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Article

Single-Drop Blood Detection of Common *G6PD* Mutations in Thailand Based on Allele-Specific Recombinase Polymerase Amplification with CRISPR-Cas12a

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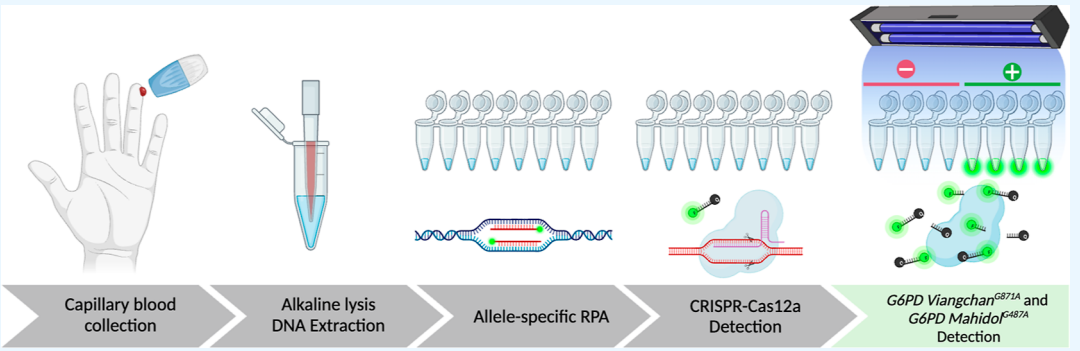

ABSTRACT: Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited enzymopathy. Identification of the G6PD deficiency through screening is crucial to preventing adverse effects associated with hemolytic anemia following antimalarial drug exposure. Therefore, a rapid and precise field-based G6PD deficiency diagnosis is required, particularly in rural regions where malaria is prevalent. The phenotypic diagnosis of the G6PD intermediate has also been a challenging issue due to the overlapping of G6PD activity levels between deficient and normal individuals, leading to a misinterpretation. The availability of an accurate point-of-care testing (POCT) for *G6PD* genotype diagnosis will therefore increase the opportunity for screening heterozygous cases in a low-resource setting. In this study, an allele-specific recombinase polymerase amplification (AS RPA) with clustered regularly interspaced short palindromic repeats-Cas12a (CRISPR-Cas12a) was developed as a POCT for accurate diagnosis of common *G6PD* mutations in Thailand. The AS primers for the wild type and mutant alleles of *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} were designed and used in RPA reactions. Following application of CRISPR-Cas12a systems containing specific protospacer adjacent motif, the targeted RPA amplicons were visualized with the naked eye. Results demonstrated that the *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} assays reached 93.62 and 98.15% sensitivity, respectively. The specificity was 88.71% in *Mahidol*^{G487A} and 99.02% in *G6PD Viangchan*^{G871A}. The diagnosis accuracy of the *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} assays was 91.67 and 98.72%, respectively. From DNA extraction to detection, the assay required approximately 52 min. In conclusion, this study demonstrated the high performance of an AS RPA with the CRISPR-Cas12a platform for *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} detection assays and the potential use of *G6PD* genotyping as POCT.

INTRODUCTION

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited enzyme defect in human erythrocytes, leading to hemolytic anemia.[1] *G6PD* gene mutations cause enzyme deficiency and anemia ranging from mild to severe. In 2008, G6PD deficiency was estimated to affect more than 400 million people worldwide, mostly in Africa, the Mediterranean, the Middle East, and Southeast Asia.[2] The *G6PD* variants are classified by the World Health Organization (WHO) into five classes based on G6PD activity and hemolytic levels.[3] In Southeast Asia, the prevalence of G6PD deficiency was 11.0% in Thailand,[4] 8.1% in Laos,[5] 8.9% in Vietnam,[5] 10.0% in Myanmar,[6] and 15.8% in Cambodia.[7] Currently, more than 186 mutations have been identified in the *G6PD* gene, of which nearly half are associated with reduced activity or stability of the G6PD enzyme.[8] The most common *G6PD* mutations in Southeast Asia, particularly Thailand, are *G6PD*

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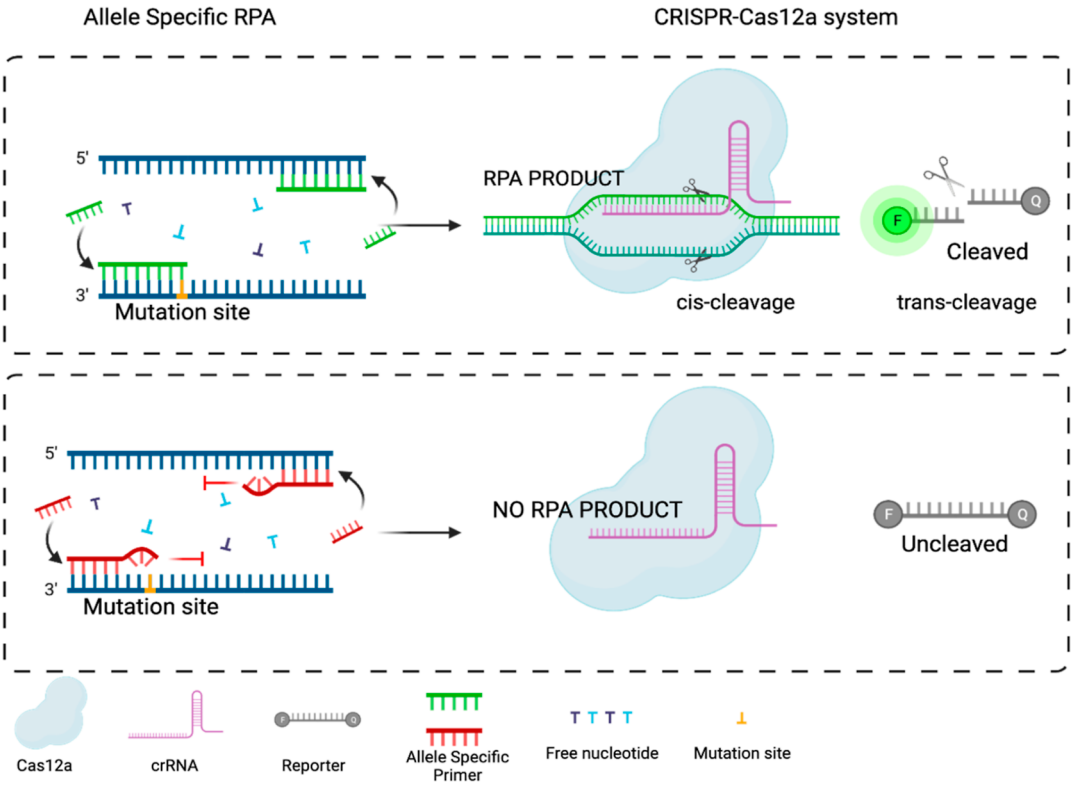

Figure 1. Schematic representation of specific primers created with https://www.biorender.com/. Target DNA was amplified by AS primers using RPA, whereas nonspecific DNA cannot be amplified. The CRISPR-Cas12a system recognizes and cleaves the complementary sequence at a specific PAM of each mutation using a specific crRNA, a procedure known as cis-cleavage. Following the cis-cleavage, Cas12a activates its trans-cleavage, which cleaves any nonspecific single-strand DNA in the vicinity of the target DNA, such as a fluorescence reporter.

Mahidol^{G487A} and *G6PD Viangchan*^{G871A}, which are classified as moderate and severe deficiency, respectively.[7,9] Patients with G6PD deficiency are susceptible to severe hemolysis when exposed to infections, foods, and medications. Consequently, the WHO recommends screening for G6PD deficiency prior to administering therapeutic dosages of specific medications when practicable.[10] In tropical countries, field-based G6PD diagnostics must be rapid and accurate, especially in rural areas where malaria is endemic. Therefore, several quantitative and qualitative assays were developed for point-of-care testing (POCT) for phenotypic G6PD diagnostics.[11−15] The phenotypic diagnosis of G6PD intermediate, primarily observed in heterozygous females,[16−20] has been a challenge due to the overlapping G6PD activity levels between deficient and normal individuals, leading to a misinterpretation of the findings. This urges the need for the development of a diagnostic platform for POCT for *G6PD* genotyping.

