THE DEVELOPMENT OF FIELD FRIENDLY TOOLS FOR PHARMACEUTICAL ANALYSIS

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment if the Requirements
for the Degree of

Doctor of Philosophy

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Notre Dame, Indiana
December 2014
THE DEVELOPMENT OF FIELD-FRIENDLY TOOLS FOR PHARMACEUTICAL ANALYSIS

Abstract

By

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Substandard and falsified medicines that find their way into the pharmaceutical supply chain short circuit the efforts of health care systems around the world. On a global health scale, medicines that deliver inappropriate amounts of active ingredient(s) can lead to increased mortality and morbidity. Medicines with reduced active ingredients can encourage antimicrobial resistance and can increase the likelihood of transmission for communicable diseases. On an individual scale, this translates to time, money and lives lost to treatable diseases. My thesis research addresses this problem and has focused on the development of analytical tools for pharmaceutical screening in low resource settings, such as developing countries where the problem is most prevalent. My work pairs an inexpensive paper platform with both chemical and biological detection methods. Chemical test cards combine twelve different colorimetric tests arranged in lanes to produce unique “color bar codes” for several pharmaceuticals, including beta lactam antibiotics and anti-tuberculosis medications. Working with the United States Food and Drug Administration, I was able to use these test cards to identify a collection of counterfeit pharmaceuticals by recognizing deviations from authentic formulations. While the chemical test cards are rapid,
sensitive, and specific with an assortment of pharmaceuticals, there remains a group of
pharmaceuticals that do not produce a unique color bar code with the test cards. To
complement the chemical tests, I have also developed a biological paper-based test card
that incorporates engineered yeast, which have the ability to respond to compounds
with high specificity. My work details the development, optimization and evaluation of
this biologically based test that can identify the presence of doxycycline in
pharmaceutical dosage forms. It is my hope that these analytical tools and others like
them can be used in low resource settings to better define the problem of substandard
and falsified medicines, to identify poor quality medicines before they reach patients,
and to address global health problems where they exist.
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMPATH</td>
<td>Academic Model Providing Access to Healthcare</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BioPAD</td>
<td>Biological-based Paper Analytical Device</td>
</tr>
<tr>
<td>CD3</td>
<td>Counterfeit detection device</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CHMP</td>
<td>Centrale-Humanitaire Mecido Pharmaceutique</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>E, Eth</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform-Infrared Spectroscopy</td>
</tr>
<tr>
<td>FCC</td>
<td>Forensic Chemistry Center</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>I, Iso</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>MTRH</td>
<td>Moi Teaching and Referral Hospital</td>
</tr>
<tr>
<td>NGO</td>
<td>Non-Governmental Organization</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared Spectroscopy</td>
</tr>
<tr>
<td>NQS</td>
<td>1,2-naphtholquinone-4-sulfonate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>P, Pyr</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>PAD</td>
<td>Paper Analytical Device</td>
</tr>
<tr>
<td>PPC</td>
<td>4-pyridyl pyridinium chloride</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>Quamed</td>
<td>Quality Medications for All</td>
</tr>
<tr>
<td>R, Rif</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operator Characteristic</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic Complete (yeast media)</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>US EPA</td>
<td>Unites States Environmental Protection Agency</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Peptone Dextrose (yeast media)</td>
</tr>
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ACKNOWLEDGMENTS

From the beginning there were a number of people that shaped and contributed to the work presented in this thesis. Thanks to Dr. Marya Lieberman for getting me back into a research lab through the Research Experience for Teachers (RET) program. I had really signed up for the program to make some extra cash, but I got more out of it than I expected. Thanks to my husband, Murray, for telling me I looked 10 years younger when I talked about school (I would have taken 5), and to Evelyn and Ian who make me smile when they include, “My mom is a scientist” on lists of things people should know about them. Thanks to my mom, who has always been ready to help with childcare, so I could have time to get work done. Without the support of my family I couldn’t have completed this work, nor would I have tried.

I need to thank my advisors, Drs. Marya Lieberman and Holly Goodson, for the guidance, patience and freedom that they provided me as a graduate student, as well as my committee members Drs. Norm Dovichi and Josh Shrout, who always had open doors when I came to them with questions. Gathering this helpful foursome to listen and give feedback about my project always made my committee meetings a privilege and a treat.
Many people played a role in the development of the chemical and BioPAD test cards. This project might not have existed without the contributions of Dr. Toni Barstis of Saint Mary’s College, and my yeast based research benefitted from many discussions with Dr. Don Paetkau about yeast and genetic circuitry. Also, behind the test card development is a large group of undergraduate researchers from both the University of Notre Dame and Saint Mary’s College who put in many hours of work to establish this research project.

I want to thank Dr. Mark Witkowski who went above and beyond the call of duty to arrange my time spent at the United States Food and Drug Administration’s Forensic Chemistry Center (FCC). It felt like everyone at the FCC was willing to go out of their way to help and welcome me during my short stay there, and I am grateful for this positive experience. I am particularly grateful to the FCC’s Trace Analysis group and my collaborators Drs. Mark Witkowski, Cheryl Flurer, Nico Ranieri and Sara Andria for their assistance in my work with chemical test cards and counterfeits from the global supply chain.

Lastly, I want to thank my lab members from the Goodson and Lieberman Labs for their daily company on this journey. This group was always there to point out the holes and shortcomings in my presentations, so I could make every talk better and become a better presenter. Thanks to Valerie, Nick, Michelle, Jill, Aranda, Julia, Ben, Emily, Lori and Erin for the many lessons you have taught me and for celebrating with me when things went right.
CHAPTER 1

PHARMACEUTICAL ANALYSIS IN LOW RESOURCE SETTINGS

1.1 Introduction

Substandard medicines, defined as those that do not meet pharmacopeia standards,\(^1\) increase morbidity and mortality rates for treatable diseases and can accelerate the emergence of drug resistant variants in infectious diseases.\(^2\) In recent years, the pharmaceutical supply chain has grown more complex, with the United States (U.S.) currently importing 40% of finished medications and 80% of active ingredients.\(^3\) While the U.S. Food and Drug Administration (FDA) is taking actions to oversee manufacturing and tighten regulation of this expanding supply chain, examples of harmful medicines entering the U.S. are still a reality. In developing countries the situation is much worse,\(^2,4-8\) with studies finding up to 64% of medicines failing to meet Pharmacopeia standards.\(^9\) Access to quality medications is a human right\(^10\) and while the world seems to be getting smaller, the global supply chain is growing increasingly complicated.

As an introduction to this research problem, early in my graduate career I was able to travel to Moi Teaching and Referral Hospital (MTRH) in Eldoret, Kenya to meet with collaborating pharmacists interested in the idea of an inexpensive paper test for pharmaceutical quality. These pharmacists worked together in an overcrowded office
and when the lights flicked off someone assured me, “They will be on again shortly,” not bothering to look up from their work for an all too common occurrence. Later, during a hospital tour, I saw that chemotherapy treatments were housed in an outdoor tent, because this space was available and offered the most reliable ventilation. Throughout my visit, the themes of insufficient facilities and unreliable technology permeated this health care facility, the second largest national hospital in the country of Kenya. It’s in places like Moi Hospital that low quality pharmaceuticals can lurk undetected.

One of the most difficult tasks in addressing poor quality medicines has been determining the extent of the problem, and in 2014 researchers lament that “the global extent of the problem remains unknown.”\textsuperscript{11} The quality of antimalarials has received much attention, and a recent interactive database compiling information from reports of poor quality medicines has been developed to form a better overall picture of the problem.\textsuperscript{12} In compiling this information researchers noted problems with reports on pharmaceutical quality, such as 242 of 529 studies of antimalarial quality with small sample sizes (less than 10 samples), 74 of 529 studies that did not report sample size at all and 95\% of reports using random sampling that did not describe sampling methods in the publication. Overall, this meta-analysis reports that 30.1\% of medicines tested failed either chemical or physical analysis.\textsuperscript{11} This number is not only questionable, due to the quality of the underlying data, but is likely inflated because the percentage incorporates public alerts that only report the identification of poor quality medicines. This number currently represents the most comprehensive picture of antimalarial quality to date. An earlier review of antimicrobial drug quality summarizes fourteen
studies of antibiotics in developing countries that report prevalence of substandard samples ranging from 20 - 40 %. However, concerns of low sample number and lack of random sampling exist with these studies as well. These reviews highlight the need for well-designed studies of pharmaceutical quality to better define the problem of poor quality medications.

Another barrier to reducing the number of poor quality medicines in the supply chain is that there is no global regulatory oversight of pharmaceutical quality. Regulation of pharmaceutical quality is the responsibility of national drug regulatory authorities. These agencies are tasked with licensing pharmaceutical manufacturers and monitoring pharmaceutical manufacturing, importation and distribution. This type of oversight requires access to costly laboratory equipment and trained technicians, necessary for state of the art quality analysis and is difficult to establish and maintain in developing countries. The World Health Organization recommends, “Countries that do not have full–fledged drug quality control laboratories should look for simpler and less resource demanding testing/screening methods for the identification of counterfeit drugs.” Pharmaceuticals thought to be counterfeit following screening, would require further pharmacopoeia testing for confirmation. My thesis research has focused on the development of analytical tools that could be useful for pharmaceutical screening at points within the global supply chain when pharmacopoeia methods are not available.

Developing tools for use in resource limited settings presents unique challenges when compared to developing tools for use in a typical lab in a developed country. The World Health Organization developed the ASSURED acronym as a benchmark for field
friendly tests for the diagnosis of sexually transmitted diseases (Fig. 1.1a). This acronym serves as a useful guide for the development of other analytical tools for use in remote and low resource settings. To test medicines in rural Kenyan pharmacies or clinics as well as in remote locations within developed countries, tests must be more than just sensitive and specific. These tests must reach remote areas, perform without specialized equipment or highly trained technicians, and be available at a fraction of the cost of other analytical methods.

\[\text{Affordable} \quad \text{Sensitive} \quad \text{Specific} \quad \text{User-friendly} \quad \text{Rapid and robust} \quad \text{Equipment free} \quad \text{Deliverable to end-user}\]

**Figure 1.1 Field-friendly technology helps monitor drug authenticity.** a) ASSURED criteria outlined by the World Health Organization that has been used by many as a guide for the development of field friendly technology for use in developing countries. b) The Sproxil pharmaceutical verification method that uses a scratch off label to reveal a code that is phoned into a system to confirm that the sample is produced by a registered manufacturer.

Among current methods being employed for pharmaceutical analysis in low resource environments are handheld X-ray fluorescence,\(^{16}\) Raman,\(^{17}\) and infrared\(^{18}\) spectroscopy devices, which have strengths and weaknesses as field-ready tools. These handheld devices are portable, easy to use, and generally produce results in less than a minute. A
recent study of one handheld Raman spectrometer concluded that it is limited to qualitative analysis of counterfeits, appropriate only for indicating medicines containing substitute or no active pharmaceutical ingredient (API).\textsuperscript{19} These portable spectrometers come with a high initial investment cost and must be preloaded with a library of standard spectra of approved pharmaceuticals. To remain up to date, this library should ideally be updated on a yearly basis, as the list of approved pharmaceuticals from manufacturers fluctuates from year to year. While this technology is used by regulatory agencies, such as the FDA for on-site testing, it is too costly to be the answer for clinics and pharmacies scattered in rural regions of developing countries.

A less expensive approach, taken by many manufacturers, incorporates security features into product packaging and labeling, such as fluorescent inks, microprinting, holographic stickers and scratch off labels (Fig 1.1b).\textsuperscript{20,21} However, labeling-based systems provide no chemical information about a sample, require the collaboration of manufacturers and regulatory agencies, and are themselves vulnerable to imitation.\textsuperscript{22}

A third approach, The Global Pharma Health Fund Minilab\textsuperscript{\textregistered},\textsuperscript{23,24} combines packaging analysis with dissolution testing, colorimetric testing\textsuperscript{25–29} and/or thin layer chromatography. While the cost per test is low, complete testing takes hours, requires the handling of hazardous chemicals and results must be interpreted by a trained technician. My work has focused on developing an analytical tool for pharmaceutical quality that meets the ASSURED criteria better than the existing technology.

At the foundation of my research is the use of paper. Given advances in printing and patterning,\textsuperscript{30,31} paper can produce versatile and sophisticated testing platforms at a low
cost. Paper test strips recognizing chemical functional groups, such as the litmus test for acids and bases, have a long history. The advantages of these tests are that they are cheap, easy to use, and don’t require a lab setting or instrumentation. However, they are generally restricted to use with liquids and lack chemical specificity. Current research has creatively expanded the capabilities of paper based tests to carry out analyses of complex samples by incorporating biochemical selectivity (enzymatic reactions, antibody/antigen binding) into paper millifluidic devices.\textsuperscript{32–36}

My research takes two approaches, one chemical and one biological, to develop tests for pharmaceutical analysis. Pairing paper with classic color producing chemistry\textsuperscript{37} has led to the development of an analytical tool described in this thesis, that produces a profile of a pharmaceutical sample based on the presence of chemical constituents. Chemical testing has the power to rapidly classify compounds without using lab equipment. The specificity of this tool is gained through a series of color producing chemical reactions that build a chemical profile of each sample. To expand on the research with chemical PADs, I have also worked to develop a paper analytical device incorporating a whole cell biosensor. Biological systems have the ability to respond to very low concentrations of analyte such as tetracycline at 1 µg/mL\textsuperscript{38} and iron at 5 ng/mL\textsuperscript{39} with high specificity. Incorporating whole cell biosensors onto a paper platform has the potential to produce a portable, highly sensitive and specific analytical tool. My work using yeast as the sensing organism expands the field of paper-based biosensors which, to date, is limited to the use of bacterial systems.\textsuperscript{40,41}
Historically, color producing chemistry has been a useful tool for chemical identification and yeast have been a common workhorse microorganism for tasks such as making bread and brewing beer. I have found in my work that coupling simple components to a paper platform can fashion simple, but eloquent analytical tools. Such tools, described in the following chapters, have the potential to carry chemical analysis out of the lab and into settings, such as warehouses, small clinics, pharmacies and remote locations in both developed and developing countries.

1.2 References


17. Ricci C, Nyadong L, Yang F. Assessment of hand-held Raman instrumentation for in situ screening for potentially counterfeit artesunate antimalarial tablets by FT-


CHAPTER 2

PAPER ANALYTICAL DEVICES FOR FAST FIELD SCREENING OF BETA-LACTAM ANTIBIOTICS
AND ANTI-TUBERCULOSIS PHARMACEUTICALS

2.1 Preface

This chapter has been reprinted with permissions from Analytical Chemistry (Weaver et al., 2013, Analytical Chemistry (85) 6453-6460). This manuscript is based on work from the following authors: Abigail Weaver, Hannah Reiser, Toni Barstis, Michael Benvenuti, Debarati Ghosh, Michael Hunkler, Brittney Joy, Leah Koenig, Kellie Raddell and Marya Lieberman. The original concept of PADs was developed by TB and ML. Validation of PADs for identification of beta-lactam medications was carried out by AW. Validation of PADs for identification of anti-tuberculosis medications was carried out by AW, HR and LK. MH contributed to test card fabrication methods. Storage stability studies were carried out by MB. Lane test development was carried out by HR, DG, BJ, ML and AW. Average amount of substance deposited on test cards was determined by KR. This manuscript was written by AW, ML and HR.
2.2 Introduction

The problem of substandard and counterfeit medicines is worldwide,\textsuperscript{1,2} but most acute in developing countries.\textsuperscript{3-8} State of the art pharmaceutical testing, such as high performance liquid chromatography or mass spectrometry, provides powerful tools to identify active pharmaceutical ingredients (APIs), but is expensive and requires skilled technicians, reliable laboratory infrastructure, and resources to buy, operate, repair, and maintain instruments. To address the problem of substandard medicines around the world, detecting poor quality medication in low resource settings is an urgent public health issue in need of inexpensive, user-friendly tools to increase screening capacity within the pharmaceutical supply chain. This chapter looks at the ability of paper analytical devices (PADs) to provide a qualitative chemical profile of pharmaceutical formulations (Figure 2.1).\textsuperscript{9}
The goal of this first generation of PADs (Figure 2.2) is rapid field screening: qualitative determination of the presence of APIs, detection of substitute APIs, and detection of unapproved excipients. Such a tool could be used at various points in the supply chain to detect very low quality pharmaceuticals that have the most potential to harm patients. The PADs described in this manuscript are the size of a playing card and cost around $0.40 USD. They provide a portable, inexpensive means of pharmaceutical chemical analysis that fills a niche no other pharmaceutical authentication technology has been able to achieve. Combining a library of tests that use classic colorimetric chemistry onto one device, these PADs detect active pharmaceutical ingredients and excipients to pro-

**Figure 2.1 Schematic of PAD testing procedure.** A tablet is “swiped” to deposit sample (step 1). The bottom edge of the device is set into water to bring together reagent and analyte by capillary flow (step 2). Colors produced by an authentic versus a substandard drug will vary in one or more lanes, reflecting differences in chemical make-up.
vide a qualitative chemical profile of a medicine and require only a cup of water to carry out testing. A pharmaceutical screening test that does not require a lab would be useful to hospitals, clinics, pharmacies and non-governmental organizations as added quality assurance during the purchasing process. Health care workers could use the devices when patients do not respond to a medication to determine if the cause might be administration of a substandard medicine as opposed to drug resistance. Additionally, such a tool could be used by regulatory agencies to implement low-cost, higher frequency testing of pharmaceuticals along the supply chain.
Figure 2.2 Examples of beta-lactam PADs (top panel) and TB PADs (lower panel). (a) 12-Lane beta-lactam PAD spotted with reagents. (b) Beta-lactam PAD with 100% amoxicillin gives dark green in lane A, green in lane B, and orange in lane G. (c) Beta-lactam PAD with a 2:1 w/w mixture of ampicillin and calcium carbonate gives blue-green in lane A, orange in lane B, and no color in lane G (indicating ampicillin rather than amoxicillin) and dark orange at the swipe line in lanes I and J (indicating carbonate). (d) Beta-lactam PAD with maize flour gives no API colors and dark purple at the swipe line in lane H (indicating starch). (e) TB PAD spotted with reagents. (f) TB PAD with isoniazid/ethambutol combo gives royal blue at the top of lane A (ethambutol), green near swipe line in lane B as well as bright orange-red in lane E (isoniazid). (g) TB PAD with 4-drug combo formulation produces a complex result, with orange across the entire swipe area and black in lanes C and I (rifampicin), orange and orange-red coloration in lanes D and E indicating pyrazinamide and isoniazid respectively. Rifampicin interferes with detection of ethambutol in lane A. (h) TB PAD with diphenhydramine produces a bright aqua color in lane K, indicating an API not expected in TB medications.
2.3 Experimental

The Appendix D contains complete description of the materials used for the fabrication of the PADs and the lab validation study as well as details of test development and stability testing.

2.3.1 Lab validation-generation of fringe diagrams

For each lane, positive and negative outcomes were first defined based on images of standard test results obtained with known chemicals in a lab setting. Once these standards had been generated, unknown samples were run and two readers compared each lane to the standards to determine the presence or absence of specific functional groups in the unknowns. In the case of lane tests with binary or tertiary outcomes, such as the starch or talc tests, positive results were identified by the presence or absence of the appropriate color found in the specified location (Figure 2.3, upper panel). Lane results that did not resemble a positive or negative outcome were classified as negative for the analyte in question. Some lane tests, such as the copper (II) on the TB PAD, gave different colors (sometimes in different locations) depending on which pharmaceutical was applied. These tests could no longer be treated as binary tests, and a more complex set of standards known as the “fringe” analysis (due to the image’s resemblance to carpet fringe) was used to categorize results (Figure 2.3, lower panel and Figure S2.2, available at http://pubs.acs.org/journal/ancham). The “fringe” analysis was generated by running multiple samples containing pure pharmaceutical ingredients, combinations of pharmaceutical ingredients (in the case of the TB drugs, all possible binary, ternary, and quater-
nary combinations were used, see Table D.2) and pure excipients. Replicate tests were carried out using light, medium and heavy swiping of the analyte, generating six test results per sample. Light swipes consisted of a barely visible dusting of the powdered pharmaceutical onto the PAD; gravimetric analysis with an analytical balance showed deposition of an average of about 0.5 mg of material in each lane. Heavy swipes consisted of a layer of sample thick enough to obscure the grey color of the wax lines beneath the “swipe”, which provided 2-6 mg of material in each lane (Table D.3). Medium swipes were intermediate in coverage. These loading levels were chosen based on our observation of the variability of the amount of powder participants in a 500-person field test had loaded on PADs for detection of acetaminophen.\textsuperscript{10} PADs were run in deionized water until the timer spot appeared and were allowed to dry flat for 5 minutes for optimal color development. Each PAD was imaged with a cell phone or digital camera and individual lane images were extracted for the different samples. Images from each lane were laid side by side to produce a colorful “fringe” of outcomes based on the identity and quantity of the sample. The readers could then match an observed lane outcome with colorimetric outcomes associated with the known samples.
Figure 2.3 Validation standards used by readers for the analysis of PAD colorimetric results. Tests with binary or ternary outcomes are compiled into a small color key (top panel). Tests of the TB PAD present a more complex array of results and are evaluated using the “fringe” diagram. Represented here is a portion of the fringe diagram for the copper sulfate test for ethambutol (lower panel). The “fringe” diagram is a collection of outcomes from testing different quantities of all 15 combinations of the four main TB drugs, plus a library of excipients and substitute APIs. The reader matches each lane of the PAD to the most similar group of images to identify analytes present. See Figure S2.2, available at http://pubs.acs.org/journal/ancham, for the complete fringe diagram.
2.3.2 Lab validation-analysis of unknowns

For laboratory validation of PADs, a light to medium swipe of each known sample was applied to the swiping region of the PAD. Sample identities were coded, so readers did not know what sample had been run on the PADs they were analyzing. PADs were run in distilled water until the appearance of the timer spot. Following removal from the water, images of the PADs were taken within 3-5 minutes using a cell phone or digital camera. These images were analyzed by two expert readers with access to standard results and “fringe” diagrams for comparison. If the expert readers did not agree on test outcomes, a third reader was used as a tie-breaker. The tie-breaker was only required for about 10% of the 12-lane PADs, so overall, only about 1% of the lanes were ambiguous enough to require extra attention.

2.4 Results

2.4.1 Fabrication of the 12-lane PAD design is fast and inexpensive.

Eight PADs fit onto each 8.5 x 11" sheet of paper without wastage, and for accurately cut pages, the three layers of printing (front color laser, front wax, and back wax) aligned within the 0.5 mm tolerance 93% of the time. Batches of pages were baked in an oven for 9 minutes to give reliable hydrophobic barriers. We found that the slowest and most error-prone step in fabrication, spotting the 20-30 reagents in the correct lane locations, could be accelerated to a rate of 1/min and an error rate under 6% by a liquid delivery robot. A batch of 60 PADs can be made start to finish in under 2 hours and at a materials cost of under $0.12 per PAD. With labor at $10/hour, the cost per PAD is about $0.45.
2.4.2 Paper analytical devices show high sensitivity and specificity for active pharmaceutical ingredients and excipients

In order to determine whether the PADs could reliably detect the presence or absence of target APIs and excipients, lane tests from both TB PADs and beta lactam PADs were carried out in a lab setting to determine operational parameters and expected outcomes, as described in the experimental sections, and then evaluated in a blinded validation study. The samples used in the validation study are listed in Table 2.1 and Table D.4. Each sample was coded so the person evaluating the PAD did not know the sample identity, and the serial numbers of the PADs were randomized so readers would not encounter clusters of identical PADs. A medium swipe of each sample (0.5-2 mg per lane) was applied to PADs with a spatula in the appropriate location. The PADs were run in deionized water until the timer spot was fully formed, and each PAD was laid flat for 3-5 minutes for color development, and then imaged with a cell phone camera or digital camera. The photos were acquired in a home-built light box to provide uniform illumination and focal length. Two readers evaluated the PAD images lane by lane, comparing the appearance of each lane to standard positive/negative images or to a fringe diagram. Lanes were scored as positive or negative for each of the analytes they were designed to detect (eg, the copper (II) test was scored for detection of any beta lactam antibiotic, but not for detection of carbonate). Ambiguous or weak test results were scored as negatives (see scoring examples in Figure D.3). After un-blinding the samples, the sensitivity of each lane was determined based on the number of samples that should have been detected by that lane and the number actually detected, and the selectivity by the num-
number of samples that should not have been detected by that lane and the number detected as positives.

### TABLE 2.1

SENSITIVITY AND SELECTIVITY FOR DETECTION OF PURE API'S AND EXCIPIENTS

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Test</th>
<th>Type</th>
<th>Sensitivity</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactam 100% (20 ampicillin, 20 amoxicillin)</td>
<td>SaturatedCu(II)</td>
<td>Whole PAD</td>
<td>100% (40/40)</td>
<td>100% (48/48)*</td>
</tr>
<tr>
<td>Ampicillin 100%</td>
<td>Ninhydrin</td>
<td>Whole PAD</td>
<td>90% (18/20)</td>
<td>100% (66/66)b</td>
</tr>
<tr>
<td>Amoxicillin 100%</td>
<td>Ninhydrin</td>
<td>Whole PAD</td>
<td>100% (18/18)</td>
<td>100% (71/71)c</td>
</tr>
<tr>
<td>Amoxicillin 100%</td>
<td>Nitroaniline</td>
<td>Whole PAD</td>
<td>89.5% (17/19)</td>
<td>100% (71/71)d</td>
</tr>
<tr>
<td>Ethambutol 100%</td>
<td>1M Cu (II)</td>
<td>Lane</td>
<td>100% (24/24)</td>
<td>98% (101/102)</td>
</tr>
<tr>
<td>Rifampicin 100%</td>
<td>Iron (III)</td>
<td>Lane</td>
<td>100% (30/30)</td>
<td>88% (86/98)</td>
</tr>
<tr>
<td>Pyrazinamide 100%</td>
<td>SNP</td>
<td>Lane</td>
<td>92% (24/26)</td>
<td>98% (47/48)</td>
</tr>
<tr>
<td>Isoniazid 100%</td>
<td>NQS</td>
<td>Lane</td>
<td>100% (24/24)</td>
<td>98% (47/48)</td>
</tr>
<tr>
<td>Chalk 100% (CaCO₃)</td>
<td>FeCl₃</td>
<td>Whole PAD</td>
<td>50% (6/12)*</td>
<td>100% (84/84%)e</td>
</tr>
<tr>
<td>Baking soda 100% (NaHCO₃)</td>
<td>FeCl₃</td>
<td>Whole PAD</td>
<td>100% (36/36)</td>
<td>100% (120/120)e</td>
</tr>
<tr>
<td>Starch 100%</td>
<td>Iodine</td>
<td>Whole PAD</td>
<td>94% (34/36)</td>
<td>96.2% (75/78)</td>
</tr>
<tr>
<td>Talc 100%</td>
<td>Eosin Red</td>
<td>Lane</td>
<td>97% (35/36)</td>
<td>100% (36/36)g</td>
</tr>
<tr>
<td>Acetaminophen 100%</td>
<td>Nitroaniline</td>
<td>Whole PAD</td>
<td>100% (6/6)</td>
<td>100% (12/12)g</td>
</tr>
</tbody>
</table>

*All instances of light swiping produced a false negative with this test. "Whole PAD" data were taken from PADs with 12 different lanes; "lane" data were taken from PADs with multiple copies of certain lanes. Additional footnotes for Table 2.1 are provided in Table D.1A.
In the blinded validation tests, every pure API and every excipient tested with a moderate to heavy application of analyte was detected with sensitivity of 92%-100% and selectivity of 88%-100% on specific lanes. For the beta lactam antibiotics ampicillin and amoxicillin, the basic Cu(II) lane gave 100% sensitivity and specificity for detection of the beta lactam functionality. Ampicillin and amoxicillin have very similar chemical structures, but were reliably distinguished both by the different colors produced in the ninhydrin lane and by the positive nitroaniline test obtained for amoxicillin. The four major TB drugs rifampicin (Rif, R), isoniazid (Iso, I), pyrazinamide (Pyr, P) and ethambutol (Eth, E) were detected with high sensitivity and selectivity. Isoniazid, in addition to giving a 100% sensitive and 98% selective response on the napthaquinone sulfonate lane, also showed a characteristic green color in the Cu(II) lane used in beta-lactam analysis. This proved to be a reliable secondary test, although we did not score it for lack of standard images, which were subsequently assembled. Acetaminophen forms a grey or brown colored complex with iron; all acetaminophen samples gave false positive results for rifampicin on the Fe(III) lane. In a whole-PAD analysis, acetaminophen can be reliably distinguished from rifampicin by its positive nitroaniline test and by the lack of the orange color of rifampicin.

