Abstract

A Novel Approach for Detection of Several Tuberculosis Markers Using Diffractive Optics

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Tuberculosis (TB) is an important disease worldwide. Currently, one-third of the world’s population is infected with TB, and it is a leading cause of death among people living with HIV. Immediate but also accurate diagnosis is required for disease control, yet available diagnostics cannot do both simultaneously. Therefore, designing a technique that can diagnose the disease correctly in the shortest possible time is in great demand in order to stop its spread. Diffraction-based sensing is a novel technique for measuring of biomolecular interaction that has potential for disease diagnosis. In this study, diffraction-based sensing successfully demonstrated its usefulness for diagnostics of TB using recombinant TB antigen, or by detection of interferon-γ that is produced from white blood cells when the immune system activates. The feasibility of the technology was also evaluated in terms of providing real time observation, reducing diagnostic duration, and increasing sensitivity of detection.
Acknowledgements

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<th>Description</th>
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<tr>
<td>ABTS</td>
<td>2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFP</td>
<td>Culture filtrate protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DI</td>
<td>Diffraction intensity</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dot</td>
<td>Diffractive-optics technology</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ELISPOT</td>
<td>Enzyme linked immunosorbent spot assay</td>
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<tr>
<td>ESAT</td>
<td>Early secretory antigenic target</td>
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<tr>
<td>GAM</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>GAR</td>
<td>Goat anti-rabbit</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon-γ release assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
</tr>
<tr>
<td>mab(s)</td>
<td>monoclonal antibody(ies)</td>
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<tr>
<td>M. TB</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>MTB/RIF</td>
<td>Mycobacterium tuberculosis/rifampin</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cutoff</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NTM</td>
<td>Nontuberculosis mycobacteria</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
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<tr>
<td>RAM</td>
<td>Rabbit anti-mouse</td>
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<tr>
<td>RD</td>
<td>Region of difference</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampin</td>
</tr>
<tr>
<td>RRDR</td>
<td>Rifampin resistance determining region</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Total internal reflection</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
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<tr>
<td>UV/Vis</td>
<td>Ultraviolet/visible</td>
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WHO  World health organization
Chapter 1 Introduction

1.1 *Mycobacterium tuberculosis (M. TB)*

Tuberculosis (TB) has been recognized as a global health problem since an official announcement made by the World Health Organization (WHO) in 1991.\(^1\) It became a leading cause of morbidity and mortality worldwide, with about one-third of the world’s population infected with TB. The most prevalent regions are found primarily in Africa and South-East Asia. The latest WHO report released in 2009 stated that in 2007, 13.7 million individuals suffered and 1.76 million died from active TB infection.\(^2\) TB spreads through aerosolized droplets generated by infected patients, so when combined with the common active symptoms of coughing and sneezing, the disease is highly infectious.\(^1\) If a patient with active TB is left untreated, the patient could infect an average of 10 to 15 other people per year. Incidence, prevalence, and mortality are especially high in many poverty-affected countries where population is dense but access to medical care is often limited.

A number of factors can contribute to TB epidemiology. TB infection divides into two sub-categories based on the state of the disease: active and latent TB. An active TB state is when pathogens are active and results in spreading of the disease to other individuals. Patients with latent TB may not exhibit any physical symptoms, but have a 5 to 10% chance of reactivation of the pathogen and become infectious in some point of their lifetimes.\(^1\) In addition, people infected with human immunodeficiency virus (HIV) are 20 to 30 times more likely to develop active TB. Co-infection of TB and HIV is one of the major causes of death among people in high HIV settings.\(^3\) The emergence of a drug resistant strain brings another serious threat to TB control.
Thus, investigations on novel drug-resistant TB diagnostic techniques are inevitable. However, such novel techniques and others currently used in third-world countries are lacking in speed and accuracy, resulting in often delayed and occasionally false diagnosis. Therefore, development of a technique that can provide rapid but also accurate results is absolutely necessary towards better control of the disease.

1.1.1 Immunological TB Diagnostics

Immunological TB diagnostics strategies are based on the patients’ immune response to TB antigens. Previous or current infections may result in positive response depending on the patients’ immune status. The most well known methods are the tuberculin skin test (TST), TB antibody detection, and interferon-γ release assay (IGRA).

1.1.1.1 Tuberculin skin test (TST)

Upon the development of TST by the Austrian pediatrician, Clemens von Pirquet in the early 20th century, it has become a standard for TB diagnosis in many clinical settings. TST is based on the delayed hypersensitivity reaction. Purified protein derivative (PPD) is intradermally injected, and the response is observed after 48 to 72 hours when the patient re-visits the clinic. Results are dependent on the patient’s immunological reaction with PPD, which is normally reported as the diameter of swelling around the injection site. A swelling size greater than a cut-off value is considered a positive response. Such cut-off value ranges between 5 and 15 mm depending on the patient’s immune status, and is determined empirically based solely on the physician’s subjective judgement.
The advantage of TST comes from its simplicity; the test does not require heavy equipment and the cost is economical. The overall sensitivity of the test is 77% for active tuberculosis, but the percentage dramatically decreases for certain groups of people with compromised immune systems: Infants or elderly persons whose immune systems are weak, individuals with congenital or acquired immunodeficiencies, patients taking corticosteroids or other immunosuppressive drugs, and patients with renal failure, cancer, and even malnutrition. Regardless, the major drawback of the TST is its cross reactivity with other mycobacterium species because PPD is a crude mixture of poorly defined mycobacterial antigens, some of which are shared among pathogenic mycobacteria, such as *M. tuberculosis*, *M. bovis*, and *M. africanum*, environmental non-tuberculosis mycobacteria (NTM), and *M. bovis* bacilli Calmette-Guérin (BCG), a substrain commonly used as a vaccine for tuberculosis. Consequently, the specificity of the technique is low (59%). Infection of other mycobacteria species or prior exposure to BCG vaccine can result in false positive signal.

Recent advances in TST focused on the improvement of specificity of the test by using antigens coded in the region of difference (RD), a TB-specific genomic sequence that is not shared with other species. Recombinant Early Secretory Antigenic Target (ESAT)-6 and recombinant Culture Filtrate Protein (CFP)-10 are coded in RD1 region and they can be used as alternatives for PPD. Specificity of the test can be increased by using RD1 antigens because they are only specific to *M. tuberculosis* and not shared with any other Mycobacteria species and BCG vaccine. Recently, results of phase I trial of the test with ESAT-6 have confirmed its safety and tolerability, and it may be available in the market in the near future.
1.1.1.2 Detection of TB specific antibody

Upon TB infection, lymphocytes produce antibodies against TB antigens. Similarly, TB antibodies in a patient’s blood sample can be detected using recombinant TB antigens. Detection of TB specific antibody is also an immunology-based diagnostic test that is more advantageous than TST. The test is performed in-vitro using immunoassay techniques, such as Enzyme Linked ImmunoSorbent Assay (ELISA), and it can provide the result relatively quickly, often within one day while the patient is still in the clinic.

RD antigens can also be used to detect TB specific antibodies due to their specificity only to the *M. tuberculosis* but not to other mycobacteria species and BCG. Beside recombinant ESAT-6 and CFP-10 from RD1, a number of studies evaluated sensitivity and specificities of other recombinant antigens for serodiagnosis of TB, including 38 kDa antigen, malate synthase, DPEP, TbF6, and MPT51.17 Although these antigens could achieve high specificities, ranging from 93% to 97%, they are not currently advocated clinically2 because of their low sensitivities, from 47% to 75%,17 and possibly even lower for certain groups of individuals whose immune systems are impaired. In the studies of humoral immune responses to TB, single antigen based assays resulted in low sensitivities and did not demonstrate satisfactory serodiagnostic performances.18 However, higher sensitivity could be achieved with a combination of several antigens and this may be more useful for the diagnostic purposes because of large inter-individual variation in serological responses.18,15 With regards to the disease states, this technique cannot discriminate between latent and active TB, but an RD antigen, Rv1978, largely associated with latently infected individuals, could be a promising antigen to be utilized in disease state discrimination, especially for latent TB.15
Although plenty of evidence has been found showing that the serological test is superior to TST due to its greater specificity, it is still not a good replacement for TB culture, the gold standard for active TB diagnosis, and should not be recommended as a diagnostic tool unless a novel strategy to improve the sensitivity of the test is developed.