Existing *G6PD* molecular genotyping methods are laborious and require specialized equipment and maintenance services, making them unsuitable for *G6PD* mutation screening in a low-resource environment. *G6PD* genotyping can be achieved in several ways, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP),[4,6,9] *Taq*Man SNP assay,[9] AS-PCR,[21] reverse dot blot hybridization (RDB),[22] amplification refractory mutation system,[23,24] gold nanoparticle-based test,[25] high-resolution melting curve analysis (HRM),[26−28] and DNA sequencing.[4,29] In addition, a high-throughput multiplex AS-PCR-based assay is available for the diagnosis of *G6PD* mutations. DiaPlexC *G6PD* genotyping kits based on a single PCR step with gel electrophoresis are capable of identifying the *G6PD* mutations, which are specific to Asians[30,31] and Africans.[32,33] Recently, isothermal amplification methods using locked nucleic acids (LNAs) and the Yaku-Bonczyk principle primers for identifying substitution mutations for *G6PD* mutations at the point of care have been explored, as well. A LNA is a nucleic acid analogue that improves mismatch discrimination over DNA-only primers by locking the ribose moiety into a C3′-endo conformation using a 2′-O, 4′-C methylene bridge.[34,35] In accordance with the Yaku-Bonczyk principle,[36] the AS primer design method was applied to increase the performance of primers that distinguish mutant from wild type DNA. The principle underlines that the last nucleotide at the 3′ terminus should be SNP-specific. In addition, a designed mismatch in the primer sequence at the third nucleotide from the 3′ end implies that the primer will never anneal to the other nucleotide except in the sequence with a perfect match at the 3′ terminus.[37−40]

The AS-recombinase polymerase amplification (RPA), combined with the CRISPR-Cas12a system,[41] was developed to minimize processing time. It also simplifies the procedure and allows the outcomes to be seen with the naked eye. Furthermore, the clustered regularly interspaced short palindromic repeats-Cas12a (CRISPR-Cas12a) system is frequently exploited to broaden the applications for highly sensitive and specific molecular detection. However, this technology is yet to be developed to detect common *G6PD* mutations in Thailand. Hence, this study aimed to develop an AS-RPA with CRISPR-Cas12a with high sensitivity and specificity in detecting common *G6PD* mutations in Thailand, namely, *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A}.

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Table 1. Primers for RPA[a]

mutation	primer name	sequence of primer (5′ → 3′)	reference position (NC_000023.11)
Mahidol	Mahidol G mismatch forward	GGG TAA CGC AGC TCC GGG CTC CCA GCA **a**AG	154,534,524−154,534,495
	Mahidol A LNA forward	GGG TAA CGC AGC TCC GGG CTC CCA GCA GA**A**	154,534,524−154,534,495
	Mahidol reverse	CAC CAT GAG GTT CTG CAC CAT CTC CTT ***t***CC CAG GT	154,534,343−154,534,377
Viangchan	Viangchan G mismatch forward	CAT TCT CTC CCT TGG CTT TCT CTC AGG TCA **t**GG	154,533,154−154,533,122
	Viangchan A LNA forward	CCC ATT CTC TCC CTT GGC TTT CTC TCA GGT CAA **G**A	154,533,156−154,533,122
	Viangchan reverse	CAT AGA GGA CGA CGG CTG CAA AAG TGG CGG T	154,532,963−154,532,993

[a]Lowercase bold letters: mismatch sites; underlined capital letters: mutation sites; capital bold letters: LNA sites; and italic lowercase bold letters: mutagenesis sites.

Table 2. crRNA Sequence for G6PD Mutations[a]

crRNA name	sequence of primer (5′ → 3′)
Temp-crRNA_Mahidol	CTACCGCATCGACCACTACCTGG *ATCTACACTTAGTAGAAATTA* **CCCTATAGTGAGTCGTATTA**
Temp-crRNA_Viangchan	CCGATGGAGAGGGCGAGGCCAC *ATCTACACTTAGTAGAAATTA* **CCCTATAGTGAGTCGTATTA**

[a]Underlined letters: spacer sequences (target sequence); italic letters: scaffold sequences; and bold letters: T7 promoter sequences.

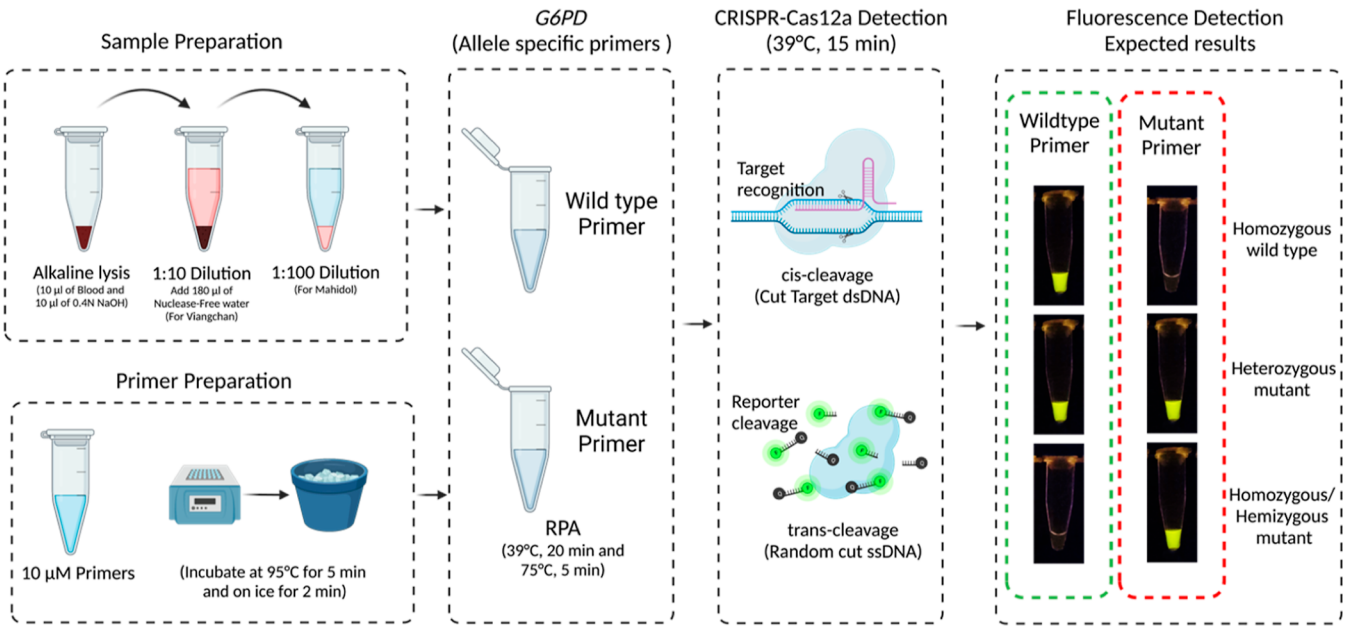

Figure 2. Workflow of the overall process for rapid detection based on single-drop blood detection of AS-RPA with CRISPR-Cas12a created with https://www.biorender.com/.

METHODS

Study Subjects and Sample Collection. The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Chulalongkorn University (COA no. 1355/2020, IRB no. 570/63, and COA no. 1002/2022, IRB no. 0426/65). Leftover EDTA blood samples from 156 *G6PD* mutation carrier individuals collected from the annual health checkup at King Chulalongkorn Memorial Hospital from February to April 2021 with written informed consent prior to enrollment were used in this study.

Measurement of G6PD Activity. Briefly, 20 μL of the hemolysate from packed red blood cells was added to 1 mL of distilled water and vortexed for 5 min. The G6PD activity was then measured at 37 °C according to the previously described protocol using a clinical chemistry analyzer (BS-360E, Mindray Medical International).[42] The rate of absorbance change (ΔA/min) was calculated by [ΔA/min − sample] − [(ΔA/min blank) and normalized with Hb (g/dL), according to the manufacturer's instructions. A sample of G6PD activity was considered valid only if the control G6PD activity fell within the reference range.

