A Droplet Microfluidics Platform for Highly Sensitive and Quantitative Detection of Malaria Causing *Plasmodium* Parasites Based on Enzyme Activity Measurement

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Supplementary Information

S1

Recombinant hTopI and pTopI were purified to homogeneity essentially as described in[28]. The resulting protein fractions were analyzed in SDS-PAGE and visualized by Coomassie stain for purity and Western-blotting using a poly-clonal anti-TopI antibody for identification. The DNA recognition potentials of the two enzymes were compared by incubating each of them with 5´-end P^{32}-labelled double-stranded DNA fragments (OL37/OL56 or OL62/OL63) as described in the Methods section below. To allow detection of cleavage, the anti-cancer drug camptothecin (CPT), which specifically inhibits the religation step of TopI catalysis was added to the reaction mixtures. The religation reaction could be observed by omitting CPT from the reaction. The result of this analysis demonstrated that pTopI recognizes and cleaves the sites cleaved by hTopI. However, in addition pTopI is capable of cleaving double-stranded DNA a few bases upstream to a 3´-end of the substrate followed by ligation of a protruding 5´-end, which hTopI is not.

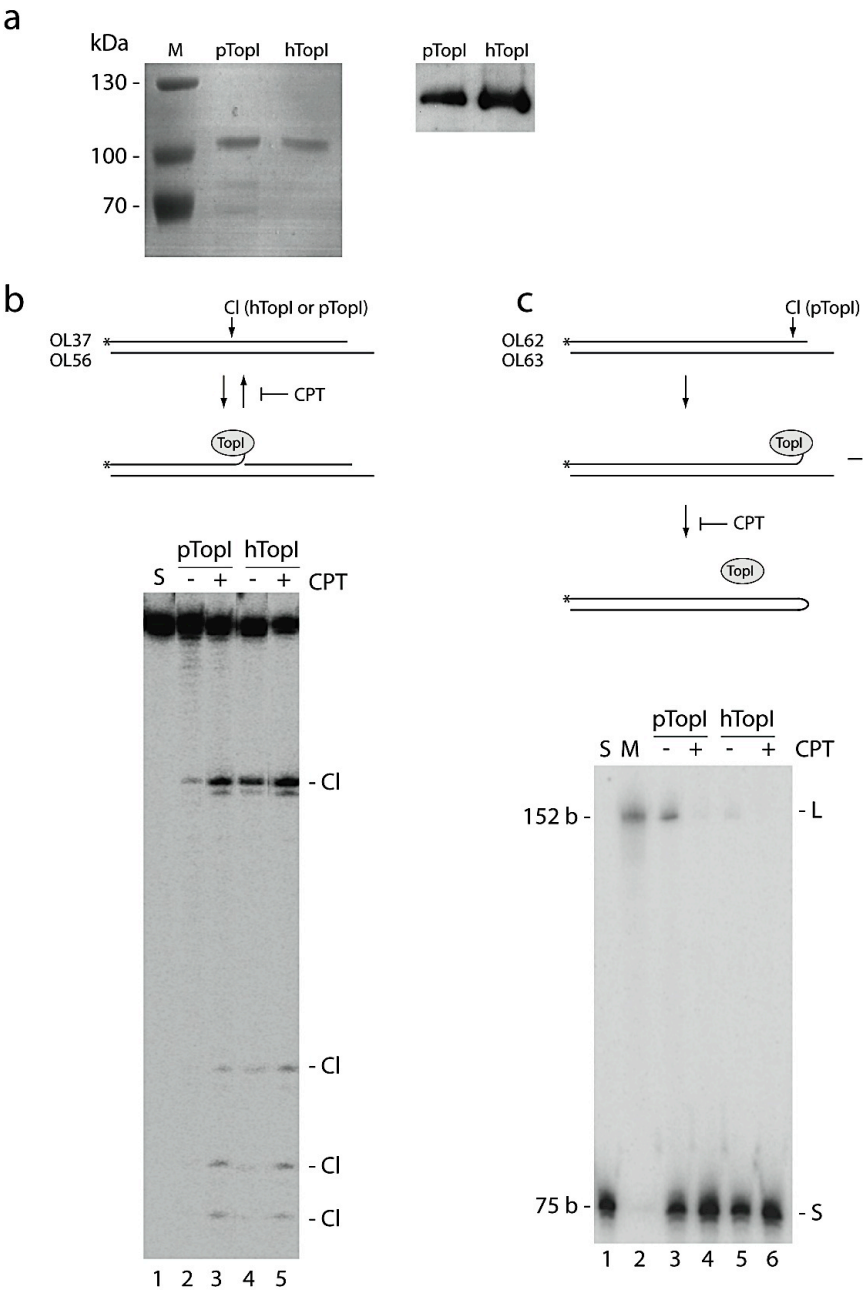

Figure S1. Comparison of DNA recognition by pTopI and hTopI. **a,** left panel; shows the result of analyzing purifed pTopI or hTopI by SDS-PAGE followed by coomassie stain. Lane 1, size marker with sizes of specific bands indicated to the left of the figure. Right panel, same as left panel except that the bands corresponding to pTopI or hTopI were visualized by Western blotting using a poly-clonal anti-TopI antibody. **b**, top panel, schematic illustration of cleavage-ligation reactions shared by pTopI and hTopI (an example of a cleavage site is indicated by an arrow marked Cl). Bottom panel, shows the result of incubating either pTopI or hTopI with an end-labelled double-stranded DNA fragment (OL37/OL56) in the absence or presence of CPT followed by denaturing gel-electrophoretic analysis of the results. Note that CPT inhibits the religation reaction of nuclear typeIB topoisomerases leading to accumulation of cleavage complexes. The radioactive reaction products were visualized by Phosphor-Imaging. Bands representing the most pronounced cleavage products generated by both pTopI and hTopI are indicated with Cl to the right of the gel picture. **c**, top panel, schematic illustration of the cleavage-ligation reaction mediated by pTopI but not hTopI at the end of a double-stranded DNA fragment having a protruding 