1 REVISED

2
3
4 Comparison of point-of-care-compatible lysis methods for
5 bacteria and viruses
6

7 Short title: Pathogen Lysis in Low-resource Settings
8

9 Erin K. Heiniger^{1*}, Joshua R. Buser¹, Lillian Mireles¹, Xiaohong Zhang¹, Paula D. Ladd¹, Barry R. Lutz¹,
10 and Paul Yager¹

11 ¹ Department of Bioengineering, University of Washington, Seattle, Washington, United States of
12 America

13 *Corresponding Author

14 Email: erinkh@uw.edu (EKH)

15 Address:
16 Foegel Building
17 3720 15th Ave NE
18 Seattle WA 98195
19 United States
20

Abstract

Nucleic acid sample preparation has been an especially challenging barrier to point-of-care nucleic acid amplification tests in low-resource settings. Here we provide a head-to-head comparison of methods for lysis of, and nucleic acid release from, several pathogenic bacteria and viruses—methods that are adaptable to point-of-care usage in low-resource settings. Digestion with achromopeptidase, a mixture of proteases and peptidoglycan-specific hydrolases, followed by thermal deactivation in a boiling water bath, effectively released amplifiable nucleic acid from *Staphylococcus aureus*, *Bordetella pertussis*, respiratory syncytial virus, and influenza virus. Achromopeptidase was functional after dehydration and reconstitution, even after eleven months of dry storage without refrigeration. Mechanical lysis methods proved to be effective against a hard-to-lyse *Mycobacterium* species, and a miniature bead-mill, the AudioLyse, is shown to be capable of releasing amplifiable DNA and RNA from this species. We conclude that point-of-care-compatible sample preparation methods for nucleic acid tests need not introduce amplification inhibitors, and can provide amplification-ready lysates from a wide range of bacterial and viral pathogens.

Key words: sample preparation, RNA, DNA, AudioLyse, POC, low resource settings

Introduction

In the modern clinical laboratory, detection of a myriad of pathogens with extreme sensitivity and specificity allows rapid diagnosis of disease and recommendations for treatment. However, these techniques are not always compatible with low-resource settings, or the point-of-care (POC). This disparity in technology availability affects not only health outcomes, but increases the burden of infectious disease in laboratory-poor areas of the developing world (Yager et al., 2006). In order to narrow this divide, our group is developing the MAD NAAT (Multiplexable Autonomous Disposable for Nucleic Acid Amplification Testing) platform (Fig. 1A). The launch point for the MAD NAAT project was the use of two-dimensional networks of paper (2DPNs) to automate an isothermal nucleic acid (NA) amplification process, isothermal strand displacement amplification (iSDA) (Bhushan J. Toley et al., 2015b). To enable full sample-to result NAAT automation, advances were required with regard to sample acquisition and transfer into the device (Panpradist et al., 2014), valving to automate and sequence the assay (Bhushan J Toley et al., 2015a; Toley et al., 2013), nucleic acid purification and concentration (Byrnes et al., 2015), isothermal nucleic acid amplification chemistry (Bhushan J. Toley et al., 2015b), and non-electric temperature-regulated heaters (Buser et al., 2015a; Shah et al., 2015; Singleton et al., 2013). Most sample preparation techniques in the literature are predicated upon use in a laboratory. Point-of-care-compatible methods have previously been described in isolation; to evaluate nucleic acid preparation efficacy they must be compared head-to-head.

Fig. 1. Point-of-care-compatible lysis of a broad range of pathogens. A) The MAD NAAT platform is a disposable instrument-free pathogen detection device currently being developed for point-of-care medical applications. To solve sample preparation hurdles for this device, we investigated a broad range of pathogen lysis methods. B) The AudioLyse device as used in previous work (Buser et al., 2015b) C) The general lysis procedure followed in this work.

A sample preparation protocol must consider a number of constraints to be compatible with a lab-on-a-chip (LOC) device such as the MAD NAAT, informed by conditions found in the developing world where many of the mainstays of laboratory methods are absent (Archibald and Reller, 2001; Mabey et al., 2004). In these environments, trained personnel are rare. Electricity, and thus equipment such as refrigerators and centrifuges, may be unavailable or unreliable, and disposables such as pipette tips and

centrifuge tubes may be cost-prohibitive. The same constraints are found in environmental monitoring and diagnosis of veterinary and plant diseases in the field. Currently available rapid diagnostics for non-human pathogens detect pathogen-specific antibodies or antigens using immunochemistry (Yetisen et al., 2013). Nucleic acid amplification tests are capable of low-copy detection, leading to greater sensitivity for the target of interest, but are often also more sensitive to inhibitors present in the specimen (Rådström et al., 2004). Currently, NAAT technologies for non-human pathogens are under development, but none is commercially available in a sample-to-result format (Biswas and Sakai, 2014; Teles and Fonseca, 2015).

Constraints on diagnostics at the point-of-care are encompassed by the World Health Organization's ASSURED criteria: tests must be **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free, and **D**elivered to those in need (Mabey et al., 2004). In order to meet these conditions for the MAD NAAT platform, we sought to develop a procedure to release amplifiable DNA or RNA from as broad a range of bacteria and viruses as possible, and allow direct input of lysed sample into the amplification reaction *without a purification step*. To meet, to the extent possible, the "user-friendly" ASSURED principle, we have also tried to minimize the number of user steps necessary for MAD NAAT, including sample preparation.

Lysis methods that use extreme pH, chaotropic salts, organic solvents, or detergents can interfere with subsequent nucleic acid amplification if these chemicals cannot be removed (Atshan et al., 2012; Chomczynski and Rymaszewski, 2006; Eckert and Kunkel, 1990; Sung et al., 2003; Wilson, 1997). Contaminant mitigation relies on purification using laboratory equipment (*e.g.* centrifuge), or drastically dilution of the sample, consequently reducing test sensitivity. We extensively considered the use of FTA paper, a product available from Whatman that was developed to capture and preserve nucleic acids from a variety of sample types, and has been used for prototype point-of-care NAATs (Bearinger et al., 2011).

However, we excluded FTA papers from this study because they must be washed before use in amplification reactions like iSDA (Rajendram et al., 2006), and thus were difficult to integrate into MAD NAAT. Remaining lysis techniques include **mechanical** lysis, **thermal** lysis, and **enzymatic** lysis.

Mechanical methods generally use grinding or shearing forces to lyse bacteria; they do not rely on chemicals that could inhibit nucleic acid amplification. A gold-standard mechanical method, bead beating, has been shown to release more DNA from hard-to-lyse microbes, including *S. aureus*, over other routine clinical laboratory techniques (de Boer et al., 2010). Point-of-care-compatible mechanical lysis methods evaluated in this study include the OmniLyse – a single-use, battery-powered miniature bead mill that has been shown to disrupt difficult-to-lyse bacteria and produce amplifiable DNA (Vandeventer et al., 2011) – and our own laboratory’s AudioLyse – a miniature bead mill that uses a portable audio device and electromagnetic coil to rotate a spherical magnet in a bead slurry, grinding the sample and causing lysis (shown in Fig. 1B) (Buser et al., 2015b).