Identification of *G6PD* Mutations. Genomic DNA samples were extracted from blood samples using a Nucleospin Blood kit (MACHEREY-NAGEL GmbH & Co., KG) according to the manufacturer's recommendations. *Taq*Man SNP genotyping assay (Applied Biosystems) was performed to screen *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A}. Amplification, real-time detection, and result analysis were performed following the manufacturer's instructions for StepOnePlus real-time PCR (Applied Biosystems). PCR-RFLP was performed to detect *G6PD Canton*^{G1376T}, *G6PD Union*^{C1360T}, *G6PD Kaiping*^{G1388A}, *G6PD Chinese-4*^{G392T}, *G6PD Chinese-5*^{C1024T}, and *G6PD Coimbra*^{C592T} in G6PD deficient and intermediate samples according to previously described procedures.[6] *G6PD Aures*^{T143C} and *G6PD Songklanagarind*^{T196A} were amplified using primers for *G6PD* exon 3−4 as previously reported,[43] and their genotypes were determined using *Bgl*II and *Bst*XI (New England Biolab), respectively. *G6PD*

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Table 3. Sample Characteristics

G6PD mutations	genotype			total
	hemizygous	homozygous	heterozygous	
Mahidol	25	0	66	**91**
Viangchan	9	3	43	**55**
others[a]	4	1	5	**10**
total	**38**	**4**	**114**	**156**

[a]Others: hemizygous *Canton*; hemizygous *Kaiping*; hemizygous *Union*; hemizygous *Valladolid*; homozygous *Chinese-4*; heterozygous *Aures*; heterozygous *C519T*; heterozygous *Songklanagarind*; heterozygous *Coimbra*; and heterozygous *Chinese-5*.

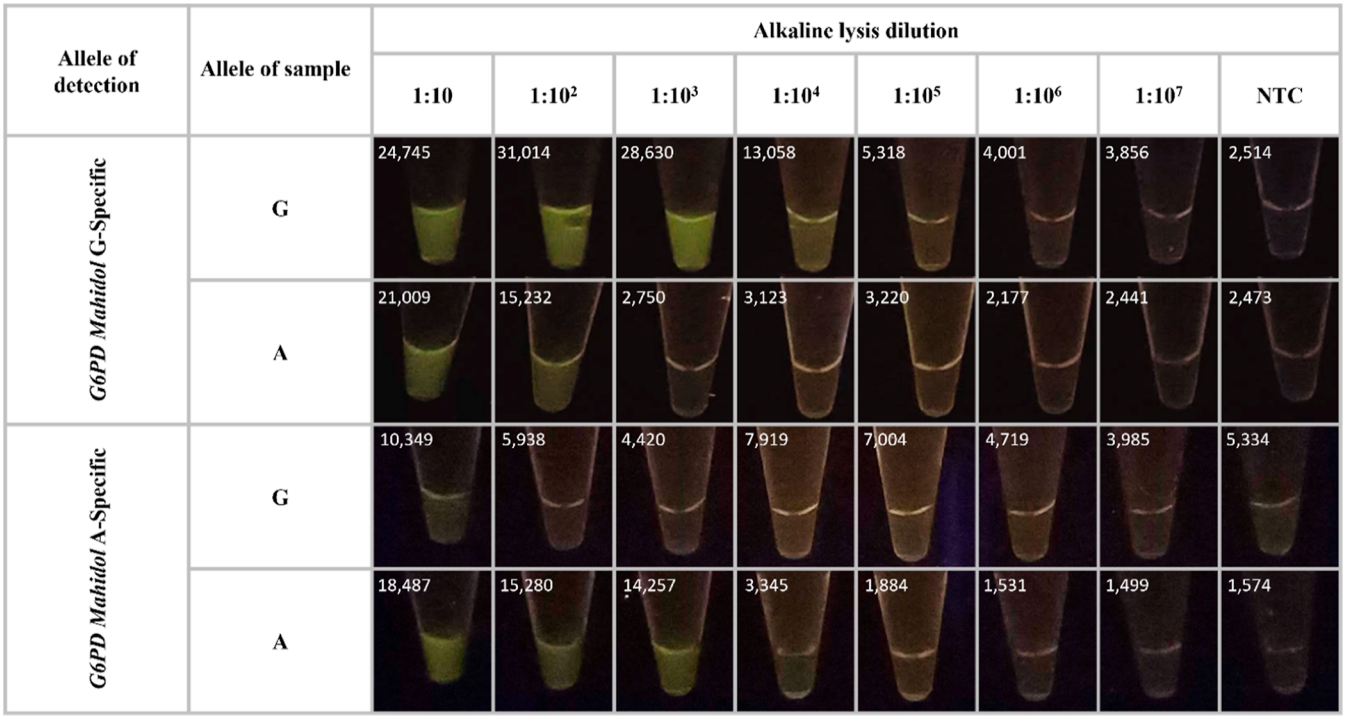

Figure 3. LOD for *G6PD Mahidol*^{G487A} based on AS-RPA with the CRISPR-Cas12a assay in each dilution. Number represents the fluorescence intensity in each tube.

Valladoli^{C406T} was amplified and genotyped using primers for *G6PD* exon 5 and *Aci*I (New England Biolab), respectively. Direct DNA sequencing was performed to confirm all mutations (Figure S1).

Alkaline Lysis Method for DNA Extraction. The whole blood was used for the extraction of genomic DNA. Briefly, 10 μL of whole blood was lysed by pipetting up and down in 10 μL of 0.4 N NaOH. Then, the solution was incubated at room temperature for 5 min.[34] Before being introduced directly to RPA reactions, the solution was diluted in a 10-fold serial dilution for *G6PD Viangchan*^{G871A} and a 100-fold serial dilution for *G6PD Mahidol*^{G487A} with nuclease-free water. A no-template control was prepared using DNase-free water instead of NaOH. The concentrations (mean ± S.D.) in the 10-fold dilution were 513.31 ± 85.26 μg/μL (ranging from 232.20 to 741.00 μg/μL) and 40.42 ± 9.89 μg/μL (ranging from 16.15 to 79.75 μg/μL) for the 100-fold dilution.

Allele-Specific Recombinase Polymerase Amplification. AS with LNAs and mismatch primers (Figure 1) were designed using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as previously reported,[34,39] to detect specific point mutations (Figures S2 and S3). DNA sequence of *G6PD* with GenBank accession NC_000023.11 was used as a reference. All RPA primers are shown in Table 1.

Prior to the RPA, primer annealing was performed by heating at 95 °C for 5 min, followed by cooling on ice for 2 min to unwind the secondary structure (Figure S4). Regarding the TwistAmp Basic Kit (TwistAmp) instruction, the RPA reaction master mix consisting of 0.48 μM forward primer, 0.48 μM reverse primer (Table 1), and rehydration buffer were mixed with the lyophilized reaction. After that, 1 μL of DNA template for *Mahidol*^{G487A} or 1.65 μL of DNA template for *Viangchan*^{G871A} and 14 nM MgOAc were finally added to the tube cap.[41] Then the tube was spun down. The reaction mixture was incubated at 39 °C for 20 min, followed by thermal inactivation at 75 °C for 5 min (Figure S5).

Preparation of CRISPR RNA. To generate crRNA, 25 μL of reaction mixture consisted of 250 μM synthetic crRNA template (Table 2) containing the T7 promoter, scaffold sequence, and spacer (target sequence). A 250 nM of T7 promoter and 1× T4 DNA Ligase Buffer (Thermo Scientific) were incubated at 95 °C for 3 min, 65 °C for 3 min, 42 °C for 5 min, and 37 °C for 45 min. Next, the crRNA was transcribed using Ribomax In Vitro Transcription Systems (Promega) according to the manufacturer's protocol. Then, a miRNA isolation kit (Geneaid) was used to purify transcribed crRNA, and the concentration of crRNA was quantitated by the Qubit