Tuberculosis medications for uncomplicated TB are almost always given as combination tablets containing two, three, or all four of the first-line drugs (Table D.2). The two-drug combination of ethambutol and isoniazid was properly detected 30 out of 30 times with no false positives for this combo. The three- and four-drug combinations both contain rifampicin, whose strong orange color interfered with the ethambutol test. Rifam-
picin produced a strong purple color in the Cu(II) lane that partially masked the green from isoniazid, but the isoniazid reaction with NQS was not affected by rifampicin. While isoniazid and pyrazinamide were both reliably detected in the presence of each other and the presence of rifampicin, ethambutol was missed 30% of the time in the presence of rifampicin, and in the presence of rifampicin, ethambutol was detected 17% of the time even when it wasn't there. The ethambutol test is therefore not sufficiently reliable to detect presence/absence of ethambutol in formulations that contain rifampicin. Neglecting the ethambutol, formulations containing RIP were properly identified in 58/60 cases (Table D.4).

Very low quality drugs are often adulterated with fillers such as calcite, talc, or starch, or expensive APIs may be substituted with inexpensive drugs including acetaminophen, quinine, diphenhydramine, or chloroquine. The presence of unapproved fillers or substitute APIs is a critical warning sign. Several of the analytes were designed to simulate falsified drugs. Samples of ampicillin and amoxicillin were cut with 50% w/w talc to simulate an adulterated antibiotic; the PADs detected the talc in 81% of the samples. To mimic poor quality TB medications, rifampicin was mixed with 90% w/w potato starch; even though the dark orange color of rifampicin makes it difficult to see the starch color, 73% of the adulterated samples were detected. One sample of pure pyrazinamide was cut with 50% w/w turmeric to mimic the orange color of rifampicin ex-

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1 "Some circumstantial evidence is very strong, as when you find a trout in the milk" (H. D. Thoreau, Journal, Nov. 11 1854)
pected in an RP combination medicine. We had not tested turmeric on the PADs and found that it gave unusual colors in several lanes; all of the readers agreed that the suspect formulation did not match any of the possible TB drug combinations and flagged 100% of them as suspicious on the whole-PAD analysis. Finally, a set of substitute pharmaceuticals was tested, including the analgesic acetaminophen, antimalarial drugs quinine sulfate and chloroquine phosphate, and the antihistamine diphenhydramine. All of these inexpensive pharmaceuticals have been used as substitute APIs in counterfeit drug formulations, and each of these substitute APIs was detected with 100% sensitivity and specificity.

2.4.3 Time restrictions for PAD analysis

Once the sample is applied, the PAD is set in water up to the blue line and held upright for about four minutes. Capillary action carries water up the hydrophilic test lanes and brings together test reagents and analyte. The outcome is a “color bar code” at the top third of the PAD, providing the basis of sample characterization. The variety of chemical tests and transient nature of many colorimetric tests provide a challenge to PAD analysis. To obtain the best test results the timing of 1) test run duration and 2) test imaging are both important factors. One lane houses a control reaction that generates a strong pink color; this lane serves as a timer. When the pink blaze at the top of the lane is fully formed, it signals the user that the test run is complete and the PAD should be removed from the water.
To indicate the best time for analysis of the “color bar code” we measured color intensity of the two tests whose color outputs 1) decayed most rapidly (the starch/iodine test) and 2) appeared most slowly (the talc/eosin red test). The optimum time for capturing the image of the PAD for color analysis was determined by the intersection of the color development of these two tests, and was found to be between 5-7 minutes (Figure D.4). The blue cobalt thiocyanate reagent turns pink when it is eluted up the lane by the water, but dries to a blue color that begins at the top of the lane. A distinct blue rim is visible at 15 -20 minutes after removal from water, and indicates that a user has waited too long to take the image (Figure D.5). In PAD analysis, the appearance of the timer blaze and pink at the top of the cobalt thiocyanate lane both indicate that the user is compliant with PAD testing requirements.

2.4.4 Development of lane tests for the beta-lactam and TB PADs

Many color-generating reactions have been used in low-resource settings for the detection of APIs and excipients.\textsuperscript{13–16} The World Health organization (WHO) has published a series of monographs on simple color reactions and precipitation test procedures for pharmaceutical substances and dosage forms, intended for use in developing countries.\textsuperscript{17,18} Several pharmaceutical associations and organizations have developed testing specifications that can be carried out in a laboratory with limited instrumentation (eg, UV-vis spectrometer, analytical balance, and potentiometer).\textsuperscript{19,20} Many of the classic color tests used in these procedures originated before the 1960's, and survive today as TLC development procedures.\textsuperscript{21} The adaptation of inorganic and organic color tests to
microliter scale using spot plates and filter or reagent papers, is comprehensively described in Fritz Feigl’s monograph. However, many of these laboratory-based spot tests involve digestion in strong acids, heating, and toxic, volatile or unstable reagents, and are not suitable for field use. The paper test devices must operate reliably in low resource settings, and this imposes a very stringent set of requirements on prospective color reactions:

1) The reaction must work at room temperature. Controlled heating is not common in many field settings, and a chemical heater would increase the cost of the PAD.

2) All reagents and the overall reaction must tolerate water, as non-aqueous solvents (even ethanol) are not reliably available in the developing world. Including solvents in a kit form would increase costs and introduce hazard and waste disposal issues.

3) All reagents must be stable during storage on paper, which eliminates use of concentrated acids (which eat away the cellulose), volatile materials such as iodine (but see below), or highly reactive species such as nitrous acid (see below).

4) The quantity of reagents on each PAD must be small enough that the PAD would not be treated as hazardous waste according to US EPA standards, limiting use of most heavy metals and highly toxic reagents.

5) And finally, the array of reagents used in the available lane tests has to provide good sensitivity and specificity for identification of not only the targeted APIs, but for excipients and substitute APIs that might be present in low quality pharmaceuticals.

As an example of the process of adapting literature color reactions as colorimetric tests in a lateral flow paper device, it is useful to focus on two copper-based lane tests.
which were selected for use on the beta lactam and TB drug PADs. A mixture of copper(II), hydroxide, and potassium tartrate is a useful test reagent for beta lactam antibiotics. The dark green compounds that form are not crystallographically characterized, but spectroscopic data has been used to justify a variety of chelation modes between the copper and nucleophilic groups in ampicillin and amoxicillin.\textsuperscript{23–27} Cu(II) also serves as an indicator for the substituted ethylenediamine group present in ethambutol; a dark royal blue color results from formation of a chelated complex ion.\textsuperscript{17} Both of these literature color tests are conducted by adding solutions of copper salts to solutions of the pharmaceuticals, which requires volumetric measurements, balances, and a modicum of skill and training for the operator. In order to translate these tests to PAD format, aliquots of copper salts had to be stored in stable form on the paper and some base had to be introduced as well to increase the solubility of the drugs (the beta lactams all become much more soluble in basic solution). An additional complication is that copper (II) reacts with both carbonate and hydroxide to form insoluble colored precipitates, which might block fluid flow up the paper, compete with the colors of the test results, or use up the copper before it could reach the pharmaceutical. Spot testing and paper chromatography were used to identify reagents that gave strong colors with ampicillin, amoxicillin, or ethambutol. Then, a 12-lane PAD was used to systematically vary the positions and concentrations of these components (copper sulfate and basic copper tartrate as copper sources, potassium carbonate and sodium hydroxide as bases) and lateral flow tests were conducted using light swipes of seven APIs that contain amine groups.
Two slightly different lanes were selected for incorporation into the antibiotic PADs and the TB PADs. The optimal formulation for detection of beta lactams is saturated \( \text{K}_2\text{CO}_3 \) on the swipe line and \( 1\text{M Cu(SO}_4\text{)}_2 \cdot 5\text{H}_2\text{O} \) just above the swipe line; this gives an intensely colored forest green band nearly 0.5 cm long at the top of the lane with both ampicillin and amoxicillin, and a light blue-green precipitate of basic copper carbonate in the absence of beta lactams. Some difficulties were encountered in spotting this lane, as the potassium carbonate solution had a tendency to ball up on the paper, particularly if the droplet was not deposited precisely in the center of the lane. Improvements in alignment of the PAD blanks eliminated this problem. Placing the copper below the swipe line nearly eliminated the green color because most of the copper precipitated before it could encounter the pharmaceutical. When NaOH was used as the base in place of carbonate, large amounts of turquoise colored copper hydroxide precipitated where the copper encountered the base, and capillary flow was slowed compared to other lanes. In contrast to the beta lactam lane, the optimal formulation for detection of ethambutol was to place copper below the swipe line and either carbonate or hydroxide on or above the swipe line; in the presence of ethambutol, a royal blue compound that moved at the solvent front was formed as the copper and pharmaceutical traveled through the basic zone of the lane. Both of these optimal copper lanes also give a green color with low chromatographic mobility when isoniazid is present at the swipe line (both isoniazid and ethambutol are independently detected by the same lane). In later testing of these lanes in the final antibiotic PAD formulation we found that a minty-green mobile color is also formed from salicylic acid (degraded aspirin).
2.4.5 Differentiation of ampicillin and amoxicillin with ninhydrin

Ninhydrin in lane B was used to discriminate between ampicillin and amoxicillin. Primary amines readily add to the electrophilic central ketone in ninhydrin, giving Schiff base compounds that undergo Schiff exchange reaction and dimerization to form an intense purple chromophore on heating. At room temperature in water ampicillin forms an orange colored species, while amoxicillin gives a forest-green color, presumably due to formation of Schiff base intermediates (Figure 2.2). Ninhydrin has poor solubility in water and must be spotted in acetonitrile. Excess potassium carbonate was placed below the swipe line to neutralize any primary amines present in protonated form and to help dissolve the antibiotics. During storage, the lanes often develop faint purple smears near the potassium carbonate spots (possibly due to adventitious amines reacting with traces of ninhydrin) but they retain the ability to differentiate the two antibiotics for at least 2 months at room temperature (Figure D.6), at least 104 days at 37°C and at least 30 days at 60°C (Figure D.7). Control lanes that contained ninhydrin and base, plus 4 ul spots of either ampicillin (13 mg/ml) or amoxicillin (4 mg/ml) were intended as standards to show the proper colors of the ninhydrin reaction, but proved ineffective. Actual swipe samples always show three nearly identical color outcomes (all green or all orange) because the relatively high dosing provided by even a light swipe deposits so much ampicillin or amoxicillin in all three ninhydrin lanes that the control reactions are masked. The competitive reaction between drugs stored in the lane and drugs applied to the swipe area is still an area of active study because of the potential for quantification of the amount of ampicillin or amoxicillin in the swipe line.
2.4.6 Detection of electron-rich phenols with nitroaniline.

Both amoxicillin and acetaminophen (a substitute API that has been found in several counterfeit anti-infective drugs\textsuperscript{12,16,28}) contain electron-rich phenols. This functional group can be detected by its reaction in basic solution with diazotized aromatic groups, which are in turn formed by reacting anilines with nitrous acid. While nitrous acid is itself unstable, it can be formed in situ from the reaction of NaNO\textsubscript{2} and a strong acid. Thus, this lane contains four reagents: at the bottom, tosic acid (a strong acid that is stable on paper), then NaNO\textsubscript{2}, nitroaniline, and at the swipe line, sodium hydroxide. As the water moves up the lane by capillary action it successively mixes these reagents, forming HONO, diazotizing the nitroaniline, and finally reaching the basic region where the excess acid is neutralized and any phenol groups in the sample are deprotonated. This lane produces a strong orange color with amoxicillin (Figure 2.2) or acetaminophen; the two drugs can be differentiated by cross-checking with both the ninhydrin lane and the copper lane (both are negative for acetaminophen). The lane shows good stability following 2 months at room temperature with a clear, though fainter, positive result (Figure D.6). This test remains viable for at least 104 days at 37°C and can withstand 5 days of storage at 60°C (Figure D.7).

2.4.7 Detection of nucleophilic functional groups with pentacyanoaquoferroate

Sodium pentacyanoaquoferroate Na\textsubscript{3}Fe(CN)\textsubscript{5}H\textsubscript{2}O is a moderately labile complex that undergoes replacement of the coordinated water by strong nucleophiles in basic solution. The inorganic reagent is formed by hydrolysis or photolysis of Na\textsubscript{2}Fe(CN)\textsubscript{5}NO (sodi-
um nitroprusside or SNP). Isoniazid, which contains an acyl hydrazine, gives a strong yellow-orange color which develops within five minutes, while pyrazinamide, like pyridine, gives a reddish-orange color (Figure D.2) which is stronger than the yellow isoniazid color. Tests remain viable for at least 104 days at 37˚C (Figure D.7). The presence of isoniazid can be confirmed by checking copper lane tests for a characteristic light green precipitate at the swipe line, and the NQS lane for a dark red-orange addition compound that forms at the top of the lane.

2.4.8 Detection of primary amines and hydrazines with 1,2-napthoquinone-4-sulfonate (NQS)

NQS undergoes coordinated Michael addition/elimination reactions and/or Schiff base substitution reactions with amines and hydrazines in basic solution. At room temperature, the reaction with strong nucleophiles like the acyl hydrazine group in isoniazid is rapid, while primary amines like ampicillin or amoxicillin react slowly. The NQS must be spotted below the drug swipe and a spot of concentrated NaOH (6M works best) placed at the swipe line. Isoniazid gives a strong orange-red color; rifampicin shifts the color towards red-black.

2.4.9 Detection of tertiary amines with cobalt thiocyanate

A modification of the Scott test for alkaloids was used as a colorimetric indicator of compounds with tertiary amines. A solution of purple cobalt thiocyanate deposited below the swipe line turns pink as the water carries it up the lane, but gives intense blue or
green colors with pharmaceutical compounds such as quinine sulfate, chloroquine, amodiaquine, and diphenhydramine. Following the testing of unknowns with the TB PAD this test was further optimized to improve the detection of quinine sulfate. Placement of a 4 µL aliquot of 2M cobalt thiocyanate below the swipe line with a 4µL spot of 1M tosic acid immediately below the cobalt thiocyanate improved the color formation in response to the tertiary amine in quinine sulfate (Figure 2.3).

2.4.10 Detection of starch with triiodide

The blue starch/tri-iodide complex has been used as an indicator in iodometric titrations for more than 100 years. Tri-iodide is formed from iodine in the presence of excess iodide. However, tri-iodide is only stable on the paper lanes for a few weeks, because iodine is both volatile and soluble in the hydrophobic wax barriers on the PAD. Addition of povidone (polyvinylpyrrolidone, 1% w/w in water) stabilized the tri-iodide, probably by hosting it in the hydrophilic polymer coil. Starch is detected as a dark blue or black coloration that may occur at the swipe line (for insoluble starches like those found in flour) or above the swipe line (for soluble starches). Even trace quantities of starch, like those used as a binder in commercial wallboard, are strongly detected. The tri-iodide/povidone lane is stable for at least 2 months at room temperature, though a yellowing of the paper occurs when PADs are stored open to air for this length of time (Figure D.6). The iodine test remains viable for 66 days at 37°C (Figure D.7).
2.4.11 Detection of talc by selective dye binding

Talc is a magnesium silicate mineral with formula $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$. It is insoluble in water and has only sparing solubility in strong mineral acids, so detection is a challenge. Each unit cell of talc contains two surface exposed hydroxyl groups, which are potential binding sites for dye molecules. A library of 48 dye molecules was screened to identify 12 dyes that were either immobilized on solid talc swiped on Ahlstrom 319 paper, or changed color after encountering the talc. These dyes were further tested in lateral flow mode on PAD blanks. Eosin Red gives a vibrant pink color on the paper, but if it passes over a “swipe” of talc, a vibrant red color develops at the swipe mark after about 5 minutes. Freshly spotted tests performed well in lab validation, however, in PADs that were tested one or more days following fabrication eosin red did not migrate well. In the TB lab validation carried out over 2-7 days following fabrication, 43% (65/150) of eosin red tests did not run to completion.

2.4.12 Timer lane for consistent run times

A spot of nioxime or dimethyl glyoxime is placed below the swipe line, and a spot of Ni(II) is deposited at the very top of the lane. The nickel has a very pale color and is not visible in the lane. When the chromogenic ligand reaches the metal, an intense pink spot is formed, which indicates to the user that the PAD should be removed from the water. This lane also serves to detect user errors such as failing to run the PAD completely (no spot appears) placing the PAD in the water upside-down (spot shows up in
the wrong location) or dunking the entire PAD into water (reagents wash off and spot does not appear).

2.5 Conclusions

The presence of substandard and fake medicines in many parts of the world attests to the need for more accessible and inexpensive methods of pharmaceutical screening. PADs developed in our lab use inexpensive paper-based technology coupled with robust colorimetric chemistry to create an eloquent solution to this problem. Overall, these tests show high sensitivity and specificity for analytes and provide a useful chemical profile of APIs and excipients with a few minutes. The analytical metrics are good enough to detect very low quality drugs in wide-scale field screening. Although the tests do not directly address the issues of patent violation and unauthorized manufacturing—a counterfeit brand name product that contained the correct APIs and excipients in the correct amounts would not be detected as suspicious—and do not detect slight under- or overdosing, they are able to detect medications with substitute APIs, unapproved fillers, and adulterated APIs. The minimal training for users and ease of manufacturing scale-up makes these devices a viable option for large-scale pharmaceutical quality screening in low-resource settings.

The scale of testing necessary to detect a given prevalence of "bad" drugs can be estimated statistically.31 If the fraction of pharmaceuticals in a region that are very low quality is \( \theta \) and one wishes to measure the prevalence to within \( \pm u \), the number of sam-

35
ples \( m \) that must be examined in order to estimate the prevalence with 95% confidence is given by:

\[
m = \frac{4\theta(1-\theta)}{u^2}
\]

(1)

If the true prevalence of very low quality drugs is 3%, prevalence can be measured with 95% confidence in the interval 3±2% by testing 291 samples. The low rates of false positives for the excipient tests is important because the prevalence of very low quality or fake pharmaceuticals is, in most studies\(^{11}\), in the 0-7% range, so a high rate of false positives would cause needless alarm and expensive follow-up testing. For small prevalence rates, the chance that a bad drug will be missed due to a false negative can be compensated for by increasing both the sample size and the observed prevalence by a factor of 1/sensitivity. Assuming a PAD sensitivity of 75% (the worst value in our testing) a randomly selected sample\(^{32}\) of under 400 would enable reliable detection of very low quality pharmaceuticals across a region. The fabrication and labor costs of making a single PAD are under $1, so this scale of testing is feasible. In practice, a “suspicious” PAD result would be followed up by retesting the sample with a fresh PAD; two suspicious PAD tests should trigger confirmatory testing by an analytical lab.

Though the current generation of PAD tests are qualitative rather than quantitative, they are capable of detecting very low quality pharmaceuticals of the types that have been previously found in many locations in the developing world. We believe that it will be possible to add quantitative detection capabilities to detect substandard pharmaceuticals and use mobile phone technology to automate the analysis of the "color bar
codes" in the next generation of devices. An inexpensive method for field screening of medicines could provide additional quality assurance within the supply chain and exert economic pressures on manufacturers and distributors involved in the widespread trade in falsified pharmaceuticals.

2.6 References


CHAPTER 3

PAPER ANALYTICAL DEVICES FOR SCREENING ANTIMALARIAL MEDICATIONS

3.1 Introduction

3.1.1 The good, the bad, and the ugly: the state of medications in the global supply chain and how they are defined.

Access to high quality medication is part of the basic human right of access to health care.\(^1\) Previous studies of the quality of medications in the developing world\(^2\)–\(^4\) have revealed unacceptably high prevalence of low quality pharmaceuticals. These low quality medicines can be classified into three categories; substandard, falsified (or counterfeit) and degraded. Substandard refers to pharmaceuticals made by a registered manufacturer that do not meet pharmacopeia standards, while falsified pharmaceuticals are those not produced by a registered manufacturer. Falsified products may contain substitute pharmaceutical ingredients, or active ingredients are cut with inexpensive fillers like chalk or starch. In some cases, however, falsified products may contain the proper ingredients. Degraded products are pharmaceuticals that do not meet pharmacopeia standards due to improper handling or storage.\(^5\) Recent reviews of published work and "grey literature" have shown that about a third of antimalarials in sub-Saharan Africa are substandard or falsified.\(^3\)
Abiding by the above definitions, low quality medications may be either substandard or falsified dependent on the status of the manufacturer. In this chapter, I will refer to all medications that do not meet pharmacopeia standards as chemically substandard. Of special concern are medications that are significantly under-dosed, contain substitute Active Pharmaceutical Ingredients (APIs), or contain unapproved excipients.\textsuperscript{6–9} For some specific drugs, locations, and times, such as artemisinin monotherapies in Laos in the early 2000s,\textsuperscript{10} over 80\% of the products on shop shelves were found to contain little or no active pharmaceutical ingredient. Not surprisingly, very low quality medicines have been linked to poor patient outcomes,\textsuperscript{11–14} but in a health care setting where complications and death rates are high and resources for patient follow up are low, these poor outcomes may only be detected when there are a lot of the low quality products in use in the same time and location. Increased pharmaceutical screening would increase the probability of finding low quality medicines before poor patient outcomes occur and draw attention to the problem.

This chapter describes a paper test card that could serve as a screening tool to identify low quality antimalarial medicines before they reach a patient’s bedside. Because there are several published methods now for field screening of artesunate antimalarials,\textsuperscript{15–18} this work focused on the older antimalarial drugs important for clinical care developing countries, namely chloroquine, doxycycline, quinine, sulfadoxin, pyrimethamine, and primaquine. Accounts of low quality versions of these medications have been found in studies all over the world.\textsuperscript{2–4}
3.1.2 Paper millifluidics is a field-friendly platform for pharmaceutical quality screening tests.

Laboratory color tests are useful for qualitative analysis of many organic functional groups and inorganic species. Previous field tests for pharmaceuticals have utilized small-scale versions of such reactions conducted using suitcase laboratories or conventional laboratory facilities, but the equipment, chemicals, and skilled technicians needed to carry them out are often not available in low resource settings. Here, I have combined the necessary reagents needed for a dozen of these color tests on a millifluidic paper device the size of a playing card. In a previous study, a library of eleven color tests was found to detect beta-lactam antibiotics, first-line tuberculosis (TB) medications and some pharmaceutical excipients with good sensitivity and selectivity. In this study, the test card was modified to identify aspirin (acetylsalicylic acid), which has been reported as a substitute pharmaceutical in falsified pharmaceutical formulations. I also added a second version of a cobalt thiocyanate test to increase specificity, especially in the case of pharmaceuticals containing tertiary amines. Samples of pure APIs used in antimalarial drugs, formulations of APIs cut with fillers, and formulations containing substitute pharmaceuticals were applied to the test cards and readers evaluated the blinded results to test the card’s ability to identify formulations which corresponded to very low quality medications.

The porous paper provides a sturdy, light substrate for test cards that stores the needed reagents in dry form, wicks fluids through capillary action to mix reagents and samples, and chromatographically separates products. The test cards are used as shown
in Figure 3.1. The user loads about 10 mg of powder taken from a capsule or a crushed tablet by swiping it across the test card with a wooden paddle (i.e. coffee stirrer) to press small quantities of the sample into each of 12 reaction zones, as shown in Figure 3.2. The bottom edge of the card is set into water. As the water ascends the test card via capillary action, it dissolves and combines the dried reagents stored on the card and carries them to the sample. Within three minutes, colors form at or above the swipe line, developing maximum intensity after another 3-5 minutes. The user records the test results by taking a photo with a cell phone camera, which documents the primary data from each test. Different mixtures of ingredients in the pill form distinct patterns of colors or “color bar codes”, as seen in Figure 3.3.

![Figure 3.1](image.png)

**Figure 3.1 Testing a pharmaceutical with a paper test card.** A crushed tablet or the content of a capsule is applied to the card. The card is stood upright in water, which wicks up the individual lanes to activate the color tests. After 3 minutes, the card is removed and laid flat, and a photograph is recorded in 3-5 minutes. Differences between the image of the result and images of test cards run with authentic pharmaceuticals are used to identify suspicious samples.
Figure 3.2 The 12 lane test card being loaded with a pharmaceutical sample.

Figure 3.3 Representative outcomes of the 12 lane test card analysis. Samples include water only (a) and top portions of the test cards displaying the color output following testing with anti-malarial standards (b-g). Outcomes of interest that differ from the water blank—the "color bar codes"—are indicated with red boxes and described in Table 3.4.
Antimalarial medications have been an area of interest from the beginning of paper test card development, because of the high frequency of poor quality version found in markets around the world. The question addressed here is whether test card color bar codes would allow the user to distinguish full strength antimalarial APIs from formulations cut with different fillers. A lab validation study was performed on chloroquine and doxycycline, which are non-artesunate drugs often used to treat malaria. Using a blinded study methodology, I measured the sensitivity and specificity of the test for discrimination between authentic APIs and formulations that were diluted with fillers or contained substitute APIs.

