Supporting Information

Multiple SNPs detection for antimalarial pyrimethamine resistance via allele-specific PCR coupled with gold nanoparticles-based lateral flow biosensor

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Table S1 Unlabelled primer candidates utilized for single nuclear polymorphisms detection in *pfdhfr* gene.

Name		Sequences and modifications (5' to 3')	Description
Pfdhfr-N51I_Rev		GCTTTCCCAGCTTGTTCTTCCC	Common primer
Pfdhfr-N51_Fwd1_WT	W	AGGAGTATTACCATGGAAATGT*A*A	Allele specific primer
Pfdhfr-51I_Fwd1_Mut	M	AGGAGTATTACCATGGAAATGT*A*T	
Pfdhfr-59_108_ Fwd		GATGGAACAAGTCTGCGACGT	Common primer
Pfdhfr-C59_Rev1_WT	W1	TCATTCACATATGTTGTAACTGCAC*A	Allele specific primer
Pfdhfr-C59_Rev2_WT	W2	TCATTCACATATGTTGTAACTGC**T**C*A	
Pfdhfr-C59_Rev3_WT	W3	TCATTCACATATGTTGTAACTGCA**A***A	
Pfdhfr-C59_Rev4_WT	W4	TCATTCACATATGTTGTAACTGC**T***C*A	
Pfdhfr-59R_Rev1_Mut	M1	TCATTCACATATGTTGTAACTGCAC*G	
Pfdhfr-59R_Rev2_Mut	M2	TCATTCACATATGTTGTAACTGCA**A***G	
Pfdhfr-59R_Rev3_Mut	M3	TCATTCACATATGTTGTAACTGCA***A***G	
Pfdhfr-59R_Rev4_Mut	M4	TCATTCACATATGTTGTAACTGC**T***C*G	
Pfdhfr-59R_Rev5_Mut	M5	TCATTCACATATGTTGTAACTGC**G***C*G	
Pfdhfr-59R_Rev6_Mut	M6	TCATTCACATATGTTGTAACTGC**C***C*G	
Pfdhfr-S108_Rev1_WT	W1	TTTTGGAATGCTTTCCCAG*C	Allele specific primer
Pfdhfr-S108_Rev2_WT	W2	TTTTGGAATGCTTTCCC**T**G*C	
Pfdhfr-S108_Rev3_WT	W3	TTTTGGAATGCTTTCCC**T***G*C	
Pfdhfr-108N_Rev1_Mut	M1	TTTTGGAATGCTTTCCCAG*T	
Pfdhfr-108N_Rev2_Mut	M2	TTTTGGAATGCTTTCCC**T**G*T	
Pfdhfr-108N_Rev3_Mut	M3	TTTTGGAATGCTTTCCC**T***G*T	

Note: Artificial mismatches are in bold. * indicates the location of phosphorothiate modification. Fwd and Rev stand for forward and reverse, respectively. WT and Mut represent wild type and mutation, respectively. The same common primer utilized when used in allele-specific amplification C59R and S108N of Pfdhfr.

Table S2 Comparison between Nested PCR with sequencing and AS-PCR-LFA for single nuclear polymorphisms detection in *pfdhfr* gene with clinical isolates of *Plasmodium falciparum*.

