Supporting information

Pyrococcus furiosus Argonaute-Mediated Dual-Recognition Enables the Detection of Trace Single-nucleotide-Mutated Fungicide-resistant Fungal Pathogens

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Experimental section

Materials and reagents

The plasmid pET23a-6×His-*Pf*Ago was provided from Lixin Ma's group in Hubei University. Triadimefon-resistant and triadimefon-sensitive *Puccinia striiformis f. sp. tritici* (*Pst*) and the plasmids carrying the mutant-type (MUT) or wild-type (WT) genes of cyp51 were provided by Northwest A&F University. Oligonucleotides were bought from Sangon (Shanghai, China) and were listed in Table S1. LbCas12a and TOLO buffer 3 (cat. no. 32108) was purchased by Tolo Biotech (Shanghai, China). T7 RNA polymerase (cat. no. EP0111) were bought from Thermo Fisher Scientific (Beijing, China). T4 Polynucleotide Kinase (cat. no. M0201S), Adenosine 5'-Triphosphate (ATP) (P0756S), DNase I (cat. no. M0303S), dNTP mix (cat. no. N0447S), and rNTP mix (cat. no. N0466S) were purchased from New England Biolabs (Beijing, China). SanPrep Column DNA Gel Extraction Kit (cat. no. B518131), 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) kKit (cat. no. C671103), Protein Stains H Kit (cat. no. C510041), DNA Marker (25~500 bp) (cat. no. B600303), DNA Marker (100~2000 bp) (cat. no. B500350), Agarose (cat. no. A620014), TAE Buffer (cat. no. B548101), TBE Buffer (cat. no. B548102), Tris-Glycine SDS-PAGE Running Buffer (cat. no. C520001), Protein Loading Buffer (No Reducing Buffer) (cat. no. C516031), and RNase-free ddH2O (cat. no. B541018) were all bought from Sangon (Shanghai, China). Gelred dye (cat. no. TSJ003), 5× DNA loading buffer (cat. no. TSJ010), Prestained Protein Ladder (10-180 kD) (cat. no. TSP021), Escherichia coli BL21 (DE3) pLysS (cat. no. TSC-E05) and Escherichia coli DH5α (cat. no. TSC-C14) were got from Tsingke Biotechnology (Beijing, China). TwistAmp™ Basic Kit (containing TwistAmp® Basic reaction, Primer Free Rehydration buffer and 280 mM Magnesium Acetate) was purchased from TwistDx Co., Ltd. (Cambridge, UK). EndoFree Mini Plasmid Kit II (cat. no. 4992422) was bought from Tiangen Biotech (Beijing, China).

Expression and purification of *Pf*Ago

The plasmid pET23a-6×His-*Pf*Ago was transformed into *Escherichia coli* BL21 (DE3)

pLysS. Then, 200 μL of transformant was placed on solid LB media with 100 μg/ml ampicillin, followed by an overnight cultivation at 37 ℃. The culture was inoculated in liquid LB medium containing ampicillin and was incubated in a shaker at 37 ℃, 220 rpm until the OD_{600} reached to 0.6-0.8. Then, 1 mM isopropyl β-D-thiogalactopyranoside was added into the culture to induce the expression of *Pf*Ago. The procedure was performed at 37℃, 180 rpm for 16-18 h. Cells were collected by centrifugation for 5 min at 4,000 rpm and were resuspended in Buffer I (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2 mM $MnCl_2$). The collected cells were lysed by ultrasonic sterilizer (Scientz, Ningbo, China). The lysate was centrifuged at 12,000 rpm for 30 min. Next, the supernatant was heated immediately at 75 ℃ for 30 min. The treated solution was centrifuged at 12,000 rpm for 30 min to harvest the supernatant, after which the supernatant was purified with Ni-NTA affinity purification. Then, the washing buffer (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2 mM $MnCl_2$, and 25 mM imidazole) was added to remove unwanted proteins. The purified protein was eluted using elution buffers (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2 mM $MnCl_2$, 250 mM imidazole) with 3-time column volume and collected in tubes. The eluted fractions were verified by SDS-PAGE and the fraction containing target protein was collected. The collection was concentrated with a Millipore 30 kD membrane and resuspended in the storage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5 mM $MnCl_2$, 50% [v/v] glycerol). The purified *Pf*Ago was frozen rapidly in liquid nitrogen and stored at -80 ℃.