1.1.1.3 Interferon-γ Release Assay (IGRA)

IGRA is regarded by many scientists as an important innovation in the diagnosis of TB infection over the last decade. Interferon-γ (IFN-γ), a type of cytokine molecule primarily released from T helper 1 (Th1) lymphocytes, is a critical molecule in initiating immune responses when foreign substances enter into body. The main function of the molecule is to kill pathogenic microorganisms by activating macrophages, and also to produce more antibodies by signalling B lymphocytes. IFN-γ release is dependent upon the presence of a signalling molecule called interleukin-12 (IL-12), which is produced from macrophage activated by microbial attack, such as Listeria, mycobacteria, and protozoa. IGRA measures the amount of IFN-γ produced from T cells when they are stimulated with two RD antigens, ESAT-6 and CFP-10, and presence of the disease can be differentiated by the amount of IFN-γ released. If T cells have been sensitized with TB due to past or current infection, they produce a significantly higher amount of IFN-γ than cells that have not been sensitized with TB. In addition, high specificity can be reached with ESAT-6 and CFP-10 because these antigens are TB specific and can possibly discriminate between active TB patients and BCG vaccinated individuals. Many studies have concluded that the specificity is higher for IGRA (93-96%) than that for TST (59%).
There are two commercial systems available for IGRA. QuantiFERON TB-Gold (Cellestis Ltd, Carnegie, Australia) measures the amount of IFN-γ produced using ELISA after incubating patient’s blood with TB antigens for ~12 hours. T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK) is based on an Enzyme Linked ImmunoSorbent spot (ELISPOT) assay that allows visualization of IFN-γ producing white blood cells when the cells are exposed to the TB antigens. (Figure 1.1) Both commercial tests give high sensitivity and specificity. However, IGRA is incapable of distinguishing between active and latent TB because the test is based on the cellular immune response, even though the initial intent of IGRA was to diagnose latent TB infection.\textsuperscript{21} The exact prevalence of latent TB cannot be calculated because of the lack of a gold standard test for latent TB; diagnosis of latent TB infection is based on positive IGRA results without clinical symptoms and with normal chest x-ray scan.\textsuperscript{1,21} Studying IGRA with immunocompromised patients, such as HIV infected individuals, is gaining interest because TB is the main cause of death where HIV is prevalent.\textsuperscript{3} Generally, IGRA was more responsive in
immunocompromised patients when compared to the TST\(^2\) and active TB cases have been associated with greater production of IFN-\(\gamma\) compared to latent TB patients.\(^1\)

Both sensitivity and specificity are higher for IGRA than for TST, but research still needs to be done on latent TB and TB with HIV infection.

### 1.1.2 Non-immunological tests

Besides immunological diagnostic tests relying on patient immunology, some techniques are independent of the immune response but involve culturing of the bacteria from sputum sample or imaging patient lungs in the case of pulmonary tuberculosis. The conventional method is sputum smear microscopy, which examines the presence of acid fast bacillius in sputum sample that can be seen under a light microscope. This method is rapid, inexpensive and less technically demanding, but low in sensitivity (44%) and cannot differentiate between TB and other mycobacteria species.\(^2\) Recent findings suggest using fluorescence microscope can enhance the sensitivity of detection, but due to the cost of the equipment, this method is not feasible in developing countries.\(^2\) Culturing TB is considered the gold standard technique and can provide a definite proof of active TB, but results are not readily available because visible bacterial growth normally takes up to 6 weeks.\(^2\)

Molecular methods enable fast and accurate detection of tuberculosis. One of recent advances in such techniques is called Xpert MTB/RIF assay which simultaneously detects the presence of TB and its susceptibility to the rifampin (RIF) resistance by doing polymerase chain reaction (PCR) of the rifampin resistance determining region (RRDR) of \(rpoB\) gene. Rifampin is the important first-line antibiotic for TB treatment and mutation in the RRDR makes TB resistant
to the drug. Overall sensitivity using clinical samples was 98% to 100% and none of the NTM species were falsely identified as *M. TB*, suggesting high specificity as well.\(^2\)

There are other imaging methods for screening of pulmonary TB, such as chest X-rays and high-resolution computed tomography (CT), to determine activity of the disease by detecting early parenchymal lesions or mediastinal lymph node enlargements.\(^2\) Recent findings suggest that [(18)F]-2-fluoro-deoxy-D-glucose positron emission tomography is a promising non-invasive method for monitor disease activity and response to anti tuberculosis chemotherapy, but it might not a suitable for developing countries due to its high cost.\(^{23,24}\)
<table>
<thead>
<tr>
<th>Technique</th>
<th>Method</th>
<th>Sensitivity/specificity</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Duration</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunological</td>
<td>TST</td>
<td>• Sensitivity: 77% for active TB • Specificity: 59%</td>
<td>• Inexpensive • Easy to use</td>
<td>• Cross reaction with BCG and NTM</td>
<td>48 to 72 hours</td>
<td>• Standard procedure</td>
</tr>
<tr>
<td></td>
<td>TB antibody detection</td>
<td>• Sensitivity: 47-75% • Specificity: 93-97%</td>
<td>• High specificity</td>
<td>• Low sensitivity</td>
<td>2 hours</td>
<td>• Currently not advocated</td>
</tr>
<tr>
<td></td>
<td>IGRA</td>
<td>• Sensitivity: similar to TST (73%)&lt;sup&gt;13&lt;/sup&gt; • Specificity: 93-96%</td>
<td>• High specificity • No cross reactivity with BCG and NTM</td>
<td>• Requires 1 day of incubation</td>
<td>1 day</td>
<td>• Substitutes TST where BCG vaccine is prevalent</td>
</tr>
<tr>
<td>Imaging</td>
<td>Chest X-ray</td>
<td>N/A</td>
<td>• Economical cost</td>
<td>• Wide spectrum of differential diagnosis&lt;sup&gt;2&lt;/sup&gt;</td>
<td>minutes</td>
<td>• Standard diagnostic&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CT scan</td>
<td>N/A</td>
<td>• High resolution</td>
<td>• Not pathognomonic for TB&lt;sup&gt;2&lt;/sup&gt; • High exposure to radiation</td>
<td>1 hour</td>
<td>• Improves the evaluation of treatment success&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-immunological</td>
<td>Sputum microscopy</td>
<td>• Sensitivity: 44%</td>
<td>• Inexpensive</td>
<td>• No differentiation between &lt;em&gt;M. TB&lt;/em&gt; and NTM</td>
<td>2 hours</td>
<td>• Standard diagnostic&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Bacterial Culture</td>
<td>• Specificity: 100%</td>
<td>• Definitive proof of active TB</td>
<td>• Results are not readily available</td>
<td>2-6 weeks</td>
<td>• Gold standard for active TB</td>
</tr>
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<td></td>
<td>Xpert MTB/RIF assay</td>
<td>• Sensitivity: 98-100%&lt;sup&gt;21&lt;/sup&gt; • Specificity: 100%&lt;sup&gt;21&lt;/sup&gt;</td>
<td>• Easy to use • Detection of drug resistance TB</td>
<td>• High financial demand</td>
<td>2 hours</td>
<td>• Detection of rifampin resistance TB</td>
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Table 1.1 Summary table of current TB diagnostic
1.2 Diffractive Optics Technology

1.2.1 Diffraction-based sensing

As increasing concerns in biomolecular and protein-protein interaction, it is necessary to develop a device that can explore the nature of the protein interactions on observable scales. Since protein analysis in biological samples deal with concentrations in the picogram per millilitre range, good sensitivity of the device is required for instrument development. In addition, specificity of the technique is essential because biological samples are often a mixture of many different macromolecules and only select proteins are targeted for analysis.

Diffraction-based sensing, introduced by Goh and coworkers in the Department of Chemistry, University of Toronto, has the potential for both sensitive and specific detection of analyte molecules. It utilizes light diffraction to measure molecular interactions in both quantitative and qualitative ways.

