

NOVEL METHODS FOR ON-SITE AND COST-EFFECTIVE QUANTIFICATION OF
MULTIPLE TARGETS USING FUNCTIONAL DNA AND PORTABLE INSTRUMENTS

BY

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THESIS

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ABSTRACT

This thesis aims at developing novel methods of cost-effective on-site quantitative assays of multiple targets using functional DNA and portable instruments. Such assay methods are important for personal care as well as analyte detection processes. Recent advancements such as the personal glucose meter method developed can achieve fast but not low-cost or user-friendly tests. Two simple and novel methods have been developed. The lateral flow device method is based on Millipore High-flow Test Kit™, where sample is applied to a dipstick pre-treated with aptamer-conjugated magnetic beads, leading to the release of invertase-DNA strands. The strands then flows up with the liquid to an absorption pad pre-treated with sucrose, where the invertase linked on the strands converts sucrose into glucose in amounts proportional to the concentration of the target molecule. The glucose is then detected by a personal glucose meter. The pH meter method is based on aptamer-conjugated magnetic beads and a portable pH meter, where target

molecule triggers the release of glucose-oxidase-conjugated DNA strand and the oxidation of glucose in the buffer solution, therefore lowering the pH of the solution proportionally to the concentration of the target molecule. Both methods are cost-effective and convenient for on-site assays.

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CHAPTER 1: INTRODUCTION

1. Background

Researches have long focused on the development of low-cost and quantitative detection devices for various targets¹⁻⁵. Conventional methods for quantitative assays of analytes usually require expensive antibodies, dedicated analysis equipment, and/or the special preparation of samples by professionals in analytic chemistry^{6,7}. For instance, a conventional method of detecting cocaine requires the use of either a colorimetric reader combined with special antibodies or MS-GC technique⁸. These requirements limit the detection of small molecules, especially their field detection, resulting in difficulties for non-professional users to operate.

Aptamers are artificially-selected nucleic acids that bind specific targets with high affinity⁹. The use of aptamers has proven to be a powerful tool in many fields such as diagnostics as well as therapeutics¹⁰⁻¹². Aptamers that bind cocaine as the ligand have been widely used and incorporated into various designs of detection¹³⁻¹⁶, voiding the necessity of expensive antibodies. Moreover, recently there has been a method by Xiang et al.¹⁷ which couples structure switching aptamer sensors with portable glucose meters to quantify cocaine, featuring a user-friendly method and rendering the use of expensive and bulky instruments unnecessary. However, these

designs still include the preparation of samples by professionals or complicated manufacturing processes which still prevents them from performing fast and in-field detection.

2. Solutions

To address the above-mentioned problem, a solution of two methods for such on-site and cost-effective assays was developed.

The first method involves the aforementioned aptamer-involving detection method¹⁷, the use of personal glucose meters (PGMs) and lateral flow devices (LFDs). The targets are cocaine, a recreational drug, and a protein, streptavidin (STV), an important molecular biology protein. LFDs have seen wide use in rapid detection methods of different targets, but usually they are qualitative or semi-quantitative¹⁸⁻²². This method therefore is fast, low-cost, and user-friendly. It does not require expensive and delicate antibodies, delicate and bulky analysis instruments, or professional operation of devices when performing the assay in-field.

The second method involves the use of portable pH meters and functional-DNA conjugated magnetic beads. The target is cocaine. This method utilizes the target-binding releasing of glucose oxidase (GOx)-conjugated DNA strand to oxidize glucose into concentrations related to concentrations of target solutions. The oxidation product will eventually hydrolyze into gluconic

acid and lower the pH of the solution, leading to pH changes that can be detected by portable pH meters.

CHAPTER 2: THE METHOD USING LATERAL FLOW DEVICES AND PORTABLE GLUCOSE METERS

1. Cocaine detection

The scheme of the method to quantify cocaine using lateral flow devices is shown in Figure 1. In the design to quantify cocaine, a reagent pad, a Hi-Flow™ nitrocellulose membrane and a reaction pad are assembled onto a membrane backing to form the LFDs. The reaction pad is pre-treated with sucrose solution and dried before assembly. Magnetic beads (MBs) functionalized with cocaine aptamer and DNA-invertase conjugates¹⁷ are then deposited on the reagent pad. When quantifying cocaine, LFDs are dipped into target solutions. As the solution travels up along the LFDs, functionalized MBs in the reagent pad interact with targets and release DNA-invertase conjugates, which then catalyzes the sucrose in the reaction pad into glucose in amounts proportionate with the concentrations of cocaine in the solutions. Hi-Flow™ nitrocellulose membrane prevents the MBs from traveling into reaction pad and interfering with the assay results. A portable glucose meter is used to quantify the concentrations of glucose in the reaction pad, which can be finally transformed into the concentrations of cocaine in solutions.

