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Optimizing CRISPR-Cas12-based detection methods for drug-resistant *Plasmodium falciparum* with an advanced gene visualization pipeline

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ABSTRACT: Malaria, a life-threatening disease caused by the parasite *Plasmodium falciparum*, poses a significant health challenge, particularly in tropical regions where it is most prevalent. This disease is traditionally treated with various medications, including chloroquine, quinine, artemisinin, sulfadoxine-pyrimethamine, and combinations of artemisinin-based therapies. However, the emergence of drug-resistant strains of P. falciparum has necessitated the development of more sophisticated diagnostic and treatment strategies to effectively manage and combat this disease. In response to this growing concern, our research project endeavors to pioneer a novel approach to malaria diagnosis and treatment. We focus on the design of Loop-Mediated Isothermal Amplification (LAMP) primers, which are engineered to specifically identify treatment-resistant strains of P. falciparum. The project strongly emphasizes optimizing the guide RNA (gRNA) efficacy, which is pivotal for enhancing the specificity and sensitivity of the CRISPR-Cas12 system in detecting these resistant strains. Our aim is to significantly improve the efficiency and accuracy of malaria treatments, particularly in areas heavily impacted by drug resistance. By leveraging the advancements in CRISPR technology, we anticipate creating a robust tool for the on-field diagnosis of drug-resistant Malaria based on CHOPCHOP and E-CRISPR in silico gene visualization tools. This research addresses a critical gap in the current treatment paradigm and contributes to the broader initiative of employing innovative genetic technologies for public health. Ultimately, the successful implementation of this project could lead to more effective malaria control and treatment strategies, reducing the global burden of this devastating disease.

KEYWORDS: Malaria; CRISPR, LAMP, Cas12; gRNA; Plasmodium

1. INTRODUCTION

Isothermal amplification reaction development for detecting drug-resistant strains of *Plasmodium falciparum* poses a crucial challenge in malaria research. Currently, the common method of drug resistance in Plasmodium strains requires trial and error, which is costly in time and resources. Therefore, a more efficient system that is able to detect drug resistance-associated genes in *P. falciparum* is important.

Loop-mediated isothermal amplification, also recognized as LAMP, is a method for DNA amplification where four up to six primers bind to different regions of the target genome, making it highly specific [1]. The benefit of utilizing this method above regular PCR is the isothermal amplification capability that then simplifies the nucleotide amplification process as there is no need to cycle the temperatures compared to the regular PCR amplification [2]. LAMP primers are composed of multiple primers; however, based on direction, they could be divided into forward and backward primers [3]. Mechanically, the forward primers first bind to the DNA, leading to the creation of the product and the forward LAMP primer looping on itself, successively after the backward primer also binds to DNA and thus creates another loop for the backward primer; as such, it creates a dumbbell structure allowing amplification to occur throughout the

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primer sites creating to faster amplifications [4]. Therefore, the LAMP primer design needs to abide by the appropriate standard for LAMP, which is generalized into Tm, GC content, and most importantly, 5' and 3' stability [5].

Plasmodium falciparum is a protozoan parasite that spreads to humans with the vector of Anopheles mosquitoes. This parasite targets and infects red blood cells, thus causing Malaria. The deaths caused by Malaria were about 619,000 cases in 2021 wit, with an estimated 247 million cases of infection worldwide [6,7]. The current techniques to reduce the number of cases is mostly preventative measures, such as reducing the number of mosquito bites. At the same time, curative measures include rapid diagnostic tests and drug-based treatments. However, some strains were found to be resistant to drugs commonly used for treatment [8]. This research aims to develop further methods to detect these treatment-resistant strains of P. falciparum utilizing LAMP and gRNA-based CRISPR systems, specifically the 3D7 strain, the most common isolate found in patients.

