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Paper-based Biosensor for Colorimetric Detection of PSA Biomarker

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HONS 497
Honors Thesis

Paper-based Biosensor for Colorimetric Detection of PSA Biomarker

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Department: _____________________________
ABSTRACT

Prostate cancer is commonly screened in the bloodstream by detection of excess PSA, prostate-specific antigen which is a protein the prostate produces in large quantities when cancerous tumors are present. In order to facilitate inexpensive and disposable detection, diagnostic tools have begun to be created out of paper and wax which wick solutions through channels based on their hydrophobicity. George M. Whitesides, who pioneered the majority of this work, designed a paper-based LFD, lateral flow device, effective for detecting liver function which we are modifying to detect PSA. In this paper, we designed an oPAD, origami paper analytical device, specific to PSA using GNPs, gold nanoparticles, which provide a colorimetric, qualitative, inexpensive, efficient, and disposable design to detect prostate cancer through the bloodstream. Our device separates the sample laterally to prevent back-mixing and vertically to enable addition of novel particles. The design was optimized for channel thickness, size, proper heating, type of paper involved, and amount of sample solution needed. We are currently concept-testing of PSA qualitative detection via sandwich assay to target detection levels < 100ng/mL, but we hope to design a device that can be easily modified to detect many other biomarkers for disease.
INTRODUCTION

*PSA, a cancer marker*

PSA, or prostate-specific antigen, is a marker that was identified in 1971 and is today used as a marker for prostate cancer.¹ This cancer affects many people in the United States and across the world and is a fairly dominant cancer among the male population. Because of its frequency, regular testing is recommended once a man hits the age of about 40.² The testing currently involves visiting a physician and having either a manual exam which is notorious for its uncomfortable and awkward experience or a chemical test which involves the analysis of the level of PSA present. As a normal functioning human being, PSA is already present in the male body and is not a negative nor bad thing to have. It exists in levels of about 4ng/mL and aids in healthy prostate maintenance³.

PSA only becomes a marker of a serious disease like cancer once it elevates because of a hyperactive prostate. As cancer mainly works through the methods of uncontrolled and rapid growth, there are many antigens, proteins, and other markers that will become elevated in the body once cancer spreads to that region. But PSA is most commonly used to detect prostate cancer because of its dramatically elevated levels in cancer-ridden prostates, and its specificity to the prostate alone. Any other molecules, proteins, or antigens may also be produced by other regions of the body and thus their resulting overproduction could be a sign of another hyperactive organ or cancer present elsewhere in the body. Any amount above 4ng/mL is considered to be an indicator that prostate cancer may be present. This is not to alarm anyone with PSA levels of above 4ng/mL, as the human body normally fluctuates and PSA changes can be due to several other factors including poor overall health, a naturally low or high level, or

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¹ Heyns, 2014.
² Heyns, 2014.
³ Heyns, 2014.
perhaps just the daily fluctuations of the body in accordance with temperature, activity, and nutrition.

In many recent publications, it has been found that the testing of PSA is not as useful as perhaps originally thought. Because of the body’s normal fluctuation and the fact that PSA already exists at very low concentrations, 4ng is a microscopic amount and cannot be seen by the naked eye, the recorded changes in PSA often set off false positives, alarming healthy people and causing undue stress without the presence of cancer. It is estimated that about half of the positives given from PSA level testing, are false alarms and due to noncancerous developments in health. While a positive thing for the family whose loved one is now no longer thought to have cancer, the undue stress provided can be avoided by manual testing and a plethora of other chemical diagnostics which are now preferred by many physicians as a way of avoiding a false positive. It is important to note that false negatives, while more rare, are much more harmful to the individual and have occurred using this type of screening. This is likely to occur when an individual has a naturally low PSA level and has not been tested prior to acquiring prostate cancer, which then causes their PSA levels to rise to a “normal” level of around 4ng/mL, which can deceive the clinicians and physicians using this method. It is recommended to take care of your health and have regular checkups, regardless of your skepticism of this process because the chemical balance of the body is a very telltale sign of disease and even without cancer, a sick prostate is something to be attended to and taken care of as soon as possible.

