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## Cellular Phone-Based Image Acquisition and Quantitative Ratiometric Method for Detecting Cocaine and Benzoyllecgonine for Biological and Forensic Applications

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**Abstract:** Here we describe the first report of using low-cost cellular or web-based digital cameras to image and quantify standardized rapid immunoassay strips as a new point-of-care diagnostic and forensics tool with health applications. Quantitative ratiometric pixel density analysis (QRPDA) is an automated method requiring end-users to utilize inexpensive (~ \$1 USD/each) immunotest strips, a commonly available web or mobile phone camera or scanner, and internet or cellular service. A model is described whereby a central computer server and freely available IMAGEJ image analysis software records and analyzes the incoming image data with time-stamp and geo-tag information and performs the QRPDA using custom JAVA based macros (<http://www.neurocloud.org>). To demonstrate QRPDA we developed a standardized method using rapid immunotest strips directed against cocaine and its major metabolite, benzoylecgonine. Images from standardized samples were acquired using several devices, including a mobile phone camera, web cam, and scanner. We performed image analysis of three brands of commercially available dye-conjugated anti-cocaine/benzoylecgonine (COC/BE) antibody test strips in response to three different series of cocaine concentrations ranging from 0.1 to 300 ng/ml and BE concentrations ranging from 0.003 to 0.1 ng/ml. This data was then used to create standard curves to allow quantification of COC/BE in biological samples. Across all devices, QRPDA quantification of COC and BE proved to be a sensitive, economical, and faster alternative to more costly methods, such as gas chromatography-mass spectrometry, tandem mass spectrometry, or high pressure liquid chromatography. The limit of detection was determined to be between 0.1 and 5 ng/ml. To simulate conditions in the field, QRPDA was found to be robust under a variety of image acquisition and testing conditions that varied temperature, lighting, resolution, magnification and concentrations of biological fluid in a sample. To determine the effectiveness of the QRPDA method for quantifying cocaine in biological samples, mice were injected with a sub-locomotor activating dose of cocaine (5 mg/kg; i.p.) and were found to have detectable levels of COC/BE in their urine (160.6 ng/ml) and blood plasma (8.1 ng/ml) after 15–30 minutes. By comparison rats self-administering cocaine in a 4 hour session obtained a final BE blood plasma level of 910 ng/ml with an average of 62.5 infusions. It is concluded that automated QRPDA is a low-cost, rapid and highly sensitive method for the detection of COC/BE with health, forensics, and bioinformatics application and the potential to be used with other rapid immunotest strips directed at several other targets. Thus, this report serves as a general reference and method describing the use of image analysis of lateral flow rapid test strips.

**Keywords:** diagnostic, point-of-care, rapid test strip, cellular phone, self-administration, crowdsourcing

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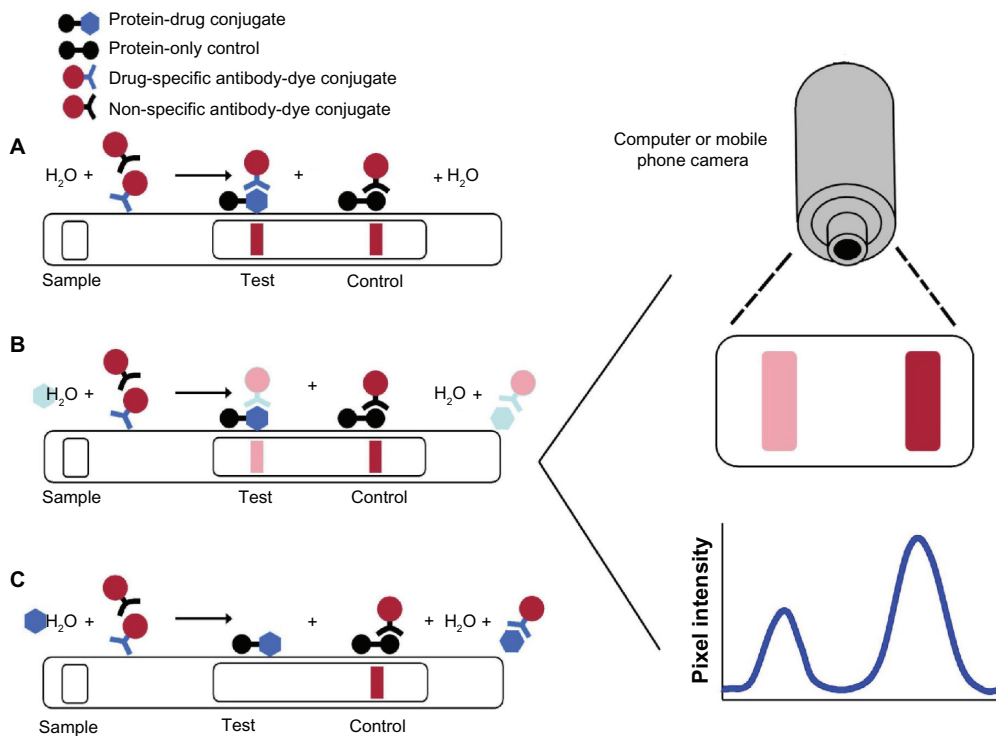
## Introduction

The concepts of “e-health” and “m-health,” or electronic and mobile health, refer to the use of technology and mobile devices to improve the availability and quality of health care. In recent years, these ideas have seen a reemergence as ways of providing health care in developing countries. In particular, increases in availability and coverage of cellular phones in developing countries make them an attractive option for the development of mobile health services. These phones have considerable computational power, connectivity, and the ability to take digital photographs, all of which provide valuable tools for scientists, doctors and patients.<sup>1-4</sup> This widespread interconnectivity allows for new point-of-care diagnostics and “crowdsourcing” in the developing world. Crowdsourcing refers to the act of outsourcing jobs traditionally held by employees or workers to a group of people or community. So far, the concept of crowdsourcing has mostly been applied to research, education, and other areas using advanced technology.<sup>5,6</sup> However, in medicine, crowdsourcing has the potential to shift the responsibilities of data collection and storage from a centralized hospital or laboratory to the patient and/or caregiver. In communities where there may be many patients but few hospitals or clinics, crowdsourcing using “smart” cellular phones provides a way to easily collect, transmit, and organize data. In this paper, we describe a quantitative ratiometric pixel density analysis (QRPA), which utilizes low-cost cameras, immunoassays, and automatic quantification to provide a new point-of-care tool for the analysis of lateral flow rapid test strip assays. QRPA can be applied to point-of-care diagnostics and a variety of substances, such as drugs of abuse, water contaminants, and infectious agents. To demonstrate its usefulness for quantifying lateral flow test strips we used QRPA to quantify cocaine and benzoylecgonine levels in biological fluids.