All DNA was obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). ACCU-CHEK™ Avia portable glucose meter was from Roche (Indianapolis, IN). Invertase and GOx

were purchased from Sigma-Aldrich Inc. Cocaine, invertase and other chemicals for buffers, solvents and conjugation were purchased from Sigma-Aldrich, Inc. The constitution of the PBS buffer used in these methods was: 100mM Na-P saline, 100mM NaCl, 0.05% Tween-20, pH = 7.3. The design scheme of the LFDs are shown in Figure 2 below.

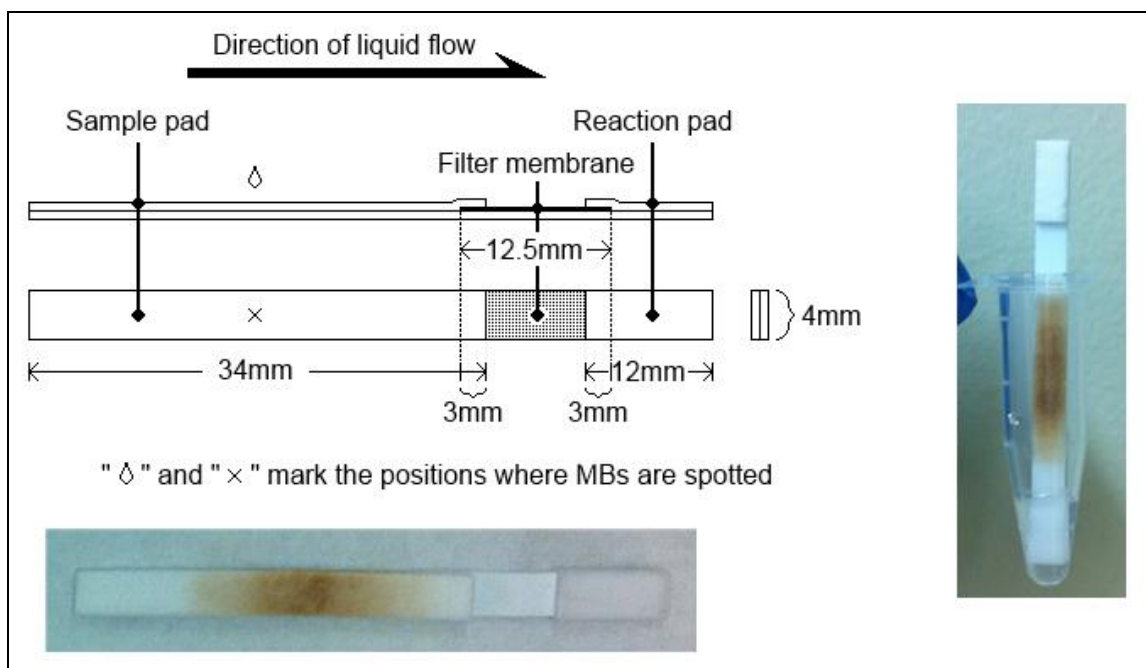


Figure 2 The design and use of a lateral flow device.

Materials for building the LFD were from Millipore High-flow Test Kit™. Each LFD was cut from a pad assembled according to the aforementioned design using an office paper cutter. The width of each device could also be altered to adapt to difference usages. To prepare the sucrose pads, spot cellulose pads with 1M sucrose water solution to ensure complete immersion and leave at room temperature to dry overnight. After drying, cut the sucrose pads into proper dimensions as mentioned in Figure 9. To prepare the sample pad, spot cellulose pads previously assembled on the backing pad with 10uL functionalized MB (8.8mg/mL) in PBS buffer and leave at room temperature for 15 minutes to dry before use.

