC content (%)	50.00	41.67
Self complementarity	3.00	2.00
Self 3' complementarity	0.00	0.00
Target template found (Homo sapiens mRNA RefSeq)	Yes – CD4 target only	Yes – CD4 target only

Tm, melting temperature; GC, guanine-cytosine.

The predicted amplification products ("Products on target templates") returned by Primer-BLAST are summarized in Table 2b. The primer pair produced consistent amplicons of 136-139 base pairs across all matched templates, including two annotated human CD4 mRNA transcript variants (variants 1 and 5), and a human genomic DNA clone containing the corresponding CD4 exonic sequence, and one non-human primate ortholog (Pongo pygmaeus CD4, transcript variant X2), reflecting the expected cross-species sequence conservation of the CD4 gene rather than a specificity concern for human clinical specimens.

Table 2b. Primer-BLAST Specificity Result of the CD4 Primer Pair

Accession	Description	Product Length (bp)
NM_000616.5	Homo sapiens CD4 molecule (CD4), transcript variant 1, mRNA	136
NM_001195017.3	Homo sapiens CD4 molecule (CD4), transcript variant 5, mRNA	136
CR932777.1	Human DNA sequence from clone XX-NCIH2171_7H20, complete sequence	136
XM_054444485.2	PREDICTED: Pongo pygmaeus CD4 molecule (CD4), transcript variant X2, mRNA	139

Sequence alignment mapping confirmed that the forward primer, probe, and reverse primer bind within a single annotated CD4 exon, spanning approximately nucleotide positions 2790-2970 of the reference CD4 mRNA sequence (NM_000616.5), and that all three oligonucleotides are oriented consistently with the expected amplicon architecture (Figure 1). The low self-complementarity scores obtained for both primers (3.00 for the forward primer and 2.00 for the reverse primer), together with the absence of 3' self-complementarity in either primer, indicated a low likelihood of primer-dimer formation under standard cycling conditions.

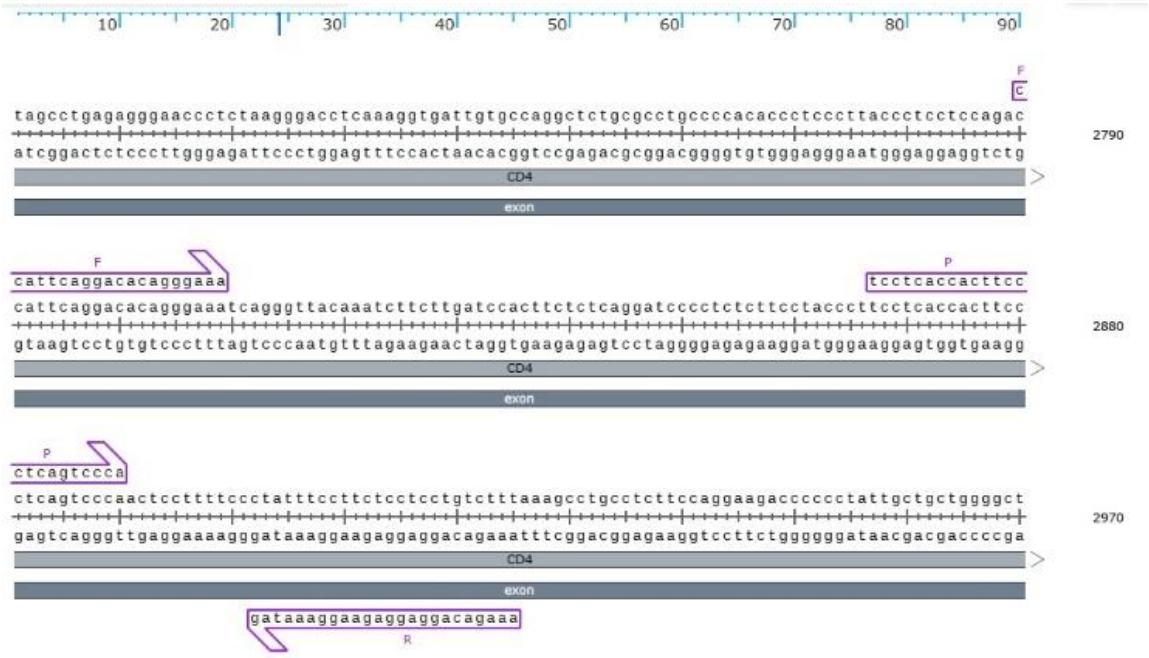

Figure 1 Alignment of the Forward Primer (F), Probe (P), and Reverse Primer (R) Binding Sites Within the CD4 Exon (Reference: NM_000616.5)

Annealing Temperature Optimization

Amplification of the pooled sample (1MF) across the eight-point annealing temperature gradient (54.0-66.0°C) produced consistent, well-defined amplification curves at every temperature tested, with Cq values ranging from 23.25 to 23.76 (Table 3, Figure 2. This narrow range indicates that the CD4 primer-probe set tolerates a broad range of annealing temperatures without a substantial loss of amplification efficiency or consistency.