Group	Sample No.	Nested PCR with sequencing			AS-PCR-LFA		
		51	59	108	51	59	108
Low	WK-Pf-21	I	R	S	I	R	S
	WK-Pf-22	I	R	S	I	R	S
	WK-Pf-42	I	R	S	I	R	S
	WK-Pf-43	I	R	S	Neg	Neg	Neg
	WK-Pf-47	I	R	S	I	R	S
	WK-Pf-56	I	R	S	I	R	S
	WK-Pf-75	N	R	S	N	R	S
	WK-Pf-83	I	R	S	I	R	S
	WK-Pf-107	I	R	S	I	R	S
	WK-Pf-111	I	R	S	I	R	S
	WK-Pf-115	I	R	S	I	R	S
	WK-Pf-129	I	R	S	I	R	S
	WK-Pf-134	I	R	S	I	R	S
	WK-Pf-151	I	R	S	I	R	S
	WK-Pf-165	I	R	S	I	R	S
	WK-Pf-166	I	R	S	I	R	S
	WK-Pf-176	I	R	S	I	R	S
	WK-Pf-185	I	R	S	I	R	S
	WK-Pf-195	I	R	S	I	R	S
	WK-Pf-202	I	R	S	I	R	S
	WK-Pf-218	I	R	S	I	R	S
	WK-Pf-219	I	R	S	Neg	Neg	Neg
	WK-Pf-228	I	R	S	I	R	S
Middle	WK-Pf-8	I	R	S	I	R	S
	WK-Pf-10	I	R	S	I	R	S
	WK-Pf-17	I	R	S	I	R	S
	WK-Pf-29	I	R	S	I	R	S
	WK-Pf-32	I	R	S	I	R	S
	WK-Pf-34	I	R	S	I	R	S
	WK-Pf-41	I	R	S	I	R	S
	WK-Pf-44	I	R	S	I	R	S
	WK-Pf-53	I	R	S	I	R	S
	WK-Pf-57	I	R	S	I	R	S
	WK-Pf-90	I	R	S	I	R	S
	WK-Pf-95	I	C/R	S	I	C/R	S
	WK-Pf-106	I	R	S	I	R	S

	WK-Pf-108	I	R	S	I	R	S
	WK-Pf-114	I	R	S	I	R	S
	WK-Pf-120	I	R	S	I	R	S
	WK-Pf-128	I	R	S	I	R	S
	WK-Pf-135	N	R	S	N	R	S
	WK-Pf-148	N	C	N	N	C	N
	WK-Pf-162	I	R	S	I	R	S
	WK-Pf-169	I	R	S	I	R	S
	WK-Pf-178	I	R	S	I	R	S
	WK-Pf-181	N	R	S	N	R	S
	WK-Pf-182	I	R	S	I	R	S
	WK-Pf-183	I	R	S	I	R	S
	WK-Pf-193	I	R	S	I	R	S
	WK-Pf-196	I	R	S	I	R	S
	WK-Pf-223	N	C	N	N	C	N
	WK-Pf-5	N/I	R	S	N/I	R	S
	WK-Pf-33	I	R	S	I	C/R	S
	WK-Pf-65	I	R	S	I	R	S
	WK-Pf-92	I	R	S	I	R	S
	WK-Pf-104	I	R	S	I	R	S
	WK-Pf-108	I	R	S	I	R	S
	WK-Pf-126	I	R	S	I	R	S
	WK-Pf-156	I	R	S	I	R	S
	WK-Pf-175	I	R	S	I	R	S
	WK-Pf-7	I	R	S	I	R	S
	WK-Pf-9	I	R	S	I	R	S
	WK-Pf-11	I	R	S	I	R	S
	WK-Pf-12	I	R	S	I	R	S
	WK-Pf-14	N	R	S	N	R	S
	WK-Pf-18	I	R	S	I	R	S
	WK-Pf-25	I	R	S	I	R	S
	WK-Pf-28	I	R	S	I	R	S
	WK-Pf-31	I	R	S	I	R	S
High	WK-Pf-36	I	R	S	I	R	S
	WK-Pf-38	I	R	S	I	R	S
	WK-Pf-39	I	C/R	S	I	C/R	S
	WK-Pf-45	I	T	S	I	T	S
	WK-Pf-48	I	R	S	I	R	S
	WK-Pf-52	I	C/R	S	I	C/R	S
	WK-Pf-59	I	C/R	S	I	C/R	S
	WK-Pf-70	I	R	S	I	C/R	S
	WK-Pf-76	I	R	S	I	R	S
	WK-Pf-77	I	R	S	I	R	S

