Design and implementation of an image based portable ELISA analyzer using EIPA and 4PLR

by

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Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

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Abstract

This thesis presents an implementation of predictive analytics on ELISA Imaging Systems in the absence of the standard laboratory equipment for field diagnostics. To that aim I developed a custom built optical setup with image processing and machine learning techniques. Using the light absorbance and transmittance properties of chemical compounds involved in hormone assays, I was able to estimate the hormone levels across reproductive stages. This work would allow for the eventual development of compact and economical closed systems which can be used for diagnostic advisory purposes in remote areas. This line of applied research, is expected to yield data that can be used to monitor health related outcomes. To test this use I focus the development of this tool on the monitoring of women's ovarian function. Experimental results demonstrate that our proposed model predicts hormone levels comparable to currently used commercial and laboratory methods.

Keywords: Enzyme Linked Immunosorbent Assay (ELISA); Colorimetry; Spectrophotometry; Immunoenzyme Techniques; Image Processing; Computer-Assisted

Dedication

I would like to dedicate this thesis to my loving and supportive family, my supervisor Dr. Ash Parameswaran, and to all those for whom this research will benefit.

Acknowledgements

I would like to thank my senior supervisor Dr. Ash M. Parameswaran for establishing a vibrant lab atmosphere. Words fail me in describing how supportive, encouraging and motivating he has been throughout my research. I have gained a wealth of knowledge, experience and opportunities under his supervision. I would also like to thank Dr. Pablo A. Nepomnaschy and Dr. Katrina G. Salvante for granting me access to work in their lab and also for their keen interest and support in the research along with guidance and suggestions.

My deepest gratitude goes to my mother (Rama Devi Ogirala) and father (Sri Hari Sarma Ogirala) - no words can describe what you have done for me.

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List of Acronyms

4PL	4 Parameter Logistic Regression
5PL	5 Parameter Logistic Regression
EIPA	ELISA Image Processing Algorithm
ELISA	Enzyme-Linked Immunosorbent Assay
HT	Hough Transformation
NRMSE	Normalized Root Mean Square Error
RMSE	Root Mean Square Error
ROI	Region of Interest
S/N	Signal-to-Noise
SER	Society, Environment and Reproduction

Glossary

ELISA	Enzyme Linked Immunosorbent Assay: laboratory test that uses antibodies and changes in intensity of color or fluorescence to quantify the concentration of the target substance, e.g., hormones
PdG	pregnanediol glucuronide: a urinary metabolite of progesterone

Chapter 1. Introduction

1.1. Background – Field Analysis

Frequent collection and analysis of bio specimens in naturalistic (i.e., field) conditions is typical of longitudinal naturalistic study designs [1]. As traditional hormone assays are conducted in a laboratory setting, field-collected bio specimens need to be frozen and properly stored in the field and then transported frozen to laboratory facilities where specialized equipment is available. In nonurban field sites, freezer space is often scarce and transport of frozen specimens is logistically difficult and expensive. Additionally, individuals need to wait long periods of time to learn the results of their laboratory analyses. Therefore, methods that reduce the need for specimen storage and transport and accelerate the generation of results, are important to field-research dependent disciplines such as biology, ecology and epidemiology as well real time monitoring of health markers.

Available laboratory techniques, such as for example, enzyme-linked immunosorbent assays (ELISA) are designed for detecting and quantifying a variety of substances such as proteins, antibodies and hormones. Such techniques generally utilize microtiter plate readers to detect visual changes, which are generally a by product of chemical reactions in microtiter plates. The quantification of these chemical changes has a direct (or inverse) relation with the quantity of the substance in the bio specimen we intend to measure. The chemical reactions in our field of study corresponds to colorimetric changes for most part of this research, and deal with detection of changes in color of the reactions in microtiter plates. These studies can also be extended to fluorescence and chemiluminescence which works on light emissions instead of change in color due to a chemical reaction. Hence, it can be assumed that the accuracy of the system to detect the changes of the chemical reactions plays a significant role in hormone predictions. Most often than not, microplate readers are used as a standard laboratory equipment to detect these changes. The operation of the microtiter plates is described in the later parts of this chapter. The plate readers are expensive and require resources like line-electrical power supply to operate. These resources are not readily available in the field diagnostic situations. Thus, a portable and cost-effective method to visualize and quantify

colorimetric assays would represent a step forward towards making these techniques available to field scientists, rural health providers and the general public.

Using a smartphone or a webcam to accomplish the task of quantifying the colorimetric ELISA would allow for a portable as well as an economical solution which could be deployed in the field. Images of the microtiter plate could be taken using a smartphone or a webcam, and standard Digital Image Processing techniques could then be used to analyze the pictures. Digital Image Processing is a form of signal processing where the input is an image (a photograph or a video frame); the output can be either an image or a set of characteristics or parameters related to the image. Most image-processing techniques involve treating the image as a two-dimensional signal and applying standard signal-processing techniques to it [2]. These techniques can be combined with a wide range of algorithms to be applied to the captured image. By doing so, a considerable amount of information can be extracted and used for diagnostic purposes. These features offer a new paradigm in image processing applied to biochemical analysis on a portable platform. Image processing techniques can be applied using an application developed exclusively for the smartphone's operating system platform or in a PC/laptop computer application. A web based application could also be deployed for similar purposes.

Here we compare the ability of novel smartphone and webcam systems along with custom image processing algorithm and 4 parameter logistic regression techniques to accurately quantify women's reproductive hormone levels. The results are then compared against a standard laboratory microtiter plate reader.

1.2. Biomedical Techniques

1.2.1. ELISA

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique in biochemistry designed for detecting and quantifying a variety of substances such as proteins, antibodies and hormones. The basic overview of an ELISA is, an antigen must be immobilized to a solid surface and then convoluted with an antibody linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity with a substrate to produce a quantifiable product. A very crucial element in the detection

strategy is the extremely precise antibody-antigen interaction. Most commonly, ELISAs are performed in 96-well polystyrene plates, which will passively bind antibodies and proteins immobilizing reagents.

Direct ELISA:

In the presence of an antigen, direct ELISA refers to a method in which only a labelled primary antibody is used and indirect ELISA refers to a method in which the antigen is bound by the primary antibody which then is detected by a labeled secondary antibody. When the primary antibody is of interest, as in the case of immunization analyses, this antibody is directly detected by the secondary antibody and the term direct ELISA applies to a setting with two antibodies.

A buffered solution of the antigen is adhered to each of the 96 wells through charge interactions. A solution of non-reacting protein is added to wells which remains uncoated by the antigen. The primary antibody with a conjugated enzyme is added, which binds specifically to the antigen coating of the well. A substrate for this enzyme is then added which changes color upon reaction with the enzyme. The stronger the color change the higher the concentration of the primary antibody. A spectrometer is then used to give quantitative values for color strength.

Sandwich ELISA:

A known quantity of capture antibody is bound to a surface with any nonspecific binding sites blocked. The antigen-containing sample is then applied to the plate, and captured by antibody followed by a wash to remove any unbound antigen. A specific antibody is added which binds to antigen. Secondary antibodies are applied as detection antibodies that also bind specifically to the antibody's nonspecific region. The plate is washed to remove the unbound antibody-enzyme conjugates. A chemical is then added to be converted by the enzyme into a color or fluorescent or electrochemical signal. The absorbance or fluorescence or electrochemical signal is measured to determine the presence and quantity of antigen.

Competitive ELISA:

A third use of ELISA is through competitive binding. Unlabeled antibody is incubated in the presence of its antigen. These bound antibody/antigen complexes are then added to an antigen-coated well followed by a wash to remove any unbound

antibodies. A secondary antibody, specific to the primary antibody, is then added which is coupled to the enzyme. A substrate is added, and remaining enzymes produce a color or fluorescent or electrochemical signal. The reaction is stopped to prevent eventual saturation of the signal. Some of the competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody.

1.3. Standard Processes

There are a number of biochemical processes that are used in an assay for hormone analysis. One of those popular process that is of relevance in this research is described below:

1.3.1. Serial Dilution

The process of serial dilution involves preparing standard solutions and adding buffer of indicated volume to these samples. In the highest standard, add the indicated volume of the original standard solution. Then take the indicated volume of the mixture, and add it to the second tube. Then from the 2nd tube, transfer the indicated volume of the mixture to the 3rd tube, and so on. The goal of this procedure is to reduce a concentration to a more usable concentration of an analyte in a series of sequential dilutions.

The advantages in using serial dilution process can be understood in a wider perspective in chapter 4 while plotting the concentrations on the standard curve

1.4. Laboratory Apparatus

1.4.1. Microtiter Plate readers

In ELISA, generally a plate with 96 wells is used which are coated with antibodies. The microtiter plate is a flat tray with multiple wells that are used as small test tubes. The microtiter plates are commonly manufactured in a 2:3 rectangular mix with 96, 384, or 1536 wells. A 96 well plate is a standard tool in analytical research and clinical diagnostics [3]. After the enzyme action, absorbance is read using a microplate reader.

Microplate readers are available for fixed wavelength and variable wavelength systems. In most plate readers, the concentration of the substance assayed automatically calculated with the help of proprietary software.

1.5. Adopted Procedure for Hormone Analysis

The Quansys multiplex female hormone array is a valid alternative method to individual immunoassays for the quantification of stress, reproductive and energetic hormones and metabolites in human urine samples and can be used to examine the dynamic interactions between these hormones [28, 29]. A competitive ELISA was used to quantify urinary concentrations of pregnanediol glucuronide (PdG), a urinary metabolite of progesterone, a reproductive steroid hormone, in urine samples of unknown concentrations using the protocol described by O'Connor and colleagues [9]. Anti-PdG monoclonal antibodies, PdG-MAb, clone 330, Quidel Corporation, were diluted to 3.08 mg/L in 50 mmol/L coating buffer, pH 9.6. Diluted antibodies were dispensed 50 µL/well into Nunc Immunosorp 96-well microtiter plates and incubated overnight at 4˚C. Plates were washed three times using wash buffer containing 0.15 mol/L NaCl and 0.5 mL/L Tween 20. 50 µL blocking buffer consisting of 0.1 mol/L sodium phosphate solution containing 8.7 g/L NaCl and 1 g/L bovine serum albumin, pH 7.0, was added to each well. Following a one hour incubation at room temperature, 20 µL of serially diluted commercial PdG standard (Cat. No. P3635; Sigma), a negative control comprised of deionized distilled water, a positive control that consisted of a pool of urine samples collected from women in the luteal phase of the menstrual cycle diluted 1:5, and samples of unknown concentration diluted 1:5 were added to individual wells. Immediately following this step, 50 µL of horseradish peroxidase-linked PdG competitor (PdG-HRP) was diluted in blocking buffer and added to each well. All samples, controls and standards were run in duplicate. Deionized, distilled water was used to dilute standards, control, and samples just before they were added to plate wells. Following a 4˚C overnight incubation, the plates were washed three times. 100 µL of substrate solution consisting of 50 mmol citrate buffer, 1.6 mmol/L hydrogen peroxide and 0.4 mmol/L 2,2-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid), pH 4.0, was added to each well and allowed to develop for one hour at room temperature.

All standards, controls and samples were run in duplicate. In this assay, each microtiter well is coated with an anti-PdG monoclonal antibody (clone 220, Quidel). The PdG in each sample or standard competes with horseradish peroxidase -conjugated PdG to bind to the antibodies. Following the addition of substrate to each well, the horseradish peroxidase activity and resulting color intensity is inversely proportional to the concentration of PdG in the sample or standard. The PdG concentration of each sample was quantified by comparing the color intensity of each sample to that of an eight-point standard curve generated using a four-parameter logistic regression. The standard curve ranged in concentration from 19.53 to 2500 ng/mL.

