Natural Resources for Human Health
eISSN: 2583-1194

Original Research

Received 12 March 2026
Revised 20 April 2026
Accepted 18 May 2026

Natural Resources for Human Health 2026, Vol. 6 (2s): 196–203

KEYWORDS:
LAMP; colorimetric assay; beta-thalassemia; molecular screening; point-of-care diagnostics

Development and Optimization of a Colorimetric Loop-Mediated Isothermal Amplification (C-LAMP) Assay for Screening of Beta-Thalassemia Mutations

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ABSTRACT:

Objective: To develop and optimize a colorimetric loop-mediated isothermal amplification (C-LAMP) assay for equipment-minimal screening of four common HBB mutations (CD15, CD26/HbE, CD41/42, IVS1-5) prevalent in the Ciayumajakuning population, West Java, Indonesia.

Material and Methods: Allele-specific LAMP primers were designed for each target, with discriminating bases at the 3' terminus of the FIP/BIP segments; a dual-mismatch strategy was additionally applied for CD26/HbE to widen allele discrimination. Synthetic gBlock templates served as positive controls. Four colorimetric indicators (phenol red, malachite green, propidium iodide, and a commercial fluorescence dye) were compared for visual interpretability, equipment dependency, and cost. Reactions were incubated at 65°C for 60 minutes in a closed-tube format to minimize carryover contamination.

Results: Primer sets for all four targets, plus wild-type, produced specific amplification confirmed by gel electrophoresis, with no signal in wild-type or no-template controls. Phenol red produced the clearest unaided-eye color transition at the lowest cost without auxiliary equipment, outperforming the three comparator indicators, each limited by specificity or equipment dependency. Reproducibility, lyophilized reagent stability, and time-to-color kinetics were not assessed in this developmental phase.

Discussion/Conclusion: The optimized C-LAMP protocol offers a low-cost, instrument-minimal alternative for first-line beta-thalassemia screening, intended to complement centralized confirmatory diagnostics (PCR, RDB, Sanger sequencing) within a tiered screening framework. Reproducibility, lyophilized reagent-stability, and reaction-kinetics studies are planned as follow-up work. Primer sequence details are withheld pending patent application (Universitas Indonesia, Direktorat Inovasi/DJKI).

INTRODUCTION

Beta-thalassemia is one of the most common monogenic disorders globally, with particularly high carrier prevalence across Southeast Asia. In Indonesia, the Ciayumajakuning region of West Java (Cirebon, Indramayu, Majalengka, Kuningan) represents a high-prevalence area where population-level carrier screening remains constrained by limited access to centralized molecular diagnostic infrastructure. Reference diagnostic methods — polymerase chain reaction (PCR)-based assays, reverse dot blot

(RDB) hybridization, and Sanger sequencing — require thermal cyclers, trained molecular biology personnel, and centralized laboratory capacity that are frequently unavailable at the primary or peripheral healthcare level where at-risk populations are most accessible.

Loop-mediated isothermal amplification (LAMP), first described by Notomi et al.[1], offers a methodological alternative suited to this access gap. As an isothermal amplification technique employing four to six primers recognizing six to eight distinct target regions, LAMP achieves high amplification specificity without requiring thermal cycling equipment, and its high tolerance to common biological inhibitors reduces dependency on extensive sample purification. When coupled with a colorimetric, pH-sensitive visual readout, LAMP-based assays (C-LAMP) become interpretable by minimally trained personnel without instrumentation — properties well aligned with point-of-care (POCT) screening needs in decentralized settings.

Adapting LAMP for detection of single-nucleotide and small indel mutations — as required for the four HBB mutations of interest in this population (CD15, CD26/HbE, CD41/42, IVS1-5) — introduces design demands beyond conventional pathogen-detection LAMP assays, which typically require only template presence/absence discrimination. Allele-specific primer placement, together with sequence-context-dependent challenges arising from locally AT-rich or GC-rich regions flanking each mutation site, required specific design strategies to achieve reliable single-nucleotide discrimination within an isothermal, fixed-temperature reaction format. This report describes the development, reaction optimization, and colorimetric indicator selection process underlying the resulting C-LAMP protocol, intended as a first-line, decentralized screening tool complementary to centralized confirmatory diagnostics within a tiered screening framework.

