Volume 1 (2026)
https://doi.org/10.22452/jtoh.vol1.4

JOURNAL OF TROPICAL One Health

SHORT COMMUNICATION

Modification of the rPLU5 Primer to Address Primer-Template Mismatch in a *Plasmodium* 2 Genus-Specific Nested PCR Assay

Yik-Zheng Lim, Nurul-Farhanah Hanuar, Noor-Syahida Azizan, Cheng-Hong Chua, Ummi-Syafiqah Rosmini, Wan-Nur-Athirah Muhammad-Ghazali, Juraina Abd-Jamil, Chee-Sieng Khor, Wei Yin Vinnie-Siow, Sing-Sin Sam, Van-Lun Low, Sazaly AbuBakar, Boon-Teong Teoh*

Tropical Infectious Diseases Research & Education Centre, Higher Institution Centre of Excellence, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author: boonteong@um.edu.my

ARTICLE HISTORY

Received: 28 Nov 2025
Revised: 31 Mar 2026
Accepted: 14 Apr 2026
Published 30 Jun 2026

ABSTRACT

A primer-template mismatch was identified at the second nucleotide from the 3′ end of the nest 1 reverse primer (rPLU5) used in the *Plasmodium* genus-specific nested PCR assay developed by Singh et al. (1999). The mismatches near the 3′ end of primer have a strong destabilizing effect on primer annealing. Therefore, rPLU5 was modified (C→T substitution) and nested PCR was optimized using annealing temperature gradients: 55–65 °C for nest 1 and 60–70 °C for nest 2. Optimal amplification was observed at 59–63 °C for nest 1 and 60–66 °C for nest 2, with the lowest temperatures recommended to maximize sensitivity and specificity. Furthermore, the performance of original and modified rPLU5 was compared using *Plasmodium*-positive DNA samples extracted from human clinical specimen and macaque specimen. The original rPLU5 amplified only the macaque sample, whereas the modified rPLU5 successfully amplified both samples, indicating improved detection. However, given the limited number of samples and absence of species identification, this modification should be interpreted as a precautionary optimization to enhance assay robustness rather than a performance limitation of the original rPLU5. Overall, this work presents an optimized nested PCR assay incorporating an improved primer design that minimizes mismatch-related amplification issues.

Keywords: malaria, mosquito, parasite, annealing temperature

Introduction

Plasmodium is a genus of parasitic protozoa that causes malaria in humans and other animals. Transmission occurs through the bite of an infected female *Anopheles* mosquito. The diagnosis of malaria can be based on the parasite detection using microscopy, antigen detection using rapid diagnostic tests or DNA detection using molecular methods (Sato, 2021). Singh et al. (1999) developed a nested PCR assay for detecting *Plasmodium* DNA. Nested PCR involves two sequential PCR amplifications using two primer sets: 1) nest 1 (first PCR amplification) primers amplify a larger region of the target DNA, and 2) nest 2 (second PCR amplification) primers bind within the first amplicon and amplify a smaller internal fragment. This two-step approach enhances sensitivity and specificity by allowing detection of low DNA level and minimizing non-specific products (Green & Sambrook, 2019; Singh et al.). We performed an *in silico* analysis of the *Plasmodium* genus-specific primer sets (Singh et al., 1999) to assess primer-template mismatches against nine *Plasmodium* species. A primer-template mismatch was identified at the second nucleotide from the 3′ end of the nest 1 reverse primer (rPLU5). Previous studies showed that mismatches near the 3′ end of primer have a strong destabilizing effect on primer annealing (Klungthong et al., 2010; Stadhouders et al., 2010). Therefore, we modified the rPLU5 primer, and subsequently optimized the nested PCR setup using the revised primer.