1.6. Ethics

Urine specimen collection in 2000-2001 and analysis were approved by the University of Michigan's Institutional Review Board. Informed consent was obtained from all individual participants included in the study. Secondary analysis of these specimens was approved by Simon Fraser University's Ethics Review Board.

1.7. Archived Samples

First morning urine specimens were collected between 2000 and 2001 in the context of the Society, Environment and Reproduction (SER) study [7, 8]. Briefly, urine specimens were collected three times per week from women in two rural Kaqchikel Mayan communities in the southwest highlands of Guatemala who were parous, not using any form of chemical contraception, and at least six months from the birth of their last child. Sample were frozen at -10ºC for up to six months in the field and then shipped on dry ice from Guatemala to the laboratory at the University of Michigan, where they were archived at -80ºC. In 2010 samples were shipped on dry ice to the Maternal and Child Health laboratory at Simon Fraser University, where they were again stored at -80ºC until analysis for the quantification of reproductive hormone levels.

1.8. Significance of Research

This thesis presents an implementation of colorimetric ELISA using a mobile and standalone imaging instrumentation setup and regression techniques for determination of women's reproductive steroid hormone profiles. Using the light absorbance and transmittance properties of the chemical compounds that make up the hormone assay, we were able to estimate the hormone levels across women's ovarian cycles. This setup would allow for the eventual development of a compact and economical system which can be used for fertility research as well as clinical and individual monitoring of reproductive function in urban or remote areas. This line of applied research, in the long run, is also expected to provide necessary information for examining the extent to which reproductive function varies within and between populations and how it is influenced by psychosocial, energetic and environmental challenges.

1.9. Publications

This research led to the publication of a manuscript in the Journal of Medical & Biological Engineering & Computing (MBEC) titled, "Smartphone based colorimetric ELISA implementation for determination of women's reproductive steroid hormone profiles [10]" and a conference paper titled, "Adaptive illumination backlight panel for ELISA imaging systems [11]" was presented at the Canadian Conference on Electrical and Computer Engineering (CCECE) conference in Vancouver, British Columbia.

1.10. Organization of Thesis

Chapter 2 presents a detailed overview of the experimental setup which includes various light sources tested, calibrations for finding an ideal source, and microtiter plate layout which we have adopted.

Chapter 3 is focussed on Image Analysis with a detailed overview on optical roadblocks in biomedical imaging, image processing techniques reviewed and adopted to counter the roadblocks followed by the image processing algorithm which has been developed.

Chapter 4 describes various techniques of statistical analysis and predictive analytics using some of the machine learning techniques. The later part of this chapter includes the results and comparisons of our implementation with the laboratory standards.

Chapter 5 presents a conclusion along with the overview of the results, roadblocks faced, and limitations in the research followed by the insights into possibilities in future work.

Chapter 2. Optical Setup

The purpose of this research was to provide a determination of women's reproductive hormone profile which could be used as a field diagnostic equipment without the necessity for expensive laboratory instrumentation. The focus in this chapter would be in building an optical equipment which would eventually replace the use of the microtiter plate readers. This essentially implies the replacement of the laboratory apparatus mentioned in the previous chapter. The goals in building a custom optical setup are:

1. Provide a portable and economic replacement to the laboratory equipment which could be used for field diagnostic purposes

2. Ability to capture reliable images that are able to differentiate minute changes in color of the chemical reaction

The intuition in building the optical setup can be understood by the flow chart below:

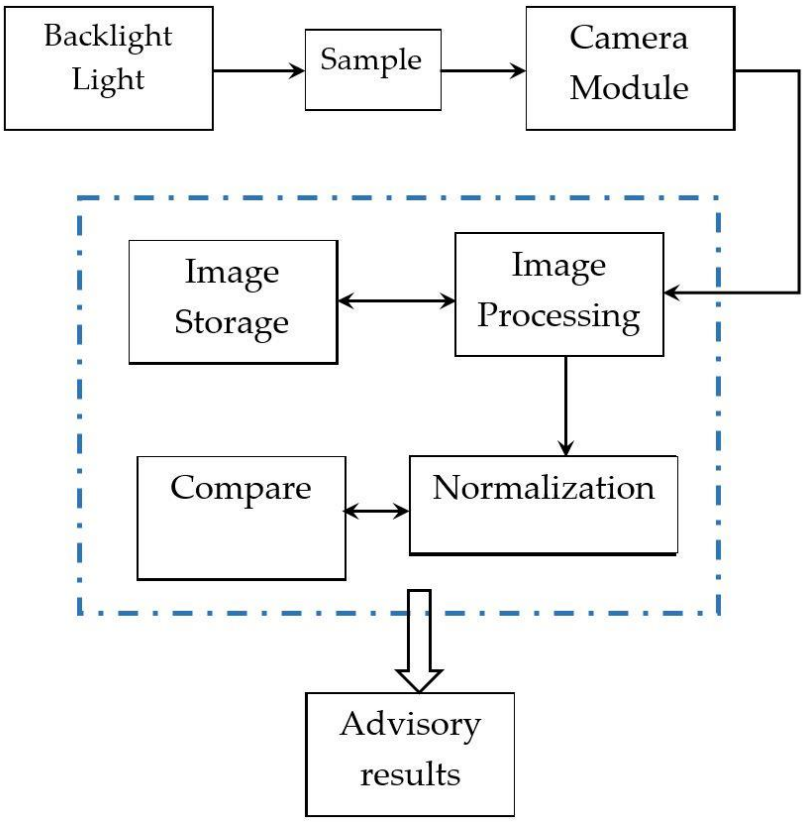


Figure 1. Flow chart of the image based implementation of colorimetric ELISA

As the light passes through the microtiter plate, variation in hormone concentration among the standards, controls and samples altered the amount of light transmitted through each well. Based on the hormone concentration present in the sample, the corresponding modulation in the intensity of the color was recorded by the camera [10]. The concentration of the hormone was inversely proportional to the intensity of the observed color. Color intensities of the wells were recorded on the camera as images and were digitally processed in MATLAB using a PC.

The process of capturing images using adaptive illumination techniques for ELISA imaging systems with smartphone or a standalone imaging device such as a webcam or a digital camera is performed using an inexpensive setup which consists of the following components:

2.1. Enclosure

It was also extremely important to make sure that the images we captured were not adversely affected by the environmental lighting conditions. Hence, we used an enclosure over the entire setup. For the earlier prototype, this enclosure was built using cardboard, which was lightweight and portable. The inside walls of the enclosure were painted black to avoid any stray reflections of ambient light.

2.2. Light sources

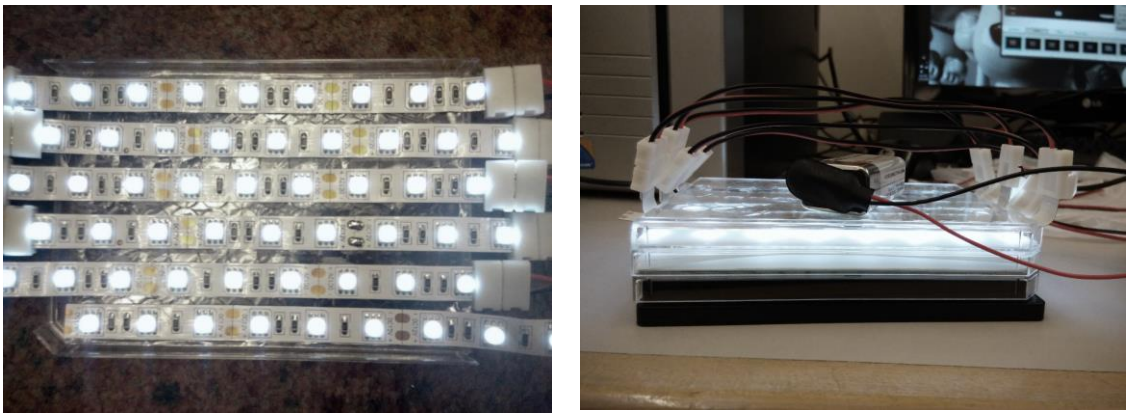
In imaging, appropriate light source is essential. The direction of the light will affect the shape and texture of the subject recorded. Similarly, the characteristics of the light source have considerable influence on the recorded image and for subsequent interpretation of the results. Therefore, it is extremely important that the source of light used for this experimentation remain constant, regardless of the time and place where the assay is performed. By using a well-calibrated, dedicated light source, we can reduce, if not eliminate, the effect of environmental lighting conditions in the field. For these purposes, a variety of light sources including LED, electroluminescent display panels and light box have been considered and tested. Some, if not all have had promising results with reliability. The light source must be stable, predictable, measurable, and reliable.

Choice of these light sources depends on the application requirement on wavelength, intensity, beam size, and beam quality of the light source

2.2.1. LED Strip

Light-emitting diode (LED) is a type of solid-state light source. It consists of a semiconductor chip doped with impurities to form a p-n junction (p-side (anode); n-side (cathode)). Current flows in the p-n junction from p-side to n-side under different voltages. Once electrons meet holes (absence of electrons), they will fall to lower energy level and release the energy in the form of light. The wavelength depends on the bandgap of the p-n junction. With the advancement in material science, now broadband LED light sources have covered the full wavelength range from UV to visible to near infrared. Because LED has a very compact size and very long lifetime, it is expected that LED light source will find broad applications in biomedical spectroscopy, particularly in absorption spectroscopy, reflectance spectroscopy, and fluorescence spectroscopy [12].

This lighting module consists of an array of two-dimensional individually controllable array of LED's. This is a custom build setup with 3 x 5 inches in dimensions approximately. The dimensions closely resemble the surface area of the microtiter plate providing optimal light to illuminate the entire top/bottom surface area of the plate.



(a) **(b)**

Figure 2. Illustration of the light source using LED's: (a) A custom built LED array stuck to the lid of the microtiter plate. (b) LED array mounted over the microtiter plate

A stack of diffusion sheets has been used to ensure that the light is soft and is uniform across the entire surface area making sure that each of the 96 wells are lit consistently with the same color temperature and intensity ensuring that the results stays comparable among all the wells. Figure 2 shows the basic setup of the custom built LED array attached to the lid of the microtiter plate along with diffusion sheets.

2.2.2. Electroluminescent display

Our experiments were performed with a white 5" x 3" electroluminescent panels from ElastoLite® which is highly flexible, waterproof and consists of a 3-dimensional, elastomeric, membranous polymer thick film. Figure 3 shows the electroluminescent panel used in a similar way to that of the LED setup.