Preparation of guide DNAs (gDNAs) and CRISPR RNAs (crRNAs)

All ssDNAs designed for target sequence were treated with T4 polynucleotide kinase (T4 PNK) and adenosine 5'-triphosphate to yield ssDNAs with 5'-terminal phosphate group as guide DNAs. The reaction was carried out with 40 μL mixture, containing 400 pmol ssDNA, 4 μL ATP(10 mM), 4 μL 10 × T4 PNK buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT) and 10 U T4 PNK. *In vitro*-transcription of crRNAs designed for CRISPR/Cas12a system was conducted by incubating 4 μL the target DNA strand (10 μM), 4 μL T7 promoter (10 μM), 4 μL buffer (10 mM Mg-acetate, 33 mM Tris-acetate, 1 mM DTT, 66 mM K-acetate, and 0.1% (v/v) Tween 20), and 14 μL RNase-free H_2O at 95℃ for 5 min and then incubated at 37℃ for 10 min. Subsequently, 2 μL T7 RNA polymerase, 2 μL rNTP mix, and 8 μL

transcription buffer (containing 200 mM Tris-HCl, 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl, and 10 mM spermidine) were added and incubated at 37 ℃ overnight to yield crRNAs. Finally, 2 μL DNase I was added to digest DNA at 37 °C for 5 h and the mixture was heated at 85 °C for 5 min to inactivate DNase I.

Plasmid extraction and sequencing

The plasmid of pET23a-6×His-*Pf*Ago, the plasmid of MUT *cyp51* gene and the plasmid of WT *cyp51* gene were transformed into *Escherichia coli* DH5α, respectively. Then, the transformants were cultured in liquid LB medium at 37 ℃, 180 rpm for 12 h to produce the plasmids. Then, plasmids were extracted by EndoFree Mini Plasmid Kit II and were sequenced by Sangon (Shanghai, China). Both of the extracted plasmid of the MUT *cyp51* gene and that of the WT *cyp51* gene were amplified by PCR using the primers, PCR-FW and PCR-RV (Table S2). The 691 bp amplicons were used as templates.

Detection of *Pst*

First, the *Pf*Ago-mediated cleavage process was conducted in a 1× *Pf*Ago reaction buffer with a volume of 20 μL, containing 15 nM *Pf*Ago, 150 nM gDNA, and the MUT template or the WT template. The mixture was heated at 95 ℃ for 20 min. According to the RPA product manual, a mixture with 1 μL product of the *Pf*Ago-mediated cleavage process, 1.2 μL RPA forward primer (10 μM), 1.2 μL reversed primer (10 μM), 14.75 μL Primer Free Rehydration buffer and 5.6 μL RNase-free H_2O was prepared and added to a TwistAmp® basic reaction. Then the solution was incubated at 37 °C for 10 min after adding 1.25 μL of magnesium acetate (280 mM). Next, 2 μL RPA product was added to a mixture containing 4 μL reporter (5 μM), 0.4 μL Cas12a (10 μM), 4 μL crRNA (2 μM), 4 μL 10× Cas12a buffer, and 25.6 μL RNase-free H_2O. The mixture was incubated at 37 °C for 30 min. Finally, the fluorescence intensity of the reagent was measured by Synergy H1 Multi-Mode Microplate Reader. The excitation wavelength was 480 nm, and the emission wavelength ranged from 510 to 650 nm.

Gel Electrophoresis Analysis

First, the gel-loading solution with a volume of 6 μL was prepared by mixing 5 μL sample and 1 μL loading buffer. Next, 2% agarose, 1× TAE buffer, and 1× GelRed dye were mixed to prepare the gel. Then, 5 μL gel-loading solution was added to the prepared gel. Gel electrophoresis was conducted in TAE buffer at 150 V for 20 min. Finally, the result was imaged and photographed using ChampGel™ 7000 gel imaging system (SinSage Technology, Beijing, China).

Table S1. Oligonucleotide sequences.