WK-Pf-87	I	R	S	I	R	S
WK-Pf-91	I	R	S	I	R	S
WK-Pf-93	I	R	S	I	R	S
WK-Pf-94	I	R	S	I	R	S
WK-Pf-97	I	R	S	I	R	S
WK-Pf-98	I	R	S	I	R	S
WK-Pf-99	I	R	S	I	R	S
WK-Pf-101	I	R	S	I	R	S
WK-Pf-102	I	R	S	I	R	S
WK-Pf-121	I	R	S	I	R	S
WK-Pf-124	I	R	S	I	R	S
WK-Pf-189	I	R	S	I	R	S
WK-Pf-192	I	R	S	I	R	S
WK-Pf-199	I	R	S	I	R	S
WK-Pf-211	I	R	S	I	R	S
WK-Pf-226	I	C	S	I	C	S
WK-Pf-230	I	R	S	I	R	S
WK-Pf-231	N	C	N	N	C	N
WK-Pf-233	N	C	N	N	C	N

Note: Neg indicate negative.

Table S3 Methodological comparison of AS-PCR-LFA and nested PCR with sequencing in different parasitemia.

Method			AS-PCR-LFA				Sensitivity (%)	Specificity (%)	False negative (%)	False positive (%)
	Genotype	Parasitemia (parasites/μl)	No.	WT	Mut	Mix				
Nested PCR with sequencing	N51I	Low (≤1,000)	23	1	20	0	91.30	100	8.70	0
		Middle (1,001-9,999)	37	4	32	1	100	100	0	0
		High (≥10,000)	38	3	35	0	100	100	0	0
		Subtotal	98	8	87	1	97.96	100	2.04	0
	C59R	Low (≤1,000)	23	0	21	0	91.30	100	8.70	0
		Middle (1,001-9,999)	37	2	33	1	100	97.30	0	2.70
		High (≥10,000)	38	4	30	3	100	97.37	0	2.63
		Subtotal	98	6	84	4	97.96	95.92	2.04	4.08
	S108N	Low (≤1,000)	23	0	21	0	91.30	100	8.70	0
		Middle (1,001-9,999)	37	2	35	0	100	100	0	0
		High (≥10,000)	38	2	36	0	100	100	0	0
		Subtotal	98	4	92	0	97.96	100	2.04	0

Note: WT, Mut and Mix represent wild type, mutation and mixed type respectively.