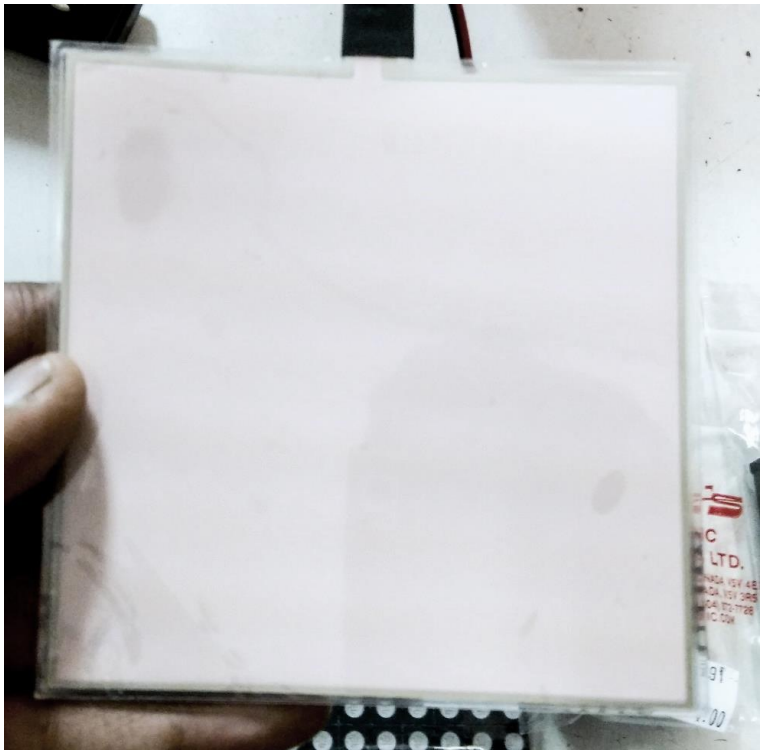


Figure 3. Electroluminescent panels from ElastoLite®

2.2.3. Light Box

A metal container with a light bulb inside and frosted glass on one side with uniform light strength across the glass pane connected to the power supply. It is very similar to the X-Ray Illuminators but with controlled intensity

2.2.4. Light Source Calibration

The color calibration experiments were performed using food color as the substance and MATLAB code to extract the red, green and blue components. Using the same concentration of the food color across all the wells, the consistency across the physical dimensions of the wells were observed. By comparing the results from the above mentioned light sources, we found that the LED's and the electroluminescent panels were comparable in color and the difference between the wells was below 5% in margin of error variations. However the light box produced over saturated images which were discarded. Due to the versatility and market availability LED's were used as primary light source during the experiments. They also have an advantage to calibrate the light intensity and color temperature based on the assay by limiting the power supplied.

Calibrating the light source to make sure that intensity remained with the margin of error was the objective of running some of the calibration experiments. The focus of the experiment was to ensure that we had properly light exposure. The images captured with the camera system in this case should not be over exposed or under exposed. Using water as the solution in the microtiter plates, images were captured with LED's running at various power (in Watts) covering a range of under exposed and over exposed images. The intensity vs power plots were for 4 different experiments as shown in the figure below:

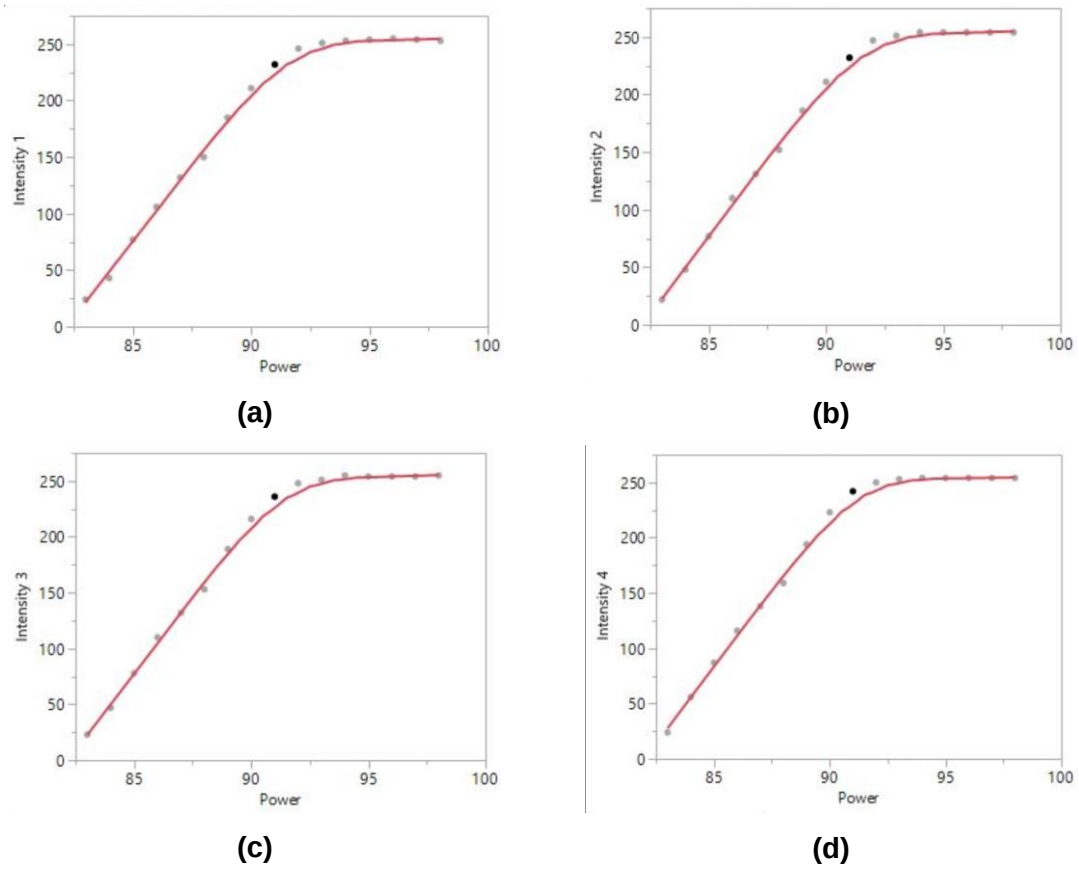


Figure 4. Light source calibration experiments using power vs intensity plots for LED light source (repeated 4 times as shown in a, b, c and d)

A kernel smoother with a Gaussian weight function was used to fit the data points and the inflection points was used as the determinant for the ideal power (in Watts) for exposure compensation. A power of about 91 W was set as the ideal condition during the course of further experiments.

2.3. Camera

To capture the images, we used an iPhone 4S, which is an older mid-tier or lower-end smartphone by today's market standards, and a Logitech c920 webcam to capture the images. Using two types of portable cameras allowed us to compare the quality of our results and interpret them for configuring the portable analysis systems. Images from both the iPhone and webcam were saved in JPEG lossy compression format.

The microtiter plate was positioned between the light source and the camera in such a way that the camera recorded the light transmitted through the plate. The distance

between the LED's and the plate was approximately 1 cm, and the distance between the plate and the camera lens was approximately 30 cm. The optical axis of the camera passed through the center of the LED's and the microtiter plate.

Figure 5. A cardboard enclosure along with the light source

2.4. Microtiter Plate Layout

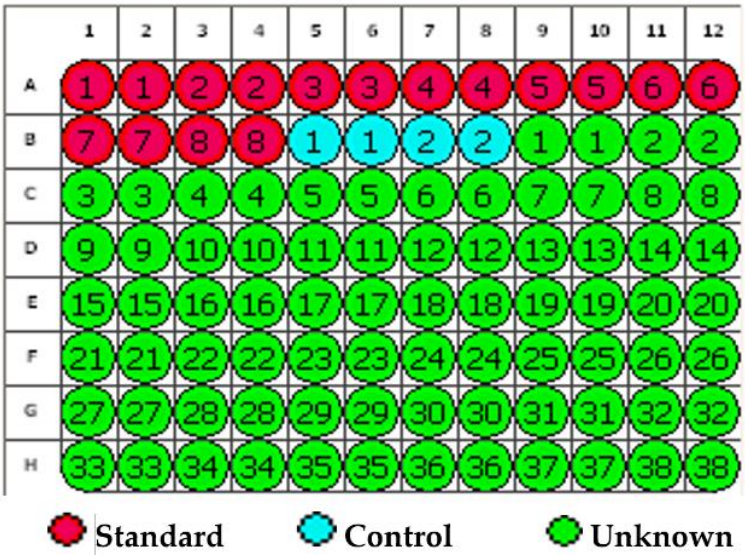


Figure 6. Layout of the microtiter plate used in colorimetric ELISA

Standards:

A set of eight known concentrations of the substrate serially diluted in duplicates as shown in the figure 6. These values are used to plot the standard curve.

Controls:

The negative control is a solution without antibody or antigen. The controls help to normalize or standardize each plate. Controls are also used to validate the assay and to calculate sample results. In most tests, the controls are pre-diluted and ready to use.

Unknowns:

The samples whose concentrations are not known and are yet to be estimated using the techniques mentioned in the next two chapters.

Chapter 3. Image Analysis

The optical apparatus described in the chapter 2 of this thesis serves as an experimental device setup to take images of the colorimetric ELISA's in the microtiter plates. The images captured by the camera are processed into data points using the ELISA Image Processing Algorithm (EIPA) as described during the course of this chapter.

3.1. ELISA Image Processing Algorithm based on Region Growing Image Segmentation

Algorithms developed for image processing are very specific for a particular use case scenarios and these learnings cannot be adopted directly for ELISA imaging with the optical setup explained in the chapter 2. The necessity still exists for a specific image processing algorithm as there are no universally accepted imaging methodologies which produces satisfactory data points for all images under examination, nor are all methods equally good for our optical imagery setup [16, 17]. The ELISA Image Processing Algorithm (EIPA) based on Region Growing Image Segmentation was fairly successful in producing consistent data points for the economic and efficient optical setup employed. An overview of the algorithm (EIPA) which remains the same for the images taken by on the Logitech C920 or the iPhone 4S keeping the algorithm adoptable for a wide variety of camera sensors can be visualized using Figure 7.

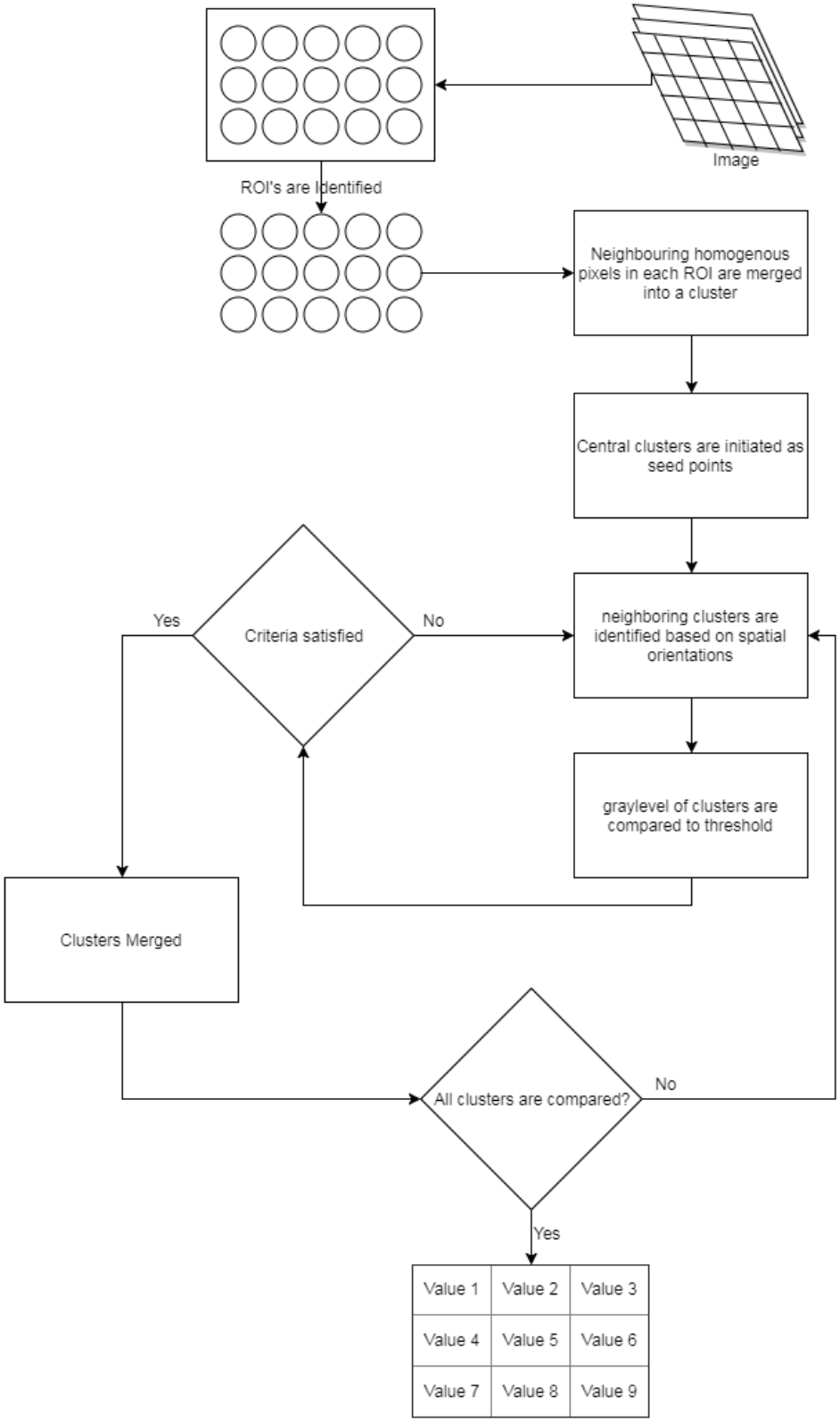


Figure 7. An overview of ELISA Image Processing Algorithm based on Region Growing Image Segmentation (EIPA)

EIPA consists of five steps. The initial step is the ***isolating individual wells*** using pattern recognition and Hough transform. EIPA uses edge based pattern recognition and Hough Transform to restrict the computations within the wells forming spatially heterogeneous regions of interests. The second stage would involve ***clustering*** of neighbouring pixels in a process called pixel binning. The third step, ***seed generation*** involves recognizing an ideal pixel cluster in each of the 96 wells. The neighbouring clusters to the seeds are grouped together as homogeneous segments in a process of ***pixel aggregation***. The final stage consists of ***normalization*** of data points for the predictions as described in the chapter 4.