For the detection process, an LFD was placed in a 1.5-mL Eppendorf tube with 200uL of sample solution inside for at least 15 min. The capillary effect of the sample pad would drive sample solution up along the filter membrane into the sucrose pad. After 15 minutes of reaction, the solution in the sucrose pad was squeezed out and applied to a portable glucose meter to quantify the concentration of glucose.

DNA sequences used in the method using LFDs (from left to right: 5' to 3'):

Coc-Apt:

TTTTTTACTCATCTGTGAATCTCGGGAGACAAGGATAAATCCTTCAATGAAGTGGGTC
TCCC

Biotin-DNA (biotin-modified):

TCACAGATGAGTAAAAAAAAAAAAA-biotin

DNA-Inv (Thiol-modified):

HS-AAAAAAAAAAAAAGTCTCCCGAGAT

In this design, since the inverted-conjugated DNA strand can catalyze sucrose into glucose in concentrations proportionate to that of the invertase-conjugated DNA strand¹⁷, the connection between the concentration of target molecule and concentration of glucose quantified by the portable glucose meter is from the concentration of released invertase-conjugated DNA strand. To verify this, a 6-carboxyfluorescein (FAM) labeled DNA strand with no invertase conjugated

was used as a probe to show the relationship between the concentration of released invertase-conjugated DNA strand and the concentration of target molecule. The test was undergone with PBS buffer (pH=7.3) and FAM fluorescence intensity at 518nm by excitation light of 490nm wavelength was measured. The reaction time was 30min. Results are shown below as in Figure 3.

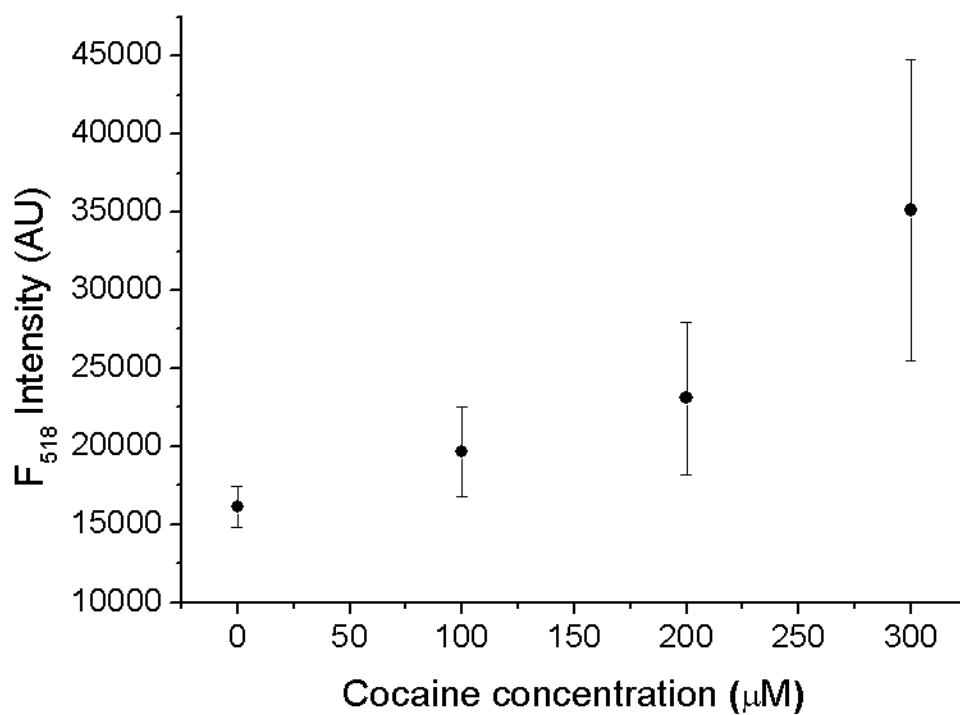


Figure 3 Releasing of FAM-labeled DNA strand after 30 min of reaction.

From the graph above, it is verified that the concentration of released invertase-conjugated DNA strand is positively related to the concentration of cocaine. Therefore, in the next step, several LFDs were assembled and spotted with magnetic beads functionalized with the aptamer DNA and invertase-conjugated strands. The LFDs were then immersed in different concentrations of cocaine in PBS buffer, and a portable glucose meter was used to quantify the glucose concentrations in the reaction pads of the LFDs. The reaction time was 30min. Results are shown below in Figure 4.