3.2 Experimental

3.2.1 Materials

Acetylsalicylic acid, 4-pyridyl pyridinium chloride, amodiaquine, calcium carbonate, sulfadoxin, primaquine bisphosphate, pyrimethamine, atovaquone, chloroquine phosphate, quinine, polyvinylporrolidone and proguanil hydrochloride were obtained from Sigma-Aldrich (St. Louis). Artesunate was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Doxycycline was obtained from MP Biomedical (Santa Ana, CA). Corn starch was obtained from a local grocery store.

The test cards were printed as described in a previous publication. A spoke inoculating manifold (or "frog" device) was obtained from Dan-Kar (Woburn, MA) and used to transfer spots of reagents stored in two 96-well plates to the printed cards.
3.2.2 Test fabrication

The sources of chemicals, printing and reagent deposition process used in assembling the 12 lane test card are described previously,\textsuperscript{25} with the exception of lanes that test for aspirin and sulfadoxine and a cobalt thiocyanate test modified by the presence of a pH 8.0 buffer. Unless otherwise stated, all reagents were deposited from aqueous solution. Test cards can be produced on a small scale using simple equipment, which could be suitable for local production in some countries affected by low quality medications. Labels, instructions, fiducial marks, security features, and color standards are first printed onto the cards with a laser printer. Wax printing\textsuperscript{26,27} followed by baking in a 100°C oven is used to create hydrophobic wax barriers on the card; these barriers define reaction areas for the different tests.

Chemical reagents in twelve lane test cards were spotted in 2 µL aliquots in locations indicated in Figure 3.3a. The compositions and locations of the reagents are shown and described in detail in Table 3.1. After air drying the cards until the pink color of hydrated cobalt thiocyanate in lanes C and D had changed to the blue color of dehydrated cobalt thiocyanate, the cards were transferred to plastic zipper-lock bags for storage. In this study, cards were used within four weeks of fabrication.
# TABLE 3.1

**LAYOUT OF TEST REAGENTS OF 12 LANE TEST CARDS USED FOR ANTI-MALARIAL TESTING**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Row</th>
<th>Reagent</th>
<th>Conc (in water unless stated otherwise)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timer</td>
<td></td>
<td>$\text{NiCl}_2$</td>
<td>0.2M</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Dimethylglyoxime</td>
<td>0.2M in acetonitrile</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>A</td>
<td>ninhydrin</td>
<td>50 mg/ml in acetonitrile</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ninhydrin</td>
<td>50 mg/ml in acetonitrile</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ninhydrin</td>
<td>50 mg/ml in acetonitrile</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$\text{K}_2\text{CO}_3$</td>
<td>2M</td>
</tr>
<tr>
<td>Biuret reagent</td>
<td>B</td>
<td>Biuret test reagent*</td>
<td>10x</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Biuret test reagent*</td>
<td>10x</td>
</tr>
<tr>
<td>Tertiary amines</td>
<td>C</td>
<td>$\text{Co(SCN)}_2$</td>
<td>1 M $\text{Co(SCN)}_2$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>$\text{Co(SCN)}_2$</td>
<td>1 M $\text{Co(SCN)}_2$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tosic acid (HOTs)</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Tosic acid (HOTs)</td>
<td>1M</td>
</tr>
<tr>
<td>Basic $\text{Co(SCN)}$</td>
<td>D</td>
<td>$\text{Co(SCN)}_2$</td>
<td>1 M $\text{Co(SCN)}_2$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>$\text{Co(SCN)}_2$</td>
<td>1 M $\text{Co(SCN)}_2$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tris buffer pH8.0</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Tris buffer pH8.0</td>
<td>1M</td>
</tr>
<tr>
<td>beta lactams (Cu)</td>
<td>E</td>
<td>$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$\text{K}_2\text{CO}_3$</td>
<td>2M</td>
</tr>
<tr>
<td>Sodium Nitroprusside</td>
<td>F</td>
<td>Sodium nitroprusside</td>
<td>200 mg/ml</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Sodium nitroprusside</td>
<td>200 mg/ml</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NaOH</td>
<td>2M</td>
</tr>
<tr>
<td>Naphtholquinone Sulfonate</td>
<td>G</td>
<td>NaOH</td>
<td>6M</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NQS</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NQS</td>
<td>10 mg/ml</td>
</tr>
<tr>
<td>Ethambutol (Cu)*</td>
<td>H</td>
<td>NaOH</td>
<td>2M</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$</td>
<td>1M</td>
</tr>
<tr>
<td>Aspirin test</td>
<td>I</td>
<td>FeCl$_3$</td>
<td>125 mg/ml in water</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NaOH</td>
<td>6M</td>
</tr>
<tr>
<td>Starch</td>
<td>J</td>
<td>Triiodide/ povidone</td>
<td>0.5% I$_2$, excess I’, 2% povidone</td>
</tr>
<tr>
<td>p-nitroaniline</td>
<td>K</td>
<td>NaOH</td>
<td>2M</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>p-nitroaniline</td>
<td>30mg/mL in acetonitrile</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NaNO$_2$</td>
<td>30mg/mL</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Tosic acid (HOTs)</td>
<td>1M</td>
</tr>
<tr>
<td>Carbonate</td>
<td>L</td>
<td>FeCl$_3$</td>
<td>125 mg/ml in water</td>
</tr>
<tr>
<td>Sulfadoxine</td>
<td>0</td>
<td>Tosic acid (HOTs)</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Tosic acid (HOTs)</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>pyridyl pyridinium chloride</td>
<td>30 mg/mL</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NaOH</td>
<td>6M</td>
</tr>
</tbody>
</table>

*Biuret test reagent 10x recipe: 1.5 g CuSO$_4 \cdot 5$H$_2$O, 6 g Rochelle Salt, 20 ml 2M NaOH
Acetylsalicylic acid (aspirin) lane tests were spotted in the locations labeled in Figure 3.3a. The spotting reagents were: 125mg/mL iron (III) chloride (location 1) and 6M sodium hydroxide (location 3).

Sulfadoxin lane tests were spotted in the locations labeled in Figure 3.3a. The spotting reagents were: 1M tosic acid (locations 0, 1), 30 mg/mL pyridyl pyridinium chloride (PPC) (location 3) and 6M sodium hydroxide (location 4).

3.2.3 Sample preparation

The compositions of samples used for the blinded study are described in Table 3.2. Samples were massed, combined in 20 ml scintillation vials and shaken for at least 1 minute to combine the powders.

### TABLE 3.2

**COMPOSITION OF ACTIVE AND INACTIVE COMPONENTS IN MIXED SAMPLES**

<table>
<thead>
<tr>
<th>Active ingredient(s) (percent by mass)</th>
<th>Inactive ingredients (percent by mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% chloroquine</td>
<td>30% calcium carbonate</td>
</tr>
<tr>
<td>40% chloroquine</td>
<td>60% corn starch</td>
</tr>
<tr>
<td>70% sulfadoxin, 3.5% pyrimethamine</td>
<td>26.5% corn starch</td>
</tr>
<tr>
<td>70% sulfadoxin</td>
<td>30% calcium carbonate</td>
</tr>
<tr>
<td>40% sulfadoxin</td>
<td>60% calcium carbonate</td>
</tr>
<tr>
<td>10% sulfadoxin</td>
<td>90% calcium carbonate</td>
</tr>
<tr>
<td>40% doxycycline</td>
<td>60% corn starch</td>
</tr>
<tr>
<td>40% doxycycline</td>
<td>60% polyvinylpyrrolidone (PVP)</td>
</tr>
</tbody>
</table>
3.2.4 Testing methodology and image analysis

The testing methodology for visual recognition of test card results is described previously. In brief, each test card was photographed 3-5 minutes after it was removed from water, and the images were masked to conceal everything but the color bar code region. Two readers compared each card to images of standard pure samples and reported on whether they thought the claimed API was present or absent and whether they thought adulterants were present or absent. If the two readers disagreed, a third reader was brought in as a tie breaker. Image analysis was carried out by Marya Lieberman. For chloroquine (CQ) and doxycycline (DOX), selected lanes were analyzed using ImageJ to derive color intensity data related to the content of each drug. For CQ, the mean intensity of the blue color in lane C was analyzed in a region of interest extending 1 cm above and below the swipe line; for DOX, the mean greyscale intensity of the red-brown color of lane L was assessed in a region of interest extending from the swipe line to the top of the lane. This analysis is facilitated by the melting behavior of the wax ink, which consists of a mixture of colorless hydrophobic waxes and pigments; on melting, the colorless waxes permeate the paper at a faster rate than the pigments, so each grey hydrophobic barrier is surrounded by a halo about 0.5 mm wide of uncolored but hydrophobic paper, which repels colored products that are chromatographically mobile in the water phase. It is thus possible to draw a boundary box around the entire colored portion of the lane without including pixels from the grey barrier.
3.3 Results

3.3.1 Versatility in detection of antimalarial APIs, substitute APIs, fillers, and excipients.

The color tests assembled on the paper test card signaled the presence of APIs and excipient materials found in common antimalarial drugs, giving unique color bar codes for chloroquine, pyrimethamine, doxycycline, quinine phosphate or quinine free base (same color bar code), atovaquone, proguanil, amodiaquine and primaquine. The library of color tests produced different color bar codes for substitute pharmaceuticals and fillers used in some previously observed falsified formulations, such as acetylsalicylic acid, acetaminophen, starch, and calcium carbonate. Representative images of test outcomes are found in Figure 3.3, and annotated descriptions are given in Table 3.3, describing the characteristic differences in colors generated in the lanes with each material tested. In order to characterize the color bar codes, pure antimalarial compounds were run in triplicate on 12-lane test cards. Two readers judged how color production differed from a water blank, and the consensus was used to define a color bar code. Some color bar codes are strong and distinctive, such as chloroquine, which produces a strong blue color that extends above and below the sample swipe line in the two cobalt thiocyanate containing test lanes (Figure 3.3b, 3rd and 4th lanes). Other compounds produce weaker or less distinctive color bar codes. Proguanil produces a small amount of blue color on the swipe line in the acidic cobalt thiocyanate lane (Figure 3.3g third lane), but this color is not distinctive enough to constitute a unique color bar code, because other molecules that contain tertiary amine groups also produce this signal. Additional information is also provided by some APIs such as amodiaquine,
primaquine and doxycycline that are themselves colored, depositing color at the swipe line.
# TABLE 3.3

**DESCRIPTION OF LANE BY LANE OUTCOMES FOR WATER AND ANTIMALARIAL ACTIVE INGREDIENTS**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample color</th>
<th>Timer (edge)</th>
<th>Ninhydrin (lane A)</th>
<th>Biuret (lane B)</th>
<th>Cobalt thiocyanate acidic (lane C)</th>
<th>Cobalt thiocyanate pH8.0 (lane D)</th>
<th>Beta-lactams (lane E)</th>
<th>SNP (lane F)</th>
<th>NQS (lane G)</th>
<th>Ethambutol (lane H)</th>
<th>Aspirin test (lane I)</th>
<th>Starch test (lane J)</th>
<th>p-Nitroaniline (lane K)</th>
<th>Iron (III)Chloride (lane L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>none</td>
<td>pink spot</td>
<td>yellow</td>
<td>light blue</td>
<td>pink</td>
<td>pink</td>
<td>blue (does not always reach top of lane)</td>
<td>brown to top of lane</td>
<td>Dark brown spot above swipe, light brown above</td>
<td>orange</td>
<td>yellow/orange</td>
<td>yellow at swipe, faint yellow above</td>
<td>traces of orange/yellow near and below swipe</td>
<td>White at swipe and above</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>pale yellow</td>
<td>like water</td>
<td>like water</td>
<td>emerald green at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Artesunate</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>bright yellow</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>Orange at swipe line</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>blue at swipe (faintNER at lane C)</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>pale yellow</td>
<td>like water</td>
<td>like water</td>
<td>green at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>green at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Primaquine</td>
<td>bright orange</td>
<td>black at swipe</td>
<td>like water</td>
<td>dark green at swipe</td>
<td>like water</td>
<td>like water</td>
<td>yellow at swipe, above is like water</td>
<td>green at or above swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>blue at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>faint yellow at a swipe</td>
</tr>
<tr>
<td>Proguanil</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>blue at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
<tr>
<td>Quinine and Quinine Sulfate</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>blue at swipe</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>yellow at swipe</td>
</tr>
<tr>
<td>Sulfadoxin</td>
<td>white</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>green streak at top of blue</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
<td>like water</td>
</tr>
</tbody>
</table>
3.3.2 Detection of very low quality medications.

A blinded study methodology was used to assess test card outcomes of pure chloroquine (CQ) or doxycycline (DOX) that were combined with different amounts of fillers (Table 3.2) to correspond to full strength (100%) and substandard (70% and 40%) formulations. Representative test images are shown in Figure 3.4 with outcomes that differ from water boxed in red. Additional samples in the pool consisted of a substitute API (aspirin, acetylsalicylic acid) or an inert filler (chalk, CaCO₃). Two expert readers, who have read more than 100 test cards, were independently presented with images of the test cards in random order. The readers assessed the images by comparison to sets of standard images of water, pure CQ, pure DOX, pure acetylsalicylic acid, and pure calcium carbonate. A third reader was used in the 7-8% of cases where readers disagreed. In the DOX study, the readers disagreed about the excipients present in 4/60 images but agreed on all 60 images about the presence/absence of DOX; in the CQ study, they disagreed about the presence of excipients in 6/80 images and agreed on all 80 images about the presence/absence of CQ. In addition, selected lanes were analyzed using ImageJ to derive color intensity data, as described in the Methods section.
In assessing a test result, readers judge whether the claimed active ingredient is present or absent and whether an unapproved filler or substitute ingredient is present. The readers identified the presence of chloroquine by eye with 100% sensitivity (all samples containing chloroquine in any concentration were identified correctly, n=60) and 100% specificity (no false positive outcomes occurred for samples that did not contain chloroquine, n=20) (Table 3.4). This demonstrates the ability of the test to discriminate presence/absence of chloroquine with visual inspection. The mean intensities of the blue color of chloroquine in the images of lane C showed significantly

![Figure 3.4 Twelve lane test card outcomes for samples representing variations in pharmaceutical formulations. Color outcomes that differ from water are boxed in red. Chloroquine (CQ) at 100% (a) looks very similar to 70% CQ/30% calcium carbonate, however 40% CQ/60% starch (c) produces a different pattern of colors due to the lighter blue color in the third lane and the presence of starch. 100% calcium carbonate (d) gives an orange color with iron chloride, but this color is not detectable in the 30% calcium carbonate used to cut CQ in (b). The API doxycycline (DOX) at 100% produces a distinctive color bar code (e). At 40% concentration, the intensity of the brown color in the last lane is much weaker (f-g) and the appearance of starch (f) alters the overall color bar code and would indicate a variation in this formulation. Acetylsalicylic acid (h) produces a unique color bar code and is easily distinguished from anti-malarial APIs.](image-url)
different values for full strength (157±10, mean±SD) and 40% API (117 ±10) formulations, as shown in Figure 3.5a, suggesting that readers should be able to glean semi-quantitative information from test card results. An experienced reader reevaluated the blinded samples, this time rating each CQ as "strong", "weak", or "missing". All of the 18 full-strength formulations were rated as "strong", while 6/19 of the samples containing 70% API were rated as "strong" and 13/19 as "weak". Of the 20 samples containing 40% API, one was rated as "strong" and the other 19/20 as "weak". This result confirms that an experienced reader could pick out formulations containing less than 50% API by visual examination. However, the reader could not reliably distinguish full strength and 70% formulations by eye.
### TABLE 3.4
SENSITIVITY AND SPECIFICITY OF THE 12 LANE TEST CARDS
BASED ON READER EVALUATIONS

<table>
<thead>
<tr>
<th>Presence of Active Ingredient</th>
<th>Positive test samples</th>
<th>Negative test samples</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Reader disagreements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%, 70%, 40% Chloroquine</td>
<td>100% Calcium carbonate, 100% acetylsalicylic acid</td>
<td>100% (60/60)</td>
<td>100% (20/20)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100%, 70% Chloroquine</td>
<td>40% or less chloroquine</td>
<td>100% (40/40)</td>
<td>50% (20/40)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100% Pyrimethamine</td>
<td>100% Calcium carbonate, 100% acetylsalicylic acid</td>
<td>95% (19/20)</td>
<td>100% (20/20)</td>
<td>1</td>
</tr>
<tr>
<td>Sulfadoxin-pyrimethamine</td>
<td>100% Calcium carbonate, 100% acetylsalicylic acid</td>
<td>40% (8/20)</td>
<td>100% (20/20)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mixture (for pyrimethamine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% and 40% Doxycycline</td>
<td>100% Calcium carbonate, 100% acetylsalicylic acid</td>
<td>100 (40/40)</td>
<td>100% (20/20)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100% Chloroquine</td>
<td>70% Chloroquine with calcium carbonate</td>
<td>100% (20/20)</td>
<td>0% (0/20)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>100% Doxycycline</td>
<td>40% Doxycycline with starch</td>
<td>95% (19/20)</td>
<td>100% (10/10)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>100% Doxycycline</td>
<td>40% Doxycycline with PVP</td>
<td>95% (19/20)</td>
<td>10% (1/10)</td>
<td>3</td>
</tr>
<tr>
<td>Presence of Excipients</td>
<td>100% Acetylsalicylic acid</td>
<td>100%, 70% 40%, Chloroquine, 100% Calcium carbonate</td>
<td>90% (9/10)</td>
<td>100% (70/70)</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>100% Calcium carbonate</td>
<td>100%, 40%, Chloroquine, 100% Acetylsalicylic acid</td>
<td>100% (10/10)</td>
<td>100% (50/50)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>40% Chloroquine with starch</td>
<td>Chloroquine without starch (100%,70%)</td>
<td>100% (20/20)</td>
<td>100% (40/40)</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 3.5 Box plot analysis of color intensity versus percent active ingredient. a) Blue color of chloroquine in lane C region-of-interest measured as mean intensity of inverted image in red channel. b) Brown color of doxycycline in lane L region-of-interest measured as mean intensity of inverted image in grayscale. Box plots depict the median (horizontal line), second and third quartile (box), and 1.5 x interquartile range (whiskers) for each sample group; sample sizes provided below each box. Minimum and maximum outliers shown with crosses.
To aid in distinguishing dosage forms with reduced active ingredients, the 12 lane test card is equipped with several lanes that detect common binders, fillers, and substitute APIs. When an active ingredient is reduced, it is generally replaced by another compound that may lead to a change in the expected pharmaceutical color bar code. Samples of pure calcium carbonate and pure acetylsalicylic acid were identified by readers with 100% and 90% sensitivity, respectively, and with no false positives (Table 3.4). Samples of 40% chloroquine mixed with 60% starch were all identified by eye based on the presence of a strong purple-black color reaction in lane J to the starch. However, identification of adulterated drugs requires reference to known authentic samples. Some formulations of chloroquine include microcrystalline cellulose and talc as excipients, while others include starch as a binder.

Doxycycline created several characteristic color changes in different regions of interest, including pale green at the swipe line in lane H, a bright orange color in lane I, and a deep brown-red in lane L (Figure 3.3f). Through visual inspection, readers identified formulations containing doxycycline with 100% sensitivity (all of the samples containing any concentration of DOX were identified correctly, n=40) and specificity (no false positives from the samples lacking DOX, n=20). Pure doxycycline could be distinguished from 40% doxycycline cut with corn starch (representing a very low quality drug) with 95% sensitivity and 100% specificity by evaluation of the blue-black color of starch in lane J, but samples containing 40% doxycycline cut with polyvinylpyrrolidone (PVP), a filler that does not produce distinct colors with the current test card, were not easily distinguished and specificity fell to 10%. However, the brown color produced by
the reaction of doxycycline with Fe(III) in lane L provided additional information to help distinguish authentic (greyscale color intensity 189±10, mean±SD) from substandard (color intensity 132±8) formulations (Figure 3.5b). An experienced reader reevaluated lane L of the doxycycline images, scoring the brown color in each as strong, weak, or absent. Of the 20 full-strength formulations of DOX, 19 were rated "strong" and one "weak". The ten formulations containing 40% DOX cut with PVP were all rated "weak", as were eight of the ten formulations containing 40% DOX cut with starch (the other two were rated "strong").

3.3.3 Lane Test Development for Sulfadoxin

Sulfadoxin and pyrimethamine are sold in a combination dosage form. Pyrimethamine produces a strong color bar code with the test card and in its pure form could be identified with 95% sensitivity, however the sulfadoxin/pyrimethamine combination dosage form contains only 3.5% pyrimethamine by weight. In this very dilute combination form the test card could only identify 40% of the samples containing pyrimethamine (Table 3.5), so it would not be reliable for identification of sulfadoxin/pyrimethamine formulations that were missing pyrimethamine. Sulfadoxin produced a very weak color bar code on the original test card (Figure 3.1) so an additional lane was developed to indicate the presence of the primary aromatic amine in this drug. 4-Pyridyl pyridinium chloride (PPC) and sodium hydroxide are used to generate glutaronic aldehyde in situ just below the swipe line; the aldehyde condenses with the aromatic amine of sulfadoxin in the presence of acid to yield an intense orange
Schiff base, located at the top of the test lane (Figure 3.6). This test is able to detect sulfadoxin with greater than 90% sensitivity at concentrations as low as 10% in a formulation cut with calcium carbonate.

**TABLE 3.5**

SENSITIVITY AND SPECIFICITY OF SULFADOXIN LANE TEST

<table>
<thead>
<tr>
<th>Presence of Active Ingredient</th>
<th>Positive test samples</th>
<th>Negative test samples</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Reader disagreements</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Sulfadoxin</td>
<td>No API (corn starch)</td>
<td>100% (24)</td>
<td>100% (24)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100%, 70% Sulfadoxin</td>
<td>No API (corn starch)</td>
<td>98% (48)</td>
<td>100% (24)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>70% Sulfadoxin</td>
<td>No API (corn starch)</td>
<td>96% (24)</td>
<td>100% (24)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>40% Sulfadoxin</td>
<td>No API (corn starch)</td>
<td>100% (24)</td>
<td>100% (24)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10% Sulfadoxin</td>
<td>No API (corn starch)</td>
<td>92% (24)</td>
<td>100% (24)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 3.6. Lane test outcomes for the detection of sulfadoxin.* Test for primary aromatic amines produces a bright orange at the top of the test lane with 100% sulfadoxin (f) and a sulfadoxin/pyrimethamine combination (g) compared to water (a) or starch (b). Sulfadoxin cut with calcium carbonate (w/w) to concentrations of 10% (c), 40% (d) and 70% (e) also produce the positive outcome with only small reductions in sensitivity.
3.4 Discussion

There is a long history of using color reactions to characterize pharmaceutical ingredients or formulations\textsuperscript{21,28} in low resource settings, but these methods require a high level of support infrastructure and technical skill. Here, I have combined a library of chemical color reactions onto a paper test card for screening of very low quality medications. The test card described here includes thirteen color reactions and a timer lane. The library of color reactions has been shown to identify several beta-lactam antibiotics and four of the first line TB drugs,\textsuperscript{22} and in this work I show that they can identify eight common antimalarial drugs and three substitute APIs and excipient materials as well. More importantly, full-strength doxycycline or chloroquine could be distinguished from very low quality formulations where the API had been cut with fillers to concentrations of 40\%, or replaced with substitute APIs or inert ingredients.

Disadvantages of the test cards include the need for comparison to standard outcomes of authentic samples, the existence of many pharmaceuticals that do not give useful color bar codes, the lack of sufficient quantification to identify substandard medications, and the necessity to establish a confirmatory testing process. Different brands and dose levels may use different excipients, which can alter "color bar codes", increasing the number of potential standard outcomes the user must access for comparison with unknown samples. Additionally, the user may not know the putative brand of the drug that should serve as a standard for comparison, as loose pills are sometimes dispensed without packaging information in the developing world. For these drugs, detection of excipients will be of less use than for drugs where the expected
formulation is known. Some APIs, which are detected readily in concentrated form, do not produce strong color responses when they are used as minor constituents of a combination tablet, as seen with pyrimethamine present at 3.5% in sulfadoxin/pyrimethamine formulations. Other APIs, such as artemether, or excipients, such as gypsum, do not produce color bar codes with the current set of test lanes. Finally, the test results are presumptive and suspicious samples must be analyzed by a "gold standard" method to confirm a low quality pharmaceutical.

Advantages of the test cards include ease of use and ability to build and share digital libraries of standard outcomes. Paper test cards allow presumptive testing of pharmaceuticals outside of the laboratory. Users do not have to weigh or dilute anything or handle any chemicals. The test cards are inexpensive (manufacturing costs under $0.50 per card) and do not require purchase of any capital equipment, such as readers or kits of glassware, so the financial barrier to use of the cards is minimal. The turnaround time for analysis of a dosage form is under 10 minutes. Standards for comparison may include cards run with authentic samples, or stored images that may be shared commonly among test users. Presently, the default situation in many developing countries is that pharmaceuticals are credence goods--the buyer must take their quality on faith. The availability of more tools, such as paper test cards, for presumptive testing of pharmaceuticals could provide an inexpensive and sustainable mechanism to increase the rate of detection of very low quality pharmaceuticals in the global supply chain.
3.5 References


CHAPTER 4

A LAB-ON-PAPER FOR IDENTIFYING COUNTERFEIT PHARMACEUTICAL DOSAGE FORMS

4.1 Introduction

Medications whose chemical contents vary significantly from an approved formulation, either by error or design, are a global health problem. While low quality medicines can find their way into developed countries through complex supply chains and Internet sales, the prevalence of poor quality medicines is greater in developing countries. Studies have reported 35% of antimalarials in southeast Asia and 17% of anti-tuberculosis (TB) medicines in Africa failing chemical analysis.

To increase monitoring of the pharmaceutical supply chain in locations where resources are limited, inexpensive instruments and methods for field testing have been developed. Existing field testing technologies, including handheld Raman and Near Infrared (NIR) spectroscopy devices, are designed to be user-friendly, easily transported and able to provide a qualitative analysis of pharmaceuticals. However, the high cost of the instruments creates a barrier for widespread pharmaceutical testing, the utility of the devices depends on the availability of a library of authentic samples, and they do not provide reliable quantification. Colorimetric testing, thin layer chromatography and minilab testing are able to provide pharmaceutical analyses.
at a lower cost, but these methods require a trained technician. The United States Food and Drug Administration/Forensic Chemistry Center (FDA/FCC) Counterfeit Detection Device (CD3) is a relatively recent addition to the collection of field screening tools. This battery operated device uses a series of wavelengths ranging from the ultraviolet to infrared to illuminate the sample. The different wavelengths of light will interact with colored materials in the sample in different ways and allow the user to visually differentiate authentic from suspect materials.\textsuperscript{25} While not designed to provide chemical information about the contents of a pill or tablet, one of the CD3’s strengths is its ability to evaluate both packaging and dosage forms rapidly and at a lower cost than a handheld spectroscopic device.