I. Isolating individual wells

To avoid excess computation that will impinge on said resource, a region of interest (ROI) can be used [13] ensuring that computer resources are spent on necessary calculations to avoid excessive calculations which can result in longer processing times. As the standard microtiter plates have circular wells, the region of interest can be specified as a circle described by the equation:

$$(x - a)^2 + (y - b)^2 = r^2 \qquad (1)$$

Where,

a, b = coordinates of the center of the circle

r = radius of the circle.

Using this equation, Hough Transformation maps each image point (x, y) to the parameter points which lie on the surface of an inverted right angled cone whose apex is at (x, y, 0) [23, 24, 25, 26, 27] . This method involves a mapping features in an image space to sets of points in a parameter space. Each point of parameter space represents an instance of the model in image space. Image features are mapped into parameter space using a function which generates all parameter combinations compatible with both the observed image feature and the hypothesized model.

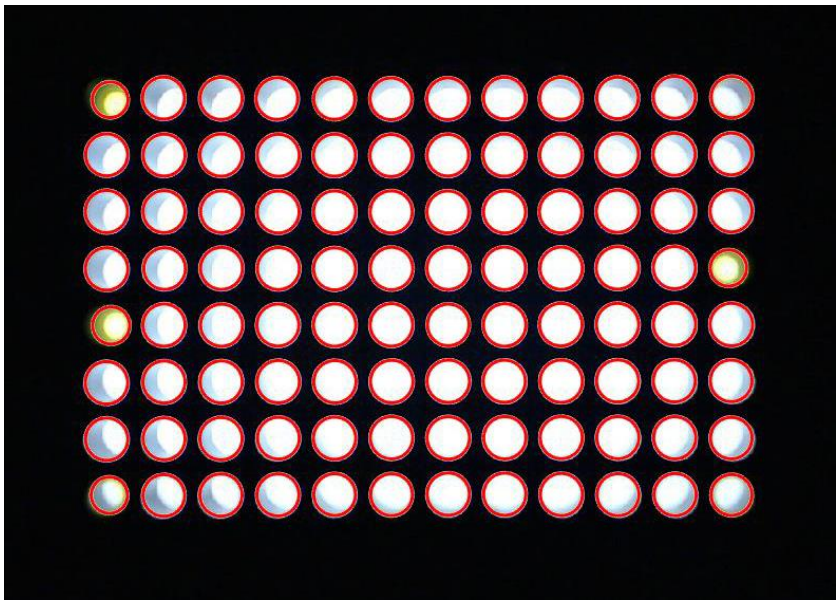


Figure 8. A sample of the region detection image of the microtiter plate with food color

Figure 8 demonstrates the identified regions of interests in the microtiter plate image. Diluted food coloring is randomly added to test the flexibility of edge based region detection algorithm.

II. Image Segmentation (or Clustering)

The region of interest (or the set of pixels in a well) represented as R is further divided into homogeneous clusters S by the process of Pixel Binning. The neighboring pixels are merged spatially averaging the data points forming multiple homogeneous segments within each of the 96 wells. These clusters of grouped pixels would save computations in the later stages of the EIPA.

The region based segmentation is partitioning of an image into homogenous clusters of connected pixels through the application of similarity criteria among candidate sets of pixels. The clusters/subsets of pixels represented as S_i is defined such that for all regions S_i,

$$R = \bigcup_{i=1}^{n} S_i \qquad (2)$$

Where,

R = Region of Interest

S_i = i^{th} cluster in R

n = number of clusters in each R

A set of n subsets of clusters in a well can be represented as (S_1, S_2,..., S_n). Each of the pixels in these regions is similar with respect to characteristics such as area, colour and intensity.

III. Seed Generation

The *n* clusters in each of the 96 wells would have different intensity values. These differences are mostly due to the limitations of the optical setup. The shadow of the wells create a darker clusters and this is a serious consideration in the wells towards the edges of the microtiter plate. The optical apparatus is designed in such a way that the center of each of the 96 wells are mostly immune to these effects. Hence, the centre clusters of the region of interests are selected as seeds for the data points for predictions.

IV. Pixel Aggregation

The neighbouring clusters in each of the wells are merged with a limited threshold so that any imperfections in the image due to discrepancies in the optical setup could be accounted for. This process of region merging is repeated until all the clusters are grouped together and the data points are averaged [14, 18]. The logical predicate P(R) of merging regions is based on comparing the differences of their feature measures with a segmentation threshold [19, 20].

$$P(R) = \begin{cases} True, & \sum_{x,y} \sqrt{(G_{xy} - G_{ij})^2} \leq \Delta \\ False, & Otherwise \end{cases} \quad (3)$$

Where,

G_{xy} is the graylevel of the neighbouring cluster

G_{ij} is the graylevel of the seed cluster

Δ is the user-defined threshold value

V. Normalization

The averaged data points, one from each of the 96 wells are then normalised and are segregated into standards, controls and unknowns. The standards are then utilized to plot the standard curve and used to estimates the hormone concentrations in the samples with unknown values.

3.2. Validation

A **method blank** is a sample containing all components except analyte, and it is taken through all steps of the analytical procedure. We subtract the response of the method blank from the response of a actual sample prior to calculating the quantity of analyte in it.

A **reagent blank** is similar to a method blank, but it has not been subjected to all sample preparation procedures. The method blank is a more complete estimate of the blank contribution to the analytical response.

A **field blank** is similar to a method blank, but it has been exposed to the site of sampling. For example, to analyze particulates in air, a certain volume of air could be sucked through a filter, which is then dissolved and analyzed. A field blank would be a filter carried to the collection site in the same package with the collection filters.

Chapter 4. Predictive Analytics and Results

4.1. Standard Curve

A standard curve is a general method for estimating the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration [21]. As mentioned in the earlier chapters, a standard curve is plotted using the data points extracted from the image using EIPA and by comparing each of the unknown samples to the standard sample reaction result, we predict the hormone concentrations of the unknown samples in the microtiter plate. A standard curve in our work is plotted with the known standard sample concentrations (from serial dilutions) on X-axis and the data points extracted from EIPA for the sample standards are plotted as intensity values on Y-axis. To achieve a scattered plot for concentrations, logarithmic scale is adopted on the X-axis. The method of least squares is used to draw the straight line through experimental data points that have some scatter and do not lie perfectly on a straight line.

Considerations are made while plotting the standard curves. It is important to ensure that the eight point standard curve is approximating the shape of the generic standard cure derived by absorbance values using standard laboratory equipment. There isn’t any compensation for the lack-of-fit component of the standard curve. The difference of the intensity values of the set of duplicates of the samples should be as low as possible which would ensure that the algorithm isn’t defective.

4.2. Plot Generation

The data points from EIPA (discussed in Chapter 3) are used to plot the standard curve using regression analysis, a statistical process employed for assessing the relationships among variables to find best-fit values with a set of parameters of a model. In this specific case, it enables us to estimate the concentrations of the unknown samples compared against the known concentrations from the standard curve. There are a number of regression analyses techniques that are used for studying the behaviour of a system.

4.2.1. A Linear Approach

Linear regression is an approach used for modeling the relationships between a dependent variable and one or more independent variables. The model specification is that the dependent variable, is a linear combination of the parameters. In vector form, linear regression is described by

$$y = X\beta + \varepsilon \qquad (4)$$

Where,

y = dependent variable vector

X = independent variable vector.

β = parameter vector

ε = noise

A wide variety of extensions for the linear regression are derived depending on the datasets and the use case scenarios.

4.2.2. Four Parameter Logistic (4PL) Regression

The 4 Parameter Logistic (4PL) Regression curve is a higher complexity model more suitable for a variety of biologic systems. It is quite useful for dose response and/or receptor-ligand binding assays, or other similar types of assays [22]. The model specification is that it has 4 parameters that need to be estimated for the curve fitting purposes and is governed by the equation:

$$y = d + \frac{a-d}{1+(\frac{x}{c})^b} \qquad (5)$$

Where,

x = independent variable

y = dependent variable

a = the minimum value that can be obtained

d = the maximum value that can be obtained

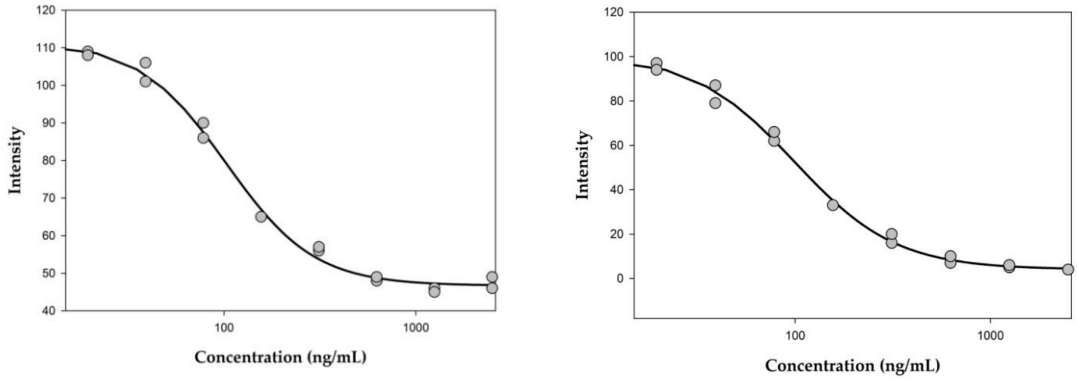
c = the point of inflection, a point on the curve halfway between a and d

b = Hill's slope of the curve (steepness of the curve at point c).

The same equation could be re-written as:

$$x = c(\frac{a-d}{y-d} - 1)^{\frac{1}{b}} \qquad (6)$$

A 4PL curve transforms to a straight line in logit–log space. We will have to solve for x, given your data y. The values of the parameters a, b, c and d shape the curve and determine the prediction accuracy.



(a) **(b)**

Figure 9. Intensity vs. Concentration plot (eight-point standard curve) using: (a) Logitech c920 webcam and (b) iPhone 4S smartphone

Figure 9 shows the relation between the standard concentrations and the Intensity data points from EIPA from images taken using the Logitech c920 webcam and the iPhone 4S respectively forming the eight-point standard curves for the respective cases. The 4PL model can be extended to a 5 Parameter Logistic (5PL) Regression by adding a fifth parameter g, which controls the degree of asymmetry of the curve.