	Oligonucleotide	Sequences (5' to 3')
gDNAs	gS(MP2)	P-ATAAACTACATCCGTA
	gS(MP4)	P-TCATAAACTACATCCG
	gS(MP6)	P-CATCATAAACTACATC
	gS(MP7)	P-ACATCATAAACTACAT
	gS(MP9)	P-GGACATCATAAACTAC
	gS(MP10)	P-GGGACATCATAAACTA
	gS(MP11)	P-CGGGACATCATAAACT
	gS(MP12)	P-TCGGGACATCATAAAC
	gS(MP14)	P-GTTCGGGACATCATAA
RPA	P1	GAATTTTTCTACAGCTACTCCTGTATTCGG
	R1	GGAATATATGATCCTCGATATAACCGATCGTC
	P2	CAGTATGTCATCTCATCTCTCGTCTTCTTC
	R2	CTCTGGAATATATGATCCTCGATATAACCG
	P3	GAAAGTGACTGTAGCTTTAGGTTTACAAGG
	R3	TATATGATCCTCGATATAACCGATCGTCTC
	P4	CGTTGTTCGTTCAATTTACGATCTATCTCC
	R4	TATATGATCCTCGATATAACCGATCGTCTC
crRNA transcription	L-crRNA1	CAAGATTGCGTTCGGGACATCAATCTACACTTAGT AGAAATTACCCTATAGTGAGTCGTATTA
	L-crRNA2	TCAAAAACTACATCCGTACCATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3/L-crRNA3(MP10)	GATGTAGTTTTTGATGTCCCATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP2)	TCGGTACGGATGTAGTTTTTATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP4)	GGTACGGATGTAGTTTTTGAATCTACACTTAGTAG AAATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP6)	TACGGATGTAGTTTTTGATGATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP8)	CGGATGTAGTTTTTGATGTCATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP12)	TGTAGTTTTTGATGTCCCGAATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP14)	TAGTTTTTGATGTCCCGAACATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP16)	GTTTTTGATGTCCCGAACGCATCTACACTTAGTAG AAATTACCCTATAGTGAGTCGTATTA
	L-crRNA3(MP18)	TTTTGATGTCCCGAACGCAAATCTACACTTAGTAG AAATTACCCTATAGTGAGTCGTATTA
	T7 promoter	TAATACGACTCACTATAGGG
PCR	PCR-FW	TAGGCTCAGCAATCTCATACGG

	PCR-RV	AGGTAGTCCAGGTAGAGCGAAA
	TG-reporter	5’-FAM-GGGTTTTTTGGG-BHQ1-3’

* The blue bases represent the hybridization regions of the Cas12a/crRNA. The introduced mismatched bases were marked in red.

Table S2. Sequences of the fungicide-resistant and fungicide-sensitive *Puccinia striiformis.*

	Sequences (5' to 3')
Fungicide-resistant *Puccinia striiformis*	TAGGCTCAGCAATCTCATACGGAATCGATCCATACGCTTTCCTA GAATCATGCAGAAAGAAATATGGGAACGTATTCACATTCATACT TATAAATAAGAAAGTGACTGTAGCTTTAGGTTTACAAGGAAACG CATTGGTTCTCAATGGGAAACTTGCTCAAGTTAATGCTGAAGAA GCCTATACCGCTCTCAGTATGTCATCTCATCTCTCGTCTTCTTC GCAATTTCCCTAAACTAATAATGAGTTCTTCTCCTTCTATTGAAT TTTTCTACAGCTACTCCTGTATTCGGTACGGATGTAGTTTTTGA TGTCCCGAACGCAATCTTGATGCAACAAAAAAAATTCATCAAAT CTGGCTTGACCACCGAAAACTTTCGTAAATACGTATCTACGATT GTCGATGAGACGATCGGTTATATCGAGGATCATATATTCCAGA GTAAGTTTGAGATTCTTTTATATCGACCATTCCCTCCCCCTTAA CTTACTTGAGCATTTATCTCAGACCCAAAAACCCAGCAAGCTGT CAAAGATGCTCTCAAGGTGGCCTCAGAGATCACGATCTGTACC GCTTCGGCTACACTTCAAGGGCCTGAAGTCAGAGAAGGACTCA ACACATCATTTGCGGATATATATCATGATTTAGATGGTGGATTT ACACCCTTACATTTCGCTCTACCTGGACTACCT
Fungicide-sensitive *Puccinia striiformis*	TAGGCTCAGCAATCTCATACGGAATCGATCCATACGCTTTCCTA GAATCATGCAGAAAGAAATATGGGAACGTATTCACATTCATACT TATAAATAAGAAAGTGACTGTAGCTTTAGGTTTACAAGGAAACG CATTGGTTCTCAATGGGAAACTTGCTCAAGTTAATGCTGAAGAA GCCTATACCGCTCTCAGTATGTCATCTCATCTCTCGTCTTCTTC GCAATTTCCCTAAACTAATAATGAGTTCTTCTCCTTCTATTGAAT TTTTCTACAGCTACTCCTGTATTCGGTACGGATGTAGTTTATGA TGTCCCGAACGCAATCTTGATGCAACAAAAAAAATTCATCAAAT CTGGCTTGACCACCGAAAACTTTCGTAAATACGTATCTACGATT GTCGATGAGACGATCGGTTATATCGAGGATCATATATTCCAGA GTAAGTTTGAGATTCTTTTATATCGACCATTCCCTCCCCCTTAA CTTACTTGAGCATTTATCTCAGACCCAAAAACCCAGCAAGCTGT CAAAGATGCTCTCAAGGTGGCCTCAGAGATCACGATCTGTACC GCTTCGGCTACACTTCAAGGGCCTGAAGTCAGAGAAGGACTCA ACACATCATTTGCGGATATATATCATGATTTAGATGGTGGATTT ACACCCTTACATTTCGCTCTACCTGGACTACCT