![Schematics of diffraction-based sensing in transmission configuration.](image)

**Figure 1.2** Schematics of diffraction-based sensing in transmission configuration.
As shown in Figure 1.2, diffraction of light happens when the incident beam strikes a regularly patterned surface. The diffraction image is created by constructive and destructive interference of light and they are represented as bright and dark spots on the screen. When a liquid sample containing analyte molecules flows through the sample cell, the detector monitors the light intensity at one of the diffracted spots. The height of the surface increases as the analyte molecules bind to the patterned protein, and the intensity of the diffracted beam increases as a result. (Figure 1.2)

![Diagram](image1.png)

**Figure 1.3** Representation of the two glass slides that make up a fluid cell.²⁶

![Diagram](image2.png)

**Figure 1.4** Schematics of the diffraction-based sensing experimental set-up.²⁶
Protein, usually an antigen, is stamped on one side of substrate facing a fluid channel. (Figure 1.3) The opposite side of the substrate is attached to a prism which allows an incident laser beam to come in at an angle of total internal reflection (TIR). (Figure 1.4) This configuration was chosen for several reasons. First, more of the laser light intensity can be retained and measured by detector whereas a non-TIR configuration causes the beam intensity to split between reflection and transmission. Second, there is less noise from the scattering of both the main and diffracted beams when incident light comes in at angle of TIR. And third, the TIR configuration prevents light from interacting with a complex medium, which would otherwise absorb and/or scatter the light beam. Instead, the incident beam will only interact with the substrate surface containing the pattern and bound proteins.

ELISA was first published in 1971, and it still constitutes a major proportion of immunodiagnostic tests. Diffraction-based sensing has the potential to be a replacement for ELISA because of its unique advantages. Diffraction-based sensing allows researchers to complete an assay within a few hours while observing the result in real time. In contrast, ELISA is only able to provide an end-point signal when enzyme substrate is added for color change. Each incubation step in ELISA requires about 1 hour with an initial overnight coating process; the whole procedure can take up to 2 days to obtain results. In addition, one can save valuable samples with the diffraction-based sensing system, as it requires small volumes, typically 50 µl to 70 µl of each reagent. Furthermore, the sensitivity of the diffraction-based sensing assay can be greatly improved with the use of the a peroxidase enzyme and its substrate, TMB (3,3’,5,5’-tetramethylbenzidine), where an insoluble precipitate formed from the enzymatic reaction accumulates on the sensor surface, amplifying the signal. This amplification strategy could result in more than a 1000-fold improvement in the limit of detection over a direct measurement without amplification. Recently, the technology was implemented by Axela Biosensors, Inc.
and commercialized into a bench top instrument called the dotLab® (Diffractive Optics Technology) system.

### 1.2.2 Axela’s dotLab® System

#### 1.2.2.1 The dotLab® instrument

The dotLab® instrument enables both detection and data analysis. As shown in Figure 1.5, all parts have been incorporated into a single standalone unit (17.11 in x 22.81 in x 27.27 in).

![Axela’s dotLab® System](image)

**Figure 1.5** Axela’s dotLab® System.

The dotLab® system introduces liquid samples into the dotLab® sensor using an automated sampling system. The fluidic panel on the upper left part of the instrument creates a pressure gradient that controls the rate of the fluid flowing through the sensor surface. The pressure created from the pump system draws the sample from the sample tray and bring it down to the sensor surface where protein incubation occurs. DotLab® software allows to program incubation mode; it can be a mix or a static mode. All the actions are pre-programmed using the software linked to the fluidic panel and the optical part of the instrument. Once incubation is
done, unbound reagents are washed off from the sensor surface by a washing solution coming from the bulk solution storage through the fluid panel, and waste materials are collected into the waste container. The dotLab® software allows users to perform a tipwash between each incubation and this removes residual proteins on the tip used to draw reagent from the sample plate, thus cross contamination between samples can be avoided.

1.2.2.2 The dotLab® sensor

![Figure 1.6](image)

**Figure 1.6** The dotLab® avidin sensor.

![Figure 1.7](image)

**Figure 1.7** The dotLab® sensor surface representation.
The dotLab® sensor (Figure 1.6) is a disposable plastic consumable, equally important as the dotLab® system. Similar to the preliminary experiment using glass slides with protein pattern stamped on the surface, pre-patterned avidin on the sensor surface allows rapid immobilization of biotin-conjugated reagents due to the high affinity of the avidin-biotin interaction ($K_a \approx 10^{15} \text{ L/mol}$). Among 8 available assay spots (Figure 1.7), up to 3 spots can be detected by illuminating the underside of each spot with a focused laser. An optical prism is attached right beneath a low-volume flow cell and diffraction of light occurs upon illumination of laser beam at the angle of TIR. The laser beam only interacts with the patterned surface for a few nanometers in height and does not penetrate into the solution medium in the flow channel, providing an ideal platform to work with complex biological samples.

Overall, the dotLab® system offers a simple and convenient alternative strategy of performing immunoassays. The amount of sample required is minimal, as little as 40 uL, whereas conventional ELISA procedure requires several milliliters of sample. The time required to complete an assay is also reduced to less than a few hours in comparison to ELISA, which usually takes up to 2 days to complete with the overnight coating process. The dotLab® system also enables observations in real time; the detector system constantly measures the changes in the diffraction intensity and displays it on a monitor. In addition, the fully automated platform minimizes human errors; therefore more accurate and precise measurements are possible. Finally, the system is not labor intensive and no extensive training is required for operating the instrument.
1.3 Purpose of research

The objective of this study is to develop a diagnostic test for infectious diseases that is cost effective, but also able to provide rapid and sensitive results.

Of the TB diagnostic techniques introduced above, TB antibody detection and IGRA will be focused on this study to show that the diffraction-based immunoassay is not only an alternative, but a better way of detecting the analyte molecules, TB antibody and IFN-γ, by comparing it to the conventional immunodiagnostic technique, ELISA. Based upon the results obtained from both approaches, advantages of the diffraction-based sensing system will be speculated on.

In Chapter 2, two immunoassay techniques, ELISA and the dotLab® system, were explored in terms of their ability to detect rabbit polyclonal TB antibody using two recombinant TB antigens, 16 kDa and 38 kDa. In Chapter 3, IFN-γ was detected using both techniques and a series of experiments were performed to optimize the assay condition for sensitive detection of IFN-γ, especially for the dotLab® experiment.
Chapter 2 TB Antibody Detection

2.1 Introduction

TB antibody can be detected using recombinant TB antigens. In this chapter, two recombinant TB antigens, 16 kDa and 38 kDa, were used to detect rabbit anti-TB antibodies using two immunoassay techniques, ELISA and the dotLab® system.

Two ELISA experiments were performed and examined their ability in detecting the rabbit anti-TB antibody. The first ELISA experiment was done with the overnight coating of monoclonal antibody (mab) to 16/38 kDa TB antigen, which would then capture recombinant TB antigens to detect rabbit anti-TB antibody. Instead of using the mab to 16/38 kDa TB antigen, the recombinant TB antigens were directly coated on a polystyrene plate in the second ELISA experiment.

A series of experiments were performed with the dotLab® to optimize the assay condition in terms of sensitivity and reducing non-specific bindings. The initial scheme was to use biotin-GAM, which would capture the mab to detect the recombinant TB antigens. However, the biotin-GAM antibody was associated with the non-specific binding but such a issue could be corrected by biotinylating the recombinant TB antigens.
2.2 Experimental

2.2.1 Materials

Mabs to 16 kDa (lot #: 24G18606) and 38 kDa (lot #: 1H22205) TB antigen, recombinant 16 kDa TB antigen (lot #: 19H22905), and rabbit TB antibody (lot #: 2A02909) were all purchased from Medirian Life Science. Recombinant 38 kDa TB antigen was purchased from Fitzgerald Industries International, Inc (lot #: A06070501). OmniPur* bovine serum albumin (BSA), fraction V (lot #: 2374B96) was used for blocking purposes. Horseradish peroxidise (HRP) conjugated goat anti-rabbit (GAR) antibody was purchased from Sigma Aldrich (lot #: 057K4802). Biotin conjugated goat anti-mouse (GAM) was purchased from Calbiochem®. Peroxidase substrates, ABTS (1-component) (lot #: 040427) and TMB membrane (1-component) (lot #: 040368), were both purchased from KPL. Both substrates were used as purchased without dilution. All protein samples were diluted in deionized water.

2.2.2 Procedure

2.2.2.1 ELISA

2.2.2.1.1 Mab coat

A 96 well transparent polystyrene plate (Sarstedt) was obtained and divided into 2 areas; the first 6 columns were used for the 38 kDa and the other 6 columns were for the 16 kDa TB antigens, respectively. The last 2 columns of each region were used for negative control. As shown in Figure 2.1, the ELISA experiment started with coating of the mab to each antigen.
Figure 2.1  Schematics of the ELISA with mab coating. (1) Mabs to 16/38 kDa TB antigen, (2) BSA block, (3) 16/38 kDa TB antigen, (4) rabbit anti-TB antibody, (5) GAR-HRP antibody, and (6) ABTS peroxidase substrate.

