Improved Tools for Point-of-Care Nucleic Acid Amplification Testing

Thesis by

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ABSTRACT

There is a critical need for improved diagnostic tools to detect infectious diseases, especially in low-resource regions. A sample-to-answer point-of-care nucleic acid amplification test (NAAT) would be incredibly valuable for many different applications (e.g. COVID-19, Chlamydia/Gonorrhoeae, Influenza, Ebola, Zika/Chikungunya/Dengue, etc.). However, sample preparation (purification of pure nucleic acids) is a challenging bottleneck. In Chapter 2, commercial NA extraction methods were studied and improved. In Chapter 3, commercial stocks of SARS-CoV-2 RNA used in FDA emergency-use authorizations were found to be inaccurate and were independently quantified using reverse transcription digital PCR. In Chapter 4, a 3D printed meter-mix device was developed for initial processing prior to the sample preparation device. In Chapter 5, a 3D printed sample-to-device interface was prototyped to facilitate loading multi-volume SlipChip devices with purified template mixed with LAMP reactants. In Chapters 6-7, advancements were made for image processing of commercial chips to study digital LAMP reactions. In Chapter 8, additional tools were developed towards sample-to-answer point-of-care NAAT including a sample preparation module, amplification module, cell-phone readout, and automated base station.

PUBLISHED CONTENT AND CONTRIBUTIONS

E. Jue, D. Witters, and R.F. Ismagilov. 2020. "Two-phase wash to solve the ubiquitous contaminant-carryover problem in commercial nucleic-acid extraction kits" ***Scientific Reports*****. 10(1940). doi:10.1038/s41598-020-58586-3**

EJ acquired funding. Planned and analyzed buffer dilution experiments for Error! Reference source not found.. Planned, ran, and analyzed all remaining experiments. Generated all figures and wrote the manuscript.

DW Ran preliminary experiments evaluating the use of TPW to reduce buffer carryover.

E. Jue, N.G. Schoepp, D. Witters, and R.F. Ismagilov. 2016. "Evaluating 3D printing to solve the sample-to-device interface for LRS and POC diagnostics: example of an interlock meter-mix device for metering and lysing clinical urine samples" ***Lab on a Chip*****. 16:1852-1860. doi:10.1039/c6lc00292g**

EJ contributed to the invention, design, and validation of the meter-mix device. This includes the invention and design of the plunger system (chambers, plungers, valves, stoppers), invention and design of the multivalve and interlock system, design of the sealing mechanisms, design of the static mixer, and the design of the urine suction tube. EJ validated device function, user operation, device assembly, urine suction tube anti-drip feature, accurate metering and dispensing, mixer efficacy, and compatibility with qPCR.

NS contributed to the invention of the multivalve and interlock system as well as testing of the meter-mix device using biological samples, including development and validation of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) assays for the detection of Ct and Ng. All device validation and control experiments extracting urine spiked with either Ct or Ng prior to mixing with the device were performed by NS. Amplification of extracted nucleic acids using either PCR or LAMP was performed by NS, including data analysis.

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*These authors contributed equally

JRM developed the idea, designed experiments, and prepared the manuscript. Took the lead for TOC, Error! Reference source not found.**-4**, Error! Reference source not found.**-9**, Error! Reference source not found., and Error! Reference source not found.. Shared responsibility for Error! Reference source not found.. Developed idea for enhanced constrast by image processing and predicting RGB ratiometric signal output based on transmittance spectra. Involved in writing ImageJ macro for image processing.

MAK developed idea, designed experiments, and helped prepare the manuscript. Took the lead for Error! Reference source not found. and Error! Reference source not found.. Shared responsibility for Error! Reference source not found. and Error! Reference source not found.. Wrote ImageJ macro for image processing and automatic counting

SB developed the idea and designed experiments. Shared responsibility for Error! Reference source not found.**-3**. Developed the idea of enhancing contrast between positive and negative reaction by image processing. Wrote imageJ macro for image processing.