Table 3. Cq Values of the CD4 Primer-Probe Set Across an 8-Point Annealing Temperature Gradient (Sample 1MF, FAM Fluorophore)

Well	Annealing Temperature (°C)	Target	Cq
A01	66.0	CD4	23.76
B01	65.3	CD4	23.72
C01	64.0	CD4	23.72
D01	61.6	CD4	23.34
E01	58.7	CD4	23.68
F01	56.4	CD4	23.67
G01	54.8	CD4	23.25
H01	54.0	CD4	23.38

Cq, quantification cycle.

The lowest Cq value (23.25) was observed at an annealing temperature of 54.8°C (well G01), indicating the most sensitive and efficient amplification across the tested temperatures. Therefore, this temperature was selected as the optimal annealing temperature for subsequent CD4 qRT-PCR assays using this primer-probe set.

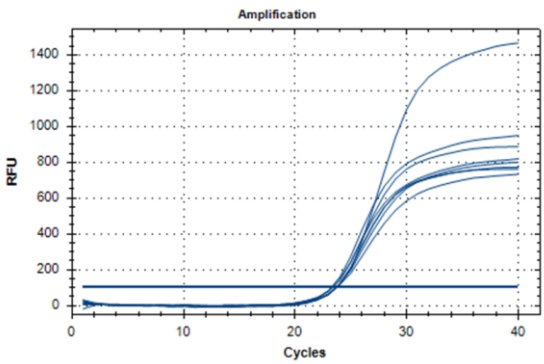

Figure 2. Amplification Curves of the CD4 Primer-Probe Set Across the 8-Point Annealing Temperature Gradient (54.0-66.0°C)

Discussion

Primer Specificity and *In Silico* Validation of the CD4 Primer-Probe Set

This study presents the design and multistage validation of a CD4-specific primer-probe set for qRT-PCR-based monitoring of $CD4^+$ T lymphocytes. *In silico* specificity

assessment using Primer-BLAST represents a critical first step in primer validation because it enables the identification of potential off-target amplification before laboratory testing (Ye et al., 2012). The confirmed specificity of the primer pair toward the intended CD4 transcript, together with low self-complementarity scores, indicates a low likelihood of non-specific amplification or primer-dimer formation, both of which are major sources of inaccurate Ct determination and reduced assay reproducibility (Bustin et al., 2009; Taylor et al., 2019).

These findings are consistent with the MIQE guidelines, which emphasize primer specificity as a prerequisite for reliable qRT-PCR quantification and recommend combining *in silico* evaluation with subsequent experimental validation to ensure analytical accuracy (Bustin et al., 2009). Similar observations have also been reported by Nolan et al., (2020), who demonstrated that rigorous primer specificity assessment substantially improves qPCR performance and minimizes false-positive amplification. Likewise, Huggett et al. (2020) emphasized that primer specificity is fundamental for ensuring the analytical validity and reproducibility of molecular diagnostic assays, particularly when qRT-PCR is intended for clinical applications.

Annealing Temperature Optimization and Assay Robustness

Annealing temperature is one of the most critical parameters influencing amplification efficiency and assay specificity. Temperatures below the optimal range may increase non-specific primer binding, whereas excessively high temperatures reduce primer-template hybridization efficiency, ultimately decreasing amplification yield (Bustin et al., 2009; Taylor et al., 2019). In the present study, the narrow Cq range observed across the temperature gradient (54.0–66.0°C; Cq 23.25–23.76) indicates that the primer-probe set maintains stable amplification performance over a relatively broad annealing temperature range.

Such thermal tolerance is advantageous because it increases assay robustness across different thermocycler platforms and laboratory conditions. The optimal annealing temperature of 54.8°C was selected because it produced the lowest Cq value, indicating the highest amplification efficiency under identical template concentrations (Bustin et al., 2009). Comparable results have been reported by Taylor et al. (2019), who noted that primer sets exhibiting minimal Cq variation across temperature gradients generally possess greater analytical robustness and improved inter-laboratory reproducibility.

Although the primer-probe set demonstrated stable amplification across the evaluated annealing temperature range, the present study did not experimentally determine PCR amplification efficiency, standard curve slope, or coefficient of determination (R^2). According to the MIQE guidelines, these parameters are essential for validating quantitative RT-qPCR assays because amplification efficiencies of 90–110%, slopes of approximately −3.3, and R^2 values ≥0.99 indicate reliable quantitative performance (Bustin et al., 2009). Recent recommendations likewise emphasize that assay validation should include amplification efficiency, analytical sensitivity, linearity, precision, and reproducibility before clinical implementation (Forootan et al., 2017; Huggett et al., 2020; Whale et al., 2023). Therefore, future studies should establish standard curves using serial template dilutions to comprehensively evaluate these analytical performance characteristics.