In this paper, we use PSA as an indicator for prostate cancer not because we hope to detect prostate cancer, but because the focus of this research project has been the design and development of a device applicable to many diseases and ailments. With the use of PSA, which has a very easily identifiable antigen, we are able to concept test our design and apply to a field

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4 Heyns, 2014.
that is still applicable in the lives of many men today. For those who have been diagnosed with prostate cancer already, taking long trips into the hospital becomes somewhat of a regular thing and developing a product like ours could potentially help to reduce the amount of visits required, and thus give more freedom to the individuals already affected by this disease. With perfection of our device, its application to other diseases is endless and we hope to expand testing to dengue fever, malaria, and just about any disease that has antibodies. Because of Dr. Kwon’s previous work with prostate cancer and knowledge of PSA, we began work on this cancer-indicating molecule and have found much of our success through its simplicity and straightforward binding properties. We hope that the current application towards prostate cancer and PSA will not overshadow our work in the development of our GNP-detecting colorimetric oPAD, origami paper analytical device, lateral and vertical flow device\(^5\). As this field expands, many researchers are racing towards new applications and innovative designs. Our chimeric design and potential for endless applications will make this device revolutionary.

\textit{Paper Biosensors}

In designing a device to detect this wide variety of applications, we knew we had to start with something simple. We began working with fluids, microfluids in particular because of the generally microscopic amounts of disease-related antigens circulating in the body and our desire to use as little human fluid as possible. In our research, we found that microfluidic analytical devices were recently found to be composed of paper, an inexpensive, stable, and widely available foundation upon which to conduct experiments\(^6\). These devices were called microfluidic paper based analytical devices and an entire field of study was opening up to focus

\[^5\text{Scida, 2013.}\]
\[^6\text{Scida, 2013.}\]
on development of these devices for various purposes. Microfluidic paper based analytical devices can be designed in a variety of ways so long as there are clear boundaries between the separate compartments. Channels need to be created for the fluids to flow through and they need to have relatively impenetrable boundaries, at least impenetrable to the fluids.

George M. Whitesides is the main powerhouse behind the creation of paper-based biosensors which have since then grown in a variety of different directions. Biosensors can be flat, folded, qualitative, quantitative, employ colorimetric or chemical detection, use horizontal or lateral flow, and many other various approaches. Our biosensor combines many of these aspects into one simple device: a folded qualitative, colorimetric, horizontal and lateral flow, and potentially chemically quantitative analytical tool. In our device, gold nanoparticles serve the purpose of colorimetric analysis via a red hue which can be traced throughout the device.

*Wax Printing*

Photolithography used to be the most common method used to create microfluidic paper based analytical devices and required quite a long list of steps and procedures. Beginning with impregnation with photo-resistant material, the process was already expensive, delicate, and not easily attainable. The device needed pre-baking before alignment under a mask which would be a negative of the intended channels and require more planning and production of the negative before work could begin. The entire set-up was then put under exposure to UV light, which would reflect onto the photo-resistant material through the openings in the mask to create the intended design. This device was then put through another process of post-baking and finally, the resulting developing of the analytical device produced a paper that has a photo-resistant barrier.

Continuing research revealed a simpler and less expensive solution. Whitesides, a revolutionist and father of the paper-based biosensors, published a paper entitled “Measuring

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7 Vella, 2012.
Markers of Liver Function Using a Micropatterned Paper Device Designed for Blood from a Fingerstick."\textsuperscript{8} This key paper outlines an innovative idea Whitesides came up with while working with microfluidics and the miniscule channeling involved therein. His idea was to create channels as simply as possible and as inexpensive as possible. The most inexpensive thing available to him at the time was paper and he began brainstorming ways to use this paper in a fluidic channel design. Utilizing paper’s hydrophilic qualities, he theorized that wax’s hydrophobic qualities could contrastingly create a channel through which fluids would flow on the paper or hydrophilic areas and would be blocked off by the wax’s hydrophobic properties. The procedure for developing a hydrophilic-hydrophobic wax-paper based channel is efficient and rather than involving a long and complicated procedure, simply requires printing of wax upon paper and the heating of that wax to melt through the paper and create a boundary.

