

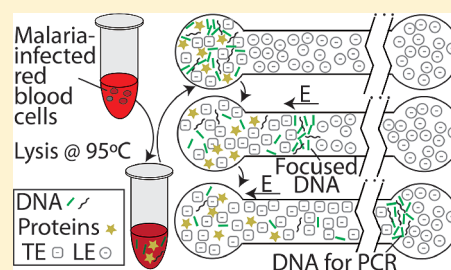
Extraction of DNA from Malaria-Infected Erythrocytes Using Isotachophoresis

Lewis A. Marshall,[†] Crystal M. Han,[‡] and Juan G. Santiago^{*,†}

[†]Departments of Chemical Engineering and [‡]Mechanical Engineering, Stanford University, Stanford, California, 94305

S Supporting Information

ABSTRACT: We demonstrate a technique for purification of nucleic acids from malaria parasites infecting human erythrocytes using isotachophoresis (ITP). We release nucleic acids from malaria-infected erythrocytes by lysing with heat and proteinase K for 10 min and immediately, thereafter, load sample onto a capillary device. We study the effect of temperature on lysis efficiency. We also implement pressure-driven counterflow during ITP extraction to extend focusing time and increase nucleic acid yield. We show that the purified genomic DNA samples are compatible with polymerase chain reaction (PCR) and demonstrate a clinically relevant limit of detection of 0.5 parasites per nanoliter using quantitative PCR.



Microfluidic platforms are an attractive alternative to benchtop solutions for diagnostic medical testing because they consume low sample and reagent volumes and offer the potential of integrating multiple assay steps. However, sample preparation in microfluidic devices is a continuing challenge because of the complexity and variety of biological samples and the low concentrations of target molecules.¹

Blood is an attractive sample for microfluidic analysis because it is collected routinely and contains information about the entire body.² However, blood contains a complex mixture of cells, proteins, and electrolytes, which can interfere with diagnostic tests. Extraction of DNA from erythrocytes for downstream polymerase chain reaction (PCR) is a particular challenge because of the abundance of hemoglobin, a PCR inhibitor.³

Microfluidic systems exist for preparation of nucleic acids from blood and other biological fluids.^{4,5} These systems typically seek to adapt benchtop-scale methods like solid phase extraction or magnetic bead purification.⁴ Such methods rely on specific channel geometries, porous structures, and/or surface chemistries and may require pumping and valves to implement wash steps. Some systems require external manipulations of magnets.

Isotachophoresis (ITP) offers an alternative to surface-based purification methods for nucleic extraction and purification. ITP-based DNA purification does not require surface chemistry-dependent DNA adsorption, rinses during extraction, or pumping of fluid streams. ITP purification is also weakly dependent on surface chemistry as it can be performed under conditions of strongly suppressed electroosmotic flow (which aids in reproducibility). ITP is a robust sample preparation method,^{6,7} can be highly selective,^{8,9} and can provide up to one million-fold preconcentration.¹⁰ We have used ITP for extraction of small RNA from cell cultures,¹¹ micro-RNA from total RNA,^{8,9} genomic DNA (gDNA) from whole blood,¹² and rRNA from bacteria in urine.⁷ With ITP, analytes are extracted and preconcentrated at the interface

between leading (LE) and trailing electrolytes (TE) of a two-buffer system. Selectivity is based on electrophoretic mobility. The strong electric field gradient at the TE-to-LE interface stabilizes and sharpens the focusing zone. Nucleic acids can be preferentially separated and preconcentrated, while excluding proteins and PCR-inhibiting molecules.

As mentioned above, we demonstrated extraction of human gDNA from whole blood.¹² That method was developed to lyse and extract nucleic acids from host cells, used a relatively gentle lysis process, was performed in a small channel volume, and used pressure-driven finite injection. Here, we demonstrate an ITP-based technique to extract pathogenic DNA from human red blood cells infected with the malaria-causing parasite *Plasmodium*. This work differs from our previous work on ITP-based extraction of host gDNA from blood,¹² as we here offer a new lysing and ITP chemistry which achieves more aggressive chemical and thermal lysing applicable to the malaria parasite. Our current method also uses larger channel volumes, semi-infinite injection,¹⁰ and pressure-driven counter flow to increase sensitivity. We start by showing that higher lysis temperatures are required to lyse malaria parasite cells. We show that ITP-based DNA preparation can extract DNA from pathogenic cells as well as host cells. The work suggests that ITP can be integrated into blood diagnostic systems for a wide range of pathogenic diseases.