An inexpensive method for quantifying cocaine using rapid test strips is useful because cocaine is second only to marijuana as the most commonly used illicit drug in the United States. Cocaine is second to marijuana as the most commonly used illicit drug in the United States, with 1.9 million adults using it in the previous month.<sup>7-9</sup> Such wide spread usage

of cocaine has consequences in the work place. In 2002, drug and alcohol abuse related problems cost U.S. businesses approximately \$128.6 billion in lost productivity.<sup>10</sup> Illegal drugs are used by about 9.8 million employees in the United States, so employers are likely to employ one or more people with a drug problem.<sup>11</sup> According to the US Postal Service, employees who tested positive on their pre-employment drug tests are 77% more likely to be discharged within the first three years of employment and are absent from work 66% more often than those who tested negative.<sup>11</sup> While a positive drug test cannot be used to prove impaired performance, nor can it be used to infer intoxication, it does function as an objective measure of recent drug use. Subjects who tested positive for cocaine were over three times more likely to be heavy users after leaving their job than those who tested negative, and they were twice as likely to be involuntarily separated from their jobs. Identifying cocaine use in the workplace via drug testing may initiate an employees' search for treatment and prevent the loss of productivity.

Many tests exist which detect the presence of cocaine and its metabolites. These tests vary in several factors, including sensitivity, ease of use, and cost. Inexpensive antibody-based test strips, which cost around \$2–10 a test and are most commonly used in urine tests, use dye-conjugated monoclonal or polyclonal antibodies directed against cocaine and/or its metabolites.<sup>12,13</sup> These test strips are easy to use and provide a signal that can be detected with the naked eye (Fig. 1). Signal bands provide a qualitative positive or negative result with a National Institute for Drug Abuse (NIDA) designated BE sensitivity cutoff of 300 ng/ml.<sup>14</sup> Quantitative techniques, such as gas chromatography-mass spectrometry (GCMS) or tandem mass spectrometry (MS/MS), are the most sensitive detectors of drug concentration with detection levels of less than 10 ng/ml.<sup>15,16</sup> However, these methods are costly in time and equipment, and they require special organic solvent extraction protocols and personal expertise. Immunoassays are commonly used for large scale drug screening, while quantitative tests are reserved for cases when confirmation is necessary or exact levels of a drug must be known.



**Figure 1.** Test strip immunoassay system. **A)** Liquid samples are placed on the test strip sample area, where capillary action draws the fluid along with dye conjugated antibodies across a test area and control area. If no drugs (blue hexagons) are present, dye will bind at both locations, causing two bands to appear. **B)** At intermediate doses, drug-dye binding is incomplete, resulting in a light test band which varies in intensity according to the drug concentration. A camera and image analysis software can then quantify the relative intensity of the two bands. **C)** If drugs are present at sufficient concentrations, they completely bind the drug specific dye and prevent a band from appearing in the test area at all.

## Methods

### Generation of cocaine and BE standard curves

In order to quantify COC/BE levels in unknown samples, a series of known concentrations were made to generate a standard curve. Unknown samples are compared to the standard curve, which allows quantification. Cocaine immunoassay strips were obtained from Craig Medical Distribution (Vista, CA), Medimpex United, Inc. (Bensalem, PA) and Q Test, Inc. (Linden, NJ). For the Craig Medical cocaine standard curve, five concentrations of cocaine solution (0, 3, 5, 10, and 30 ng/ml) were made by weighing out powdered cocaine hydrochloride and adding double distilled water (ddH<sub>2</sub>O). For the Medimpex cocaine curve, four cocaine standards were created (0, 0.1, 0.5, 5 ng/ml). For the Q Test curve, six cocaine standards were created (0, 5, 10, 20, 50, 100 ng/ml). For the Craig Medical benzoylecgonine standard curve, eight concentrations of BE hydrate (Sigma) were made (0, 0.003, 0.005, 0.01, 0.02, 0.03, 0.05, and 0.1 ng/ml)

by adding ddH<sub>2</sub>O to the bottle to create a stock solution that was later diluted with additional ddH<sub>2</sub>O into the desired amounts and applied to cocaine test strips. Concentrations from each standard curve were tested in triplicate, and the results were plotted in IGOR Pro 5.03 (Wavemetrics, Inc.). An exponential curve was used to fit the COC and BE data and tau calculated as per the equation  $f(x) = x_0 e^{-t/\tau}$ , where  $x_0$  is the initial value,  $t$  equals time and tau equals the decay constant that governs the rate of decay of the curve. As a control, two samples (10 and 20 ng/ml cocaine) were quantified by QRPDA and then verified by GC/MS analysis (Chematox Laboratory, Boulder, CO).

### Image analysis

Image analysis was performed using the free National Institutes of Health (NIH) developed image processing software, ImageJ.<sup>17</sup> Images were cropped for analysis of the colloidal gold pigmented control and test bands, and the background was with a rolling ball radius of 25 pixels. Next, a rectangular window was placed



such that it overlapped both colored bands and the pixel density of the selected area plotted to produce a graph with two peaks representing the colored bands on the test strip. Horizontal lines were drawn connecting the base of each peak to create an enclosed space and the area underneath each peak determined. Pixel density ratios (PDR) were expressed as the area under the control peak divided by the area under the test peak. For standard curves, ratios were then normalized to ddH<sub>2</sub>O controls by dividing each value by the ddH<sub>2</sub>O ratio.

### Automatic web based quantification

The ability to transmit an image and receive results in minutes is crucial for an effective point-of-care tool. To that end, we created a program which automates image analysis. ImageJ allows users to create macros, text files which contain code that results in a specific application or output from the program. A custom macro was written which automatically performed the ratio quantification process detailed in the section above. This custom macro can be found at: <http://neurocloud.org> under the software and protocol downloads tab. The macro subtracts the background, selects the signal bands, plots the pixel density ratio of the bands, draws lines enclosing each peak, and measures the area underneath each peak. Area output can be automatically set to export results into a spreadsheet program or text file.

We also describe a web based method whereby cocaine test strip images are uploaded and results of the ImageJ pixel density analysis are obtained. After receiving an uploaded image from the client, ImageJ analysis through a PHP command executes a command-line code. When run in a command-line fashion, the ImageJ program can receive arguments which specify the file to open and the macro to run.

