# Paper analytical devices for fast field screening of beta lactam antibiotics and anti-tuberculosis pharmaceuticals

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## ASSOCIATED CONTENT

Supporting Information.



**Materials.**

1,2-napothoquinone-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.

**Fabrication**

**Printing**. Beta lactam and TB PADs were based on a 12-lane wax-printed PAD (Figure S1, Supporting Information) laid out using Adobe Illustrator (see file "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 color 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 and back wax" layer 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 in order to ensure that 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.

**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.

**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 S1 (Supporting Information). 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 S1, Supporting Information). 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 numbered 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 spot-

ting was high (i.e. less than 3 improperly spotted PADs per batch 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.

**Measurement of accuracy of robot solution deposition.** The Biomek FX was used to place 96 spots of 4.00 μL $2.007x10^{-3}$ M $KIO_3$ 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 $KIO_3$ aqueous solution the average spotting volume for a nominal 4 ul spot was measured titrimetrically as 3.72± 0.07 μL.

**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 S1, Supporting Information).

**Light box fabrication.** To ensure uniformity of lighting during this study, light boxes were constructed using shoe boxes. A single hole was cut into the box to allowing imaging, but eliminate all natural light from the image. Reproducible, diffuse lighting was achieved by a white LED within the box directed at an angle of 120º away from the PAD. All images were taken without flash.

**Targets for PAD detection:**

**Selection of pharmaceutical targets.** 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 distributers, 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 (CaCO3), 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. Based on tablet weights and labeled doses, 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.[2] 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.[5] 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 S2, Supporting Information, 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.

**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 powder is then pressed into the swipe line with a spatula or popsicle stick. 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 S3, Supporting Information).

**Development and optimization of chemical tests:**

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 cop-

per(II), hydroxide, and potassium tartrate is a useful test reagent for beta lactam antibiotics.[16] 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.[40–44] 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,[16] 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 $K_2CO_3$ on the swipe line and 1M $Cu(SO_4)_2 \cdot 5H_2O$ 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).

**Differentiation of ampicillin and amoxicillin with ninhydrin.** Ninhydrin 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. 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 S6, Supporting Information), at least 104 days at 37˚C and at least 30 days at 60˚C (Figure S7, Supporting Information). 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.

**Detection of electron-rich phenols with nitroaniline.** Both amoxicillin and acetaminophen (a substitute API that has been found in several counterfeit anti-infective drugs[19,34,45]) 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 $NaNO_2$ and a strong acid. Thus, this lane contains four reagents: at the bottom, tosic acid (a strong acid that is stable on paper), then $NaNO_2$, 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 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 S6, Supporting Information). This test remains viable for at least 104 days at 37˚C and can withstand 5 days of storage at 60˚C (Figure S7, Supporting Information).

**Detection of nucleophilic functional groups with pentacyanoaquoferroate.** Sodium pentacyanoaquoferroate $Na_3Fe(CN)_5H_2O$ 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 $Na_2Fe(CN)_5NO$ (sodium nitroprusside or SNP).[39] 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 S2, Supporting Information) which is stronger than the yellow isoniazid color. Tests remain viable for at least 104 days at 37˚C (Figure S7, Supporting Information). 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.

**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.

**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 quinine sulfate (Figure 2). The acidified cobalt thiocyanate lane test was used to identify tertiary amines in the validation study.

**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.[46] 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 S6, Supporting Information). The iodine test remains viable for 66 days at 37˚C (Figure S7, Supporting Information).

**Detection of talc by selective dye binding.** Talc is a magnesium silicate mineral with formula $Mg_3Si_4O_{10}(OH)_2$. 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.[47] 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.

**Stability Studies.** PADs for stability testing were spotted as shown in supplementary figure 2 and dried in a convection oven at 60 ºC for 10 min. Storage stability tests were carried out by storing PADs in either 37ºC or 60ºC 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 included 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.

**Optimization of test 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 DI 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" location 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.

