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Specific Detection of Topoisomerase I from the Malaria Causing *P. falciparum* Parasite using Isothermal Rolling Circle Amplification

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***Abstract*- We present a Rolling-Circle-Enhance-Enzyme-Activity-Detection (REEAD) system with potential use for future point-of-care diagnosis of malaria. In the developed setup, specific detection of malaria parasites in crude blood samples is facilitated by the conversion of single *Plasmodium falciparum* topoisomerase I (pfTopI) mediated cleavage-ligation events, happening within nanometer dimensions, to micrometer-sized products readily detectable at the single molecule level in a fluorescence microscope. In principle, REEAD requires no special equipment and the readout is adaptable to simple colorimetric detection systems. Moreover, with regard to detection limit the presented setup is likely to outcompete standard gold immuno-based diagnostics. Hence, we believe the presented assay forms the basis for a new generation of easy-to-use diagnostic tools suitable for the malaria epidemic areas in developing countries.**

I. Introduction

By definition enzymes convert substrate molecules to products with changed chemical or physical characteristics without being affected by the process. Hence, at least theoretically one enzyme can create indefinite amounts of product provided with sufficient substrates and, consequently, the most sensitive detection of pathogens imaginable would rely on detection of species-specific enzymatic products. The challenge is that only few enzymatic products are readily detectable without the use of sophisticated equipment and even then most products can be detected only when produced in high numbers. For clinically relevant identification of pathogens based on species-specific enzymatic activities it is, therefore, necessary to develop new technologies.

We recently presented the so-called REEAD assay (Figure 1) allowing specific detection of human topoisomerase I (hTopI) and the mechanistically related Flp or Cre recombinase at the single cell level [1,2,3,4]. Enzymes from the TopI family all act by introducing single strand cuts in DNA followed by subsequent ligation of the generated nicks in a reaction that involves the formation of a covalent enzyme-DNA cleavage intermediate [5]. In REEAD this reaction is utilized to convert self-folding oligonucleotide substrates to closed DNA circles, which subsequently are subjected to Rolling Circle Amplification (RCA) leading to products (RCP) consisting of ~10^3 tandem repeats of a sequence complementary to the DNA circles. These RCPs are visualized at the single-molecule level in a fluorescence microscope by annealing to fluorescently labeled probes giving rise to one fluorescent spot for each RCP. Since the assay involves no thermal cycling, each RCP represents one closed DNA circle, which in turn derives from a single cleavage-ligation event. Alternatively RCPs can be coupled to Horse Radish Peroxidase (HRP) to allow colorimetric readout in terms of HRP conversion of specific substrates [6].

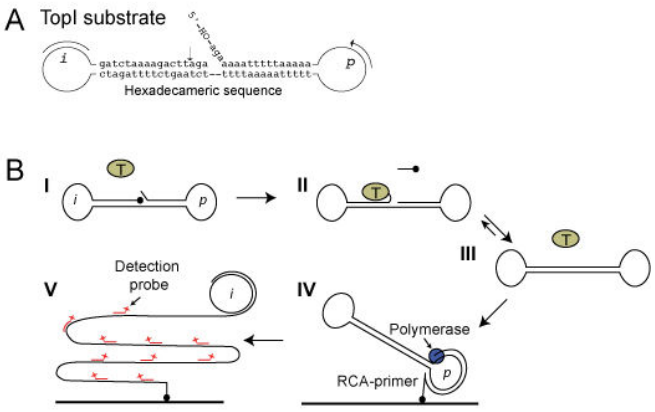

Figure 1. Schematic representation of the REEAD assay. A. The TopI DNA substrate folds into a dumbbell-shaped structure containing a double-stranded stem region with a preferred hexadecameric TopI recognition sequence. TopI cleavage site is indicated by an arrow. *i*: identifier element. *p*: primer binding sequence. B. Reaction mechanism and visualization of generated circular DNA products. I. Simplification of DNA substrate as in A. II. After TopI cleavage, the enzyme is covalently bound to the 3'-end of the DNA substrate. A tri-nucleotid is release and diffuses away, while the 5' overhang with a hydroxyl group necessary for ligation hybridizes to the double stranded region. III. TopI re-ligates thereby creating a closed DNA circle. IV. The substrates *p* region is hybridized to a 5'-amine linked primer attached to a glass surface, which allows polymerase-assisted RCA of the circularized substrates (for unreacted open substrates the reaction terminates at the strand interruption). V. Visualization is performed by hybridization of fluorescently labeled probes to the RCP region corresponding to the *i* element of the dumbbell-substrate. Yellow ellipses marked T represents TopI and blue circles represent Phi29 polymerase. The red asterisks represent fluorophores.