### Testing QRPDA with a variety of image acquisition devices

As a useful point-of-care and crowdsourcing tool, it is important that QRPDA achieve similar results using a variety of devices to acquire images. To test the effects of using different devices on the pixel density ratio, a series of 5 cocaine standards (0, 3, 5, 10, and 30 ng/ml) were created and applied to Craig Medical test strips in triplicate. Images of each test strip were taken using one of the following devices: Dino-Lite AM211 digital microscope, Kodak ESP-7 Scanner, Microsoft Lifecam

Cinema Webcam, or Sprint HTC 3.2 Megapixel Camera Phone. The maximum resolution varied among the various devices (Dino Lite, 640 × 480 pixels; Kodak ESP-7, 3470 × 2536 pixels; Lifecam, 1289 × 720 pixels; HTC camera, 2048 × 1536 pixels). Data was plotted in Igor PRO 5.03 and fit to exponential curves as above.

### Testing effects of time delay between assay and analysis

It is possible that the passage of time between applying a sample to the test strip and taking a photograph of it affects the results of a QRPDA test. To test the effects of the passage of time on the pixel density ratio, a series of 5 cocaine standards (0, 3, 5, 10, and 30 ng/ml) were created as above and applied to Craig Medical test strips in triplicate. Images of each test strip were taken using the Dino-Lite AM211 Microscope at 1 hour, 20 hours, and 30 days and quantified. Data was plotted in Igor PRO 5.03 and fit to exponential curves as above.

### Testing the limits of photo resolution

It is possible that images taken at different resolutions may yield different results. To test the effects of resolution on pixel density ratio, a 0 and 10 ng/ml cocaine sample was applied to Craig Medical test strips and scanned using the Kodak ESP-7 scanner at various resolutions (75, 150, 200, 300, and 600 dots per inch, or DPI) and then quantified as described above.

### Biological fluid effects and comparison

The addition of various amounts of biological fluids may affect test results. To test this, samples of 10 ng/ml cocaine were made as above. C57/BL6 mice were anesthetized with isoflurane and decapitated and trunk blood collected. Blood was spun in a centrifuge at 7000 rpm for 15 minutes and blood plasma collected. Blood plasma was added to the 10 ng/ml cocaine samples to create various concentrations of blood plasma (0, 2, 10, and 25% blood plasma). Samples were then applied to cocaine test strips from Craig Medical, photographed at 1 hour using the Dino-Lite AM211 Microscope, and quantified as described above.

Depending on how the cocaine is administered and metabolized, different biological fluids may contain



different levels of COC/BE. To compare COC/BE levels in blood serum and urine, mice were intraperitoneally injected with 5 mg cocaine/kg body weight. After 15 or 30 minutes, animals were anesthetized with isoflurane, decapitated, and urine and trunk blood collected. Blood was collected in a capillary tube and spun in a centrifuge at 7000 rpm for 15 minutes to collect blood plasma. Blood plasma was diluted 1:50 in ddH<sub>2</sub>O and urine diluted 1:500 in ddH<sub>2</sub>O to provide a quantifiable signal. Samples were applied to test strips and quantified using the BE standard curve to determine the concentration in each sample.

### Temperature tests

Temperature variation may affect test results. To test the effects of temperature, we performed the QRPDA test at different temperatures (4.5–32.2 °C), samples of 0 and 10 ng/ml cocaine were created and applied to Craig Medical test strips. Tests were performed in triplicate and the results quantified as described above. Next, samples of 0 and 10 ng/ml cocaine were created and applied to Craig Medical test strips after heating the sample or the test strip only, or both the strip only and sample. Tests were performed in triplicate and quantified as above.

### Camera magnification tests

The magnification or the distance between the camera and test strip could have effects on the final pixel density ratio. We tested this by applying ddH<sub>2</sub>O to Craig Medical test strips and taking images using the Sprint HTC 3.2 Megapixel camera phone at various heights (5.5, 8, 18, 30, and 60 cm) at 1 hour and quantifying as described above.

### Testing background illumination

Photos of test strips may be taken under many different lighting conditions. We tested this by applying ddH<sub>2</sub>O to Craig Medical test strips and taking images using the Sprint HTC 3.2 Megapixel camera phone at 1 hour at various levels of background illumination. The luminosity (51, 75, 100, and 154 average luminosity) was determined using Adobe Photoshop CS3, and signal bands were quantified as described above.

### Experimental animals

Male Sprague–Dawley rats weighing 275–325 g (Charles River Laboratories, Kingston, NY, USA)

were individually housed in wire cages with food and water available ad libitum. Male C57/BL6 mice weighing 22–28 g were group housed (2–5) in plastic cages with food and water available ad libitum. Experiments were conducted during the light cycle of a 12:12 hour light:dark cycle (lights on at 0700 hours) in accordance with guidelines established by the University of Colorado Animal Use and Care Committee.

### Quantifying cocaine levels following animal self-administration

We tested whether QRPDA has utility as a method of quantifying COC/BE levels in animals used in drug research. Cocaine self-administration or need injection are both procedures commonly used in drug research labs. For self-administration, animals are placed in an apparatus and allowed to intravenously administer cocaine at will by pressing a lever. Rats were allowed to self-administer cocaine as previously described.<sup>18</sup> Rats received 0.5 mg cocaine/kg body weight per infusion, with 100 µl per infusion delivered over 5 seconds on a fixed ratio 1 schedule (1 lever press equals one infusion). On the 11th day of self-administration, 15 minutes after the end of the session, blood was collected from the lateral tail vein of the six rats which self-administered the most cocaine. Blood was collected in a capillary tube and spun in a centrifuge at 7000 rpm for 15 minutes. Blood serum was then collected from each capillary tube and diluted at a concentration of 1:50 serum to ddH<sub>2</sub>O. This served to create enough liquid to apply to the test strip (130 µl) and diluted the serum to a concentration that would create a quantifiable signal on the test strip. Image analysis was performed on each assay as described above and the signal band ratio plotted on the BE standard curve (See discussion) to obtain the concentration in each sample.