Mabs to 16 kDa and 38 kDa TB antigens were diluted in sodium carbonate (Na$_2$CO$_3$) buffer (0.05 M, pH 9.6) to make 1 µg/ml solutions and added to the respective regions of the plate. An aliquot of 50 µl sample was added to each well, incubated overnight (~12 hours) at 4°C and then washed 4 times with 0.05% of Tween 20 solution in phosphate buffered saline (PBS, pH 7.4) for effective removal of unbound proteins. Bovine serum albumin (BSA) in PBS (5 mg/ml) was used as a blocking solution to reduce non-specific binding. An aliquot of 100 µl PBS-BSA was added to each well and incubated for 2-3 hours at room temperature. The plate was washed 4 times with 0.05% of PBS-Tween 20. Both TB antigens were diluted in PBS to 1 µg/ml. An aliquot of 50 µl of the reagent was added to each well of the respective region of the plate and incubated for 1 hour at room temperature. The plate was washed 4 times with 0.05% of PBS-Tween 20. The interaction between TB antigens and rabbit anti-TB antibody was examined as serially diluting the antibody along the column. 100 µl of the rabbit TB antibody (1 µg/ml) was added to the first row and 50 µl of PBS was added to remaining wells. Negative control
wells contained only 50 µl of PBS buffer solution. Using a multipipette, aliquots of 50 µl of the sample from each well in the first row were transferred to the corresponding wells in the second row. After mixing the liquid well, aliquots of 50 µl of the sample in each well in the second row was transferred to the corresponding wells in the third row. The process was repeated until the eighth row was reached and the final 50 µl was discarded. The rabbit anti-TB antibody was incubated for 1 hour at room temperature. The plate was washed 4 times with 0.05% of PBS-Tween 20. Aliquots of 50 µl of 1 µg/ml of goat anti-rabbit (GAR) HRP were added to entire plate. HRP labeled secondary antibody is required in an ELISA protocol in order to generate a readable signal, such as a color change. The plate was incubated for 1 hour at room temperature and washed 4 times with 0.05% of PBS-Tween 20. The signal is produced when HRP enzyme substrate is added in the next step. 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is one commonly used HRP substrate for ELISA and it visualizes protein interactions by absorbance at 410 nm. ABTS (50 µl per well) was added to every well and incubated for ~10 minutes until a visible colour was seen. Finally, the plate was measured at 410 nm with a microtiter plate reader (Tecan, Safire² or Infinite M1000).
2.2.2.1.2 TB antigen coat

**Figure 2.2** Schematics of the ELISA with TB antigen coating. (1) 16/38 kDa TB antigen, (2) BSA block, (3) rabbit anti-TB antibody, (4) GAR-HRP antibody, and (5) ABTS peroxidase substrate

In the second ELISA experiment, TB antigens were overnight coated instead of mabs to 16/38 kDa TB antigens. (Figure 2.2) A 96 well polystyrene plate was divided into 2 regions for 38 kDa and 16 kDa TB antigen and 2 last columns of each region were used for a negative control.

An aliquot of 50 µl of the appropriate TB antigen (4 µg/ml) in Na₂CO₃ buffer (0.05M, pH 9.6) was added to each well and incubated overnight at 4 °C. The solution was removed and washed 4 times with 0.05% PBS-Tween 20. The plate was then blocked with 100 µl per well of 5 mg/ml of BSA in PBS (pH 7.4) for 2-3 hours at room temperature. It was washed 4 times with 0.05% PBS-Tween 20. The rabbit TB antibody (4 µg/ml in PBS) was added to the first row, 100 µl in each well, and 50 µl of PBS to remaining wells. The TB antibody was serially diluted as described above and incubated for 1 hour at room temperature. The plate was washed 4 times with 0.05% PBS-Tween 20. An aliquot of 50 µl of GAR-HRP antibody (2 µg/ml in PBS) was
added to each well, incubated for 1 hour at room temperature, and washed 4 times with 0.05% PBS-Tween 20. Finally, ABTS substrate (50 µl) was added for color change and the plate was measured at 410 nm with the microtiter plate reader.

2.2.2 Diffraction-based sensing

2.2.2.1 Biotin-GAM antibody

Axela’s dotLab® system and avidin sensors were used for all the experiments. Biotinylated goat anti-mouse (GAM) antibody was used as a capture agent for mab to 38 kDa TB antigen. As shown in Table 2.1, the remainder of incubations protocols was kept the same as ELISA: mab to 38 kDa TB antigen, 38 kDa TB antigen, rabbit anti-TB antibody, GAR-HRP antibody, and HRP substrate. TMB (3,3’,5,5’-tetramethylbenzidine) was the peroxidase substrate used because it produces insoluble precipitate which deposits on the sensor surface to amplify the diffraction signal. All protein samples were diluted in PBS (pH 7.4).
Table 2.1 Proteins used to detect the rabbit anti-TB antibody using the biotin-GAM antibody. The incubation mode was mix for all reagents.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Incubation Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-GAM antibody</td>
<td>50 µg/ml</td>
<td>4</td>
</tr>
<tr>
<td>Mab to 38 kDa TB antigen</td>
<td>50 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>38 kDa TB antigen</td>
<td>50 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit anti-TB antibody</td>
<td>50 µg/ml</td>
<td>15</td>
</tr>
<tr>
<td>GAR-HRP antibody</td>
<td>50 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>TMB</td>
<td>No dilution</td>
<td>6</td>
</tr>
</tbody>
</table>

2.2.2.2.2 Biotin-TB antigens

Using biotin conjugated TB antigen would be more advantageous; it would shorten the duration of assay by eliminating the first two steps and also reduce the chance of non-specific binding between proteins.

2.2.2.2.2.1 Biotinylation

The biotin conjugation reaction was mediated by 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide (EDC) to form active ester functional groups with N-Hydroxysuccinimide (NHS) from carboxylic acid groups present on the TB antigens. The active NHS esters were then readily reacted with the amine on the target molecule, biotin. (Figure 2.3)
Figure 2.3  EDC and NHS mediated conjugation reaction.$^{30}$

An excess amount of biotin-hydrazide (2-4 mg) was dissolved in 40 μl of dimethyl sulfoxide. Two TB antigens were kept in separate centrifuge tubes and diluted in PBS (pH 7.4) to make up total volume of 200 μl with concentration of ~1 mg/ml. About 10 mg each of NHS and EDC were added to a tube and dissolved in 50 μl of PBS. An aliquot of 20 μl of the biotin-hydrazide solution was added to each tube containing TB antigens and mixed well. An aliquot of 20 μl of the NHS and EDC solution was added to each tube containing TB antigens and biotin-hydrazide and mixed well. The final volume of each tube was 240 μl; this includes 200 μl of protein, 20 μl of biotin-hydrazide in DMSO, and 20 μl of EDC and NHS in PBS. Both tubes were placed on an orbital shaker (IKA, KS basic) and incubated with gentle agitation overnight (~12 hours) at room temperature.
2.2.2.2.2 Purification

Molecular weight cutoff (MWCO) spin columns (Millipore) were used to purify the biotinylated proteins. Columns with MWCO 3-5 times smaller than the molecular weight of the molecule to be retained were chosen as suggested from the manufacturer guidelines. Columns with MWCO at 3 kDa and 10 kDa were used for 16 kDa and 38 kDa TB antigens, respectively. The rotor was equipped to a centrifuge (Sorvall, Legend RT) and counterbalanced with a tube containing the same volume of liquid (240 μl). Samples were centrifuged at 14,000 x g for 20 minutes for the 16 kDa TB antigen and 6 minutes for the 38 kDa TB antigen. Both protein samples were centrifuged for 5 times at room temperature. After each centrifugation, 200 μl of PBS (pH 7.4) was added to restore the volume. After last spin, 200 μl of PBS was added to sample reservoir, mixed gently, and transferred into a clean centrifuge tube. Filtrate solution was also collected after each spin and kept in a separate tube. Later characterization confirmed that the 38 kDa TB antigen was successfully purified, while the 16 kDa TB antigen was not.