Following my investigations of the 12 lane test card with beta-lactam antibiotics, anti-tuberculosis medications\textsuperscript{26} and anti-malarials, I wanted to measure the performance of the tests using dosage forms. The predominant questions that I wanted to investigate were: 1) what additional compounds can the test cards identify and 2) can test cards identify low quality/counterfeit medicines pulled from the supply chain. To address these questions, I contacted the United States Food and Drug Administration (US FDA) Forensic Chemistry Center (FCC). The FCC is the analytical chemistry hub that inspects all suspect pharmaceuticals pulled from the supply chain. I was able to spend four weeks at the FCC facility testing a variety of standards as well as authentic and counterfeit dosage forms. To me, this chapter represents a great break-through in my understanding of the versatility of the test cards to be used with a diverse collection of pharmaceutical components.
4.2 Experimental

4.2.1 Materials

Pyrazinamide was obtained from Acros Chemicals (Geel, Belgium). Dimethylglyoxime 99%, 1,2-cyclohexanedione-dioxime (“nioxime”), and potassium iodide were obtained from Alfa Aesar (Ward Hill, MA). Sodium hydroxide was obtained from Amresco (Solon, OH). Primary grade potassium iodate, soluble potato starch, and sodium nitrite were obtained from J.T. Baker (Phillipsburg, NJ). Metformin was obtained from Enzo Life Sciences (Farmingdale, New York). Cobalt (II) nitrate hexahydrate, ethambutol dihydrochloride, potassium carbonate, sodium thiosulfate pentahydrate, nickel chloride, quinine sulfate, iron (III) nitrate and iron (III) chloride were obtained from Fisher Scientific (Fairlawn, NJ). Erythromycin and isoniazid were obtained from Fluka (Buchs, Switzerland). Cephalexin and ampicillin trihydrate were obtained from MP Biomedicals, LLC (Solon, Ohio). Chloroquine diphosphate, diphenhydramine hydrochloride, polyvinylpyrrolidone average molecular weight 360,000 (“povidone”) and rifampicin were obtained from Sigma (St. Louis, MO). Vardenafil was obtained from Santa Cruz Biotechnology (Dallas, Texas). 1,2-Naphthoquinone-4-sulfonic acid sodium salt (NQS), p-toluenesulfonic acid (“tosic acid”) and sodium nitroferricyanide (III) dihydrate (sodium nitroprusside or SNP), phentermine hydrochloride, acetaminophen, amoxicillin, amodiaquine, calcium carbonate, copper sulfate pentahydrate, ninhydrin, dextromethorphan hydrobromide monohydrate, oxytetracycline, R-phenylephrine, chlorpheniramine, diclofenac sodium salt, penicillin G sodium salt, doxylamine succinate, p-nitroaniline and polyethylene glycol were obtained from Sigma-Aldrich.
(Saint Louis, Missouri). Sibutramine hydrochloride monohydrate was obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). Ciprofloxacin, streptomycin and tramadol were obtained from the United States Pharmacopeia (Rockville, Maryland).

Sildenafil citrate and tadalafil standards, authentic atorvastatin (10mg), oseltamivir (75mg), oxycodone (80mg), sildenafil (100mg), tadalafil (20mg) and vardenafil (20mg), dosage forms containing dipyrone, and all counterfeit pharmaceutical samples were kindly provided by the US FDA Forensic Chemistry Center. Counterfeit samples were identified by the U.S. FDA/FCC by one or more of the following methods: Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectroscopy (LC-MS), microscopy, packaging analysis and Fourier Transform-Infrared (FT-IR) and Raman spectroscopy.

Paper test cards were printed using an HP Color LaserJet printer (CP3525x) and a Xerox ColorQube 8570. 1-20uL tips racked for use in the Biomek FX were obtained from USA Scientific (Ocala, FL). 96 well flat bottom microtiter plates were purchased from Sarstedt (Numbrect, Germany). Ahlstrom® 319 Cellulose Sheets obtained from Midland Scientific, Chicago, USA were used as the substrate for PAD fabrication.

4.2.2 Methods

Test card printing, reagent deposition and pharmaceutical testing were carried out as previously described\textsuperscript{26} using reagents and spotting locations tabulated in Figure 4.1 and Table 4.1.
Figure 4.1. General purpose test card layout. The labeled test card lay-out indicates the position of reagent spotting as listed in Table 4.1.
Pharmaceutical color bar codes were determined based on visual inspection of outcomes from triplicate testing. Identification of counterfeit versus authentic medications was carried out by a blind study in which test card outcomes from assembled authentic and counterfeit medications were assessed by two readers. Each reader received four visual standards of authentic samples. A third reader was used for cases in which the initial readers’ interpretations were not in agreement (13% of tests). The number of paper test cards run for samples in the identification of authentic and counterfeit dosage forms were as follows: authentic sildenafil 100 mg (16), F012 (4),...
F014 (4), F015 (4), F022 (4), authentic vardenafil 20 mg (4), F011 (10), authentic oxycodone 80 mg (10), F008 (5), F020 (5), authentic tadalafil 20 mg (13), F010 (5), F0081 (5), F0091 (5), authentic oseltamivir 75 mg (13), F001 (4), F007 (6), authentic atorvastatin 10 mg (12), and F009 (9).

Both handheld Raman and FT-IR analysis of authentic and counterfeit samples were carried out in duplicate. Raman spectroscopic measurements were performed using a handheld Thermo Scientific TruScan instrument. The Raman spectra were searched using the TruScan software and a Raman library of authentic dosage forms assembled by the FDA/FCC. FT-IR spectroscopic measurements were performed using a handheld Thermo Scientific TruDefender FT-IR instrument. The FT-IR spectra were exported from the TruDefender and searched using Omnic software (Thermo v.8.0.342) and a library of FT-IR spectra for authentic dosage forms assembled by the FDA/FCC. A CD3, provided by the FDA/FCC, was used to image dosage forms under a variety of different wavelengths of light. Identification of counterfeit samples using CD3 images was carried out through visual inspection by two evaluators comparing the appearance of a potentially counterfeit sample with an authentic sample at the same wavelength settings.

4.3 Results

4.3.1 Paper-based test cards for pharmaceutical identification and differentiation

Previous work with paper-based test cards has shown that they enable users to differentiate beta-lactam, anti-tuberculosis medications and anti-malarials from
substitute ingredients or altered formulations with unexpected fillers with greater than 90% success. The test card’s chemical profile of a compound is reflected in a color bar code formed by the 12 test lanes in response to the presence of specific functional groups. The color bar code is a reproducible pattern of results reflecting positive and negative outcomes of lane tests, characterized by both hue and location of color production. To a lesser extent, color intensity and ratios of color intensities were also found to be useful in defining pharmaceutical color bar codes.

Working in collaboration with the FDA, I was delighted to have the opportunity to test authentic and counterfeit versions of pharmaceuticals, however prior to my arrival at the FCC facility I was not able to obtain detailed information about samples that would be available to me. Without knowing what pharmaceuticals I might be testing, I assembled a general purpose test card (Table 4.1) by choosing 12 lanes tests that had performed well in prior testing. I hypothesized that with these 12 tests, detecting a range of functional groups, I would be able to produce color bar codes for a wide variety of pharmaceutical products. At the FCC, I examined a total of 45 pharmaceutical active ingredients, excipients, and dosage forms to determine which ones would produce unique color bar codes.

Differentiation among members of the beta-lactam family of antibiotics was a strength of the general purpose test card. My testing included ampicillin, amoxicillin, penicillin G, clavulanic acid and cephalexin (Figure 4.2). All of these beta-lactams produce a forest green in lane B with copper sulfate, with the exception of clavulanic acid. Penicillin G produces a single positive lane outcome in lane B, while ampicillin
triggers two additional positive outcomes because of its primary amine: orange in lane C with ninhydrin and a blue/black in lane D with biuret reagent. Amoxicillin is identical structurally to ampicillin, except the phenyl group present in ampicillin is a phenol group in amoxicillin. In this case biuret reagent (lane D) forms the same blue/black, but ninhydrin (lane C) produces an olive green and the nitroaniline test (lane F) produces a bright orange in response to the phenol group. Cephalexin is a cephalosporin and structurally similar to ampicillin, differing at the thiazole ring. Lane B for cephalexin produces green that is characteristic of most beta-lactams, while lane C, ninhydrin, develops a red/orange and lane D, biuret reagent, produces a royal blue; in addition, cobalt thiocyanate (lane E) produces blue/green at the sample swipe line and iodine (lane H) forms black at the swipe line. Production of these unique outcomes for such structurally similar compounds demonstrates the considerable potential the test card chemistry has to distinguish between related molecules based on small variations in functional groups.
Figure 4.2. A variety of medicines produce a chemical color bar code with the general purpose test card. Patterns of colors produced by the general purpose test cards for a collection of pharmaceuticals, imaged by cell phone, showing the distinguishing power of this paper-based testing. Four structurally similar beta-lactam antibiotics (amoxicillin, ampicillin, penicillin G and cephalexin) can be easily differentiated. Paper test cards also produce chemical color bar codes for a variety of other medicines including, but not limited to other antibiotics (ciprofloxacin, erythromycin and oxytetracycline), the antihyperglycemic drug metformin, and the antihistamine chlorpheniramine. Positive test outcomes are circled in red to highlight differences in the chemical color bar codes.
4.3.2 Versatility of the paper-based test cards

Outside of the beta-lactam antibiotics, the general purpose test card produces unique color bar codes for fluoroquinolones, tetracyclines, a macrolide antibiotic, and an aminoglycoside (Figure 4.2). Ciprofloxacin and levofloxacin, from the fluoroquinolone family, both contain a 1,3-dicarbonyl group that forms a bidentate complex\(^{28}\) with iron(III), producing orange with ciprofloxacin and brown with levofloxacin in lane G. Tertiary amines in both of these drugs produce a green/blue at the swipe line with cobalt thiocyanate in lane E. Two members of the tetracycline family, oxytetracycline and doxycycline, also react with iron (III) chloride to produce a deep brown in lane G. However, doxycycline (but not oxytetracycline) produces green in lane I and the pharmaceutical color itself is a distinguishing characteristic; oxytetracycline is brown while doxycycline is yellow. Erythromycin, which is a macrolide antibiotic, produces a different color bar code consisting of green/blue at the swipe line with cobalt thiocyanate (lane E). A member of the aminoglycoside family, streptomycin, was found to produce a dark brown at the top of the lane with ninhydrin in lane B and a unique orange color with 2,3-naphthoquinone sulfonate in lane J, though the latter has only been reproduced using freshly fabricated tests.

In addition to antibiotics, the general purpose test card produces color bar codes with a variety of other pharmaceuticals. The diabetes medication metformin generates a unique pink color with copper in lanes B and I (Figure 4.2). The phenol group in phenylephrine forms a bright orange with the o-nitroaniline test and black with iron (III) chloride. Chlorpheniramine’s tertiary amine produces blue with cobalt thiocyanate (lane
distinctive due to its location both at and above the swipe line. 39 pharmaceutical products, ranging from antibiotics to weight loss aids, were found to produce color bar codes, 25 of which were unique, using this single test card design (Table 4.2). A description of the characteristic color outcomes for these compounds can be found in Table 4.3.

TABLE 4.2
PHARMACEUTICALS THAT PRODUCE A “COLOR BAR CODE” DIFFERING FROM WATER ON THE GENERAL PURPOSE TEST CARD

<table>
<thead>
<tr>
<th>Pharmaceutical Components</th>
<th>Dosage Forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Levofoxacin</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>Metformin</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Oxytetracycline</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Penicillin G</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>Phentermine&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>R-phenylephrine</td>
</tr>
<tr>
<td>Chloroquine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Polyethylene glycol&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Quinine&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dextromethorphan&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>Diphenhydramine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sibutramine&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doxylamine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sildenafil&lt;sup&gt;#,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Starch</td>
</tr>
<tr>
<td>Erythromycin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Tramadol&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Vardenafil&lt;sup&gt;#,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Denotes that visibly indistinguishable color bar codes are produced by these compounds.

<sup>a</sup>Due to low quantities of sample vardenafil was tested once and sildenafil twice to produce these outcomes that are consistent with their chemical structures. 
### Table 4.3

<table>
<thead>
<tr>
<th>Pharmaceutical name</th>
<th>Sample Color</th>
<th>Beta-lactam (lane B)</th>
<th>Ninhydrin (lane C)</th>
<th>Biuret (Lane D)</th>
<th>Tertiary amines (lane E)</th>
<th>Phenols (Lane F)</th>
<th>Carbonate (Lane G)</th>
<th>Starch (Lane H)</th>
<th>Ethambutol (Lane I)</th>
<th>NQS (Lane J)</th>
<th>SNP (Lane L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetaminophen</td>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amodiaquine</td>
<td>yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoxicillin</td>
<td>white</td>
<td>dark green</td>
<td>olive green</td>
<td>dark blue/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampicillin</td>
<td>white</td>
<td>dark green</td>
<td>orange</td>
<td>dark blue/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cephalexin</td>
<td>white</td>
<td>dark green</td>
<td>red/orange</td>
<td>dark blue</td>
<td>green/blue at swipe</td>
<td>orange</td>
<td></td>
<td>black at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorpheniramine</td>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>orange</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4.3 (continued)

<table>
<thead>
<tr>
<th>Pharmaceutical name</th>
<th>Sample Color</th>
<th>Beta-lactam (lane B)</th>
<th>Ninhydrin (lane C)</th>
<th>Biuret (Lane D)</th>
<th>Tertiary amines (lane E)</th>
<th>Phenols (Lane F)</th>
<th>Carbonate (Lane G)</th>
<th>Starch (Lane H)</th>
<th>Ethambutol (Lane I)</th>
<th>NQS (Lane J)</th>
<th>SNP (Lane L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tadalafil dosage form, varenafil dosage form, sildenafil dosage form, polyethylene glycol, quinine, chloroquine, sibutramine, erythromycin, phentermine, sildenafil, tramadol, vardenafil</td>
<td>white</td>
<td>green or aqua on swipe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>white</td>
<td>green/blue at swipe</td>
<td>orange</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dextromethorphan, diphenhydramine, oxycodone dosage form</td>
<td>white</td>
<td>blue or green at swipe</td>
<td>orange/brown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>doxycycline</td>
<td>yellow</td>
<td></td>
<td>dark brown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>doxylamine and dipyrene</td>
<td>white</td>
<td>green/blue at swipe-migrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethambutol</td>
<td>white</td>
<td></td>
<td>royal blue at top of lane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmaceutical Name</td>
<td>Sample Color</td>
<td>Beta-lactam (Lane B)</td>
<td>Nitroprusside (Lane C)</td>
<td>Biuret (Lane D)</td>
<td>Phenols (Lane E)</td>
<td>Carbonate (Lane G)</td>
<td>Tertiary Amines (Lane H)</td>
<td>Ethambutol (Lane I)</td>
<td>Starch (Lane J)</td>
<td>SNP (Lane L)</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
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<td>------------------------</td>
<td>---------------------</td>
<td>----------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>White</td>
<td>White</td>
<td>Faint</td>
<td>Pink</td>
<td>Faint/Blue</td>
<td>Yellow at swipe</td>
<td>Tertiary orange/brown</td>
<td>Pink</td>
<td>Pink</td>
<td>Red/orange</td>
<td></td>
</tr>
<tr>
<td>Atorvastin</td>
<td>White</td>
<td>White</td>
<td>Green/blue at swipe</td>
<td>Yellow</td>
<td>Orange/brown</td>
<td>Black at swipe</td>
<td>Tertiary orange/brown</td>
<td>Black at swipe</td>
<td>Black</td>
<td>Red/orange</td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>White</td>
<td>White</td>
<td>Pink</td>
<td>Pink</td>
<td>Green</td>
<td>Pink at top of lane</td>
<td>Tertiary orange/brown</td>
<td>Black at swipe</td>
<td>Black</td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>Brown</td>
<td>Brown</td>
<td>Forest green</td>
<td>Brown</td>
<td>Orange/brown</td>
<td>Green at swipe</td>
<td>Tertiary orange/brown</td>
<td>Black at swipe</td>
<td>Black</td>
<td>Green</td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td>White</td>
<td>White</td>
<td>Green</td>
<td>Forest green</td>
<td>Orange/brown</td>
<td>Black at top of lane</td>
<td>Tertiary orange/brown</td>
<td>Black at swipe</td>
<td>Black</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>R-phenylephrine</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange/brown</td>
<td>Orange at swipe</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange/brown</td>
<td>Black at top of lane</td>
<td>Tertiary orange/brown</td>
<td>Black at swipe</td>
<td>Black</td>
<td>Red/orange</td>
<td></td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>White</td>
<td>White</td>
<td>Orange</td>
<td>White</td>
<td>Orange</td>
<td>Green at top of lane</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange</td>
<td>Orange</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange</td>
<td>Orange</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange</td>
<td>Orange</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Orange</td>
<td>Orange</td>
<td>Tertiary orange/brown</td>
<td>Orange at swipe</td>
<td>Orange</td>
<td>Orange</td>
<td></td>
</tr>
</tbody>
</table>
Six active ingredients and fillers did not produce reliable color bar codes with the general purpose test card. These included clavulanic acid, diclofenac, tadalafil, hydrochlorothiazide, orlistat, and microcrystalline cellulose. This elusive group is characterized by functional groups such as secondary amines, carbonyls, and carboxylic acids, which are not identified by any of the current lane tests and provide potential targets for future investigation.

4.3.3 Test cards as screening tools for identification of low quality pharmaceuticals

I found that the ability of the general purpose test card to distinguish a counterfeit pharmaceutical from its authentic version is dependent on the nature/composition of the authentic dosage form as well as the composition of the counterfeit sample. In previous testing pharmaceutical standards either pure or cut with one filler were generally used for testing. This had worked well in the case of amoxicillin, in which the capsule contains primarily pure active ingredient. I learned at the FCC that amoxicillin is an exception rather than the rule and that for most medicines testing standards versus dosage forms is like testing apples versus apple pie. Dosage forms are a complex mixture of active ingredients, excipients and coatings that determine factors such as overall pill mass, dissolution rate, taste and pill physical strength. The color bar code produced by dosage forms is generally, though not always, more complex and more informative than for active ingredients alone. I found several cases discussed below in which changes in excipients were the key indicators of a suspicious sample.
Paper test cards were used to analyze authentic samples obtained directly from the manufacturers as well as counterfeit samples, known from independent testing at the FCC to be unapproved formulations (Figure 4.3). The best results for identification of counterfeit dosage forms were obtained with vardenafil 20mg, sildenafil 100mg and oxycodone 80mg, all of which resulted in 100% correct identification of both authentic and counterfeit samples (Figure 4.4). The test cards detected four out of four counterfeit sildenafil samples that contained substandard quantities (46-87%) of sildenafil. These pharmaceuticals all 1) produce strong colorimetric outcomes with authentic dosage forms and 2) had counterfeit dosage forms containing a substituted ingredient. In each of these examples, the counterfeit samples contained starch. This unapproved filler was easily identified by the appearance of black at the swipe line in the iodine test (lane I). In one sample of counterfeit oxycodone, the tablet core material underneath the coating of a dosage form was a different color from the authentic, making it obvious that this formulation varied from the approved version.
Figure 4.3. General purpose test card identification of counterfeit pharmaceuticals. Color production in lane tests can indicate an active ingredient or filler. The general purpose test card identifies low quality medicines based on missing or substituted ingredients detected in the chemical color bar code. Positive outcomes are circled in red to highlight differences between chemical color bar codes obtained with authentic and counterfeit samples. Low quality versions of sildenafil, vardenafil, and oxycodone dosage forms were identified by the addition of an unexpected filler (starch), while oseltamivir and a different variety of counterfeit oxycodone dosage forms show the loss of a signal, indicating the absence of active ingredient.
Figure 4.4. Accuracy of pharmaceutical screening tools compared to state of the art analysis. Percent agreement of pharmaceutical screening using the general purpose test card and handheld Raman and FT-IR devices compared to state of the art chemical analysis carried out by the FDA/FCC indicates that all methods identify a majority of the counterfeit medicines.

*Handheld Raman testing was repeated after initial scans, using a partial pill, resulted in a “fail” or not authentic result.

*Due to noise in the F017 FT-IR spectra obtained with the handheld instrument, this sample was matched most closely with a high quality counterfeit tadalafil dosage form.

*Analysis methods may include, but are not limited to GC-MS, LC-MS, microscopy, packaging analysis and FT-IR and Raman spectroscopy.
The authentic oseltamivir 75mg dosage form (capsule) produced strong positive outcomes with both cobalt thiocyanate and tri-iodide. The counterfeit product, which lacks active ingredient, also produced positive outcomes in both of these lanes, though color in lane E was faint and color in lane I was strong. Taking into account the relative color intensity of these responses to one another, 9 of 10 counterfeit samples were properly identified. One sample of an authentic oseltamivir dosage form was improperly labeled as poor quality due to the unexpected color produced in lane G, iron(III), likely caused by a fabrication error.

Tadalafil 20mg and atorvastatin (authentic 10mg and counterfeit 20mg strength) dosage forms produced weak color responses with cobalt thiocyanate that were not visible in cases of light sample dosing. For this reason, five tests run with authentic tadalafil tablets were misidentified as having little or no active ingredient. This led to a 61% sensitivity for authentic tadalafil dosage forms, but 100% of the 3 different counterfeit varieties, each containing sildenafil that was adulterated with starch, were correctly identified. Authentic atorvastatin dosage forms produced positive test outcomes with cobalt thiocyanate (lane E) and iron (III) chloride (lane G) tests that were not visible in cases of lighter sample dosing and this also led to false negatives (authentic identified as little or no active ingredient). The counterfeit atorvastatin dosage form produced a faint positive from starch, an unexpected filler, in the iodine test lane, which was not visible with light to medium sample application. Test cards identified authentic atorvastatin dosage forms with a 75% success rate and the counterfeit sample with 67% success (Figure 4.4). Therefore, in cases of light sample
application, authentic and counterfeit produced indistinguishable signals. These examples highlight the need for a standardized sample dosing tool, which is currently in development. Identification of poor quality pharmaceuticals could be improved through additional test development for pharmaceutical components such as atorvastatin and additional fillers.

4.3.4 Comparison of test cards to other pharmaceutical screening methods

Authentic and counterfeit samples of six different pharmaceuticals were tested using handheld Raman, FT-IR and paper test cards (Figure 4.4). The Raman device identified an authentic oxycodone as non-authentic when a partial pill was tested, however follow-up testing with a complete tablet produced two “pass” outcomes for the authentic product. Handheld FT-IR results for 18 of 19 samples agreed with previous FCC laboratory analysis carried out using a variety of state of the art testing methods including High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), and bench top FT-IR spectroscopy. The authentic tadalafil dosage form was indicated as being non-authentic. In this case, the authentic tadalafil dosage form spectrum was matched most closely with a high quality counterfeit version of this drug on file in the FDA/FCC FT-IR library. Another screening tool recently developed by the Forensic Chemistry Center is the Counterfeit Detection Device or “CD3”, which can evaluate the authenticity of a pharmaceutical product in minutes based on the fluorescence or reflectance of packaging, dosage forms coatings or chemical components when exposed to a variety of light ranging from infrared (IR) to ultraviolet
(UV). Counterfeit samples were placed next to authentic samples and imaged under several different wavelengths to determine if a difference could be visualized. The two readers formed the same conclusions for all of the CD3 tests. Of the counterfeit products tested with the CD3, 11 of 13 samples were identified as appearing different from the authentic (Table 4.4).
<table>
<thead>
<tr>
<th>Name and sample number of counterfeit drug (Superscripts show characterization methods)</th>
<th>Gold standard analysis findings</th>
<th>Percent of samples identified by test cards</th>
<th>Counterfeit dosage forms identified in CD3 image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin-20 mg-F009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 mg atorvastatin/tablet, unapproved formulation</td>
<td>67% (6/9)</td>
<td>Yes</td>
</tr>
<tr>
<td>Oseltamivir-F007&lt;sup&gt;a, c&lt;/sup&gt;</td>
<td>No API</td>
<td>83% (5/6)</td>
<td>Yes</td>
</tr>
<tr>
<td>Oseltamivir-F001&lt;sup&gt;a, c&lt;/sup&gt;</td>
<td>No API, carbohydrate observed by IR</td>
<td>100% (4/4)</td>
<td>Yes</td>
</tr>
<tr>
<td>Tadalafil-F0091&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>38 mg/tablet sildenafil</td>
<td>100% (5/5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Tadalafil-F0081&lt;sup&gt;a, c&lt;/sup&gt;</td>
<td>92 mg/tablet sildenafil</td>
<td>100% (5/5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Tadalafil-F010&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>15.5 mg/tablet tadalafil, also contains sildenafil</td>
<td>100% (5/5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxycodone-F020&lt;sup&gt;a, b, d&lt;/sup&gt;</td>
<td>8.4 mg codeine/tablet</td>
<td>100% (5/5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxycodone-F008&lt;sup&gt;a, b, d&lt;/sup&gt;</td>
<td>22 mg acetaminophen, 19 mg tramadol, 18 mg codeine, and 5.4 mg phenylpropanolamine/tablet</td>
<td>100% (5/5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Vardenafil-F011&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>33 mg/tablet sildenafil</td>
<td>100% (10/10)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sildenafil-F022&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>87.4 mg/tablet sildenafil</td>
<td>100% (4/4)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sildenafil-F015&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>48 mg/tablet sildenafil</td>
<td>100% (4/4)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sildenafil-F014&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>46 mg/tablet sildenafil</td>
<td>100% (4/4)</td>
<td>No</td>
</tr>
<tr>
<td>Sildenafil-F012&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
<td>57 mg/tablet sildenafil</td>
<td>100% (4/4)</td>
<td>No</td>
</tr>
</tbody>
</table>

Given that the paper test card was not optimized for the identification of the variety of pharmaceuticals involved in the testing of authentic and counterfeit products, the device performed well. All but 2 counterfeits were identified at a 100% success rate. Authentic samples were identified as non-authentic with three of the six pharmaceuticals, generally due to weak color production in the pharmaceutical color.
bar code with lighter dosing. As shown by the example of beta-lactams, further development of tailored chemical tests would enhance the ability of these cards to produce more robust color bar codes and better distinguish authentic samples in additional pharmaceutical families.