Thermal lysis methods have been used to lyse bacteria, but often require the use of detergents such as Triton X-100 or SDS, or organic solvents such as phenol and chloroform (Atshan et al., 2012; Sung et al., 2003) which may inhibit nucleic acid amplification if not removed. Due to the constraints of our device, we chose to test boiling in a hypotonic solution free of these detergents and solvents as our thermal lysis condition.

Enzymatic lysis routinely utilizes proteinases (e.g., proteinase K) and/or lysozyme, but many important pathogens are resistant to these techniques. For example, *S. aureus*, our initial pathogen target in development of the MAD NAAT device, makes an enzyme—a peptidoglycan O-acetyltransferase, OatA—that determines its resistance to lysozyme (Bera et al., 2005). We chose to test the broadly-

applicable achromopeptidase (ACP), a cocktail of proteases and peptidoglycan-specific hydrolases (Li et al., 1997; Masaki and Nakamura, 1978; Masaki et al., 1981). Like other proteases, ACP is an inhibitor to downstream amplification. However, those components of ACP that affect both iSDA and PCR can be irreversibly deactivated at temperatures above 80°C (Buser et al., 2016). A boiling water bath enables thermal deactivation of ACP without any other amenities. ACP activity can be stabilized by dehydration in the presence of preservatives (Buser et al., 2016), and thus shipped worldwide in the absence of refrigeration. Prior to this work, it was unknown how tolerant ACP lysis would be to a range of conditions that might be present in an integrated device, such as exposure to varying ambient temperatures and duration of ACP enzyme deactivation by thermal deactivation allowable before DNA degradation began.

The three bacterial human pathogen targets tested represent a broad range of bacterial types. *Staphylococcus aureus* is a Gram-positive, opportunistic bacterial pathogen of great clinical relevance, especially in its methicillin-resistant form (MRSA) (Cosgrove et al., 2003; Lowy, 1998). *Staphylococcus* can be difficult to lyse compared to other bacterial species, in part due to its thick peptidoglycan cell wall, as is typical for Gram-positive bacteria (Atshan et al., 2012; Hogg et al., 2008). *Bordetella pertussis* is a Gram-negative bacterial respiratory pathogen, the causative agent of whooping cough, which takes 4-14 days to diagnose by cell culture, or a laboratory DNA purification and amplification method for diagnosis (Spilker et al., 2014; Tatti and Tondella, 2013). Like all Gram-negative bacteria, *B. pertussis* has a cell wall protected from lytic enzymes by a lipid outer membrane, and consequently has lysis properties that are distinct from those of Gram-positive bacteria like *S. aureus*. *Mycobacterium marinum* has, like other mycobacteria, a unique cell wall structure, containing long-chain branched fatty acids and mycolic acids. This durable cell wall excludes lytic enzymes and makes *Mycobacterium* very difficult to lyse (Brennan and Nikaido, 1995; Niwa et al., 2005). In this study, we use *M. marinum* as a model organism for *Mycobacterium tuberculosis*, which causes a large portion of deaths worldwide, especially among HIV-

infected individuals (Archibald and Reller, 2001). *M. marinum* was chosen because it has been used previously at our institution as a model organism for TB in fish pathogenesis studies (Cosma et al., 2006), and because of its ease and speed of culture in our BSL-2 laboratory facilities (Gao and Manoranjan, 2005).

Symptoms associated with bacterial infections often overlap those caused by viral infections, and MAD NAAT was designed to enable detection of both bacteria and viruses. Many common viral pathogens, such as respiratory syncytial virus (human RSV A) and influenza, contain RNA genomes. To ensure that the relatively rigorous conditions and enzymes needed to extract DNA from bacteria did not destroy RNA from easier-to-lyse viruses, integrity of amplifiable RNA targets introduced subsequent to the ACP lysis method was evaluated. Traditionally, RNA viruses are lysed using chaotropic salts or detergents, which require nucleic acid purification before amplification. A method enabling DNA and RNA assays for bacteria and viruses without purification would be hugely beneficial for integrated devices like MAD NAAT.

We performed a head-to-head comparison of point-of-care-compatible lysis methods for three diverse bacterial species and two viral species. Using these methods, DNA and RNA released from pathogens is quantified without purification. Furthermore, AudioLyse is evaluated for room-temperature release of RNA from bacterial cells, this work's primary DNA-releasing targets.. The suite of lysis methods described here will be applicable to the development of a wide range of point-of-care diagnostic devices.

Materials and Methods

Bacterial growth

S. aureus (strain RN4220(Kreiswirth et al., 1983)) was cultured in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ, USA) at 37°C with shaking (250 rpm). Overnight cultures were diluted 1:100 in fresh medium and grown to mid-log phase ($OD_{600} = \sim 2$). *B. pertussis* (Moreno-Lopez ATCC 9797) was cultured in Stainer-Scholte (BD Difco) broth as described previously (Hulbert and Cotter, 2009). Cultures were grown for two days at 37°C with shaking (250 rpm), then diluted 1:100 in fresh medium, and grown 16-20 hours to mid-log phase ($OD_{600} = \sim 1$). *M. marinum* (Aronson ATCC 927) was cultured in Middlebrook 7H9 broth with ADC enrichment (BD Difco) at 30°C with shaking (250 rpm) for 4-7 days. Cultures were diluted 1:100 in fresh medium and grown for 48 hours to mid-log phase ($OD_{600} = \sim 1$).

Virus source

Cultured RSV was obtained from the UW Clinical Virology Laboratory, Seattle, WA. Influenza A grown in embryonated chicken eggs was purchased from Virapur, LLC, San Diego, CA.

Lysis methods

The general lysis procedure used in this work is illustrated in Fig. 1C. Bacterial cell suspensions were diluted to $OD_{600} = 0.02$ in 10 mM Tris, pH 8.0. RSV virion concentration was determined by quantification of genomic RNA prior to dilution in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For the no-lysis negative control condition, the bacterium or virus was added to the qPCR reaction (described below) without further processing, quantifying the sum of extracellular DNA/RNA and DNA/RNA released by the denaturation step of the qPCR reaction.