In our research, we have decided to use the wax printing method of creating a barrier and have found that wax prints much like ink and binds to a variety of paper types. The reduced costs of materials helps factor into our grand-scheme mass production frame of mind, and help us to conduct numerous research experiments to fine tune our procedure without the restraints of costly materials. Because wax melts horizontally as well as vertically, we have to factor that part into our designs and print channels that will be larger than the final desired product. This, along with the consideration of having a prepped hot oven, is really the only concerns that need to be addressed before a device like this is prepared and ready to receive its reagents.

\textit{Our Project}

After researching the various approaches to paper-based biosensors, we came to the conclusion that there exist micropatterned papers that uses blood filters to examine liver

\textsuperscript{8} Vella, 2012.
function, a basic design which we could adapt to our device. There are also various methods of preparing a wax and paper channel, as well as origami techniques that can make efficient use of space. Chirathaworn showed us that colorimetric detection via use of gold nanoparticles was relatively easy to see and related well to disease detection when coupled with specific antigens, as well as introducing us to the use of nitrocellulose in immunochromatographic test strips. Photolithography was contrasted with wax printing and other various methods of channel creation by Whitesides. Lateral flow design was show by several people, including in the simultaneous detection of nucleic acid and protein using gold nanoparticles and lateral flow device paper by Mao. We found that the detection level of PSA in the body can be linked to prostate cancer when it is around 60ng/mL. Finally, we learned that electrochemical analysis is also possible as a future direction that this experiment can take. In this paper, we combined aspects of the many current ideas on the marked and came up with a device that incorporates the best aspects of each of them. We designed an oPAD, origami paper analytical device, specific to PSA using GNPs, gold nanoparticles, which provide a colorimetric, qualitative, inexpensive, efficient, and disposable design to detect prostate cancer through the bloodstream. Our device separates the sample laterally to prevent back-mixing and vertically to enable addition of novel particles.

METHODOLOGY

Design

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10 Scida, 2013.
11 Chirathaworn, 2014.
12 Martinez, 2010.
13 Mao, 2014.
14 Heyns, 2014
15 Shao, 2008.
Our design was based on the necessity of separating solutions for detection using similar methods to Xun Mao, but in a vertical format with only PSA detection. This six-region device separates the sample laterally to prevent back-mixing and vertically to enable addition of novel particles. The design was tested for necessary channel thickness, size, proper method and endurance of heating to melt the wax, type of paper involved, and amount of sample solution needed to seep through all necessary layers. A simplistic design was concept-tested with acid and base using pH indicators and necessary sample volume adjusted appropriately. Current studies include the concept-testing of PSA qualitative detection via sandwich assay to target detection levels of less than 100ng/mL.

*Print*

After completing the design and specifying its exact diameters and proportions, taking into account the expansions it will undergo upon heating, the design is sent to a computer connected to the wax printer. This design is printed with a wax compatible printer using wax ink which is laid down on the surface of the intended paper. In our case, we are using chromatography paper and nitrocellulose paper. If the paper size is smaller than normal, the paper is attached to a regular legal-sized paper and then printed together. The design to be printed is generated by Canvas 11 and printed in black ink. Multiple devices are printed at the same time as they are small and many can fit onto one page comfortably.

*Heat*

The next step is to cut out the designed device around the outside, rectangular perimeter. Once all the devices are cut, the number of devices to be used immediately are selected and placed in a preheated oven. These are then heated for two minutes at 130°F in the oven. After two minutes, the devices are examined to see if the wax has thoroughly melted through the paper. Both sides of the device should be a dark grey, a little lighter than the black originally printed on them. The top side will be a little darker than the bottom but otherwise
indistinguishable and evenly blackened on both sides. While in the oven, the wax spread not only vertically but horizontally as well and the wells are both thicker in outer diameter and smaller in lumen, as well as the channels that connected them. It is not uncommon to see a few dark specks of wax on the paper after removing it from the oven. This is normal and will not affect the experiment.