II. Materials and Methods

Cell Preparations and Nuclear Extracts

Human embryonic kidney HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100

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units/mL penicillin and 100 mg/mL streptomycin. Cells were incubated in a humidified incubator (5% CO_2/95% air atmosphere at 37 °C).

Cells were harvested with a cell scraper. Media was discarded and the cell lysed in 1 mL of Lysis buffer (0.1% NP-40, 10 mM Tris, pH 7.9, 10 mM $MgCl_2$, 15 mM NaCl, 0.1 mM phenylmethyl sulfonyl fluoride). After cell lysis the nuclei were extracted in 80 µL of 0.5 M NaCl, 20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM phenylmethyl sulfonyl fluoride. The amount of cells used for extract preparation was estimated prior to harvesting.

Recombinant enzymes

The yeast *Saccharomyces cerevisiae* Top1 null strain RS190 was a kind gift from R. Sternglanz (State University of New York). Plasmid for expression of recombinant full-length hTopI and pfTopI in yeast and purification of the expressed enzymes were described previously [7,8].

Cleavage/religation of hexadecameric sequence

Oligonucleotide CL25 (5'-GAAAAAAGACTTAGAAAA ATTTTTA-3') was 5'-end radiolabeled with [γ32P] ATP. The CP25 complementary strand (5'-TAAAAATTTT TCTAAGTCTTTTTTC-3') was phosphorylated at the 5'-end with unlabeled ATP. The two strands were annealed at a 2-fold molar excess of CP25 to CL25. DNA cleavage/equilibrium reactions were carried out by incubating 20 nM of radio labeled DNA substrate CL25/CP25 with 100 ng of hTopI or pfTopI in 15 µL cleavage/religation reaction buffer containing 20 mM Tris pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 µg/ml acetylated BSA and different NaCl concentration as stated in the text. Reactions were incubated for 30 min at 25 ºC, stopped by adding 0.5% SDS, and digested with trypsin after ethanol precipitation. Reaction products were resolved in 16% (v/v) acrylamide 7 M urea gels in TBE (48 mM Tris, 45.5 mM Boric Acid, 1 mM EDTA). Products were visualized by phosphorimaging.

Synthetic DNA Substrates, Probes, and Primers

Oligonucleotides for construction of the TopI substrate, the RCA primer (*p*), and the identification probes (*i*) were purchased from DNA Technology A/S, Aarhus, Denmark and synthesized on a model 394 DNA synthesizer from Applied Biosystems. The sequences of the oligonucleotides are identical to [2].

Rolling Circle Amplification

The previously presented single molecule TopI activity assay is based on enzyme-mediated circularization of synthetic DNA substrates, followed by isothermal signal enhancement *via* Phi29 DNA polymerase-driven RCA (MBI Fermentas) on a solid support (CodeLink Activated Slides from SurModics). The TopI reactions were carried out in a 10 µL reaction volume containing a divalent cation depletion buffer (5 mM Tris-HCl, pH 7.5 2 mM EDTA, 8 mM NaCl, 0.6% beta-mercaptoethanol, 10% PEG6000) supplemented with 100 nM of substrate. Reactions were initiated by the addition of one or more of the purified enzymes hTopI, pfTopI, or HEK293T nuclear extracts as stated in the text. Incubation was carried out for 30 min at 37 °C before inactivating the enzyme(s) for 5 min at 95 °C. Subsequently, hybridization to the covalently coupled primer was performed for 60 min at room temperature. Slides were washed for 1 min at room temperature in wash buffer 1 (0.1 M Tris-HCl, 150 mM NaCl, and 0.3% SDS) and for another 1 min at room temperature in wash buffer 2 (0.1 M Tris-HCl, 150 mM NaCl, and 0.05% Tween-20). Finally, the slides were dehydrated in 99.9% ethanol for 1 min and air-dried. Rolling circle DNA synthesis was performed for 45 min at 37 °C in 1X Phi29 buffer supplemented with 0.2 µg/µL BSA, 250 µM dNTP, and 6 units Phi29 DNA polymerase. The reactions were stopped by addition of wash buffers 1 and 2. The RCPs were detected by hybridization of 0.2 µM of each fluorescently labeled detection probe in a buffer containing 20% formamide, 2X SSC, and 5% glycerol for 30 min at 37 °C. The slides were washed in wash buffers 1 and 2, dehydrated, mounted with Vectashield (Vector Laboratories) and visualized as previously described [1].