For ACP lysis, ACP (20 U/ μ L, Sigma A3547) in TE buffer + 5% trehalose was added to 0.2 mL polypropylene tubes (Cat #951010006, Eppendorf North America, Hauppauge, NY, USA) and dried in a miVac (Genevac, Stone Ridge, NY, USA) for 1.5 hours. Dried-down tubes were stored in a desiccator (Cat #420721115, Bel-Art Scienceware, Wayne, NJ, USA) at room temperature for 1-15 days before use. Bacterial or viral suspensions (80 μ L) were added to the dried ACP pellet to give a final concentration of 0.5 or 1.5 U ACP/ μ L. After gentle mixing by agitating the tube, the lysis reaction was incubated at 19°C (ambient room temperature) for 2 minutes unless otherwise noted. For incubation temperatures other than 19°C, tubes containing dried ACP and tubes containing cells to be lysed were equilibrated to the noted temperature using a digital heatblock (Cat #97043-610, VWR, Radnor, PA, USA) for 2 minutes before cells were added to ACP and incubation began. ACP was deactivated by placing the tube in a boiling water bath (measured >95°C) for five minutes, unless otherwise noted. Previously, we have shown that temperatures above 80°C are sufficient for thermal deactivation of ACP used to lyse *S. aureus* cells (Buser et al., 2016).

Thermal lysis was performed by placing a tube containing cell suspension in a hypotonic buffer (10 mM Tris, pH 8.0) in a boiling (measured >95°C) water bath for five minutes, unless otherwise noted.

Bead beating lysis—used here as a gold-standard, positive control method—was performed by adding 800 μ L cell suspension and 0.8 g of 0.1 mm sterile glass beads (Cat #11079101, BioSpec Products, Bartlesville, OK, USA) to a 2 mL screw-top tube (Cat #02-682-558, Fisher Scientific, Waltham, MA, USA). Tubes were agitated at maximum speed using a Mini-BeadBeater-16 (BioSpec Products, Bartlesville, OK, USA) for 3 cycles of 60 seconds on, 60 seconds off. Lysate was separated from the bead slurry by pipetting from above the settled beads. OmniLyse Cell Lysis Kits (Claremont Biosolutions, Upland, CA, USA) were used as directed by the manufacturer, manually drawing 500 μ L cell suspension

into and out of the device three times, at a rate of 500 μ L per 30 seconds, for a total processing time of 1.5 minutes (Vandeventer et al., 2011). Construction of the OmniLyse retains glass beads within the bead mill, separating them from the lysate when liquid is ejected from the device. AudioLyse lysis was implemented using a 500 μ L cell suspension with 250 mg 0.1 mm sterile glass beads and a 1/4" diameter NdFeB spherical magnet (S4, K&J Magnetics, Inc., Pipersville, PA, USA) in a 2 mL screw-top tube as previously described (Buser et al., 2015b). A 30 Hz sine wave was supplied by an mp3 player (Sansa Clip+ 4 GB, SanDisk, Milpitas, CA, USA) for 10 minutes as previously described. Lysate was separated from the bead slurry by pipetting from above the settled beads and magnet.

Genome quantification

Amplifiable genomic DNA and RNA were quantified using real time quantitative PCR or RT-PCR. *S. aureus* DNA was quantified using the MRSA/SA ELITE MGB *ldhI* assay (ELITechGroup Inc. Molecular Diagnostics, Bothell, WA, USA). The thermal profile used was: 50°C hold for 2 minutes, 93°C hold for 2 minutes, 45 cycles of 93°C for 10 seconds, 56°C for 30 seconds, and 72°C for 15 seconds, ending with a final elongation step at 72°C for 5 minutes. *B. pertussis* DNA was quantified using the assay against IS481 as described previously (Tatti and Tondella, 2013), substituting the Bioline SensiFAST Probe No-Rox kit (Bioline, Taunton, MA, USA) for the master mix used previously, using the thermal protocol: 95°C hold for 5 minutes, 40 cycles of 90°C for 10 seconds, and 60°C for 20 seconds. *M. marinum* DNA was quantified using a previously described assay against the 23S rDNA region (Jacobs et al., 2009), substituting the master mix with the Bioline SensiFast kit, and using the thermal protocol described above for *B. pertussis* quantification. All amplifications were run on a CFX96 (BioRAD, Hercules, CA, USA) and copy number values were generated by the CFX96 machine software from C_t using a standard curve of genomic DNA purified from lysates by ethanol precipitation and quantified using spectrophotometry

(Nanodrop 1000, ThermoFisher, Waltham, MA, USA). Amplifiable DNA recovery for each experiment was calculated as a percentage of the copy number measured from the noted control sample.

RSV copy number was determined using the Ultrasense quantitative RT-PCR assay mix (Life Technologies, Carlsbad, CA, USA), the ATCC derived RSV genomic RNA (ATCC, Manassas, VA, USA), and primers and probe sequences published previously (Kuypers et al., 2004). Influenza copy number was determined similarly, using the protocol developed by the UW clinical virology laboratory (Munro et al., 2013) and influenza A control RNA (a kind gift from PATH, Seattle, WA, USA).

Bacterial RNA quantification

In order to quantify RNA release from lysed bacterial targets, several strategies were employed. First, cells of *S. aureus* or *M. marinum* were lysed as described above, using the BeadBeater, the AudioLyse, ACP at 0.5 U/μL, and a “no-lysis” control (cells incubated in hypotonic 10 mM Tris pH 8.0 at room temperature). Unpurified lysates were then subjected to the following treatments: untreated samples (4 μL) were diluted in 7 μL 10 mM Tris pH 8.0. DNase I treatment consisted of sample (4 μL) combined with 10X DNase I reaction buffer (1 μL), DNase I (1 μL, Amplification Grade, Cat #18068015, ThermoFisher), and water to a volume of 10 μL. After a 15 minute incubation at room temperature (19-21°C), 1 μL 25 mM EDTA was added, and the sample was incubated at 65°C for 10 minutes to inactivate the DNase, as per the manufacturer’s instructions. Mock-treated samples were prepared as for DNase-treated samples, substituting water for the DNase I enzyme solution.

Untreated, DNase-treated, and mock-treated lysates were quantified for nucleic acid (DNA + RNA) using the Ultrasense quantitative RT-PCR assay mix. Primers and probes for *S. aureus* and *M. marinum* were as described above for DNA quantification, at concentrations recommended by the Ultrasense

manufacturer's protocol. This assay includes SuperScript III Reverse Transcriptase for cDNA generation and Platinum *Taq* DNA Polymerase for DNA amplification. To measure only DNA, the Ultrasense kit was modified by replacing the RT/DNA polymerase Enzyme Mix provided in the kit with Platinum *Taq* DNA Polymerase (Cat #10966018, ThermoFisher), used at 0.1 μ L per 20 μ L reaction (1 U).

Long-term ACP storage

ACP was dried as described above in a 0.2 mL PCR tube. Dried ACP tubes were stored at room temperature (19-20°C) under uncontrolled humidity for 11 months. Lysis efficiency was measured by comparison to freshly-resuspended ACP lysis of *S. aureus* cells in Tris buffer, with amplifiable DNA quantified by qPCR, as described above.