4.4 Discussion

The adaptation of classic colorimetric chemistry to a paper format allows for the production of a user-friendly, inexpensive panel of tests that builds a chemical profile for a range of pharmaceuticals. The 12-lane general purpose test card presented cannot definitively identify a pharmaceutical, but can generate a color bar code for at least 39 pharmaceutical ingredients ranging from antibiotics to antihistamines to diabetes medications and analgesics. Through the appearance of colors from unexpected fillers or the lack of signals from active ingredients, dosage forms that vary from an approved formulation can be identified. These test cards could be expanded for general use by addition of more lanes and more chemical tests, or tailored for a specific class of drugs. In addition, the chemical characterization provided by the test cards is complementary to the optical reflectance information provided by the CD3 device as well as handheld spectroscopic methods. Results from the test cards combined with other screening devices led to the detection of 100% of the counterfeit pharmaceuticals. Given its usefulness with such a range of medicines this fast and inexpensive technology offers a viable option for pharmaceutical screening in remote or resource limited settings.
4.5 References


CHAPTER 5

DEVELOPMENT OF THE FIRST PAPER-BASED, WHOLE-CELL YEAST BIOSENSOR DEVICE

5.1 Introduction

Paper-based tests have a long history of use providing answers to analytical problems outside of the laboratory, and can be especially useful in developing countries or settings where resources are limited. Recent examples include paper-based, colorimetric tests for glucose, proteins, liver function and beta-lactam antibiotics. In earlier research, paper-based tests developed in the Lieberman lab focused on chemical means for qualitative analysis of beta-lactam antibiotics and anti-tuberculosis medicines. These chemical tests are rapid and user-friendly but, as is generally true of chemical tests, rely on the recognition of functional groups or chemical motifs, and therefore multiple test lanes must be combined for results to be specific. To increase specificity, paper-based tests incorporating antibodies have become a staple of medical diagnostic testing. The use of antibodies, however, requires isolation steps, and these purified biological components can be unstable during long term storage. The use of whole microorganisms in a biosensor device eliminates the need for isolated components, simplifying fabrication methods and potentially increasing test longevity.
The primary aim of this research is to harness robust biological recognition and response displayed by whole cell biosensors by incorporating genetically engineered yeast into paper analytical devices (PADs), thus making “BioPADs.” Living organisms like yeast possess the innate capacity to respond to many pharmaceuticals including antibiotics, and they can be made responsive to others through genetic engineering. Redirecting this responsive genetic machinery to produce a reporter molecule transforms yeast into a sensor for specific medications. Incorporating these biosensors into a paper-based test could produce an inexpensive and specific test, increasing the repertoire of current paper-based tests.

While several whole-cell biosensors have been developed, most are bacteria based, and those converted to paper-based tests are limited to bacterial systems, for example, in the detection of arsenic and quorum sensing molecules. These examples showed that reporters could be produced by bacterial biosensors on a paper substrate. However, yeast offer some advantages for use in a biosensor including a) tolerance to pH and temperature fluctuations, b) established procedures for long term storage, c) ability to survive over long periods of time in a dried state, d) an extensive genetic toolkit, e) a non-threatening public perception, and f) eukaryotic nature, such that response to many pharmaceutical agents and/or toxic substances is similar to higher eukaryotes. Previously developed yeast biosensors have relied on electrode measurements of solution pH or oxygen levels that reflect the increased metabolism of a substrate in the presence of the analyte. For example, as yeast metabolize glucose, the drop in pH of the surrounding solution is measured to reflect glucose concentration.
Concentrations of small molecules, such galactose and copper, have also been measured by these means.\textsuperscript{12–14} This chapter explores the possibility of using yeast as the whole cell biosensor embedded in paper and improves upon previous biosensors on paper by a) defining test zones to combine multiple tests on one device, b) trapping cells onto paper with a hydrogel matrix, c) evaluating a visual interpretation of tests used with a pharmaceutical dosage form and d) carrying out a long term study measuring test viability following storage.

5.2 Experimental

5.2.1 Materials

Doxycycline hydrochloride was obtained from MP Biomedicals (Santa Ana, CA). Difco YPD (yeast peptone dextrose) broth was obtained from BD (Franklin Lakes, NJ). Dextrose was obtained from J.T. Baker (Center Valley, PA). Yeast nitrogen base without amino acids and Magnesium sulfate hexahydrate were obtained from Bioworld (Dublin, OH). Granulated agar was obtained from Research Products International Corp. (Mt. Prospect, IL). Amino acid drop-out media from USBiological (Salem, MA). Trehalose dehydrate was obtained from Calbiochem (Darmstadt, Germany). Calcium chloride, sodium phosphate monobasic anhydrous, sodium phosphate dibasic anhydrous, 2-mercaptoethanol, N-N-dimethylformamide (DMF) and potassium chloride were obtained from Fisher Scientific (Waltham, MA). Ahlstrom 319 cellulose sheets were obtained from Midland Scientific (Chicago, IL) 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (X-gal) was obtained from IBI Scientific (Peosta, IA). Parafilm and
Whatman #1 filter paper was obtained from VWR (Radnor, PA). Sodium alginate-medium viscosity and yeast synthetic drop-out medium supplement without uracil was obtained from Sigma-Aldrich (St. Louis, MO). Drierite desiccant was obtained from W.A. Hammond Drierite Company (Xenia, OH). Scotch Brite Pads, zipper lock plastic bags, Nuk® brand baby bottle, cellulose sponges and aluminum foil were obtained from a local grocery store. The *Saccharomyces cerevisiae* strain, CML282 (MATa CMVp(tetR-SSN6)::LEU2 ura3-1 ade2-1 leu2-3,112 his3-11,15 trp1-Δ2 can 1-100) derived from BMA64-1A strain, and the pCM176 (tetO7-lacZ TRP1) plasmid were a kind gift from the Herrero Lab at the University of Lleida.\textsuperscript{15}

5.2.2 Fabrication methods

The 12-lane lateral flow devices were fabricated as described previously.\textsuperscript{4} Stationary or “spot” BioPADs were fabricated on Whatman #1 filter paper printed with a Xerox ColorQube 8570. Printed stationary tests were incubated in a 100°C oven for 45 seconds to seal the hydrophobic barrier prior to application of yeast to the paper substrates, as described below.

5.2.3 Yeast preparation and immobilization on PADs

Lithium acetate yeast transformation was carried out according to Becker and Guarente.\textsuperscript{16} Yeast cultures grown in SC-trp media\textsuperscript{17} and ranging from 1.2 to 4.2 OD\textsubscript{600nm} were concentrated by centrifugation for 10 minutes at 3700g, the supernatant was aspirated away, and the yeast pellet resuspended in 2% sodium alginate (aq) (low-
viscosity) containing 5% trehalose to give a final concentration of yeast equivalent 2.0 OD$_{600\text{nm}}$ units per 10µL solution. The yeast-hydrogel slurry was spotted in 10µL aliquots onto PADs as described above and cross-linked by immersion in a 4% CaCl$_2$ solution for 15 minutes. Papers were rinsed by immersion in de-ionized water and air dried for at least 2 hours at room temperature before use in testing.

5.2.4 Analyte exposure and reporter color development with BioPADs (stationary tests)

Stationary BioPADs embedded with yeast, as described above, were exposed to 10µL aliquots of doxycycline in YPD media$^{17}$ at concentrations of 0.1-10,000µg/mL. These tests were set on a Scotch Brite pad and sealed in a culture dish, using Parafilm, for 16 hours at 30ºC with 1in$^3$ hydrated sponge squares to maintain humidity. Yeast were lysed by dipping paper devices into liquid nitrogen twice for 5 seconds. Five microliters of Z-buffer$^{18}$ containing 0.3mg/mL X-gal (5-bromo-4-chloro-3-indoly]-β-D-galactopyranoside) was pipetted onto each “spot,” and the BioPADs were incubated at 30ºC for 4 hours in a parafilm sealed culture dish with hydrated 1 cm$^3$ sponge squares to allow color to develop.

5.2.5 Colorimetric analysis

BioPADs were imaged in RGB mode using a Hewlett-Packard HP PhotosmartC4150 All-in-one scanner. Images were inverted in ImageJ, and the mean intensities of test regions were measured in the red channel. In lateral flow testing, mean intensity from a neighboring testing region of paper was subtracted as background. In this manuscript I
report the adjusted mean intensities for each experimental condition, defined as the average of the experimental samples minus negative controls.

5.2.6 Determination of yeast growth stage for optimal BioPAD fabrication

Yeast cultures were grown to OD$_{600nm}$ of 1.2, 2.2 and 4.2, fabricated into “spot” BioPADs, as described above, and stored at 4ºC prior to testing. BioPADs were exposed to doxycycline at concentrations of 0.1, 1.0, 10, 100 and 1,000µg/mL, reporter color developed and ImageJ analysis carried out as discussed above.

5.2.7 Determinations of concentrations of doxycycline toxic to yeast

Overnight cultures of the dual tetracycline responsive *Saccharomyces cerevisiae* from standard glycerol stocks, as described above, were grown in SC-trp media at 30ºC with shaking at 195 rpms. 100µL aliquots of the resulting culture were diluted 1:10 (v/v) using SC-trp to produce 1mL cultures that contained 0, 0.1, 0.3, 1.0, 3.0 and 10mg/mL doxycycline. These cultures were placed on a rotator and incubated at 30ºC for one hour. Cultures were then diluted 1:1000 in SC-trp media. Then 10µL of the final dilution were plated onto SC-trp plates and incubated at 30ºC for 3 days, after which the number of colony forming units was recorded.
5.2.8 Measuring BioPAD responsive range for doxycycline

Stationary BioPADs were fabricated as described above and exposed to doxycycline ranging from 0-10,000µg/mL (4 spots per concentration). BioPAD exposure, reporter development and ImageJ analysis were carried out as described above.

5.2.9 Measuring hydrogel effect on bacterial doxycycline response

DH5α bacterial cells containing the pUC18 plasmid were grown in LB media containing 100µg/mL ampicillin. To prepare the hydrogel conditioned medium, hydrogel beads were produced by cross-linking 150 aliquots of 2% sodium alginate (aq) (low-viscosity) containing 5% trehalose for 15 minutes with 4% CaCl₂. The hydrogel mixture and all solutions used were sterilized by autoclaving or filter sterilization. Beads were allowed to dry overnight. 1mL of LB/amp media containing 30µg/mL doxycycline was pre-incubated with and without a hydrogel bead. 5 µL aliquots of bacterial cell culture was combined with 495µL of each of the pre-incubated LB/amp/doxy mix as well as with 495 µL of LB/amp. Cells were cultured at 37°C for an hour, plated on LB/amp plates, incubated overnight at 37°C and resulting colony forming units were counted. Tests were carried out in duplicate in three separate trials.

5.2.10 Measuring BioPAD ability to identify presence/absence of doxycycline in dosage forms

The test solution was produced by dissolving a 100 mg doxycycline dosage form in approximately 100 mL of tap water with 5 g of YPD dry media. To carry out the testing
procedure without laboratory measuring tools, measurement was carried out in a baby bottle. The 100mL marking of the bottle was found to correspond to 104.3 ± 1.2mL (n=3). Lateral flow BioPADs were placed in cups containing either the test solution (n=64) or a control solution (n=56), containing hydrated media only, to the blue indicator line found on the BioPAD. Cups were sealed using Parafilm and incubated at 30ºC overnight. Cells were lysed by two 5 second freeze thaw cycles in liquid nitrogen and BioPADs were placed onto blotter paper, saturated with Z buffer containing 0.33mg/mL X-gal dissolved in DMF and in a culture dish. Hydrated cellulose sponge cubes were added to prevent test drying and the culture dishes were sealed using paraffin film and incubated at 30ºC for 4 hours. Lateral flow BioPADs were imaged in the same manner as stationary BioPADs as described above.

5.2.11 Measuring BioPAD viability following extended storage

A 200mL culture of tetracycline responsive yeast was grown to an OD$_{600nm}$ of 1.7 for BioPAD storage at -20ºC, 4ºC and 25ºC, and 2.7 for storage at 54ºC, 37ºC, 30ºC, and a second trial of 25ºC. BioPADs were fabricated from these cells as described above, wrapped in aluminum foil and sealed in a zipper-lock baggie with 7-10 grams of Drierite. BioPADs were set upright in a beaker of YPD media containing 100µg/mL doxycycline at a volume sufficient to reach the blue indicator line marked on the paper device. Negative controls were similarly exposed to media only. Testing procedure and reporter visualization was carried out as described above for lateral flow BioPADs. Hydrated sponge squares (1 cm$^3$) were added to the color development step on day 120 to
prevent test drying. Significant color production indicating BioPAD viability was confirmed by two-tailed Student T-test comparison of color intensity of yeast with and without exposure to doxycycline. Viable BioPADs produced significant color compared to negative controls with >95% confidence levels.

5.2.12 Preparation of glycerol stocks

Yeast obtained from the Herrero Lab containing CMVp(TetR-Ssn6) and transformed with the pCM176 plasmid \(^{15}\) were grown overnight in SC-trp (synthetic complete media minus tryptophan) media at 30º C and diluted in SC-trp media and 50% glycerol to give a final glycerol concentration of 15% and yeast concentration equivalent to an OD \(_{600nm}\) of 0.8. These glycerol stocks were stored at -80ºC and subsequently used to inoculate all cultures used in this manuscript, with the exception of cold temperature stability testing.

5.2.13 Induction of reporter in liquid cultures

To produce positive controls used in BioPAD testing, yeast glycerol stocks, as described above, were used to inoculate 200mL of SC-trp media and were incubated at 30ºC with shaking at 195 rpm to overnight. Doxycycline hydrochloride was added to a concentration of 100µg/mL and cells returned to the incubator for 6 hours. Cells were isolated by centrifugation for 10 minutes at 3700g and liquid media discarded. Aliquots of yeast, equivalent to 2.0 OD \(_{600nm}\) units, were deposited into microcentrifuge tubes and
stored at -20°C. These samples were subsequently lysed by two 5 second freeze thaw cycles in liquid nitrogen and pipetted onto BioPADs prior to color development.

5.3 Results

5.3.1 BioPAD yeast component considerations

I envisioned that an analytical device composed of simple components, i.e., yeast, paper and hydrogel, would be a new and useful tool to address analytical questions in low resource settings. In developing this paper-based biosensor I wanted a test design that was easy to make and easy to use. I also recognized at the outset that a yeast strain should respond to analyte with robust expression of a reporter molecule, while tightly repressing reporter expression in the absence of analyte. Since the ultimate goal was use of this device in a resource limited setting, I chose to use yeast able to produce a visible reporter signal, eliminating the need for specialized equipment for reporter detection. Additionally, the analyte recognized needed to be relevant to an analytical problem in a low resource setting. For test fabrication, I wanted to embed yeast into paper using a “yeast-friendly” environment that would fix cells to a location on paper while allowing the analyte to pass freely through the matrix. Finally, I hoped to determine a straightforward method of preservation that would allow tests to remain viable during storage and transport.

Yeast incorporated into the BioPAD are able to produce reporter in response to antibiotics in the tetracycline family. In the absence of tetracycline antibiotics, repressors bind a series of tetracycline operators placed in tandem upstream of the
promoter region of the reporter gene and block transcription. Tetracycline antibiotics, when present, interact with both activators and repressors, causing the release of repressors promoting binding of activators to the Tet operators. This dual control of repression and activation produce a tightly regulated expression system.\textsuperscript{15,19}

To fix the yeast into paper I chose a calcium alginate hydrogel matrix, a cross-linking carbohydrate polymer previously used with \textit{Saccharomyces} in the food industry\textsuperscript{20} and shown to encapsulate cells without inhibiting cell function.\textsuperscript{13,21} A 2\% medium viscosity sodium alginate solution (w/v) containing 5\% trehalose as a cryoprotectant,\textsuperscript{22} cross-linked with calcium ions, was able to contain yeast cells, while allowing exposure and response to doxycycline in the test solution.

5.3.2 BioPAD test design considerations

Two test designs, exposing yeast to analyte through either a lateral flow design\textsuperscript{4} (Figure 5.1a) or a stationary system (Figure 5.1d), were employed in this study. Images of test regions following exposure to either 0 or 1 mg/mL doxycycline are shown in Figures 5.1b and 5.1c, respectively. Yeast in the stationary format, exposed to a range of doxycycline concentrations, are shown in Figure 5.1e. While the stationary BioPADs provide a well-defined testing area, the lateral flow BioPADs are more user-friendly (wicking replaces the need for sample deposition) and therefore are the more desirable format for a qualitative analytical tool for use outside of the laboratory setting. In this study, stationary tests were used to define a responsive range of BioPADs and lateral
flow testing was employed to determine test stability following storage as well as presence/absence of active ingredient in doxycycline pills.

![Figure 5.1 BioPAD in lateral flow (a-c) and stationary (d-e) formats. (a) Lateral flow BioPADs (6 x 11 cm) as described in Weaver et al. 4 have a QR code corner fiducials and a color box for computer image analysis in development. Test lanes are separated by hydrophobic barriers and yeast spotted in the lower third of the test lanes. Following exposure to doxycycline a blue pigment is produced following exposure to color developing solution (c) compared to yeast without doxycycline exposure, which appear pink (b). The stationary format (8.5 x 11 cm) isolates testing regions using wax barriers. Hydrophilic lines or “moats” are included to redirect liquid that may contaminate a neighboring test region (d). Yeast test zones exposed to doxycycline concentrations ranging from 0-10,000 µg/mL are shown in image e.](image)

### 5.3.3 Growth phase considerations

Yeast liquid cultures of logarithmic (log), late log, and approaching stationary growth phase were exposed to doxycycline in concentrations of 0.1, 1, 10, 100 and 1,000 µg/mL overnight at 30°C. Log phase yeast having an OD$_{600nm}$ of 1.2 were found to be most responsive to doxycycline, especially at 100 µg/mL concentrations, while BioPADs fabricated with yeast at an OD$_{600nm}$ of 4.2 were generally least responsive (Figure 5.2). Therefore, subsequent BioPAD fabrication was carried out using log phase yeast. In
preliminary studies I found that two OD$_{600nm}$ units of yeast at log phase, concentrated and embedded into paper in a hydrogel matrix were sufficient to visualize reporter production in response to 1-10,000 µg/mL analyte (not shown); this amount of yeast was used for fabrication of all further BioPADs unless otherwise indicated.

Figure 5.2 Yeast response to doxycycline. (a) Yeast cultures were grown to OD$_{600nm}$ of 1.2, 2.2 and 4.2, incorporated into a stationary BioPAD, exposed to doxycycline and reporter intensity measured using ImageJ software. BioPADs incorporating yeast at OD$_{600nm}$ 1.2 and 2.2b were fabricated 14 days prior to testing and BioPADs at OD$_{600nm}$ 2.2a and 4.2 were fabricated 4 days prior to testing. Yeast grown to culture densities of 1.2 were most responsive to doxycycline, particularly as the concentration approaches 100µg/mL. (b) Colony forming units were counted following a 1 hour exposure of the tetracycline responsive yeast in actively growing liquid cultures to various concentrations of doxycycline. Concentrations of 3mg/mL and higher were found to be lethal.
5.3.4 BioPAD response

To test the responsive range of stationary BioPADs, these tests were exposed to 0 - 10,000 µg/mL doxycycline. At concentrations between 1 and 10 µg/mL, BioPAD response was too variable to clearly indicate the presence of doxycycline, while following exposures from 30 - 10,000 µg/mL, reporter intensities were distinguishable from negative controls with a >95% confidence level, with the strongest color response at 100 µg/mL (Figure 5.3). I noted the moderate loss of signal at higher doxycycline concentrations, and hypothesized that it might be due to yeast death induced by doxycycline.

![Figure 5.3 BioPAD reporter intensity in response to doxycycline exposure.](image)

Figure 5.3 BioPAD reporter intensity in response to doxycycline exposure. The increase in reporter intensity of stationary BioPAD in response to doxycycline/media mixture, as compared to media only samples, is reported over a range of concentrations. Doxycycline concentrations between 30-10,000 µg/mL can be distinguished from negative controls by a Student T-test with p < 0.05. The standard deviation of media only samples is indicated by the dashed red line.
To test the effects of high levels of doxycycline on yeast mortality, actively growing cultures were exposed to doxycycline for 1 hour with 30°C incubation and then plated. The resultant drop in colony forming units (Figure 5.2b) shows that doxycycline at high concentrations (> 3 mg/mL) is lethal to actively growing cultures of this strain of yeast. This observation provides a potential explanation for why the reporter signal drops off at high levels of doxycycline, but comparison of these results to those in Figure 5.2 show that yeast in the BioPAD produce significant amounts of reporter up to concentrations of 10 mg/ml, indicating that they are less susceptible than yeast in suspension culture.

A remaining question is why the yeast in the BioPAD are more resistant to doxycycline than are yeast in suspension culture. One explanation might be that the hydrogel matrix sequesters or interferes with the doxycycline. I found that pre-incubation of doxycycline with hydrogel did not reduce the effect of the antibiotic solution (Figure 5.4). Previously, it has also been shown that glucose and α-lactoalbumin diffuse freely from 2% calcium alginate beads.23 Based on this evidence, I find it unlikely that this problem is due to the hydrogel. It is interesting to speculate that the reduced doxycycline susceptibility in the BioPAD is similar to the microbial response seen in bacterial and fungal biofilms, a phenomenon that is not well understood.24 These issues warrant future investigation.
To test the BioPAD using a field relevant sample, I measured its ability to identify the presence/absence of doxycycline in pharmaceutical dosage forms obtained from a local pharmacy. Doxycycline dosage forms (100 mg) used in this study were analyzed for purity by Michelle Joyce by high performance liquid chromatography (HPLC), comparing the predominant peak with that from a doxycycline standard. This peak was confirmed to be doxycycline by an exact mass measurement from electrospray mass spectrometry (Figure 5.5). A pill was dissolved in approximately 100 mL of complete yeast media to comprise the test solution. With an eye towards BioPAD use in resource limited
settings, testing was carried out with the more user-friendly lateral flow. 64 lateral flow test lanes were exposed to the doxycycline test solution and 56 to media only. Evaluation of the test results was carried out both visually and by ImageJ analysis to determine the predictive power of the BioPAD.

Figure 5.5  High performance liquid chromatography analysis of doxycycline dosage form. HPLC chromatograms at 350 nm of (a) 2.5 µg doxycycline standard injected on column and (b) 10.8 µg tablet 1 injected on column. HPLC was performed on a Waters Alliance 2695 chromatograph equipped with a 2998 Photodiode Array Detector. The column used was a Waters XBridge 5 µm, 3.0 x 50 mm C18. The mobile phases consisted of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient was run at 1 mL/min and began at 95% A: 5% B which was held for 3 minutes. The mobile phase was ramped to 35% B over the next 2 min and held for 1 minute. The mobile phase was then immediately ramped to 50% B and held for 1 minute. The column was re-equilibrated to initial mobile phase conditions at the end of the run. Standard doxycycline was directly infused on a Bruker micrOTOF II ESI-mass spectrometer for the exact mass measurement. This data is representative of chromatograms of all three pills analyzed.
Test evaluations by visual and ImageJ analysis were found to be in agreement. A blind visual inspection, carried out by 2 readers comparing outcomes to standard images, identified 92% of samples containing doxycycline, and correctly identified 100% of samples containing no doxycycline (Table 5.1) with only one sample disagreement, requiring a third reader as a tie-breaker. The second method, measuring reporter intensity using ImageJ software, found 95% of positive samples to have reporter intensities higher than samples not exposed to doxycycline. Intensity values from this second method were used to assemble a receiver operating characteristic (ROC) plot (Figure 5.6), able to measure the distinguishing power of a diagnostic. A ROC plot graphs the lateral flow BioPAD sensitivity (fraction of positive samples identified correctly) against 1-selectivity (fraction of false positives) for a series of possible thresholds that differentiate positive from negative outcomes. Ideal results in a ROC plot will show sensitivity approaching 100% before false positives are detected. Determining the area under the curve (AUC) is a common evaluation for the ROC plot with areas near .90 or greater indicating a useful diagnostic. In this case the AUC is .994 (n=120), using the Mann-Whitney U–test. These measurements support the idea that the BioPAD is a useful indicator of the presence/absence of doxycycline at concentrations of 1 mg/mL.
TABLE 5.1

ACCURACY OF LATERAL FLOW BIOPAD BY VISUAL INSPECTION

<table>
<thead>
<tr>
<th></th>
<th>True positive</th>
<th>True negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioPAD+</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>BioPAD-</td>
<td>6</td>
<td>56</td>
</tr>
<tr>
<td>N=</td>
<td>64</td>
<td>56</td>
</tr>
</tbody>
</table>

92.2% sensitivity  100% selectivity

Figure 5.6 Receiver operating characteristic (ROC) plot of BioPAD response. A ROC plot was assembled reflecting the distinguishing power of the BioPAD for identifying the presence/absence of doxycycline at ~1mg/mL concentrations. The area under the curve (AUC) determined by Mann-Whitney is .994, indicating that the BioPAD is a useful tool for this analytical task.
5.3.5 BioPADs stability in storage

One of the greatest advantages of yeast is their stability during storage. Lateral flow BioPADs, wrapped in aluminum foil and sealed in a zipper-locked baggie containing desiccant, were stored in both warm and cold environments. Viability of the BioPAD was measured by comparing the mean intensity of reporter, measured in ImageJ, for test lanes exposed to 100 µg/mL doxycycline in media versus media only. BioPADs were considered viable when the average reporter intensity was determined to be significantly higher than the negative control (p<0.05) by the Student T-test. BioPADs kept at 4ºC were found to be responsive to analyte for at least 6 months (no later samples were available for testing) while those at -20ºC were viable for > 1 year (415 days with no later samples for testing) (Figure 5.7). BioPADs were found to remain viable following 12 days of storage in 54ºC conditions before losing activity, while those stored at 37ºC were responsive for at least 56 days with no further tests available. These results indicate that cool storage is preferred, but tests can remain reliable following exposure to warm or even hot temperatures.
Figure 5.7 Days of BioPAD viability following storage. Test response was measured following storage in aluminum foil and sealed in a zip-lock storage bag at various temperatures. Filled blue markings note viable BioPAD activity, while open markings indicate a failure of BioPAD response. Test validity was measured by a significant increase in reporter intensity as compared to the negative control by Student T-test, p<0.05. Two separate trials were carried out at room temperature, one trial remaining viable for 56 days, while the second trial remained viable for greater than 200 days. Note that day 56 at -25°C has a p value=0.057.