III. Results and Discussion

Here we present the development of a REEAD assay for specific identification of the malaria causing parasite *P. falciparum* in crude clinical samples based on the specific detection of single pfTopI cleavage-ligation events. First a synthetic gene encoding pfTopI was cloned and the recombinant protein expressed in and purified from *Saccharomyces Cerevisiae* to allow characterization of the enzyme [7]. The ability of pfTopI to cleave the classical hexadecameric sequence known as a preferred cleavage site for nuclear TopI enzymes from other species was investigated using a synthetic 25-mer substrate (CL25/CP25). pfTopI cleaved this substrate between nucleotides -1 and +1, which is the preferred cleavage site for other nuclear TopI, including hTopI, as well as several addition sites located downstream of this position (Figure 2).

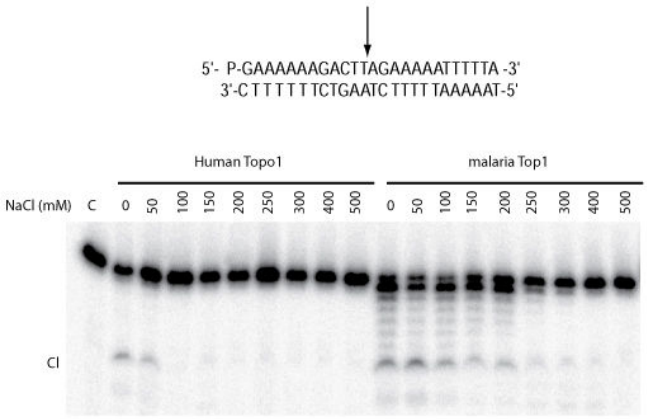

Figure 2. The effect of salt concentration on TopI DNA cleavage. hTopI or pfTopI was incubated with the 5´-radiolabeled DNA substrate (CL25/CP25) (top panel) at the indicated salt concentrations. The reactions were terminated by SDS and the products analyzed by denaturing PAGE. The lane marked C is a control without added enzyme. The main cleavage product is indicated by "Cl" in the figure.

Based on this result it was anticipated that single cleavage-ligation events mediated by pfTopI could be detected by REEAD using the classical dumbbell substrate originally developed to detect hTopI activity (Figure 1A). As demonstrated in Figure 3 this expectation held true.

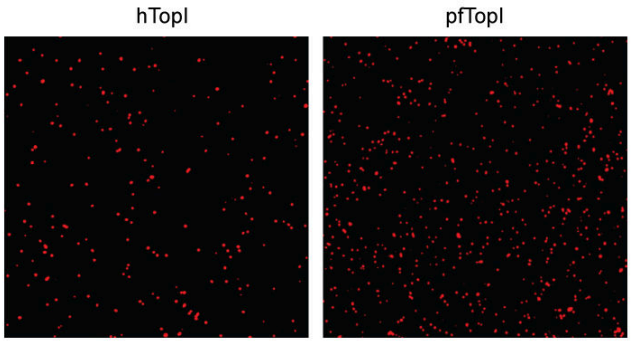

Figure 3. REEAD detection of purified hTopI or pfTopI. Left panel, microscopic view obtained by REEAD of hTopI using the TopI specific dumbbell substrate. Right panel, as left panel except that hTopI was replaced with pfTopI. Each red dot represents a single TopI cleavage-ligation event.