### Using QRPDA to for a pharmacokinetic model of cocaine self-administration

Cocaine and BE metabolism data from Sun and Lau<sup>19</sup> was entered into IGOR Pro 5.03 and a curve fit to the data to provide the metabolism rates for both COC and BE following a 2 mg cocaine/kg body weight i.v. injection. A pharmacokinetic model was then created which used these metabolism rates to calculate the COC, BE, and combined COC and BE levels in an animal after repeated infusions to



simulate a self-administration session. The pattern of rat self-administration for the four hour session was simulated by introducing a random value between 1 and 20 minutes from a Gaussian distribution with a standard deviation set to be 2. The Gaussian distribution is achieved using a Box-Muller transformation of uniform random numbers. These parameters produced simulated infusion intervals that matched actual rat responses. After adjusting the infusion intervals such that the average interval and standard deviation matched the average rat self-administration data, the per infusion BE and COC blood serum levels were adjusted to match the level of BE measured after a 4 hr session (910 ng/ml). To reflect the increased sensitivity for BE on the test strips, COC levels were first divided by 250 prior to combining COC levels with BE levels to create the average combined COC/BE curve (Fig. 9). The model was run 100 times and the average number of infusions and average COC/BE levels calculated. Finally, self-administration timing data from a rat self-administration session was run in the model to confirm its accuracy.

## Results

### Cocaine and benzylecgonine standard curves

Each COC and BE standard provided colored signal bands (Fig. 2) which were quantified and used to create a standard curve. For the test strips obtained from Craig Medical, an exponential function provided the best fitting curve for both the COC and BE data with a tau of 6.324 and 0.017, respectively (Fig. 3). Sensitivity for COC ranged from 3 to 30 ng/ml, whereas sensitivity for BE ranged from 0.003 to 0.1 ng/ml. Thus, the Craig Medical test strips were 250 times more sensitive towards BE than COC. This is contrary to the cross-reactivity listed by the manufacturer, which lists an equal cross-reactivity for both BE and COC. For the test strips obtained from Medimpex and Q Test, an exponential function provided the best fitting curve for both sets of data with a tau of 19.84 and 0.38, respectively. Cocaine sensitivity for Medimpex test strips ranged from 0.1 to 2 ng/ml, whereas sensitivity for Q Test strips ranged from 5 to 100 ng/ml. Thus, the Medimpex test strips were approximately 10 times more sensitive

to cocaine compared to those from Craig Medical and the Q Test strips approximately 3 times less sensitive to cocaine compared to those from Craig Medical. 10 and 20 ng/ml cocaine samples sent off for GCMS analysis returned slightly higher results than the QRPDA method (14 and 30 ng/ml versus 11 and 21 ng/ml, respectively), possibly due to the extraction process necessary in GCMS analysis.

### Effects of image acquisition device and time delay

To assess whether using different devices to acquire the images affected the final pixel density ratio, four devices were used to capture images from a single set of assay strips. Data from each device was fit to an exponential curve. The tau for the scanner curve, mobile phone curve, webcam curve, and Dino-Lite curve were 7.793, 6.872, 7.298, and 7.007, respectively (Fig. 4).

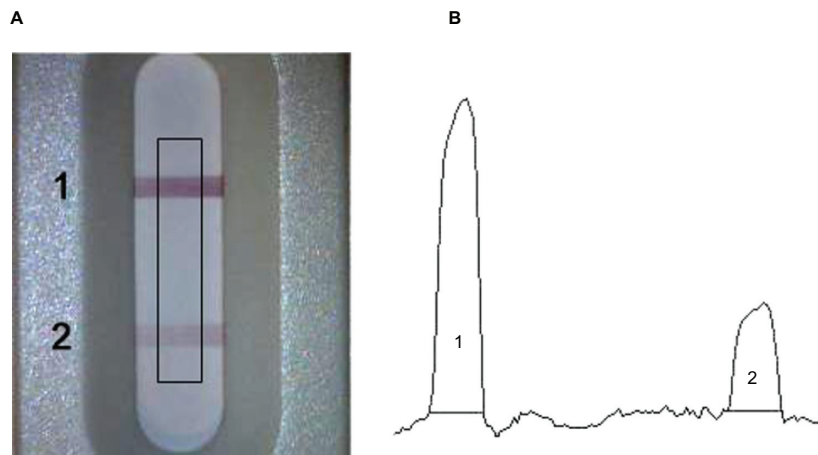
Next, images of a single set of concentrations were taken at 1 hour, 20 hours, and 30 days to assess the effects of a time delay on the results of the image analysis. Each time point was fit to an exponential curve with a tau of 7.05, 11.248, and 11.841, for 1 hour, 20 hours, and 30 days. The passage of time results in an increase in the pixel density ratio. However, the magnitude of this increase diminishes over time. Comparing tau scores, the 1 hour and 20 hour time points are 62.68% similar, whereas the 20 hour and 30 day are 94.99% similar. Therefore, the Craig Medical test strips taken at the 20 hours time -point yields a stable pixel density ratio.

### Effects of image resolution

QRPDA was stable across a range of resolutions. Across all resolutions (75–600 DPI), the ddH<sub>2</sub>O samples provided a pixel density ratio ranging from 1.20 to 1.25, with an average of 1.24 and an average SEM of 0.07 (Fig. 5). Across all resolutions, the 10 ng/ml cocaine samples provided a pixel density ratio ranging from 0.41 to 0.45, with an average of 0.43 and an average SEM of 0.10.

### Influence of biological fluid on QRPDA

Spiking a 10 ng/ml sample of cocaine with various amounts of mouse blood plasma (0, 2, 10, and 25%) provided pixel density ratios ranging

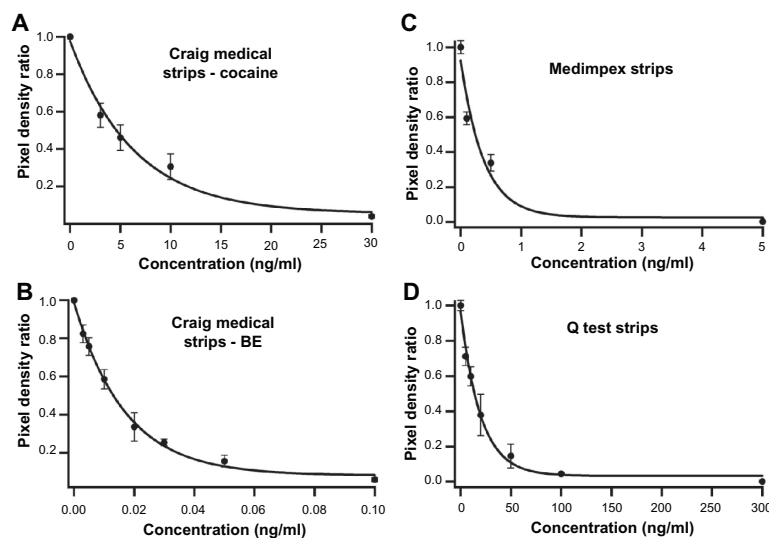


**Figure 2.** Antibody Test Strip Analysis. **A)** Example of a photo of a test strip used for image analysis prior to background subtraction. The black rectangle displays the area selected for pixel density ratio analysis. 1, control band. 2, test band. **B)** The pixel density of the area in the black rectangle in a was graphed and a line drawn under each peak to create an enclosed area. The area was then quantified and the area under peak 2 divided by the area under peak 1 to yield the pixel density ratio. 1,2 as in a.