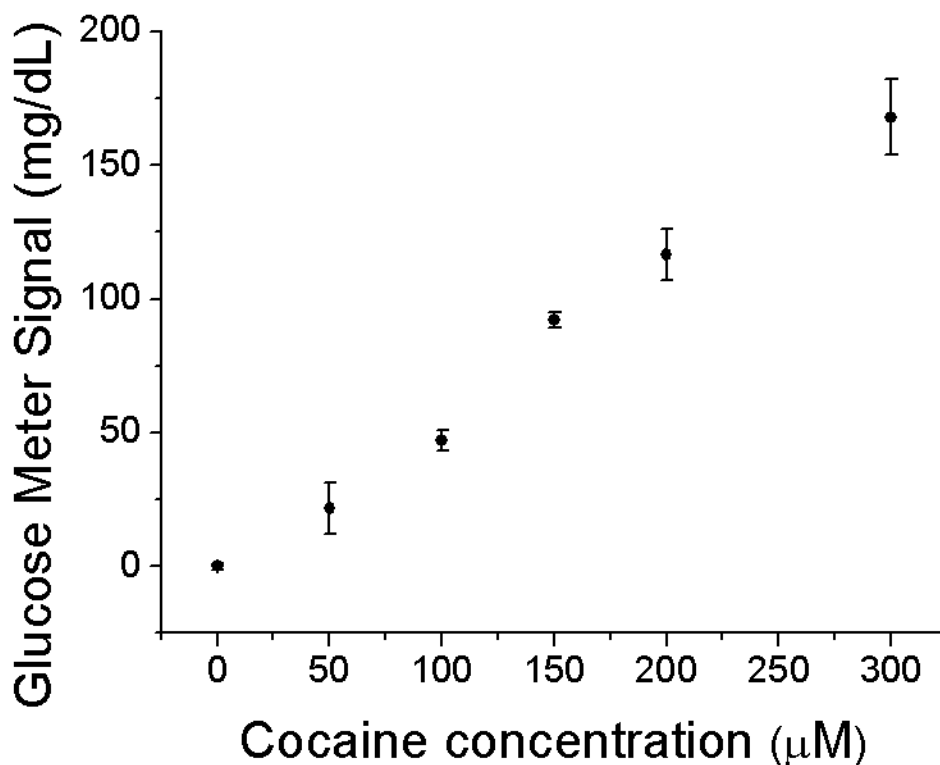


Figure 4 Glucose meter readings for different cocaine concentrations. Reaction time is 30min.

From the graph above, it is shown that the LFDs can quantify different concentrations of cocaine, because the readings of the portable glucose meter increases as the concentrations of cocaine increases. The detection limit was $7.67\mu\text{M}$ (3σ). As the cost of assembling such LFDs are very low (calculated to be at around \$3 per test), and the required time of reaction is short (30min), the required volume of sample solution is low ($200\mu\text{L}$), this is a cost-effective and on-site method of quantification of cocaine.

2. Streptavidin detection

In the design to quantify STV, same LFDs are assembled and MBs coated with STV as well as biotinylated invertase are deposited on separate regions of the reagent pad. When quantifying STV, the LFDs are dipped into target solutions. As the solution travels up along the LFDs, STV in the solutions will bind a portion of the biotinylated invertase. The STV-bound invertase travels further along the LFDs into the reaction pad, where it catalyzes sucrose into glucose in amounts that are proportionate with the concentrations of cocaine in the solutions. The rest of the biotinylated invertase which are not bound to STV will be immobilized on the STV-coated MBs, preventing them from traveling further into the reaction pad. A portable glucose meter is used to quantify the concentrations of glucose in the reaction pad, which can be finally transformed into the concentrations of cocaine in solutions. The scheme of the design is shown in Figure 5.

All DNA was obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). ACCU-CHEK™ Avia portable glucose meter was from Roche (Indianapolis, IN). Invertase and GOx were purchased from Sigma-Aldrich Inc. STV-coated magnetic beads were from Bangs Laboratories Inc. STV and other chemicals for buffers, solvents and conjugation were purchased from Sigma-Aldrich, Inc. The constitution of the PBS buffer used in these methods was: 100mM Na-P saline, 100mM NaCl, 0.05% Tween-20, pH = 7.3.

