Validation of a CRISPR-Cas Nuclease-Based Diagnostic Test for the Rapid Detection of Antimalarial Drug Resistance

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Abstract

Reduction of malaria disease burden and transmission is dependent upon diagnosis and treatment of the parasitic infection caused by *Plasmodium spp.* However, the emergence of *P. falciparum* drug resistance threatens the progress of ongoing control efforts. Assays that produce simple, accurate readouts for the detection of drug-resistant *P. falciparum* parasites would be a useful tool for informing treatment decisions for infected individuals, as well as for assessing the efficacy of antimalarial drug products in clinical trials. Assays that employ CRISPR-Cas nucleases have shown great promise for the development of next-generation molecular diagnostics technology for their ability to specifically target DNA via strong collateral and indiscriminate small molecule cleavage activity. Recently, Cas nucleases have been utilized in malaria CRISPR diagnostic methods (PMIDs: 32958655) with greater sensitivity and specificity than traditional RT-PCR. Here, we validate a "single-pot" CRISPR-Cas12a rapid assay for the detection of a single nucleotide polymorphism (DHODH, PF3D7_0603300, C276F) that confers reduced sensitivity to *Pf*DHODH inhibitors (PMID: 33361312). By combining Recombinase Polymerase Amplification (RPA) with Cas12a nucleic acid-targeting in a "single-pot" assay that quantitatively measures the presence of either the wild-type or mutated *Pf*DHODH gDNA via a fluorescent reporter, we aim to detect this novel biomarker with greater sensitivity and more rapidly than existing methods for detecting drug-resistant malaria parasites. Future work will involve determining the diagnostic sensitivity, specificity, and limit of detection within a human serum matrix, as well as expanding the assay to include additional biomarkers for drug resistance to other widely used classes of antimalarial compounds. Optimization of these assays will enable rapid and sensitive detection of drug-resistant Plasmodium parasites in clinical samples, which may be used for evaluation of both antimalarial drug and vaccine efficacy and as a novel point-of-care diagnostic in endemic countries, particularly where reported emergence of drug resistance impedes the use of front line antimalarials.

CRISPR-Cas Target Detection Workflow

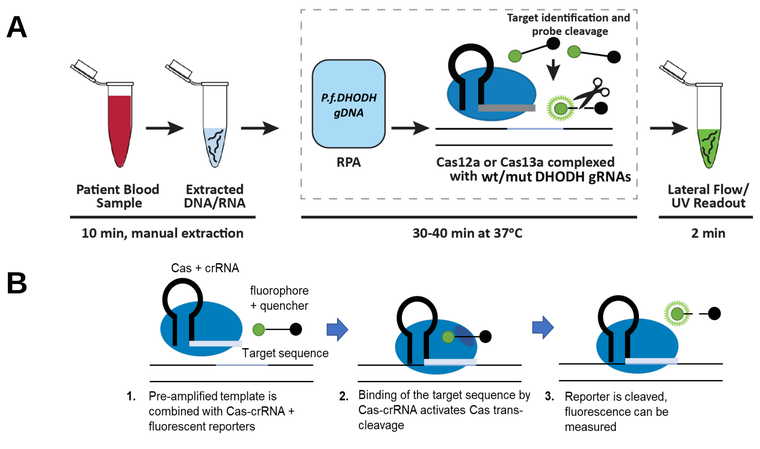

Figure 1. Overview of the CRISPR detection method with an RPA pre-amplification step. **A**) Following extraction of *P. falciparum* genomic DNA from infected blood, a pre-amplification step is performed to amplify the target sequence using RPA (TwistDx). The amplification product is then detected via gRNA guided Cas endonuclease activity resulting in a fluorescent signal. **B**) Detection of the target will occur after recognition of the target sequence by the gRNA, upon which an activated Cas12a nuclease will begin to indiscriminately cleave small molecules. This activity can be measured with the addition of fluorescent reporters. Upon cleavage of the reporter, fluorescence can be measured as an indicator for the presence of the target.