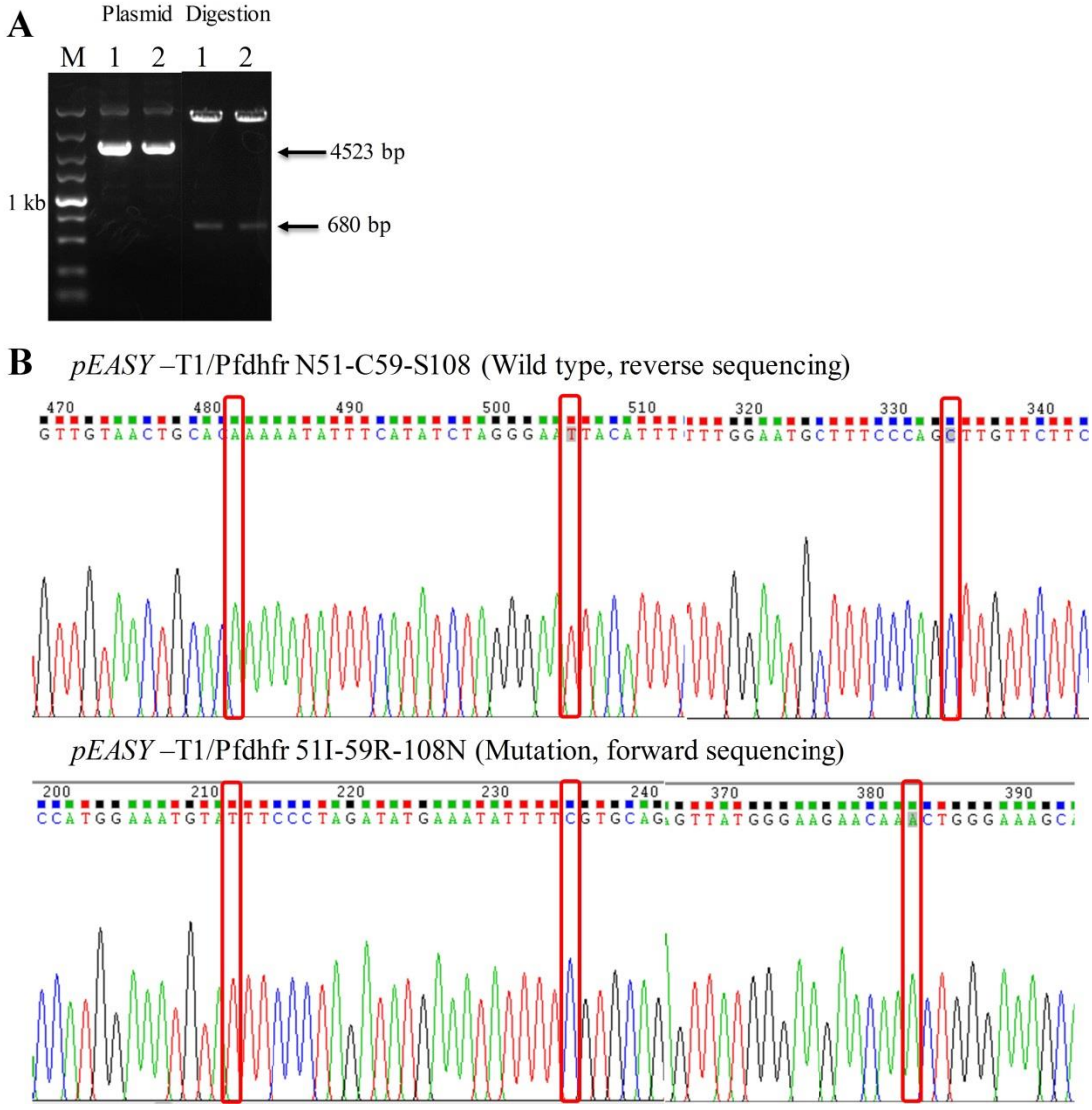

Figure S1 Recombinant plasmids construction and identification. (A) Double digestion with restriction endonuclease *Bam*H I and *Xho* I. M represent DL5000 DNA ladder (100 bp, 250 bp, 500 bp, 750 bp, 1 kb, 2 kb, 3 kb, 4 kb and 5 kb). 1, 2, represent recombinant plasmid of *pEASY*-T1/Pfdhfr N51-C59-S108 (wild type), *pEASY*-T1/Pfdhfr 51I-59R-108N (mutation), respectively. (B) DNA sequencing.