Statistics

Statistics were performed using Excel (Microsoft, Redmond, WA, USA). Means and standard deviations were calculated using the AVERAGE and STDEV functions of Excel, respectively, for the array of values generated from at least three independent experiments. Standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of n, the number of data points collected. Unless otherwise noted, n = 9 for all data points. P-values were calculated using the Excel TTEST function, assuming a two-tailed distribution and equal variance.

Results

280
281 Several point-of-care-compatible lysis methods were compared by quantifying release of amplifiable
282 DNA or RNA from the chosen representative pathogens. Amplifiable DNA was normalized to the copy
283 number obtained from the bead beating lysis condition (the reference, “gold-standard” method for
284 mechanical lysis of recalcitrant organisms (Amita et al., 2002)). Table 1 shows that 0.5 U/μL ACP,
285 followed by boiling water thermal deactivation, released amplifiable DNA from both the Gram positive
286 bacterium *S. aureus* ($90.4 \pm 6.6\%$, $p=3.6 \times 10^{-10}$ vs. no-lysis control), as expected given previous
287 literature, and from the Gram negative bacterium *B. pertussis* ($89.4 \pm 5.9\%$, $p=1.4 \times 10^{-10}$ vs. no-lysis
288 control). ACP lysis did not release significant DNA from the mycobacterium *M. marinum* ($0.42 \pm 0.1\%$,
289 $p=0.79$ vs. no-lysis control). Increasing the ACP concentration did not significantly improve the release of
290 amplifiable DNA from *S. aureus* ($p=0.66$), or *M. marinum* ($p=0.24$).

291
292 ACP lysis was not predicted to be effective against Gram negative organisms due to the presence of the
293 outer membrane between the environment and the peptidoglycan, which is presumed to be the primary
294 target of ACP. The majority of *B. pertussis* lysis can be attributed to boiling thermal lysis ($79.0 \pm 5.8\%$ vs
295 bead beating, compared to $3.6 \pm 0.6\%$ for no-lysis vs bead beating). This 20-fold increase in DNA
296 recovery by boiling lysis over the no-lysis condition is statistically significant ($p=1.0 \times 10^{-6}$). Lysis of *B.*
297 *pertussis* by boiling was not statistically different from lysis using 0.5 U/μL ACP ($p=0.22$). However,
298 increasing ACP concentration to 1.5 U/μL increased DNA recovery from *B. pertussis* ($104 \pm 7.4\%$ vs bead
299 beating lysis, compared to $89.4 \pm 5.9\%$ in the 0.5 U/μL condition), distinguishing this condition
300 statistically from boiling lysis ($p=0.02$). Lysis in a boiling water bath with hypotonic solution released
301 seven times less amplifiable DNA from *S. aureus* than ACP/boiling deactivation lysis ($12.1 \pm 0.6\%$,
302 $p=2.7 \times 10^{-9}$ vs. 0.5 U/μL ACP condition). Boiling lysis released almost 38 times less amplifiable DNA
303 from *M. marinum* than did bead beating lysis ($p=3.6 \times 10^{-20}$ vs. bead beating condition).

The OmniLyse, which was previously shown to work well on *Mycobacterium bovis* (Vandeventer et al., 2011), also worked well on *M. marinum* ($41.2 \pm 3.2\%$, $p=9.7 \times 10^{-10}$ vs. no-lysis control). The AudioLyse released a significant amount of amplifiable DNA from *M. marinum* ($17.2 \pm 2.6\%$, $p=9.8 \times 10^{-6}$ vs. no-lysis control) – approximately half as much as the OmniLyse..

Table 1. Comparison of DNA recovery between point-of-care lysis methods for selected bacterial pathogens.

	No lysis	0.5 U/ μ L ACP	1.5 U/ μ L ACP	Boiling	OmniLyse	AudioLyse
<i>S. aureus</i>	0.81 ± 0.12	90.4 ± 6.6	86.9 ± 4.2	12.1 ± 0.6	74.8 ± 2.5	41.3 ± 4.9
<i>B. pertussis</i>	3.6 ± 0.6	89.4 ± 5.9	104 ± 7.4	79.0 ± 5.8	39.5 ± 7.4	19.9 ± 4.9
<i>M. marinum</i>	0.37 ± 0.2	0.42 ± 0.1	0.30 ± 0.1	2.65 ± 0.2	41.2 ± 3.2	17.2 ± 2.6

Organisms were lysed using a range of concentrations of ACP, boiling, OmniLyse, or AudioLyse. Results are reported as amplifiable DNA recovered, as measured by qPCR, normalized to bead beating as the control ($\pm$ standard error of the mean). Sample size was $n = 9$ for all data points. P-values are given in the main text.

ACP lysis released the greatest amount of amplifiable DNA from *S. aureus* and *B. pertussis*, though more ACP was required for ACP to outperform boiling lysis of *B. pertussis*. Amplifiable DNA release from *M. marinum* was low for ACP/boiling, and improved with the mechanical OmniLyse and AudioLyse methods. ACP lysis was investigated further due to its predicted ease of integration into point-of-care devices such as the MAD NAAT device (Fig. 1A).

To extend the ACP shelf life in the absence of refrigeration, ACP was dehydrated in the presence of trehalose as a preservative. ACP can be stored in this dry form for up to 11 months without the loss of *S. aureus* lysis activity ($106 \pm 1.6\%$ amplifiable DNA compared to the freshly-made control, $p=0.26$, $n=2$,

data not shown). Fig. 2A shows that after 2 minutes of ACP treatment at 19°C, 3 minutes of boiling is sufficient for maximum amplifiable DNA release from an *S. aureus* sample. Amplifiable DNA remained above 80% of maximum for boiling times from 3-30 minutes, with only a slight decrease below maximum at 30 minutes ($86.8 \pm 3.3\%$, $p=0.01$). However, a significant amount of amplifiable DNA is produced by the one-minute deactivation as compared to no-boiling deactivation ($68.5 \pm 2.3\%$, $p=1.5 \times 10^{-15}$). DNA purified by ethanol precipitation from a no-boiling control resulted in a much lower DNA recovery than from the ACP/boiling condition ($9.6 \pm 2.4\%$, $p = 4.1 \times 10^{-5}$ vs ACP/boiling condition, $n = 3$ data not shown). This suggests that while ACP is an amplification inhibitor, the boiling aspect of the ACP/boiling method is crucial for effective lysis and DNA recovery. Ambient temperature can vary widely in point-of-care settings without climate control, so the effect of temperature on the ACP digestion was quantified. Fig. 2B shows that ACP effectively lyses *S. aureus* at a wide range of ambient temperatures.