Dialysis was an alternative strategy to purify the 16 kDa TB antigen. A dialysis membrane (Spectrum Laboratories Inc.) with MWCO of 3.5 kDa was pre-soaked in PBS until it fell apart into a hollow cylinder with two open ends. One side of the membrane was sealed tightly with a membrane clip and the filtrate solution was placed inside of the cavity. The other end was then sealed with another membrane clip. The bag was then dialyzed against 250 ml of PBS (pH 7.4), and the buffer was changed every 2 hours for 4 times.
2.2.2.2.3 Characterization

The concentration of the purified protein was calculated based on a calibration curve constructed from UV/Vis absorbance measurement of non-conjugated TB antigens at 280 nm. The concentration range of the calibration curve was from 2 μg/ml to 250 μg/ml for the 38 kDa TB antigen and 0.2 μg/ml to 100 μg/ml for the 16 kDa TB antigen. All protein samples were placed in a 96 well half-area UV/Vis transparent plate (Greiner) and measured with the microtiter plate reader. The purified protein samples were diluted ten folds in PBS (pH 7.4) before measuring. The UV/Vis absorbance measurement of the filtrate indicated the protein passed through the membrane. Biotin conjugated protein was also characterized with the dotLab® system. The activity of the protein was confirmed by co-incubation with mab to each TB antigen. Each biotin-TB antigen (30 μg/ml) was introduced first to the avidin sensor and detected by the mab (50 μg/ml) to the antigen. Incubation of the mab to the antigen should cause signal increase if the biotin-TB antigen retained its activity.
2.2.2.2.4 The dotLab® measurement

Instead of using biotin-GAM antibody and mab to 38 kDa TB antigen, the biotin-38 kDa TB antigen was used to detect rabbit anti-TB antibody. The incubation sequences after the biotin-38 kDa TB antigen were kept the same as before: rabbit anti-TB antibody, GAR-HRP antibody and TMB (Table 2.2). The negative control assay was incubated with PBS (pH 7.4), without the rabbit anti-TB antibody. All protein samples were diluted in the PBS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Incubation Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-38 kDa antigen</td>
<td>25 µg/ml</td>
<td>4</td>
</tr>
<tr>
<td>Rabbit anti-TB antibody</td>
<td>50 µg/ml</td>
<td>15 (+ve control) 5 (-ve control)</td>
</tr>
<tr>
<td>GAR-HRP antibody</td>
<td>25 µg/ml</td>
<td>10 (+ve control) 5 (-ve control)</td>
</tr>
<tr>
<td>TMB</td>
<td>N/A</td>
<td>6 (+ve control) 5 (-ve control)</td>
</tr>
</tbody>
</table>

Table 2.2 Proteins used to detect the rabbit anti-TB antibody using the biotin-38 kDa TB antigen. The incubation mode was mix for all reagents.
2.3 Results

2.3.1 ELISA

2.3.1.1 Mab coat

**Figure 2.4** Result of ELISA to detect rabbit anti-TB antibody when mab to 16/38 kDa TB antigen was coated. Error bars represent standard errors (n=4).

Absorbance values measured at 410 nm were plotted against concentration of rabbit anti-TB antibody. Figure 2.4 clearly shows that neither the 16 kDa nor 38 kDa TB antigen was able to detect the rabbit anti-TB antibody, as no apparent linearity can be observed from the plot. All absorbance values were similar regardless of the concentration of the rabbit anti-TB antibody. Negative controls had absorbance values of 0.023 ± 0.001 for the 16 kDa and 0.015 ± 0.001 for the 38 kDa TB antigens, and they were similar to the measurements made with the highest concentration (1 μg/ml) of the rabbit anti-TB antibody. Failure in the ELISA might be due to the
low sensitivity of the technique. In addition, both mabs might have lost their activity due to a prolonged storage at 4 °C for 3 years.

2.3.1.2 TB antigen coat

![ELISA](image)

**Figure 2.5** Result of ELISA to detect the rabbit anti-TB antibody when TB antigens were coated. Error bars represent standard errors (n=4).

Even with direct coating of the TB antigens, ELISA was not able to detect binding of the rabbit anti-TB antibody. As shown in Figure 2.5, there was no correlation between the absorbance values and concentration of the rabbit anti-TB antibody. Negative control measurements, 0.121 ± 0.003 for the 16 kDa and 0.108 ± 0.007 for the 38 kDa TB antigens, also indicated that the ELISA experiment was not successful as those values were similar to ones with high rabbit TB antibody concentration.
The rabbit anti-TB antibody was a polyclonal antibody; it can potentially bind various different TB antigens, including the 16 kDa and the 38 kDa TB antigens. Even if there was a binding event happening between the TB antigens and the rabbit anti-TB antibody, the amount was too small for detection by ELISA. Therefore, a device or technique capable of sensitive detection of the rabbit anti-TB antibody is necessary.

2.3.2 Diffraction-based sensing

2.3.2.1 Biotin-GAM antibody

![Diagram](image)

**Figure 2.6** The dotLab® measurement with the biotin-GAM antibody for the rabbit anti-TB antibody detection.
Figure 2.6 is a typical result curve obtained with the dotLab® system. The binding event causes diffraction intensity (DI) to increase. The beginning of an incubation step is represented by a spike, which is due to air drawn into the microtubing before loading reagent solutions. Because air has lower refractive index than that of liquid samples, the DI increases as air passes over the sensor surface. An air gap also prevents mixing of two reagents as it separates a sample that was already in the microtubing and new reagent that is going to be drawn into the microtubing.

A commercial biotin-GAM antibody was used for the first layer and it is represented by a big signal increase in the beginning of the plot (Figure 2.6) due to its high molecular weight (150 kDa). Similar to ELISA, a BSA block was required to reduce non-specific binding of proteins. Interestingly, the only visible signal increases in the graph were biotin-GAM antibody, mab to 38 kDa TB antigen, and TMB amplification, whereas 38 kDa TB antigen and rabbit anti-TB antibody signals remained at the baseline even though they had high molecular weights. This indicates that the captured surface was not saturated with proteins and only a few protein molecules were bound to the surface. Nevertheless, the signal could be amplified by TMB, which was shown as the big DI change when it was added at the end.

However, a significant amount of background signal was observed in the negative control assay (Figure 2.7), without rabbit anti-TB antibody, possibly due to the non-specific binding event of GAR-HRP antibody to other proteins and/or on the polystyrene surface exposed between patterns. Having a false positive signal would result in low specificity of the technique, hence it cannot be used as a satisfactory diagnostic test. In order to eliminate non-specific binding of proteins, the first two steps were omitted by directly conjugating a biotin molecule to the TB antigen.
2.3.2.2 Biotin-TB antigen

2.3.2.2.1 Characterization of 38 kDa TB antigen

After the biotinylation reaction, both TB antigens were purified with MWCO spin columns and characterized by UV/VIS absorption measurement at 280 nm. Based on the best fit equation of the calibration curve of 38 kDa antigen shown in Figure 2.8, the purified sample had a 96% recovery; the reaction was done with 1.2 mg/ml and 1.15 mg/ml of the protein was recovered. A dotLab® measurement with an avidin sensor also showed that the protein was successfully biotinylated as DI increased with the addition of the biotin-38 kDa TB antigen. (Figure 2.9) This also demonstrates that the activity of the protein was retained, as the biotinylated antigen was able to capture the mab to 38 kDa TB antigen.
Figure 2.8  Calibration curve of the 38 kDa antigen.

Figure 2.9  The dotLab® characterization of the biotin-38 kDa TB antigen with its mab.
2.3.2.2 Characterization of 16 kDa TB antigen

The 16 kDa TB antigen was not successfully purified. According to the UV/Vis measurement after MWCO spin column purification, only 15% of the initial concentration was recovered; the reaction was started with 1.16 mg/ml of the protein but only 0.17 mg/ml was recovered. The concentration was calculated based on the best fit equation of the calibration curve shown below. (Figure 2.10) High UV/Vis measurement obtained from the filtrate collected from spin column showed that the 16 kDa antigen was not retained, but passed through the membrane. The filtrate was then dialyzed against PBS and characterized with UV/Vis and the dotLab® measurements. The protein concentration calculated from UV/Vis measurement was even lower than measurement made after the MWCO spin column purification, as it contained only 6% of the initial concentration; 0.067 mg/ml of the 16 kDa TB antigen was recovered. Although the dotLab® measurement showed a slight DI increase in the biotin-16 kDa TB antigen, subsequent incubation with the mab to 16 kDa TB antigen caused no signal increase. (Figure 2.11)
**Figure 2.10** Calibration curve of the 16 kDa antigen.