Materials and methods

Small subunit ribosomal RNA (ssrRNA) gene sequences of nine *Plasmodium* species, including *P. coatneyi* (NC_033560), *P. vivax* (NC_009910), *P. cynomolgi* (NC_020397), *P. fieldi* (AB287282), *P. inui* (EU400386), *P. knowlesi* (NC_041784), *P. falciparum* (NC_004326), *P. malariae* (NC_041784) and *P. ovale* (L48987) were retrieved from NCBI database. Multiple sequence alignment of these reference

sequences was generated using ClustalX2 and visualized in GeneDoc. *In silico* analysis revealed a primer-template mismatch at the second nucleotide from the 3′ end of rPLU5 primer, where the cytosine (C) was non-complementary to the adenosine (A) in all *Plasmodium* species (Figure 1). The original rPLU5 primer (5'-CCTGTTGTTGCCTTAAACTCC-3') was thereby modified by substituting the mismatched C with thymine (T). For nest 1 PCR, rPLU1 forward primer sequence was 5'-TCAAAGATTAAGCCATGCAAGTGA-3' (24 bp), and the modified rPLU5 reverse primer sequence is 5'-CCTGTTGTTGCCTTAAACTTC-3' (21 bp), with an expected product size of 1,627–1,720 bp. The C→T substitution in the rPLU5 was underlined. For nest 2 PCR, rPLU3 forward primer sequence was 5'-TTTTTATAAGGATAACTACGGAAAAGCTGT-3' (30 bp), and rPLU4 reverse primer sequence was 5'-TACCCGTCATAGCCATGTTAGGCCAATACC-3' (30 bp), with an expected product size of 233–243 bp. MyTaq Red Mix (Meridian, USA) was used in place of the PCR kit originally referenced by Singh et al. (1999). The nested PCR setup was adapted from Singh et al. (1999) with slight modifications based on the manufacturer's protocol of MyTaq Red Mix kit. The nest 1 PCR reaction mixture with a final volume of 20 µL comprised: 10 µL of 2× MyTaq Red Mix, 0.5 µL of 10 µM rPLU1, 0.5 µL of 10 µM modified rPLU5, 2 µL template DNA (*Plasmodium*-positive DNA sample spiked into human serum) and 7 µL of nuclease-free water. Thermocycling conditions were as follow: an initial denaturation at 95 °C for 4 min; 35 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing at 55–65 °C for 1 min (55, 57, 59, 61, 63 and 65 °C were tested), and extension at 72 °C for 1 min; and a final extension at 72 °C for 4 min. The nest 1 amplicons were then analyzed by agarose gel electrophoresis, and the amplicon showing a clear target band of expected size (1,627–1,720 bp) was used as DNA template for nest 2 PCR. The nest 2 PCR reaction mixture with a final volume of 20 µL comprised: 10 µL of 2× MyTaq Red Mix, 0.5 µL of 10 µM rPLU3, 0.5 µL of 10 µM rPLU4, 2 µL of DNA template (nest 1 amplicon) and 7 µL of nuclease-free water. Thermocycling conditions were as follows: an initial denaturation at 95 °C for 1 min; 35 cycles of amplification consisting of denaturation at 95 °C for 15 s, annealing at 60–70 °C for 15 s (60, 62, 64, 66, 68 and 70 °C were tested), and extension at 72 °C for 10 s. The nest 2 amplicons were then analyzed by agarose gel electrophoresis. Furthermore, the performance of original and modified rPLU5 primers were compared using *Plasmodium*-positive DNA samples extracted from a human clinical specimen and macaque specimen.