The primary issue regarding drug-resistant Plasmodium is that current methods require a significant amount of time, and as such, treatments could be less effective than desired [9]. The current method utilizing regular PCR also requires equipment and other materials that are relatively resource-intensive [10]. Furthermore, the identification of target regions along with binding sites leads to the pivotal role of CRISPR-based gRNA computational programs [11], especially in identifying specific target regions with the *Plasmodium falciparum* genome associated with drug resistance. Commonly, these targets exist as SNPs within Plasmodium's genome. The gRNA computational program provides information about the binding sites, including the target DNA sequence or region of interest and their efficacy for binding [12]. The efficacy of gRNA is paramount in ensuring the precise and accurate placement of LAMP primers on the genomic sequences so that the SNPs can be isolated and amplified to be detected. Moreover, fine-grained design of gRNA is crucial to avoid potential off-target CRISPR expression system that is proven in the human cell lines [13]. The hard placement of primers on the LAMP techniques also necessitates specific and accurate positioning [14].

To effectively address the challenge of detecting drug-resistant *Plasmodium falciparum* strains, the development of an advanced gRNA discovery and efficacy prediction program is proposed. The program will predict gRNA efficacy and actively identify optimal gRNA sequences. It will be seamlessly integrated with reference genomes and annotation data, which will hold the potential to significantly enhance the precision and efficiency of drug resistance detection in *Plasmodium falciparum* [15]. The computational programs of CHOPCHOP and E-CRISP visualization tools, in synergy with the predicted gRNA, ensure that the LAMP primers are designed more precisely, enabling them to bind well with exceptional specificity to the drug resistance-associated regions. gRNA's adaptability allows researchers to customize the target sequence, changing the genomic target of the Cas protein as needed, simply by modifying the gRNA sequence [16].

This research aims to develop advanced diagnostic tools using LAMP and CRISPR technology for detecting and addressing drug-resistant *Plasmodium falciparum* strains with an in-silico gene visualization pipeline based on CHOPCHOP and E-CRISP programs. Additionally, we aim to create a gRNA find and efficacy prediction program to improve LAMP primer placement. Our objective is to reduce the prevalence of treatment-resistant cases in Indonesia and support the development of more effective treatment strategies. This integrated approach holds great promise for advancing malaria diagnosis and treatment in affected regions.

2. RESULTS AND DISCUSSION

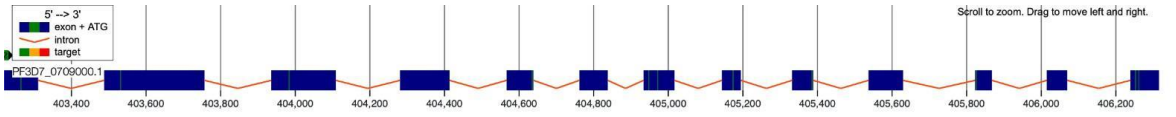
2.1 Web-Based Tools gRNA Program

Web-based tools have revolutionized the field of gRNA programming, offering researchers user-friendly platforms for designing guide RNAs with precision and efficiency. These tools leverage the power of cloud computing and extensive biological databases to provide researchers with a streamlined approach to target selection, off-target analysis, and other parameters. Four primary approaches have been selected: alignment-based, hypothesis-driven, learning-based, and posterior experimental analysis. Each of these approaches caters to different research needs and preferences, allowing scientists to tailor their gRNA design process accordingly [17].

The CRISPR-Cas12 program and approach are still in a limited number that align with the criteria specific to the system. One of the possible reasons could be the unique nature of CRISPR-Cas12 compared to

other CRISPR-Cas systems, such as Cas9. The distinct features and requirements of CRISPR-Cas12 might limit the number of available tools and approaches that are well-suited for its specific functionality. There are currently 2 main programs widely available on the web suitable for CRISPR-Cas12 with hypothesis-driven and learning-based approaches: CHOPCHOP and E-CRISP. Other programs are available for well-known organisms, such as humans, but they still need to be implemented for *Plasmodium falciparum*.