EXPERIMENTAL METHODS

A schematic of our extraction process is shown in Figure 1a. We used heat and chemical treatment to lyse malaria parasites infecting erythrocytes. We then mixed the resulting lysate with a

Received: September 27, 2011

Accepted: November 10, 2011

Published: November 10, 2011

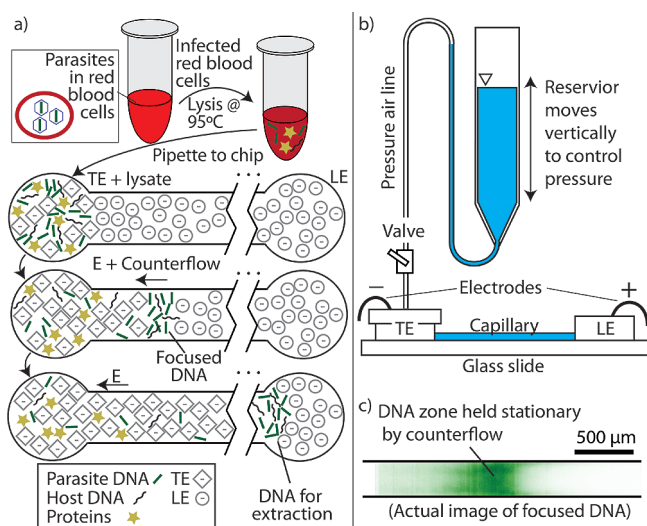


Figure 1. (a) DNA extraction process. A culture sample containing *P. falciparum* parasites infecting (within) red blood cells was diluted, mixed with proteinase K, and lysed at 95 °C. During lysis, parasite cells released their DNA into the cell lysate. The cell lysate was pipetted directly into the microfluidic well containing the TE. An electric field was applied, and the DNA was pulled into a capillary, where it focused at the ITP interface. In this process, PCR inhibitors (including proteins) remain unfocused in or near the TE well. The focused ITP zone containing purified nucleic acids eventually reached the LE well, where it was extracted for off-chip PCR. (b) Experimental setup. TE and LE wells are connected by a $300 \times 30 \mu\text{m} \times 2.5 \text{ cm}$ rectangular cross-section capillary. Pressure in the TE well is controlled using an elevated water chamber connected to an air-filled pressure line. (c) CCD camera image of extracted DNA focused at the ITP interface. DNA is visualized using SYBR Gold fluorescent dye.

TE buffer and pipetted the mixture onto a simple capillary setup. We applied an electric field to perform ITP. The nucleic acids were separated via electric field and traveled from the TE sample well to an LE well. The purified nucleic acids were pipetted from the LE well for off-chip PCR.

***Plasmodium falciparum* Samples.** We received *P. falciparum* W2 cells cultured in human erythrocytes from the Stanford Blood Center (Stanford, CA). The cells were cultured using the method described by Trager and Jensen.¹³ Culture samples were taken with culture parasitemia of 3.5% and 9%. These samples were stored at $-20 \text{ }^\circ\text{C}$ for later use. (For further detail, see Supporting Information, Section 1.)

Capillary Preparation. We used a free-standing, rectangular-cross-section, borosilicate capillary (not a microfluidic chip) for these experiments. We glued a 2.5 cm long, $30 \times 300 \mu\text{m}$ (inner dimensions) capillary (Vitrocom Inc., Mountain Lakes, NJ) to a $2.5 \text{ cm} \times 7.5 \text{ cm}$ glass slide (VWR, West Chester, PA) using UV-cure optical adhesive (Norland, Cranbury, NJ). The capillary axis was aligned with the 7.5 cm axis of the slide, and the $300 \mu\text{m}$ by 2.5 cm face of the capillary was laid flat against the glass slide with the $30 \mu\text{m}$ dimension parallel to the optical axis. We cut the threaded rings from two plastic screw-top 1.5 mL microcentrifuge tubes (Applied Scientific, South San Francisco, CA) and adhered these over the capillary ends as wells (using the same adhesive). We taped a $2 \times 2 \text{ cm}$ piece of aluminum foil (Reynolds Wrap, Richmond, VA) over the capillary to improve heat dissipation. An image of the capillary setup is shown in Figure S-1, Supporting Information. This simple, free-standing capillary

offers an inexpensive, easy-to-reproduce channel geometry with large cross section relative to most etched microchannels. Large cross section increases the sample volume from which we extract DNA.