Since pfTopI recognizes and circularizes the same substrate as does hTopI and since all clinical samples contain hTopI, the challenge in using the same REEAD substrate for detection of pfTopI and hTopI relies on indentifying reaction conditions supporting the activity of pfTopI and not hTopI. As evident from Figure 2, pfTopI exhibits a higher salt optimum in a standard DNA cleavage assay than does hTopI. Therefore, to identify reaction conditions allowing the activity of pfTopI to be distinguished from that of hTopI, 10 units of purified pfTopI or hTopI were incubated with the dumbbell substrate in a standard reaction-buffer containing NaCl concentrations ranging from 0-500 mM (data not shown). The results demonstrate that whereas the activity of hTopI is completely abolished at NaCl concentrations above 300-400 mM pfTopI retains activity at 400 and 500 mM of NaCl. Hence, addition of NaCl to the reaction mixture may provide a simple tool for the specific detection of pfTopI in crude clinical samples containing vast numbers of human enzymes, including hTopI. Note that circularization of the DNA substrate, by enzyme activities other than TopI present in human cells e.g. ligases or DNA repair enzymes is prohibited by chelating divalent cations with EDTA, which is a component of the standard reaction buffer used here [1,2].

The ability to detect specifically the activity of pfTopI in a background of the human cell content simply by adding high NaCl concentrations and EDTA to the reaction buffer was confirmed by assaying substrate circularization in HEK293T cell extracts before or after addition of 10 units of hTopI or pfTopI. At high salt concentrations substrate circularization was only observed after addition of pfTopI to the cell extract, demonstrating the possibility of detecting specifically this enzyme activity even when present in cell extract (Figure 4).

The *P. falciparum* parasite is found primarily in blood of infected patients and, hence, blood samples are used for diagnosis of malaria by traditional means. We, therefore, investigated the ability of REEAD to detect malaria on the basis of pfTopI activity in the blood of individuals diagnosed with malaria by standard methods. As a negative control the blood of non-infected individuals were used. Representative results from these tests are shown in Figure 5. Consistent

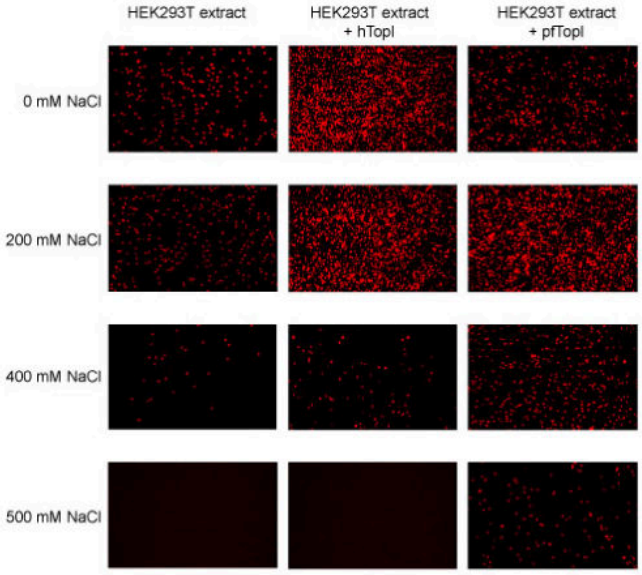

Figure 4. REEAD detection of naturally expressed hTopI, or spike-in hTopI or pfTopI added to crude extracts from HEK293T cells. The microscopic view of the results obtained by measuring hTopI activity by REEAD at increasing salt concentrations (indicated left of the figure) is depicted. Left panel, crude extracts from HEK293T cells. Middle panel, spike-in hTopI was added to the HEK293T cell extract before REEAD. Right panel, pfTopI was used as spike-in to the HEK293T cell extracts instead of hTopI. Each red dot represents a single TopI cleavage-ligation event.

with the ability of pfTopI but not hTopI to operate at high salt concentrations, signals were observed only when blood extracts prepared from malaria positive patients were used for circularization of the substrate at 400 mM NaCl. At lower salt concentrations, in which hTopI can support circularization of the substrate, signals were generated by extracts prepared from blood samples of both infected and non-infected individuals validating the quality of the utilized samples in terms of hTopI activity originating from nuclei containing blood cells.

IV. Conclusion

We demonstrate the specific detection of malaria in clinical relevant samples by visualizing single cleavage-ligation events mediated by pfTopI. This is achieved by the REEAD system in which each TopI catalytic reaction is converted to a micrometer-sized product visible at the single-molecule level. Since each pfTopI, at least in theory, can perform thousands of catalytic reactions without loosing activity, the detection limit of the presented REEAD assay is expected to outcompete current immunohistochemical based diagnostic tools and may even be improved to a level that allow diagnosis based on non-invasive samples such as mucus or saliva, which typically contain only a few parasites. In longer terms the microscopic readout used for the current initial experiments is expected replaced by

simple colorimetric readout as the presented HRP mediated conversion of TMB. This readout is suitable for at-place-of-care diagnosis even in third world countries currently suffering the major burden of malaria epidemics.