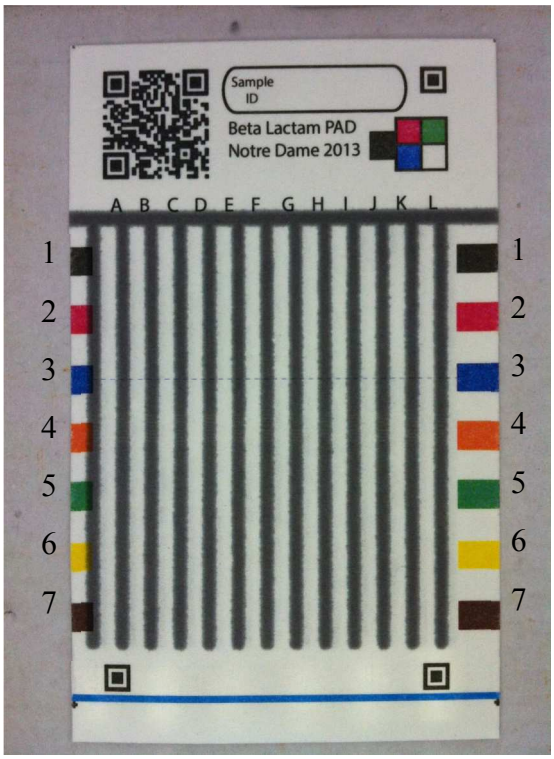



**Figure S1. Reagents and spotting locations for beta lactam PAD and TB PAD**

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 handling 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: 2M NaOH (A2), 1M copper (II) sulfate (,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 (A4, L4), saturated potassium carbonate (B2), 0.12M copper sulfate (B4, E2, E3), 1M Iron (III) 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.

**Figure S2. Fringe diagram**

This diagram contains images of lanes on the TB PAD run with different analytes at different loadings, and is included as a zip file due to its large size.

**Table S1. Compatible solvent volumes for reagent deposition that are contained by wax barriers.**

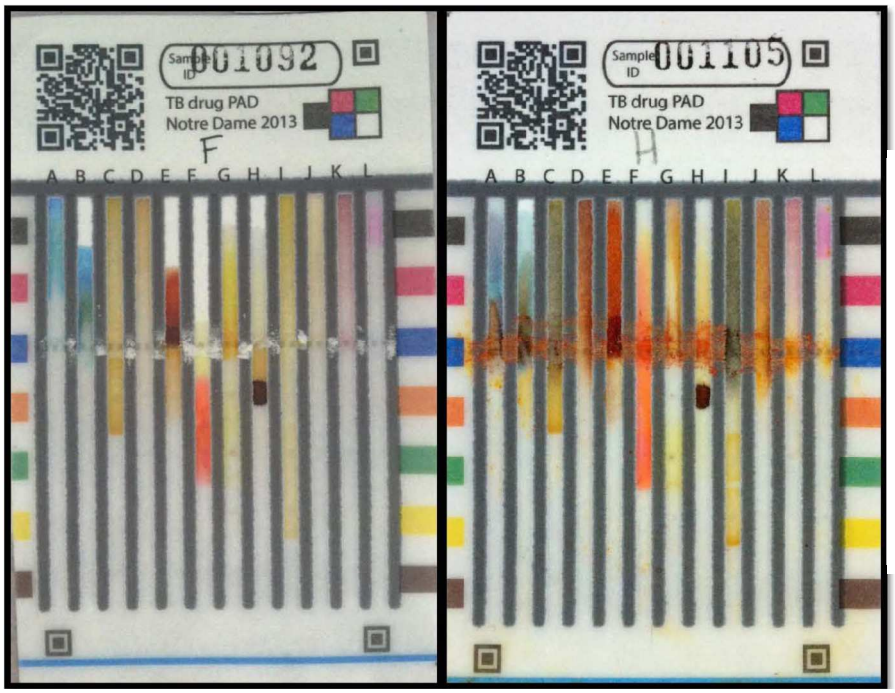
| Not compatible | Compatible up to 2µL | Compatible up to 5µL | Compatible up to 10µL |
|---|---|---|---|
| Ethyl acetate | Methanol | Methylene chloride | DMSO |
| Triethylamine | Ethanol | | Acetonitrile |
| | Acetone | | Water |