Fig. 2. Robustness of the ACP + boiling method. A) DNA recovery as function of thermal deactivation time after ACP incubation at 19°C for two minutes. *S. aureus* samples were placed in boiling water for

342 varying periods. DNA yields are reported as qPCR copy number normalized to the three-minute thermal
343 deactivation (mean $\pm$ SEM), n = 9. B) DNA recovery as a function of ACP incubation temperature.
344 Incubations were performed for 2 minutes at various temperatures, followed by a 5-minute thermal
345 deactivation in a boiling water bath (measured $>95^{\circ}\text{C}$). DNA qPCR yields are normalized to the 20°C
346 ACP incubation reported as mean $\pm$ SEM, n = 9. Statistical comparisons are described in the text.

347
348 In an effort to understand whether the ACP/boiling method could be applied to samples containing RNA
349 viruses as well as bacteria, this lysis method was performed in the presence of one of two RNA viruses,
350 RSV or influenza A. ACP treatment followed by thermal denaturation was expected to degrade the RNA
351 template, as this was performed without addition of RNase inhibitors. However, quantitative reverse
352 transcription coupled with PCR (qRT-PCR) revealed amplifiable product. When either virus sample was
353 incubated with ACP for five minutes prior to 95°C thermal denaturation, there was significant amplifiable
354 RNA from samples of both viruses (Fig. 3). When 10,000 RSV virions (as measured by gRNA copy
355 number of the stock) were treated with 12.5 units of ACP, an average of 7,500 RNA copies were detected
356 in the 95°C denatured sample, representing 75% recovery (Fig. 3, $p=3.78 \times 10^{-5}$ compared to no-
357 deactivation control). Similar recovery was seen for Influenza A, although the copy number was 2.5
358 times higher. Starting with 270,000 influenza A virions (as measured by gRNA copy number of the
359 stock) and 12.5 units of ACP results in an average of 190,000 RNA copies, or $\sim 70\%$, after thermal
360 denaturation (Fig. 3, $p=8.52 \times 10^{-4}$ compared to no-deactivation control). This result demonstrates that
361 we can use a single lysis approach to identify the presence of both DNA and RNA in a sample.

Fig. 3. Comparison of viral RNA recovery in the presence of ACP with and without 95°C thermal deactivation. Each assay used 12.5 units of ACP with 10,000 RSV virions (as measured by gRNA copies, dark bars) or 270,000 influenza A virions (as measured by gRNA copies, light bars), respectively. Samples were incubated at 95°C or 26°C prior to direct qRT-PCR. Results normalized to input copy number, reported as mean $\pm$ SEM, n=3. Statistical comparisons are discussed in the text.

Since RNA was more stable in the presence of these lysis methods than expected, the amount of bacterial RNA released from *S. aureus* or *M. marinum* lysed with ACP, the BeadBeater, the AudioLyse, or a no-lysis control was quantified. Untreated, DNase-treated or mock-treated lysates were measured by qPCR, both in the presence and absence of reverse transcriptase (RT). In the absence of RT, DNase-treated BeadBeater and ACP lysates for both species contained <1 % as many copies target DNA as the mock-treated lysates. The same low DNA recovery (<1% as many copies DNA as mock-treated) was true of the

no-lysis control for *M. marinum*. DNase-treated lysates from the AudioLyse contained $8.6 \pm 3.1\%$ (*M. marinum*) or $8.2 \pm 2.2\%$ (*S. aureus*) as many copies as the mock-treated lysates. DNase-treated lysates from the *S. aureus* no-lysis control contained $46 \pm 6.8\%$ as many copies as the mock-treated lysates., Copy numbers measured in the presence of RT from the DNase-treated condition were adjusted by the copy number measured in the absence of RT (DNA copies remaining), to arrive at RNA copies present. RNA copy numbers were normalized to the bead beating condition, and are shown in Figure 4.

The RNA copy number recovered from AudioLyse-treated *M. marinum* was lower than that recovered from bead beating ($37.2 \pm 4.6\%$, $p = 0.00012$). ACP and no-lysis treatments resulted in very little recovery of RNA from *M. marinum* ($0.4 \pm 0.2\%$, $p = 2.1 \times 10^{-7}$ and $0.7 \pm 0.3\%$, $p = 8.0 \times 10^{-7}$ vs bead beating, respectively). RNA copy level recovered from AudioLyse-treated *S. aureus* was roughly equivalent to that recovered from bead beating ($134 \pm 29\%$, $p = 0.27$), while that recovered from ACP lysis was roughly 4 times higher ($437 \pm 23\%$, $p = 1.7 \times 10^{-10}$). The no-lysis control for *S. aureus* showed an RNA copy number approximately $1/4^{\text{th}}$ as large as bead beating ($25.6 \pm 6.0\%$, $p = 4.3 \times 10^{-7}$).

Fig.4. RNA released from *S. aureus* or *M. marinum* using various lysis techniques. Cell suspensions were lysed and copy numbers of RNA from *M. marinum* (dark grey bars) and *S. aureus* (light grey bars) were measured. All RNA values were normalized to that species' RNA-from-bead-beating value (set at 100%). Thin bars represent standard error of the mean, n = 9 for all data points, except the *M. marinum* no-lysis condition, where n = 8.

Discussion and Conclusions

Identifying effective, easy-to-operate, and inexpensive methods of recovering amplifiable nucleic acids from bacteria, viruses and other pathogens in samples is an important step toward improving detection of them at the point-of-care or in the field. The optimum method would balance easy operation by untrained personnel, low cost, no reliance on laboratory equipment, and sufficient release of amplifiable DNA and/or RNA. We performed a head-to-head comparison of several methods of lysis, in order to evaluate their suitability for point-of-care sample preparation in low-resource settings.

In the course of this work, we used bead beating as a gold standard compared to all other methods. Because of the broad range of bacterial types used, we could think of no better common way to ensure disruption of the cell wall and cell membrane. It is possible that the DNA recovery efficiencies of the bead beater differ among the organisms tested. In order to answer this question, one would need to measure accurately the genome copies per colony forming unit (CFU), using the best possible lysis technique for each of the separate organisms, then relate that number to DNA copies per CFU from bead beating.

ACP lysis followed by heating to $>95^{\circ}\text{C}$ by boiling is effective for more bacteria and viruses than expected. Though ACP lysis has previously been shown effective for Gram-positive organisms, especially *S. aureus*, it was unknown whether the enzyme would work on a Gram-negative organism. Gram-negative bacteria like *B. pertussis* have an outer membrane that should protect the peptidoglycan cell wall from the peptidases that are the major components of ACP (Li et al., 1997). ACP/boiling deactivation lysis of *B. pertussis* using 0.5 U/ μL ACP did not significantly increase amplifiable DNA recovered over the boiling lysis condition. However, increasing ACP concentration to 1.5 U/ μL did result in a significant increase in amplifiable DNA recovery, suggesting that ACP participates in lysing *B. pertussis* when used at a high enough concentration. As ACP is a cocktail of enzymes including proteases (Masaki et al.,

1981), outer membrane perforation by ACP proteases may allow exposure of peptidoglycan to ACP peptidases, which degrade the bacterial cell wall and allow increased lysis. Increased lysis over the boiling condition caused by a sufficiently high concentration of ACP was also observed in preliminary experiments on the Gram-negative bacterium *Salmonella typhi* (data not shown).