MATERIAL AND METHODS

Study design and sample source

This is a methodological development and optimization study describing the design, reagent optimization, and colorimetric indicator selection process for a C-LAMP assay targeting four HBB mutations prevalent in the Ciayumajakuning population (Cirebon, Indramayu, Majalengka, Kuningan), West Java, Indonesia: CD15 (TGG→TAG), CD26/HbE (GAG→AAG), CD41/42 (delTTCT), and IVS1-5 (G→C). Diagnostic performance (sensitivity, specificity) of the finalized assay against a clinical cohort of 279 samples is reported separately[2]; this report focuses on assay development and optimization methodology.

DNA EXTRACTION AND TEMPLATE PREPARATION

Genomic DNA was extracted from EDTA whole blood using a commercial column-based mini-kit according to manufacturer protocol. DNA concentration was quantified spectrophotometrically, with a minimum working concentration threshold of 20 ng/μL; samples below this threshold underwent re-concentration or re-extraction. Extracted DNA was stored at −20°C until use.

Synthetic double-stranded DNA fragments (gBlocks; Integrated DNA Technologies), 300–400 bp in length, were used as positive controls for each mutation target during primer and reaction optimization. Each gBlock was designed to contain the complete primer-binding region for its corresponding target, enabling reproducible positive-control amplification independent of patient sample availability — particularly valuable for rare homozygous genotypes (e.g., homozygous CD41/42 or CD15) that are difficult to source clinically.

PRIMER DESIGN PRINCIPLES

LAMP primers were designed following the standard four-to-six primer architecture described by Notomi et al.[1], comprising a Forward Outer Primer (F3), Backward Outer Primer (B3), Forward Inner Primer (FIP), Backward Inner Primer (BIP), and loop primers (LoopF/LoopB) added to accelerate amplification kinetics[3]. Primers were designed to recognize six to eight distinct regions flanking each mutation site, a configuration that underlies LAMP's high amplification specificity relative to two-primer PCR-based methods. Candidate primer sets were generated in silico (PrimerExplorer) using standard parameters — annealing temperature 60–65°C, GC content 40–60%, and screened to exclude significant hairpin or dimer-forming structures — followed by BLAST verification against the human genome to exclude non-specific binding sites.

Given that LAMP specificity is fundamentally primer-dependent (unlike PCR, where a thermal-cycling program provides an additional layer of stringency), mutation-discriminating strategies at the sequence and chemical-modification level were incorporated to achieve single-nucleotide allele discrimination. Full primer sequences and gBlock construct designs are withheld

from publication pending patent application through Universitas Indonesia (Direktorat Inovasi/DJKI); researchers interested in methodological collaboration may contact the corresponding author.

DESIGN CONSIDERATIONS FOR SEQUENCE-CONTEXT-DEPENDENT (AT-RICH/GC-RICH) REGIONS

Because LAMP amplification proceeds at a single fixed temperature without a denaturation step, primer sets cannot be temperature-tuned per cycle as in conventional PCR; all primers recognizing a given target must therefore maintain balanced effective binding stability despite local variation in base composition across the six-to-eight recognition sites. This constraint becomes particularly consequential when target regions flanking a mutation site are locally AT-rich or GC-rich, and required the following general design strategies.

AT-rich regions. Low local GC content reduces primer-template duplex stability (lower melting temperature, Tm), increasing the risk of weak or transient annealing, primer-dimer formation[4], and reduced amplification efficiency at the fixed 65°C reaction temperature. Mitigation strategies applied in this study were limited to sequence-level adjustments: (i) modest extension of primer length in AT-rich segments to compensate for reduced duplex stability without shifting the recognition site away from the mutation position, and (ii) avoidance of AT-rich stretches at primer 3' termini, which are more prone to spurious extension. Chemical modification approaches such as Locked Nucleic Acid (LNA) incorporation — synthetic nucleotide analogs in which the ribose ring is conformationally locked (C3'-endo, via a 2'-O,4'-C methylene bridge), increasing hybridization affinity by approximately 2–8°C per residue without requiring additional primer length — were considered during design but were not incorporated into the primer set validated in this study; this remains a candidate strategy for future protocol refinement, particularly for short, AT-rich recognition windows.

GC-rich regions. High local GC content increases the risk of stable secondary structure formation (hairpins, self-dimers, and potential G-quadruplex-like structures) that can persist through the isothermal reaction and physically impede primer binding or Bst polymerase strand-displacement activity, since LAMP — unlike PCR — provides no high-temperature denaturation step to resolve such structures each cycle. Mitigation strategies applied included: (i) in silico secondary-structure screening of candidate primers to exclude those with significant predicted hairpin or self-dimer free energy; (ii) inclusion of betaine (5 M) in the reaction master mix, a well-established GC-rich secondary-structure destabilizer that also promotes isostabilization of A-T versus G-C base pairs; and (iii) where feasible, repositioning of primer recognition boundaries to avoid GC-rich hairpin-prone motifs while preserving proximity to the discriminating mutation base.