Results and discussion

The presence of single band of expected size (1,627–1,720 bp for nest 1 and 233–243 bp for nest 2) in the gel confirmed successful amplification of *Plasmodium* DNA. The annealing temperature of nest 1 PCR was evaluated at 55, 57, 59, 61, 63 and 65 °C. The amplicon at 55 °C showed a non-specific band (400 bp). A faint target band (1,500–2,000 bp) appeared at 57 °C, accompanied by a non-specific band similar to that observed at 55 °C. From 59 to 63 °C, clear target bands were observed, whereas no amplification was detected at 65 °C (Figure 2A). Sub-optimal annealing temperature results in non-specific amplification, whereas excessive stringency of super-optimal temperature leads to weak or no amplification (Rychlik, Spencer & Rhoads, 1990). Accordingly, the nest 1 amplicons at 59 and 63 °C were selected as templates for subsequent nest 2 PCR. The resulting nest 2 amplicons from both templates revealed similar amplification pattern across all annealing temperatures evaluated (60, 62, 64, 66, 68, and 70 °C). Nest 2 target bands (200–300 bp) were consistently observed from 60 to 68 °C, with a weaker intensity at 68 °C, whereas a non-specific band (400 bp) appeared at 70 °C (Figure 2B). Based on the results, the annealing temperatures of 59–63 °C for nest 1 PCR and 60–66 °C for nest 2 PCR were appropriate. The lowest annealing temperature within range was recommended as it maintains specific amplification without non-specific product, thereby maximizing sensitivity and specificity.

Two *Plasmodium*-positive DNA samples, one from a human clinical specimen and one from a macaque specimen, were tested to compare the performance of the original and modified rPLU5 primers. The comparison was performed using the annealing temperature described by Singh et al. (1999). As the nest 1 PCR produced weak or non-visible band, *Plasmodium* detection was determined based on the presence of target band in the nest 2 PCR. The original rPLU5 successfully amplified the macaque sample but failed to detect the human sample, whereas the modified rPLU5 successfully amplified both samples (Figure 3). This indicates that the primer modification improved detection in the tested samples. However, given the limited number of *Plasmodium*-positive DNA samples and absence of species identification, this modification should be interpreted as a precautionary optimization to enhance assay robustness rather than a performance limitation of the original rPLU5. Further validation using a larger panel of well-characterized *Plasmodium* species is required to confirm its impact on sensitivity and specificity. Overall, this work presents an optimized nested PCR assay incorporating a primer design corrected to reduce potential mismatch issues, thereby supporting more reliable *Plasmodium* detection.