* The red letter represents the single nucleotide mutation site of the drug-resistant *Puccinia striiformis*.

Table S3. Comparison of the performance on SNMs detection using various methods.

Detection method	Feature	Reaction Time	Sensitivity	Specificity	Enzyme	Reference
PARPA	Nuclease reaction and RPA	1 h	0.05% with fluorescence detection	High	*Pf*Ago, Cas12a	This work
castPCR	PCR	2 h	0.1% with fluorescence detection	Medium	-	[1]
Cold-PCR	PCR	>1 h	0.1%-1% with NGS	Medium	-	[2]
BDA	PCR	2 h	0.1% with NGS or qPCR	Medium	-	[3]
HOLMESv2	LAMP and Nuclease reaction	1 h	0.1% with fluorescence detection	High	Cas12b	[4]
SHERLOCKv2	RPA and nuclease reaction	2 h	0.1% with fluorescence detection	High	Cas12a, Cas13 and Csm6	[5]
Cut-PCR	Nuclease Reaction and PCR	>2 h	0.01% with NGS	High	Cas9	[6]
cARMS	Nuclease reaction and PCR	>2 h	0.5% with qPCR	High	Cas12a	[7]

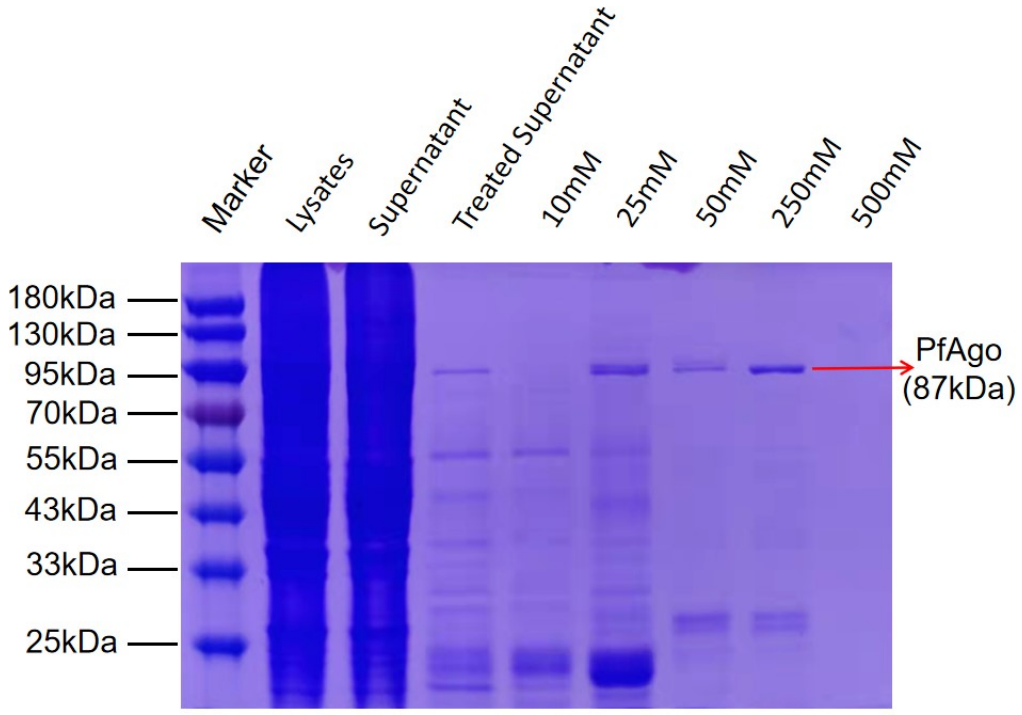

Fig. S1. The expression and purification of *Pf*Ago. The SDS-PAGE showed the products that eluted by different concentrations of imidazole. Lysates were cell lysate proteins of *Escherichia coli* BL21 (DE3). Treated supernatant was the lysate supernatant heated at 75 ℃ before purification.

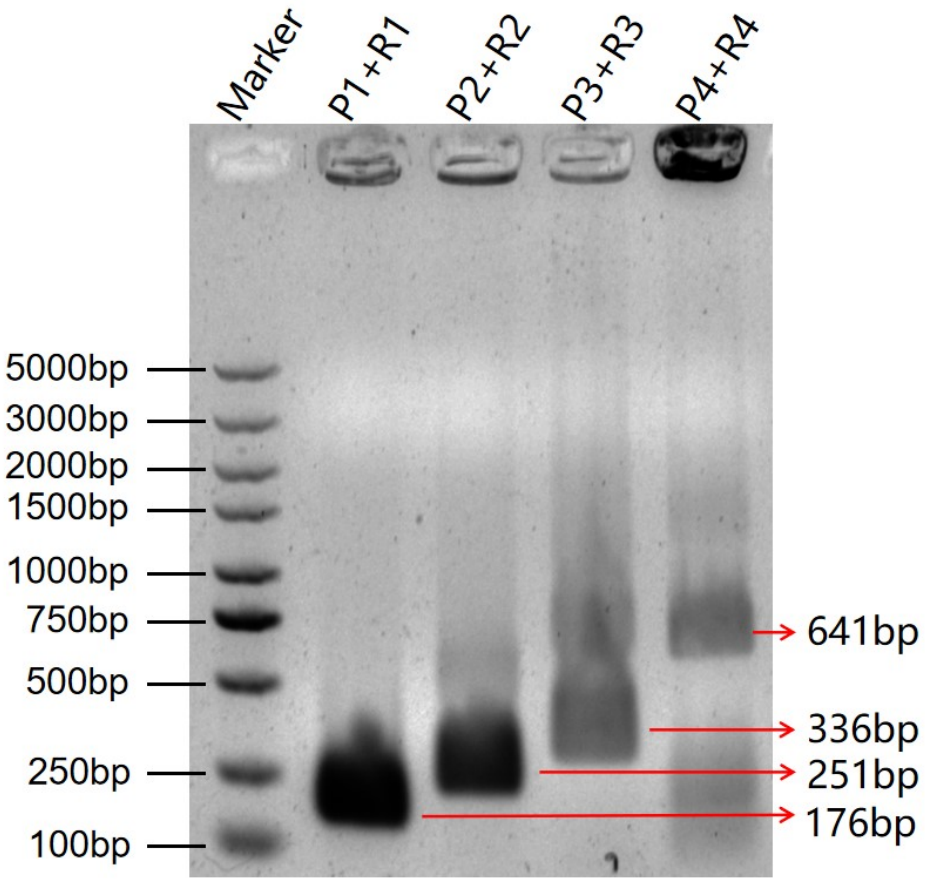

Fig. S2. Electrophoresis results of RPA reaction with different primers. M: DNA Marker (100~5000 bp).