Single-nucleotide allele discrimination (allele-specific LAMP). The discriminating mutant base for each of the four targets was placed at the 3' terminus of the F2 or B2 segment within the FIP or BIP primer, exploiting Bst DNA polymerase's inability to efficiently extend a primer bearing a 3'-terminal mismatch against a non-target template. For three targets (CD15, CD41/42, IVS1-5), this terminal-mismatch placement was used alone. For CD26/HbE, an additional artificial mismatch was introduced at the penultimate (−2) position, synergizing with the natural discriminating base at −1 to further destabilize non-target annealing — a dual-mismatch strategy applied specifically to this target. LNA incorporation was not used in the validated primer set; primer length and F2/B2 segment length were adjusted iteratively across all targets, since shortening the annealing segment can selectively lower Tm and increase mismatch sensitivity, at the cost of reduced amplification efficiency on fully matched templates. All primer and gBlock designs were validated empirically against synthetic gBlock templates prior to clinical sample testing.

REACTION MASTER MIX AND GENERAL COMPONENTS

Each LAMP reaction (25 μL total volume) consisted of isothermal amplification buffer, $MgSO_4$, betaine, dNTP mix, Bst DNA polymerase (WarmStart, strand-displacement activity), the target-specific primer set (F3, B3, FIP, BIP, LoopF/LoopB), and 2 μL of template DNA (patient sample, gBlock positive control, or nuclease-free water as no-template control). Reagent proportions were optimized iteratively to balance amplification efficiency against non-specific background signal, given that divalent cation and betaine concentrations directly influence both amplification kinetics and specificity in isothermal strand-displacement synthesis, consistent with optimization principles reported for other isothermal amplification platforms[7].

COLORIMETRIC INDICATOR SELECTION

Four colorimetric/visualization approaches were systematically compared for suitability as a point-of-care visual readout: phenol red (pH-indicator), malachite green (dsDNA-binding dye), propidium iodide (PI; fluorescent intercalating dye), and a

commercial LAMP fluorescence dye (SYBR Green-class). Each was evaluated across three dimensions: (1) ease of unaided visual interpretation, (2) dependency on auxiliary equipment (e.g., UV light source, transilluminator), and (3) reagent cost.

Phenol red was tested at 0.07 µL/µL final concentration. Its mechanism relies on the stoichiometric relationship between LAMP amplification and proton release: each polymerization cycle generates pyrophosphate and H^+ ions, progressively lowering reaction pH and shifting the indicator from red-orange (neutral, pH 6.8–8.4) to yellow (pH <6.8) in a manner directly proportional to amplicon yield, consistent with the magnesium pyrophosphate turbidity/pH mechanism described for LAMP more broadly[5,6].

Malachite green was tested at 0.16 µg/µL, relying on direct binding to double-stranded amplification products. Propidium iodide (1 µg/µL) was evaluated as a pre-reaction additive to avoid post-amplification tube-opening, and separately as a post-reaction indicator. The commercial fluorescence dye was evaluated per manufacturer instructions requiring UV transillumination for readout.

REACTION INCUBATION AND INACTIVATION

Optimized reactions were incubated in a temperature-stable water bath at 65°C for 60 minutes, followed by polymerase heat-inactivation at 95°C for 10 minutes. Tubes were allowed to cool to room temperature for 2–3 minutes prior to visual readout. All reactions were performed in a closed-tube format — reaction tubes were not reopened after template addition — to minimize aerosol-mediated carryover contamination, a recognized risk in isothermal amplification workflows given the high copy number of amplification product generated.

WORKFLOW SEGREGATION AND CONTAMINATION CONTROL

Reaction preparation followed spatially segregated workflow zones: (a) master mix preparation, (b) template addition, and (c) result reading, each performed in physically separate areas with UV decontamination of the master-mix preparation bench prior to use. Each sample was tested against all four mutation targets in parallel, alongside one gBlock positive control and one no-template (nuclease-free water) negative control per batch.