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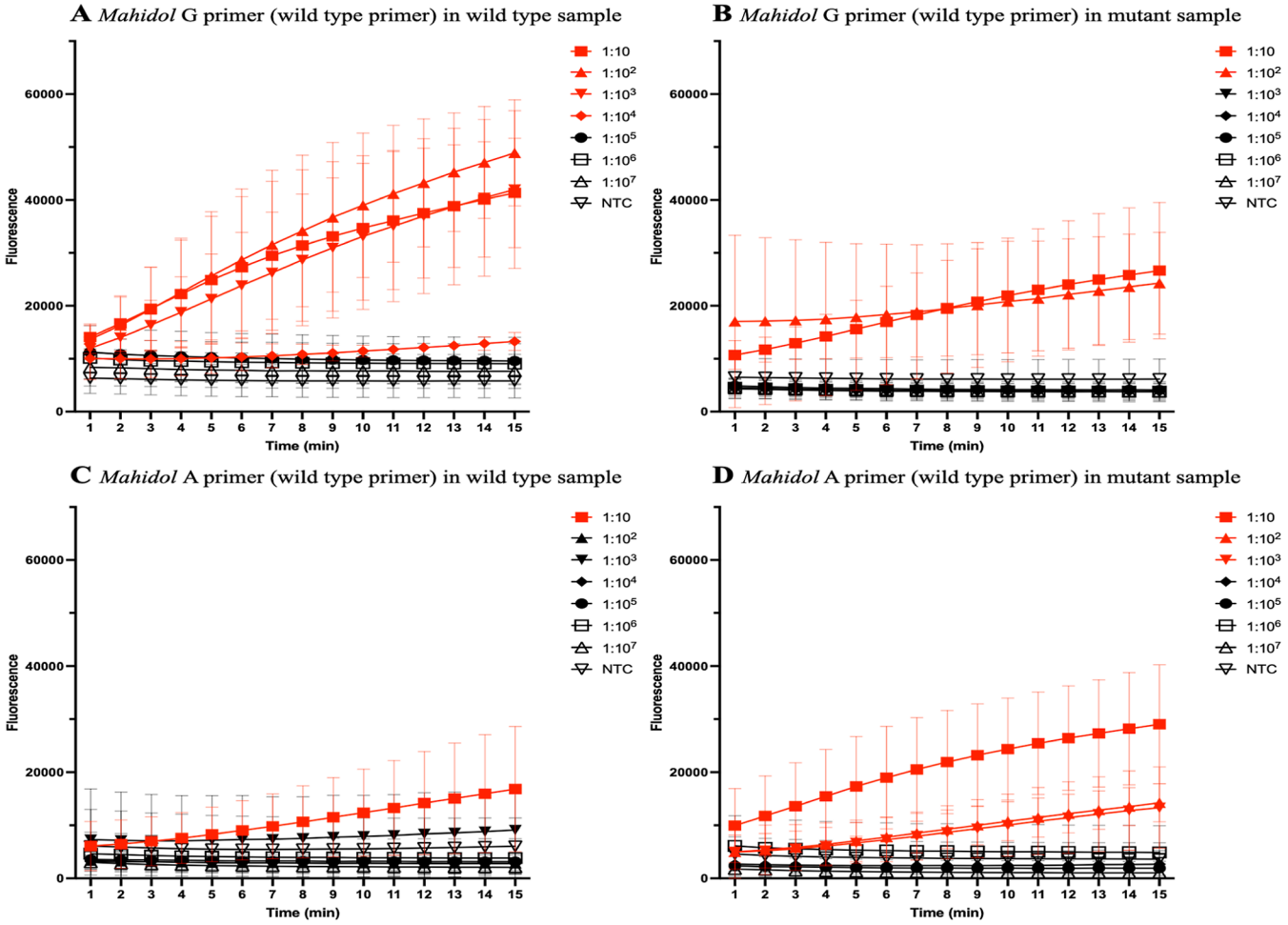

Figure 4. Fluorescent signals of different diluted crude alkaline lysates from *G6PD* AS-RPA for *G6PD Mahidol*^{G487A} detection (n = 3): (A) Mahidol G primer in the wild type sample; (B) Mahidol G primer in the mutant sample; (C) Mahidol A primer in the wild type sample; and (D) Mahidol A primer in the mutant sample.

microRNA assay kit with the Qubit 4 fluorometer (Invitrogen).[41]

CRISPR-Cas12a Reaction. The CRISPR-Cas12a reaction mixture contained RPA product (1 μL), a CRISPR-Cas12a reaction mixture (14 μL) comprising 30 nM specific crRNA, 33 nM EnGen *Lba* Cas12a (Cpf1) (New England Biolabs), a 200 nM fluorescent reporter (FAM)-quencher (BHQ1) probe: 5′ FAM-AGG ACC CGT ATT CCC A-BHQ1 3′, and 1× NEBuffer 2.0 reaction buffer (New England Biolabs). After 15 min of incubation at 39 °C, the fluorescent signal was visualized using the BluPAD Dual LED blue/white light transilluminator (BIO-HELIX),[41] as illustrated in Figure 2. The results were interpreted based on a consensus among at least two out of three technicians. Samples displaying bright fluorescence were classified as positive, while those with no fluorescence or very faint fluorescence were considered negative.

Limit of Detection and Cross-Reactivity Testing. Limit of detection (LOD) was performed using a 10-fold serial dilution of each alkaline lysis solution (ranging from 1:10 to 1:10^8 dilution) as a template for AS-RPA with CRISPR-Cas12a. The LOD was observed in reaction tubes containing the lowest concentration of alkaline lysis solution that yielded a fluorescent signal. The real-time PCR was performed to measure the fluorescence of the FAM signal reported in each dilution every 1 min for 15 min.[41] The cross-reactivity testing was validated with clinical samples containing other *G6PD* mutations, including *G6PD Aures*^{T143C}, *G6PD*^{C519T}, *G6PD Canton*^{G1376T}, *G6PD Chinese-4*^{G392T}, *G6PD Chinese-5*^{C1024T}, *G6PD Coimbra*^{C592T}, *G6PD Kaiping*^{G1388A}, *G6PD Songklanagarind*^{T196A}, *G6PD Union*^{C1360T}, and *G6PD Valladolid*^{C406T}.

Statistical Analysis. A comparison was conducted between the results of AS-RPA with CRISPR-Cas12a and the *Taq*Man SNP genotyping assay. The fluorescence intensity of the tubes in the images was quantified using the ImageJ program. The actual fluorescence intensity is the difference between solution density and tube density. The area under the receiver operating characteristic (ROC) curve (AUC) was then calculated to test the efficacy of the cutoff point for quantified fluorescence intensity in the detection of *G6PD Viangchan*^{G871A} and *G6PD Mahidol*^{G487A} (Figure S6). The clinical performance of the assay, including sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and diagnostic accuracy, was evaluated using a web-based diagnostic test evaluation calculator implemented in MEDCALC easy-to-use statistical software.[41] Sensitivity focuses on identifying true positives [sensitivity = (true positives)/(true positives + false negatives)], specificity on identifying true negatives [specificity = (true negatives)/(true negatives + false positives)], and diagnostic accuracy provides a

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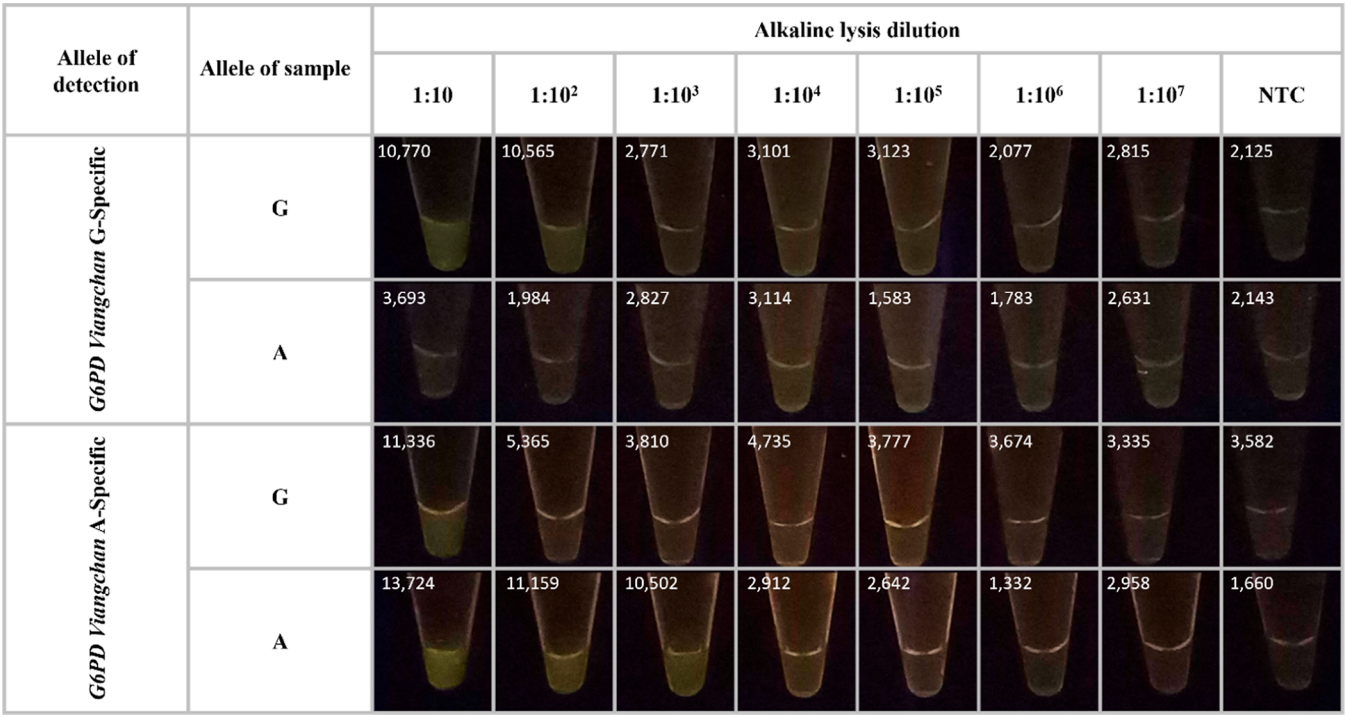