*Apply GNP and Antibody*

Once the device itself is prepared, the reagents have to be added. In chromatography paper, these reagents will not bind to the paper itself, but rather rest atop the paper as it dries and flow through the paper with the sample once the sample has been added. 10uL of GNP bound Antibody was applied to each of the four small wells in the 3rd partition from left to right and 10uL of Antibody 10-1395 was applied each of the four wells in the 5th partition from left to right. After these amounts dried, 2.4ug and 1mg of each antibody remained, respectively.

*Test Sample*

The device is then folded accordion style along the dotted lines. This was then turned so that the 1st partition left to right was exposed, the first section or sample section. 20uL of sample was then applied and once it finished wicking through the device, it was unfolded to reveal the results. Generally, a concentration of 2.5ug/mL was used, except for testing of the limitations of detection or sensitivity of the device.

RESULTS

*Designing the Device*
Following the guidelines of preparation found in Whitesides’ “Measuring Markers…”, we designed a device made of chromatography paper that had multiple folds and channels for fluids to disperse from. The main idea was to use a large sample well as the first layer, four secondary and smaller wells from which the sample would disperse out, first down and then laterally. This would incorporate a horizontal, lateral flow pattern, with origami folding, and allow fluids to disperse from a point of origin.

![Initial design diagram](image)

Fig. 1 The initial design wherein the black represented the printed wax areas, the white was the unprinted paper areas, and the device was folded in half, with the single circle facing upward.

This basic design was modified using different shapes and sizes, a dotted line to instruct exactly where to fold was added, and colors were utilized to find an aesthetically appealing design. This research proposition was abandoned, however, and a focus on the mechanical design was re-emphasized so the shapes remained circular, and the color remained black from this point on, but the dotted line remained and served as a key aspect of all following designs.
Fig. 2 The colorful, shapely, and explicitly dotted trials to determine aesthetic appeal of our biosensor device.

For much of the time spent on this project, various designs were researched and were designed by hand using a Canvas 11 program. There are many devices already designed, and by redrawing them myself, I gained experience with the program and a general feel for how the channels had to be formed, as well as the flexibility in the design. The following are small images of the designs I came up with and the modifications done to each. Though some of the more complicated designs are more aesthetically pleasing, we found that the printer could not process any shapes beyond the complexity of a circle or square at especially on a miniscule scale, the images were converted into blocked boxes. Each design had a purpose and functionality beyond aesthetics, for example: the snowflake design with the blue, yellow, and red coloring was a pH indicator test that effectively separated channels via length, tested for controls, and kept the compartments separate. The 3 blocked channels were controls for a known basicity. The 3 open channels received NaOH and tested its basicity at a certain concentration. The thin outer channels leading to outer circles tested for how far the liquid could wick through the hydrophilic paper.
Fig 3. The various designs developed from research and personal innovativeness involving wax printing on chromatography paper.

These designs eventually all failed because either they were too complex or did not fit the needs as simply as possible. We needed a device that was simple, had separate areas for sample addition, antibody 1 binding, antibody 2 binding, no room for backwash or cross-contamination, and a final absorbent pad to help wick the liquids through the device faster. As a side note, the majority of these designs was developed prior to the acquisition of the proper testing models and thus was more of an outflow of imagination than a practical development to fit the needs at hand. The bottom left corner holds the 4 most practical of the designs and shows their development from the original design, as they acquire more areas of separation, absorbent pads, and the channel thickness is tested for optimization.