The gRNA sequences were retrieved from the CHOPCHOP tools, which incorporate learning-based algorithms to assess the potential of each gRNA candidate. CHOPCHOP uses *Plasmodium falciparum* 37D genome data combined with machine learning to assess the best gRNA candidates. This resulted in three potential candidates, all associated with a positive strain (Figure 1). However, it is worth noting that their GC content remains below the optimal 30% threshold, which is essential for achieving the best melting temperature (Tm) [18]. Unfortunately, the efficiency number of these sequences remains 0, as the program could not record and count the number of efficiencies.



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Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	Efficiency	MM0	MM1	MM2	MM3
1	TTCTATAGATTATTTTCATTGTCTTCCA	Pf3D7_07_v3:403045	+	25	0	0	0	0	0	0
2	TTCTTACATATAACAAAATGAAATTCGC	Pf3D7_07_v3:403205	+	25	0	0	0	0	0	0
3	TTCATTGTCTTCCACATATATGACATAA	Pf3D7_07_v3:403059	+	29	1	0	0	0	0	0

Figure 1. CHOPCHOP gRNA design result

E-CRISP operates on a hypothesis-driven approach, meaning that it relies on predefined hypotheses or assumptions to guide its algorithm. It uses three hypotheses to identify potential gRNA candidates [19] in this context. The output revealed a set of 10 prospective gRNA candidates, all associated with malaria-caused genes but with varying efficacy scores (Figure 2). However, it is essential to note that determining the precise number of these efficacious candidates from this program is challenging because the program needs to provide the exact count. This limitation underscores the need for additional analysis and refinement to ascertain the most effective gRNA sequences for specific applications, such as for this specific project.

Name	Nucleotide sequence	SAE-Score	Target	Matchstring	Number of Hits
PlAsmodium_2_0	GATGGCTCACGTTTA GGTGG NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_0_1768	GGGAAGAAACACAGT CGTAG NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_1_2652	GGTTCTATAATTTACC GTGT NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_1_0	GCCATCTGTTAAGGT CGACA NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_2_2652	GACGTTGGTTAATTCT CCTT NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_0_2652	GGTAAATTATAGAACC AAAT NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_0_0	GAATAGATAAATCAAC CTAT NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_1_1768	GTGATGATTGTGACG GAGCA NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_0_442	GACTGAACAGGCATC TAACA NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1
PlAsmodium_0_2210	GTTACGAAATCTAATA ATCT NGG	S A E	MAL7P1.27::CRT	Matchstring Info	1

Figure 2. E-CRISP gRNA design result

2.2. Non-Web-Based-Tools gRNA Program

While web-based gRNA design tools provide convenience and accessibility, non-web-based programs remain valuable assets due to their capacity to offer advanced customization options, offline functionality, and enhanced data privacy, which is crucial for handling sensitive genomic information. This

program design mainly relies on software packages like Bioconductor to facilitate the process. Through this approach, the gRNA design could suit specific experimental requirements. The results obtained from these non-web-based tools also reveal a diverse range of gRNAs, each demonstrating varying levels of efficacy.

There are challenges unique to their specific study organisms, such as *Plasmodium falciparum*. One significant hurdle faced is downloading and converting reference genomes into BSgenome datasets. This task can be particularly challenging for *Plasmodium falciparum* due to the limited availability of high-quality genomic resources for this species. Furthermore, the creation of transcript annotation data (txdb) for P. falciparum is another intricate task. The limited resources and comprehensive transcript information for this species can make the process time-consuming and demanding. The results obtained from these programs reveal a diverse range of gRNA, each demonstrating varying levels of efficacy (**Table 1**). While progress has been made, it is important to note that the pool of available gRNAs for *Plasmodium falciparum* is still relatively small. It is acknowledged that there is a need for further enhancements in gRNA efficacy to maximize the precision and efficiency of genome editing in this organism.