RESULTS

PRIMER AND GBLOCK DESIGN OUTCOME

LAMP primer sets were successfully designed for all four target mutations (CD15, CD26/HbE, CD41/42, IVS1-5) and the corresponding wild-type sequence, each incorporating a discriminating mutant or wild-type base positioned within the 3' region of the FIP and/or BIP segment to enable single-nucleotide allele discrimination. Corresponding synthetic gBlock templates (300–400 bp) were synthesized for all five constructs (wild-type plus four mutants), each engineered to contain all six LAMP primer-binding sites (F3, FIP, BIP, B3, LF, LB) within a single defined template molecule (Table 1).

Table 1: Summary of primer design parameters per target

Target	Mutation type	No. of primers	GC content range	Discrimination strategy
CD15	Nonsense (TGG→TAG)	6	40.0–55.0%	Terminal mismatch only
CD26/HbE	Missense (GAG→AAG)	5	47.5–64.7%	Dual mismatch (terminal + penultimate)
CD41/42	Frameshift (delTTCT)	5	41.7–61.1%	Terminal mismatch only
IVS1-5	Splice-site (G→C)	6	40.0–57.5%	Terminal mismatch only

VERIFICATION OF PRIMER SPECIFICITY BY GEL ELECTROPHORESIS

LAMP amplification using gBlock templates produced the characteristic ladder-like banding pattern typical of LAMP products on 2% agarose gel electrophoresis (80 V, 45 minutes). This banding pattern was observed specifically in reactions where the primer set matched its intended target mutation, with no amplification observed in wild-type gBlock or no-template control

(NTC) reactions for the corresponding mutant-specific primer sets. This confirms the analytical specificity of the primer design at the sequence level, independent of and prior to colorimetric readout testing (Figure 1).

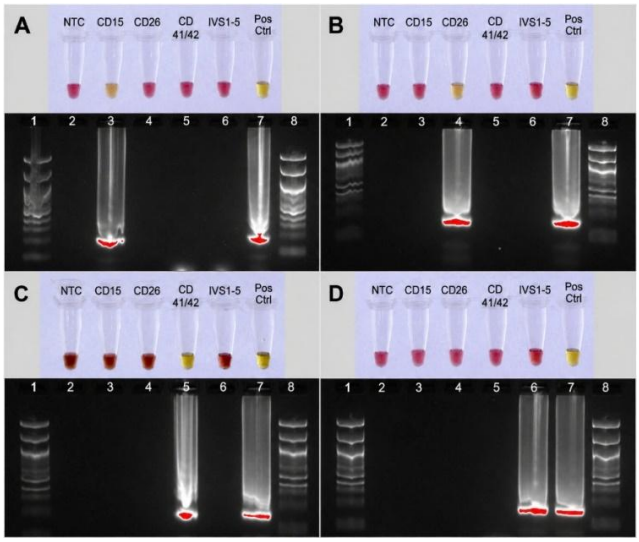

Figure 1. Concordance between colorimetric readout and gel electrophoresis for all four HBB mutation targets. (A) CD15 (G>A); (B) CD26/HbE (G>A); (C) CD41/42 (delTTCT); (D) IVS1-5 (G>C). In each panel, the upper row shows tube colorimetric results (yellow = positive, pink = negative) across lanes NTC, CD15, CD26, CD41/42, IVS1-5, and positive control; the lower row shows the corresponding 2% agarose gel electrophoresis (100 bp DNA marker in lanes 1 and 8), in which the characteristic LAMP ladder banding pattern appears only in lanes matching the intended target mutation and the positive control, with no amplification in wild-type or no-template control lanes.

COLORIMETRIC INDICATOR COMPARISON

Four colorimetric/visualization methods were evaluated head-to-head across three practical dimensions relevant to point-of-care deployment: unaided visual interpretability, dependency on auxiliary equipment, and reagent cost (Table 2). Phenol red (tested at 0.07 μL/μL) produced a consistent, unambiguous red-to-yellow color transition in positive reactions following 60 minutes of incubation at 65°C, required no auxiliary equipment, and had the lowest reagent cost among the four methods evaluated. Malachite green (0.16 μg/μL) produced visible color change in positive samples but also generated unintended signal in wild-type gBlock reactions at the concentration tested, indicating a narrow specificity window requiring further concentration optimization. Propidium iodide, evaluated both as a pre- and post-reaction additive, produced non-specific background fluorescence in no-template controls regardless of amplification status, precluding reliable unaided-eye discrimination and requiring UV-based fluorescence detection equipment. The commercial LAMP fluorescence dye produced strong positive-sample contrast but similarly required UV transillumination for interpretation, along with substantially higher per-reaction reagent cost. Based on this comparison, phenol red was selected as the colorimetric indicator for the finalized C-LAMP protocol.