**Figure 2.11** The dotLab® characterization of the biotin-16 kDa TB antigen with its mab.
2.3.2.2.3 The dotLab® measurement

The 38 kDa TB antigen was successfully biotinylated and used for the dotLab® measurement to detect the rabbit anti-TB antibody. (Figure 2.12) Biotinylation of the TB antigen could reduce non-specific binding of proteins, as the negative control did not show an increase in TMB signal. (Figure 2.13) Also, the assay could be completed in a shorter period of time as the first two incubation steps were eliminated.

Figure 2.12 The dotLab® measurement with the biotin-38 kDa TB antigen for the rabbit anti-TB antibody detection
Figure 2.13 The dotLab® measurement with the biotin-38 kDa TB antigen for negative control of detecting the rabbit anti-TB antibody.
2.4 Discussion

The dotLab® system successfully demonstrated detection of rabbit TB antibody using the biotin-38 kDa TB antigen. In general, TB antibody detection is considered a highly specific technique with the use of TB specific antigens, such as ESAT-6 and CFP-10, and shows a lot of potential for use in high TB prevalence regions. However, it has been reported that the sensitivity (47-75%) of the technique is greatly reduced in comparison to other technique, for example TST (77%) and IGRA (73%).2,17,18 Although ELISA is still a primary technique used for TB antibody detection, two ELISA experiments using two recombinant TB antigens, 16 kDa and 38 kDa, were not successful in our hands and could not detect rabbit anti-TB antibody. The rabbit anti-TB antibody was a polyclonal antibody; it has the potential to be detected by any other TB antigens including the 16 kDa and the 38 kDa TB antigens. It might have been bound to the antigens but ELISA lacked the sensitivity enough to convert the binding event into a signal in any measurable degree. The dotLab® system, however, was able to detect rabbit anti-TB antibody with the biotin-38 kDa TB antigen, indicating that this could provide a better sensitivity for TB antibody detection than ELISA.

The biotinylation of the 16 kDa TB antigen was not successful. The 16 kDa TB antigen might have been biotinylated but the purification methods used, MWCO spin column (MWCO 3 kDa) and dialysis (MWCO 3.5 kDa), were not suitable for the 16 kDa TB antigen. High signal from the UV/Vis absorbance measurement of the filtrate solution confirmed that the 16 kDa TB antigen was not retained by the column. Additionally, low UV/Vis absorbance measurement of the filtrate solution after dialysis showed that the 16 kDa TB antigen was not retained but passed through the membrane. A membrane with smaller MWCO is required for successful purification of the 16 kDa TB antigen. However, different purification methods, such as affinity
chromatography and size exclusion chromatography, need to be incorporated because the column used was one of the smallest MWCO spin columns available in the market.

Not only are better purification methods required, but a more efficient biotinylation process might also help. Saturation of the avidin surface with a biotinylated antibody (150 kDa) would normally result in a 0.35 to 0.40 signal increase. As the 38 kDa TB antigen has a molecular weight about 4 times less than that of an antibody, one fourth of the DI increase by the biotin-38 kDa TB antigen would be expected – that is about 0.1. However, the measured value ranged from 0.03 to 0.04, which was only one third of the expected value. More efficient biotin conjugation would be possible with a commercial biotin conjugation kit, such as the ChromaLink™ Biotin Protein Labeling kit, to provide a greater density of TB antigens bound onto the avidin surface. This would increase the probability of the rabbit anti-TB antibody detection by TB antigens, thus allowing for a more sensitive detection of the rabbit TB antibody.

A number of studies have shown that the sensitivity of the TB antibody detection was enhanced when a multiple antigen system was used.17,18 A single TB antigen is not enough to be used to cover the antibody profiles of active TB patients, but combinations of antigens may yield improved levels of sensitivity, without affecting specificity.18 In addition, using multiple antigens rather than a single antigen may be more advantageous for the development of diagnostic tests because of large inter-individual variations in serological responses.18 A multiple antigen system can be applied to the dotLab® system by premixing various TB antigens or using the dotLab® Panel sensor for multiplex detection of TB antibody. More studies need to be done on the dotLab® system to determine whether TB antibody detection can be improved in this manner. Nevertheless, the dotLab® system was advantageous in terms of providing rapid results and its
ease to use. It is a promising tool for developing countries where TB is prevalent and is worthy of further investigation.
Chapter 3 Interferon-γ release assay

3.1 Introduction

Measuring the amount of IFN-γ is another way of diagnosing TB infection. In this chapter, two immunoassay techniques were used to detect IFN-γ in low concentrations.

ELISA was performed to detect IFN-γ. Mab to IFN-γ was coated on a polystyrene plate to capture IFN-γ molecules. The dotLab® system was another approach of detecting IFN-γ, which possibly had the limit of detection below nanogram per milliliter range. Similar to what had done in the Chapter 2, a series of experiments were performed to enhance the sensitivity of the IFN-γ detection, but also to reduce the non-specific binding occurring between proteins.

3.2 Experimental

3.2.1 Materials

All IFN-γ proteins, mouse mab to IFN-γ (clone #: M32185), rabbit polyclonal antibody to IFN-γ (lot #: X09121003), and human recombinant IFN-γ antigen (lot #: A09121030) were purchased from Fitzgerald. OmniPur® bovine serum albumin (BSA), fraction V (lot #: 2374B96) was used for blocking purposes. GAR-HRP was purchased from Sigma Aldrich (lot #: 057K4802). Biotin-GAM was purchased from Calbiochem®. Peroxidase substrates, ABTS (1-
component) (lot #: 040427) and TMB membrane (1-component) (lot #: 040368), were both purchased from KPL and used without dilution. Tris-Tricine-SDS (sodium dodecyl sulfate) running buffer (20X), LDS (lithium dodecyl sulfate) sample buffer (4X), and DTT (dithiothreitol) reducer (10X) were all from C.B.S. scientific. Proteins used for the electrophoresis experiment, GAM-HRP antibody (lot #: 13869) and rabbit IgG HRP were purchased from Rockland Immunochemicals Inc., and rabbit anti-mouse (RAM) (lot #: 315-005-046) was purchased from Jackson ImmunoResearch Laboratories, Inc. Molecular weight marker (lot #: 00064086) was purchased from Fermentas Life Sciences. All protein samples were diluted in deionized water.

3.2.2 Procedure

3.2.2.1 ELISA

All protein samples were diluted in PBS (pH 7.4) unless specified, and the plate was washed for 4 times with 0.05% PBS-Tween 20 between incubation steps. As shown in Figure 3.1, the procedure started with the mab to IFN-γ to capture IFN-γ molecules. The mab to IFN-γ was diluted in Na₂CO₃ buffer (0.05M, pH 9.6) to make 2 µg/ml solution and a 96 well polystyrene plate was coated with 50 µl of the solution. The plate was incubated overnight (~12 hours) at 4 °C. The coat was removed the next day and the plate was then incubated with 100 µl of BSA (5 mg/ml) for 3 hours at room temperature. IFN-γ was only added to the first 6 columns and it was serially diluted along the column. Aliquots of 50 µl of PBS were added to all wells except the first row, which contained 100 µl of IFN-γ with concentration 0.5 µg/ml. Aliquots of 50 µl of the sample from each well in the first row were transferred to the corresponding wells in the second row. The process was repeated until the eighth row was reached. Remaining 6 columns did not contain IFN-γ but only 50 µl of PBS.
After 1 hour of incubation with IFN-γ, 1 µg/ml of rabbit polyclonal antibody to IFN-γ, followed by an addition of 1 µg/ml of GAR-HRP antibody. Both proteins were incubated with 50 µl of the solution for 1 hour at room temperature. ABTS (50 µl per well) was finally added to all wells and measured at 410 nm with the microtiter plate reader.