**Table S2. Common formulations of combination anti-TB therapies.**[5]

| Adult Combinations | Isoniazid (mg) | Rifampicin (mg) | Ethambutol (mg) | Pyrazinamide (mg) |
|---|---|---|---|---|
| 2-way | 150 | - | 400 | - |
| 2-way | 150 | 150 | - | - |
| 2-way | 75 | 150 | - | - |
| 3-way | 75 | 150 | 275 | - |
| 3-way | 75 | 150 | - | 400 |
| 4-way | 75 | 150 | 275 | 400 |
| | | | | |
| Pediatric Combinations | Isoniazid (mg) | Rifampicin (mg) | Ethambutol (mg) | Pyrazinamide (mg) |
| 2-way | 30 | 60 | - | - |
| 2-way | 60 | 60 | - | - |
| 3-way | 30 | 60 | - | 150 |

**Table S3. Mass of powdered materials deposited into lanes with light and heavy swipes.**

| Reagent | Light (g/lane) | Heavy (g/lane) |
|---|---|---|
| Chalk | $5.(1) \times 10^{-4}$ | $2.5(2) \times 10^{-3}$ |
| Talc | $3.7(7) \times 10^{-4}$ | $2.1(6) \times 10^{-3}$ |
| Baking Soda | $3.2(8) \times 10^{-3}$ | $1.3(2) \times 10^{-2}$ |
| Acetaminophen | $3.2(4) \times 10^{-4}$ | $1.4(2) \times 10^{-3}$ |




**Key**

eth= ethambutol

rif = rifampicin

pyz = pyrazinamide

iso = isoniazid

neg = negative result

| PAD # | A | B | C | D | E | F | G | H | I | Overall | Reader | Final |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1092 | eth | iso or salicylic acid | neg | neg | iso | neg | e- rich phenol | neg | neg | iso, eth | 1 | isoniazid ethambutol |
| 1092 | eth | iso or salicylic acid | neg | neg | iso | neg | e- rich phenol | neg | neg | iso, eth | 2 | |
| 1105 | neg | iso or salicylic acid | rif | pyz | iso | neg | e- rich phenol | neg | rif | rif, iso, pyz | 1 | Rifampicin isoniazid pyrazinamide |
| 1105 | eth | iso or salicylic acid | rif | pyz | iso | neg | e- rich phenol | neg | rif | rif, iso, pyz, eth | 2 | |
| 1105 | neg | iso or salicylic acid | rif | pyz | iso | neg | e- rich phenol | neg | rif | rif, iso, pyz | 3 | |

**Figure S3. PAD validation scoring example**

Figure S3 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 S4a. Sensitivity and selectivity for detection of pure analytes with PADs**

| Analyte | Test | PAD Type* | Sensitivity | Selectivity |
|---|---|---|---|---|
| Beta-lactam 100%<br>(20 ampicillin, 20 amoxicillin) | SaturatedCu(II) | 12-lane | 100% (40/40) | 100% (48/48)[a] |
| Ampicillin 100% | Ninhydrin | 12-lane<br>Single lane<br>Total | 90% (18/20)<br>100% (11/11)<br>94% (29/31) | 100% (66/66)[b] |
| Amoxicillin 100% | Ninhydrin | 12-lane<br>Single lane<br>Total | 100% (18/18)<br>100% (12/12)<br>100% (29/29) | 100% (71/71)[c] |
| Amoxicillin 100% | Nitroaniline | 12-lane<br>Single lane<br>Total | 89.5 (17/19)<br>100% (12/12)<br>93.5% (29/31)) | 100% (71/71)[d] |
| Ethambutol 100% | 1M Cu (II) | Single lane | 100% (24/24) | 98% (101/102) |
| Rifampicin 100% | Iron (III) | Single lane | 100% (30/30) | 88% (86/98) |
| Pyrazinamide 100% | SNP | Single lane | 92% (24/26) | 98% (47/48) |
| Isoniazid 100% | NQS | Single lane | 100% (24/24) | 98% (47/48) |
| Chalk 100% ($CaCO_3$) | $FeCl_3$ | 12-lane<br>Single lane<br>Total | 50% (6/12)<br>100% (12/12)<br>75% (18/24) | 100% (84/84%)[e]<br>100% (36/36)[e]<br>100% (120/120)[e] |
| Baking soda 100% ($NaHCO_3$) | $FeCl_3$ | 12-lane<br>Single lane<br>Total | 100% (36/36) | 100% (84/84%)[e]<br>100% (36/36)[e]<br>100% (120/120)[e] |
| Starch 100% | Iodine | 12-lane<br>Single lane<br>Total | 94% (34/36) | 92.9% (39/42)[f]<br>100% (36/36)[f]<br>96.2% (75/78)[f] |
| Talc 100% | Eosin Red | Single lane | 97% (35/36) | 100% (36/36)[g] |
| Acetaminophen 100% | Nitroaniline | 12-lane<br>Single lane<br>Total | 100% (6/6)<br>100% (24/24)<br>100% (30/30) | 100% (12/12)[h]<br>100% (71/71)[h]<br>100% (83/83)[h] |