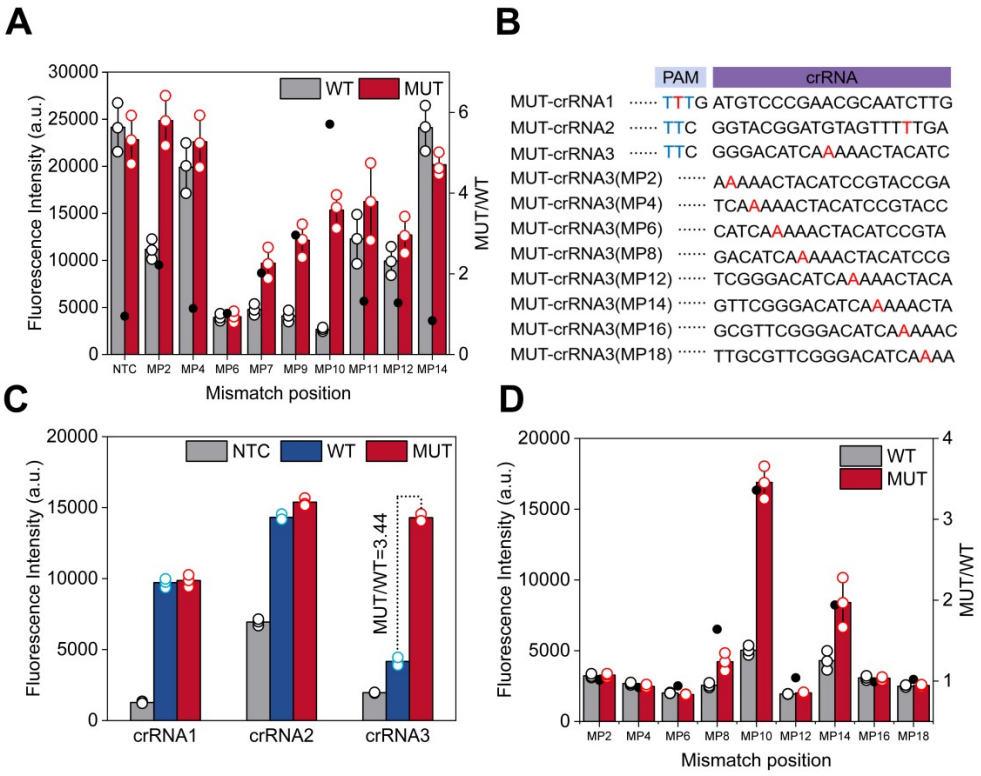

Fig. S3. The optimization of gRNAs and crRNAs of the PARPA assay. (A) Fluorescence analysis for optimization of gDNAs of the PARPA assay. (B) The design of the crRNAs used in (C) and (D). Nucleotides marked in blue were PAM recognition sites of crRNAs. Nucleotides marked in red were introduced mismatched bases. (C) Fluorescence analysis for the optimization of crRNAs. (D) Fluorescence analysis using crRNAs incorporated mismatched nucleotides in different positions.

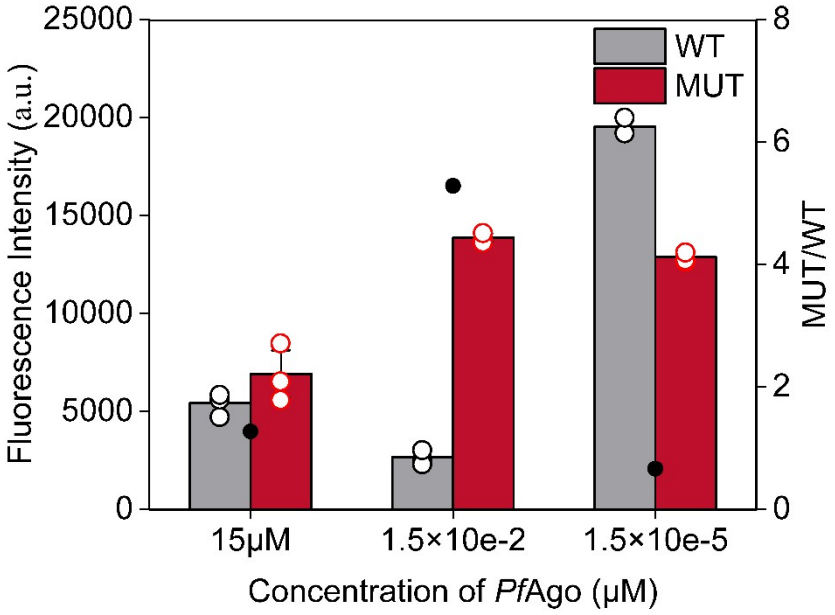

Fig. S4. Fluorescence intensity of wild-type and mutant-type template treated with *Pf*Ago/gDNA in different ratios.