https://doi.org/10.22452/jtoh.vol1.4

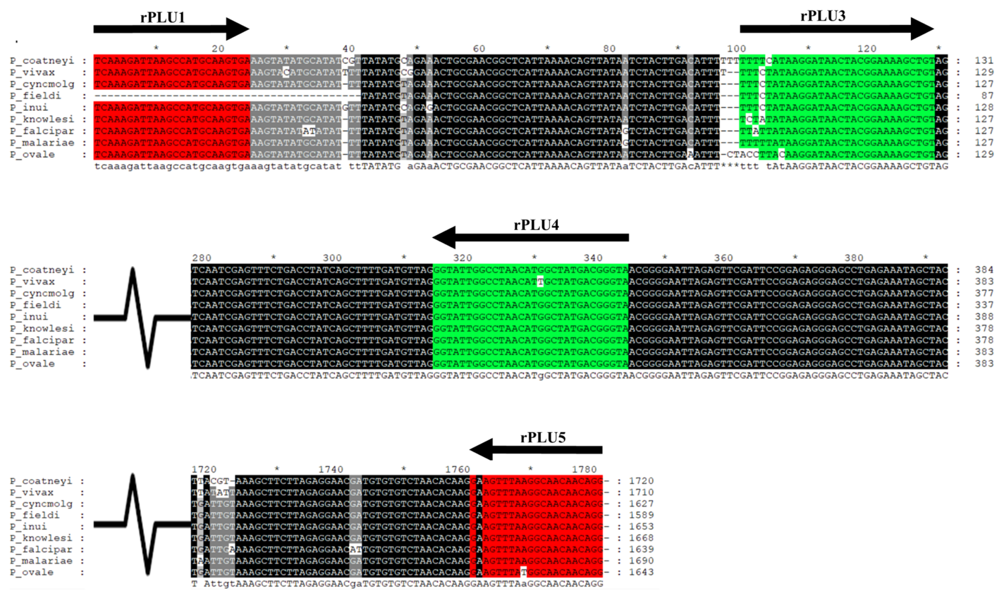

Figure 1. Multiple sequence alignment of nine *Plasmodium* species (*P. coatneyi, P. vivax, P. cynomolgi, P. fieldi, P. inui, P. knowlesi, P. falciparum, P. malariae* and *P. ovale*).
The arrows indicate orientation (5'→3') of nest 1 primers (rPLU1 and rPLU5) and nest 2 primers (rPLU3 and rPLU4). Highlighted nucleotides at the primer binding region indicate primer-template matches, whereas unhighlighted nucleotides indicate primer-template mismatches. The adenosine (A) at alignment position 1,762 in all *Plasmodium* species is mismatched with the 3' end of rPLU5 primer.

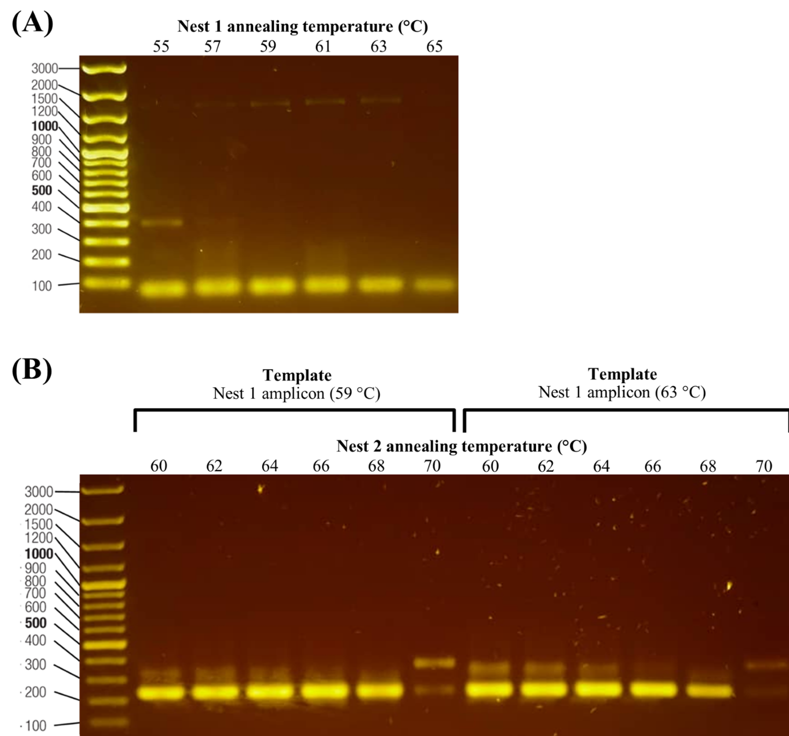

Figure 2. Optimization of annealing temperatures for (A) nest 1 and (B) nest 2 PCR.
(A) Nest 1 amplicons amplified at annealing temperatures of 55, 57, 59, 61, 63 and 65 °C are shown. Clear target bands (1,500–2,000 bp) are observed from 59 to 63 °C, while non-specific bands (400 bp) are visible at 55 and 57 °C. No amplification is detected at 65 °C.
(B) Nest 2 amplicons amplified at annealing temperatures of 60, 62, 64, 66, 68, and 70 °C, using nest 1 amplicons amplified at 59 °C and 63 °C as templates, are shown. Clear target bands (200–300 bp) are observed from 60 to 68 °C, with weaker intensity at 68 °C, while a non-specific band (400 bp) appears at 70 °C.