*All PAD testing was carried out and on the same printed devices loaded with either 12 different tests, noted as "12-lane" or in some cases repeats of a "single lane" test.

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 (12-lane)

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 (12-lane)

c) 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), amp (18/18), amp:talc (9/9)=71/71 (12-lane)

d) 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 (12-lane)

e) Analytes tested as negative controls included: chalk 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 (12-lane)

f) Analytes tested as negative controls included: 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 (12-lane)

g) Analytes tested as negative controls included: chalk 12/12, starch 12/12, baking soda 12/12 =36/36 (single lane)

h) 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 (12-lane)

**Table S4b. Sensitivity and selectivity for detection of pharmaceutical combination with PADs**

| Analyte | Test | PAD Type | Sensitivity | Selectivity |
|---|---|---|---|---|
| **Beta-Lactam 50%** (11 amoxicillin, 10 ampicillin) Talc 50% | Cu(II) test for beta lactam | 12-lane | 76% (16/21) (2 lanes with fabrication errors included) | 100% (48/48)[a] |
| **Ampicillin 50%** Talc 50% | Ninhydrin test for ampicillin | 12-lane Single lane | 9/10 (1 lane with fabrication error included) 100.0% (24/24) | 100% (66/66)[b] --- |
| **Amoxicillin 50%** Talc 50% | Nitroaniline test for phenol<br>Ninhydrin test for amoxicillin | 12-lane<br>12-lane<br>Single lane | 78% (7/9) (2 lanes with fabrication errors included)<br>60% (3/5)<br>100% (24/24) | 100% (30/30)[c]<br>100% (69/69)[d]<br>--- |
| Beta-lactam 50% (11 amoxicillin, 10 ampicillin) **Talc 50%** | Eosin Red test for talc | Single lane | 97% (35/36) | 100% (36/36)[e] |
| **Rifampicin 10%** Potato Starch 90% | Iron (III) test for rifampicin | 12-lane | 100% (15/15) | 100% (75/75)[f] |
| Rifampicin 10% **Potato Starch 90%** | Iodine test for starch | 12-lane | 73.3% (11/15) | 87.4% (118/135)[g] |
| **Pyz 50%** Turmeric powder 50% | SNP test for pyrazine | 12-lane | 33.3% (5/15) Note: See overall TB PAD analysis for identification of suspicious TB combos | 80% (60/75)[h] 0% (0/15) with rif, 100% (60/60) without rif |
| 2-drug combo IE: **Eth 73%,** Iso 27% | Cu(II) test for ethambutol | 12-lane | 100% (30/30) | 96.7% (87/90)[i] |
| 2-drug combo IE: Eth 73% , **Iso 27%** | NQS test for isoniazid | 12-lane | 100% (30/30) | 83.3% (50/60)[j] 33.3% (5/15) with rif 100% (45/45) without rif |
| 3-drug combo RIP: **Pyr 64%,** Rif 24%, Iso 12% | SNP test for pyrazine | 12-lane | 100% (30/30) | 80% (60/75)[h] 0% (0/15) with rif, 100% (60/60) without rif |
| 3-drug combo RIP: Pyr 64%, **Rif 24%,** Iso 12% | Iron (III) test for rifampicin | 12-lane | 100% (30/30) | 100% (75/75)[k] |
| 3-drug combo RIP: Pyr 64%, Rif 24%, **Iso 12%** | NQS test for isoniazid | 12-lane | 100% (30/30) | 83.3% (50/60)[j] 33.3% (5/15) with rif 100% (45/45) without rif |
| 4-drug combo RIPE: **Pyr 44%,** Eth 31%, Rif 17%, Iso 8% | SNP test for pyrazine | 12-lane | 100% (30/30) | 80% (60/75)[h] 0% (0/15) with rif, 100% (60/60) without rif |
| 4-drug combo RIPE: Pyr 44%, **Eth 31%,** Rif 17%, Iso 8% | Cu(II) test for ethambutol | 12-lane | 60% (18/30) | 96.7% (87/90)[i] |
| 4-drug combo RIPE: Pyr 44%, Eth 31%, **Rif 17%,** Iso 8% | Iron (III) test for rifampicin | 12-lane | 100% (30/30) | 100% (75/75)[k] |
| 4-drug combo RIPE: Pyr 44%, Eth 31%, Rif 17%, **Iso 8%** | NQS test for isoniazid | 12-lane | 100% (30/30) | 83.3% (50/60)[j] 33.3% (5/15) with rif 100% (45/45) without rif |
| Tertiary Amines : 100% Quinine sulfate(24) or 100% Diphenhydramine (12) or 100% Chloroquine (12) | Cobalt thiocyanate test for tertiary amines | Single lane | 100% (48/48) | 100% (36/36)[l] |
| Suspicious TB Combo (substitute API) 100% Quinine sulfate (15) or 100% Diphenhydramine (15) | Overall TB PAD analysis | 12-lane | 100% (30/30) | 100% (90/90)[m] |