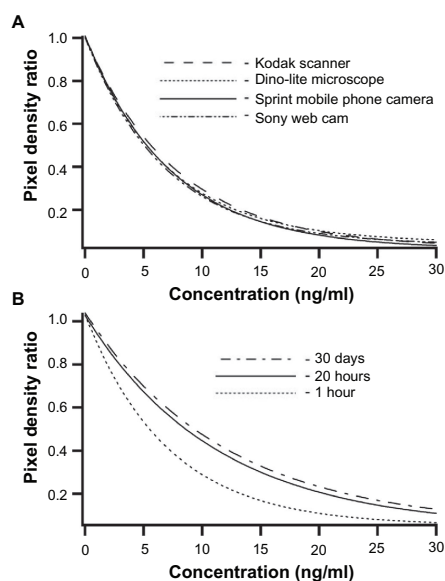
from 0.28 to 0.39, with an average of 0.33 and an average SEM of 0.04 (Fig. 6). We conclude that the addition of blood plasma up to 25% does not significantly alter pixel density ratio. It should be noted that for all experiments in this paper using blood plasma, sample concentrations did not rise above 4%.

### Temperature tests

For the 3 temperature conditions tested (4.5, 22.8, and 32.2 °C), ddH<sub>2</sub>O samples provided pixel density ratios of 1.03, 1.35, and 1.64, respectively, with an average SEM of 0.11 (Fig. 7). The 10 ng/ml cocaine samples provided pixel density ratios of 0.10, 0.33, and 0.44 with an average SEM of 0.04. Therefore,



**Figure 3.** Craig medical, medimpex, and Q test standard curves. **A)** To allow quantification of cocaine and BE in unknown samples, standard curves were created. First, a series of cocaine standards (0, 3, 5, 10, and 30 ng/ml) were created and applied to test strips obtained from Craig Medical in triplicate. 20 hours after applying the standards to the strip, images of each strip were taken using a Dino-Lite microscope, quantified in ImageJ, and the results plotted on a curve using IGOR Pro 5.03. **B)** To determine if the test strips have the same sensitivity towards cocaine and BE, a series of BE standards (0, 0.003, 0.005, 0.01, 0.02, 0.03, 0.05, and 0.1 ng/ml) were created and applied to Craig Medical test strips in triplicate. Results were obtained and plotted as in a. Craig Medical Test strips were able to detect BE levels approximately 300 times lower than that of cocaine. **C)** Different brands may have different sensitivities to COC/BE. To test this, strips were obtained from Medimpex United, Inc. Four cocaine standards (0, 0.1, 0.5, and 5 ng/ml) were created and applied to test strips in triplicate. Results were quantified and plotted as in a. Medimpex test strips were approximately 30 times more sensitive to cocaine than the Craig Medical test strips. **D)** As in c, except using Q Test strips and seven cocaine standards (0, 5, 10, 20, 50, 100, and 300 ng/ml). Results indicate that Craig Medical test strips are approximately 3 times more sensitive to cocaine than Q Test strips.



**Figure 4.** Effects of image acquisition device and time on pixel density ratio. **A)** Effects of using four image acquisition devices on the pixel density ratio. Four instruments (Dino-Lite microscope, Kodak scanner, Microsoft webcam, and Sprint camera phone) were used to take images of a single set of cocaine standards at 1 hour and quantified. Each device had no significant effect on the final pixel density ratio. **B)** Effects of the passage of time prior to image analysis on the pixel density ratio. Images of a single set of cocaine standards were taken using the Dino-Lite microscope at 1 hour, 20 hours, and 30 days and quantified. Data points omitted for clarity.

lower temperatures lower the PDR, while higher temperatures raise it. Temperature affected the cocaine signal more, with the cold water sample being 76% as large as the room temperature water signal, compared to the cold cocaine signal being only 31% as large as the room temperature cocaine signal. Heating the sample and test strip independently demonstrated that the increase in PDR came entirely from heating the strip and not from heating the sample (Data not shown).

### Effect of camera magnification and background illumination

To determine how magnification affects pixel density ratio, and thus the optimal distance or magnification to use, photos of a test strip were taken at several distances (60, 30, 18, 8, and 5.5 cm away) using a Sprint HTC 3.2 megapixel camera phone. At both 60 and 5.5 cm, the pictures were unquantifiable, because at 60 cm the picture is too small and at 5.5 cm shadows from the camera interfered with the pixel density ratio. At the remaining distances, 30, 18, and 8 cm, pictures provided a PDR of 1.33, 1.34, and 1.53 respectively, with an average SEM of 0.06 (Fig. 8). When taking

pictures of a test strip using a mobile phone camera, optimal PDR stability occurs between 18 and 30 cm.

To determine how different levels of background illumination affects the pixel density ratio, photos of a test strip were taken at several levels of lighting (154, 100, 75, and 51 average luminosity, as measured by Photoshop CS3). These photos resulted in pixel density ratios of 1.39, 1.30, 1.63, and 1.23, respectively, with SEMs of 0.07, 0.10, 0.11, and 0.40. Luminosities of less than 154 required turning off lights in the room, while the average luminosity of 51 was almost complete darkness.

### Blood plasma and urine comparison

To make a direct comparison of COC/BE levels in urine and blood plasma, mice were injected with 5 cocaine mg/kg body weight and their COC/BE levels quantified at 15 and 30 minutes. There were no significant differences between mice at 15 and 30 minutes, and they grouped ( $n = 6$ ). Mice had a range of 2.90 to 11.55 ng/ml COC/BE in blood serum samples with an average of 8.12 ng/ml. Urine samples contained between 98.00 and 233.50 ng/ml COC/BE, with an average of 160.58 ng/ml. Overall, urine was found to contain approximately 24 times more COC/BE than blood plasma samples.