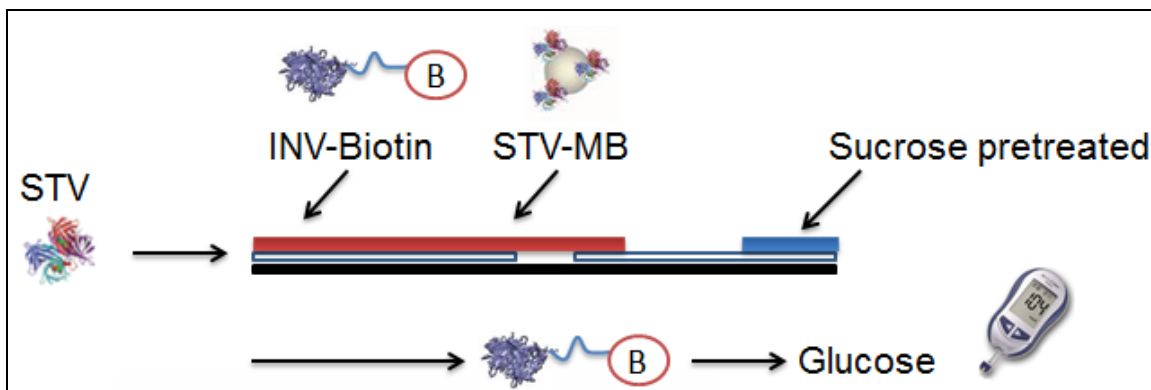


Figure 5 The scheme of the design for the STV-detection method using lateral flow devices.

Since biotin is known to be able to bind to streptavidin with a very low dissociation constant²³

, we tested the quantification of this kind of lateral flow devices with different concentrations of STV. The LFDs are assembled and spotted according to the aforementioned methods. All the tests were conducted in PBS buffer (pH = 7.0) and reaction time was 20min. Results were shown in Figure 6 below.

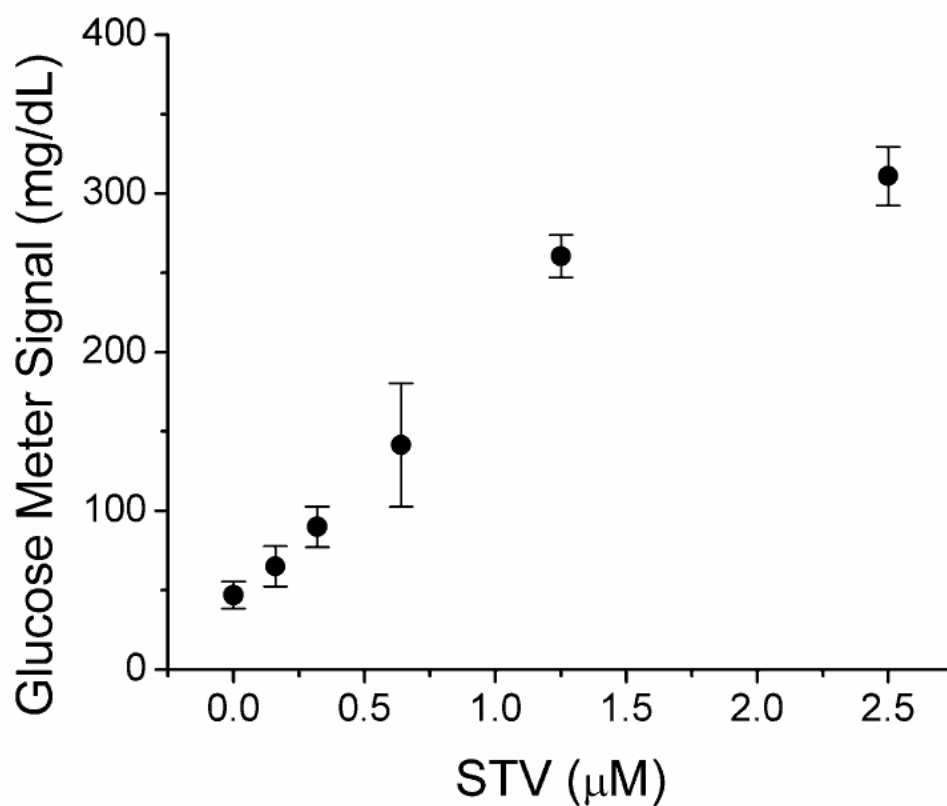


Figure 6 Results of STV quantification using the method of lateral flow devices. The reaction time was 20min. Tests were conducted in PBS buffer (pH = 7.0).