| | | | | |
|---|---|---|---|---|
| Suspicious TB Combo (API cut with a filler) Pyz 50% turmeric 50% (15) | Overall TB PAD analysis | 12-lane | 100% (15/15) | 96.7% (87/90)[n] |
| Suspicious TB Combo (API cut with a filler) Rif 10% potato starch 90% (15) | Overall TB PAD analysis | 12-lane | 73% (11/15) | 97.8% (88/90)[n] |
| 4-drug TB combo RIPE: Pyr 44%, Eth 31%, Rif 17%, Iso 8% | Overall TB PAD analysis | 12-lane | 70% (21/30) 30% (9/30) RIP | 95.8% (115/120)[o] |
| 3-drug TB combo RIP: Pyr 64%, Rif 24%, Iso 12% | Overall TB PAD analysis | 12-lane | 76.7% (23/30) 16.7% (5/30) RIPE 6.7% (2/30) Rif + starch | 88.3% (106/120)[p] |
| 2-drug TB combo IE: Eth 73%, Iso 27% | Overall TB PAD analysis | 12-lane | 100% (30/30) | 100% (120/120)[q] |

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 (12-lane )

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 (12-lane)

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 (12-lane)

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), Amp (18/18), amp:talc (9/9)=71/71 (12-lane)

e) Analytes tested as negative controls included: chalk (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(12-lane)

g) Analytes tested as negative controls included: pyz + turmeric (0/15), quinine sulfate (15/15), RIP (28/30), IE (30/30), RIPE (29/30), diphenhydramine (15/15) Note: Turmeric reliably causes false positives. Selectivity without turmeric= 118/135 (12-lane)

h) Analytes tested as negative controls included: quinine sulfate (15/15), IE (30/30), Rif & starch (0/15), diphenhydramine (15/15) = 60/75 (12-lane).

i) 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 (12-lane)

j) Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (5/15), diphenhydramine (15/15) = 50/60 (12-lane)

k) Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), IE (30/30), diphenhydramine (15/15) = 75/75 (12-lane)

l) Analytes tested as negative controls included: baking soda (12/12), talc (24/24) = 36/36 (Single lane)

m) Analytes tested as negative controls included:, RIP (30/30), RIPE (30/30), IE (30/30) = 90/90 (12-lane)

n) Analytes tested as negative controls included: RIP (28/30), RIPE (29/30), IE (30/30) = 87/90 (12-lane)

o) Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (15/15), RIP (25/30), IE (30/30), diphenhydramine (15/15) = 115/120 (12-lane)

p) Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (3/15), RIPE (21/30), IE (30/30), diphenhydramine (15/15) = 99/120 (12-lane)

q) Analytes tested as negative controls included: pyz + turmeric (15/15), quinine sulfate (15/15), Rif & starch (15/15), RIP (30/30), RIP (30/30), diphenhydramine (15/15) = 120/120 (12-lane)