4.3. Evaluation

The estimates of the unknown concentrations derived from the equation 6 are compared against the laboratory standard apparatus to determine the accuracy of the system. For

these purposes, Victor X5 2030 multilabel microplate reader (Perkin-Elmer) was used as a standard microtiter plate reader where absorbance of each well was measured using a test wavelength of 405 nm and reference wavelength of 595 nm. The difference in the absorbance values from both the wavelengths was plotted on the Y axis with concentrations on X axis.

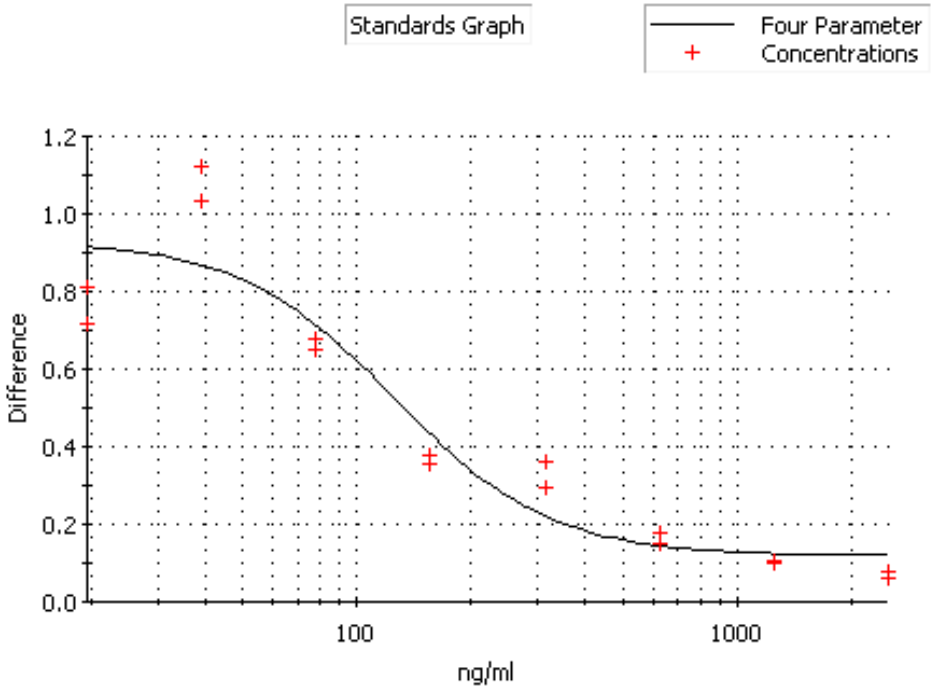


Figure 10. Absorbance vs. Concentration plot (eight-point standard curve) for the VICTOR™ X5 Multilabel Plate Reader

PdG concentrations were estimated from the difference of the eight-point standard curve from samples with known concentrations as shown in the figure 10 in Workout software (Perkin-Elmer). These values are used as the baseline for all the further comparisons. To validate the quantifications of PdG obtained by our method we will compare the standard curves using our method and the one obtained using the Victor X5 multiplate reader.

4.4. Analyzing Results

This section presents the results obtained by the comparing the hormone estimates from the Perkin-Elmer's workout software to that of the predictions made from ELISA images taken using Logitech c920 webcam and the iPhone 4S.

4.4.1. Root Mean Square Error

Root Mean Square Error (RMSE) is typically the measure of differences between the predicted values by our model and the observed values from the workout software. The calculation for this value is governed by the equation:

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \tilde{y}_i)^2}{n}} \qquad (7)$$

Where,

y_i = Predicted Estimates

$\tilde{y}_i$ = Observations from workout software

n = number of unknowns predicted

The RMSE for the predictions from the Logitech c920 webcam images is 59.36 (approx.) and the predictions from the iPhone 4S images is 62.34 (approx.). To scale these values, the Normalised Root Mean Square Error (NRMSE) is calculated using the equation:

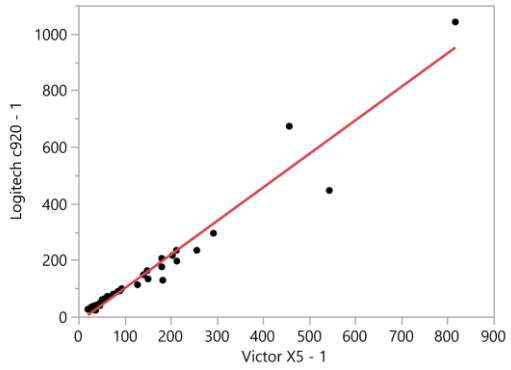
$$NRMSE = \frac{RMSE}{range} \qquad (8)$$

The NRMSE values derived from the equation 8 are approximated to 0.0745 and 0.0783 for the images taken by the webcam and iPhone4S respectively.

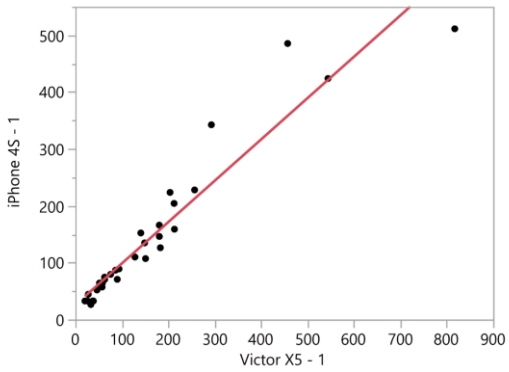
4.4.2. Coefficient of Determination

Coefficient of Determination (R^2) is a numerical fraction between 0 and 1 with no units. The higher values indicate higher accuracy of curve fitting which implies that the estimates are closer to the observed values. R^2 is computed from the sum of the squares

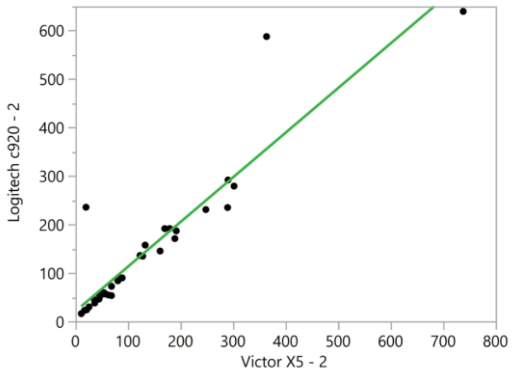
of the distances of the points from the best-fit curve. The results are normalised to turn R^2 into a fraction between 0 and 1 [22].