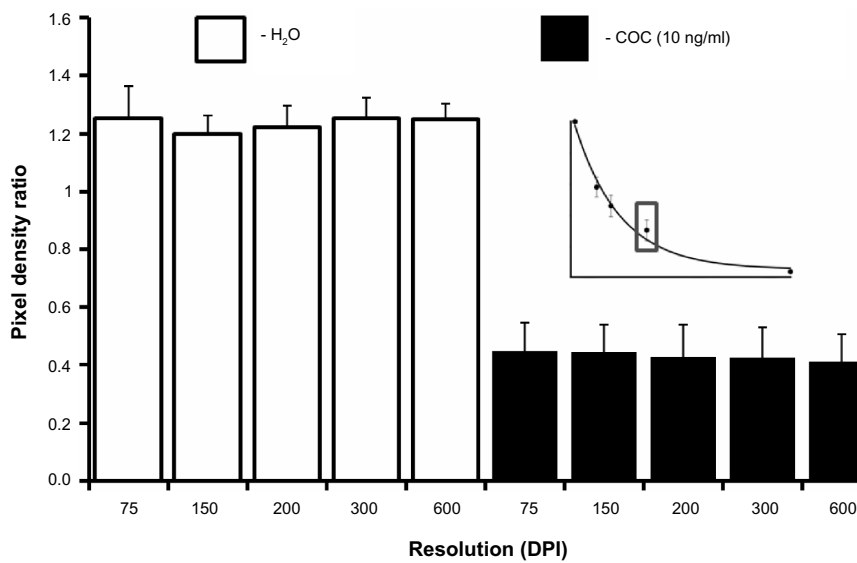
### Rat self-administration results

To verify QRPDA's utility for measuring drug metabolites, we quantified the blood plasma of rats undergoing self-administration of cocaine. Rats ( $n = 6$ ) self-administered an average of 31.25 mg cocaine/kg body weight over the entire session in an average of 62.5 infusions and had an average quantified blood plasma level of COC/BE of 910 ng/ml. Rats pressed the active lever significantly more than the inactive lever ( $P < 0.003$ ), having pressed the active lever an average of 107.83 times versus an average of 3.5 times for the inactive lever.

### Self-administration modeling

As a proof of concept, we created a program which modeled a rat self-administering cocaine using the results obtained from the QRPDA technique. An exponential curve with a tau of 20.59 provided the best fit for the cocaine metabolism curve while a sigmoidal curve with a half maximum at 125.6 minutes fit the BE metabolism data best (Fig. 9). After running the cocaine modeling program 100 times, simulating



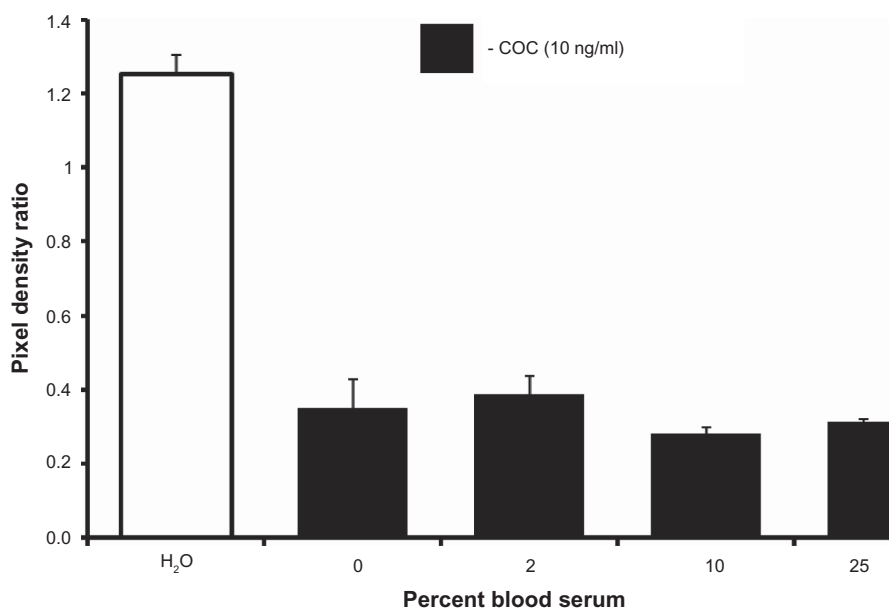


**Figure 5.** Images acquired at different resolutions do not affect pixel density ratio. Changes in resolution had no significant effect on pixel density ratio, Inset shows the standard curve across a range of cocaine concentrations. The rectangle shows the 10 ng/ml data point that was characterized as shown in the black bars.

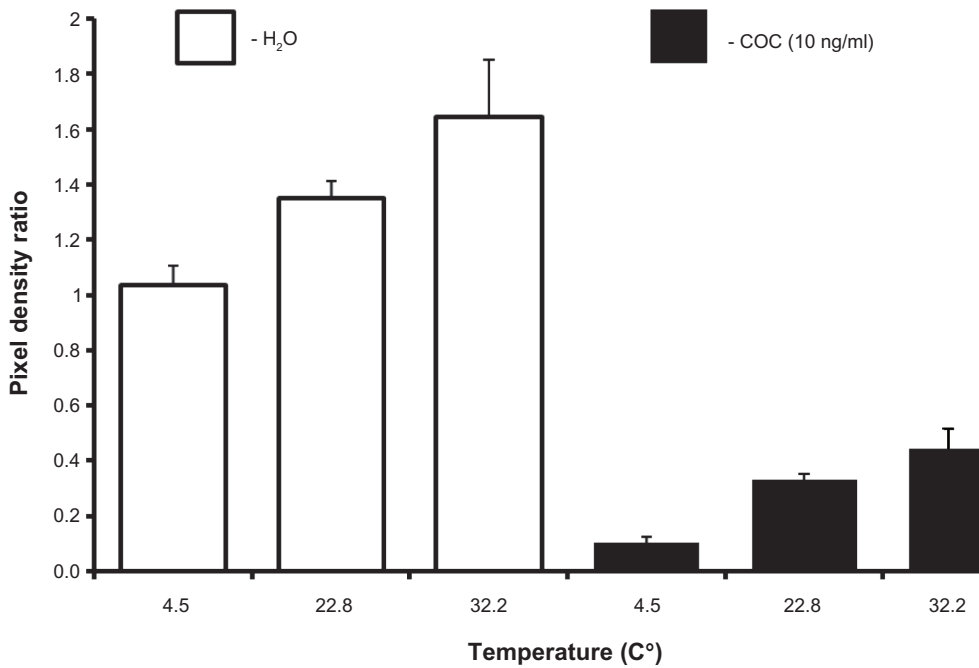
**Note:** QRPDA analysis produces similar results across a wide range of resolutions (dots per inch, DPI).