5´-OH end. The pTopI cleavage site is indicated by an arrow marked Cl. Note that CPT inhibits the ligation reaction as indicated. Bottom panel, shows the result of incubating pTopI or hTopI with the end-labelled DNA substrate (OL62/OL63) in the absence or presence of CPT as indicated on the figure. A ligation product is only observed upon incubation of the substrate with pTopI in the absence of CPT (lane 3). The mobility of this product correspond to 152 bases, which in turn corresponds to pTopI mediated cleavage 3 bases upstream to the 3´-end of OL62 of the substrate followed by ligation of the protruding 5´-OH end of the non-cleaved strand (OL63). The cleavage product itself could not be observed directly (lane 4) due to a gel-electrophoretic mobility very close to the substrate band. This is caused by a trypsin-resistant peptide that remains bound to the cleavage product leading to a slight gel-electrophoretic retardation of this product. Hence, cleavage products, arising from cleavage a few bases upstream to the 3´-end of the 75-mer (OL62), are hidden by the substrate band. CPT, camptothecin; Cl, cleavage product; L, ligation product; S, substrate control; M, size marker. The sizes of the marker bands are indicated to the left of the gel-pictures.

S2

Based on the different cleavage patterns for pTopI and hTopI reported in Supplementary Fig. 1 and summarized in "part **a**" below, five different oligonucleotides (S1-S5) (part **b**) with potential of being circularized specifically by pTopI cleavage-ligation were designed. These oligonucleotides all folded into a hairpin structure containing a probe- and a primer-annealing sequence in the single-stranded loop and a potential pTopI recognizable sequence at the end of the double-stranded stem region. The ability of S1-S5 to be circularized by pTopI or hTopI was tested in the REEAD setup by incubation one at a time with each of the purified enzymes, followed by solid-support RCA of closed circles according to the basic protocol described in[12]. RCPs were visualized microscopically at the single-molecule level upon hybridization of fluorescent probes. As a positive control for the performance of the procedure single-stranded DNA control-circles with a unique probe-annealing sequence was added to the reaction mixtures before RCA. Analysis of the circularization efficiency of S1-S5 by pTopI in terms of the frequency of S1, S2, S3, S4 or S5 specific signals relative to control-circle specific signals revealed that S1 was the most efficient substrate for pTopI (part **c**). None of the oligonucleotides S1-S5 were circularized by hTopI (data not shown).