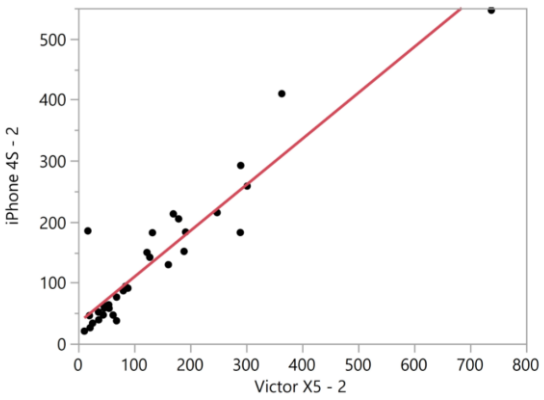
1a



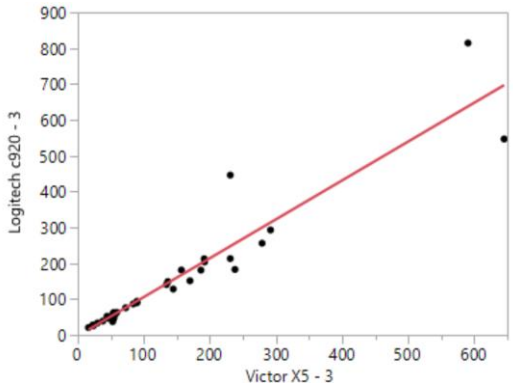
1b



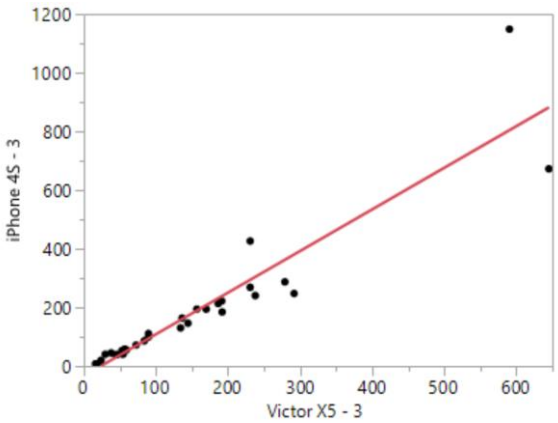
2a



2b



3a



3b

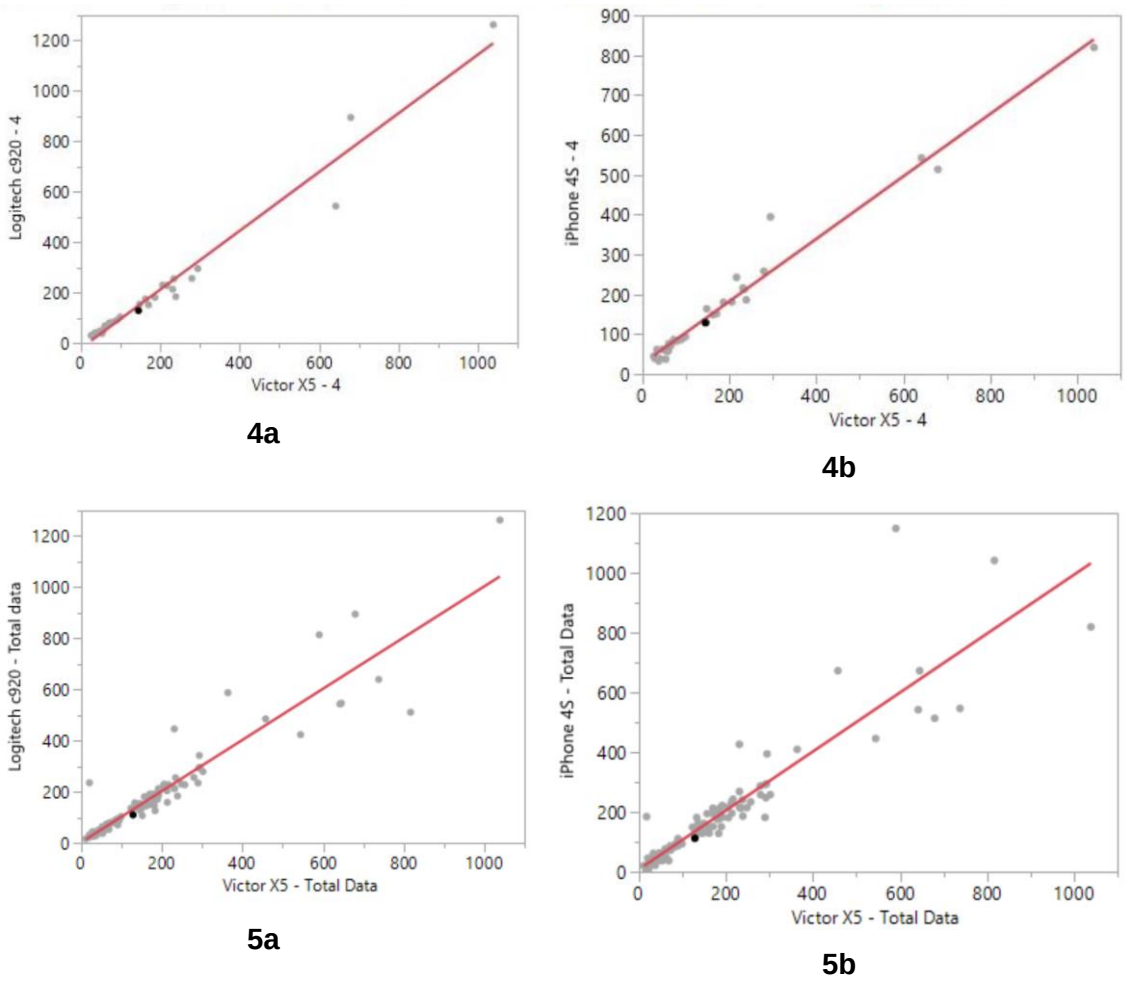


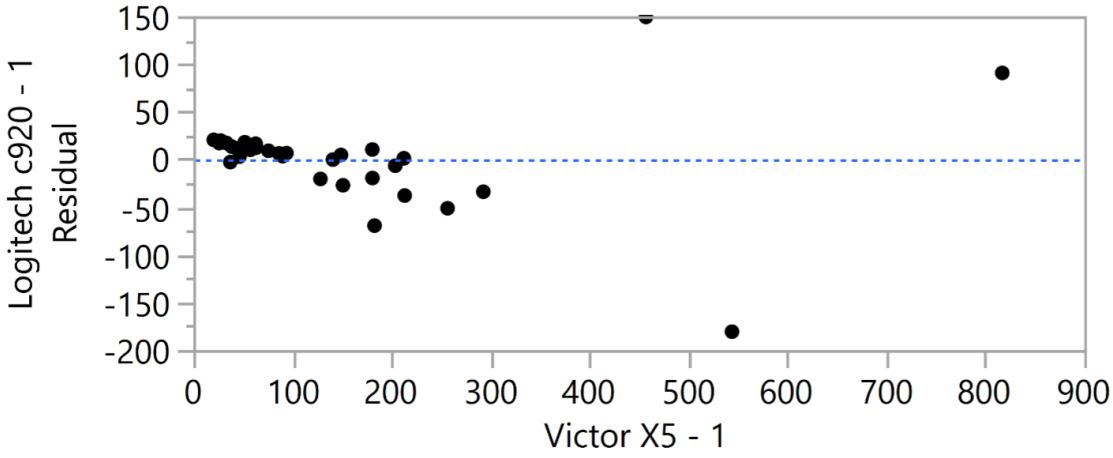
Figure 11. Regression comparison of the predictions using proposed method to that of the Victor X5 multiplate reader from data from a set of 4 experiments.

Notes: (1a) Logitech Webcam data from experiment 1
(1b) iPhone 4S data from experiment 1
(2a) Logitech Webcam data from experiment 2
(2b) iPhone 4S data from experiment 2
(3a) Logitech Webcam data from experiment 3
(3b) iPhone 4S data from experiment 3
(4a) Logitech Webcam data from experiment 4
(4b) iPhone 4S data from experiment 4
(5a) Logitech Webcam - total data
(5b) iPhone 4S - total data

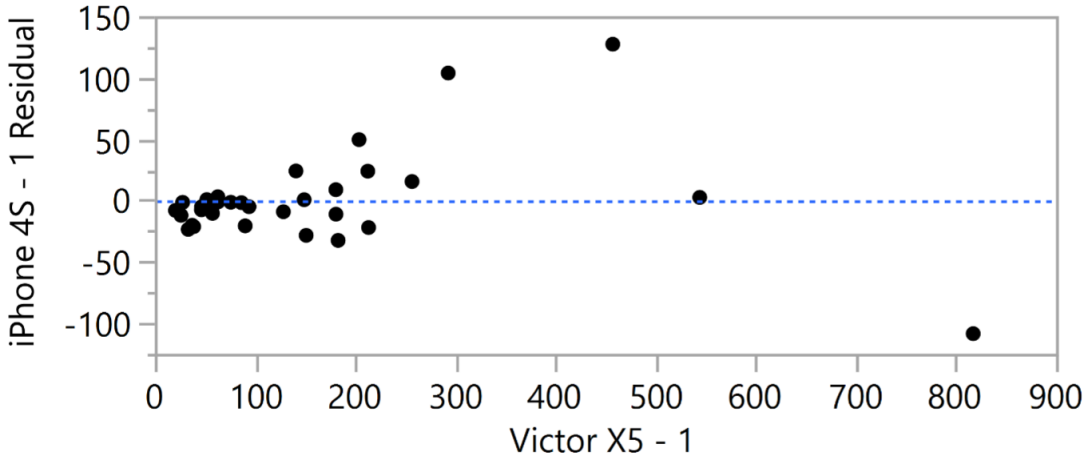
The R^2 value comparing the predictions using webcam and iPhone4s images is 0.891 (approx.) and 0.846 (approx.) respectively for a combined data from 4 different experiments.

4.4.3. Residuals

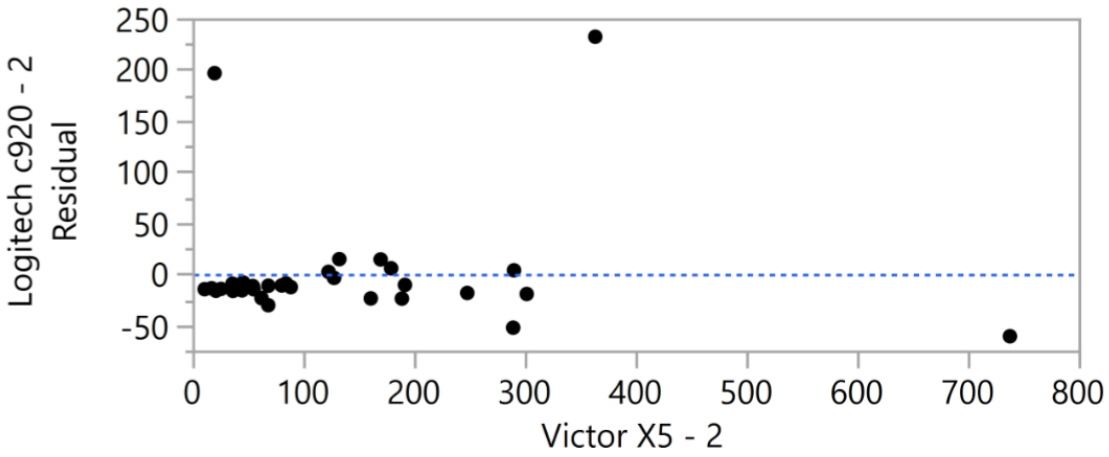
Residual is the amount by which an observation differs from its expected value. The expect value of the unknown concentration estimates using proposed model compared against the concentration estimates from the Victor X5 multiplate reader. The residual plots of the predictions using proposed method to that of the Victor X5 multiplate reader from data from a set of 4 experiments are shown in Figure 12.



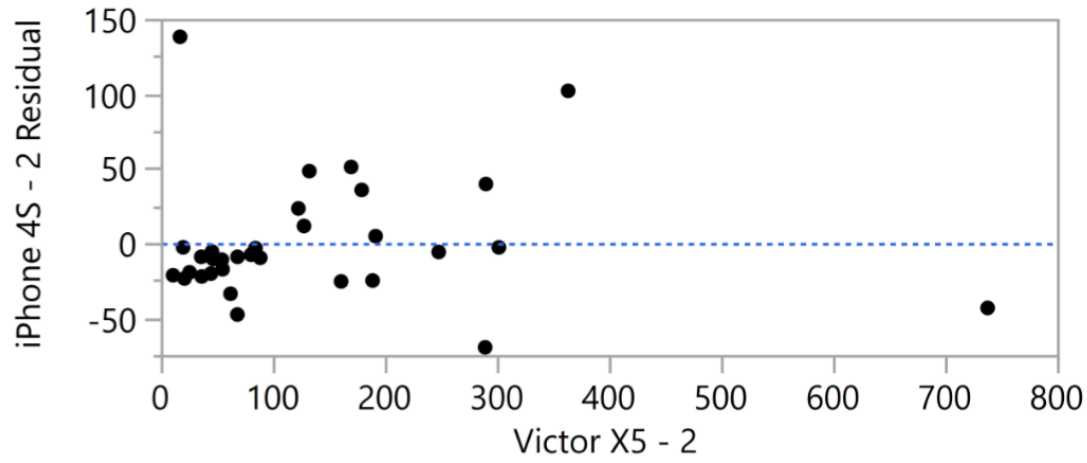
1a



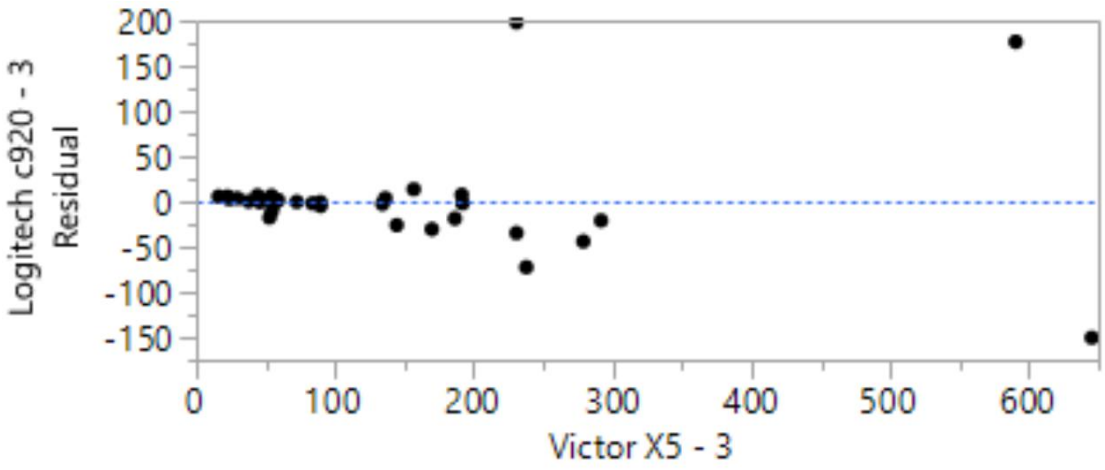
1b


Logitech c920 - 2
Residual
250
200
150
100
50
0
-50
0
100
200
300
400
500
600
700
800
Victor X5 - 2

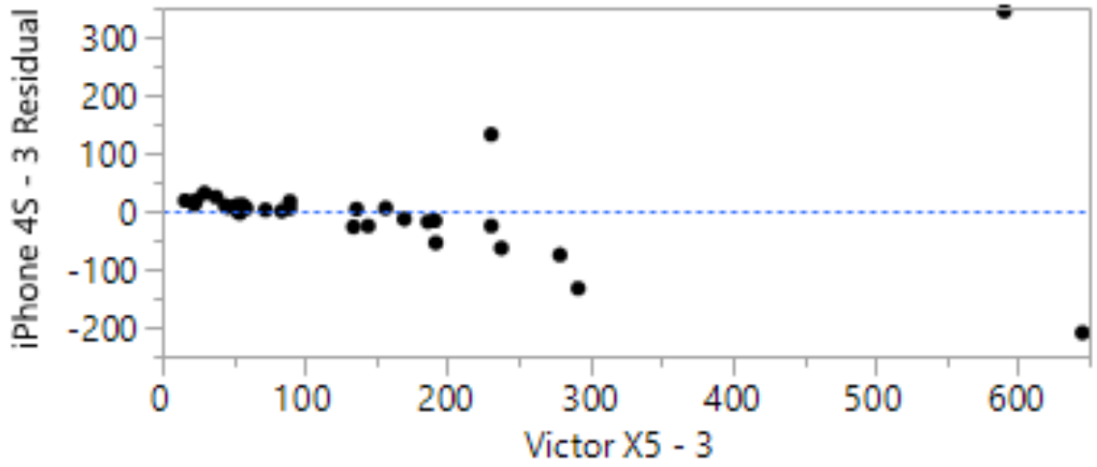
2a


iPhone 4S - 2 Residual
150
100
50
0
-50
0
100
200
300
400
500
600
700
800
Victor X5 - 2