From the graph above, it is shown that the LFDs can quantify different concentrations of STV, because the readings of the portable glucose meter increases as the concentrations of STV increases. As the cost of assembling such LFDs are very low (calculated to be at around \$2.5 per

test), and the required time of reaction is short (20min), the required volume of sample solution is low (200 μ L), this is a cost-effective and on-site method of quantification of STV.

CHAPTER 3: THE METHOD USING PORTABLE PH METERS

Glucose oxidase (GOx) is an enzyme that can catalyze the oxidation of glucose, whose final product in water is gluconic acid. The produced gluconic acid will lower the pH of the solution to a certain extent, which can be detected with a portable pH meter. If the concentration of glucose oxidase is positively related with the concentration of target molecules, then the pH change will also be positively related with the concentration of target molecules. Based on this, and the aforementioned method¹⁷, a method of quantification of cocaine was designed to use aptamer-functionalized magnetic beads and portable pH meters to achieve the detection. The scheme of the detection process is shown as below in Figure 7. The assembly of aptamer-conjugated magnetic beads was mentioned before¹⁷. The same DNA sequences (Biotin-DNA, Coc-Apt, DNA-Inv) were used as in the method using lateral flow devices. All DNA was obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). ACCU-CHEK™ Avia portable glucose meter was from Roche (Indianapolis, IN). Invertase and GOx were purchased from Sigma-Aldrich Inc. The methods for preparing the Invertase/GOx-DNA conjugates and functionalizing MBs were adapted from a paper by Yu Xiang¹⁷.

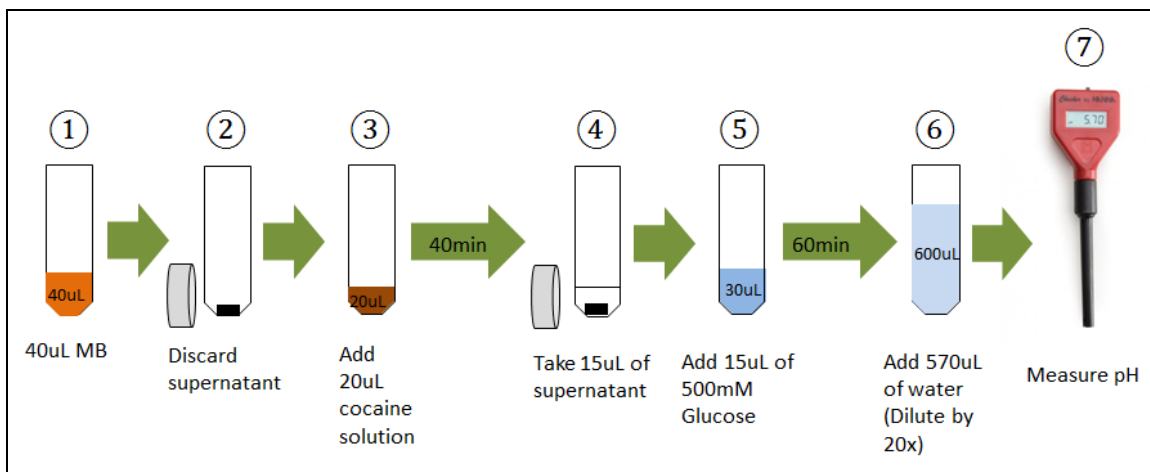


Figure 7 The detection process of the method to quantify cocaine using portable pH meters.

A FAM-labeled releasing strand was used as the probe molecule to test if its releasing by binding of cocaine to the aptamer is positively related to the concentration of cocaine solutions. The test was carried out in PBS buffer ($\text{pH} = 7.3$), with a reaction time of 30min. The results are shown below in Figure 8.