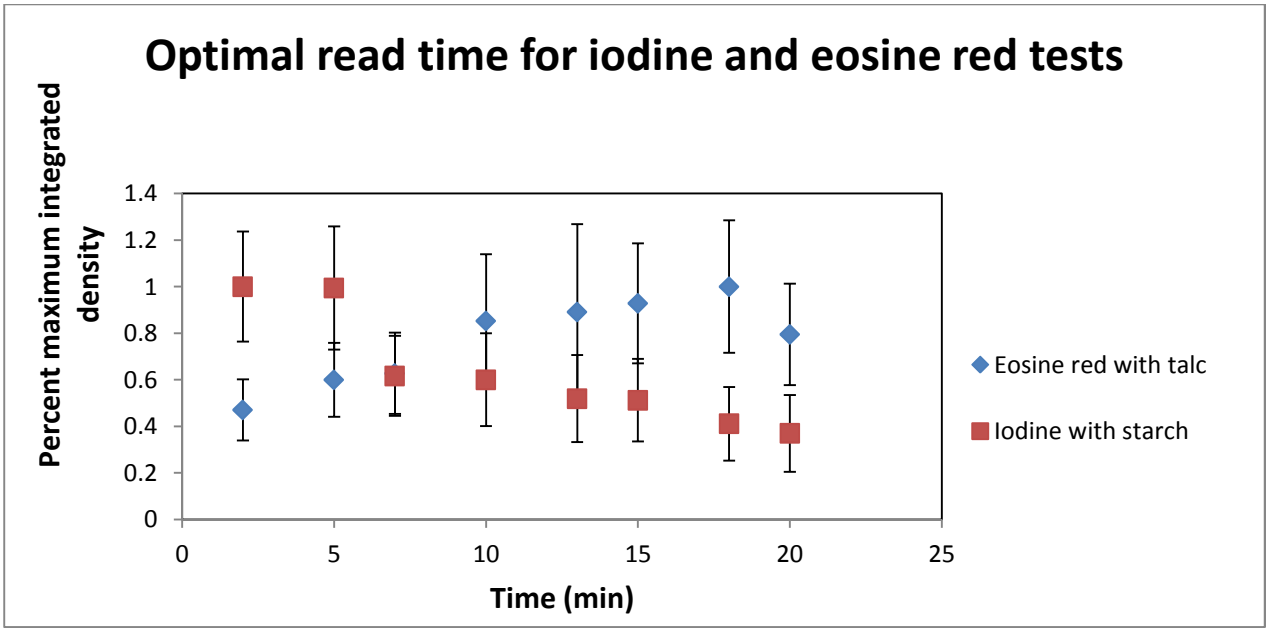



**Figure S4. Optimal read time for PADs.**

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 S5. 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.

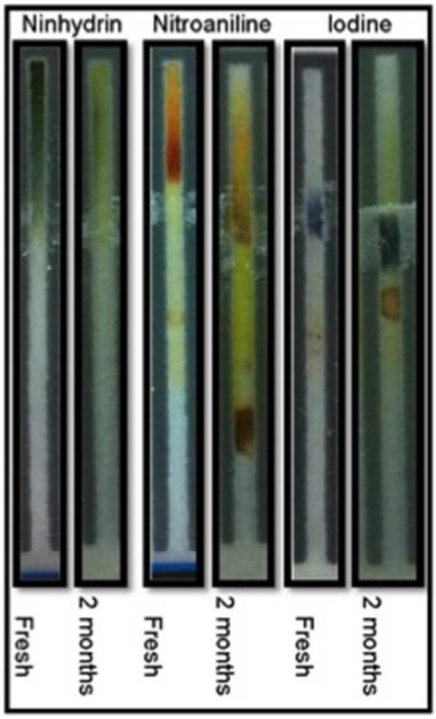



**Figure S6. Effects of storage on PAD performance: sample lane images**

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.