Device Prototype 1

The first major device used in testing was a six-sectioned device. The first or top section held a singular circular opening for the application of the sample, the right side folded down into the second section which had an identical circular well that opened out into four separate wells to disperse the sample before Ab addition. The third section holds four small wells identical to those on the second section but without the channels or center well, it is here that the gold nanoparticles (GNPs) which are bound to PSA antibodies are initially added until evaporation and attach to PSA upon operation of the device. The fourth section features the four small wells again but also a channel from each, leading outwards to a secondary small well allowing for
further dispersion in order to discourage backwashing and cross contamination. The fifth section contains only the furthest four wells and is where the second antibody to PSA is added. Upon binding with the previous complex of PSA-GNP-Ab, the collective molecule becomes too large to pass through the chromatography paper and thus this is the test section. The sixth layer serves only as an absorbent pad to wick out all remaining liquids and, if PSA is not present, to hold the unbound Abs and sample. Preliminary results indicated that ideal measurements are as follows: necessary channel thickness (>1pt), size (for 3cmx18cmx0.1mm, sample well d=.736cm, other wells d=.528cm, 8pt channels), proper method and endurance of heating to melt the wax (oven, 130°C -140°C, 2min.), type of paper involved (chromatography), and amount of sample solution needed to seep through all necessary layers (40µL).

![Diagram](image)

Fig. 4 a) Schematic diagram of a lateral and vertical flow device based on the antibody-functionalized gold nanoparticle probes for detection of PSA. b) This schematic is folded accordion style along the dotted lines prior to sample application. c) Unfolded schematic diagram of a lateral and vertical flow device based on the antibody-functionalized gold nanoparticle probes for detection of PSA showing the molecules as they are bound to the various different sections.

**Preparation of the Device**

Once the design is decided upon and drawn into Canvas 11, the file is sent to be printed from a wax compatible printer using wax ink and chromatography paper. This printed file is then cut out around the outer perimeter of the design and placed in a hot oven for two minutes at 130°C. A hot plate method of melting was also attempted but deemed more potentially dangerous and less evenly melted when compared with the oven method.
Hot Plate Testing at 120°
Effectiveness (% Penetrance)

<table>
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<th>Time (s)</th>
<th>Small, Pt 2</th>
<th>Small, Pt3</th>
<th>Small, Pt4</th>
<th>Large, Pt2</th>
<th>Large, Pt3</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
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<tr>
<td>30</td>
<td>0</td>
<td>0</td>
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<td>100</td>
<td>100</td>
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<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 5 Tabulated and graphed data from hot plate and oven testing, at 120°F and 130°F - 140°F respectively, of variously sized devices with differing channel thicknesses to measure the thoroughness of the wax melting through, recorded in % penetrance as observed from the back of the device post-heating.

The use of an oven makes this preparation fast and easily repeatable. This is an inexpensive design procedure as the wax and chromatography paper are both fairly inexpensive and the printer used to administer the wax is comparable to the price of ink printers. These procedures are very simple to follow and do not require expensive nor fragile equipment thus they can be transported to third world countries or easily mass-produced.

**Concept Trials: NaOH**

In order to test our concept inexpensively, we proposed the testing of the channels, the materials, and the folding technique on a real experiment consisting of measuring pH with
various pH markers. The pH of a solution measures its acidity or basicity, where a low pH is acidic and a high pH is basic. The traditional scale is from 1-14 and measured in increments of powers of 10 (1= 10, 3= 1000). In this experiment, we would measure NaOH which is a strong base, in methylene blue, phenolphthalein, eriochrome black T (in the later experiments), and water as a control. The necessary sample volume adjusted appropriately to 40µL.

<table>
<thead>
<tr>
<th>NaOH – Small Design</th>
<th>soln</th>
<th>5N (13.76pH)</th>
<th>2.5N (13.47pH)</th>
<th>Water (pH 11.68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Phenolphthalein</td>
<td>-1</td>
<td>-1</td>
<td>-.5</td>
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<table>
<thead>
<tr>
<th>NaOH - Large Design</th>
<th>soln</th>
<th>5N (13.76pH)</th>
<th>2.5N (13.47pH)</th>
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<td>Blue</td>
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<tr>
<td>Phenolphthalein</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
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</table>

Fig. 6 Tables and graphs depicting the data received from testing NaOH pH indicators according to effectiveness rated -1 for a color disappearing, 0 for no change, and 1 for a color increasing in intensity. Control indicates water, Blue indicates methylene blue, and Phenolphthalein indicates phenolphthalein. Eriochrome black T