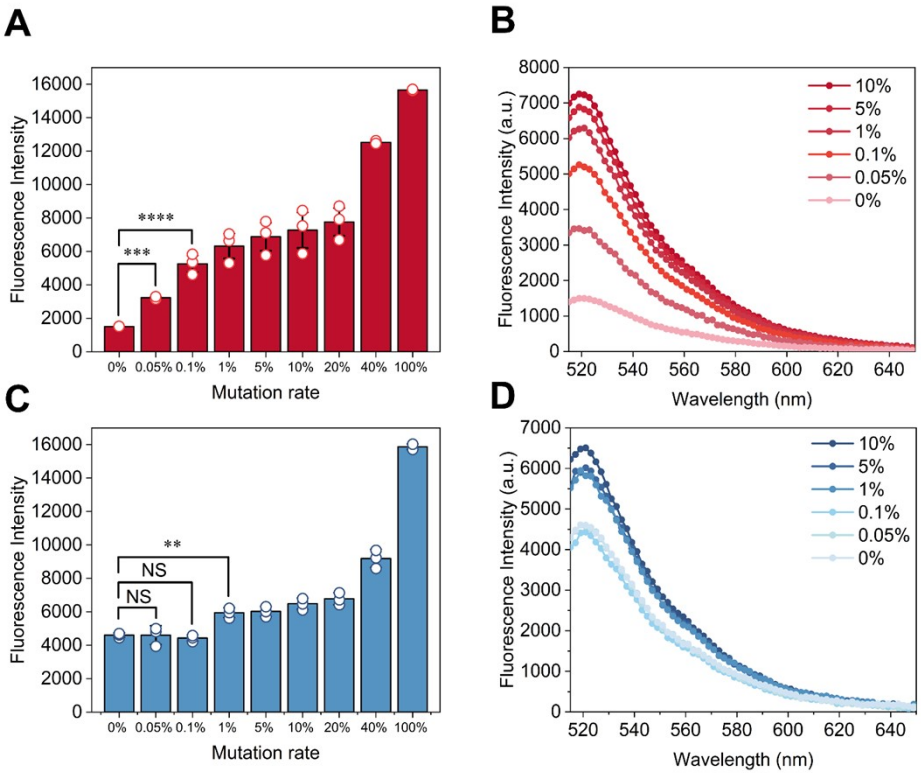

Fig. S5. Comparison of the sensitivity on SNM detection between the PARPA assay and the CRISPR-based detection. The testing samples were prepared by blending the WT template with the MUT template at various ratios, with the MUT cyp51 gene made up 0.05, 0.1, 1, 10, 20, 40, and 100 % of the total, respectively. (A) and (B) Fluorescence analysis using the PARPA assay. (C) and (D) Fluorescence analysis using the CRISPR-based assay. Two-tailed unpaired Student's t-test was carried out: **P < 0.01, ***P < 0.001, ****P < 0.0001 and NS: P > 0.05.

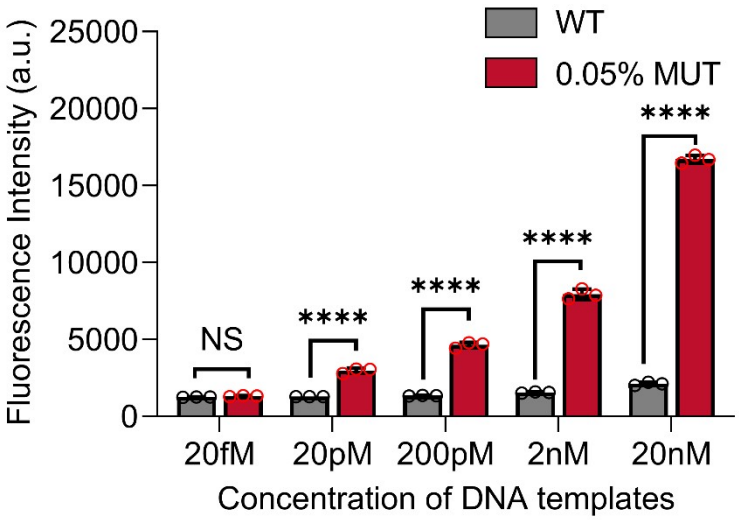

Fig. S6. Detection performance of the PARPA assay to detect 0.05% single-nucleotide mutations across varying DNA concentrations. Two-tailed unpaired Student's t-test was carried out: *P < 0.05, ****P < 0.0001.

References

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