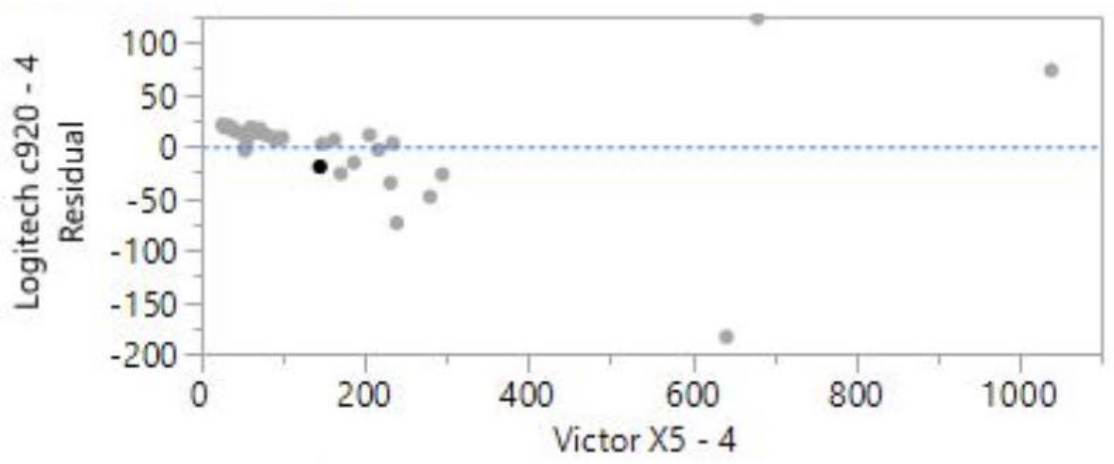
2b


Logitech c920 - 3
Residual
200
150
100
50
0
-50
-100
-150
0
100
200
300
400
500
600
Victor X5 - 3

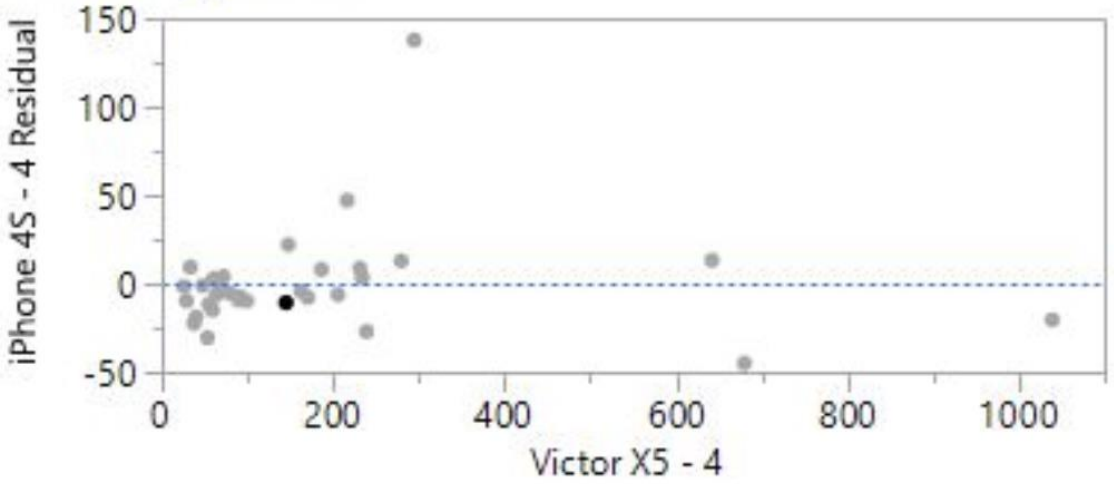
3a


iPhone 4S - 3 Residual
300
200
100
0
-100
-200
0
100
200
300
400
500
600
Victor X5 - 3

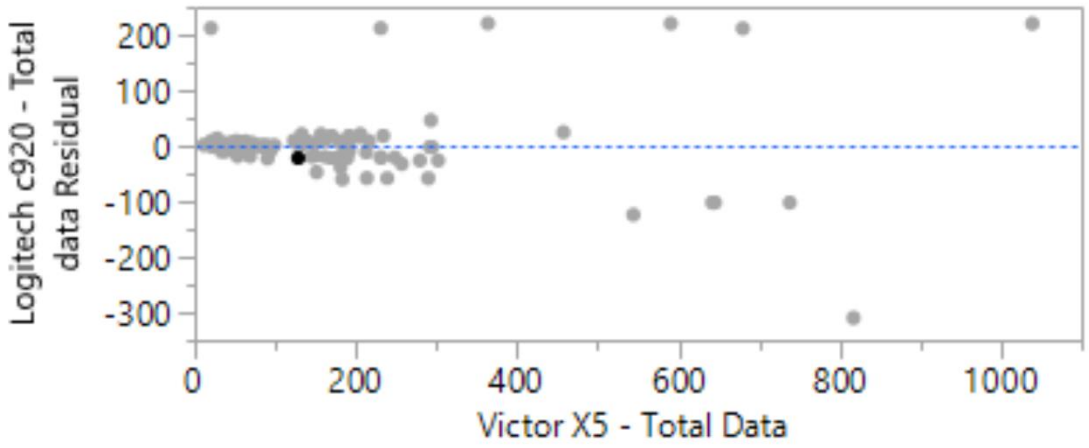
3b


Logitech c920 - 4
Residual
100
50
0
-50
-100
-150
-200
0
200
400
600
800
1000
Victor X5 - 4

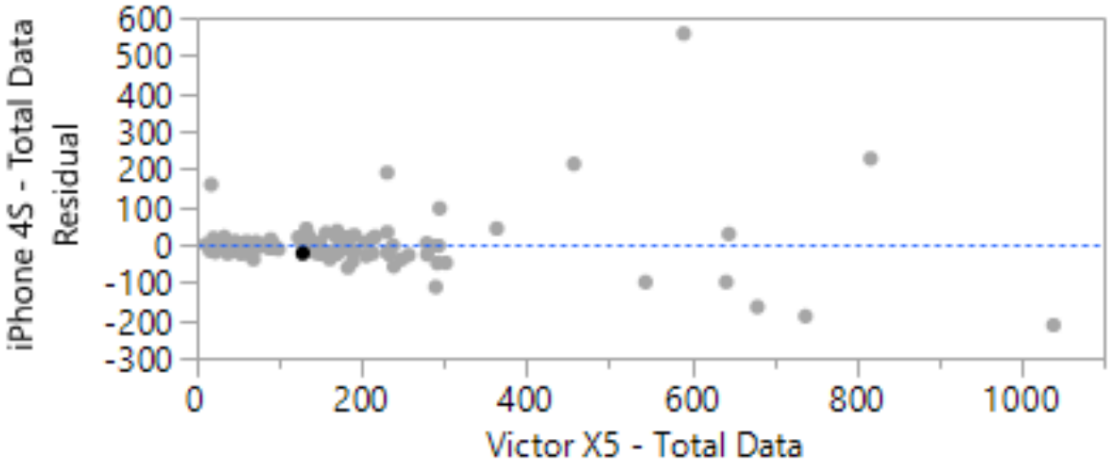
4a


iPhone 4S - 4 Residual
150
100
50
0
-50
0
200
400
600
800
1000
Victor X5 - 4

4b



5a



5b

Figure 12. Residual plot of the predictions using proposed method to that of the Victor X5 multiplate reader from data from a set of 4 experiments.

Notes: (1a) Logitech Webcam data from experiment 1
(1b) iPhone 4S data from experiment 1
(2a) Logitech Webcam data from experiment 2
(2b) iPhone 4S data from experiment 2
(3a) Logitech Webcam data from experiment 3
(3b) iPhone 4S data from experiment 3
(4a) Logitech Webcam data from experiment 4
(4b) iPhone 4S data from experiment 4
(5a) Logitech Webcam - total data
(5b) iPhone 4S - total data

4.5. Inference

From the results, it can be concluded that the proposed model fits the expected results better at the lower concentrations. At the higher concentrations the variation in the residual data is higher than that of the lower concentration estimates. Outliers in the experiment also influence the model but not to a very high degree. The overall results generated from the model proposed in this thesis are still very usable.

Chapter 5. Conclusions

5.1. Overview

The goal of this research was quantification of reproductive hormones for advisory diagnostic purposes for field diagnosis. In this work, we presented a multi-step process for hormone predictions using a custom built portable and economic optical setup to detect changes in color using any smartphone or other available cameras.

The first step involved sample collection from women in two rural Kaqchikel Mayan communities in the southwest highlands of Guatemala and the transportation of the samples to the Maternal and Child Health laboratory at Simon Fraser University followed by working out the procedure for the hormone analysis using competitive ELISA to quantify urinary concentrations of pregnanediol glucuronide (PdG).

The second step is to build a light weight, portable and economic optical rig which in theory could be built and operated by the users themselves if necessary. The objective here was to make use of everyday tools to build the setup which would be easy to replicate with minimum technical knowledge. We believe that using a regular smartphone to capture and processing images subsequently resulting in estimations would make the field diagnosis easier and efficient.

With the help of image processing and machine learning techniques, these images are converted into data points and are used for hormone predictions. These algorithms developed in the form of a smartphone, standalone or cloud based application would provide considerably consistent results regardless the physical limitations.

We have demonstrated a working prototype for the estimation of PdG with comparable results to that of the laboratory equipment. We hope that our work would show considerable on other areas of field diagnostics as well in the developing nations and aid their respective health care systems.

5.2. Roadblocks

Working with the urine specimens at every level would require resources and a basic skill level in handling bio-specimens. Due to the lack of such laboratory skills, food color was used in various occasions of the preliminary tests. This helped us in calibrating most of the steps before working with actual samples.

As the optical setup revolved around choosing a proper lighting condition, a majority of the time was spent calibrating the light sources and ensuring that the right color temperature and intensities were maintained uniformly across the wells.

5.3. Limitations

The size of the optical setup could not be shrunk beyond a point as the shadows from the wells would interfere with the color intensities therefore influencing the data points towards the edge and corner wells. Also, the light source, microtiter plate and the camera should lie along the same axis to avoid perspective distortion.

5.4. Future Work

There are a number of dimensions this research could be taken further. One among them is to streamline the optical setup experimenting on a wider range of light sources and calibrating the datasets according to the specifications of these sources. We also to extend this research in detection of various proteins, hormones and antibodies using other bio-chemical techniques such as fluorescence and other biochemical assays. Perhaps the most crucial works in this field would involve further investigation into machine learning and deep learning techniques for a higher accuracy prediction models.

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Appendix A.

Samples and Reagents

A1. **Coating Buffer** (0.05 M Na2CO3, pH 9.6)

Ingredients:

1.59 g Na_2CO_3 (Sodium carbonate), 2.93 g $NaHCO_3$ (Sodium bicarbonate) and 1 L ddH_2O

Procedure:

1. Dissolve Na_2CO_3 and $NaHCO_3$ in 900 mL ddH_2O, then pH to 9.6.
2. Top up to a final volume of 1 L with ddH_2O.
3. Store at 4°C (refrigerator) for up to 3 months.