100 self-administration sessions, the model returned an average of 64.56 infusions with a standard deviation of 19.39 infusions. This is comparable to the real rat data, in which animals took an average of 62.5 infusions with a standard deviation of 22.5 infusions. Average BE and COC levels at the end of the session were both 910 ng/ml. This data is again comparable to the real rat data, in which the average BE blood serum level

across rats was 910 ng/ml. Average combined scaled BE/COC levels at the end of the session were 920 ng/ml. To account for the increased sensitivity towards BE we divided the COC curve by 250 prior to adding the curves together to create the COC/BE curve. BE levels were found to be 99.6% similar to the combined scaled BE/COC levels. Thus, BE levels alone were representative of the combined BE/COC levels.



**Figure 6.** Blood plasma/H<sub>2</sub>O concentration does not alter pixel density ratio. Samples of 10 ng/ml cocaine were spiked with varying amounts of blood plasma to create four concentrations (0, 2, 10, and 25% blood plasma). These concentrations were then applied to test strips and quantified.

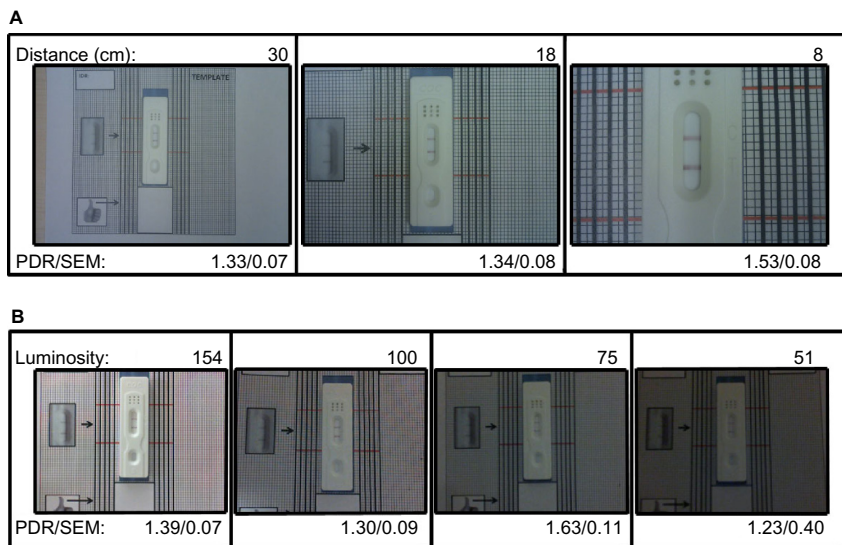


**Figure 7.** Temperature effects on measured pixel density ratio. Effect of performing QRPDA tests under three different temperature conditions, cold (4.5 °C), room temperature (22.8 °C), and hot (32.2 °C). White bars, H<sub>2</sub>O. Black bars, 10 ng/ml cocaine.

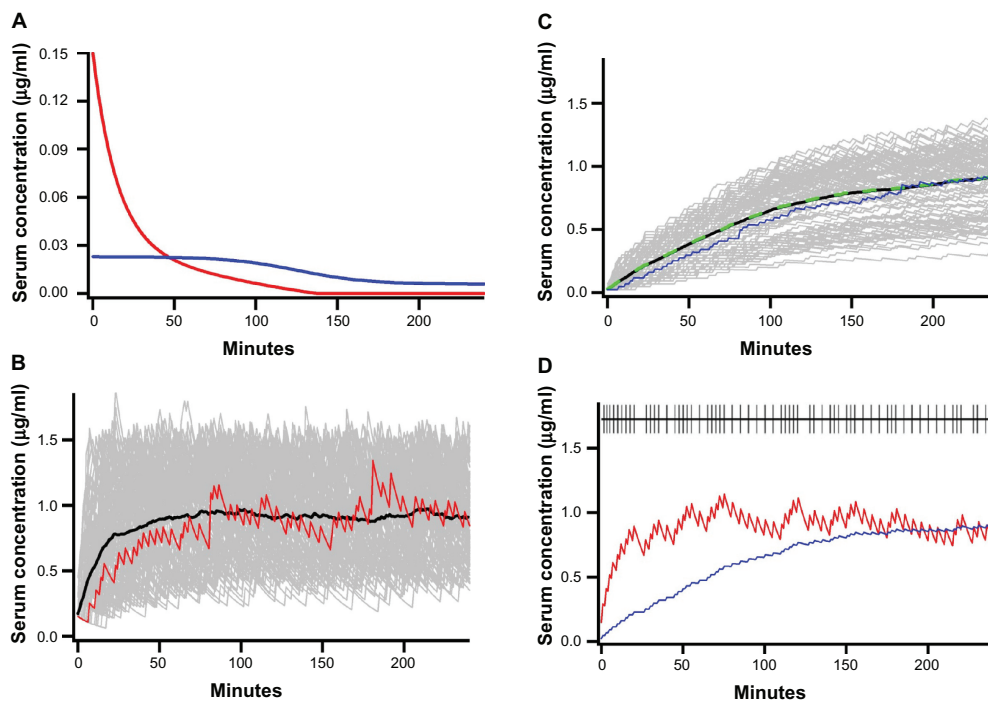
### Discussion

In this paper, we provide the first description of a technique that uses low cost immunoassay strips, a mobile phone camera, and automated image analysis to create an assay with many possible

health care, bioinformatics, and forensics applications. Foremost, QRPDA represents a powerful tool for developing countries where resources and trained personnel are limited. Immunoassay test strips and cell phones are relatively inexpensive and



**Figure 8.** Optimal magnification and lighting. **A)** Effects of magnification on pixel density ratio. Images of a test strip were taken using the Sprint HTC 3.2 megapixel camera phone at various distances and quantified. Results indicate significant changes in pixel density ratio at very close distances (less than 8 cm). Additional images were taken at 60 and 5.5 cm but were unquantifiable. Average luminosity of these pictures was 111. **B)** Effects of luminosity on pixel density ratio. Several images of a test strip were taken using the Sprint HTC 3.2 megapixel camera phone at varying levels of background illumination. Results indicate a significant effect at very low levels of light (average luminosity of less than 75) and increased standard error of the mean (SEM).



**Figure 9.** Pharmacokinetic model of Cocaine (COC) and Benzoylcegonine (BE) using QRPDA analysis. **A)** COC (red) and BE (blue) metabolism rates. These rates determined how much each infusion (0.5 mg cocaine/kg body weight) increased blood serum levels and how quickly each drug was metabolized. **B)** Blood COC levels were simulated by running the model 100 times consecutively. The gray lines represent each individual run of the model, while the red line highlights one example for clarity. The black line represents the average cocaine level across all 100 modeled sessions. **C)** Projected BE blood serum levels after running the model 100 times. As in B, each gray line represents one run of the model. The blue line highlights one example trace and the black line represents the average across all 100 sessions. The dashed green line represents the combined COC and BE averages after the COC average has been divided by 250 to account for the test strip's increased sensitivity towards BE. **D)** Output of the self administration model using real rat timing data from the eleventh day of self administration. The COC (red) and BE (blue) curves in this graph were generated by replacing the random number generators with infusion timing data from a real rat self administration session. Each dash on the line above the graph indicates one infusion. The rat took 64 infusions and had an estimated BE blood serum level of 880 ng/ml at the end of the 4 hour session.