DAS took the lead for Error! Reference source not found. and shared responsiblitiy for Error! Reference source not found. and Error! Reference source not found.. Helped with fluorescent images for Error! Reference source not found. and Error! Reference source not found.. Developed idea of enhancing constrast between positive and negative reaction by image processing. Developed idea for predicting RGB ratiometric signal output based on trasmittance spectra.

DZ took the lead for Error! Reference source not found.**-11,** Error! Reference source not found.**-16**. Designed, optimized, and fabricated rotational multivolume SlipChip device for Error! Reference source not found.**-5**. Designed and fabricated the two-step SlipChip device for Error! Reference source not found.. Helped JRM for multivolume experiments.

EJ designed and tested C-clamp interface for the pumping lid. Used the C-clamp pumping lid to help load the multivolume device for a handful of experiments until JRM was trained. Designed and fabricated multivolume device with insertable washer features to aid slipping. Multivolume device with washer was used for TOC, Error! Reference source not found.**-5**.

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JCR conceptualized the method, generated and analyzed data. Wrote the paper, constructed figures, and performed all revisions.

EJ wrote the MATLAB software script for automated analysis of digital LAMP image sequences. Provided minor input to experimental design; and minor edits and inputs to the figures and manuscript.

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EJ wrote the MATLAB software script for automated analysis of digital LAMP image sequences.

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OVERVIEW

Background

The global burden of communicable disease has been estimated at 3.2 billion DALYs (disability-adjusted life years) per year for 2017.[1] Notable examples of communicable disease include ongoing pandemics COVID-19[2] and HIV/AIDS;[3] recent outbreaks of 2014-2016 Ebola,[4] 2015-2016 Zika virus,[5] and 2009-2010 H1N1 Influenza;[6] and ongoing challenges including respiratory diseases (Tuberculosis, Pertussis, Measles, etc.), diarrheal diseases (Rotavirus, E. coli, Norovirus, etc.), mosquito-borne pathogens (West Nile Virus, Dengue, Chikungunya, Malaria, etc.), and sexually transmitted infections (Chlamydia, Gonorrhoeaea, Syphilis, etc.).

The appropriate treatment and containment of communicable diseases requires timely and effective diagnostics to identify the causative pathogen. A strong screening response enables pandemic tracking, quarantining of individuals, guidance of appropriate treatments, contact tracing, public policy advising, and proper resource allocation. Because pandemics do not discriminate by international borders, an effective containment approach necessitates testing on a global scale. However, current infrastructure and supplies are lacking for such wide-scale testing capabilities, even in the most well-equipped countries.

Diagnosing pathogens is not an effortless endeavor. Communicable diseases take many forms such as bacterial, viral, parasitic, and fungal infections. Furthermore, pathogens can be located in different sample matrices such as water, food, respiratory droplets, urine, blood, saliva, sputum, stool, and more. An ideal diagnostic platform would have the flexibility to detect different pathogens in different types of sample matrices. Furthermore, routes of transmission also vary by pathogen and include direct contact, indirect contact, respiratory droplets, food contamination, insect bites, and sexual transmission. Because an unknown sample could contain an unknown pathogen (different than the pathogen being tested for)

with an unknown route of transmission, all human-derived samples should be handled with care (e.g. minimum BSL-2). While some pathogens can be easily diagnosed by clinical presentation, many pathogens cannot be detected without diagnostics.[7] This occurs when clinical presentations are shared among diseases, there is a high asymptomatic rate, or there exists a long incubation period.

Accessibility is also a critical factor to consider when evaluating the effectiveness of a diagnostic tool. The 2013-2016 Ebola Virus outbreak demonstrated the vital role of diagnostics for confirming cases, monitoring disease progression, and resource allocation planning. Notably, long transport times (several days) and the lack of rapid diagnostics in both resource-rich and resource-limited settings contributed to the initial failure to contain the outbreak.[8]

In considering the requirements for global-scale testing, the ideal test would be applicable at the point-of-care (POC, at the site of sample collection) and in limited-resource settings (LRS). Globally distributed testing imposes stringent requirements on diagnostics devices that can be summarized by the ASSURED criteria published by the World Health Organization.[9] The ASSURED criteria stands for affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable. It is worth taking some time to elaborate on each requirement.