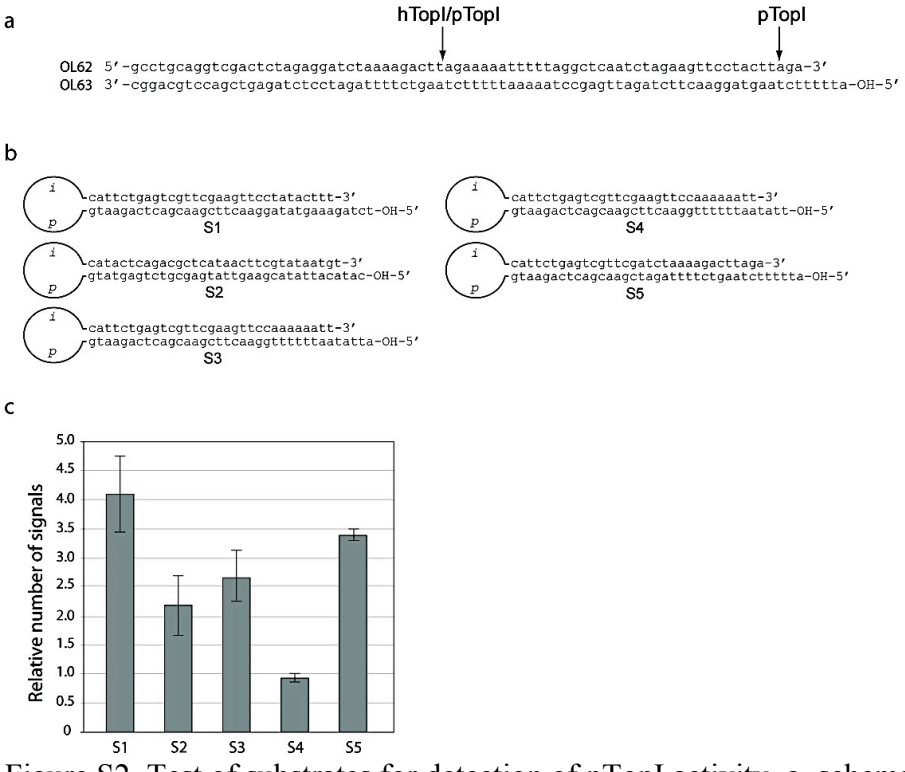

Figure S2. Test of substrates for detection of pTopI activity. **a**, schematic illustration of hTopI and pTopI cleavage sites on a double-stranded DNA fragment. Cleavage sites are indicated by an arrow. Cleavage site shared between hTopI and pTopI is indicated by "hTopI/pTopI", while the cleavage site that was specific for pTopI is indicated by "pTopI". **b**, schematic illustration of REEAD substrates (S1-S5) tested for reactivity with pTopI. Each potential substrate folds into a hairpin structure. The single-stranded loop region contains a *p*-sequence matching a primer used to template RCA and an *i*-sequence allowing annealing of a specific fluorescent probe to generated RCPs. The double-stranded stems of S1-S5 contain different nucleotide sequences matching the degenerate consensus recognition sequence of nuclear typeIB topoisomerases. **c**, quantitative depiction of the results obtained when incubating S1-S5 one at a time with purified pTopI followed by RCA and microscopic visualization of RCPs. The number of signals corresponding to individual RCPs originating from circularized S1-S5 or added control-circle were counted in 12 microscopic views of each experiment. The

bar chart shows the number of S1-S5 specific signals divided by the number of control-circle specific signals counted in three individual experiments.

S3

patient no	Place of col-lection	Date of collec-tion	Type of sample	Treat-ment	Species	Parasites/μL
#1	Denmark	11.03.26	frozen blood	No	*P. falciparum*	55,000
#2	Denmark	12.04.07	frozen blood and saliva	No	*P. falciparum*	6,793
#3	Denmark	11.11.10	frozen blood and saliva	No	*P. falciparum*	14,899
#4	Denmark	11.11.07	frozen blood and saliva	No	*P. falciparum*	9,466
#5	Denmark	11.09.16	frozen blood and saliva	No	*P. vivax*	10,000

Table S3. Overview of samples from patient #1-#5. These samples were collected in Denmark from patients returning with malaria from endemic areas and stored as frozen blood and/or saliva samples. Giemsa stained thick and thin blood films were examined by microscopy. Negative samples used as a reference in all analyses were blood and/or saliva samples from uninfected individuals (#6-#16) collected in Denmark (during the period September-October, 2011) and stored as frozen blood or saliva. They were confirmed *Plasmodium* negative using PCR as described in[31] (data not shown).