Typically, gRNAs with an efficacy score close to 1 are considered highly effective [20]. As such, the gRNAs obtained through non-web-based tools are ranked based on their efficacy scores. This ranking approach could help in identifying the most promising gRNAs for their experiments. The top three gRNAs, ranked by their efficacy scores, are selected for each mutated gene. Importantly, the results also include information about the precise location of each gRNA within the target sequence, along with their respective weights. This additional data provides researchers with the necessary details to locate and utilize the most effective gRNAs efficiently, streamlining the genome editing process

Table 1. Non-web-based gRNA design result

Mutated Gene	Extended Sequence	gRNA Efficacy	top 5 Off Target Total Score	top10 Off Target Total Score	top Off target 1 MM distance 2 PAM
PfCRT	CGAGCGTTATAGAGAATTAGATAATTTAGTA	0.261303895	4.8	4.8	20,19,13,2
	TTTAAAGAGATTAAGGATAATATTTTATTT	0.1771760709	7	11.6	19,10,6,2
	AAATGGCTTGTTCGTTCATAAATATTTATAT	0.1521748932	2.3	2.3	20,19,17,13
Pfdhdr	ATTGAAGATTTTACAATTTCGGATTTTACAA	0.3683344463	5.2	5.2	18,7,5,3
	CTGAATACCAATATTTAAATATTATTTATGA	0.3070262351	11.3	19.8	16,8,7
	AACGATTTAGGACCTATTTATGGTTTTCAAT	0.2789513669	2.6	2.6	16,12,3,2
Pfdhps	GCAGTTTCTAGAATCAACACAGCGTTTCTTC	0.6344671756	1.1	1.1	17,14,3,1
	ATTTCTTGTGGATCTCTAAAAGAATTTATAT	0.3113716903	NA	NA	
	ATATCATAAACTAGATTATC	0.2843747643	8	12.9	18,17,5
Pfkelch13	TTTTCTTCATATACTTCAATAGAATTTAATC	0.4390953291	6.9	11	20,19,10,5
	GATTCTAATAGGCTATCTTTAACATTTCCAT	0.427759228	4.9	4.9	17,8,2
	GAAAAAATGAATAAATGGGAACAATTTCCAT	0.1626317195	9.3	16.4	19,8,7
PfMDR1	AATATTATCATTAGTATCTAT AGGTTTAGTA	0.7206699313	10.2	18.2	18,2,1
	TGGTGGAATAAGAATAAATAAAAATTTAATA	0.3314292832	19.6	31.4	18,10
	ATCATTTTTTTTACCGTTTAAATGTTTACCT	0.3250374501	25.9	34.2	9,1

2.3. gRNA efficacy optimization

The efficacy of guide RNAs (gRNAs) represents a pivotal determinant in the achievement of successful genome editing experiments. Within this context, non-web-based tools for gRNA programming emerge as favored choices for the author seeking to optimize gRNA efficacy. Unlike their web-based counterparts, non-web-based programs often afford the author greater flexibility in fine-tuning various parameters to tailor gRNA designs precisely to their specific experimental needs. This adaptability allows

the researcher to address the nuanced intricacies of their target genomes more effectively. It is crucial to acknowledge that even with the advantages of non-web-based tools, the quest for enhanced gRNA efficacy remains a continuous pursuit. In response to this challenge, the author of this study, with the CRISPR Seek package, further improved gRNA efficacy. The CRISPR Seek package, known for its advanced computational capabilities, plays a vital role in this endeavor by facilitating the exploration of additional factors that can enhance gRNA performance [21].

The results revealed several promising outcomes after enhancing the gRNA efficacy scores (**Table 2**). However, it is important to note that some gRNA sequences produced scores exceeding the desired threshold of 1, particularly in the case of PfCRT and PfKelch13, with scores of 1.0319190 and 1.4413559, respectively. Exceeding this threshold may indicate a potential issue, as an ideal gRNA efficacy score should typically remain at or below 1, according to the CRISPRseek scoring criteria [22]. Further analysis and adjustment may be necessary to ensure optimal gRNA selection for effective genome editing.