Table 2: Comparison of colorimetric indicator methods for C-LAMP visual readout

Method	Visual ease (unaided eye)	Equipment dependency	Reagent cost
Phenol red	Easy (red → yellow)	None	Very low
Malachite green	Moderate (colorless/pale → green); specificity concerns observed	Minimal (natural light)	Low
Propidium iodide	Difficult (non-specific background fluorescence)	Moderate (UV light source)	Moderate
Commercial fluorescence dye	Difficult (all tubes fluoresce without discrimination by eye)	High (UV transilluminator/camera)	High

PERFORMANCE OF THE FINALIZED PROTOCOL

Using the finalized reaction conditions (65°C incubation for 60 minutes, followed by 95°C polymerase inactivation for 10 minutes, in a closed-tube format with phenol red as colorimetric indicator), consistent red-to-yellow color transition was observed in positive reactions across all four mutation targets, concordant with the gel electrophoresis specificity results described above. Formal reproducibility assessment across independent replicate runs or multiple operators was not performed as part of this development study; this is noted explicitly as a limitation below, and readers should interpret the reported color-transition consistency as observational rather than as a validated reproducibility metric.

DISCUSSION

RATIONALE FOR AN ISOTHERMAL, COLORIMETRIC APPROACH

Centralized molecular diagnostic methods for beta-thalassemia — PCR-based assays, reverse dot blot (RDB), and Sanger sequencing — remain the diagnostic gold standard but require thermal cyclers, trained molecular biology personnel, and centralized laboratory infrastructure that are often unavailable in decentralized or peripheral healthcare settings. LAMP's reliance on isothermal, strand-displacement amplification eliminates the need for thermal cycling equipment, while its use of four to six primers recognizing six to eight target regions confers amplification specificity that is generally considered comparable to, or exceeding, conventional two-primer PCR. These properties, combined with LAMP's demonstrated tolerance to common biological inhibitors, make it well suited to point-of-care (POCT) applications in resource-limited settings — a rationale consistent with prior LAMP-based diagnostic development for both infectious targets, including rapid RT-LAMP assays developed during the SARS-CoV-2 pandemic[8], and genetic targets.

COLORIMETRIC INDICATOR: COMPARATIVE RATIONALE

Among the four indicator systems evaluated, phenol red demonstrated the most favorable overall profile, combining unaided visual clarity (a distinct red-to-yellow transition), zero dependency on auxiliary equipment, and the lowest reagent cost. This aligns with prior reports establishing pH-based colorimetric LAMP as broadly adaptable across diagnostic targets with sensitivity comparable to real-time PCR-based detection[9], and with recommendations favoring phenol red specifically for field-deployable diagnostics in low- and middle-income country settings based on its favorable cost-benefit profile[10].

Malachite green, while mechanistically plausible as a direct dsDNA-binding indicator, showed unintended amplification signal in wild-type gBlock templates at the concentration tested (0.16 μg/μL), indicating a narrow optimal concentration window highly sensitive to master mix composition — a practical limitation for standardized field deployment without extensive per-batch re-optimization.

Propidium iodide was limited by non-specific background fluorescence: signal was observed even in no-template controls, indicating that PI fluorescence in this system did not reliably discriminate true amplification from background, and that its interpretation depends on dedicated fluorescence-reading equipment unavailable in most peripheral settings — undermining its suitability as an unaided-eye colorimetric method. Commercial fluorescence dyes, while providing strong contrast under UV transillumination, introduce dependency on specialized illumination equipment and add substantial per-reaction reagent cost, both of which run counter to the decentralization goals underlying C-LAMP's intended use case.

OPTIMIZATION CONSIDERATIONS AND CONTAMINATION MITIGATION

The closed-tube reaction format adopted in this protocol directly addresses one of the principal practical limitations of isothermal amplification methods: carryover contamination arising from the very high amplicon yield LAMP characteristically produces. Combined with spatially segregated workflow zones for master mix preparation, template addition, and result reading, this design choice is intended to minimize both cross-contamination between samples and false-positive signal arising from environmental DNA carryover — a consideration directly relevant to field deployment where strict laboratory containment infrastructure may not be available.