*Concept Trials: PSA*

Below are the PSA (.1ug/mL) testing setup and results with 5uL of 20OD GNP and .1mg/ml Antibody 10-1395 added. These figures show that our design is effective in wicking the sample through the various hydrophilic wells on both a lateral and horizontal plane.
Nitrocellulose Redesign

Nitrocellulose has an interesting property of being able to bind to antibodies which are dried on its surface while it does not bind to antibodies present in liquids on its surface (unless the liquid is left there for a long time and dries). Because of this ability, we redesigned the device to incorporate nitrocellulose into the testing section. Nitrocellulose is also a much finer filter paper than chromatography paper and thus the results received would be filtered much stronger and hopefully the color would be more apparent than in a chromatography experiment. Nitrocellulose is a 0.22um GS filter and in this experiment we used PSA (2.5ug/mL), GNP bound Antibody (20OD), and Antibody 10-1395(.1mg/mL).

DISCUSSION

Conclusions

This design is effective with pH and PSA testing and the methods of preparation are inexpensive and provide an efficient, disposable device. It is small, easily transportable, conducts the entire experiment in under a minute, and has great potential to be very inexpensive if mass produced. The most exciting part of this endeavor is that it works. The antibodies bind accordingly with the antigens and thus the very foundation of our premise was proven to be
correct. There are great applications waiting to be enacted upon and many exciting new fields to be explored with this device. Malaria, dengue fever, and almost any other disease can be detected with this simple system. The PSA testing that we are currently conducting measures a lower limit of 80ng/mL but we need to reduce it at least to 60ng/mL, the diagnosable amount according to Heyns.\(^\text{16}\) Regardless of the direct applicability of the PSA, the device itself works and we have to only test it in multiple diverse arenas to prove its worth.

**In the Field**

With the incorporation of the methods and techniques used by others in the field, we have produced a superior device that can do many things we were unable to do in the past. It has combined paper-based diagnostic tools with wax printing, gold nanoparticle detection, antibody applications to disease, origami, lateral, and vertical flow designs, and even incorporated PSA as a biomarker for prostate cancer. The field is rapidly expanding and yet with such sudden spurts of growth, few others have taken the time to thoroughly research others in the field and incorporate as many diverse aspects into their projects as we have been able to do in our own project. Our focus was on building a device that is superior to others in its class, and we have succeeded.

**Future Work**

In the future, we hope to continue this research project and work on developing a quantitative, sensitive analysis of PSA amounts via colorimetric analysis. From there, we can incorporate multiple markers and add a blood or urine filter for direct application of human bodily fluid as a sample. Further down the road, this project may be incorporated into a phone-based application so that analysis can occur without a lab technician present, without a visit to the hospital, and hopefully without a large cost to the individual themselves. The final frontier to

\(^{16}\) Heyns, 2014.
conquer is the expansion of this technology into other disease markers and antibodies. Once accomplished, this small inexpensive device will be an incredible diagnostic tool and useful to everyone from the youngest child to the oldest adult. Vella cites a study that records the risk of death due to drug toxicity in medicine as similar to the risk of death from the disease it was trying to prevent. Diagnosis is an essential part of our lives and having access to inexpensive yet effective detection devices might make the difference between life and death.

Questions to Ask

With the new development of inexpensive do-it-yourself diagnostic tools, perhaps developers and researchers should also consider what these kits might take away in terms of the patient’s interaction with physicians. Will less people go to the doctors, thus allowing other illnesses to go unchecked for even longer, perhaps causing more destruction than the diagnostic tools intended to prevent? Or perhaps, more people will take these tests and attempt to treat themselves based on online cures or remedies. When dealing with disease, there are a plethora of various responses that humanity can have to knowing that they are sick and not all of them are positive. Perhaps a faulty batch of diagnostics will cause mass hysteria, panic, or even long lasting decisions like euthanasia. Of course warning labels will be printed, but knowing humanity’s sinful nature, perhaps the only real way to prevent harm from being done by these tools is to make them limited or severely regulated. These regulations would undermine the purpose of such inexpensive diagnostic tools in the first place- making easy and early diagnosis available without the cost or time commitment of going in to see a doctor.

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17 Vella, 2012
LITERATURE CITED