Table 2. gRNA efficacy optimization

Mutated Gene	Extended Sequence	gRNA efficacy
PfCRT	CGAGCGTTATAGAGAATTAGATAATTTAGTA	0.9542054
	TTTAAAGAGATTAAGGATAATATTTTTATTT	1.0319190
	AAATGGCTTGTTCGTTCATAAATATTTATAT	0.6613402
Pfdhdr	ATTGAAGATTTTACAATTTCGGATTTTACAA	0.8269825
	CTGAATACCAATATTTAAATATTATTTATGA	0.5220245
	AACGATTTAGGACCTATTTATGGTTTTCAAT	0.2789569
Pfdhps	GCAGTTTCTAGAATCAACACAGCGTTTCTTC	0.8323945
	ATTTCTTGTGGATCTCTAAAAGAATTTATAT	0.7924392
	ATATCATAAACTAGATTATCATAATTTGTTA	0.4095830
Pfkelch13	TTTTCTTCATATACTTCAATAGAATTTAATC	0.9282878
	GATTCTAATAGGCTATCTTTAACATTTCCAT	1.4413559
	GAAAAAATGAATAAATGGGAACAATTTCCAT	0.6699726
PfMDR1	AATATTATCATTAGTATCTATAGGTTTAGTA	0.9982637
	TGGTGGAATAAGAATAAATAAAAATTTAATA	0.8643291
	ATCATTTTTTTTACCGTTTAAATGTTTACCT	0.7346218

2.4 DNA Sequence of Genes Related to Treatment Resistance

The genes related to drug resistance of *P. falciparum* 3D7 that were focused on were the Pfdhfr and Pfdhps, which could be found on chromosomes 4 and 8, respectively. With the resistance of these genes

towards Sulfadoxine-Pyrimethamine (SP) [23–26]. Further research was done to obtain information regarding the sites of mutation in each gene, which were presented in Table 3

The whole sequence of the chromosomes where the genes were present was first obtained from Genbank. The sequences that contained the gene encoding for the proteins related to resistance were cut and made into a new file in Benchling for ease of further research purposes. The information regarding the genes is presented in Table 4.

Table 3. Mutation in genes related to malaria treatment resistance

Drug	Gene	Mutations	Sequence (wt)	Mutated sequence	Orientation
Sulfadoxine-pyrimethamine (SP)	pfdhfr	A16V(Ala16Val)	gca	gta	Forward
		C59R(Cys59Arg)	tgt	CGT	
		S108N/T	agc	aac(N)	
		I164L(Ile164Leu)	ata	tta/ttg	
	pfdhps	A437G(Ala437Gly)	gct	gg	
		K540E(Lys540Glu)	aaa	gaa	
		A581T(Ala581Thr)	gcg	ggg	

Table 4. Information on genes related to resistance to treatments

Chromosome	Gene	Accession Number	Locus Tag
4	Pfdhfr	NC_004318.2	PF3D7_0417200
8	Pfdhps	NC_004329.3	PF3D7_0810800

2.5. Conventional Primer Design

The SNP sites were annotated, and the primers for amplification using LAMP were designed using Benchling.

2.5.1. Primer Requirements

The requirements for the pair primers to be suitable for the amplification process are that the primers should be able to isolate the mutation sites, and the primers should have 30% to 60% GC content. The length of the product was targeted to be between 200 - 2000 bp for further LAMP primer design. The primer designing process could be done using a feature present in Benchling or using external resources such as Primer3

2.5.2. Primer Sequences

Using the Benchling software, the primers were designed based on the specifications and requirements mentioned earlier. The pair of primers found were listed in **Table 5**.

Table 5. List of found primer pairs

Drugs	Gene	Mutation	Primers		Product Size
			Forward	Reverse	
Sulfadoxine-	Pfdhfr	A16V(Ala16Val)	5′-ATTACCGWC	5′-CTACATTAA	529 bp

pyrimethamine (SP)		C59R(Cys59Arg)	GATGGAACAAG TCTGCGACG-3′	TTAAAAATTCT TGATAAACAA CG-3′	
		S108N/T			
		I164L(Ile164Leu)			
	Pfdhps	A437G(Ala437Gly)	5′-ATTATCCTTCA TGATAAATGAAG GTGC-3′	5′ATTGGTATCC GAATATCCAAT AAAAAGTGG- 3′	580 bp
		K540E(Lys540Glu)			
		A581T(Ala581Thr)			