5.4 Discussion

A biologically-based Paper Analytical Device (BioPAD) was developed and is the first example of a yeast whole-cell, paper-based biosensor. This research establishes the fabrication techniques and distinguishing power of this paper-based analytical device, composed of yeast, hydrogel and paper. Both stationary and lateral flow test designs allow multiple tests to be carried out on one paper device. BioPADs were able to indicate the presence of doxycycline in the range of 30 - 10,000 µg/mL with a peak in
response around 100 µg/mL. Concentrations of doxycycline above 100 µg/mL show a reduced reporter response and 3mg/mL doxycycline was shown to be lethal to actively growing yeast cultures. In this study, tests proved to be an effective analytical tool, positively identifying active ingredient in doxycycline tablets with 92% and 95% success when interpreted by eye and computer image analysis, respectively without false positives. BioPAD viability, stored in aluminum foil and sealed in plastic zipper lock bags, was found to be at least 56 days at room temperature and at least 415 days when stored at 4ºC making them suitable for use in remote locations that require test transport and storage. While current testing requires a cell lysis step, too cumbersome for field testing, future work using fabrication methods presented here could be applied to other yeast biosensors. Given the current collection of genetically engineered yeast that exist, research in this vein could give rise to a series of yeast whole-cell, paper-based biosensor devices able to address a range of analytical questions that exist outside of the laboratory.

5.5 References


CHAPTER 6

FUTURE DIRECTIONS

6.1 Chemical test future directions

My work with chemically-based paper analytical devices was driven by the real world problem of poor quality medicines. At the end of my Ph.D. research, I can step back and assess the field-friendly nature of the chemical test cards against the ASSURED criteria (Figure 6.1) discussed in the introduction of this thesis. Though the market price of these test cards is still unknown, it’s estimated that they can be produced and sold for under one US dollar, making them more affordable than other methods available. The two step procedure that takes 6 minutes from start to finish makes the chemical test card both user-friendly and rapid. With an image analysis program in the works, evaluating the test card will soon be more user-friendly without requiring specialized equipment. One area of improvement for the paper test cards, is inconsistent sample dosing. Without the use of analytical tools, we have not been able to find a method or dosing device that can deposit a controlled amount of sample onto the test card. Without controlled dosing, the test cards cannot carry out reliable quantitative analysis. Against the ASSURED criteria, however, these test cards score very well. My vision of future research on this project includes ideas for further development of the paper-
based test cards as well as some exploration into some fundamental chemistry questions encountered during my Ph.D. research.

6.1.1 Analysis of test card products

In the development of lane tests for the test cards, colorimetric reactions that were previously carried out as “wet chemistry” in test tubes were adapted for chromatography paper. Converting from glassware to paper prohibits the use of strong acids and bases, most organic solvents and heating. Adapting a color producing test in this way sometimes led to a non-functional version of the test, but in the case of ninhydrin it created a lane test that produced a collection of colored responses that gave more information than expected, producing unique colors in the presence of both ampicillin (orange) and amoxicillin (green). These colors are most likely the result of the ninhydrin and beta-lactam forming a Schiff base (Figure 6.2), however searches in the

Figure 6.1 ASSURED criteria outlined by the World Health Organization that has been used by many as a guide for the development of field-friendly technology.
current literature did not unearth any publications addressing the chemistry occurring in this lane. Testing with the “paper-friendly” version of the ninhydrin test, described in chapter 2, could be carried out in a test tube using amoxicillin, ampicillin and additional beta-lactam antibiotics. The structure of products formed could be analyzed by nuclear magnetic resonance or infrared spectroscopy and absorption and emission spectra could be determined by spectrophotometry.

![Chemical Structure](image)

Figure 6.2 The proposed Schiff base product formed by amoxicillin and ninhydrin on the test card.

6.1.2 Safeguarding against misuse of the test

A more practical question related to the chemical test card concerns how easy (or difficult) it would be for a counterfeiter to “fool the test.” Given that 1) we have published information explaining how the test card works and 2) making counterfeit
pharmaceuticals is a million dollar industry, it is not unlikely that a skilled counterfeiter could produce a formulation that is cheaper but looks authentic to the test card. Such a formulation could then use the paper test cards to validate the counterfeit. For new technologies to be adopted, they must be trustworthy and resistant to these kinds of attempts. Therefore, I think it is important to take pro-active steps to protect the technology from being corrupted in such a manner. There are a few tactics that I believe would make this task more difficult.

Already, adding more test lanes to the card is in the plan. The current card contains 12 lanes, but more than 12 lane tests have been developed. Adding more tests would expand the profile that the card provides and make the expected outcome for a formulation more difficult to reproduce. As the test card expands, adding lane tests that can distinguish between medicines that currently produce redundant signals is of particular interest. For example, the cobalt thiocyanate test lane is triggered by a large number of pharmaceuticals as well as the filler polyethylene glycol, making this test lane unspecific. To differentiate between the many compounds that react with cobalt thiocyanate, I added a variation of that lane test to the card. In the second version, a buffer at pH 8.0 was added and only a subset of compounds producing color in the original lane produced a response with the modification, increasing test specificity. Another lane test variation that might be advantageous is to vary the concentration of reagents placed on the test cards. With a limited number of test lanes, my inclination was to optimize tests for greatest color production. Once a lane test was found to produce color, I often increased the quantities of reagents that were limiting to increase
color production. This tactic allowed the test card to detect smaller quantities of target analyte, but if additional lanes were available then lanes with reduced reagents could produce semi-quantitative information. These lanes could add specificity to the test through the identification of samples containing sufficient analyte to trigger a response with reduced reagent. This experimentation would need to be carried out using measured amounts of analyte and would not be useful in the field until a uniform dosing method is found.

While at the FDA, I realized that a combination of talc with a small amount of polyethylene glycol would mimic Viagra on the test card being used at the time. Viagra was never a target pharmaceutical in the initial development stages of the test card, so when those samples were tested I wasn’t surprised that the color bar code was simple and easily replicated. This discovery led to further test development. Currently there are still several pharmaceuticals that do not produce a complex “color bar code” with the test card. As a counterpoint to the above work focused on developing a “harder to beat” test, I think it would be interesting to have an undergraduate student, who is given test cards along with information about the test cards, and set to the task of making counterfeits that could beat the test. This “nefarious” researcher could start with one of these pharmaceuticals that produces a simple color bar code and see how easy or difficult it is to make a counterfeit that would be authenticated by the test cards. This project would point out weaknesses that exist in the test and help direct lane development. This “cat and mouse” research set-up would help advance test card development, while confronting problems that could arise in the future.
6.1.3 Exploring semi-quantitative analysis

Chemical tests are good indicators of the absence or substitution in active ingredients and/or fillers, however identifying medicines with reduced active ingredients is more difficult and would require semi-quantitative information. The potential for these tests to provide semi-quantitative analysis should be investigated for lane tests that give a dose dependent response. Preliminary work has been done showing significant reductions in color intensity between samples of 100%, 60% and 0% doxycycline with iron (III) chloride. It would be interesting to carry out repetitions this experiment to see how reproducible semi-quantitative analysis would be using the current testing method. Dosing could be controlled in this case by uniform application of a pre-determined amount of sample measured on an analytical balance. A second set of experiments stems from the observation that color intensity of 60% doxycycline changes dependent on the filler present in the sample. It would be worthwhile to investigate the cause of this variation. It may occur due to a difference in chemical reactivity or as an artifact of sample dosing. Repeat testing of a controlled quantity of 60% doxycycline with different fillers could be done in the lab using an analytical balance to measure the sample to be applied uniformly to the test card. If the same amount of doxycycline produces different intensities with different excipients, then this should be repeated with additional active ingredients, such as amoxicillin, to see if other test/sample combinations also show this effect.

Another way to gather semi-quantitative information is through the image analysis software. This image analysis software is an active area of research in collaboration
with computer scientist, Dr. Chris Sweet. The steps of image analysis are outlined on Figure 6.3. Software is being developed to identify multiple areas of interest in the test card lanes followed by a principal component analysis to determine which pieces of information are most useful in distinguishing one pharmaceutical ingredient from another. As this work progresses, I would suggest that in addition to looking at color hue and intensity within each lane, that the ratio of color intensities between lanes should also be considered. Test cards can produce signals from multiple active ingredients and/or fillers. The appearance (or disappearance) of these signals identifies poor quality medicines based on the absence or substitution of compounds in a medication. An analysis of the relative intensities of signals to one another can provide additional semi-quantitative information reflective of the ratio of pharmaceutical components to one another.
Figure 6.3 Steps of image analysis and color detection. An image of the test card is taken with a cell phone and sent for computer analysis (a). The image is first transformed using corner fiducial markings to correct for skewed pictures (b). Images are then rescaled to a standard size (c). Using wax fiducials (not present in earlier test card versions as seen in a-c), lanes are identified here as shown by the appearance of green lines identifying the wax barriers (d) and difference in color intensities is used to identify “blobs” of color in each lane. Examples of blobs are outlined in white in the image above (e). Color detection is discussed in detail in appendix 4. This description is a snapshot of the program in progress and by evaluating the ability of the system to identify unknowns test card analysis can be optimized by varying the color detection methods. These steps allow a computer program to detect and define the colorimetric information found in each test card lane. Information on color intensity and location for a collection of authentic samples is currently being collected and will serve as standards to which unknown samples are compared. Note that test card images vary because lane and color detection has only taken place with test cards containing lane test replicates.
6.1.4 Using test cards with the CD3 to gain layers of information

While at the FDA, I was able to work with the CD3, counterfeit detection device, in collaboration with Nico Ranieri and Mark Witkowski, the inventors of the device. We became interested in the idea that the CD3 might work with test cards in a synergistic manner, by allowing the colorimetric output of the paper test cards to be analyzed under a variety of excitation wavelengths. Several pharmaceutical components, such as artesunate, ciprofloxacin and talc are known to fluoresce, but it was unknown how this would affect test card results. To investigate this question, I imaged a series of test card outcomes with a variety of pharmaceuticals. In this preliminary testing I found that fluorescent compounds, such as ciprofloxacin, could be visualized at the swipe line and in a few cases, interesting patterns of fluorescence were produced in the test lanes (Figure 6.4). This line of investigation incorporating fluorescence would strengthen the ability of the test cards to work with fluorescent compounds that have not been responsive to colorimetric tests, like artemisinin used for treating malaria. It also opens the door for new lane tests that would produce fluorescence, such as Calcium Green-1 for the identification of calcium.²
6.1.5 Kenyan field study and data analysis

To begin to shed more light on the prevalence of poor quality medicines, a collaboration was formed with members of the Academic Model Providing Access to Healthcare (AMPATH). AMPATH is an organization, originally formed in 1989 in response to the Human Immunodeficiency Virus (HIV) pandemic in Kenya. This organization includes Moi University, Moi Teaching and Referral Hospital and a consortium of North American universities, including the University of Notre Dame. AMPATH’s mission is to support patient care, personnel training and medical research to help address both short and long term health issues in Kenya. Working with pharmacists in Eldoret, Kenya under the umbrella of this organization, we began a field study.

Figure 6.4 Visualization of test cards using the CD3. The fluorescence of samples, such as ciprofloxacin, can now be seen. Other samples, such as chlorpheniramine, produce a unique pattern of fluorescence with the tests. Evaluating the test card outcomes using the CD3 reveals an added layer of information concerning sample identity.
study that would serve to evaluate the performance of the test cards in the field as well as evaluate the quality of a group of medicines in the Eldoret, Kenya region.

To date, nearly a thousand medications have been purchased and tested with the chemical test card (Table 6.1). The field study, focused on the medications amoxicillin, ampicillin, ciprofloxacin and azithromycin, will evaluate samples with the chemical test cards in Kenya as well as at the University of Notre Dame and compare the test card evaluations to results obtained using standard pharmacopeia HPLC analysis (Figure 6.4).
**TABLE 6.1**

TEST CARD IMAGES ACQUIRED IN KENYA

<table>
<thead>
<tr>
<th>Test Card Group</th>
<th>Pharmaceutical Type</th>
<th>Kenyan Test Card Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012 Design Kenya: Pre-Lilly</td>
<td>Acetaminophen</td>
<td>35</td>
</tr>
<tr>
<td>2012 Design Kenya: Pre-Lilly</td>
<td>Amoxicillin</td>
<td>30</td>
</tr>
<tr>
<td>2012 Design Kenya: Pre-Lilly</td>
<td>Amoxicillin-clavulanic acid</td>
<td>30</td>
</tr>
<tr>
<td>2012 Design Kenya: Pre-Lilly</td>
<td>Ampicillin</td>
<td>30</td>
</tr>
<tr>
<td>2013 Design Kenya: Phase I</td>
<td>Acetaminophen</td>
<td>29</td>
</tr>
<tr>
<td>2013 Design Kenya: Phase I</td>
<td>Amoxicillin</td>
<td>31</td>
</tr>
<tr>
<td>2013 Design Kenya: Phase I</td>
<td>Amoxicillin-clavulanic acid</td>
<td>29</td>
</tr>
<tr>
<td>2013 Design Kenya: Phase I</td>
<td>Ampicillin</td>
<td>36</td>
</tr>
<tr>
<td>Lilly: Phase II</td>
<td>Acetaminophen</td>
<td>123</td>
</tr>
<tr>
<td>Lilly: Phase II</td>
<td>Amoxicillin</td>
<td>180</td>
</tr>
<tr>
<td>Lilly: Phase II</td>
<td>Amoxicillin-clavulanic acid</td>
<td>138</td>
</tr>
<tr>
<td>Lilly: Phase II</td>
<td>Ciprofloxacin</td>
<td>180</td>
</tr>
<tr>
<td>Lilly: Phase II</td>
<td>Azithromycin</td>
<td>108</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>979</td>
</tr>
</tbody>
</table>
Figure 6.5 Flow chart of Kenyan field study. This chart steps through the collection of pharmaceuticals in Kenya and methods of pharmaceutical evaluation that take place at each step of the Kenyan field study. This study will evaluate the sensitivity and specificity of chemical test cards in the hands of pharmacists, as well as measure the prevalence of poor quality antibiotics in the Eldoret, Kenya region.
6.1.6 Statistical analysis

Carrying out the statistical analysis of this data involves coordinating a wide variety of qualitative and quantitative information collected in Kenya and at Notre Dame. I recommend the use of “R” statistical software for the data analysis stage of this study. “R” is a free software programming language that would be accessible to collaborators in developing countries. Additionally, this program offers more versatility than other statistical software and is favored by the University of Notre Dame Center for Social Research, who offer training courses and continued support for students, staff and faculty using the software. An outline of suggested studies to carry out using the field study data are outlined in Table 6.2. These studies will not only indicate prevalence of poor quality medicines and test card performance, but also will identify the amounts and retailers of unregistered and/or expired medicines.
## TABLE 6.2

**SUMMARY OF FACTORS TO BE EVALUATED IN THE KENYAN FIELD STUDY ANALYSIS**

<table>
<thead>
<tr>
<th>Factor Evaluated</th>
<th>Field Study Aim</th>
<th>Evaluation method or metric</th>
<th>Database information to be correlated</th>
</tr>
</thead>
</table>
| PAD              | Evaluate PAD inter-operator variability (Kenya versus Notre Dame testers)        | 1. Report number of disagreements in the overall evaluation between PADs run in Kenya versus Notre Dame  
2. Visually inspect test cards with disagreements. | Overall PAD evaluations from Kenyan testing (1 Kenyan read, 1 Notre Dame read, possibly a tie-breaker)  
Overall PAD evaluation from Notre Dame testing (1 Kenyan read, 1 Notre Dame read, possibly a tie-breaker)  
Images of tests of the same sample that have reader disagreements |
| PAD              | Evaluate PAD accuracy (Using USP standards and <50% API as cut-off)             | Sensitivity, specificity, false positives and false negatives                                  | Test card evaluations of Notre Dame and Kenyan testing HPLC analysis                                   |
| Reader           | Determine reader variability                                                    | Number of tie-breakers                                                                     | PAD reads-UND  
PAD reads-Kenya  
Tie-breaker decisions                                                                 |
| Pharmacy practices | Number of drugs expired at purchase                                  | Fraction expired at purchase time                                                          | Purchase date  
Expiration date                                                                                   |
| Pharmaceutical   | Prevalence of substandard medicines (USP standards)                             | 1. Fraction of each pharmaceutical type that failed by HPLC  
2. Fraction of each pharmaceutical that failed by PAD testing | PAD test overall outcomes  
HPLC outcomes (1/3 randomly tested by HPLC)                                                        |
<table>
<thead>
<tr>
<th>Factor Evaluated</th>
<th>Field Study Aim</th>
<th>Evaluation method or metric</th>
<th>Database information to be correlated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmaceutical</td>
<td>Prevalence of very substandard medicines (&lt;50% expected API)</td>
<td>1. Fraction of each pharmaceutical type with &lt;50% API by HPLC 2. Fraction of each pharmaceutical that failed by PAD testing</td>
<td>1. PAD test overall outcomes 2. HPLC outcomes</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>Medicines with physical errors</td>
<td>Fraction of each type that fail physical inspection</td>
<td>Physical inspection data</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>Pill to pill variation within a package</td>
<td>Report range of API content (by percent expected from package labeling)</td>
<td>HPLC analysis of 5 random packages, 3 pills each (amoxicillin)</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>Pill to pill variation in mass (reflects inconsistencies in formulation)</td>
<td>Relative standard deviation</td>
<td>Mass of pills of the same package (also the same type and manufacturer can be compared)</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>Pharmaceutical Registration</td>
<td>Fraction of each type and whole group that are not registered</td>
<td>1. Manufacturer name 2. KPPB list (different spreadsheet)</td>
</tr>
<tr>
<td>Manufacturers</td>
<td>Source of substandard medicines Domestic or imported? (Kenyans have reported not trusting domestic pharmaceuticals)</td>
<td>Fraction of each group that is substandard</td>
<td>1. Manufacturer location (Kenya or not) 2. Over drug quality classification by PADs (also can be done by HPLC)</td>
</tr>
<tr>
<td>Manufacturers</td>
<td>Quality of registered vs nonregistered drugs</td>
<td>Fraction of each that is substandard (USP standard)</td>
<td>1. Registration status 2. Drug evaluation by PAD (also can be done by HPLC)</td>
</tr>
</tbody>
</table>
6.1.7 A tool to help understand prevalence and factors influencing illegal trade in the global pharmaceutical supply chain

In 2010, Paul Newton, a leading researcher in the field of substandard and falsified medicines said, “The best “guesstimate” is that 1% of drugs in the developed world are counterfeit. In developing nations, between 10 and 50% of drugs are thought to be fake. But we really have no idea of the full extent of the problem.” A few years later, this statement still holds true. I think test cards can play a role in better defining this problem, an idea that is already playing itself out in the form of field testing in Kenya. Along with the issue of prevalence, there are several questions about falsified medicines that the test cards could help address. Are countries in conflict being flooded with substandard medicines? Are the poorest of the poor the primary victims? Are there regional hotspots? What practices/governmental policies correlate with highest percentages of substandard medicines? It would be informative to design field studies that could survey locations, globally, to begin to flesh out contributing factors of this problem in order to hone in on effective tactics that could alleviate it. The University of Notre Dame has members of the anthropology department and Kroc Peace Institute interested in human development (related to economics and standard of living), illegal trade practices and repercussions of conflict and war who would make interesting collaborators, helping consider this problem in light of social and political practices that influence it. The test card could facilitate this type of work better than other analytical methods, because the tests are inexpensive and fast (making larger sample sizes easier to achieve) as well as portable and easy to use (non-chemists could test samples in the
field). Once test card results can be analyzed by computer, the cards provide an accessible method for non-chemists to carry out qualitative pharmaceutical analysis anywhere in the world.

6.1.8 Possible routes for implementation

While many locations around the world may suffer from poor quality pharmaceuticals in the supply chain, not every location is ripe for implementation of a new technology, like the chemical test cards presented in this thesis. Poor planning at the implementation stage could lead to failure and resistance to future attempts at implementation. I think the test cards were successfully introduced in the Eldoret, Kenya area because of three critical components that are present at that location. First, there is an acknowledgement by government officials, pharmacists and locals that the problem of poor quality medicines exists within the community. Second, government officials and pharmacists within the community are interested in finding a solution to this problem and willing to work towards a solution. Lastly, AMPATH and Moi Teaching and Referral Hospital are two stable institutions within the community that can serve as a hub for training and test card dispersal.

I have struggled with the question, “Who will use the test cards?” over the past few years. Though the test is simple to use, I see it as a tool for institutions rather than individuals. I see it in the hands of medical workers rather than mothers of sick children. This arrangement would help ensure an adequate amount of training for users and would provide support for follow-up testing of suspicious medications. Ideally, the test should be used in conjunction with an orthogonal screening method, such as the CD3 or
handheld spectroscopy device. However, any chemical screening is better than none at some points in the supply chain. One location where the test card was used during my visit to Kenya was in the hospital warehouse, where samples are brought to be inspected before purchasing. In this setting, test cards could supplement visual inspection for a range of pharmaceuticals. In the same way, clinics and pharmacies could use test cards during the purchasing process to ensure the quality of their products. One possibility that I could envision is a program housed from a hospital that would train local pharmacists to use the test cards. If this kind of model were successful, a next step could be to establish a local test card manufacturer to supply the surrounding area. Chemists Without Borders is a not for profit group interested in collaborations to establish sustainable change in developing countries, such as establishing locally owned pharmaceutical manufacturing facilities in Africa. Chemists without Borders or a similar NGO might embrace a project that further implements the test cards in Eldoret, Kenya because it delivers a health care solution, sustainability and economic development for the local community.

Alternatively, I think these test cards might be of interest to NGO’s focused on providing high quality medicines. Already Miti Health is using the test cards in east Africa to screen drug quality, but other groups such as CHMP-Kenya (Centrale-Humanitaire Medico Pharmaceutique) or Quamed (Quality Medicines for All) might also be interested. Both CHMP-Kenya and Quamed rely on analyses carried out in developed countries to ensure the quality of medicines sent to developing countries. This “us helping them” approach taken by many organizations in developed countries can work,
but is expensive to maintain and does not empower local communities. These organizations may be interested in establishing methods for drug quality screening within developing countries. Another group, Meds (Mission for Essential Drugs and Supplies), has established a WHO certified quality analysis lab in Nairobi and also incorporates use of the Global Pharma Health Fund Minilab. This lab boasts analysis of more than 50 samples a month,\(^7\) which could translate to about 2.5 samples per weekday. Test cards could expand the work of the Meds program to include a larger screening program for medicines. Screening an increased number of samples with the test card would increase the body of information collected on local drug quality and also serve as a filter to indicate samples that are potentially of low quality for further analytical testing. Determining the prevalence of poor quality medicines, as discussed in chapter 2 of this document, requires a sample size based on the fraction of poor quality medicines and the desired confidence interval. If the number of poor quality medicines is estimated at 30%, a lab could determine the prevalence within the range of ±5% with 95% certainty by testing 336 random samples. At a rate of 50 samples per month, it would take more than 6 months for the Meds lab to acquire this type of prevalence information. Since the prevalence of poor quality medicines still looms as an unknown, I think that groups trying to provide high quality medicines in developing countries could better serve this cause if they played an active role in defining the problem. The rapid and inexpensive test card, could serve as a vehicle to allow several groups to take on this role.
6.2 BioPAD: hitting the “ASSURED” mark

When it comes to hitting the “ASSURED” mark (Figure 6.1), the BioPAD fulfills the requirements of being affordable, sensitive (within a designated range), specific and deliverable to the end-user. This test falls short in its ability to be user-friendly, rapid and robust and equipment free. The following section outlines experiments that will begin to address these short-comings and investigate ways to expand the responsive range of the test.

6.2.1 New reporters for user-friendly tests

One factor that makes the current BioPAD testing protocol less user-friendly is the need for a cell lysis step, requiring liquid nitrogen, to visualize the beta-galactosidase reporter signal. Incorporating a different reporter, such as a fluorescent protein, that does not require lysis, would simplify the testing procedure. However, finding a field-friendly method for visualizing fluorescence is a potential obstacle to this approach. The recent introduction of the FDA’s CD3 technology, used for detection of counterfeits through reflectance, could provide a low cost tool for fluorescence visualization. Preliminary experiments have shown that the CD3 can visualize Red Fluorescent Protein (RFP) and Yellow Fluorescent Protein (YFP) in yeast cultured in plates (Figure 6.6). Additional work completed by Sarah Halweg, an undergraduate researcher, indicates that visualization of fluorescence is compatible with the BioPAD test and that visualization of RFP can also be carried out using a green laser pointer and inexpensive plastic filters used for theatre lighting (Figure 6.7).
6.2.2 BioPAD: understanding BioPAD reduced response to antibiotics

One characteristic of the BioPAD compared to yeast in liquid culture is a reduced response to antibiotics. The cause of this response is not fully understood. Previously published studies⁸ and my own experiments suggest that neither hydrogel nor paper
provide a physical or chemical barrier that reduces antibiotic exposure. I initially considered that yeast were in a less responsive state due to BioPAD drying and storage, however yeast have been shown to exit a quiescent state within 10-15 minutes following exposure to a carbon food source. One factor that might be reducing BioPAD response is decreased diffusion of the analyte into areas of high local cell concentrations. In an effort to maximize BioPAD color response, I apply a concentrated slurry of approximately $6 \times 10^7$ yeast cells in hydrogel with a total volume of 10µL to the paper. When this slurry is dehydrated for storage, these cells are estimated to fill 48% of the approximately 50mm$^3$ space occupied by paper, yeast and hydrogel. Cell clustering within the supporting paper/hydrogel matrix could block diffusion of antibiotics to yeast buried within the cluster and shield the effects of antibiotics for a portion of the cells. This effect could be visualized using a BioPAD fabricated with engineered yeast that produce fluorescent proteins in response to galactose. Fluorescence microscopy following exposure of galactose would reveal patterns of cell response within the BioPAD.

6.3 References


A.1 Background information (PAD design)

1. **PAD fabrication:** 12 lane PAD design.
   Five lanes tests for drug identification (a-g)
   Five lanes for beta-lactam LOD determination using the ninhydrin test (h-l)

2. **Educational PAD layout:**

   Tests include:

   A) **Timer**
   B) Copper sulfate test for beta-lactams
   C) Ninhydrin test to discriminate ampicillin from amoxicillin
   D) Nitroaniline test for electron rich phenols
   E) Iodine test for starch
   F) Iron (III) chloride test for carbonates
   G) Cobalt thiocyanate for tertiary amines (ex. diphenhydramine, quinine)
   H-L) Ninydrin test for LOD testing with ampicillin or amoxicillin

3. **Samples that can be tested:** ampicillin, amoxicillin, acetaminophen, starch, chalk, baking soda, degraded aspirin (salicylic acid), diphenhydramine (antihistamine).