We used the commercial silanizing agent, Sigmacote (Sigma-Aldrich, St. Louis, MO), to reduce electroosmotic flow and chemical adsorption to the glass channel walls.¹² (For further detail, see Supporting Information, Section 2.)

Prior to the first use and between runs to avoid cross-contamination, we rinsed the capillary with $50 \mu\text{L}$ of 10% bleach followed by $200 \mu\text{L}$ of deionized water and dried with vacuum for 2 min. Immediately before each experiment, we filled and rinsed with $50 \mu\text{L}$ of LE.

We controlled pressure-driven counterflow using a water column attached to the TE well. A schematic of this setup is shown in Figure 1b, and it is described in more detail in the Supporting Information (cf. Figure S-1). Briefly, we used a three-port Luer connector to connect a 1 m long tube to the TE well. This tube acted as a hydrostatic water column open to atmosphere. It was held in place with a small magnet and a ring stand. This let us apply vacuum to the capillary by lowering the water column.

Lysis. We diluted infected culture samples with uninfected erythrocytes and deionized water. This provided samples containing 9%, 1%, 0.1%, 0.01%, and 0% parasitemia and 50% hematocrit to simulate infected human blood samples while also providing well-controlled dilutions of parasite loading. We further diluted $30 \mu\text{L}$ of each sample with $185 \mu\text{L}$ of deionized water and added $17 \mu\text{L}$ of proteinase K (Invitrogen, Carlsbad, CA). We mixed each sample by pipetting and incubated at 65 °C for 1 min and then 95 °C for 9 min using a PCR thermocycler (Techne, Burlington, NJ).

We performed manual cell counts to examine the efficiency of our lysis process as a function of temperature. We prepared lysis mixtures containing $10 \mu\text{L}$ of erythrocyte sample with 0.9% nominal parasitemia, $10 \mu\text{L}$ of proteinase K, $5 \times$ SYBR Gold (Invitrogen, Carlsbad, CA), and nuclease-free water to bring the mixture to $100 \mu\text{L}$. We divided the samples into lysed and unlysed groups. We counted the cells in the unlysed samples immediately. We incubated the lysed samples at elevated temperature for 10 min in a benchtop thermocycler. As a comparison case, we incubated one lysed sample for 10 min at 56 °C, in the same manner as Persat et al.¹² We held all other samples for 1 min at 65 °C for proteinase K digestion and then an additional 9 min at elevated temperatures ranging between 65 and 95 °C.

We counted the cells using disposable hemocytometers (Cell-Vu, New York, NY). Malaria cells were visualized with SYBR Gold fluorescent dye, which fluoresces strongly when bound to nucleic acids. We prepared the hemocytometer for counting as described in the Cell-Vu operation manual. (For further detail, see Supporting Information, Section 3.)

Extraction. We prepared aqueous LE and $2 \times$ TE buffers prior to each experiment. The LE contained 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and $1 \times$ SYBR Gold (Invitrogen, Carlsbad, CA) in 100 mM Tris and 60 mM hydrochloric acid at pH 7.9. The $2 \times$ TE contained $2 \times$ SYBR Gold in 40 mM Tris and 40 mM HEPES (pH 7.9). To prepare the TE, we mixed the cell lysate 1:1 with $2 \times$ TE, for a final buffer concentration of 20 mM Tris and 20 mM HEPES.

At the start of each experiment, we filled the capillary with LE and emptied the wells with vacuum. We pipetted $50 \mu\text{L}$ of LE into one well and $50 \mu\text{L}$ of TE into the other well. We placed platinum wire electrodes into each well (and connected to

high voltage leads). We applied +600 V to the LE well, grounded the TE well, and recorded applied current over time using the Keithley voltage source and a computer running custom Matlab code. Current traces for experiments under counterflow are shown in Figure S-2, Supporting Information.