It must be considered that the primer pairs found were only for the Pfdhfr and Pfdhps genes. These primers would likely not be the most suitable or efficient primer pairs for the sequence. However, these primer pairs were utilized to create a digital PCR product in order to design the LAMP primer, which means these primer pairs are designed to capture the SNPs correlated with drug resistance, so the focus of these primer pairs is to reduce the size of the overall gene into a size that could be targeted during the LAMP primer design process. However, it still stands that further optimization could be done to create a more efficient primer pair that could further assist the necessary parameters for the LAMP primer design.

2.6 LAMP Primer Design

Using Primerexplorer V5, the LAMP primers were designed from the digital PCR products of the previously designed primers. The forward and reverse primers are listed in Tables 6 and 7. Parameters for these LAMP primers can be checked in the previously curated research [27].

Table 6. List of LAMP primers for the Pfdhps mutations (dG = -2.13)

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	123	143	18	56.9	-3.69	-4.51	0.28	TTCAAAAAGAATGGAATG
B3	345	362	18	50.18	-3.29	-4.71	0.39	TTGTATGTGGATTTCCTC
FIP			43					TCAACTAAATCATTATCAACACATT-AAATGTGAT GCGAAACCA
BIP			44					TAGTGCTTGTACAAATAATCCAG-TTTTATGCAT TAGAACTACAC
F2	164	181	18	54.29	-3.71	-4.67	0.39	AAATGTGATGCGAAACCA
F1c	222	246	25	55.21	-4.41	-4.21	0.24	TCAACTAAATCATTATCAACACATT
B2	324	3344	21	50.95	-2.4	-4.23	0.29	TTTTATGCATTAGAACTACAC
B1c	262	284	23	56.06	-4.98	-4.74	0.35	TAGTGCTTGTACAAATAATCCAG

Once more, it has to be considered that these primers may need to be optimized to give the most efficient result. However, these LAMP primers were designed to ensure that the SNP mutations are properly amplified and could be detected through other methods. For the Pfdhps primer, both in the B2 and BIP, the red underlined color refers to the site of a mutation, which could cause problems such as misaligning the primers. These primers were selected due to having the highest dG value, which measures the quality of these primers. Furthermore, the 5'dG and 3'dG refer to the end stability for these LAMP primers.

2.7. Opportunities for Further Exploration

Our study has laid a robust foundation for advancing the detection of drug-resistant malaria strains. However, several promising avenues for further exploration remain. One potential area for further research is the integration of CRISPR-Cas12 technology with other innovative gene-editing tools such as CRISPR-Cas9. The review by Ansori et al. highlights the versatility of CRISPR-Cas9 across various fields, suggesting that combining these technologies could enhance the precision and efficiency of genetic modifications in *Plasmodium falciparum* [28]. This integration could lead to more robust diagnostic tools and therapeutic strategies. Hence, the CRISPR-LAMP system could be deployed to identify genes that could serve as a target for drug candidate assays, especially the novel compounds [29-30]. Investigating such synergies might open new paths for optimizing CRISPR-Cas12 applications and improving detection sensitivity and specificity.

Furthermore, expanding the gene visualization pipeline to include machine learning algorithms could significantly improve data analysis and interpretation. As noted in various studies, the application of machine learning in genomic research can enhance the prediction of off-target effects and optimize experimental designs [28]. Incorporating these computational techniques could lead to more accurate and reliable detection methods. These opportunities for further exploration underscore the potential to refine and expand the capabilities of CRISPR-Cas12-based detection methods, ultimately contributing to more effective management of drug-resistant Malaria.