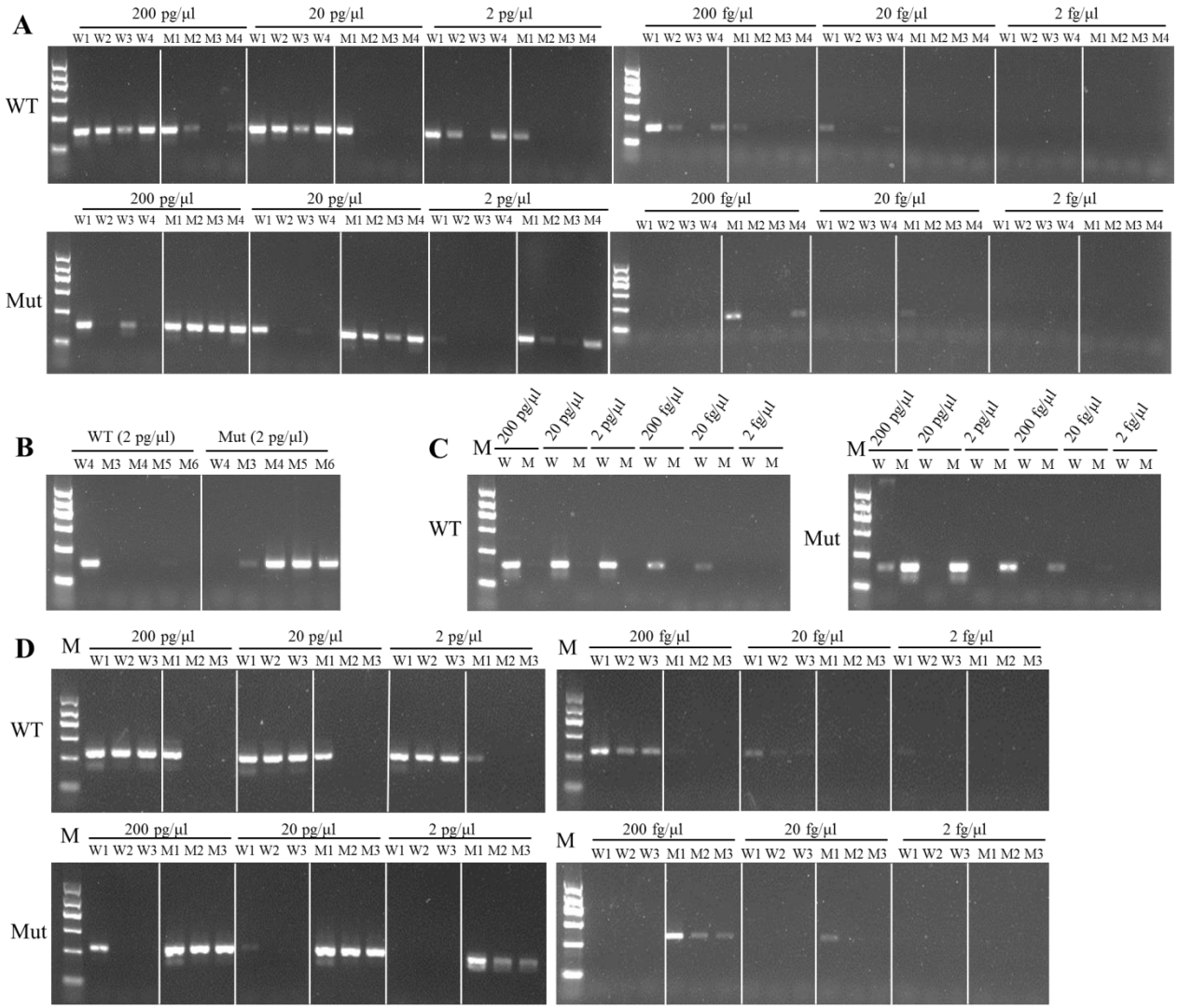

FIG S2 Primer screening for single nucleotide polymorphisms (SNPs) detection in *pfdhfr* gene. (A) The distinguishing primer for wild type and mutations at positions C59R of Pfdhfr; (B) Confirmation of mismatch position and base type for allele-specific primer (take Pfdhfr C59R as an example) provided by the agarose gel electrophoresis; (C) The distinguishing primer for wild type and mutations at positions N51I of Pfdhfr; (D) The distinguishing primer for wild type and mutations at positions S108N of Pfdhfr. WT and MUT represent wild type template and mutation template, respectively. For the final concentrations of serial dilutions of plasmid p*EASY*-T1/Pfdhfr in the PCR reaction system (20 μl), there were from 200 pg/μl to 2 fg/μl.