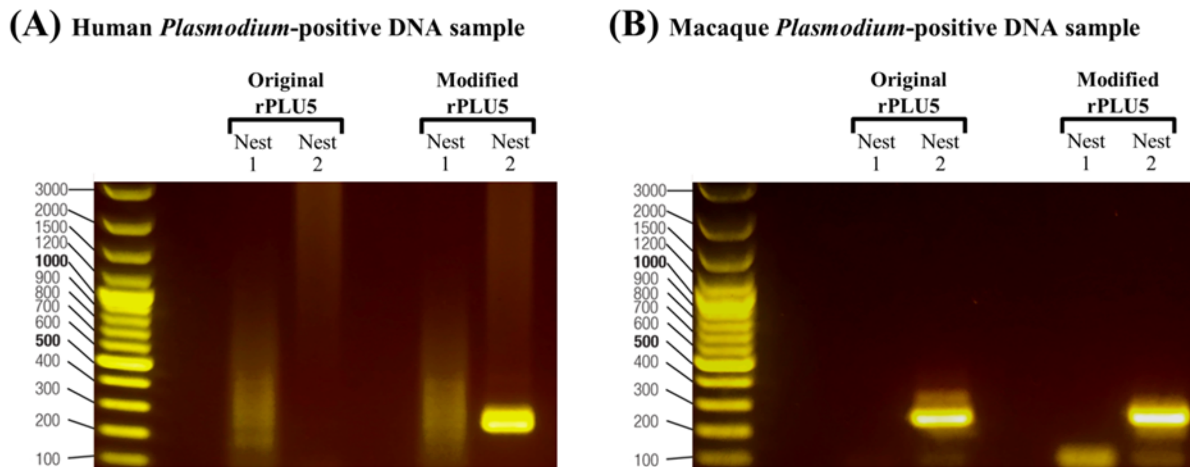

Figure 3. Comparison of performance between original and modified rPLU5 primers.
Nested PCR was performed using *Plasmodium*-positive DNA samples extracted from (A) a human clinical specimen and (B) a macaque specimen. Nest 2 target bands (200–300 bp) are observed in both samples using modified rPLU5 whereas no amplification was detected for human sample using original rPLU5.

Acknowledgements

This study was supported by the funding from the Ministry of Higher Education, Malaysia for niche area research under the Higher Institution Centre of Excellence (HICoE) Program (MO002-2019 & TIDREC-2023). We acknowledge Dr. Yap Nan Jiun (Department of Parasitology, Faculty of Medicine, University of Malaya) for providing *Plasmodium*-positive DNA samples.

Conflicts of interest statement

The authors declared no conflicts of interest.

References

Green, M. R., & Sambrook, J. (2019). Nested polymerase chain reaction (PCR). Cold Spring Harbor Protocols, 2019(2), Article pdb-prot095182. https://doi.org/10.1101/pdb-prot095182

Klungthong, C., Chinnawirotpisan, P., Hussem, K., Phonpakobsin, T., Manasatienkij, W., Ajariyakhajorn, C., Rungrojcharoenkit, K., Gibbons, R. V., & Jarman, R. G. (2010). The impact of primer and probe-template mismatches on the sensitivity of pandemic influenza A/H1N1/2009 virus detection by real-time RT-PCR. Journal of Clinical Virology, 48(2), 91–95. https://doi.org/10.1016/j.jcv.2010.03.012

Rychlik, W. J., Spencer, W. J., & Rhoads, R. E. (1990). Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Research, 18(21), 6409–6412. https://doi.org/10.1093/nar/18.21.6409

Sato, S. (2021). Plasmodium—a brief introduction to the parasites causing human malaria and their basic biology. Journal of Physiological Anthropology, 40(1), Article 1. https://doi.org/10.1186/s40101-021-00254-0

Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdullah, M. S., & Rahman, H. A. (1999). A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. The American Journal of Tropical Medicine and Hygiene, 60(4), 687–692. https://doi.org/10.4269/ajtmh.1999.60.687

Stadhouders, R., Pas, S. D., Anber, J., Voermans, J., Mes, T. H. M., & Schutten, M. (2010). The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. The Journal of Molecular Diagnostics, 12(1), 109–117. https://doi.org/10.2353/jmoldx.2010.090035