The ACP plus boiling lysis protocol provides effective lysis from Gram-positive and Gram-negative bacteria over a range of temperatures and thermal deactivation times. As long as one can provide a thermal denaturation step $>80^{\circ}\text{C}$ (such as by boiling water), for at least 3 minutes, the method can release amplifiable DNA. The ACP incubation step can also be performed at a broad range of ambient temperatures. We would not have expected an enzyme from a mesophilic organism (*Achromobacter lyticus*) to be active at 5°C , as seen in Figure 2B. It is possible that the ACP enzymes retain some activity at 5°C , or that the transient intermediate temperatures while heating the sample to 95°C give the enzymes sufficient time to do their work. We do not have data showing exactly when in the process nucleic acids released or degraded. Our group has shown thermal deactivation with a standalone disposable heater, and demonstrated ACP plus boiling amplifiable DNA recovery from *S. aureus* in the presence of patient nasal samples, confirming that this method can still function in the presence of a clinical patient sample (Buser et al., 2016). We have also integrated an ACP plus boiling lysis into a standalone sample-to-result NAAT (manuscript in preparation).

Based on work done with a similar organism, *Mycobacterium smegmatis* (Niwa et al., 2005), we did not expect ACP to be effective for *M. marinum* lysis. This proved true in our hands, and we conclude that for *Mycobacteria*, mechanical methods such as OmniLyse and AudioLyse are the preferred point-of-care-compatible lysis techniques. The OmniLyse is difficult to multiplex (one pair of hands can only process one sample every 2 minutes or so) compared to the other methods. The AudioLyse does not require user

intervention during the lysis operation, and may prove simpler to integrate into point-of-care diagnostic devices (Buser et al., 2015b). The AudioLyse was less efficient at lysing *M. marinum* than either the Bead Beater or the OmniLyse device. The Bead Beater is a large and powerful instrument running on mains electricity. The OmniLyse is battery powered, but uses a disposable rotary motor to agitate glass beads in a bead mill. Additionally, the entire sample is moved through the OmniLyse at a slow rate, giving all bacteria in the sample ample exposure to the site of grinding. We have observed mixing in the AudioLyse device, bringing portions of the sample into the area where grinding and lysis occurs, but we have performed only preliminary optimization of the mixing.

We have demonstrated that RNA can be amplified from minimally-processed virus-containing samples. Boiling virus releases RNA from within the viral capsid, making the RNA accessible to amplification enzymes. This result was surprising because RNA is believed to be much more thermally labile than DNA, and great care is generally taken when preparing RNA samples in the clinic and laboratory settings (Fleige and Pfaffl, 2006; Sambrook et al., 1989). However, should the sample be moved quickly from lysis to amplification and detection, as could be the case in an integrated device, RNA degradation is less extensive than anticipated. Possibly, the boiling lysis processing inactivates endogenous RNases; this merits further investigation, as RNases are typically considered heat-tolerant. Much of our knowledge of RNase activity is derived from commonly-used RNase A, thermal denaturation of which is reversible (Zale and Klibanov, 1986). We cannot rule out the possibility that proteases found in the ACP cocktail inactivate RNases, providing greater stability to the target RNA.

The AudioLyse device was used to prepare amplifiable RNA from two bacterial species as well. We noted that the AudioLyse performed comparably to the BeadBeater in RNA recovery from *S. aureus* and about 1/3rd as well as the BeadBeater in RNA recovery from *M. marinum*. This difference in performance

between the two organisms could be explained by the difference in lysis efficiency, as was observed for DNA in Figure 1. There is a 4-fold difference in RNA recovery from *S. aureus* between the ACP/boiling condition and the BeadBeater condition, in contrast to the rough equivalence in DNA recovery. This result suggests that recovery of RNA from the BeadBeater is lower than the total number of RNA molecules present within the cells. This loss is perhaps due to destruction of RNA by elevated temperature in the BeadBeater. This highlights the potential importance of icing steps between bead beating cycles when targeting RNA for isolation. While the AudioLyse is half as good at releasing DNA from *S. aureus* as ACP/boiling, it is approximately 1/4th as good at releasing RNA. This two-fold difference between the DNA release efficiency and the RNA release efficiency may indicate some RNA destruction in the AudioLyse, but not to as great an extent as seen in the BeadBeater. Another explanation for the loss of RNA to both the AudioLyse and the BeadBeater could be the differential adsorption of DNA and RNA to the silica beads (Poeckh et al., 2008). Future work on RNA sample preparation using the AudioLyse will address methods to minimize this possible adsorption.

We have quantitatively compared several simple point-of-care-compatible lysis methods capable of releasing amplifiable nucleic acids from a wide range bacterial and viral pathogens. While the organisms chosen were all human respiratory pathogens or models thereof, their structural characteristics are different enough that we anticipate these results will be broadly applicable to non-pathogenic targets as well.

The methods used here do not inherently introduce amplification inhibitors not already in the samples, or deactivate added inhibitors (ACP enzymes) prior to amplification, and may allow us to move point-of-care technologies into the field more quickly than adoption of conventional laboratory methods or *de novo* development of other lysis techniques. It is inevitable that patient samples will introduce

amplification inhibitors not assayed here. However, our group has already shown DNA detection of MRSA from ACP/boiling lysed patient nasal swab samples in the absence of purification (Buser et al., 2016). We have also integrated this technique into a standalone sample-to-result NAAT (manuscript in preparation). For more challenging samples, such as blood and urine, we are developing paper network technologies to quickly and simply purify nucleic acids (Byrnes et al., 2015). It is our hope that others may investigate the applicability of these lysis methods to their own microbial detection processes, whether for POC use or not. We have also shown a framework for considering what makes a lysis technique inherently point-of-care-compatible. We suggest that this or similar frameworks be used when designing sample preparation for diagnosis at the point-of-care. Coupling the sample preparation methods identified by this framework with low-power or power-free disposable amplification and detection devices could rapidly change how we diagnose disease in low-resource settings.

Acknowledgments

Our thanks to Nuttada Panpradist, Samantha Byrnes, and Alec Wollen of UW Bioengineering for initial work and helpful discussions. Thanks also to Walt Mahoney, Nicolaas Vermeulen, Boris Alabyev and Yevgeniy S. Belousov from ELITechGroup Inc. Molecular Diagnostics for supplying qPCR reagents. We thank our other collaborators at ELITechGroup Inc., at GE Global Research, and at PATH for their feedback and advice, as well as their generous donation of influenza A RNA. Thanks to the remaining members of the Yager Lab and the Lutz Lab at University of Washington. Finally, thanks to Claremont BioSolutions for their generous donation of a portion of the OmniLyse Lysis devices used for this work. Heiniger, Buser, Zhang, Lutz and Yager were supported by DARPA DSO/BTO HR0011-11-2-0007, awarded to Yager. Mireles was supported by the Louis Stokes Alliance for Minority Participation (NSF #1410465). Ladd was supported by NIH 1 R01 AI 096184-01 awarded to Yager.