4. **Also test “cut” samples of API with excipients or a substitute API (diphenhydramine)**

Background discussion could include:

1. Pharmaceutical formulations (APIs and excipients)
2. The problem of counterfeit and substandard pharmaceuticals around the world
   a. Counterfeit/Falsified versus substandard pharmaceuticals
   b. Types of counterfeits
3. Solving analytical problems in low resource environments
4. Colorimetric reactions

A.2 General procedure suggestions

**Pharmaceutical identification:**

1. Divide (cut) PADs into qualitative (a-g) and semi-quantitative (h-l) portions
2. Students will run known samples on a-g portion. Sample is “swiped on the blue line.
   Samples could include; amoxicillin, ampicillin, acetaminophen, chalk, starch etc... (Image sample 5 minutes after removing from water)
3. Compare to standard images to “read” that the PAD outcome matches the sample tested.
4. I would have students “read” the results of other students to become familiar with interpreting the colorimetric results.

5. Test an “unknown” or “suspicious” drug to determine if it looks like a genuine beta-lactam or a substandard/fake. (Image sample 5 minutes after removal from water)
   Samples could include 1) genuine pharmaceutical, 2) No API, 3) API cut with unexpected excipients, 4) API cut with a substitute API (diphenhydramine)
   a. Identify genuine or substandard
   b. Identify what they believe the contents to be

Detection of reduced API concentration:

1) Each student runs 5 lanes of amoxicillin on ninhydrin test with x% amoxicillin. We have an undergrad looking at this, but I don’t actually know what he has seen. A variety of concentrations would be good. (ex. 0, 2, 5, 7, 10, 20, 50, 100%) Note: it important that students are able to swipe a consistent amount of pharmaceutical. We describe these swiping/dosing methods in the submitted paper, but images for everyone to refer to would be best here.

2) Students should run tests until the solvent from reaches the top of the paper. The tests should be removed from water and color developed for 5 minutes before imaging. Imaging in a light box with a cell phone camera would be good, but also could be done in a location with consistent lighting. A scanner might be a better option to eliminate adjustments that cameras seem to make automatically.

3) Students can analyze color intensity using ImageJ. They should download the program through an NIH site. For analysis they will open the image in imageJ, invert the image, and box in the portion of the lanes that produce the green color. Measure the mean intensity of the color in either the red or blue channel (I’m not sure which will give the greatest change with increasing amoxicillin).

4) Students can combine their data (with a small group I would have them run a few concentrations) to assemble a ROC plot.

Anticipated problems:

1. Non-standardized sample application. Include images of standard sample applications (light, med, heavy)
2. Imaging with a cell phone can produce variation in lighting. A scanner might be best for the second portion of the lab.
APPENDIX B

COLOR DETECTION USING BLOB ANALYSIS AS CARRIED OUT BY

DR. CHRIS SWEET AND SANDIPAN BANERJEE

1. Test cards fabricated with nine replicates of the same lane test and dosed with light, medium and heavy qualities of the analyte (3 of each dosing type). Heavy dosing obscures the wax barriers between test lanes. A light dosing will not show analyte on the wax lanes and medium dosing is an intermediate of light and heavy dosing. Standard images are used as a reference to guide dosing. These test cards represent a range of outcomes that tests may produce if dosing is not uniform between users. These test cards were used to build a library of standard images for analysis and comparison to unknown samples.

2. Five “seed” locations above the swipe line in each lane have been chosen manually.

3. Moving outward from the “seed” location the program measures Red/Blue/Green (RGB) intensities at adjoining pixels. If the RGB intensities are within a specified range compared to the initial seed, the pixel is incorporated into the “blob”. The program continues to grow the blob in all directions until the area of the blob is at least 1/5th of the area above the swipe line.

4. Neighboring blobs that are determined to overlap in location by 30% or greater are combined.

5. The mean intensity values for red, blue and green (R, G, B) are calculated for each blob.

6. The background is assumed to be white, having red, blue and green intensities that are equal to each other, while areas of color production will have differences between red, green, and blue intensities. A difference is calculated using the formula below in which
“max” is the greatest of the RGB values within a blob, and R, G, and B are the mean intensities. This difference determines which blob within each lane has the greatest color production.

\[ \text{Difference} = \max |R-G| \times |R-B| \times |G-B| \]

7. Mean RGB values for 9 blobs, representing the outcome for a specific lane test:analyte combination (totaling 27 values per card) are compared to analogous values generated for other lane tests. Principal component analysis will be used to determine which tests are most informative for identifying each analyte.

8. This information will be used to define a group of possible outcomes for pharmaceutical components to be used with the test card.
APPENDIX C

DEVELOPMENT OF AN ARSENIC SENSING BIOPAD

C.1 Introduction

*Environmental Contamination:* The World Health Organization (WHO) estimates that more than 40 million people worldwide are exposed to arsenic above the “guideline value” of 10µg/L.\(^1\) Arsenic poisoning in Bangladesh from shallow tube wells has been deemed by the WHO as the largest case of mass poisoning in history.\(^2\) Studies revealed that this country alone had 9100 deaths from arsenic in 2001 \(^3\) and has more than ~3 million wells that require testing.\(^4\)

*Existing Technology:* Field tests for arsenic contamination in water suffer from multiple shortcomings. In a recent study, drawbacks included high cost, production of a poisonous arsine gas, unreliability and difficulty of use.\(^5\) The WHO has identified the need for improved field measurements of arsenic in water as an urgent requirement.\(^4\) Currently, field tests would be most helpful to evaluate efforts to mitigate arsenic in water and test new deep wells.\(^6\)

*Low-Cost Point-of-Use-Tests:* In my thesis research, I will make field tests for arsenic in ground water using a whole-cell biosensor as an inexpensive analytical test. A biosensor uses biological components to sense an analyte and produce a signal. Sensors made from antibodies or other biological components require steps to isolate the needed materials and tests must be carefully stored to maintain test viability. Whole-cell microbial biosensors forego these requirements by converting the entire organism
into a sensor. Isolation steps are no longer needed and microbes are resilient to varied storage conditions. Paper, which has a history of use for simple diagnostics, can provide an inexpensive platform for these biosensors. Incorporating responsive yeast cells onto paper analytical devices (PADs) has the potential to produce inexpensive and field-friendly tests to address the need for diagnostics in technology-limited situations.7

Yeast as the Biosensing Microbe: Previously developed yeast biosensors rely on electrode measurements of solution pH or oxygen levels that reflect the increased metabolism of a substrate in the presence of the analyte. For example, as yeast metabolize glucose the drop in pH of the surrounding solution is measured to reflect glucose concentration. Metabolites, glucose and galactose, and metals, such as copper, have been measured by these means.8,9,10 Advantages of using yeast in a biosensor include 1) rapid growth 2) a well characterized system 3) tolerance to pH and temperature fluctuations 4) established procedures for long term storage 5) ability to survive over long periods of time in a dried state and 6) eukaryotic nature, such that response to toxic substances is similar to higher eukaryotes.11

Current Arsenic Biosensors use the bacterial ArsR promoter12 and rely on fluorescence or bioluminescence measurements.13 The only attempt at a colorimetric sensor requires the growth of fresh E. coli and an incubation step at 30°C.12 A truly field-friendly paper analytical test has yet to be designed for arsenic.

C.2 Project goals summary

My research goal is to produce a quantitative or semi-quantitative paper analytical device with a whole-cell biosensor (BioPAD) for detection of water contaminants. This
will be constructed using responsive genetic elements from yeast or other organisms coupled to a reporter gene and expressed in yeast. This biosensor would be embedded onto paper using a cross-linked hydrogel matrix and yeast would produce a color output that would be captured by cell phone imaging and analyzed by a cell phone app designed by a collaborator, Patrick Flynn, a computer scientist with expertise in image recognition and phone app software.

C.2.1 Specific Aims

1. Identify Arsenic-Responsive Promoters: My initial research focused on arsenic, since it is known that multiple genes in *Saccharomyces cerevisiae* respond to this stimulus with a 16-fold increase in gene expression. The responsive promoters will be characterized by their expression of the LacZ gene, a commonly used reporter. The level of induction in the presence of the stimulus can be assayed using the X-gal substrate, which is converted to a blue precipitate. This assay is convenient in the lab, but it requires freezing the yeast in liquid nitrogen and is not field practical. Specific Aim 2 addresses this problem. From this, I hope to find a promoter that reacts quickly and with a high level of induction to serve as a candidate for the responsive portion of the biosensor.

2. Identify Appropriate Colorimetric Reporter Systems: A BioPAD suitable for visualization by eye or camera phone requires a colorimetric change in living yeast. The literature contains examples of possible genes, such as a red fluorescent protein (RFP) optimized for yeast as well as genes shown to work in bacteria, such as melanin, that
might be applicable to yeast.\textsuperscript{16} Once a reporter is chosen image analysis procedures using ImageJ will be developed.

3. Choosing a Hydrogel Matrix: Hydrogels have been used for several years to encapsulate yeast for the fermentation of beer and wine\textsuperscript{17} and more recently have been shown to support microbes contained in activated sludge for the degradation of materials as part of the water treatment process.\textsuperscript{18} Sodium alginate, a carbohydrate polymer from brown seaweed, produces a hydrogel when cross-linked with calcium chloride and has been used extensively with \textit{Saccharomyces} in the food industry.\textsuperscript{18,19} An early yeast biosensor for glucose coated a probe with yeast cells in a calcium alginate matrix to metabolize sugars. Glucose levels in the sample were determined by the solution’s change in pH.\textsuperscript{8} My work with hydrogels requires the optimization of hydrogel, which includes determining the concentration and viscosity (polymer length) of alginate needed to hold yeast solidly, yet not block the flow of analytes. To maximize color visualization I will need to determine a yeast concentration able to reveal color without over-crowding yeast, which could lead to an accumulation of toxins. Lastly, a deposition method that is 1) able to maintain proper yeast concentration and is 2) easily up-scaled for manufacturing will be established.

4. Packaging and Preserving the BioPAD in a kit will require testing different methods of yeast preservation. Drying yeast for long term storage has been done for many years and has shown yeast to be resilient organisms after long intervals of storage.\textsuperscript{20} Yeast viability after freeze-drying in gelatin is increased by use of trehalose or saccharose as a cryoprotectant in combination with short freeze times.\textsuperscript{21} BioPADS can
be tested with multiple packaging options, for example foil packed with nitrogen, to determine the best conditions for storage and test viability. Optimized deposition of hydrogel and storage techniques developed through specific aims 3 and 4 could be applied to any yeast biosensor strain for application to paper and long-term storage.

5. Field Testing a BioPAD has a two-fold aim. First, the reliability and consistency of the BioPAD would be tested on a variety of water samples, seeking local wells with varying arsenic concentrations and/or other compounds that might trigger the biosensor. Secondly, the test would be put into the hands of untrained users to evaluate the simplicity of BioPAD usage and create instructions that make this diagnostic accessible to anyone.

C.3 Preliminary results

1. Identify Arsenic-Responsive Promoters: Gene regulatory regions in yeast are generally found within 1 KB of the protein’s ATG start site. To identify regions responsive to arsenic that are able to drive reporter expression, I PCR amplified 1 KB DNA segments upstream of yeast genes shown in microarray studies to increase translation at least 16 fold in the presence of arsenite or arsenate, the predominant forms of arsenic in water. These promoter segments were inserted in front of the LacZ reporter in the shuttle vector, yEP357R (Fig C.1, and transformed into E. coli. Plasmids were confirmed by restriction digest and transformed into S. cerevisiae.
Measuring levels of beta-galactosidase in response to arsenic induction requires lysing the cells. Inductions were carried out at 400 μM As to mimic microarray studies. Cells were exposed to As at this concentration for two hours of growth; equal aliquots were pelleted by centrifugation, decanted, lysed by freeze/thaw cycles and resuspended in an X-gal assay buffer. A rapid formation of blue precipitate indicates high levels of β-gal expression from ARR2(1000) and slower color development in OYE3(1000) and AR123 constructs indicates a weaker As response (Figure C.2).

Figure C.1. Representation of promoters controlling LacZ expression. Names represent the gene replaced by LacZ and numbers in parenthesis represent the length of the promoter region included. Orange arrows show additional open reading frames within the promoter of interest. ARR123 replaces ARR3 with LacZ and contains the entire open reading frame for both ARR1 and ARR2.
ARR1, ARR2 and ARR3 are located in a gene cluster, in which genes of related function are found together and coordinately regulated. ARR2(1000) shows both strong induction, but also leaky expression in the absence of arsenic. The ARR gene cluster is unusual in yeast and both ARR2 and ARR3 are regulated by the transcription factor, Arr1p. Because of its strong induction, ARR2 is a candidate for use with a biosensor. It is interesting to note that ARR3(1000) does not respond to arsenic in this assay, while ARR123, which differs by having a full copy of the ARR1 regulator, is weakly induced. Arr1p is reported to be bound to the shared ARR2/ARR3 promoter in the presence and absence of arsenic. In light of this, a copy of ARR1 has been amplified.
for insertion into the pRS425 yeast expression vector to allow co-transformation of ARR1 with ARR2(1000). This should increase the regulation of ARR2 expression and may tighten its leaky expression. Early assays, with all 1KB promoters, were optimistically carried out at arsenic concentrations of 50 ppb, five times the WHO limit, but these assays were not able to detect production of beta-galactosidase (not shown).

2. Identify Appropriate Colorimetric Reporter Systems: Two well-established colorimetric reporters in yeast are LacZ expression and ADE2 mutations. The use of LacZ as a reporter in a BioPAD is not feasible, since lysis of cells is necessary for color output. Strains deficient in the adenine anabolic pathway accumulate a pink intermediate.²⁵ This pigment is not well suited to our needs either because it relies on a basic metabolic pathway and is slow to accumulate.

Melanin is another colorimetric reporter used in bacteria. The monophenol monooxygenase of *Rhizobium etli* produces a deep brown or black melanin pigment in *E. coli* using tyrosine as a substrate.¹⁶ We have this organism in a frozen stock, however using this gene is complicated by intellectual property constraints and because the DNA is found on a plasmid instead of genomic DNA and may not be present in our organisms. Using the National Center for Biotechnology Information (NCBI) website, I conducted a Basic Local Alignment Search Tool protein (BLASTp) search using the *Rhizobium etli* monophenol monooxygenase protein sequence as my query. It revealed the *Nitrobacter winogradskyi* tyrosinase was homologous to the *Rhizobium* protein with an E value of 2E-169. The Expect (E) value reflects the number of times a sequence of homology would appear randomly from a database search. An E value of 1 indicates that a
sequence would appear once, simply by chance. Low or zero E values indicate that the appearance of a sequence in a BLAST result is not likely to be random and has high homology. I investigated the possible use of this tyrosinase from *Nitrobacter*, but was unable to PCR amplify the correct gene using whole cell PCR amplification.

Another gene identified for colorimetric output is an RFP optimized for yeast that was obtained from Stony Brook University, New York. Currently, this gene is under the control of the TDH3 constitutive promoter and is able to produce a pink color visible to the eye. In yeast, I have found that a minimum of approximately 10 million yeast must be embedded on paper for visualization. Large numbers of yeast can be easily deposited onto paper (Figure C.3), making this a viable reporter for colorimetric output. I am beginning to investigate methods of measuring color intensity on a paper background using ImageJ software.

**Figure C.3.** Yeast cells containing yEpGAPcherry, equivalent to an optical density of 3.0 at 600nm (approximately 30 million cells) on Whatman #1 and coated with a cross-linked calcium alginate hydrogel.
3. Choosing a Hydrogel Matrix: Because of their ability to hold living organisms in a non-toxic environment that allows diffusion of small metabolites, I am investigating the ability of hydrogels to embed yeast onto paper. Original studies were done using polyvinyl alcohol (PVA), calcium alginate and mixtures of the two hydrogels. Mixing the hydrogels led to a more solid, brittle hydrogel that often trapped bubbles during its formation. Comparisons revealed that PVA and alginate hydrogels were alike in flexibility and visualization of encapsulated blue microspheres. Because of its milder treatment of cells, 2% sodium alginate cross-linked calcium chloride has been used for all recent work with hydrogels. Yeast embedded in paper using this alginate hydrogel are induced by a three hour exposure to arsenic (Figure C.4).

4. Assembling the bioPAD kit and Field testing: With an arsenic responsive yeast strain in hand, I am now able to test freeze-drying and packaging methods. These experiments will use recommended methods from literature to preserve BioPADS for field testing.

C.4 Future directions
Promoter Induction Studies: Once an ARR1 expression vector has been assembled I plan to further characterize the induction of ARR2(1000) in the presence of the Arr1p transcription regulator. Arsenic induction at lower concentrations and shorter exposure times are also of interest to test the limits of promoter response. I would like to place the ARR2(1000) in front of an appropriate colorimetric reporter that does not require cell lysis. The Mel1 gene, commonly used in the yeast two hybrid system, would be appropriate for this purpose.

Colorimetric Output: I would like to identify additional colorimetric reporters or reporter systems with the capacity for faster reaction times and/or greater amplification of signal. A melanin reporter is still an option to be investigated.

Assembling the BioPAD Kit and Field Testing: Using arsenic responsive yeast, I will investigate yeast viability following different methods of preservation and packaging. Viability can be measured by both enzyme assay and yeast viability kit. Field tests can be carried out by undergraduate volunteers using both natural and spiked samples to identify the probability of false positives and negatives given by the test.

Antibiotics as targets: The Lieberman lab is also looking into developing tests for counterfeit drugs including antimalarials and antibiotics. I have started using the well characterized tetracycline responsive system taken from bacterial strains resistant to this antibiotic. The testing of tetracycline and its derivatives is of interest because they are often counterfeited. Doxycycline, a tetracycline derivative, is often used as prophylaxis for malaria.\textsuperscript{26}
Immediate Goals: I am currently investigating the use of the constitutively expressed RFP, mentioned previously, used in combination with a tetracycline operator (TetO). In tetracycline resistant bacteria, TetO is bound by the tetracycline repressor (TetR) and expression of the TetA gene, which forms an export channel, is repressed (Figure C.5).

![Figure C.5. Native regulation of the TetA gene in bacteria in the presence and absence of tetracycline.](http://nfs.unipv.it/nfs/minf/dispense/immunology/trgen.html)

Addition of TetO sequences to the constitutive TDH3 promoter will allow binding of the TetR repressor and block RFP expression in the absence of tetracycline of the native bacterial TetR gene. Placing a single copy of the tetracycline operator, TetO, 7 bases prior to the ATG start codon has been previously shown to repress gene expression in *Saccharomyces cerevisiae* with the gal1 promoter and result in a 50 fold induction in the presence of tetracycline.\(^{27}\)

I plan to insert both one and two copies of TetO 6 bases prior to the ATG start codon. This insertion is facilitated by an EcoRI site just prior to the RFP start codon. Oligonucleotides containing both one and two copies of the TetO sequence have been designed with flanking EcoRI restriction enzyme sites to allow for cleavage and insertion into this location. Multiple copies of TetO prior to the ATG have been shown to increase the level of repression and thus allow a greater fold induction in the presence of tetracycline.\(^{28}\) The tetracycline repressor, TetR, also needed for this system will be
integrated into the yeast genome and expressed constitutively under the cytomegalovirus (CMV) promoter. Plasmid constructs from *E. coli* will be screened by PCR and yeast co-transformation can be identified by replica plating yeast in the presence and absence of tetracycline, looking for increased RFP with tetracycline.

Additional Biosensor Targets: An estrogen BioPAD could be developed from a fluorescent estrogen yeast reporter currently used in the Goodson Lab. I plan to identify additional yeast biosensor targets by searching previously published microarray data as well as conducting my own microarray studies to identify potential drug targets, such as retrovirals.

C.5 References


APPENDIX D

PAPER ANALYTICAL DEVICES FOR FAST FIELD SCREENING OF BETA-LACTAM ANTIBIOTICS
AND ANTI-TUBERCULOSIS PHARMACEUTICALS SUPPLEMENTARY MATERIALS

D.1 Materials

1,2-naphthoquinone-4-sulfonic acid sodium salt, p-toluenesulfonic acid ("tosic acid") and sodium nitroferricyanide (III) dihydrate (sodium nitroprusside or SNP) were obtained from Aldrich Chemistry (St. Louis, MO). Dimethylglyoxime 99%, 1,2-Cyclohexanedione-dioxime ("nioxime"), tetracyanoquinodimethane (TCNQ) and potassium iodide were obtained from Alfa Aesar (Ward Hill, MA). Ampicillin sodium salt, copper sulfate pentahydrate, acetaminophen USP, ninhydrin, lactose monohydrate powder USP, calcium carbonate, acetylsalicylic acid and isoniazid, were purchased from Sigma-Aldrich (St. Louis, MO). 2-nitroaniline was obtained from Acros Organics (Geel, Belgium) Chalk (in stick form) was purchased from a bookstore in Nairobi and wheat flour was obtained from a supermarket in Eldoret, Kenya. Neutrad ® hand detergent was obtained from Decon Laboratories, Inc (King of Prussia, PA). Baking soda (sodium bicarbonate) and turmeric (food grade) was obtained from a supermarket in Indiana. Sodium hydroxide was obtained from Amresco (Solon, OH). Acetonitrile, cobalt (II) nitrate hexahydrate, dimethylsulfoxide (DMSO), ethambutol dihydrochloride, ethanol, Talc USP, potassium carbonate, salicylic acid sodium salt, pyrazinamide 99%, sodium thiosulfate pentahydrate, sulfuric acid, ethyl acetate, methanol, methylene chloride,
nickel chloride, quinine sulfate, triethylamine, iron (III) nitrate, eosine red b and iron (III) chloride were obtained from Fisher Scientific (Fairlawn, NJ). Crystalline amoxicillin, chloroquine diphosphate, diphenhydramine hydrochloride, polyvinylpyrrolidone (“povidone”) and rifampicin were obtained from Sigma (St. Louis, MO). Ery-Tab erythromycin was made by Abbott Laboratories (Abbott Park, IL), Wallboard was obtained from Lowe’s building supply store. 2% starch indicator was obtained from BDH-VWR International (West Chester, PA). Primary grade potassium iodate, acetone, soluble potato starch, sodium nitrite were obtained from J.T. Baker (Phillipsburg, NJ). Aspirin and acetaminophen tablets were “Up and Up” Brand, purchased at Target®.

1-20uL tips racked for use in the Biomek FX were obtained from USA Scientific (Ocala, FL). 96 well flat bottom microtitre plates were purchased from Sarstedt (Numbrect, Germany). Several types of chromatography paper were tested for wicking speed and compatibility with chemical tests.¹ Ahlstrom® 319 Cellulose Sheets obtained from Midland Scientific, Chicago, USA were used as the substrate for PAD fabrication. Validation tests were run with deionized water.

D.2 Printing

Beta lactam and TB PADs were based on a 12-lane wax-printed PAD (Figure D.1) laid out using Adobe Illustrator (see http://pubs.acs.org/journal/ancham, "12-lane PAD.ai" in supporting information) and printed with color laser and thermal wax printers. The lanes are designed to be compatible with a 96-well plate spotting robot. The Illustrator files needed for the three print layers are included in the supplemental information. The col-
or laser printer layer, which contains color standards, text, a QR Code, fiducial marks, and lane loading indicators, was printed with an HP Color LaserJet printer (CP3525x) using manual feed, which required 40 sec/page. The printer was set to print the image full scale in “emulsion side up” mode to avoid reversing the image.

Hydrophobic lanes were created by printing the “front wax” and “back wax” layers on each page using a Xerox ColorQube 8570 wax printer and manually feeding the pages. Printing each wax layer took under 5 seconds. The “front wax” design was printed on top of the color laser printed features using the same full scale and “emulsion side up” settings, with fine alignment in X and Y axes controlled from the Illustrator print interface. The “back wax” design was printed on the obverse in “emulsion side down” mode, with fine alignment controlled from the Illustrator print interface. The wax lines on the front and back sides of the paper should align with no more than 0.50 mm of white space between the edges of lines on the front and back sides of the page. The wax forms an adequate seal through the thickness of the paper during thermal processing. The alignment of the front and back wax on each page was visually assessed (by holding it up to a strong light). Using accurately cut 8.5 x 11" pages, wastage at this stage was 7%. The time to print 50 “good” PAD blanks was approximately 12 minutes.

D.3 Baking the wax layers

To create hydrophobic regions, pages containing eight PAD blanks were placed in a single layer on paper towels in a drying oven for 8-9 minutes at 100º C, or until drops of water placed in lanes in several locations were contained by the wax barriers. As long as
the wax lines were properly aligned (<0.5 mm of “white space” visible between lines on front and back sides of PAD) the wax reliably penetrated the thickness of the paper to form reliable barriers between lanes. Baking homogeneity was generally good—if one page was sufficiently baked, the others in the oven were good as well. The final width of the wax lines was 1.5-2 mm, and the width of the hydrophilic paper lanes was 2.5-3 mm.

D.4 Loading reagents on to PAD blanks

While it is possible to load the reagents by hand using an automatic pipette, it is much faster and more accurate to use a spotting robot. Reagent stock solutions were loaded into two 96 well plates according to the loading charts given in Figure D.1. A Biomek® FX Model 717001 was used to spot the PADs with nominal 4.0 ul aliquots of reagents dissolved in a wax compatible solvent (see Measurement of accuracy of robot solution deposition, Solvent compatibility with wax lanes and Table D.1. 360 µl portions of each reagent were sufficient to load 60 PADs. Some of the reagents were prepared in acetonitrile rather than water, and it was critical to monitor these solution levels closely during spotting because of the dual problems of low viscosity (leading to larger than expected uptake by the robot) and evaporation of the stock solutions. Individual PADs were affixed to eight 96 well plates with double-sided tape so that the lanes on the PAD were aligned with the wells. A custom Biomek program loaded 96 tips, spotted 4 µl volumes of the reagents from the first reagent plate into the even numbered lanes, rinsed, blotted, washed, and unloaded the tips. After changing the tip box to avoid sample carryover, solutions from the second reagent plate were then spotted into the odd num-
bered lanes and the tips were washed and unloaded. The complete cycle took 8 minutes to load 8 PADs, during which time the operator aligned the next batch of 8 PAD blanks on 96-well plates so they are ready for loading. PADs were allowed to air dry on cookie racks for about 10 minutes and were stored in wide-mouth canning jars or zipper-lock plastic bags. Occasional incidences of poor spotting were found during first rounds of loading and after about an hour of loading, generally due to clogged or bent tips, but the typical yield for accurate spotting was high (i.e. less than 3 improperly spotted PADs of 50) and one tip box can be used to spot several hundred PADs. The time to spot 50 PADs was 50 minutes, plus 10 minutes to load the reagent plates.