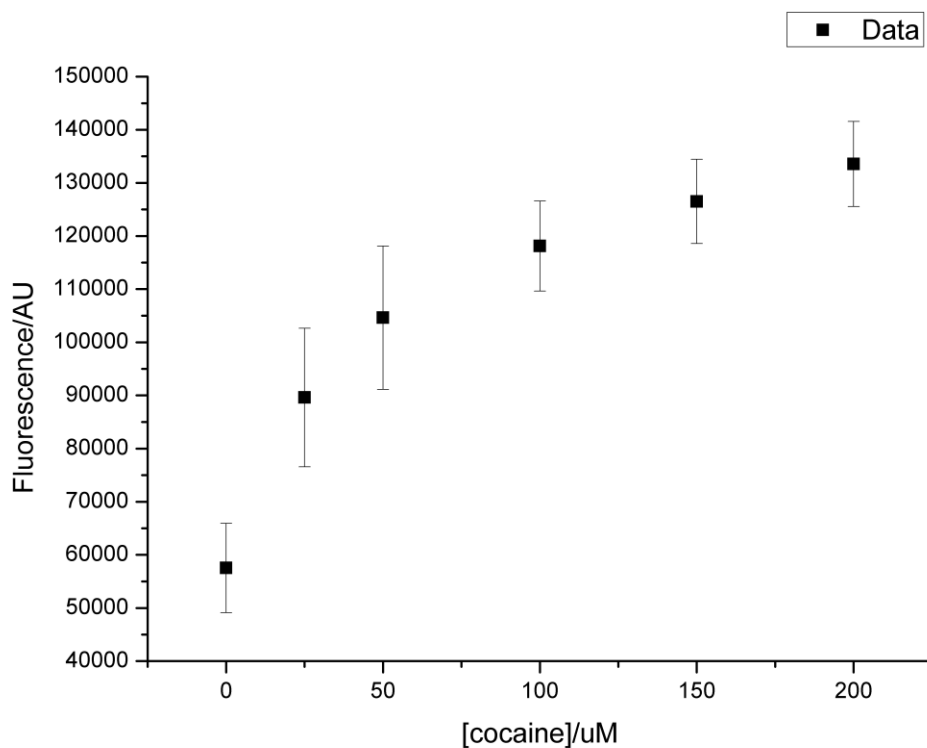


Figure 8 The results of FAM-labeled strand releasing by binding of cocaine to the aptamer.

From this graph, it is shown that the releasing of labeled strand is positively related to the concentrations of cocaine. Therefore, we proceed to the next step of using GOx-conjugated DNA strand as the releasing strand to see if different concentrations of cocaine can cause different pH changes in solutions. The reactions were carried out in PBS buffer, except the buffer solution used in the reaction of GOx catalyzing glucose being 10mM HEPES buffer (pH was measured

before and after the reaction of glucose oxidation). The resulting pH changes were plotted against the concentrations of cocaine solutions as in Figure 9 below.