Table 7. List of LAMP primers for the Pfdhfr mutations (dG = -2.16)

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	9	26	18	55.08	-4.51	-6.43	0.5	GAATGGAACAAGTCTGCG
B3	185	203	19	51.82	-4.46	-5.4	0.37	TCACATATGTTGTAACTGC
FIP			43					TCCCCTCATTTTTGCTTTCA-ACGTTTTCGATATT TATGCCATA
BIP			48					TGAGGTTTTTAATAACTACACATTT-AGGGAATT ACATTTCCATGGTAA
F2	27	49	23	56.17	-4.99	-4.98	0.3	ACGTTTTCGATATTTATGCCATA
F1c	67	86	20	57.26	-6.04	-4.02	0.4	TCCCCTCATTTTTGCTTTCA
B2	143	165	23	57.53	-5.2	-4.25	0.35	AGGGAATTACATTTCCATGGTAA
B1c	94	118	25	55.1	-5.25	-3.71	0.24	TGAGGTTTTTAATAACTACACATTT

3. CONCLUSION

This research proposes developing an advanced gRNA discovery program to enhance drug resistance detection in *Plasmodium falciparum*. Web-based gene visualization tools like CHOPCHOP and E-CRISP offer user-friendly platforms but require improvements specific to CRISPR-Cas12. Non-web-based tools provide customization options but face challenges related to genomic resource availability. Continuous optimization using computational methods like CRISPR Seek has already improved the efficiency of gRNA, though there are outliers with efficacy scores exceeding. Collaboration, data sharing, and quality control are key to advancing research in this field.

Furthermore, some LAMP primers for *P. falciparum* were also proposed and discovered to ensure that the SNPs related to drug resistance would be within the target for the LAMP primers for further downstream detection methods. To address the challenges, it is recommended that web-based tools tailored for CRISPR-Cas12 be expanded, non-web-based tools' access to genomic resources should be improved, and gRNA efficacy prediction methods should be continuously optimized. Collaboration among researchers and institutions and quality control measures will further accelerate progress in gRNA design and genome editing for drug resistance detection in *Plasmodium falciparum*. Furthermore, further optimization could be done for the primer isolates to create a better and more stable LAMP primer for the genes involved

4. MATERIALS AND METHODS

The development of the gRNA discovery and efficacy prediction program is initiated by reading several literature reviews about in silico gRNA design and the CRISPR-Cas-12 systems overall. The sequences of *Plasmodium falciparum* strains and the reference genome data were then retrieved from the Genbank - National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/genbank/). The reference genome data will then be converted into the BsGenome dataset for the Bioconductor package using the default value (https://bioconductor.org/packages/release/bioc/html/BSgenome.html). Besides that, the transcript annotation data were also retrieved from Plasmodium Informatics Resources (PlasmoDB) (https://plasmodb.org/plasmo/app), which will then be converted into TxDb Bioconductor package [31]. The assessment of gRNA efficacy involves a multi-step workflow that combines the strengths of web-based and non-web-based tools. To further optimize the efficacy of the selected gRNAs, the crisprseek package is incorporated into the workflow by invoking default value (https://bioconductor.org/packages/release/bioc/html/CRISPRseek.html). The tools utilized off-target scoring schemes and mismatch penalty matrix algorithms to enhance the precision and success rate of gRNA design, particularly for off-target results [32].

The LAMP primer was created by a literature review regarding genomic mutations for P. falciparum and their respective locus. The reference genome data were then imported from Genbank - National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/genbank/) into Benchling, which is a web-based platform for research involving genomics and has tools for sequence analysis and primer, designs (https://www.benchling.com/). Following those steps, the SNP locus were then annotated and isolated using regular primers based on their location since LAMP primers function when the targeted sequence length is between 200 - 2000 bp [32,33]. Utilizing benchling, a digital PCR product containing the isolates for the SNPs was acquired [34]. These isolates were then imputed into Primerexplorer V5, which creates LAMP primers based on the target regions (http://primerexplorer.jp/elamp4.0.0/index.html).

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Author contributions: Concept – AAP, RH; Design – AAP, RH, ES; Supervision – AAP, RH; Resources – SA, AAP; Materials – RH, ES; Data Collection and Processing – DF, DH; Analysis and Interpretation – DF, DH; Literature Search – AAP, DH, DF; Writing – AAP, DH, DF; Critical Reviews – SA, RH, ES.

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