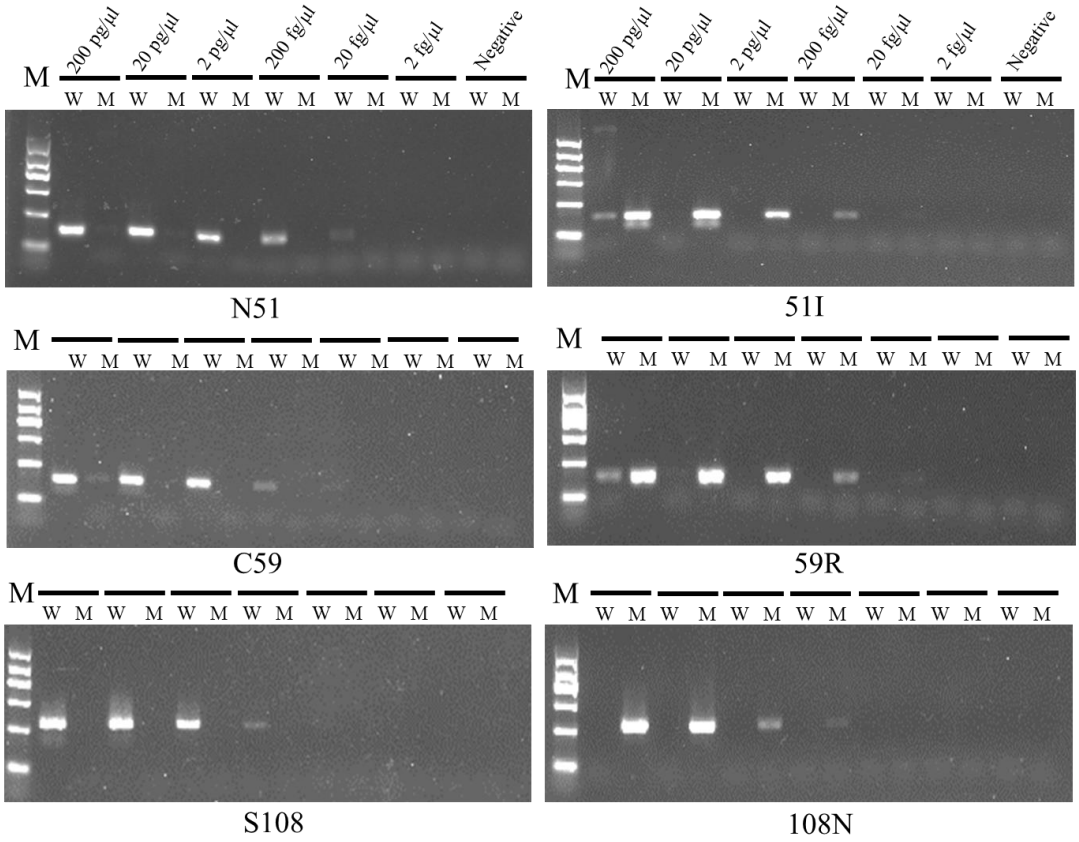

FIG S3 Sensitivity and specificity evaluation of selected primers for *pfdhfr* gene at different concentrations of plasmid by agarose gel electrophoresis. W and M on the sample lanes represent wild type and mutation primer, respectively. For the final concentrations of serial dilutions of plasmid p*EASY*-T1/Pfdhfr in the PCR reaction system (20 μl), there were from 200 pg/μl to 2 fg/μl.