POSITION WITHIN A TIERED SCREENING FRAMEWORK

Consistent with the Thalassemia International Federation's tiered screening paradigm[11], the C-LAMP protocol described here is intended as a first-line, decentralized screening tool rather than a replacement for centralized confirmatory diagnostics. Its design priorities — unaided visual readout, minimal equipment dependency, low per-reaction cost, and tolerance to non-

specialist operation — directly target the accessibility gap in beta-thalassemia carrier screening programs in high-prevalence, resource-limited regions such as Ciayumajakuning.

LIMITATIONS

This protocol, as with LAMP-based mutation detection generally, determines mutation presence or absence but cannot resolve zygosity or cis/trans phase of compound mutations; confirmatory testing via RDB or Sanger sequencing remains necessary for definitive genotyping and genetic counselling. Colorimetric interpretation, while accessible, retains an element of subjectivity in borderline color transitions, and remains sensitive to master mix buffering capacity and environmental contamination as discussed above — underscoring the continued importance of rigorous positive/negative control inclusion in every reaction batch. Smartphone-based RGB color analysis, previously demonstrated for other colorimetric LAMP applications[12], represents a promising approach to reduce this subjectivity in future protocol iterations. Primer sequence-level design strategies for enhanced single-nucleotide discrimination, while incorporated into this protocol's development, are not detailed here pending patent application; this limits full independent reproducibility of the assay pending publication of the patent or licensing arrangements for academic collaboration. Formal inter-run and inter-operator reproducibility of the colorimetric readout was not assessed in this development study and should be established before the protocol is considered validated for routine field use. Reagent stability under lyophilized (freeze-dried) storage and reaction time-to-color kinetics were likewise not formally assessed in this development phase; all reagents in this study were used in liquid form and stored under standard cold-chain conditions.

FUTURE WORK

Two follow-up studies are planned to further characterize assay performance ahead of, or alongside, broader field deployment. First, a reagent stability study will evaluate lyophilization (freeze-drying) of the C-LAMP master mix — primers, Bst polymerase, dNTPs, betaine, and phenol red indicator — as a strategy to eliminate cold-chain dependency, following protocols established for other colorimetric LAMP/RT-LAMP diagnostic kits[13]. Lyophilized master mix aliquots will be stored at multiple temperatures representative of field conditions (4°C, ambient room temperature, and accelerated-stability conditions at 37°C) over a series of time points, with amplification performance and colorimetric readout compared against freshly prepared liquid master mix using gBlock positive controls, to establish shelf-life and storage-temperature tolerance suitable for decentralized deployment in tropical field settings such as West Java. Second, a time-to-color-change kinetics study will characterize the minimum incubation time required for reliable visual discrimination, with the goal of shortening total assay turnaround time where possible without compromising specificity. Together with formal reproducibility testing across operators and reaction batches, these studies are intended to move the protocol from developmental characterization, as reported here, toward field-validated diagnostic readiness.

CONCLUSION

Systematic optimization of reaction components and comparative evaluation of colorimetric indicators identified phenol red as the optimal visualization method for a closed-tube, isothermal C-LAMP assay targeting four common HBB mutations. Primer specificity was confirmed by gel electrophoresis across all four targets. The resulting protocol offers a low-cost, equipment-minimal alternative suited to first-line beta-thalassemia screening in decentralized settings, positioned to complement rather than replace centralized confirmatory diagnostics within a tiered screening strategy; lyophilized reagent stability, reaction kinetics, and formal reproducibility remain to be established in planned follow-up studies.

ACKNOWLEDGEMENTS

The authors thank Faculty of Medicine Universitas Swadaya Gunung Jati & Yayasan Satria Budi Dharma Setia (YSDS)] for supporting this research. This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo National General Hospital (Approval Number: KET-921/UN2.F1/ETIK/PPM.00.02/2022). Written informed consent was obtained from all participants prior to enrolment.

CONFLICT OF INTEREST

A patent application covering primer sequences and gBlock construct designs described in this manuscript has been filed through Universitas Indonesia (Direktorat Inovasi/DJKI).

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Total: 13 references, all confirmed/cross-checked (11 matched against your dissertation's audited 68-reference list; 2 newly sourced and verified via web search — the TIF guidelines and the lyophilization precedent). Well within JHSMR's 40-reference limit for a full report, leaving room to add more from your dissertation's audited list if reviewers request broader context (e.g., refs on beta-thalassemia epidemiology in Indonesia, #3, #4).