Considering the global nature of pandemics, affordability is required to enable wide-scale testing, especially in the most vulnerable populations. The cost of a test must be weighed against the benefits, and is typically measured by disability-adjusted life years, or DALYs, avoided.[10] The advantages of an on-site POC diagnosis include reduced required infrastructure, laboratory workload, and turn-around-time. Overall, the patient experiences improved quality of care. However, cost comparison of POC tests to centralized laboratory testing is a controversial issue. Perspectives vary among patients, insurance companies, laboratory employees, and hospital employees.[11] While labor costs and the cost per test are quantifiable values, improvements in quality of care are more difficult to assess. Considering

the tricky cost landscape, it is prudent for diagnostic developers to weigh the cost of a new POC test against its clinical benefit.

The combined properties of sensitivity and specificity refer to the accuracy of the test. It is clear that test accuracy is the benchmark feature of a diagnostic test; and yet, a number of diagnostic tests have been developed and sold with poor accuracy.[12-14] While no test will be 100% accurate, it is important to strive for high sensitivity and high specificity from the beginning of diagnostic assay design. Generally, a test with over 90% sensitivity and 90% specificity has good diagnostic performance, but the utility of a test also depends on the specific application, prevalence of the pathogen, and rate of testing.

The ideal POC test would be user-friendly, equipment-free, and deliverable – an example of an ideal POC test (though not designed for disease detection) is the pregnancy test. The pregnancy test can be performed in the comfort of one's home by an untrained user provided with a simple page of instruction. In contrast, many gold-standard diagnostic tests require a trained user working in a temperature-controlled laboratory with access to expensive equipment.[15-17] However, much of the developing world does not have access to centralized lab testing. Even in developed countries, during times of high diagnostic demand such as a global pandemic, centralized lab facilities cannot meet testing demands. In the recent COVID-19 pandemic, we have observed long shipping times, lack of trained personnel to process samples, and a shortage of diagnostic testing supplies.[18] This has resulted in both a shortage of testing and long time-to-results for patients that are tested.

The ideal POC test should also be robust. This is especially important for globally distributed diagnostics because reagents must withstand longer shelf-times, various temperature conditions, and operation by untrained users. If the POC test is not sufficiently robust, the accuracy and utility of the test would suffer.

Lastly, the ideal POC should be rapid. This is an important requirement for on-site testing. For example, it is highly preferably for a doctor's office or mobile clinic to test patients within the timespan of their visit. If this were the case, the health provider could prescribe

the appropriate treatment and consult the patient during the visitation (rather than in a follow-up call). The shorter the test, the more likely it is that patients will wait, the less space is required for waiting rooms, and the more tests can be processed for any given amount of time.

In addition to the ASSURED criteria, we have learned that it is important for tests to be highly parallelizable. In recent years, tests have been developed with turn-around time as fast as 45 min,[19] but throughput (and associated costs) is still a concern since only one specimen can be tested at a time.

For disease diagnostics, there are three commonly employed test strategies: (1) cell culture, (2) protein-based testing (lateral-flow immune assay or enzyme-linked immunosorbent assay, LFA or ELISA), and (3) nucleic acid amplification testing (NAAT).

Cell culture is one of the earliest developed methods of testing, and involves growing the target pathogen and diagnosing the pathogen using some other method (visually, microscopy, ELISA, NAAT). However, cell culture requires long incubation times of many hours (rapid systems are still 8 h) which is not suitable or testing at the POC.[15]

There are two common implementations of protein-based testing: the enzyme-linked immunosorbent assay (ELISA) and the lateral-flow immunoassay (LFA). Both of these methods rely on antibodies which bind to the target antigen or protein of interest. Usually these antigens are peptides displayed on the surface of the pathogen or antibodies developed by the host (serology testing). A common concern for protein-based testing is non-specific protein adsorption or off-target binding, which leads to low specificity of the assay.[20,21] For ELISA specifically, assay times are long, and there are many user steps making ELISA unsuitable for POC testing.[22,23] Meanwhile, the LFA, which is commonly utilized in the off-the-shelf pregnancy test is ideally suited for POC testing, but lacks the sensitivity required for many diagnostic applications.[24-28]