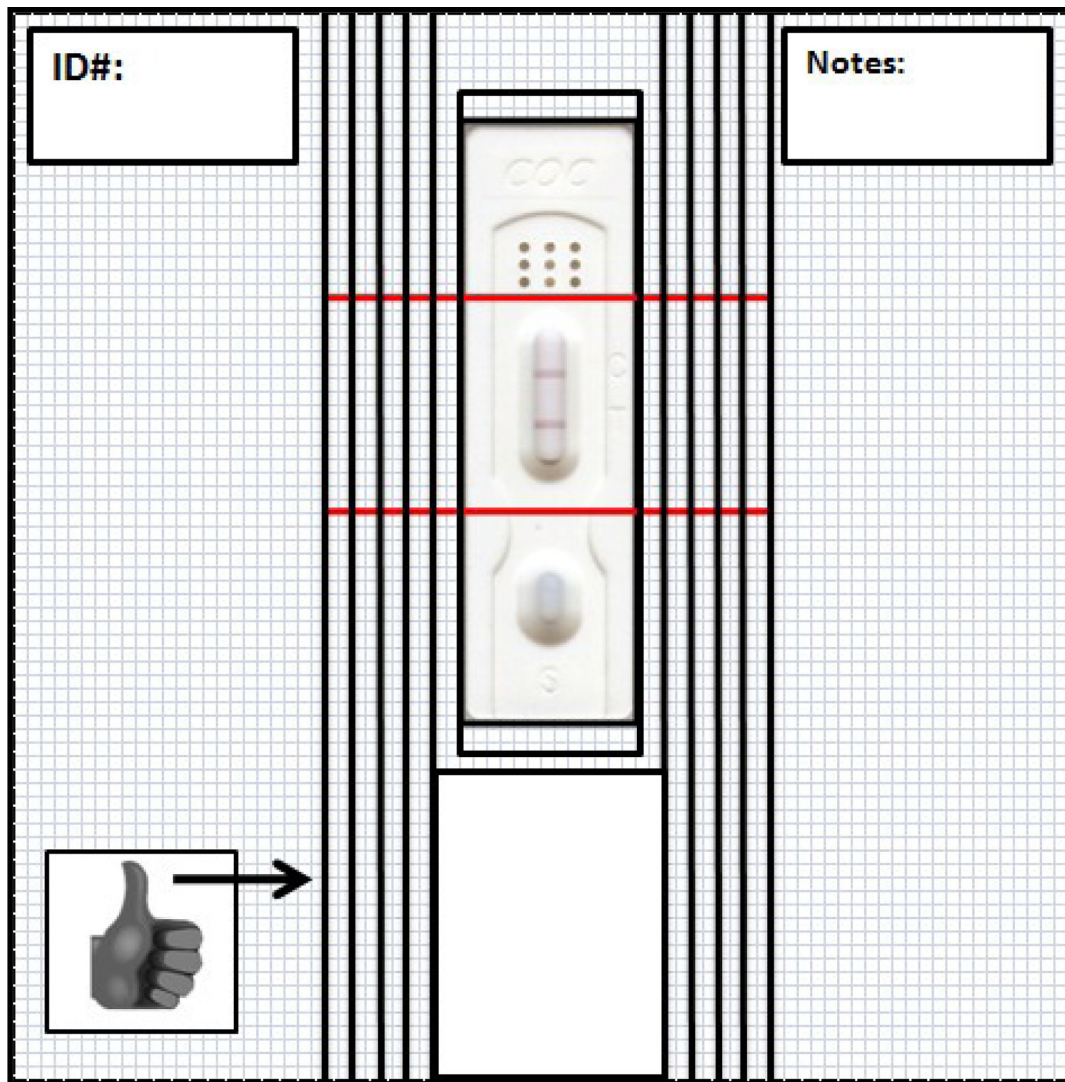
require no special training to use. Combined with automated image analysis, this allows health care workers to “crowdsource” assays and data collection to individuals. Results can be photographed by individuals, transmitted to a central server for archiving and analysis, and the results sent back within minutes. Newer phones even automatically tag photos with coordinates, allowing health care workers to track results geographically.

As a proof of concept, we used anti-COC/BE immunoassay test strips to quantify COC/BE levels in rodents after injections or self-administration of cocaine. In addition, we tested the effects of a variety of parameters on QRPDA results, including using different devices to acquire images, the passage of time, magnification, lighting, resolution, temperature, and the addition of biological fluids to samples. Overall the assay proved robust, providing consistent results across several different parameters. We also designed a printable template to further assist uniform image acquisition (Fig. 10). A barcode or other

unique identifier can be used on the test strip or template to provide information about the strip, such as the lot number, calibration curves and quality control checks.

It is important to note that this technique does have some limitations. Some substances may have cross reactivity that interferes with the immunoassay test strip. For example, the drugs pyrilamine and metoclopramide interfere with COC/BE test strips. Inconsistencies in test strip sensitivity also pose a problem, requiring the use of only a single brand or the creation of multiple standard curves.

QRPDA has many possible applications. Future directions involve adapting the technique for use with a variety of immunoassay test strips. Many immunoassay test strips exist which test for anything from drugs of abuse to water contaminants and infectious agents, such as infectious agents, such as bacteria or parasites.<sup>20</sup> The need for quality control quality and for infectious agents is of particular importance in developing countries. In summary, QRPDA



**Figure 10.** A Premade Template for Test Strip Photography and Identification (Actual Size). To assist with uniform picture acquisition and automatic quantification outside a laboratory setting, a printable template was designed. The template provides a space for the test strip, an identification number, barcode, notes and a fingerprint. The test strip space is designed to ensure proper placement of the test window, while the outside lines and boundaries provide guide markers to ensure users take pictures at the proper height. Use of an identification number or thumbprint provides unique identifiers that can be used to track results without spreading sensitive personal information.

is an innovative, easy to use, and low cost method with many applications in health care and research.

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### Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under

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## Supplemental Information

The code for both the automatic image analysis macro and self-administration model can be found at:<http://neurocloud.org>

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