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*Pf*DHODH: Resistance Biomarker

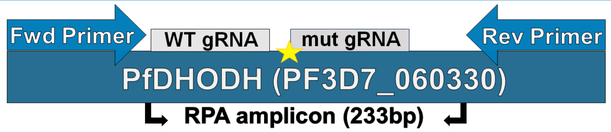


Figure 2. RPA Primer and guide RNA (gRNA) design for *Pf*DHODH. The RPA primers used in this assay were designed for a conserved DNA coding region of *Pf*DHODH and are expected to result in a band of 233bp. Cas12 gRNAs were designed for target detection within the RPA amplicon of the wild-type (WT) *Pf*DHODH sequence, as well as the mutated (mut) *Pf*DHODH sequence containing the single nucleotide polymorphism, C276F, that confers reduced sensitivity to DHODH inhibiting antimalarials. The mut gRNA is designed such that the PAM sequence for Cas12a detection is only present if the mutated sequence is present (yellow star), thus target detection of mutant *Pf*DHODH by Cas12 can occur.

*Pf*DHODH Target Amplification

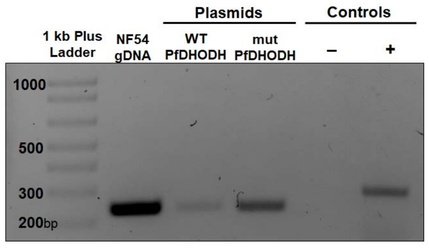


Figure 3. Amplification of *Pf*DHODH with RPA. *Pf*DHODH was amplified from NF54 gDNA, wild-type (WT) *Pf*DHODH plasmid, or mutant (mut) *Pf*DHODH plasmid compared to a "No DNA" control using RPA primers described in **Fig. 2**. and TwistAmp™ Liquid Basic RPA kit (TwistDx™ Limited, Maidenhead, UK). Amplicons were cleaned up using the QIAquick PCR Purification Kit (Qiagen) before undergoing electrophoresis on a 2% agarose gel to confirm amplification of a fragment of DHODH. A product of the expected size (233bp as indicated in **Fig. 2**), was observed in the three lanes containing *P.f.* template, and not observed in the No DNA negative control lane (-). A positive control from the TwistAmp™ RPA kit was added to the farthest right lane, resulting in a band of 289bp, consistent with the expected band size for this control.

*Pf*DHODH gRNA Specificity

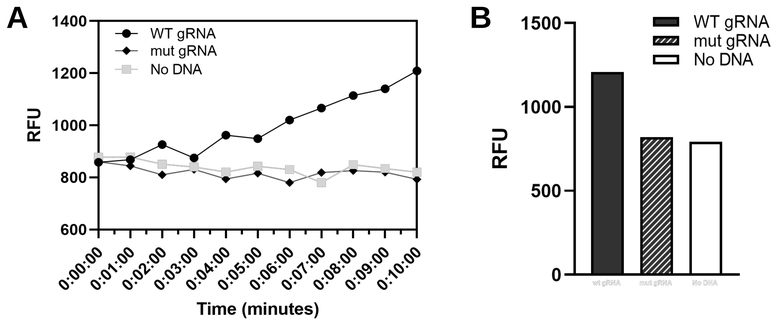
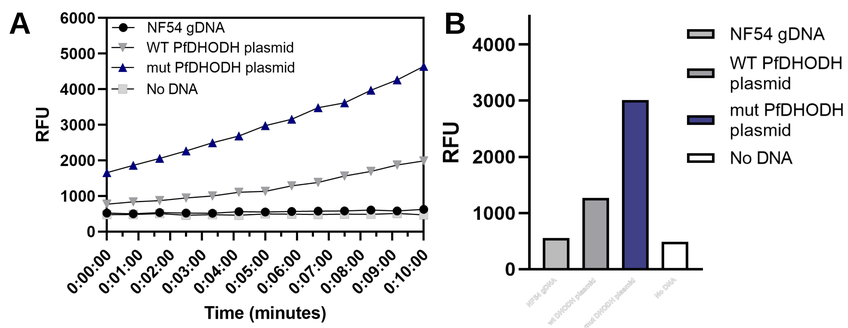