NAAT is perhaps the most powerful of these three approaches, with its relatively short time-to-result, high sensitivity, and high specificity. For many pathogens, NAAT is the preferred gold-standard testing method. There are 3 components to a NAAT: (1) nucleic acid extraction, (2) nucleic acid amplification, and (3) readout. First, nucleic acids (NAs) are purified and concentrated from the patient sample to eliminate inhibitors present in the sample matrix. Secondly, the purified nucleic acids (NAs) are amplified, typically using polymerase chain reaction (PCR), to generate billions of DNA copies. This approach works by using conserved primer sequences that are specific to the pathogen of interest. Therefore, if the pathogen is present, many DNA copies will be generated. Lastly, the amplified NAs can be detected in a number of ways such as fluorescence or visual readout.

While NAAT excels with its high sensitivity and specificity, NAAT does not meet any of the remaining POC requirements (requires highly trained users in centralized labs with expensive and bulky equipment). In recent years, research labs have made developments towards POC NAAT.[29-33] The diagnostics industry has also been developing integrated POC NAAT assays, the two most POC-amenable being the Cepheid GeneXpert system and the Abbott ID Now.[19] Nevertheless, more improvements can still be made to ensure fulfillment of all the ASSURED criteria.

Outline

In this doctoral thesis, I document my contributions towards improved POC NAAT.

In Chapter 2, I identify extraction buffer carry-over in commercial NA extraction kits as a major culprit for inhibition in downstream NA amplification.[34,35] I address this concern with the addition of a two-phase wash (TPW) which acts to reduce carry-over. By improving sample preparation purification, this enables the use of greater volumes of purified eluent, thereby improving sensitivity and limit-of-detection of downstream tests.

In Chapter 3, I identify a problem with the titer reported for commercial stocks when examining the analytical sensitivity of the CDC-recommend RT-qPCR protocol for diagnosing SARS-nCoV-2. I used an ultrasensitive and precise assay, reverse transcription digital PCR (RT-dPCR), to independently quantify the concentrations of commercial nucleic acid stocks. This work has broader implications because one of the commercial stocks was genomic RNA from BEI and measured 270% and 300% compared to the label concentration. This stock has been used in at least 11 approved FDA Emergency Use Authorizations as of April 27, 2020. The precise and accurate reporting of stock concentrations is necessary to improve the evaluation of different NAAT kits.

In Chapters 4 and 5, I develop tools for solving the sample-to-answer interface problem. While much of the published literature has focused on individual components (NA extraction, NA amplification, or readout), relatively less work has been shown towards the integration of these components. I designed and developed a meter-mix device (Chapter 4) which addresses urine sample transfer to a NA extraction device while simultaneously lysing the sample (thereby simplifying the NA extraction).[36,37] I also designed a 3D printed C-clamp (Chapter 5) which facilitated sample loading into glass microfluidic devices for nucleic acid amplification.[38,39]

In Chapter 6 and 7, I developed MATLAB image processing scripts to automatically analyze images of a digital nucleic acid amplification tracked over time. Each experiment set contained 20,000 wells and 100s of images. The first script (Chapter 6) analyzed images with a single fluorescent channel,[40] and the second script (Chapter 7) utilized dual-channel fluorescence to enable melt-curve analysis.[41]

Lastly, in Chapter 8, I describe additional tools developed for integrated sample-to-answer NAAT diagnostics.[42] In particular, I developed a parallel-filled amplification module for conducting lyophilized and multiplexed loop-mediated isothermal amplification reactions (LAMP). I designed and developed an automated base station featuring Arduino-controlled motor rotation and a heating element to automatically operate a nucleic acid extraction device and nucleic acid amplification device. Lastly, I showed a cell-phone modified for

fluorescence readout and automated MATLAB image analysis for readout of the parallel-filled amplification module.

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