Figure 3.1 Schematics of ELISA for IFN-γ assay. (1) Mab to IFN-γ, (2) BSA block, (3) IFN-γ, (4) rabbit polyclonal antibody to IFN-γ, (5) GAR-HRP antibody, and (6) ABTS peroxidase substrate

3.2.2.2 Diffraction-based sensing for IFN-γ assay

3.2.2.2.1 First Approach: biotin-GAM antibody

Axela’s dotLab® system and avidin sensor were used for the experiment. Biotin-GAM antibody was loaded first to provide a binding surface for mab to IFN-γ and the remaining steps (Table 3.1) were kept the same as the ELISA experiment, except for the use of TMB instead of using ABTS as the peroxidase substrate. TMB substrate was used because it allows for a physical height change when insoluble precipitate accumulates on the sensor surface. All protein samples were diluted in PBS (pH 7.4).
Table 3.1 Proteins used for IFN-γ assay with the biotin-GAM antibody. The incubation mode was mix for all reagents.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Incubation Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-GAM antibody</td>
<td>50 µg/ml</td>
<td>4</td>
</tr>
<tr>
<td>Mab to IFN-γ</td>
<td>15 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 µg/ml (+ve control)</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to IFN-γ</td>
<td>2.5 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>GAR-HRP antibody</td>
<td>1 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>TMB</td>
<td>N/A</td>
<td>6</td>
</tr>
</tbody>
</table>

3.2.2.2.2 Second Approach: biotin-mab to IFN-γ

Using biotin-GAM antibody, however, involved total of six steps, which not only lengthened the assay but also caused false positive signal due to non-specific binding. Direct conjugation of biotin to the mab to IFN-γ could remove the first step, the biotin-GAM antibody.

3.2.2.2.1 Biotinylation

Similar to what had been done for the TB antigens, the biotin conjugation reaction was mediated by EDC to form an active ester with NHS. A small amount of biotin-hydrazide (1-2 mg) was dissolved in 40 µl of DMSO and added to a tube containing 200 µl of the mab with a concentration of 2.05 mg/ml. An equal amount of EDC and NHS (10 mg each) were dissolved in
50 μl of PBS (pH 7.4) and added to the tube containing biotin-hydrazide and the protein sample. The reaction mixture was left on an orbit shaker with gentle agitation and incubated overnight (~12 hours) at 4°C.

3.2.2.2.2.2 Purification

MWCO spin column (Millipore) was used to purify the reaction mixture using the procedure described in the manufacturer’s User Guide. The reaction mixture was carefully transferred into a column with MWCO of 30 kDa, which was 5 times smaller than the typical size of antibody, and centrifuged for 4 times at a speed of 14,000 x g for 24 minutes per spin. Protein degradation was minimized by maintaining temperature at 4°C throughout the centrifugation. After each spin, the volume was restored with 200 μl of PBS (pH 7.4) and filtrate solution was also collected in a separate tube. The final protein volume was restored in 200 μl of PBS and stored in a new tube.

3.2.2.2.2.3 Characterization

The purified protein was characterized by UV/Vis absorption at 280 nm and the dotLab® system. The concentration of the purified protein was calculated based on fitting to a linear regression data of a calibration curve of mab, which had a concentration range of 1.6 μg/ml to 100 μg/ml. The purified protein was diluted 20 fold in PBS before the measurement. Characterization using the dotLab® system could provide two types of information: efficiency of the biotin conjugation reaction and the activity of the purified protein. The amount of signal increase due to the biotin-mab to IFN-γ would estimate how much biotin has been conjugated. The activity of the protein could be confirmed by subsequent incubation with the GAM-HRP
antibody. The dotLab® characterization with avidin sensor used 70 μg/ml of the biotin-mab to IFN-γ and 50 μg/ml of GAM-HRP antibody.

### 3.2.2.2.2.4 The dotLab® measurement

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Incubation Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-mab to IFN-γ</td>
<td>100 μg/ml</td>
<td>10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 μg/ml and below</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to IFN-γ</td>
<td>20 μg/ml</td>
<td>10</td>
</tr>
<tr>
<td>GAR-HRP antibody</td>
<td>0.5 μg/ml</td>
<td>10</td>
</tr>
<tr>
<td>TMB</td>
<td>N/A</td>
<td>6</td>
</tr>
</tbody>
</table>

| **Table 3.2** | Protein used for IFN-γ assay with the biotin-mab to IFN-γ produced by EDC/NHS conjugation reaction. The incubation mode was mix for all reagents. |

The biotin-mab to IFN-γ was introduced first to provide a binding surface for IFN-γ. As shown in the Table 3.2, the remaining steps were kept the same as previous experiment when the biotin-GAM antibody was used as a capturing antibody. All protein samples were diluted in PBS (pH 7.4). Different IFN-γ concentrations were tested and a calibration curve was constructed by plotting the normalized signal (TMB signal divided by biotin-mab to IFN-γ signal) against the concentration of IFN-γ.
3.2.2.2.3 Third Approach: Rabbit polyclonal antibody to IFN-γ HRP

Instead of using GAR-HRP, the rabbit polyclonal antibody could be labeled with HRP and this might help to increase the detection limit of IFN-γ.

3.2.2.2.3.1 HRP conjugation reaction

SureLINK HRP conjugation kit (KPL) was used and following the procedure described in the manufacturer’s manual. The rabbit polyclonal antibody to IFN-γ (50 μg) was reconstituted in 100 μl of HRP conjugation buffer to make a 0.5 mg/ml solution. The amount of SureLINK Activated HRP required was prepared based on the Table 2 in the manual and added to the antibody solution. The protein mixture was incubated in 4°C overnight (~12 hours) in gentle agitation. The next day, 10 μl of reducing agent (NaCNBH₃) was added and incubated at room temperature for another 15 minutes. An equal reaction volume of HRP Storage Buffer, 210 μl, was added to the reaction vial and incubated for 15 minutes at room temperature.

The rabbit polyclonal antibody to IFN-γ HRP was then characterized by gel electrophoresis and ELISA.

3.2.2.2.3.2 Gel electrophoresis

Protein samples used for the electrophoresis were rabbit polyclonal antibody to IFN-γ HRP, rabbit IgG HRP, activated HRP, and rabbit anti-mouse (RAM) antibody. Electrophoresis was performed under reducing conditions where a small amount of DTT reducer was added to the protein samples. This reduced the disulfide bonds of the antibody and separated the antibody into heavy and light chains. A total volume of 20 μl for each sample was prepared, which
consisted of 65% of protein sample in deionized water, 25% of LDS sample buffer (4X), and 10% of reducing agent (10X). The mixture was then incubated at 70 °C for 10 minutes and used within 2 hours to prevent reoxidation.

<table>
<thead>
<tr>
<th>Well #</th>
<th>Protein</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (6)</td>
<td>Molecular Weight Marker</td>
<td>N/A</td>
</tr>
<tr>
<td>2 (7)</td>
<td>rabbit polyclonal HRP antibody to IFN-γ HRP</td>
<td>30 µg/ml</td>
</tr>
<tr>
<td>3 (8)</td>
<td>Rabbit IgG HRP</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>4 (9)</td>
<td>Activated HRP</td>
<td>150 µg/ml</td>
</tr>
<tr>
<td>5 (10)</td>
<td>RAM antibody</td>
<td>180 µg/ml</td>
</tr>
</tbody>
</table>

Table 3.3 Proteins used for SDS-PAGE experiment.

ClearPAGE™ Tris-Tricine-SDS Running Buffer was used to run the gel. 12% Precast Gels (BioRad Laboratories) was mounted on an electrophoresis apparatus (C.B.S. Scientific Co.) and the running buffer was added to the anode and cathode chambers, which were separated from each other such that electrical current only flowed through the gel. All 10 wells of the gel were pre-wetted with the running buffer before loading the samples. 10 µl of each sample was carefully added to a well and its duplicate to 4 wells away from it; for example, well number 1 and 5 contained the same sample. (Table 3.3) Once all samples were loaded, the apparatus was run at 120 V until the LDS sample buffer reached the bottom of the gel.

The gel was removed from the plastic applicator and stained in Coomassie blue solution (Coomassie brilliant blue R250 in 40% methanol and 10% acetic acid) for 5 minutes with gentle
agitation. The gel was then washed in destaining solution (40% methanol, 10% acetic acid) to remove excess dye, and immersed in the solution overnight with gentle agitation.