Figure 4. Cas12a Detection of WT *Pf*DHODH from *P.f.* strain NF54 gDNA. *Pf*DHODH was amplified from NF54 gDNA compared to a "No DNA" control as described in **Fig. 3**. Amplicons were combined with *Lba*Cas12a (New England Biolabs), WT gRNA and mut gRNA described in **Fig. 2**, and ssDNA-FQ reporters labeled with a 5' 6-FAM and a 3' Iowa Black® FQ quencher (IDT). Reactions were then incubated at 37°C for 10 minutes while fluorescence was measured via kinetic read with 1-minute intervals on a plate reader. Measurements for each time interval are plotted **(A)** as well as endpoint fluorescence values **(B)**. RFU=relative fluorescence units.

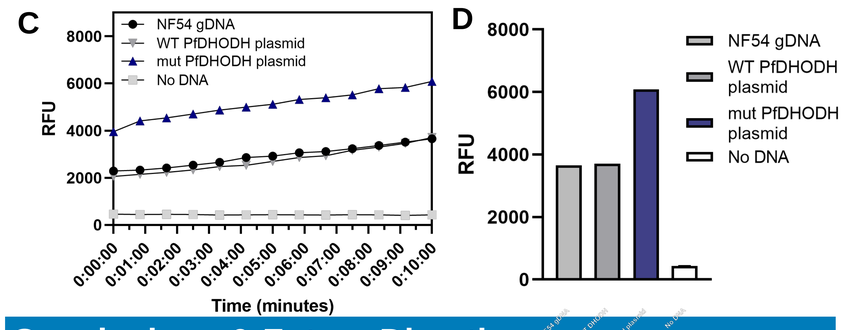
Cas12a Detection of C276F Mutation in *Pf*DHODH

Figure 5. gRNA Specific detection of Mutant and Wild-type *Pf*DHODH. *Pf*DHODH amplicons generated from NF54 gDNA, or plasmids containing WT or mut *Pf*DHODH as described in **Fig. 3,** then were combined with *Lba*Cas12a (New England Biolabs), ssDNA-FQ reporters labeled with a 5' 6-FAM and a 3' Iowa Black® FQ quencher (IDT), and either mut gRNA or WT gRNA described in **Fig. 2**. Reactions were then incubated at 37°C for 10 minutes while fluorescence was measured via kinetic read with 1-minute intervals on a plate reader. Fluorescence over time is plotted (**A and C**) as well as endpoint fluorescence values (**B and D**). RFU=relative fluorescence units. Target detection corresponds well with the gRNA being used in each assay with little to no cross reactivity between mutant or wild-type *Pf*DHODH targets and gRNAs. Thus, demonstrating the specificity of mut gRNA for the C276F single nucleotide polymorphism and verifying its utility and potential for detection of this resistance biomarker.

Mutant *Pf*DHODH gRNA



Wild-type *Pf*DHODH gRNA



Conclusions & Future Directions

- **Here we demonstrate the activity of a novel CRISPR-based assay for the detection *Pf*DHODH from *P.f.* gDNA for the purpose of detecting the presence of drug-resistance mutation.**
- **We have established that a gRNA-guided CRISPR activity can differentiate the presence of a single point mutation in *Pf*DHODH.**
- **Recombinase Polymerase Amplification of the *Pf*DHODH target fragment is a viable and rapid alternative to PCR.**
- **Future work will involve determining the diagnostic sensitivity, specificity, and limit of detection within a human serum matrix.**