We monitored the ITP zone using epifluorescent microscopy (see below). For experiments requiring extended focusing time, we induced counterflow by applying vacuum to the TE well with our water-column system. We held the interface in the channel for 10 min and then approximately eliminated pressure-driven flow by returning the water column to its original height. When the ITP interface entered the LE well, we turned off the electric field and pipetted 4 μL of the LE from the region near the capillary exit into a 200 μL PCR tube for analysis. We note visual inspection and measured current traces; each provide feedback which can be used to hold the ITP zone stationary (see Supporting Information).

Imaging System. We performed on-chip visualizations using an inverted epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 4 \times objective (Plan, NA 0.10, Nikon, Tokyo, Japan). A blue LED light source (Thor Laboratories, Newton, NJ) was used for excitation. We used a filter cube optimized for detection of FITC (FITC-A-Basic, Semrock, Rochester, NY) and a 0.63 \times demagnification lens (Diagnostic Instruments, Sterling Heights, MI). We captured images using an intensified CCD camera (PI-MAX: 512, Princeton Instruments, Trenton, NJ). The DNA fluorescent dye was SYBR Gold included in the lysis buffer as described above.

PCR. We used off-chip quantitative PCR to validate our ITP extraction method. We added 4 μL of DNA extract from ITP to a PCR tube containing 10 μL of 2 \times Fast SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), 6 μL of DNase free water, and 150 nM primers targeting the PFCS. Validated primers¹⁵ for the circumsporozoite protein gene in *P. falciparum* were used to verify the presence of *P. falciparum* DNA. The primers were PFCS79, 5'-GGAAGTCGTCAAACACAAG-3', and PFCS233, 5'-CCATCATCATTTTCTCCAAG-3'.

We performed off-chip quantitative PCR using a real-time PCR thermocycler (7500 Fast, Applied Biosystems, Carlsbad, CA) with the following thermal profile: 20 s initial hold at 95 $^{\circ}\text{C}$ and 40 cycles composed of 3 s denaturation at 95 $^{\circ}\text{C}$ and 30 s annealing and extension at 60 $^{\circ}\text{C}$. We obtained post-PCR dissociation curves using the same instrument.

RESULTS AND DISCUSSION

Lysis. We used heat and chemistry to lyse the malaria parasites. Proteinase K aids in lysis and may assist in removal of nucleic acids from packing proteins.¹² We chose this method to avoid adding high-concentration ions, which may interfere with ITP separation. For example, high concentrations of chaotropic salts are often used for lysis and denaturation during nucleic acid extraction.¹⁶ However, high ionic strengths (e.g., >500 mM) can be challenging to integrate with ITP and/or require significant dilution steps.

Visualizations of malaria with SYBR dyes have been used to detect malaria infection in blood.¹⁷ DNA-specific dyes provide a high-contrast way of visualizing the DNA-containing malaria parasite inside red blood cells (which contain no DNA). We used SYBR Gold to visualize intact parasite cells to determine lysing efficiency (cf. Figure S-3, Supporting Information).

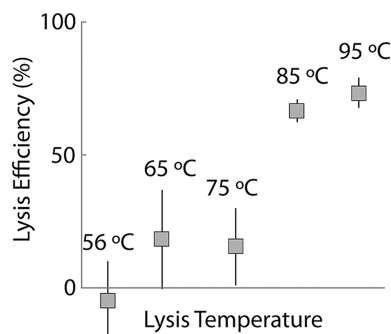


Figure 2. Lysis efficiency over maximum lysis temperatures between 56 and 95 $^{\circ}\text{C}$. We compared prelysis and postlysis parasite density by manual cell counting on disposable Cell-Vu hemocytometers. Parasite cells were visualized using SYBR Gold. Error bars indicate propagated 90% confidence interval based on Student t-distribution ($N = 14\text{--}18$ at each temperature).

Measured cell lysis efficiencies are shown in Figure 2. We defined cell lysis efficiency as:

$$\text{Efficiency} = 1 - \frac{\text{LysedCellCount}}{\text{UnlysedCellCount}}$$

We did not observe significant cell lysis at temperatures below 85 $^{\circ}\text{C}$. To estimate the uncertainty in lysis efficiency, we propagated the 90% confidence intervals from Student t-distributions of the lysed and unlysed cell count using the equation above assuming that these cell counts were uncorrelated.¹⁸ Lysing efficiency is ideally a positive quantity. However, the slight negative values in our measured estimate of lysing efficiency (for data at 56 $^{\circ}\text{C}$) is an expected result of experimental uncertainties in the lysed vs unlysed cell estimates. The lysing data shows a monotonic increase in lysing efficiency with increasing temperature from 56 to 95 $^{\circ}\text{C}$. We therefore chose 95 $^{\circ}\text{C}$ for the second, higher-temperature incubation step of our assay.