References

- Amita, J., Vandana, T., Guleria, R.S., Verma, R.K., 2002. Qualitative evaluation of mycobacterial DNA extraction protocols for polymerase chain reaction. *Mol. Biol. Today* 3, 43–50.
- Archibald, L.K., Reller, L.B., 2001. Clinical microbiology in developing countries. *Emerg. Infect. Dis.* 7, 302–305. doi:10.3201/eid0702.700302
- Atshan, S.S., Shamsudin, M.N., Lung, L.T.T., Ling, K.H., Sekawi, Z., Pei, C.P., Ghaznavi-Rad, E., 2012. Improved method for the isolation of RNA from bacteria refractory to disruption, including *S. aureus* producing biofilm. *Gene* 494, 219–224. doi:10.1016/j.gene.2011.12.010
- Bearinger, J.P., Dugan, L.C., Baker, B.R., Hall, S.B., Ebert, K., Mioulet, V., Madi, M., King, D.P., 2011. Development and initial results of a low cost, disposable, point-of-care testing device for pathogen detection. *IEEE Trans. Biomed. Eng.* 58, 805–808. doi:10.1109/TBME.2010.2089054
- Bera, A., Herbert, S., Jakob, A., Vollmer, W., Götz, F., 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.* 55, 778–787. doi:10.1111/j.1365-2958.2004.04446.x
- Biswas, G., Sakai, M., 2014. Loop-mediated isothermal amplification (LAMP) assays for detection and identification of aquaculture pathogens: Current state and perspectives. *Appl. Microbiol. Biotechnol.* 98, 2881–2895. doi:10.1007/s00253-014-5531-z
- Brennan, P.J., Nikaido, H., 1995. The envelope of mycobacteria. *Annu. Rev. Biochem.* 64, 29–63. doi:10.1146/annurev.bi.64.070195.000333
- Buser, J., Zhang, X., Byrnes, S., Ladd, P.D., Heiniger, E.K., Wheeler, M.D., Bishop, J.D., Englund, J.A., Lutz, B.R., Weigl, B.H., Yager, P., 2016. A disposable chemical heater and dry enzyme preparation for lysis and extraction of DNA and RNA from microorganisms. *Anal. Methods* 8, 2880–2886. doi:10.1039/C6AY00107F
- Buser, J.R., Diesburg, S., Singleton, J., Guelig, D., Bishop, J.D., Zentner, C., Burton, R., LaBarre, P., Yager, P., Weigl, B.H., 2015a. Precision chemical heating for diagnostic devices. *Lab Chip* 15, 4423–4432. doi:10.1039/C5LC01053E
- Buser, J.R., Wollen, A., Heiniger, E.K., Byrnes, S., Kauffman, P.C., Ladd, P.D., Yager, P., 2015b. Electromechanical cell lysis using a portable audio device: enabling challenging sample preparation at the point-of-care. *Lab Chip* 15, 1994–7. doi:10.1039/C5LC00080G
- Byrnes, S. a, Bishop, J.D., Lafleur, L., Buser, J., Lutz, B., Yager, P., 2015. One-step purification and concentration of DNA in porous membranes for point-of-care applications. *Lab Chip* 15, 2647–2659. doi:10.1039/C5LC00317B
- Chomczynski, P., Rymaszewski, M., 2006. Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. *Biotechniques* 40, 454–458. doi:10.2144/000112149
- Cosgrove, S.E., Sakoulas, G., Perencevich, E.N., Schwaber, M.J., Karchmer, A.W., Carmeli, Y., 2003. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clin. Infect. Dis.* 36, 53–59. doi:10.1086/345476
- Cosma, C.L., Swaim, L.E., Volkman, H., Ramakrishnan, L., Davis, J., 2006. Zebrafish and Frog Models of *Mycobacterium marinum* Infection, in: *Current Protocols in Microbiology*. pp. 10B.2.1–10B.2.33.

doi:10.1002/0471729256.mc10b02s3

de Boer, R., Peters, R., Gierveld, S., Schuurman, T., Kooistra-Smid, M., Savelkoul, P., 2010. Improved detection of microbial DNA after bead-beating before DNA isolation. *J. Microbiol. Methods* 80, 209–211. doi:10.1016/j.mimet.2009.11.009

Eckert, K.A., Kunkel, T.A., 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res.* 18, 3739. doi: 10.1093/nar/18.13.3739

Fleige, S., Pfaffl, M.W., 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Aspects Med.* 27, 126–139. doi:10.1016/j.mam.2005.12.003

Gao, L.-Y., Manoranjan, J., 2005. Laboratory Maintenance of *Mycobacterium marinum*, in: *Current Protocols in Microbiology*. pp. 10B.1.1–10B.1.8. doi:10.1002/9780471729259.mc10b01s00

Hogg, G.M., McKenna, J.P., Ong, G., 2008. Rapid detection of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* directly from positive BacT/Alert® blood culture bottles using real-time polymerase chain reaction: evaluation and comparison of 4 DNA extraction methods. *Diagn. Microbiol. Infect. Dis.* 61, 446–452. doi:10.1016/j.diagmicrobio.2008.03.012

Hulbert, R.R., Cotter, P.A., 2009. Laboratory Maintenance of *Bordetella pertussis*. *Curr. Protoc. Microbiol.* Chapter 4, Unit 4B.1. doi:10.1002/9780471729259.mc04b01s15

Jacobs, J., Rhodes, M., Sturgis, B., Wood, B., 2009. Influence of environmental gradients on the abundance and distribution of *Mycobacterium* spp. in a coastal lagoon estuary. *Appl. Environ. Microbiol.* 75, 7378–7384. doi:10.1128/AEM.01900-09

Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O'Reilly, M., Shlievert, P.M., Bergdoll, M.S., Novick, R.P., 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305, 709–12. doi:10.1038/305709a0

Kuypers, J., Wright, N., Morrow, R., 2004. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. *J. Clin. Virol.* 31, 123–9. doi:10.1016/j.jcv.2004.03.018

Li, S., Norioka, S., Sakiyama, F., 1997. Purification, staphylolytic activity, and cleavage sites of alpha-lytic protease from *Achromobacter lyticus*. *J. Biochem.* 122, 772–778.