Figure 5. LOD for *G6PD Viangchan*^{G871A} based on AS-RPA with CRISPR-Cas12a assay in each dilution. Numbers represent the fluorescence intensity in each tube.

comprehensive measure of the performance of a test considering both the presence and absence of the mutation in a given population [diagnostic accuracy = sensitivity × prevalence + specificity × (1 − prevalence)].[44]

RESULTS

Sample Characteristics. After molecular genotyping, results revealed that 91 samples had *G6PD Mahidol*^{G487A} (25 hemizygous and 66 heterozygous mutations), 55 samples had *G6PD Viangchan*^{G871A} (9 hemizygous, 3 homozygous, and 43 heterozygous mutations), and 10 samples had other *G6PD* mutations, including heterozygous *G6PD Aures*^{T143C}, hemizygous *G6PD Canton*^{G1376T}, homozygous *G6PD Chinese-4*^{G392T}, heterozygous *G6PD Chinese-5*^{C1024T}, heterozygous *G6PD Coimbra*^{C592T}, hemizygous *G6PD Kaiping*^{G1388A}, heterozygous *G6PD Songklanagarind*^{T196A}, hemizygous *G6PD Union*^{C1360T}, hemizygous *G6PD Valladolid*^{C406T}, and heterozygous *G6PD*^{C519T}, as shown in Table 3.

LOD of *G6PD* AS-RPA with CRISPR-Cas12a Assay. The lowest alkaline lysis dilution that could be detected by *G6PD* AS-RPA with the CRISPR-Cas12a assay was determined. Results revealed that the LOD for the *Mahidol* G-specific primer (wild type primer) in the *G6PD Mahidol*^{G487A} wild type sample was approximately 1:10^4 (Figures 3 and 4A). In contrast, the *G6PD Mahidol*^{G487A} mutant sample was detected at 1:10^2 dilution with the *Mahidol* G-specific primer (Figures 3 and 4B). Additionally, the *Mahidol* A-specific primer (mutant primer) in the *G6PD Mahidol*^{G487A} wild type sample was detected at a dilution as low as 1:10^1 (Figures 3 and 4C), whereas the LOD for the *Mahidol* A-specific primer in the *G6PD Mahidol*^{G487A} mutant sample was approximately 1:10^3 dilution (Figures 3 and 4D). The CRISPR-Cas 12a assay for *G6PD Mahidol*^{G487A} correlated with fluorescent detection using qPCR (Figure 4A−D).

The LOD for the *Viangchan* G-specific primer (wild type primer) in the *G6PD Viangchan*^{G871A} wild type sample was approximately 1:10^2 dilution (Figures 5 and 6A). This G-specific primer did not amplify the *G6PD Viangchan*^{G871A} mutant, resulting in undetectable signals in samples containing the *G6PD Viangchan*^{G871A} mutant (Figures 5 and 6B). Meanwhile, the *Viangchan* A-specific primer (mutant primer) in the *G6PD Viangchan*^{G871A} wild type sample showed the LOD at 1:10 dilution (Figures 5 and 6C) and the *Viangchan* A-specific primer in the *G6PD Viangchan*^{G871A} mutant sample displayed the signal at 1:10^3 dilution (Figures 5 and 6D). The CRISPR-Cas 12a assay for *G6PD Viangchan*^{G871A} correlated with the fluorescent detection using the qPCR (Figure 6A−D).

Cross-Reaction Test of the *G6PD* AS-RPA with the CRISPR-Cas12a Assay. The alkaline lysis solutions for the other 10 mutations represented as the wild type samples of both *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} were tested for cross-reactivity analysis. Figure 7 shows that the *G6PD* AS-RPA with CRISPR-Cas12a assay was able to detect the difference between the non-*G6PD Mahidol*^{G487A} and the *G6PD Mahidol*^{G487A} samples and the non-*Viangchan*^{G871A} and the *Viangchan*^{G871A} samples with high specificity and no cross-reactivity.

Performance of *G6PD* AS-RPA with CRISPR-Cas12a Assays in the Detection of *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} Mutations. The performance of the AS-RPA with CRISPR-Cas 12 assays in the detection of *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} was evaluated (Table 4, Figures 8 and 9, Tables S1 and S2, and Figures S7 and S8). In this study, the detection of the *G6PD Mahidol*^{G487A} genotype had a sensitivity of 93.62%, a specificity of 88.71%, and a diagnostic accuracy of 91.67%. The sensitivity of the *G6PD Mahidol* wild type (G) and mutant (A) alleles was 100.00 and 97.80%, respectively, whereas the specificity was 88.00 and 89.23%, respectively. Furthermore, the diagnostic

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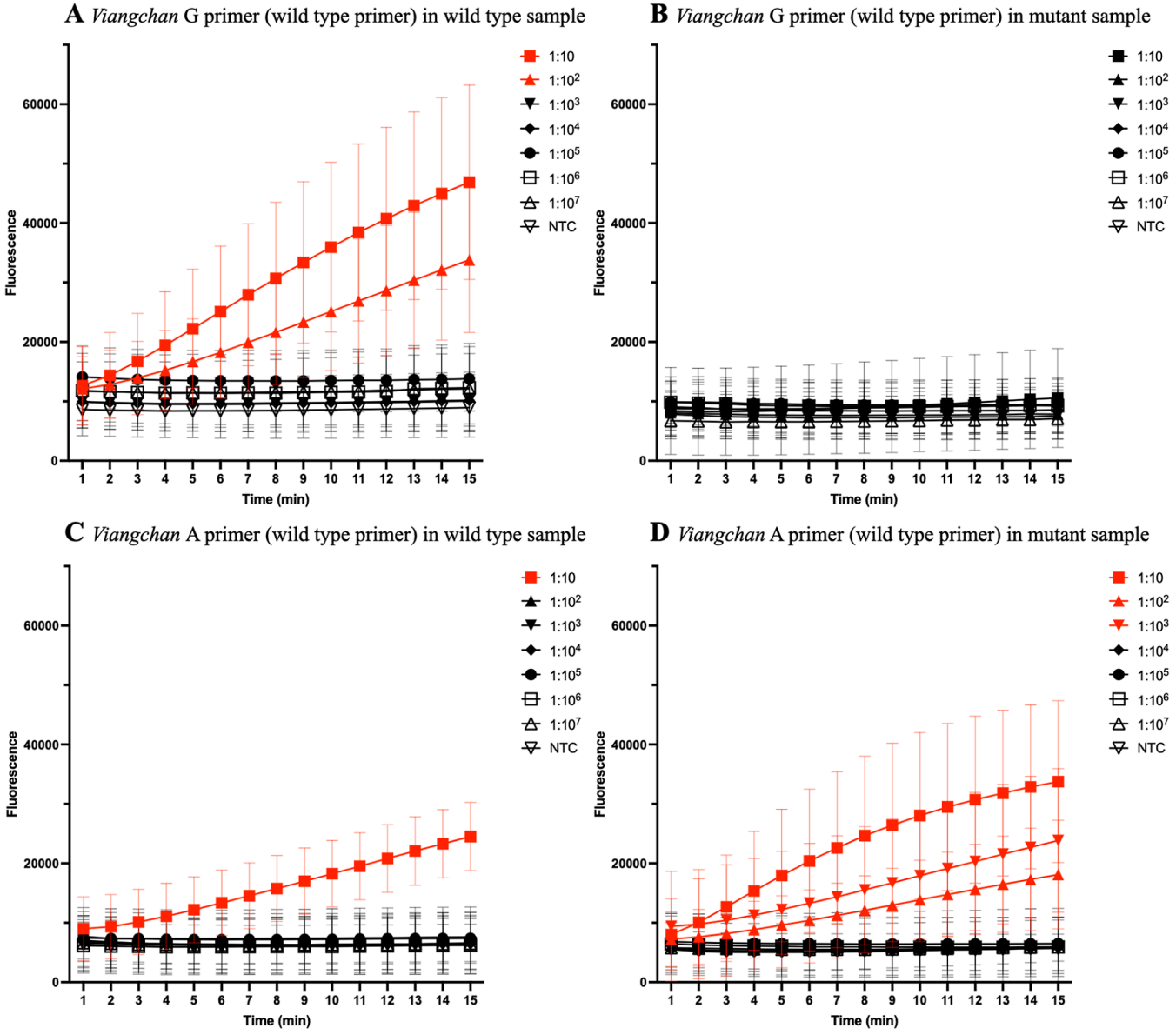