Visualization. We visualized total DNA extracted from infected erythrocyte samples during the ITP process by observing the scalar fluorescence of SYBR Gold dye. Example images of this fluorescence are shown in Figures 1c and S-4, Supporting Information. DNA visibly accumulates during the counterflow period, when the ITP zone is stationary in the channel.

PCR. Figure 3 shows measured PCR threshold cycles for circumsporozoite protein gene primers of DNA extracted from human erythrocytes with ITP. We explored parasite densities ranging 4 orders of magnitude from 0.5 to 500 parasites per nanoliter. We show example raw data from PCR runs in Figures S-5 and S-6, Supporting Information. Counterflow extends focusing time and enables a decrease in the limit of detection by an order of magnitude. We did not observe amplification during 40 thermal cycles in negative control PCR reactions in which we analyzed unlysed malaria-infected erythrocytes and nuclease-free water as templates.

Dissociation curves of the PCR product are shown in Figure S-7, Supporting Information. Amplified sequences dissociated at 69.5 $^{\circ}\text{C}$. This matched the dissociated temperature measured for PCR product of *P. falciparum* DNA as extracted from cell cultures using a commercial solid phase extraction kit (Qiagen, Valencia, CA) (a value of 69.5 $^{\circ}\text{C}$). As a second comparison, we calculated a theoretical dissociation temperature of 69.7 $^{\circ}\text{C}$ for this target

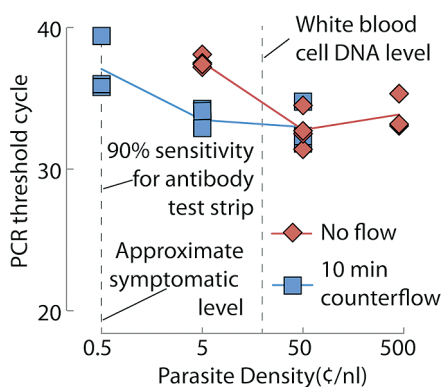


Figure 3. PCR threshold cycles from DNA extracted from malaria-infected erythrocytes using isotachopheresis (with and without counterflow to improve sensitivity). PCR primers targeted the circumsporozite gene in *P. falciparum*. Extending focusing time to 10 min (squares) using pressure-driven counterflow allowed us to detect parasite infection at the approximate clinical symptomatic threshold level of 0.5 parasites per nanoliter.

sequence¹⁵ using numerical DNA thermodynamic tools (mFold, RNA Institute, University of Albany, Albany, NY).

Detection Limits. Manual microscopy of thick blood films can detect malaria infection at 0.05 parasites per nanoliter. However, microscopy is time-consuming and requires a highly trained operator. Commercial antibody test strip kits commonly achieve >90% sensitivity above 0.5 parasites per nanoliter and 50% sensitivity at 0.05 parasites per nanoliter.^{19,14} We detected *P. falciparum* parasites in human erythrocytes to a parasite density of 0.5 parasites per nanoliter, comparable to antibody test strips. Clinical parasite concentrations can range from 0.005 to 50 parasites per nanoliter in blood. Most symptomatic cases are above 0.5 parasites per nanoliter.¹⁴ For example, a study of a Honduran population with endemic infections of *P. falciparum* and *P. vivax* measured an average concentration of 0.59 parasites per nanoliter in infected patients, including both symptomatic and nonsymptomatic patients.²⁰

CONCLUSION

We demonstrated extraction of DNA from the malaria-causing parasite *Plasmodium falciparum* in human erythrocytes using ITP with just a few manual steps. These malaria parasites are difficult to lyse compared to the host blood cells. We improved our nucleic acid yield by choosing a high lysis temperature and increasing our extraction time using pressure-driven counterflow. We showed that the extracted DNA was purified of PCR inhibitors found in red blood cells, compatible with PCR, and achieved a clinically relevant qPCR detection limit of 0.5 parasites per nanoliter.