Lowy, F.D., 1998. *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339, 520–532. doi:10.1056/NEJM199808203390806

Mabey, D., Peeling, R.W., Ustianowski, A., Perkins, M.D., 2004. Tropical infectious diseases: Diagnostics for the developing world. *Nat Rev Micro* 2, 231–240. doi:10.1038/nrmicro841

Masaki, T., Fujihashi, T., Nakamura, K., Soejima, M., 1981. Studies on a new proteolytic enzyme from *Achromobacter lyticus* M497-1. II. specificity and inhibition studies of *Achromobacter* protease I. *Biochim. Biophys. Acta* 660, 51–55. doi: 10.1016/0005-2744(81)90107-8

Masaki, T., Nakamura, K., Isono, M., Soejima, M., 1978. A new proteolytic enzyme from *Achromobacter lyticus* M497-1. *Agric. Biol. Chem.* 42, 1443–1445. doi:10.1271/bbb1961.42.1443

Munro, S.B., Kuypers, J., Jerome, K.R., 2013. Comparison of a multiplex real-time PCR assay with a multiplex luminex assay for influenza virus detection. *J. Clin. Microbiol.* 51, 1124–1129. doi:10.1128/JCM.03113-12

Niwa, T., Kawamura, Y., Katagiri, Y., Ezaki, T., 2005. Lytic enzyme, labiase for a broad range of Gram-positive bacteria and its application to analyze functional DNA/RNA. *J. Microbiol. Methods* 61, 251–260. doi:10.1016/j.mimet.2004.12.006

606 Panpradist, N., Toley, B.J., Zhang, X., Byrnes, S., Buser, J.R., Englund, J.A., Lutz, B.R., 2014. Swab
 607 sample transfer for point-of-care diagnostics: characterization of swab types and manual agitation
 608 methods. PLoS One 9, e105786. doi:10.1371/journal.pone.0105786

609 Poeckh, T., Lopez, S., Fuller, A.O., Solomon, M.J., Larson, R.G., 2008. Adsorption and elution
 610 characteristics of nucleic acids on silica surfaces and their use in designing a miniaturized
 611 purification unit. Anal. Biochem. 373, 253–262. doi:10.1016/j.ab.2007.10.026

612 Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M., Löfström, C., 2004. Pre-PCR Processing. Mol
 613 Biotechnol. 26, 133–146. doi:10.1385/MB:26:2:133

614 Rajendram, D., Ayenza, R., Holder, F.M., Moran, B., Long, T., Shah, H.N., 2006. Long-term storage and
 615 safe retrieval of DNA from microorganisms for molecular analysis using FTA matrix cards. J.
 616 Microbiol. Methods 67, 582–592. doi:10.1016/j.mimet.2006.05.010

617 Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A laboratory manual 2nd ed. Cold
 618 Spring Harbor Laboratory Press, New York.

619 Shah, K.G., Guelig, D., Diesburg, S., Buser, J., Burton, R., LaBarre, P., Richards-Kortum, R., Weigl, B.,
 620 2015. Design of a New Type of Compact Chemical Heater for Isothermal Nucleic Acid
 621 Amplification. PLoS One 10, e0139449. doi:10.1371/journal.pone.0139449

622 Singleton, J., Zentner, C., Buser, J., Yager, P., LaBarre, P., Weigl, B.H., 2013. Instrument-free
 623 exothermic heating with phase change temperature control for paper microfluidic devices. Proc.
 624 SPIE 8615, 86150R. doi:10.1117/12.2005928

625 Spilker, T., Leber, A.L., Marcon, M.J., Newton, D.W., Darrah, R., Vandamme, P., LiPuma, J.J., 2014. A
 626 simplified sequence-based identification scheme for *Bordetella* reveals several putative novel
 627 species. J. Clin. Microbiol. 52, 674–677. doi:10.1128/JCM.02572-13

628 Sung, K., Khan, S.A., Nawaz, M.S., Khan, A.A., 2003. A simple and efficient Triton X-100 boiling and
 629 chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria.
 630 FEMS Microbiol. Lett. 229, 97–101. doi:10.1016/S0378-1097(03)00791-2

631 Tatti, K.M., Tondella, M.L., 2013. Utilization of multiple real-time PCR assays for the diagnosis of
 632 *Bordetella* spp. in clinical specimens. Methods Mol. Biol. 943, 135–147. doi:10.1007/978-1-60327-
 633 353-4_9

634 Teles, F., Fonseca, L., 2015. Nucleic-Acid Testing, New Platforms and Nanotechnology for Point-of-
 635 Decision Diagnosis of Animal Pathogens, in: Cunha, V.M., Inácio, J. (Eds.), Veterinary Infection
 636 Biology: Molecular Diagnostics and High-Throughput Strategies. Springer New York, New York,
 637 NY, pp. 253–283. doi:10.1007/978-1-4939-2004-4_20

638 Toley, B.J., Covelli, I., Belousov, Y., Ramachandran, S., Kline, E., Scarr, N., Vermeulen, N., Mahoney,
 639 W., Lutz, B.R., Yager, P., 2015b. Isothermal strand displacement amplification (iSDA): a rapid and
 640 sensitive method of nucleic acid amplification for point-of-care diagnosis. Analyst 140, 7540–7549.
 641 doi:10.1039/C5AN01632K

642 Toley, B.J., McKenzie, B., Liang, T., Buser, J.R., Yager, P., Fu, E., 2013. Tunable-delay shunts for paper
 643 microfluidic devices. Anal. Chem. 85, 11545–52. doi:10.1021/ac4030939

644 Toley, B.J., Wang, J. a, Gupta, M., Buser, J.R., Lafleur, L.K., Lutz, B.R., Fu, E., Yager, P., 2015a. A
 645 versatile valving toolkit for automating fluidic operations in paper microfluidic devices. Lab Chip
 646 15, 1432–44. doi:10.1039/c4lc01155d

647 Vandeventer, P.E., Weigel, K.M., Salazar, J., Erwin, B., Irvine, B., Doebler, R., Nadim, A., Cangelosi,
 648 G.A., Niemz, A., 2011. Mechanical disruption of lysis-resistant bacterial cells by use of a miniature,

649 low-power, disposable device. *J. Clin. Microbiol.* 49, 2533–2539. doi:10.1128/JCM.02171-10

650 Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63,

651 3741–3751.

652 Yager, P., Edwards, T., Fu, E., Helton, K., Nelson, K., Tam, M.R., Weigl, B.H., 2006. Microfluidic

653 diagnostic technologies for global public health. *Nature* 442, 412–418. doi:10.1038/nature05064

654 Yetisen, A.K., Akram, M.S., Lowe, C.R., 2013. Paper-based microfluidic point-of-care diagnostic

655 devices. *Lab Chip* 13, 2210–51. doi:10.1039/c3lc50169h

656 Zale, S.E., Klibanov, a M., 1986. Why does ribonuclease irreversibly inactivate at high temperatures?

657 *Biochemistry* 25, 5432–5444. doi:10.1021/bi00367a014

658