D.5 Measurement of accuracy of robot solution deposition

The Biomek FX was used to place 96 spots of 4.00 μL 2.007x10⁻³ M KIO₃ onto three pieces of Ahlstrom 319 paper. The 96 spots on each piece of paper were extracted by submerging the paper in water and soaking for 5 minutes, then the solution was titrated iodometrically to determine the average spot volume on that piece of paper. For a 2.007x10⁻³ M KIO₃ aqueous solution the average spotting volume for a nominal 4 ul spot was measured titrimetrically as 3.72± 0.07 μL.

D.6 Solvent compatibility with wax lanes

The solvents ethanol, methanol, ethyl acetate, triethylamine, methylene chloride, DMSO, acetonitrile, acetone and water were applied to 12-lane PADs in 2 μL, 5 μL and 10 μL aliquots to determine which could be used without damaging the integrity of the wax
lanes. Damage to wax was evaluated based on whether the solvent was able to diffuse into the neighboring lanes, which was assessed by holding the paper up to a strong light source (Table D.1).

D.7 Targets for PAD detection

These PADs are intended for rapid field screening of dry formulated medications—tablets, capsules, and powders for injection. Pharmaceuticals are highly concentrated forms of reactive materials that constitute ideal samples for the qualitative color tests used in the PADs. After consultation with pharmacists working in Kenya, we designed two PADs: one for analysis of the beta lactam antibiotics ampicillin and amoxicillin, and the other for screening the first-line TB medications isoniazid, pyrazinamide, rifampicin, and ethambutol. These medications are of great importance in public health in the developing world, and they have multiple manufacturers and distributors, which contributes to supply chain insecurity.

The targets for the antibiotic and TB PADs included both excipients (binders and fillers) and active pharmaceutical ingredients (APIs). The non-API components of a pharmaceutical can give useful information about the authenticity of a drug, particularly if an unapproved ingredient is detected in a pharmaceutical formulation. Many of the materials that replace APIs in fake medications are not chromatographically mobile, so they are difficult to detect using HPLC, GC, or TLC analysis. Based on the types of binders and fillers that are commonly used in authentic pharmaceuticals,\(^2\) or have been detected as ingredients in counterfeits,\(^3,4\) we selected chalk or calcite (CaCO\(_3\)), starch, and talc as the
excipient targets, and acetaminophen and high-volume generic drugs (eg chloroquine) as likely substitute APIs. Pharmaceutical formulations of amoxicillin and ampicillin capsules are nearly pure drug material; consisting of approximately 80-90% by weight of the antibiotic. The remainder is made up of excipients such as colloidal silicon dioxide. Tablet forms of pharmaceuticals require more binders and amoxicillin/clavulanic acid tablet formulations are approximately 50-80% by weight amoxicillin and 12-21% by weight clavulanic acid or potassium clavulanate, with the remainder consisting of excipients. Thus, the beta lactam PADs must be capable of detecting amoxicillin or ampicillin mixed with small amounts of excipients. For treatment of tuberculosis, the World Health Organization (WHO) strongly recommends the use of fixed-dose combination tablets to improve compliance, decrease medication errors, and reduce the rate of development of resistant pathogens. Therefore, the PADs for analysis of anti-TB drugs must not only be able to detect the four first-line TB drugs isoniazid, pyrazinamide, rifampicin, and ethambutol, but also combinations of these drugs in pharmaceutical formulations. Table D.2, lists the most common adult and pediatric TB combination medications. These medications also contain small quantities of binders and fillers, but the APIs make up 60-90% of the mass of the pill.

D.8 Sample application

The PADs are designed to be dosed by smearing or scraping solid material over particular zones of the paper, which we call the “swipe line” areas. We decided not to dissolve tablets in a solvent before application because this would require providing the
solvent and a measuring device, because non-aqueous solvents can damage the wax barriers between lanes, and because some binders and fillers are insoluble. For powders such as pure excipients or the contents of gel-caps, a coffee stirrer or popsicle stick is used to smear a portion of material along the swipe line. For tablets, a piece of aluminum window screen is placed over the swipe line, and the tablet is broken in half (this avoids enteric coatings on some tablets) and then rubbed against this rough surface to grate some of its constituents along the swipe line. The amount of powder could be estimated based on how much the applied sample obscured the grey wax printed lines, and after some practice by individual operators, this enabled semi-quantitative dosing of samples. If there was a visible amount of powder in each lane, but not enough powder to cover up the grey wax barriers between the lanes, gravimetric analysis showed there was about 0.5 mg of material in each lane. If there was sufficient powder to cover the grey wax barriers between the lanes, a heavy loading of 1.5-2 mg of material was present in each lane. Commercial baking soda was the exception, giving loadings about ten times larger than other powders, perhaps due to the very fine powder size of this material (Table D.3).

D.9 Stability studies

PADs for stability testing were spotted and dried in a convection oven at 60 °C for 10 min. Storage stability tests were carried out by storing PADs in either 37°C or 60°C environments in the dark in sealed wide-mouth canning jars filled with argon gas. PADs were tested for analyte response in triplicate at various time intervals. Analytes tested includ-
ed amoxicillin:starch (2:1, w/w), ampicillin:chalk (2:1, w/w), salicylic acid:talc (1:2, w/w) and the TB 4-way drug combo of pyrazinamide(44%):ethambutol (31%): rifampicin (17%):isoniazid(8%). Tests were carried out and imaged as described above. Success or failure of a test was evaluated by a single reader in comparison to standard images.

D.10 Optimization of timing for test running and color development

To obtain the best test results the timing of 1) test run duration and 2) test imaging are both important factors. To produce consistency in run time, or more accurately in the distance traveled by the solvent up the PAD, a timer is placed in the last lane of a PAD. When this lane fully forms a pink blaze at the top of the lane, this signals to the user that the test run is complete and the PAD should be removed from the water. During lane development, it was noted that the Eosin red/talc color became stronger over time, while the iodine/starch color degraded over time. To find the optimal development time, alternating lanes of a 12 lane PAD were spotted with 18mg/mL eosin red and 1% triiodide(w/v) in 4% povidone (w/v). These were dosed with medium swipes (defined as a quantity of pharmaceutical that is clearly visible against the wax lane barriers without obscuring the barrier) of carbonate and talc. Tests were run by lateral flow in deionized water until water reached the top of the lanes. The PAD was removed from water and images were taken at 2, 5, 7, 10, 12, 15, 17 and 20 minutes following test completion. Talc images were inverted in ImageJ and integrated intensity of the “swipe region” was measured using the green channel. Starch lanes images were converted to 8-bit grayscale, inverted, and integrated intensity of equal sized areas at the “swipe” lo-
cation was measured. In each case, a background intensity of an adjacent, equal sized region was subtracted from the experimental measurement to give mean intensity increase due to analyte. Intensities for each analyte were divided by the maximum intensity achieved during the 20 minutes after removing the PAD from water. The percent of maximum intensity was graphed versus time to indicate the optimal timing to image test results.

D.11 Reagent spotting for PADs

Beta-Lactam PAD reagents were spotted in 4μL aliquots on printed PAD as labeled above. Spotting locations are 9 mm from each other to accommodate our solution han-
dling robot. Other locations are also possible; the spots can be placed as close as 2 mm or as far as several cm. Pills were swiped across location 3 and all solutions are aqueous unless otherwise noted. Placement as follows: saturated copper (II) sulfate (A2-A3), saturated potassium carbonate (A7,B7,C7,D7,E7), saturated ninhydrin in acetonitrile (B2-B5,C2,C3,C5,D2,D3,D5), 10mg/mL ampicillin sodium salt (C4), 4 mg/mL amoxicillin (D4), 5 mM tetracyanoquinodimethane in acetonitrile (E2-E3), 2mM cobalt thiocyanate (F4), 2M sodium hydroxide (G3), 30mg/mL sodium nitrite (G5), 10mg/mL 2-nitroaniline in 1M tosic acid (G6), 1% triiodide (w/v) with 4% povidone(w/v) (H4), 2M Iron(III) chloride in 2% detergent solution (I4), 2M iron (III) chloride (J4), 0.1M barium chloride (K2), 25mM rhodizonate (K5), saturated nickel chloride (L1), saturated nioxime (L3). Note: Validation testing was not carried out on the lane K test using rhodizonate and barium chloride due to chemical instability.

TB PAD reagent placement requires the swipe at location 3 with reagents spotted in 4µL aliquots of aqueous solutions, unless otherwise noted. Reagents are placed as follows: 1M copper (II) sulfate (A2,A3,B2,B3), saturated potassium carbonate (B5), iron (III) nitrate (C4), 20% sodium nitroprusside (D4), 1M sodium hydroxide (D5,D6, J5, J6), 6M sodium hydroxide (E3), napthoquinone sulfonic acid saturated in 50% ethanol (E4), 7mg/mL eosine red b (F5), 2M sodium hydroxide (G3), 30mg/mL sodium nitrite (G4), 10mg/mL nitroaniline in acetonitrile (G5), 1M tosic acid (G6), 1% iodine with excess iodide in 4% povidone (H4), 2M iron(III) chloride with 5% soap solution (I5, I6), 0.1M Zinc2+(J3), 20% sodium nitroprusside (J4), 2M cobalt thiocyanate (K3), 0.2M nickel chloride (L1), dimethylglyoxime saturated in acetonitrile (L4).
Stability testing samples were swiped across location 2 and reagents were spotted in 4µL aliquots as follows: 4mg/mL nioxime (A1, L1), 0.1M nickel chloride (D, L4), saturated potassium carbonate (B2), 0.12M copper sulfate (B4, E2, E3), 1M Iron (II) nitrate (C4), 10% sodium nitroprusside (w/v) (D4), 10M sodium hydroxide (D5), 200mg/mL potassium carbonate (E7, F7), saturated ninhydrin in acetonitrile (F2-F5), 2M sodium hydroxide (G3), 10% sodium nitrite (w/v) (G4), 0.3M 2-nitroaniline in acetonitrile (G5), 1M tosic acid (G7), 1mg/mL Eosine red b (H7), 1% triiodide (w/v) with 4% povidone (w/v) (I4), 0.5M iron (III) chloride in 2% detergent (J4), 0.5M iron (III) chloride.

| TABLE D.1 |

COMPATIBLE SOLVENT VOLUMES FOR REAGENT DEPOSITION THAT ARE CONTAINED BY WAX BARRIERS

<table>
<thead>
<tr>
<th>Not Compatible</th>
<th>Compatible up to 2µL</th>
<th>Compatible up to 5µL</th>
<th>Compatible up to 10µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>Methanol</td>
<td>Methylene chloride</td>
<td>DMSO</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>Ethanol</td>
<td>Acetonitrile</td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

174
<table>
<thead>
<tr>
<th>PAD chemical analyte [test reagent]</th>
<th>Samples used for selectivity testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Beta-lactam 100% [SaturatedCu(II)]</td>
<td>Lactose (6/6), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6) acetaminophen tablet (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6)=48/48 (whole PAD analysis)</td>
</tr>
<tr>
<td>b) Ampicillin 100% [Ninhydrin]</td>
<td>Lactose 6/6), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6) acetaminophen tablet (4/4) 2 lane failures, talc(6/6), erythromycin tablet(4/4) 2 lane failures, chloroquine (6/6), Amoxi (17/17), amoxi:talc (5/5)=66/66 (whole PAD analysis)</td>
</tr>
<tr>
<td>c) Amoxicillin 100% [Ninhydrin]</td>
<td>Lactose (6/6), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6), acetaminophen tablet (4/4) 2 lane failures, talc(6/6), erythromycin tablet(4/4) 2 lane failures, chloroquine (6/6), Amp (18/18), amp:talc (9/9)=71/71 (whole PAD analysis)</td>
</tr>
<tr>
<td>d) Amoxicillin 100% [Nitroaniline]</td>
<td>Talc (12/12), (lane tests) :Lactose (5/5)(one lane failure), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6), Amp (20/20), amp:talc (10/10)=71/71 (whole PAD analysis)</td>
</tr>
<tr>
<td>e) Chalk 100% and Baking Soda 100% [FeCl₃]</td>
<td>starch (12/12), povidone (12/12), talc (12/12) =36/36 (lane tests) : Lactose (12/12), aspirin tablets (12/12), wallboard (12/12) acetaminophen tablet (12/12), talc(12/12), erythromycin tablet(12/12), chloroquine (12/12)=84/84 (whole PAD analysis)</td>
</tr>
<tr>
<td>f) Starch 100% [Iodine]</td>
<td>Chalk 12/12, baking soda 12/12, talc 12/12 (lane tests)=36/36 : Lactose (6/6), Chalk ?(3/6), aspirin tablets (6/6), acetaminophen tablet (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6)=39/42 (whole PAD analysis)</td>
</tr>
<tr>
<td>g) Talc 100% [Eosine Red B]</td>
<td>chalk 12/12, starch 12/12, baking soda 12/12 =36/36 (lane tests)</td>
</tr>
<tr>
<td>h) Acetaminophen 100% [Nitroaniline]</td>
<td>talc 12/12, (lane tests) : Lactose (5/5)(one lane failure), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6), Amp (20/20), amp:talc (10/10)=71/71 (whole PAD analysis)</td>
</tr>
</tbody>
</table>
**TABLE D.2**

COMMON FORMULATIONS OF COMBINATIONS

ANTI-TB THERAPIES CURRENTLY IN USE

<table>
<thead>
<tr>
<th>Adult Combinations</th>
<th>Isoniazid (mg)</th>
<th>Rifampicin (mg)</th>
<th>Ethambutol (mg)</th>
<th>Pyrazinamide (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-way</td>
<td>150</td>
<td>-</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>2-way</td>
<td>150</td>
<td>150</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-way</td>
<td>75</td>
<td>150</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-way</td>
<td>75</td>
<td>150</td>
<td>275</td>
<td>-</td>
</tr>
<tr>
<td>3-way</td>
<td>75</td>
<td>150</td>
<td>-</td>
<td>400</td>
</tr>
<tr>
<td>4-way</td>
<td>75</td>
<td>150</td>
<td>275</td>
<td>400</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pediatric Combinations</th>
<th>Isoniazid (mg)</th>
<th>Rifampicin (mg)</th>
<th>Ethambutol (mg)</th>
<th>Pyrazinamide (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-way</td>
<td>30</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-way</td>
<td>60</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-way</td>
<td>30</td>
<td>60</td>
<td>-</td>
<td>150</td>
</tr>
</tbody>
</table>

**TABLE D.3**

MASS OF POWERED MATERIALS DEPOSITED INTO LANES

WITH LIGHT AND HEAVY SWIPES

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Light (g/lane)</th>
<th>Heavy (g/lane)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalk</td>
<td>$5.1 \times 10^{-4}$</td>
<td>$2.5(2) \times 10^{-3}$</td>
</tr>
<tr>
<td>Talc</td>
<td>$3.7(7) \times 10^{-4}$</td>
<td>$2.1(6) \times 10^{-3}$</td>
</tr>
<tr>
<td>Baking Soda</td>
<td>$3.2(8) \times 10^{-3}$</td>
<td>$1.3(2) \times 10^{-2}$</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>$3.2(4) \times 10^{-4}$</td>
<td>$1.4(2) \times 10^{-3}$</td>
</tr>
</tbody>
</table>
Figure D.3  PAD validation scoring example. Figure D.3 shows two TB PADs tested with isoniazid (73%), ethambutol (27%) (w/w)(left panel) and pyrazinamide (64%), rifampicin (24%), isoniazid (12%) (w/w)(right panel). The table shows the expert readers’ responses. The presence of ethambutol and isoniazid is unambiguous while the presence of a colored substance, such as rifampicin, in the right hand example makes reading the colorimetric output difficult and leads to disagreement as seen in the scoring example (lower panel). A discrepancy in lane indicating the presence or absence of ethambutol necessitates the use of a third reader to serve as a tie breaker.

<table>
<thead>
<tr>
<th>PAD #</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>Overall</th>
<th>Reader</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>1092</td>
<td>eth</td>
<td>iso or salicylic acid</td>
<td>neg</td>
<td>neg</td>
<td>iso</td>
<td>neg</td>
<td>e-rich phenol</td>
<td>neg</td>
<td>neg</td>
<td>iso, eth</td>
<td>1</td>
<td>isoniazid ethambutol</td>
</tr>
<tr>
<td>1092</td>
<td>eth</td>
<td>iso or salicylic acid</td>
<td>neg</td>
<td>neg</td>
<td>iso</td>
<td>neg</td>
<td>e-rich phenol</td>
<td>neg</td>
<td>neg</td>
<td>iso, eth</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1105</td>
<td>neg</td>
<td>iso or salicylic acid</td>
<td>rif</td>
<td>pyz</td>
<td>iso</td>
<td>neg</td>
<td>e-rich phenol</td>
<td>neg</td>
<td>rif</td>
<td>rif, iso, pyz</td>
<td>1</td>
<td>Rifampicin isoniazid pyrazinamide</td>
</tr>
<tr>
<td>1105</td>
<td>eth</td>
<td>iso or salicylic acid</td>
<td>rif</td>
<td>pyz</td>
<td>iso</td>
<td>neg</td>
<td>e-rich phenol</td>
<td>neg</td>
<td>rif</td>
<td>rif, iso, pyz, eth</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1105</td>
<td>neg</td>
<td>iso or salicylic acid</td>
<td>rif</td>
<td>pyz</td>
<td>iso</td>
<td>neg</td>
<td>e-rich phenol</td>
<td>neg</td>
<td>rif</td>
<td>rif, iso, pyz</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**Key**

eth = ethambutol
rif = rifampicin
pyz = pyrazinamide
iso = isoniazid
neg = negative result
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Test</th>
<th>Type</th>
<th>Sensitivity</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beta-Lactam 50%</strong> (11 amoxicillin, 10 ampicillin) Talc 50%</td>
<td>Cu(II) test for beta lactam</td>
<td>Whole PAD Lane</td>
<td>76% (16/21) (2 lanes with fabrication errors included)</td>
<td>100% (48/48)^a</td>
</tr>
<tr>
<td><strong>Ampicillin 50%</strong> Talc 50%</td>
<td>Ninhydrin test for ampicillin</td>
<td>Whole PAD Lane</td>
<td>9/10 (1 lane with fabrication error included) 100.0% (24/24)</td>
<td>100% (66/66)^b</td>
</tr>
<tr>
<td><strong>Amoxicillin 50%</strong> Talc 50%</td>
<td>Nitroaniline test for phenol Ninhydrin test for amoxicillin</td>
<td>Whole PAD Lane</td>
<td>78% (7/9) (2 lanes with fabrication errors included) 60% (3/5) 100% (24/24)</td>
<td>100% (30/30)^c 100% (69/69)^d ---</td>
</tr>
<tr>
<td>Beta-lactam 50% (11 amoxicillin, 10 ampicillin) Talc 50%</td>
<td>Eosin Red test for talc</td>
<td>Lane</td>
<td>97% (35/36)</td>
<td>100% (36/36)^e</td>
</tr>
<tr>
<td><strong>Rifampicin 10%</strong> Potato Starch 90%</td>
<td>Iron (III) test for rifampicin</td>
<td>Whole PAD</td>
<td>100% (15/15)</td>
<td>100% (75/75)^f</td>
</tr>
<tr>
<td>Rifampicin 10% Potato Starch 90%</td>
<td>Iodine test for starch</td>
<td>Whole PAD</td>
<td>73.3% (11/15)</td>
<td>87.4% (118/135)^g</td>
</tr>
<tr>
<td><strong>Pyz 50%</strong> Turmeric powder 50%</td>
<td>SNP test for pyrazine</td>
<td>Whole PAD</td>
<td>33.3% (5/15) Note: See overall TB PAD analysis for identification of suspicious TB combos</td>
<td>80% (60/75)^h 0% (0/15) with rif, 100% (60/60) without rif</td>
</tr>
<tr>
<td>2-drug combo IE: Eth 73%, Iso 27%</td>
<td>Cu(II) test for ethambutol</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>96.7% (87/90)^i</td>
</tr>
<tr>
<td>2-drug combo IE: Eth 73%, Iso 27%</td>
<td>NQS test for isoniazid</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>83.3% (50/60)^j 33.3% (5/15) with rif 100% (45/45) w/o rif</td>
</tr>
<tr>
<td>3-drug combo RIP: Pyr 64%, Rif 24%, Iso 12%</td>
<td>SNP test for pyrazine</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>80% (60/75)^k 0% (0/15) with rif, 100% (60/60) w/o rif</td>
</tr>
<tr>
<td>3-drug combo RIP: Pyr 64%, Rif 24%, Iso 12%</td>
<td>Iron (III) test for rifampicin</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>100% (75/75)^l</td>
</tr>
<tr>
<td>3-drug combo RIP: Pyr 64%, Rif 24%, Iso 12%</td>
<td>NQS test for isoniazid</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>83.3% (50/60)^m 33.3% (5/15) with rif 100% (45/45) w/o rif</td>
</tr>
<tr>
<td>4-drug combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8%</td>
<td>SNP test for pyrazine</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>80% (60/75)^n 0% (0/15) with rif, 100% (60/60) w/o rif</td>
</tr>
<tr>
<td>4-drug combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8%</td>
<td>Cu(II) test for ethambutol</td>
<td>Whole PAD</td>
<td>60% (18/30)</td>
<td>96.7% (87/90)^o</td>
</tr>
<tr>
<td>4-drug combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8%</td>
<td>Iron (III) test for rifampicin</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>100% (75/75)^p</td>
</tr>
</tbody>
</table>
**TABLE D.4 (continued)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Test</th>
<th>Type</th>
<th>Sensitivity</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-drug combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8%</td>
<td>NQS test for isoniazid</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>83.3% (50/60)</td>
</tr>
<tr>
<td>Tertiary Amines: 100% Quinine sulfate (24) or 100% Diphenhydramine (12) or 100% Chloroquine (12)</td>
<td>Cobalt thiocyanate test for tertiary amines</td>
<td>Lane</td>
<td>100% (48/48)</td>
<td>100% (36/36)</td>
</tr>
<tr>
<td>Suspicious TB Combo (substitute API) 100% Quinine sulfate (15) or 100% Diphenhydramine (15)</td>
<td>Overall TB PAD analysis</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>100% (90/90)</td>
</tr>
<tr>
<td>Suspicious TB Combo (API cut with a filler) Pyz 50% turmeric 50% (15)</td>
<td>Overall TB PAD analysis</td>
<td>Whole PAD</td>
<td>73% (11/15)</td>
<td>97.8% (88/90)</td>
</tr>
<tr>
<td>4-drug TB combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8%</td>
<td>Overall TB PAD analysis</td>
<td>Whole PAD</td>
<td>70% (21/30)</td>
<td>95.8% (115/120)</td>
</tr>
<tr>
<td>Suspicious TB Combo (API cut with a filler) Rif 10% potato starch 90% (15)</td>
<td>Overall TB PAD analysis</td>
<td>Whole PAD</td>
<td>76.7% (23/30)</td>
<td>88.3% (106/120)</td>
</tr>
<tr>
<td>2-drug TB combo IE: Eth 73%, Iso 27%</td>
<td>Overall TB PAD analysis</td>
<td>Whole PAD</td>
<td>100% (30/30)</td>
<td>100% (120/120)</td>
</tr>
</tbody>
</table>

**a)** Analytes tested as negative controls included: lactose (6/6), chalk (6/6), aspirin tablets (6/6), wallboard (6/6), acetaminophen tablet (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6) = 48/48 (whole PAD analysis)**

**b)** Analytes tested as negative controls included: lactose (6/6), chalk (6/6), aspirin tablets (6/6), wallboard (6/6) acetaminophen tablet (4/4) 2 lane failures, talc(6/6), erythromycin tablet(4/4) 2 lane failures, chloroquine (6/6), Amoxi (17/17), amoxi:talc (5/5) = 66/66 (whole PAD analysis)**

**c)** Analytes tested as negative controls included: talc (12/12), (lane tests) Lactose (5/5) (one lane failure), Chalk (6/6), aspirin tablets (6/6), wallboard (6/6), talc(6/6), erythromycin tablet(6/6), chloroquine (6/6), Amp (20/20), amp:talc (10/10) = 71/71 (whole PAD analysis) **

**d)** Analytes tested as negative controls included: lactose (6/6), chalk (6/6), aspirin tablets (6/6), wallboard (6/6), acetaminophen tablet (4/4) 2 lane failures, talc(6/6), erythromycin tablet(4/4) 2 lane failures, chloroquine (6/6), Amoxi (18/18), amp:talc (9/9) = 71/71 (whole PAD analysis) **

**e)** Analytes tested as negative controls included: chlk (12/12), starch (12/12), baking soda (12/12) = 36/36 (lane tests) **

**f)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), IE (30/30), diphenhydramine (15/15) = 75 /75 (whole PAD analysis) **

**g)** Analytes tested as negative controls included: pyz + turmeric (0/15), quinine sulfate (15/15), RIP (28/30), IE (30/30), PIE (29/30), diphenhydramine (15/15) **

**h)** Note: Turmeric reliably causes false positives. Selectivity without turmeric = 118/135

**i)** Analytes tested as negative controls included: quinine sulfate (15/15), IE (30/30), Rif & starch (0/15), diphenhydramine (15/15) = 60/75 (whole PAD analysis) **

**j)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), RIP (27/30), Rif & starch (15/15), diphenhydramine (15/15) = 87/90 (whole PAD analysis) **

**k)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), IE (30/30), diphenhydramine (15/15) = 75 /75 (whole PAD analysis) **

**l)** Analytes tested as negative controls included: bakteing soda (12/12), talc (24/24) = 36/36 (lane test analysis) **

**m)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (5/15), diphenhydramine (15/15) = 50/60 (whole PAD analysis)**

**n)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), IE (30/30), diphenhydramine (15/15) = 75 /75 (whole PAD analysis) **

**o)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), IE (30/30), diphenhydramine (15/15) = 99/120 (whole PAD analysis) **

**p)** Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (15/15), RIP (30/30), IE (30/30), diphenhydramine (15/15) = 120/120 (whole PAD analysis)**
Figure D.4 Optimal read time for PADs. A timeline of color development for most limiting colorimetric reactions indicates that 5-7 minutes following test completion is optimal for imaging PAD results.

Figure D.5 Indicator for dried-out PAD. Lane images of the fully formed timer blaze (a) and cobalt thiocyanate at 0 (b), 10 (c), 15 (d), 20 (e), 35 (f), 60 (g) and 90 minutes (h) after removal from water.
Figure D.6  Effects of storage on PAD performance: sample lane images. Beta lactam PADs were run with amoxicillin/starch 50% w/w either fresh or after 2 month storage in ambient conditions. A general yellowing is evident, but that positive results are still distinguishable. If PADs are serialized, a reader can pinpoint the date of manufacturing and discount yellowing due to age in their interpretation of results.
Figure D.7 Effects of storage at 37°C and 60°C on different lane tests. Sensitivity of PAD lane tests to target analytes or appearance of timer spot as measured by visual inspection compared to standard results following storage at 37°C and 60°C.
D.12 References