The pressure-driven counterflow technique we use here may not be compatible with some on-chip analysis systems. However, counterflow was used only to increase DNA yield by increasing the ITP focusing time. Other methods of increasing yield may include increasing channel length and cross-sectional area, increasing applied current, and/or simultaneously extracting into multiple channels. We are currently exploring methods to increase throughput without counterflow.

This demonstration of extraction of pathogenic DNA from parasites inside human erythrocytes by ITP represents a widening of the scope of applications of ITP as a microfluidic DNA sample preparation method. Such studies are a step toward pro-

ducing clinical devices for diagnosis of infection on microfluidic platforms.

ASSOCIATED CONTENT

S Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: juan.santiago@stanford.edu. Fax: 650-723-7657.

ACKNOWLEDGMENT

We thank Prof. Mark Levenston for use of his qPCR thermocycler. We thank Prof. Niaz Banaei and Ellen Yeh for providing *P. falciparum* samples. This work was supported in part by the Defense Advanced Research Projects Agency (DARPA) N/MEMS S&T Fundamentals Program under grant no. N66001-1-4003 issued by the Space and Naval Warfare Systems Center Pacific (SPAWAR) to the Micro/Nano Fluidics Fundamentals Focus (MF3) Center. We gratefully acknowledge funding from DARPA under grant number N660001-09-C-2082. We also gratefully acknowledge funding from the Gates Foundation under contract number OPP1007350 GCE.

REFERENCES

- (1) Mariella, R. *Biomed. Microdevices* **2008**, *10*, 777–784.
- (2) Toner, M.; Irimia, D. *Annu. Rev. Biomed. Eng.* **2005**, *7*, 77–103.
- (3) Abu al-Soud, W.; Radstrom, P. *J. Clin. Microbiol.* **2001**, *39*, 485–493.
- (4) Wen, J.; Legendre, L. A.; Bienvenue, J. M.; Landers, J. P. *Anal. Chem.* **2008**, *80*, 6472–6479.
- (5) Auroux, P.-A.; Koc, Y.; deMello, A.; Manz, A.; Day, P. J. *R. Lab Chip* **2004**, *4*, 534–546.
- (6) Bocek, P. *Top. Curr. Chem.* **1981**, *95*, 131–177.
- (7) Bercovici, M.; Kaigala, G. V.; Mach, K. E.; Han, C. M.; Liao, J. C.; Santiago, J. G. *Anal. Chem.* **2011**, *83*, 4110–4117.
- (8) Persat, A.; Santiago, J. G. *Anal. Chem.* **2011**, *83*, 2310–2316.
- (9) Persat, A.; Chivukula, R. R.; Mendell, J. T.; Santiago, J. G. *Anal. Chem.* **2010**, *82*, 9631–9635.
- (10) Jung, B.; Bharadwaj, R.; Santiago, J. G. *Anal. Chem.* **2006**, *78*, 2319–2327.
- (11) Schoch, R. B.; Ronaghi, M.; Santiago, J. G. *Lab Chip* **2009**, *9*, 2145–2152.
- (12) Persat, A.; Marshall, L. A.; Santiago, J. G. *Anal. Chem.* **2009**, *81*, 9507–9511.
- (13) Trager, W.; Jensen, J. *J. Parasitol.* **2005**, *91*, 484–486.
- (14) Moody, A. *Clin. Microbiol. Rev.* **2002**, *15*, 66–78.
- (15) Wooden, J.; Kyes, S.; Sibley, C. *Parasitol. Today* **1993**, *9*, 303–305.
- (16) Chomczynski, P.; Sacchi, N. *Nat. Protoc.* **2006**, *1*, 581–585.
- (17) Smilstein, M.; Sriwilajaroen, N.; Kelley, J. X.; Wilairat, P.; Riscoe, M. *Antimicrob. Agents Chemother.* **2004**, *48*, 1803–1806.
- (18) Meyer, S. L. *Data analysis for scientists and engineers*; Wiley: New York, 1975; p 513.
- (19) Murray, C. K.; Gasser, R. A.; Magill, A. J.; Miller, R. S. *Clin. Microbiol. Rev.* **2008**, *21*, 97–110.
- (20) Quintana, M.; Piper, R.; Boling, H.-L.; Makler, M.; Sherman, C.; Gill, E.; Fernandez, E.; Martin, S. *Am. J. Trop. Med. Hyg.* **1998**, *59*, 868–871.