Sequence Alignment and Implications for Assay Specificity

Sequence alignment analysis demonstrated that the forward primer, hydrolysis probe, and reverse primer all bind within a single annotated exon of the CD4 gene (approximately nucleotide positions 2790–2970 of NM_000616.5), rather than spanning an exon-exon junction. Although this design preserves gene specificity, it does not inherently discriminate cDNA derived from CD4 mRNA from residual genomic DNA. Consequently, effective RNA purification and DNase treatment are essential to minimize genomic DNA contamination and ensure that Ct values accurately represent CD4 transcript abundance.

This observation agrees with previous recommendations that assays targeting a single exon require stringent RNA quality control or DNase treatment to avoid overestimation of gene expression (Bustin et al., 2009; Taylor et al., 2019). Similar conclusions have been reported by

Huggett et al. (2020), who highlighted the importance of minimizing genomic DNA contamination to improve the accuracy and reproducibility of RT-qPCR-based gene expression analysis. Future refinement of the assay may therefore consider exon-junction-spanning primer design to further improve transcript specificity.

Contribution of the Present Study to RT-qPCR Assay Development

Compared with previous studies, the present work extends earlier efforts by integrating computational primer design, *in silico* specificity analysis, sequence mapping, and empirical annealing temperature optimization into a single validation workflow. Previous investigations have generally focused on primer design or gene expression analysis independently, whereas the current study provides a more comprehensive analytical framework for early-stage assay development. This integrated approach establishes a robust foundation for subsequent analytical validation and may facilitate the development of standardized RT-qPCR assays for CD4 transcript quantification in molecular diagnostics.

Clinical Implications for CD4 Monitoring

Beyond analytical optimization, the validated primer-probe set has potential clinical relevance for $CD4^+$ T-lymphocyte monitoring. Flow cytometry remains the reference method for CD4 enumeration; however, its implementation is constrained in many low- and middle-income countries by high equipment costs, infrastructure requirements, and the need for specialized personnel. In contrast, many diagnostic laboratories already possess real-time PCR platforms that are routinely used for infectious disease testing (Tang et al., 2020). Therefore, a validated RT-qPCR assay targeting CD4 transcripts could provide an alternative molecular approach for estimating immune status in laboratories where flow cytometry is unavailable. Although additional clinical validation is required, this approach has the potential to complement conventional CD4 testing and improve access to HIV monitoring in resource-limited settings.

Practical Advantages and Future Development

From a practical perspective, the proposed assay may improve laboratory accessibility by utilizing existing RT-qPCR infrastructure without requiring dedicated flow cytometry instruments. This approach could potentially reduce operational costs, shorten turnaround time, and simplify implementation in regional laboratories already performing molecular diagnostics. Nevertheless, formal cost-effectiveness analyses comparing RT-qPCR with flow cytometry are still required before economic advantages can be established. Future investigations should also evaluate analytical sensitivity, limit of detection, inter-laboratory reproducibility, and diagnostic agreement with flow cytometry using larger and more diverse clinical populations.

Limitations

This study has several limitations. Primer specificity was evaluated primarily through *in silico* Primer-BLAST analysis against the human RefSeq mRNA database (Ye et al., 2012), whereas experimental confirmation using agarose gel electrophoresis, melting-curve analysis, or Sanger sequencing was not performed. In addition, annealing temperature optimization was conducted using a single pooled sample without biological replicates, limiting the generalizability of the selected optimal temperature. Furthermore, analytical performance parameters recommended by the MIQE guidelines, including amplification efficiency, standard curve slope, linearity (R^2), and limit of detection, were not evaluated in the present study.

Future Directions

Future studies should include comprehensive experimental validation of primer specificity together with standard curve analysis to determine amplification efficiency, linearity, analytical sensitivity, and reproducibility in accordance with current MIQE recommendations. Validation across larger and more diverse clinical samples, followed by comparison with flow cytometry, will be necessary to determine the diagnostic performance and clinical utility of the proposed RT-qPCR assay for $CD4^+$ T-lymphocyte monitoring.

Conclusions

A CD4-specific primer-probe set was designed and subjected to in silico specificity assessment and annealing temperature optimization. The primer pair demonstrated confirmed specificity to the intended CD4 target with low risk of self complementarity, and tolerance across a wide annealing temperature range with an optimal temperature of 54.8°C. These properties support the suitability of this primer-probe set as a foundational molecular tool for qRT-PCR-based CD4+ T lymphocyte monitoring assays, particularly in healthcare settings with limited access to flow cytometry. Further wet-laboratory specificity confirmation is recommended prior to broader assay implementation. The quantitative performance of this primer-probe set against flow cytometry-derived CD4+ T lymphocyte counts is reported in a separate manuscript currently in preparation.

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