S4

The performance of a test based on the REEAD-on-a-Chip protocol under field conditions was characterized using an experiment including blood samples of 20 infected patients from different endemic areas and a reference panel of blood samples from 11 uninfected patients (one sample per patient). The samples of this trial were analyzed using the REEAD-on-a-Chip combined with fluorescent-microscopy inspection of slides (one slide per sample). The total number of pTopI-signals/slide was determined for each of the 11 uninfected reference samples. Only few signals were observed in the 11 uninfected reference samples (mean=4 signals/slide). No significant differences between the expected numbers of signals of those samples were detected (p-value 0.9045, n=31, likelihood-ratio-test for Poisson generalized linear models[20]). The process of counting the total number of pTopI-signals/slide of an infected sample is time consuming with the current proof-of-concept implementation of the protocol. Therefore, a sequential analysis technique[32] was applied to determine whether an infected sample tested positive: first a significant level $\alpha = 10-6$ was prefixed and then a detection-threshold was calculated using the quantiles of a Poisson distribution with mean 4 (same as the mean of the uninfected samples), yielding a detection-threshold of 17 and the stopping-rule "if at least 17 pTopI-signals are found in a slide, then the counting process stops and the respective sample is declared to be positive; otherwise the sample is declared to be negative". Since all the samples tested positive with the REEAD-on-a-Chip protocol, our results are equivalent to obtain a p-value of at most $10-6$ for each sample (which becomes 1.9×10^{-5} after a Bonferroni correction for multiple testing).

Patient no	Treat-ment	Parasite	Parasitemia(%)	Parasites/μL	Thick smear	PCR	RDT	QBC	REEAD
#17	Q	*P. falciparum*	0.4	20,000	+	+	+	+	+
#18	No	*P.falciparum*	<0.01	<500 <125*	Gametocytes	+	+	+	+
#19	AL	*P.falciparum*	0.8	40,000	+	+	+	+	+
#20	Q	*P.falciparum*	0.5	25,000	+	+	+	+	+
#21	Q	*P.ovale* + *P. falciparum*	0.08	4,000 400*	+	+	+	+	+
#22	Q	*P.falciparum*	5.5	275,000	+	+	+	+	+
#23	ATVPG	*P.falciparum*	0.1	5,000	+	+	+	+	+
#24	Q	*P.falciparum*	3	150,000	+	+	+	+	+
#25	Q	*P.falciparum*	0.36	18,000 9,000*	+	+	nd	nd	+
#26	Q	*P.falciparum*	<0.01	<500 <250*	+	+	+	+	+
#27	Q	*P.vivax*	0.1	5,000	+	+	nd	nd	+
#28	CQ	*P.vivax*	0.14	7,000 700*	+	+	+	+	+
#29	Q	*P.falciparum*	0.7	35,000 3,500*	+	+	+	+	+
#30	Q	*P.falciparum*	<0.01	<500 <50*	+	+	+	+	+
#31	CQ	*P.ovale*	0.8	40,000 4,000*	Gametocytes	+	+	+	+
#32	ATVPG	*P.falciparum*	0.1	5,000	+	+	+	+	+
#33	Q	*P.falciparum*	1.5	75,000	+	+	+	+	+
#34	Q	*P.falciparum*	nd	nd	nd	+	nd	nd	+
#35	MQ	*P.falciparum*	<0.01	<500	+	+	nd	nd	+
#36	NO	*P.falciparum*	<0.01	<500	Gametocytes	+	nd	nd	+