Figure 6. Fluorescent signals of different diluted crude alkaline lysates from *G6PD* AS-RPA for *G6PD Viangchan*^{G871A} detection (n = 3): (A) Viangchan G primer in the wild type sample; (B) Viangchan G primer in the mutant sample; (C) Viangchan A primer in the wild type sample; and (D) Viangchan A primer in the mutant sample.

Table 4. Sensitivity, Specificity, and Accuracy of the AS-RPA with CRISPR-Cas12 Assays as Calculated by Genotype and Allele

mutations	sensitivity (%)	specificity (%)	PPV (%)	NPV (%)	accuracy (%)
Mahidol genotype	93.62 (86.62−97.62)	88.71 (78.11−95.34)	92.63 (86.20−96.20)	90.16 (80.79−95.23)	91.67 (86.17−95.49)
allele G	100.00 (97.22−100.00)	88.00 (68.78−97.45)	97.76 (93.79−99.21)	100.00	98.08 (94.48−99.60)
allele A	97.80 (92.29−99.73)	89.23 (79.06−95.56)	92.71 (86.32−96.24)	96.67 (88.02−99.13)	94.23 (89.33−97.33)
Viangchan genotype	98.15 (90.11−99.95)	99.02 (94.66−99.98)	98.15 (88.28−99.73)	99.02 (93.54−99.86)	98.72 (95.45−99.84)
allele G	99.31 (96.19−99.98)	91.67 (61.52−99.79)	99.31 (95.63−99.89)	91.67 (60.76−98.74)	98.72 (95.45−99.84)
allele A	100.00 (93.51−100.00)	100.00 (96.41−100.00)	100.00	100.00	100.00 (97.66−100.00)

accuracy of *G6PD Mahidol* was 98.08% for the wild type allele and 94.23% for the mutant allele. According to *G6PD Viangchan*^{G871A} genotype detection, the sensitivity was 98.15%, the specificity was 99.02%, and the diagnostic accuracy was 98.72%. The sensitivity of the *G6PD Viangchan* wild type (G) and mutant (A) alleles was 99.31 and 100.00%, respectively, whereas the specificity was 91.67 and 100.00%, respectively. In addition, the diagnostic accuracy of *G6PD Viangchan* was 98.72% for the wild type allele and 100.00% for the mutant allele.

DISCUSSION

G6PD deficiency is the most frequent enzyme insufficiency caused by *G6PD* mutations, which is particularly prevalent in people of African, the Mediterranean, and Southeast Asian heritage.[1] G6PD deficiency can cause hemolytic anemia, a

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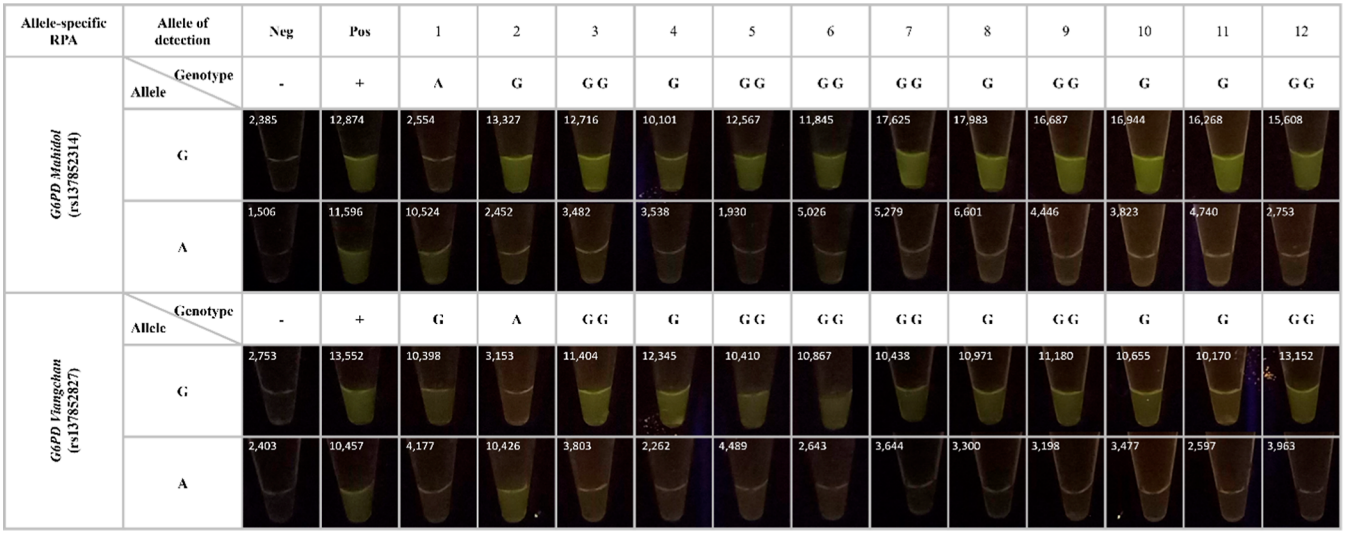

Figure 7. Cross-reaction test of the *G6PD Mahidol*^{G487A} and the *G6PD Viangchan*^{G871A} AS-RPA with the CRISPR-Cas12a assay in the detection of other *G6PD* mutations: (1) hemizygous *G6PD Mahidol*^{G487A}; (2) hemizygous *G6PD Viangchan*^{G871A}; (3) heterozygous *G6PD Aures*^{T143C}; (4) hemizygous *G6PD Canton*^{G1376T}; (5) homozygous *G6PD Chinese-4*^{G392T}; (6) heterozygous *G6PD Chinese-5*^{C1024T}; (7) heterozygous *G6PD Coimbra*^{C592T}; (8) hemizygous *G6PD Kaiping*^{G1388A}; (9) heterozygous *G6PD Songklanagarind*^{T196A}; (10) hemizygous *G6PD Union*^{C1360T}; (11) hemizygous *G6PD Valladolid*^{C406T}; and (12) heterozygous *G6PD*^{C519T}. Number represents the fluorescence intensity in each tube.