A2. **Blocking Buffer** (0.1 M PBS, pH 7.0; with 0.1% BSA)

Ingredients:

5.42 g NaH_2PO_4 -H_2O (Sodium phosphate monobasic, monohydrate), 8.66 g Na_2HPO_4 -H_2O (Sodium phosphate dibasic, anhydrous), 8.7 g NaCl (Sodium chloride) 1 g BSA (RIA grade bovine serum albumin) (IN FRIDGE) and 1 L ddH_2O

Procedure:

1. Dissolve everything in 900 mL ddH2O, then pH to 7.0.
2. Top up to a final volume of 1 L with ddH2O.
3. Store at 4°C (refrigerator) for up to 2 months.

A3. **Citrate Buffer** (0.05 M, pH 4.0)

Ingredients:

9.61 g citric acid (anhydrous), 1 L ddH2ODissolve citric acid in 900 mL ddH2O (then pH to 4.0), Top up to a final volume of 1 L with ddH2O. Store at 4°C (refrigerator) for up to 3 months.

When disposing of expired buffer, pH to between 5.5 and 12 before dumping down the drain.

A4. 10x PdG Wash Solution (0.15 M NaCl; 0.5% Tween 20)

Ingredients:

88 g NaCl, 5.0 mL Tween 20 and 1 L ddH_2O

Procedure:

1. Dissolve 88 g NaCl in 750 mL ddH2O.
2. The Tween 20 is very viscous. Measure out 5.0 mL using a serological pipette and add this to the NaCl solution. Then use ddH2O to rinse the Tween 20 that is stuck onto the inside of the pipette into the NaCl solution.
3. Top up to a final volume of 1 L with ddH2O.
4. Store at room temperature.
5. Dilute 100 mL of this 10x stock with 900 mL ddH2O to make the 1x working wash solution.

A5. ABTS (40 mM) [Enough for 83 plates]

Ingredients:

0.275 g ABTS (2,2’-AZINO-bis 3-ethylbenzthiazoline-6-sulfonic acid) and 12.5 mL ddH_2O

Procedure:

1. Dissolve ABTS in ddH2O, then pH to 6.0. This reagent is difficult to pH because of its small volume.

2. Use VERY, VERY dilute NaOH or HCl to adjust pH (mix a few drops of the regular pH solutions in about 15 mL of ddH2O to make them very dilute).

3. Store wrapped in foil to minimize exposure to light at 4°C (refrigerator) for up to 1 month.

A6. H_2O_2 (2.0% hydrogen peroxide, 0.5M [Enough for 50 plates]

Ingredients:

125 uL 30% (8.0 M) H_2O_2 (in cabinet under fume hood) and 1.875 mL ddH_2O

Procedure:

1. Combine, then vortex for 5 seconds.

2. Store in opaque container to minimize exposure to light at 4°C (refrigerator) for up to 3 weeks.

Appendix B.

ELISA Protocol

B1. Coating Protocol (Day 1)

1. Take Coating Buffer out of fridge.
2. Calculate how much coating buffer is required for the day. Required vol (µL) Coating Buffer = (# of 96-well plates x 5000 µl) + 5000 µL for error.
3. Measure out the total volume required and let it warm to room temperature.
4. Return Coating Buffer bottle to the fridge.
5. Make a 1:4000 working solution of PdG-MAb by combining PdG Monoclonal Antibody (PdG-MAb) stock and Coating Buffer:
    - Capture Antibody: Quidel 330 monoclonal. Stored undiluted in -80˚C freezer. Required vol (µL) PdG-MAb stock = volume of coating buffer (µL) / 4000
    - Combine in a flask, cover with parafilm and swirl/invert a couple of times to mix thoroughly.
6. Make a note of how much volume is left in the PdG-MAb stock tube, parafilm, and refreeze in -80˚C freezer.
7. Add 50 µL / well to all wells.
    - Tap edges of the plates until the antibody solution is distributed evenly in all wells.
8. Cover plates with sealing tape and label with date.
9. Incubate overnight (minimum 16-18 hours) or longer at 4˚C.
    - Incubating for 2 to 3 days optimizes assay.

- Plates should be used within 5-7 days.

B2. Blocking Protocol (Day 2)

1. Take Blocking Buffer out of fridge.
2. Calculate how much blocking buffer is required for the day.
    - Total required vol (mL) Blocking Buffer = (# of plates x 10 ml) + 10 mL for error
3. Measure out the total volume required and let it warm to room temperature.
4. Return Blocking Buffer bottle to the fridge.
5. Wash plates 3x w/ 300 µL of 1x PdG Wash Buffer using the multichannel pipette.
6. After third wash, invert on paper towel and knock out any remaining liquid.
7. Place up-side down on paper towels to dry.
8. Label plates with plate number on the right side and top right corner.
9. Add 50 uL / well Blocking Buffer to all wells.
    - Required Vol (mL) Blocking Buffer for this step = (# of plates x 5 ml) + 4 mL for error
10. Cover loosely with plate lids, plastic wrap or foil.
11. Incubate at room temperature for ~1 hour (min 30 minutes; max 3 hours)

B3. Positive Control Preparation Protocol (Day 2)

1. Take 1 tube Luteal Urine Pool out of -80˚C.
2. Set up 1 plastic minitube.
3. Make a 1:15 Luteal Pool solution (enough for 14 plates) by combining (in minitube)

4. Pipet up and down to mix. Discard any leftover Luteal Pool into the biohazard level 1 bag.

B4. Sample Preparation Protocol (Day 2)

1. Make sure samples are defrosted. They will be in minitubes arranged by plate.

2. Set up 96-well plates for sample dilution (1 dilution plate for every 2 assay plates).

3. Label top left and right corner with the appropriate plate numbers (found on the outside of the minitube sample box).

4. For a 1:5 sample dilution (this is what we normally use):

    - Using multichannel pipette, add 80 µL ddH2O to each well getting a sample (see dilution plate map).

    - Using multichannel pipette, transfer 20 µL samples from minitubes to corresponding wells in dilution plate.

    - Pipette up and down to mix.

5. For a 1:2 sample dilution (ONLY for re-runs where value was “OVER” the range of the assay):

    - Using multichannel pipette, add 30 µL ddH2O to each well getting a sample (see dilution plate map).

    - Using multichannel pipette, transfer 30 µL samples from minitubes to corresponding wells in dilution plate.

    - Pipette up and down to mix.

B5. PdG Standard Curve Preparation Protocol (Day 2)

1. Take 1 tube (50 µL aliquot) PdG Standard stock solution (50 µg/mL) out of -80˚C. This will be enough for 11 plates.

2. Set up 8 microcentrifuge tubes.

    - Add 950 µL ddH2O to Tube 1.

    - Add 500 µL ddH2O to Tubes 2 through 8.

3. Set up 9 plastic minitubes.

4. To make 1 mL of the top standard point (2500 ng/mL), add entire aliquot (50 µL) of PdG Standard stock solution to 950 µL ddH2O in first microcentrifuge tube.

    - Pipet up and down to mix.

    - Add 500 µL from Tube 1 to Tube 2.

    - Add remaining volume of Tube 1 into Minitube 1.

    - Pipet up and down to mix glass Tube 2.

5. Repeat with the rest of the tubes.

    - Microcentrifuge Tube 8 will have 1 mL of liquid. Only add 500 µL to Minitube 8.

6. Add 500 µL ddH2O to Minitube 9.

B6. PdG-HRP Tracer Working Stock (1:100) Preparation Protocol (Day 2)

1. Check fridge for non-expired 1:100 PdG-HRP Tracer Working Stock.

2. If none available or if expired, take 1 tube PdG-HRP Tracer undiluted stock out of -80˚C.

3. Make a 1:100 PdG-HRP Tracer Working Stock (enough for 30 plates) by combining in a microcentrifuge tube: 1 µL PdG-HRP Tracer + 99 µL Blocking Buffer

4. Wrap tube in foil to minimize exposure to light.

5. 1:100 PdG-HRP Tracer Working Stock should be stored in the opaque metal can in the refrigerator for no more than 2 weeks, and ideally only for 1 week.

6. Make a note of how much volume is left in the PdG-HRP Tracer stock tube, parafilm, and refreeze in -80˚C freezer.

B7. PdG-HRP Tracer 1:160,000 Working Solution Calculations (Day 2)

1. Make sure Blocking Buffer is at room temperature.

2. Required vol (µL) Blocking Buffer for this step = (# of plates x 5000 µl) + 5000 µL for error

3. Vol (µL) 1:100 PdG-HRP Tracer Working Stock = Total vol (µL) 1:160,000 PdG-HRP Tracer working soln / 1600

4. Combine PdG-HRP Tracer Working Stock with Blocking Buffer in a flask.

    - Parafilm and swirl/invert a couple of times to mix.

    - Transfer to loading trough and cover with foil until ready to load into assay plates.

B8. Loading Assay Plate (with standards, control and samples) Protocol (Day 2)

1. Do not dump out the blocking solution.

2. Make sure you have made the PdG-HRP Tracer 1:160,000 Working Solution.

3. Make sure the plate number on the assay plate matches the one on the dilution plate.

4. Using the multichannel pipette, transfer 20 µL diluted samples from dilution plate to the appropriate wells in the assay plate (in duplicate).

5. Using the multichannel pipette, transfer 20 µL standards, water blank and diluted luteal pool (positive control) from the minitubes to the appropriate wells in the assay plate (in duplicate).

    - For consistency, always load “top” then “bottom” wells (e.g., first row load #12 first, then #11; for second row, load #8 first, then #7).

6. Immediately add 50 µL / well PdG-HRP Tracer 1:160,000 Working Solution using the multichannel pipette.

7. Cover plates with sealing tape and label with date.

8. Incubate overnight (minimum 16-18 hours) at 4˚C.

B9. Detection Protocol – Washing (Day 3)

1. Take Citrate Buffer out of fridge and calculate how much citrate buffer is required for the day.

    - Required vol (mL) Citrate Buffer = (# of plates x 10 ml) + 5 mL for error

2. Measure out the volume required and let it warm to room temperature.

3. Return Citrate Buffer bottle to the fridge.

4. Wash plates 3x w/ 300 µL of 1x PdG Wash Buffer using the multichannel pipette. Do this in the same order that you loaded the plates yesterday.

5. After third wash, invert on paper towel and knock out any remaining liquid.

6. Place up-side down on paper towels to dry.

B10. Substrate Solution Calculations and Protocol (Day 3)

1. Vol (µL) ABTS = Total vol Citrate Buffer x 0.01

2. Vol (µL) H_2O_2 = Total vol Citrate Buffer x 0.0032

3. Combine ABTS and H_2O_2 with citrate buffer after plate washing, just prior to loading plate.

    - Wrap with parafilm and swirl/invert a few times to mix.

4. Fill plates in batches of 4. Add 100 µL / well Substrate Solution to all wells.

5. This step is time and light sensitive. Do not stop during this process.

6. After every 4 plates are filled, cover plates with lids and place on shaker. Cover entire shaker with foil.

7. Shake gently (covered with foil) for approximately 60-90 minutes, until a deep green color begins to develop.

    - Development time varies with substrate batch and temperature.

Appendix C.

Data Analysis

C1. Reading the plate

1. To read plate:

    - Click on “WorkOut” icon
    - Click on “Run or open an existing protocol”
    - Click on “MChHLab PdG”
    - Click on “Start measurements”

C2. Target optical densities (optical density = Absorb @450nm – Absorb @595)

1. These are only loose guidelines, and plates can vary quite a lot.

    - S1 (Top Standard) 0.05 to 0.075 (or as low as possible)
    - S8 (Bottom Standard) 1.0 to 1.4
    - Blank (Control 1) 1.3 to 1.6
    - Multiple reads may be necessary. If so, name data files of 2nd, 3rd, etc. reads with b, c, etc., respectively.
    - Return plates to covered shaker between reads.