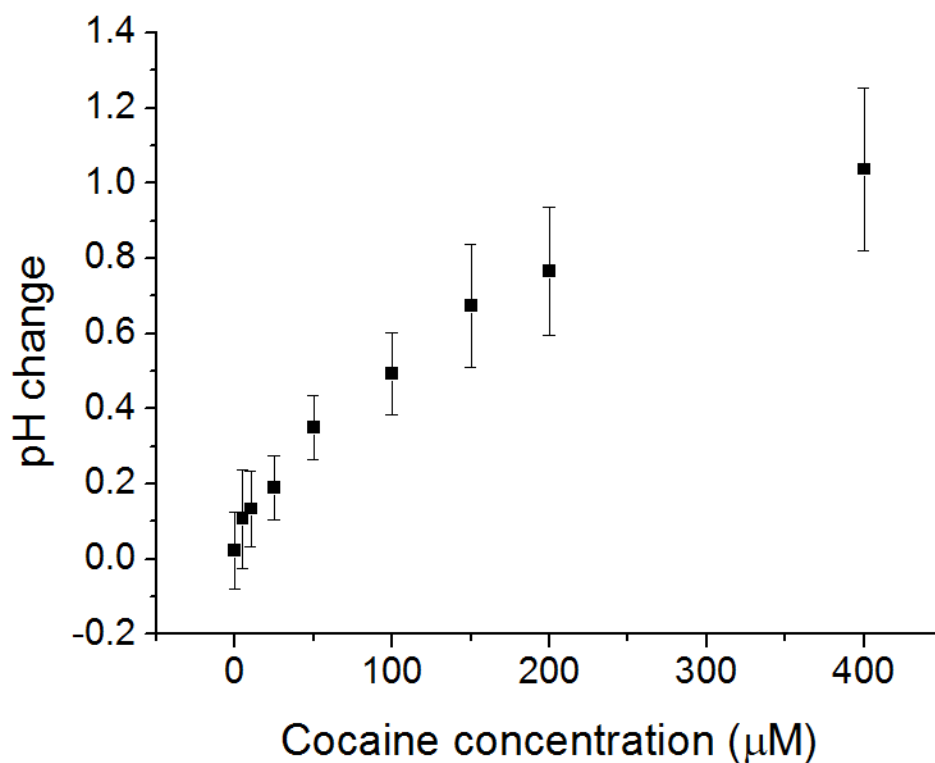


Figure 9 Results of cocaine detection using the method of portable pH meters.

From the graph above, it is shown that the method can quantify different concentrations of cocaine, because the pH changes of the sample solutions measured by the portable pH meter increase as the concentrations of cocaine increases. As the cost of this test is cheap (calculated to

be at around \$2.5 per test, and price for the portable pH meter is at around \$50, plus that the pH meter is reusable), and the instrument required (the portable pH meter) is easy to use, this method is a cost-effective method for quantification of cocaine and is good for on-site use.

CHAPTER 4: CONCLUSION AND DISCUSSIONS

In summary, this study demonstrates two cost-effective and on-site methods for quantification of multiple targets using functional DNA and portable instruments, achieving limits of detection of nM level. The target specific binding to the functional DNA triggers the releasing of enzyme-conjugated DNA strands, leading to changes in glucose concentration or pH of the solutions that can be detected and quantified by portable instruments. By more precise instruments, more advanced engineering and further optimization these methods are able to achieve even lower limits of detection. Besides, the development of aptamers for more target molecules will eventually widen the range of target molecules for these methods, making them reach out to point-of-care markets and wider use in fields such as environmental monitoring.

For the method using LFDs and PGMs, it is important to keep the LFDs wet during the detection process. Our method is to cover the LFDs with plastic tubes during the detection process. Another gist is that when squeezing the catalyzed glucose solution out of the reaction pad, we used a glass clamp assembled by our group and avoided un-even distribution of the solution. Furthermore, it is critical that the more invertase-containing solution enters the reaction pad the more sucrose is catalyzed; therefore, a pattern that will let the cocaine interact with the MBs most and let the most invertase-containing solution will be high in efficiency. Due to restrictions in manufacturing

techniques we have limited our pattern to be like mentioned above, and if more advanced manufacturing techniques permit the efficiency and overall limit of detection will be even higher.

For the method using portable pH meters, the choosing of a proper enzyme-substrate combination and a proper buffer solution is important. For the enzyme, we tested calf alkaline-phosphatase and *p*-Nitrophenylphosphate at first, but found out that this combination does not have a efficiency high enough to lower the pH to an extent detectable by portable pH meters within an acceptable time; besides, this combination also does not work as stably as glucose because the substrate, *p*-Nitrophenylphosphate, will hydrolyze in water solutions. For the buffer, it is significant to choose a buffer that has a capacity both high enough to retain the activity of the enzyme and low enough to make the pH change large enough to be detected by portable pH meters. The PBS buffer cannot meet the criteria, and after several tests we chose a dilute (10mM) HEPES buffer to use. Dilute HEPES solution is not a very strong buffer system and is good for biological macromolecules as well, which renders it ideal as a candidate.

Finally, for both methods, the choice of aptamers limits the range of target molecules. The aptamer must be a structure switching one in terms of target-binding process so that it may release the enzyme-conjugated strand when reacting with targets. Such an aptamer is not very common. Commercial aptamers are either not studied enough to determine if they are structure-switching or not, or not structure-switching at all. For those aptamers that are known to undergo

structure-switching, only part of them are known about the binding position or binding pockets for target molecules. Releasing strands must be designed not to hybridize to these binding pockets in order not to impede the binding of targets. Cocaine aptamer is one that had been studied extensively and sufficiently that has been known for structure-switching when binding to targets. Its releasing strand also has been tested according to the paper by Yu Xiaing¹⁷ so that it does not affect the target binding process. Therefore it is our choice of aptamer.

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