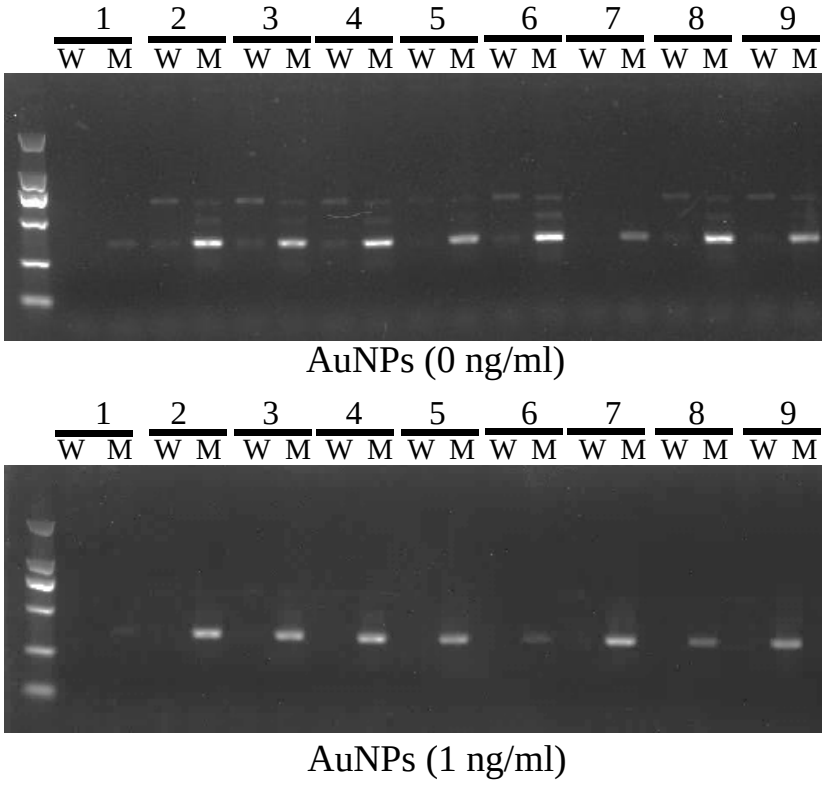

Figure S4 Agarose gel electrophoresis showing the AuNPs-enhanced allele-specific PCR strategy. Note: The 1-9 represent blood samples from falciparum malaria patients; W, M stands for optimized primer Pfdhfr-S108_Rev3_WT and Pfdhfr-108N_Rev3_Mut, respectively. W and M on the sample lane respresent wild type and mutation, respectively.

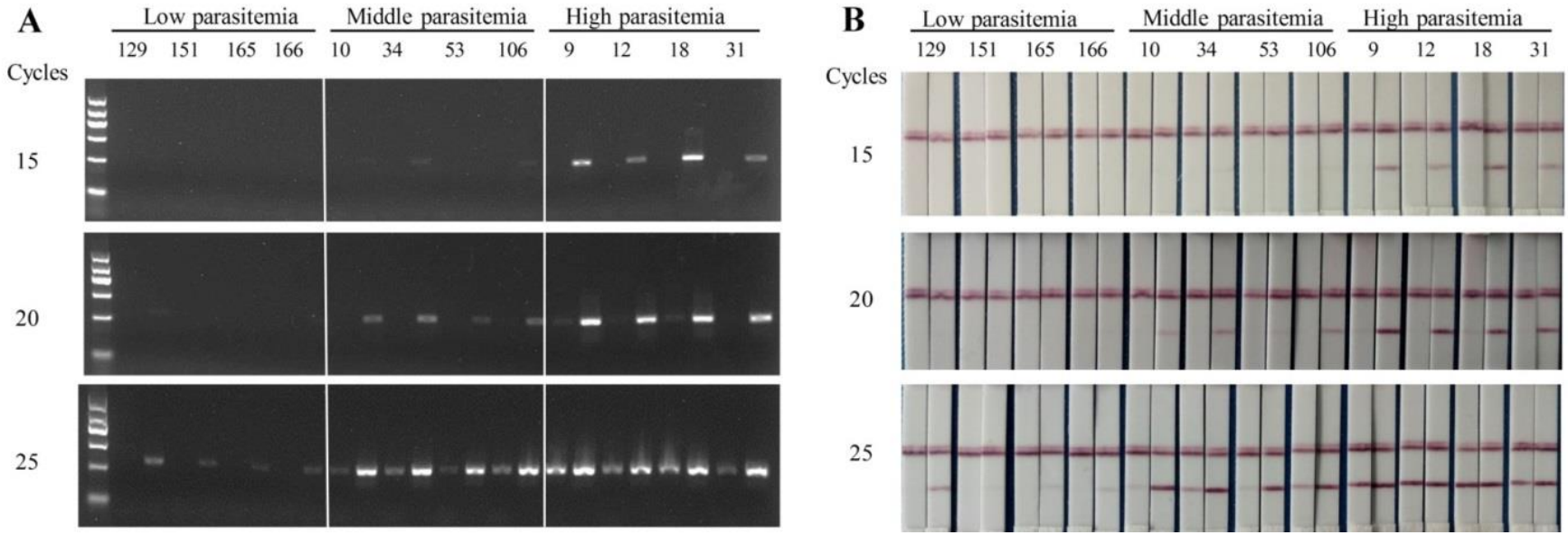

Figure S5. Cycle confirmation for AS-PCR at different parasitemia by Pfdhfr S108N. (A) Agarose gel electrophoresis, (B) The AS-PCR-LFA system through visualized interpretation.