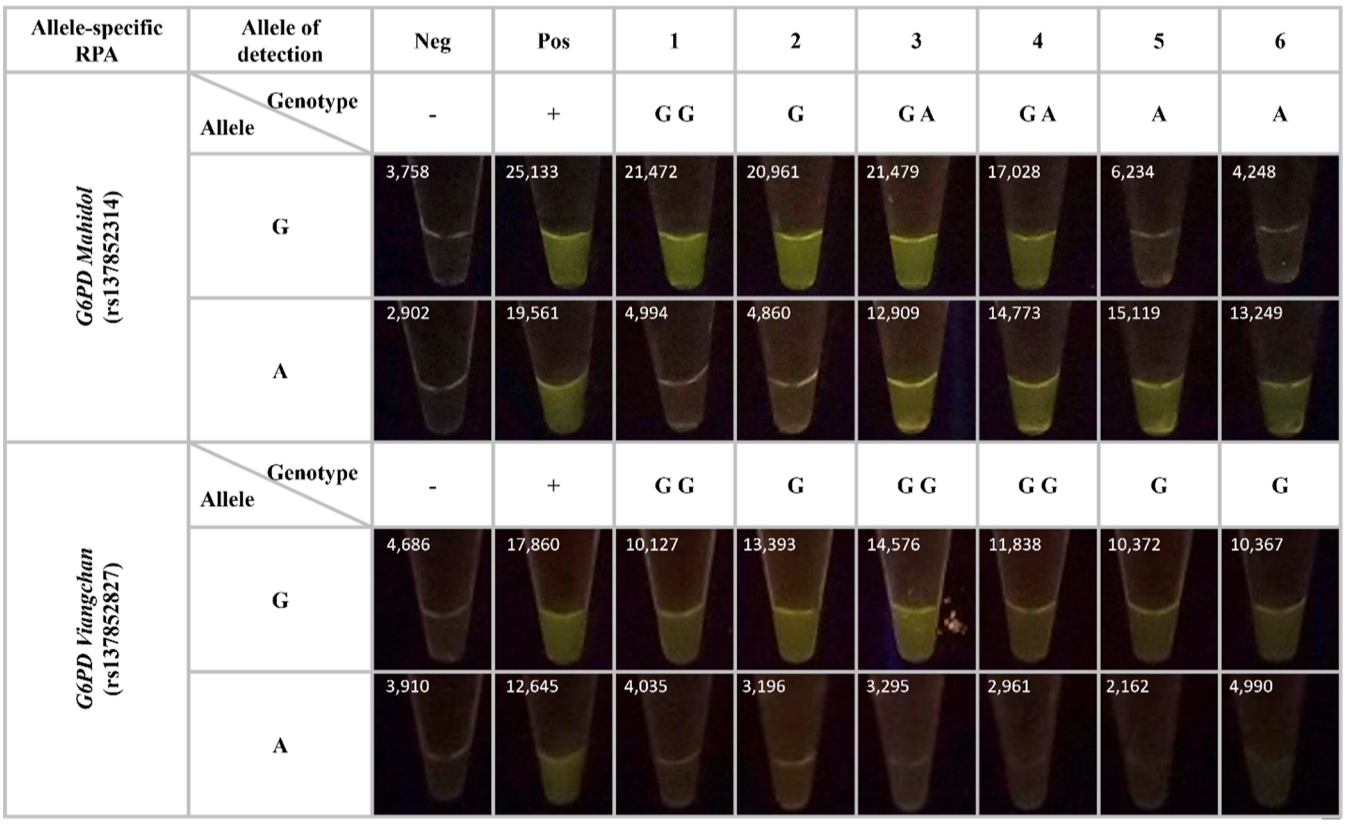

Figure 8. Representative results for the detection of the *G6PD Mahidol*^{G487A} mutation using the AS-RPA with the CRISPR-Cas 12 assays in various samples: (1) homozygous *G6PD Mahidol* wild type; (2) hemizygous *G6PD Mahidol* wild type; (3,4) heterozygous *G6PD Mahidol* mutant; and (5,6) hemizygous *G6PD Mahidol* mutant. Number represents the fluorescence intensity in each tube.

condition where red blood cells are prematurely destroyed.[1,45,46] The phenotypic variability of anemia, ranging from asymptomatic to severe, can be caused by different G6PD mutations, including *Mahidol*^{G487A} and *Viangchan*^{G871A} mutations, and exposure to stimulants, including antimalarial drugs. The WHO has suggested screening for G6PD deficiency prior to delivering antimalarial drugs. However, the difference between intermediate and normal G6PD has been a challenge for screening due to the mosaicism of heterozygous *G6PD*. Heterozygous females (one wild type and one mutant allele) have a much broader phenotype, which is mostly in the 20−80% range of G6PD activity.[16−20] This phenomenon is

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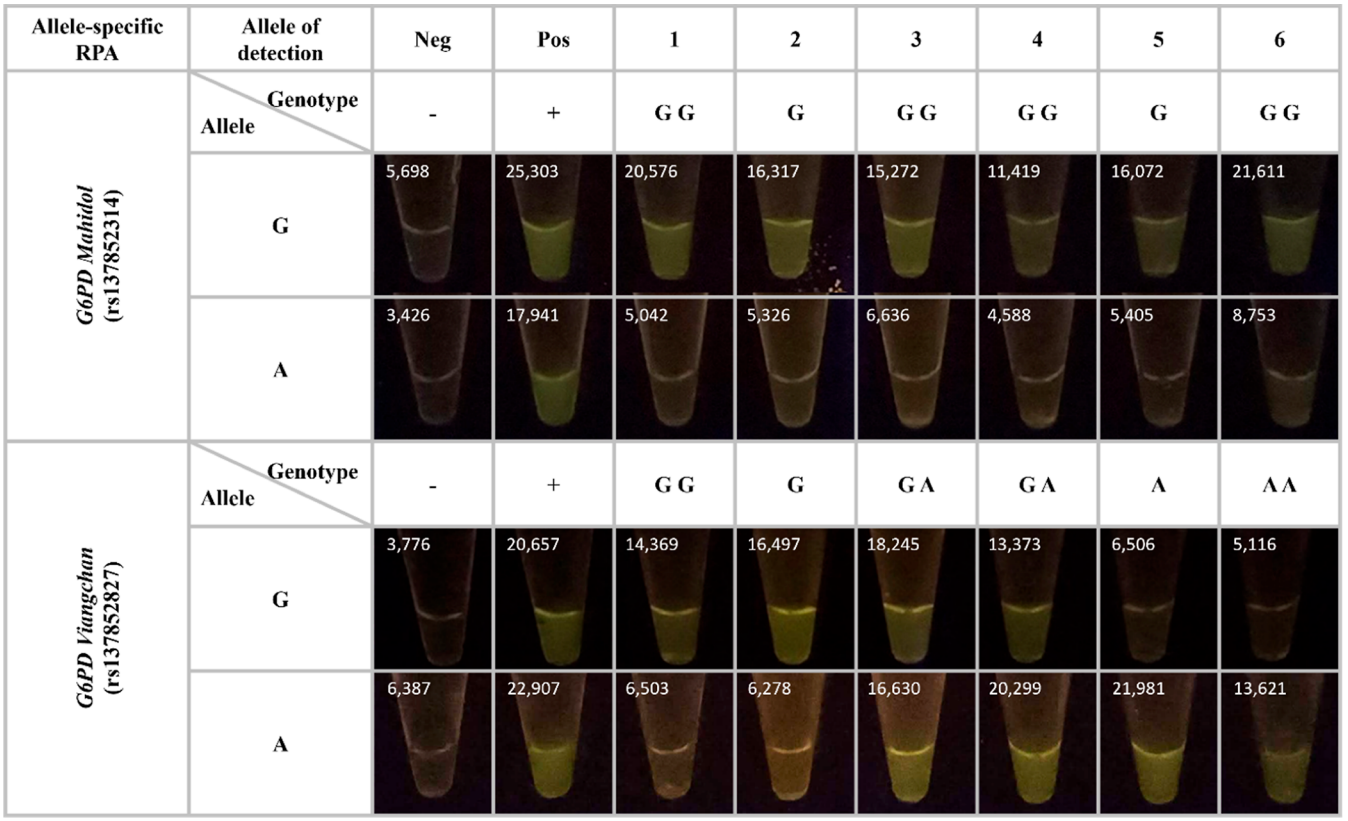

Figure 9. Representative results for the detection of the *G6PD Viangchan*^{G871A} mutation using the AS-RPA with the CRISPR-Cas 12 assays in various samples: (1) homozygous *G6PD Viangchan* wild type; (2) hemizygous *G6PD Viangchan* wild type; (3,4) heterozygous *G6PD Viangchan* mutant; (5) hemizygous *G6PD Viangchan* mutant; and (6) homozygous *G6PD Viangchan* mutant. Number represents the fluorescence intensity in each tube.

explained by the lyonization of the X chromosome, a process in which one of the two X chromosomes is randomly inactivated.[47,48] The use of genotyping can help eliminate the potential misdiagnosis of intermediate cases in heterozygotes.[1,5] The development of a suitable and reliable POCT for genotyping *G6PD* mutations will also expand the opportunities available in low-resource settings.