Table S4. Overview of samples from patients #17-#36. These samples were collected from patients infected in west Africa for *P. falciparum* and *P. ovale* cases, and India for *P. vivax* cases during the time period september-november, 2011 and stored as frozen blood samples. They were analyzed as indicated in the table prior to REEAD analyses using microscopical examination of Giemsa stained thick and thin blood smears, Quantitative buffy coat (QBC) fluorescent test, HRP2/LDH rapid diagnostic test (RDT), and real-time PCR using the Light Cycler system as previously described for species confirmation.[33] The whole panel of diagnostic methods could not be used for all patients for technical reasons, and some RDT and QBC were not determined (ND) for a few samples. Patients were treated after diagnosis and received either quinine (Q), artemether-lumefantrine (AL), atovaquone-proguanil (ATVPG), chloroquine (CQ) or mefloquine (MQ) according to clinical and biological status.
All samples tested positive (adjusted p-value of at most 1.9×10^{-5} for each sample) in a REEAD-on-a-Chip sequential analyses using a reference panel of blood samples from 11 uninfected patients (#6-#16, see S3) as described above.
*Note that samples #18, #21, #25, #26, #28-#31 were diluted in uninfected blood before REEAD-on-a-Chip analyses due to insufficient (less than 50 μL) volume of these samples. The parasite densities in the diluted samples are indicated by a star in the table.

S5

patient no	Place of collection	Date of collec-tion	Type of sample	Species	Parasites/ μL	PCR	REEAD	**p-values**
#37	Denmark	12.03.21	frozen blood	*P. malariae*	10,000	+	+	$1.0x10^{-16}$
#38	Malaysian Borneo	09.04.23	Dried blood on filter paper	*P. knowlesi*	528	+	+	0.00208

Table S5. Results of analyses of samples from patients #37 and #38. The blood sample from #37 was collected in Denmark (DK) and stored as frozen blood. Before REEAD this sample was analyzed by light-microscopy of Giemsa stained thick and thin blood films and species identity confirmed by real-time PCR using the Light Cycler system as previously described for species confirmation.[33] REEAD analysis of this sample was done using the REEAD–on-a-Chip setup and the results analyzed using a reference panel of blood samples from 11 uninfected patients as described in S4. The sample tested positive as shown in the table. The sample from patient #38 was collected in Hospital Sarikei, Malaysian Borneo, stored as dried blood on filter paper and kept at -20°C. Before REEAD, this sample was analyzed by Plasmodium species-specific nested PCR for *P. falciparum, P. vivax, P. malariae* and *P. knowlesi* and contained only *P. knowlesi* DNA.[34] REEAD analysis of this sample was done using a modified bulk extraction protocol (see the method section below) and REEAD-bulk circularization, since residues of the filter paper blocked the microfluidic chip and made analyses by REEAD-on-a-Chip difficult. A reference panel of blood samples from nine uninfected patients (#6-#14) were dried on filter paper and analyzed in a similar way. The sample tested positive as shown in the table. The p-values were obtained by likelihood-ratio-tests for Poisson generalized linear models[20] as described in Table S4.

Methods:

SDS PAGE and Western blotting: pTopI and hTopI were analyzed by electrophoresis on 10% SDS polyacrylamide gels and the proteins either stained with Coomassie brilliant blue following standard procedures or transferred to a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and 20% methanol. Western blotting was performed using standard procedures (primary antibody, polyclonal antibody to hTopI from Scleroderma Patient Serum (TopoGEN); secondary antibody, ImmunoPure Goat Anti-Human IgG-HRP (Thermo Scientific)).

Synthetic substrates for cleavage assays: All oligonucleotides were purchased from DNA Technology A/S and purified by denaturing polyacrylamide gel electrophoresis. The sequences of the oligonucleotides are as follows: OL37: 5'-CGAATTCGCT ATAATTCATA TGATAGCGGA TCCAAAAAAG ACTTAGAAAA AAAAAAAGCT TAAGCAA[35], OL56: 5'-TTGCTTAAGC TTTTTTTTTT TCTAAGTCTT TTTTGGATCC GCTATCATAT GAATTATAGC GAATTCG[35], OL62: 5'-GCCTGCAGGT CGACTCTAGA GGATCTAAAA GACTTAGAAA AATTTTTAGG CTCAATCTAG AAGTTCCTAC TTAGA, OL63: 5'- ATTTTTCTAA GTAGGAACTT CTAGATTGAG CCTAAAAATT TTTCTAAGTC TTTTAGATCC TCTAGAGTCG ACCTGCAGGC. The oligonucleotides representing the scissile strands (OL37 and OL62) were 5′-radiolabeled by T4 polynucleotide kinase (NEB) using [γ-^{32}P]ATP as the phosphoryl donor. The oligonucleotides were annealed pairwise with a 2-fold molar excess of the bottom strand over scissile strand as previously described.[36]