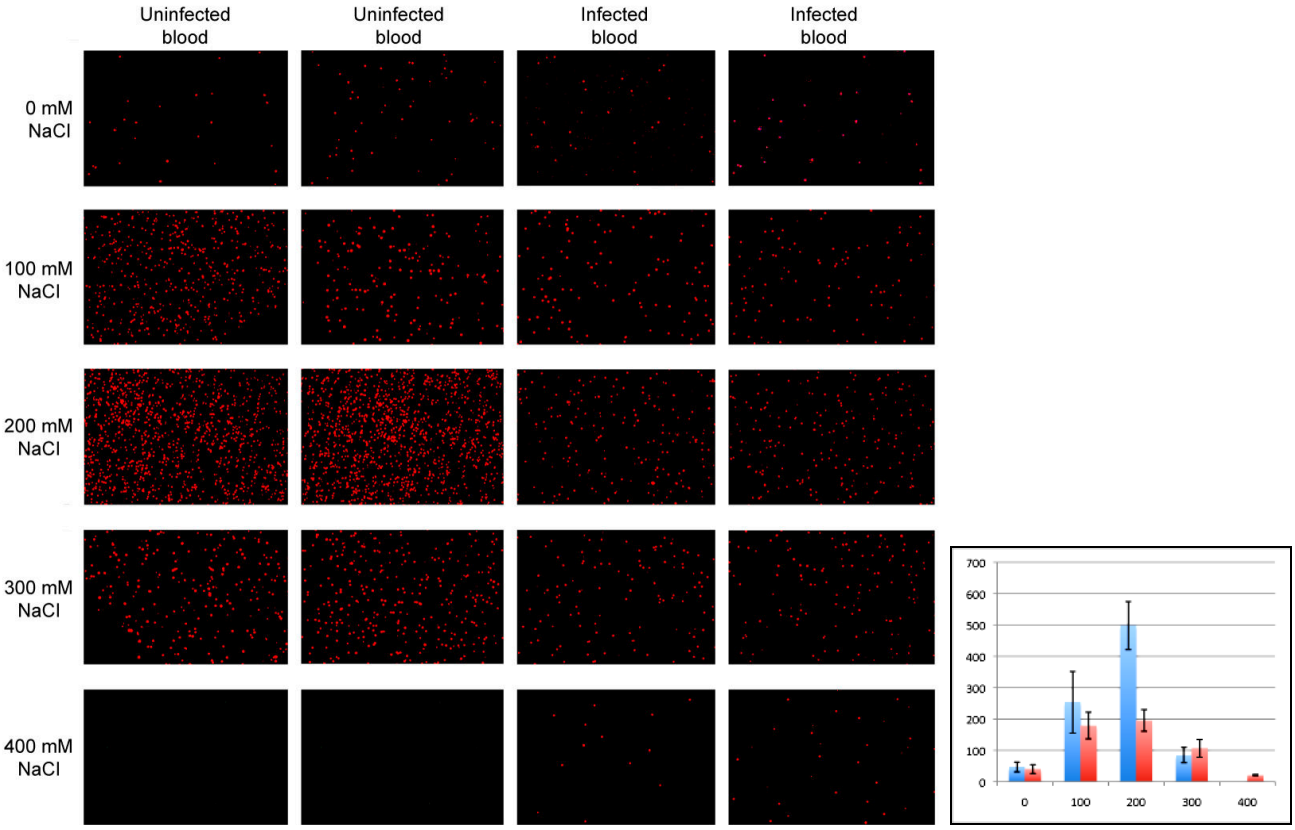

Figure 5. REEAD detection of TopI activities in extracts from uninfected or *P. falcifarum* infected blood samples. Picturese to the left. Panel 1 and 2 show the result of REEAD performed on extracts from uninfected blood at increasing salt concentrations (indicated to the left of the figures). Panel 3 and 4, same as panels 1 and 2 except that extract from *P. falciparum* infected blood was subjected to REEAD. Barchart to the right shows the results of quatifying the number of signals on five individual pictures of each sample. Blue and red bars show the number of singnals obtained using uninfected and infected blood samples, respectively. The Y-axis shows number of singnals and the salt-concentration at which each sample were analyzed is shown on the X-axis.

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