In our study, AS-RPA with the CRISPR-Cas12a process employing blood-derived genomic DNA has emerged as a feasible technique for *G6PD* mutation detection. Molecular diagnosis begins with DNA extraction using the alkaline lysis method, which is one of the simplest strategies for DNA extraction.[49] It is an efficient step in dissolving proteins such as membrane proteins and nucleases. Furthermore, the primary structure of DNA remains relatively stable in alkaline solutions.[50] The samples in an alkaline solution were diluted to enhance the primer's efficiency for each mutation, resulting in remarkable assay specificity (with a 100-fold dilution for *G6PD Mahidol*^{G487A}) and sensitivity (with a 10-fold dilution for *G6PD Viangchan*^{G871A}). The mutant regions in the *G6PD* gene can be amplified using the AS-RPA technique, which uses isothermal amplification.

The design of AS primers was a pivotal aspect of this assay. Various techniques, such as LNA addition[34,35] and the Yaku-Bonczyk method,[36] were employed to enhance AS discrimination in the primer design. First, the addition of a LNA at the base preceding the substitution site proved effective in improving mismatch discrimination. LNAs feature a methylene bridge that connects the 2′-oxygen of ribose to the 4′-carbon, thereby stabilizing the ribose in a locked 3′-endo conformation. This structural modification reduces the ribose's conformational flexibility and enhances the organization of the local phosphate backbone. Second, the Yaku-Bonczyk method emphasized that the final nucleotide at the 3′ terminus should be SNP-specific. Additionally, introducing a designed mismatch in the primer sequence at the third nucleotide from the 3′ end ensures that the primer exclusively anneals to sequences with a perfect match at the 3′ terminus[37−40] (Figures S2 and S3). Subsequently, mutation identification was accomplished using the CRISPR-Cas12a genome editing technology.[41]

LOD was applied to assess method validation, especially in the case of impurity determination. It refers to the lowest concentration/signal of an analyte that the analytical process can consistently distinguish from background levels.[51] Despite both *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} having the same nucleotide alteration (G to A transition), their LODs differed. These LOD discrepancies are attributed to variations in their surrounding DNA sequences, which in turn influence the design of primer sequences. These distinctions in primer sequences impact their ability to anneal with the DNA template and amplify the RPA product. Differences in the GC content of the sequences surrounding the mutations affect the primer stability during primer-template annealing. For instance, the Mahidol primer has a GC content of 51.28%, while the Viangchan primer has a GC content of 39.53%, leading to varying binding efficiencies. Differences in the secondary structures of primers, such as the formation of different hairpin loops within the single-stranded DNA of certain primers and the strength of hydrogen bonding in self-

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complementary, stemming from variations in GC content, contribute to disparities in amplification efficiencies between the two mutations. Given that the secondary structure of the primer-DNA binding can impact RPA reactions, the presence of a single-strand binding protein, a core component of RPA, is necessary to effectively eliminate secondary structures or dimer formation, thereby enhancing the amplification of DNA fragments.

Mutations within the crRNA-complementary sequences of the target region can potentially disrupt the effectiveness of AS-RPA with CRISPR-Cas12a. Our discovery highlights this phenomenon, as a sample featuring a heterozygous *G6PD Coimbra*^{C592T} mutation, situated within the crRNA complementary sequence of the Mahidol detection assay, exhibited pronounced fluorescence for the Mahidol G wild type allele, as depicted in Figure 7 (sample #7). This finding underscores the capability of the AS-RPA with the CRISPR-Cas12a assay to differentiate between non-*G6PD Mahidol*^{G487A} and *G6PD Mahidol*^{G487A} samples, even in cases where these samples harbor additional mutations within the crRNA-complementary sequence of the target region. Nevertheless, there were no samples available that featured mutations within the complementary sequence of crRNA for *Viangchan* detection, limiting an assessment of the capability of the AS-RPA with the CRISPR-Cas12a assay in detecting *G6PD Viangchan*^{G871A}.

Although other genotyping techniques are effective in identifying mutations, there are limitations. Direct DNA sequencing provides accurate results and the ability to identify novel or rare mutations. However, DNA sequencing is not a high-throughput platform. It is time-consuming and costly. PCR-RFLP, a widely used technique for *G6PD* genotyping, is limited by its dependence on specific restriction enzyme sites, inability to detect novel mutations, potential for incomplete digestion, and complex and time-consuming workflow. The *Taq*Man SNP genotyping assay is a high-throughput technique with specific probes to detect mutations. However, these three genotyping methods require hours (7−14 days for direct DNA sequencing, approximately 5 h for PCR-RFLP, and 2.5 h for *T*aqMan SNP assay), specialized equipment, and expertise to perform. In contrast, AS-RPA with the CRISPR-Cas12a assay can simultaneously detect two *G6PD* mutations within 52 min with simple techniques and devices, including a heat block, a microcentrifuge, and a blue/white LED light transilluminator, making it suitable for POCT. Although AS-RPA with the CRISPR-Cas12a assay may not be suitable for detecting mutations in highly polymorphic genes since two specific primers and crRNA are required for each mutation, multiplex AS-RPA with a CRISPR-Cas12a assay for detecting other common *G6PD* mutations in Southeast Asia is our further study.

In conclusion, AS-RPA with CRISPR-Cas12a using genomic DNA extracted from the blood via an alkaline lysis procedure can detect *G6PD Mahidol*^{G487A}*and G6PD Viangchan*^{G871A} in the Thailand cohort with high sensitivity, specificity, and accuracy. It also has numerous advantages, such as rapidity, cost-effectiveness, and applicability in field research and environments with limited resources, making AS-RPA with CRISPR-Cas12a suitable for accurately diagnosing and monitoring *G6PD* mutations. These findings suggest that the AS-RPA with the CRISPR-Cas12a assay is highly effective in detecting *G6PD* mutations, particularly in populations where these mutations are prevalent.

■ ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05596.

Direct sequencing chromatograms and PCR-RFLP showing the different identified *G6PD* mutations; incorporating a LNA and the Yaku-Bonczyk method; primers under various conditions; secondary structure prediction of each AS primer; incubation times for RPA at different intervals (10, 20, and 30 min); pairwise comparison between *Taq*Man SNP assay and CRISPR-Cas12a-based detection of *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A}; ROC analysis of the AS-RPA with CRISPR-Cas12a in detecting *G6PD Viangchan*^{G871A} and *G6PD Mahidol*^{G487A}; and detection of *G6PD Mahidol*^{G487A} and *G6PD Viangchan*^{G871A} based on CRISPR-Cas12a (PDF)

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Author Contributions

C.L.C. designed the research study. P.M. and P.N. performed the research. P.M., P.N., S.P., and C.L.C. analyzed and interpreted the patient's data. P.M. drafted the manuscript. C.L.C., S.P., and P.C. revised the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

Ethics approval and consent to participate: this study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (COA no. 1002/2022 and IRB no. 0426/65). The protocol of this study was performed according to the declaration of Helsinki for the

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participation of human individuals. Written informed consent was obtained from each participant.

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■ ABBREVIATIONS

G6PD, glucose 6-phosphate dehydrogenase; CRISPR-Cas12a, clustered regularly interspaced short palindromic repeats-Cas12a; WHO, World Health Organization; PCR-RFLP, polymerase chain reaction−restriction fragment length polymorphism; AS-PCR, allele-specific-PCR; RDB, reverse dot blot hybridization; HRM, high-resolution melting curve analysis; RPA, recombinase polymerase amplification; LNA, locked nucleic acid; SNP, single nucleotide polymorphism; EDTA, ethylenediamine tetraacetic acid; NTC, no template control; PAM, protospacer adjacent motif; crRNA, CRISPR RNA; LOD, limit of detection; POCT, point-of-care testing; PPV, positive predictive value; NPV, negative predictive value

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