Detection of pTopI activity using radio-labeled DNA substrates: DNA cleavage reactions were carried out by incubating 20 nM duplex OL37/OL56 or OL62/OL63 with 500 fmol of pTopI or hTopI in the absence or presence of 60 μM CPT for 20 min at 37 °C in 10 mM Tris pH 7.5, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ in a final volume of 20 μl. After 20 min. incubation, reactions were stopped with 0.5% (w/v) SDS. Samples were subjected to ethanol precipitation, resuspended in 10 μl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis following standard procedures, and radioactive bands were visualized by Phosphor-Imaging.

Sequences of REEAD substrates, primers and probes

S(TopI): 5'-AGAAAAATTT TTAAAAAAAC TGTGAAGATC GCTTATTTTT TTAAAAATTT TTCTAAGTCT TTTAGATCCC TCAATGCTGC TGCTGTACTA CGATCTAAAA GACTTAGA[12].

S1: 5'-TCTAGAAAGT ATAGGAACTT CGAACGACTC AGAATGACTG TGAAGATCGC TTATCCTCA ATGCACATGT TTGGCTCCCA TTCTGAGTCG TTCGAAGTTC CTATACTTT.

S2: 5'-CATACATTAT ACGAAGTTAT GAGCGTCTGA GTATGACTGT GAAGATCGCT TATCAGTGAA TGCGAGTCCG TCTACTCATA CTCAGACGCT CATAACTTCG TATAATGT.

S3: 5'-ATTATAATTT TTTGGAACTT CGAACGACTC AGAATGACTG TGAAGATCGC TTATCCTCAA TGCACATGTT TGGCTCCCAT TCTGAGTCGT TCGAAGTTCC AAAAAATT.

S4: 5'-TTATAATTTT TTGGAACTTC GAACGACTCA GAATGACTGT GAAGATCGCT TATCCTCAAT GCACATGTTT GGCTCCCATT CTGAGTCGTT CGAAGTTCCA AAAAATT.

S5: 5'-ATTTTTCTAA GTCTTTTAGA TCGAACGACT CAGAATGACT GTGAAGATCG CTTATCCTCA ATGCACATGT TTGGCTCCCA TTCTGAGTCG TTCGATCTAA AAGACTTAGA.
Control-circle substrate: 5'-AGAAAAATTT TTAAAAAAAC TGTGAAGATC GCTTATTTTT TTAAAAATTT TTCTAAGTCT TTTAGATCCCGA GATGTACCGC TATCGTCATG ATCTAAAAGA CTT. Control-circle was prepared as described previously.[12]
RCA primer: 5'-AMINE-CCAACCAACC AACCAAATAA GCGATCTTCA CAGT.[12]
Fluorescent probes:
For detection of S(TopI): 5'-FITC-CCTCAATGCT GCTGCTGTAC TAC.[12]
For detection of S1-S5: 5'-rhodamine-CCTCAATGCA CATGTTTGGC TCC.
For detection of control-circle: 5'-FITC- CCGAGAT GTACCGCTAT CGT.
The fluorophores rhodamine or FITC were used for red or green fluorescence detection, respectively.

Bulk preparation of extracts from blood samples dried on filter paper: 8 mm in diameter of dried blood on filter paper (corresponding approximately to 8 μL of blood) was cut into small pieces and placed in the bottom of an eppendorf tube. 40 μL of ice-cold lysis buffer with extra EDTA (10 mM Tris-HCL pH 7.5, 5 mM EDTA, 1 mM DTT, 1 mM PMSF) was added to cover the blood stained filter paper pieces and this extraction mixture was incubated on ice for 16 hours while horizontally shaking. After extraction we followed the standard REEAD-bulk protocol.

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