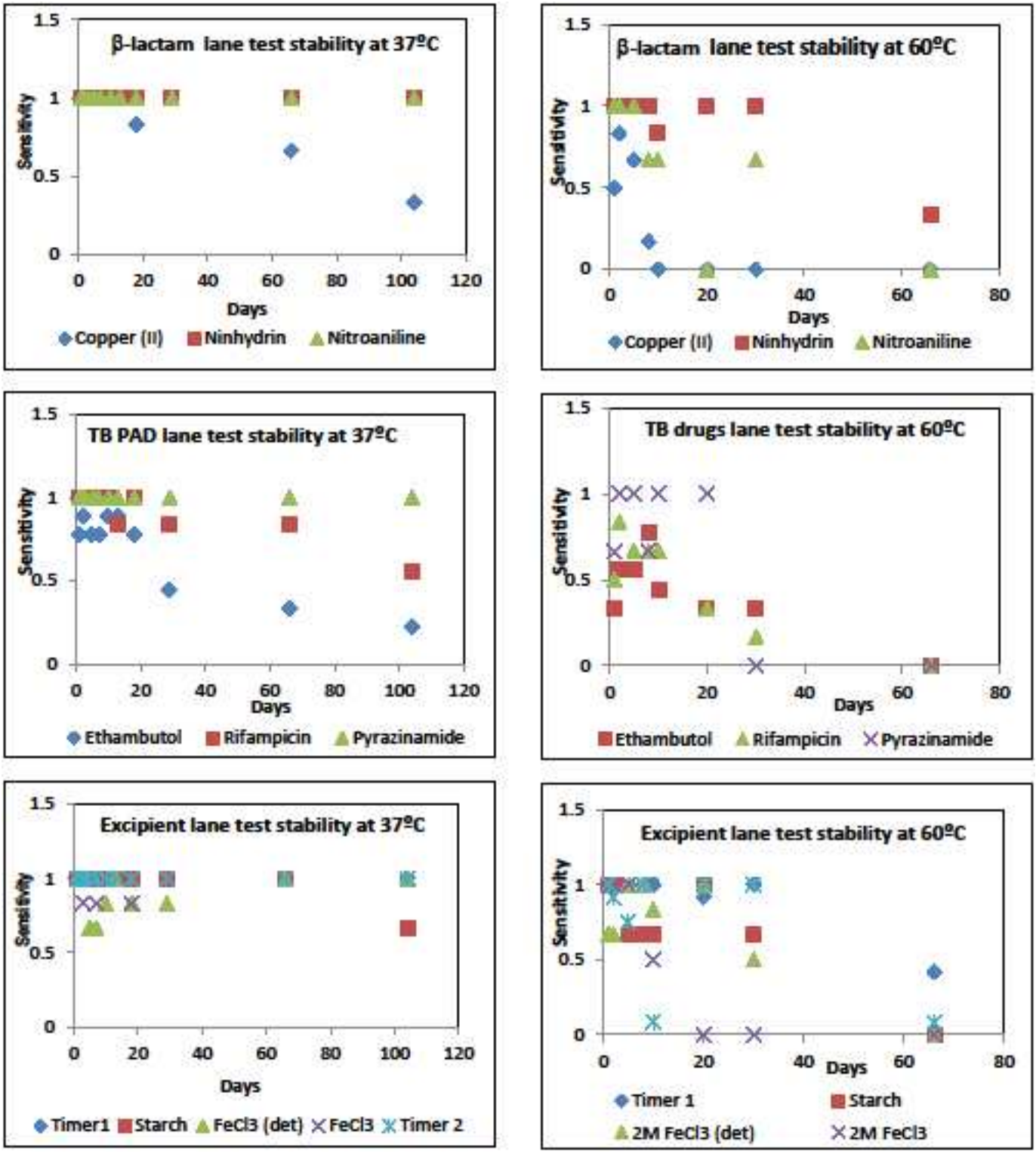



**Figure S7. Effects of storage at 37˚C and 60˚C on different lane tests**

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 37ºC and 60ºC.

**Print Files for color laser, front wax, and back wax**

These print files are included in Adobe Illustrator (.ai) format in a zip file entitled "Print files" included in the supplementary information. The .ai format allows greater control over print alignment during printing of the wax layers.

**References for supporting information.**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(2) World Health Organization Department of Essential Medicines and Pharmaceutical Policies *The International Pharmacopoeia*; 4th ed.; World Health Organization, 2011.

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(4) Kelesidis, T.; Kelesidis, I.; Rafailidis, P. I.; Falagas, M. E. *The Journal of antimicrobial chemotherapy* **2007**, *60*, 214–36.

(5) World Health Organization *WHO Model List of Essential Medicines*; Vol. 17th list.