3.2.2.3.3 ELISA

Biological activity of the rabbit polyclonal IFN-γ antibody HRP was tested using ELISA. Two ELISAs were run simultaneously on two separate 96 well plates; one plate contained rabbit polyclonal antibody to IFN-γ HRP and the other plate contained rabbit polyclonal antibody to IFN-γ and GAR-HRP antibody pair. (Figure 3.2 and Figure 3.3) Each plate was divided into 2 regions; the first 6 columns included IFN-γ antigen and the other 6 columns were used for negative control. All protein samples were diluted in PBS (pH 7.4) unless specified and the plates were washed 4 times with 0.05% PBS-Tween 20.

An aliquot of 50 μl of mab to IFN-γ (1.5 μg/ml) in Na₂CO₃ buffer (0.05M, pH 9.6) was added to each well for both plates and incubated overnight at 4°C. Coat was removed on the next day and the plate was blocked for 3 hours with 100 μl of 5 mg/ml BSA per well. In the first 6 columns, IFN-γ was serially diluted with 1:1 dilution of 0.25 μg/ml. The other 6 columns were negative control hence no IFN-γ was added but 50 μl of PBS. Both plates were left for 1 hour at room temperature. Both the HRP conjugated and non-conjugated rabbit polyclonal IFN-γ antibodies were diluted to 1 μg/ml, and a 50 μl of the solution was added to each well and incubated for 1 hour at room temperature. ABTS (50 μl) was added to the first plate and absorbance was measured at 410 nm after ~10 minutes. The second plate was incubated with 1 μg/ml of GAR-HRP, 50 μl per well, for another 1 hour. ABTS was added, 50 μl per well, for colour change and the plate was measured at 410 nm.
Figure 3.2  Plate 1: ELISA with rabbit polyclonal antibody to IFN-γ and GAR-HRP antibody. 
(1) Mab to IFN-γ, (2) BSA block, (3) IFN-γ, (4) rabbit polyclonal antibody to IFN-γ, 
(5) GAR-HRP antibody, and (6) ABTS peroxidase substrate

Figure 3.3 Plate 2: ELISA with rabbit polyclonal antibody to IFN-γ HRP. (1) Mab to IFN-γ, (2) BSA block, (3) IFN-γ, (4) rabbit polyclonal antibody to IFN-γ HRP, (5) ABTS peroxidase substrate
3.2.2.3.4 The dotLab® measurement

<table>
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<td>10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to IFN-γ HRP</td>
<td>20 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>TMB</td>
<td>N/A</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 3.4**  Protein used for IFN-γ assay with the biotin-mab to IFN-γ and the rabbit polyclonal antibody to IFN-γ HRP. The incubation mode was mix for all reagents.

The same biotin-mab to IFN-γ was used as a capture for IFN-γ. After incubating with IFN-γ, the rabbit polyclonal antibody to IFN-γ HRP and TMB peroxidase substrate was added. (Table 3.4) A huge TMB signal increase would be expected if the rabbit polyclonal antibody to IFN-γ was properly conjugated. All protein samples were diluted in PBS (pH 7.4).
3.2.2.2.4 Fourth Approach: biotinylation using commercial conjugation kit

3.2.2.2.4.1 Biotinylation

EDC mediated biotinylation was successful but the conjugation yield was low as the DI increase was ~7 fold lower than DI increased by typical biotinylated antibody. In order to resolve the issue with the efficiency of the conjugation and to enhance the sensitivity of the INF-$\gamma$ detection, a commercial biotin conjugation kit was used instead. The ChromaLink$^{\text{TM}}$ Biotin Protein Labeling kit (SoluLink) enables quantification the degree of biotinylation once the protein is purified, because the biotin molecule is conjugated to a traceable chromophore which absorbs light at 354 nm. The water soluble N-hydroxy-sulfosuccinimidyl ester functional group modifies lysine residues in a phosphate buffered system.

![Molecular structure and formula of Sulfo-ChromaLink Biotin](image)

The first buffer exchange procedure was performed with a Zeba$^{\text{TM}}$ buffer exchange spin column included in the kit. Determination of protein concentration after the first buffer exchange was necessary in order to determine the right amount of Sulfo-ChromaLink Biotin needed for the reaction. As suggested from the labeling protocol, the Bradford assay (Bio Rad Laboratories) was used to measure the concentration of the protein. However, the concentration calculated based on the Bradford assay was 0.55 mg/ml, only a quarter of the original concentration, 2.05
mg/ml. BSA was used for standard curve as described by the manufacturer’s guideline, but the mab to IFN-\(\gamma\) might contain unequal proportion of UV absorbing amino acids, tyrosine, tryptophan, and phenylalanine. The concentration was approximated using UV/Vis absorption at 280 nm in comparison to bovine IgG provided from the kit, and it turned out to be around 1.65 mg/ml.

The amount of Sulfo-ChromaLink Biotin calculated using ChromaLink Biotin protein labeling calculator was added to the mab to IFN-\(\gamma\) and incubated for 90 minutes at room temperature. The second buffer exchange step proceeded right after. Finally, the sample was scanned from 220 nm to 420 nm to quantify the amount of biotin incorporation. Concentration of the biotin-mab to IFN-\(\gamma\) was calculated based on the UV/Vis absorption at 280 nm, in comparison to the bovine IgG from the kit. It was 1.86 mg/ml, which was about 10% lower than the original concentration.

### 3.2.2.2.4.2 The dotLab® measurement

The conjugated proteins were then used for IFN-\(\gamma\) assay using the dotLab® system. Rabbit polyclonal antibody to IFN-\(\gamma\) and GAR-HRP antibody were used instead of rabbit polyclonal antibody to IFN-\(\gamma\) HRP. The same concentration of the biotin-mab to IFN-\(\gamma\) was used as before (100 \(\mu\)g/ml) but a greater DI change occurred in comparison to the DI increase by the biotin-mab to IFN-\(\gamma\) produced by the EDC mediated biotinylation. The binding curve showed that the biotin-mab to IFN-\(\gamma\) was saturating at a higher rate, almost instantly once incubation started, as the slope was steep. A reduced concentration could achieve the same amount of signal increase, but took more time to saturate; the concentration was decreased to 25 \(\mu\)g/ml and incubation time was increased to 15 minutes. In addition, IFN-\(\gamma\) was diluted in 5 mg/ml of BSA.
in PBS (pH 7.4) in order to increase complexity of the sample matrix; therefore, binding between mab and IFN-γ would be more specific. The incubation time for IFN-γ was also increased to 15 minutes. The rabbit polyclonal antibody to IFN-γ concentration was reduced to 10 µg/ml because it was already in excess compared to the antigen concentration. Concentration of the GAR-HRP antibody was increased to 1 µg/ml after confirming that it did not cause a false positive signal. The incubation mode for TMB was changed to static; the static mode allowed for more stable accumulation of TMB precipitate.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration</th>
<th>Incubation Duration (minutes)</th>
</tr>
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<tbody>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-mab to IFN-γ</td>
<td>25 µg/ml</td>
<td>15</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1 µg/ml and below</td>
<td>15</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to IFN-γ</td>
<td>10 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>GAR-HRP antibody</td>
<td>1 µg/ml</td>
<td>10</td>
</tr>
<tr>
<td>TMB</td>
<td>N/A</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 3.5** Protein used for IFN-γ assay with the biotin-mab to IFN-γ produced with the commercial biotin conjugation kit. The incubation mode was mix for all reagents except TMB. Static incubation mode was used for TMB.

Using the above concentrations stated in Table 3.5, the dotLab® system successfully demonstrated sensitive detection of IFN-γ in nanograms to sub-nanogram per milliliter, from 0.625 ng/ml to 10 ng/ml. The TMB signal was normalized by dividing it by the signal increase due to binding of the biotin-mab to IFN-γ. Finally, a calibration curve was constructed by plotting the normalized signal against the concentration of IFN-γ.
3.3 Results

3.3.1 ELISA

**Figure 3.5** Result of ELISA for IFN-γ assay. Error bars represent standard errors (n=6).

With the experimental condition used for the ELISA, IFN-γ antigen was successfully captured by the mab to IFN-γ. (Figure 3.5) A negative control assay done without the IFN-γ antigen resulted in a low absorbance value (0.043 ± 0.001). Also, each measurement was associated with a small error as indicated by narrow error bars, thus less non-specific bindings.
3.3.2 Diffraction-based sensing

3.3.2.1 First Approach: biotin-GAM antibody as a capture

The biotin-GAM antibody was introduced first and is represented by the initial DI increase in Figure 3.6. A signal increase was only observed for the biotin-GAM antibody and the mab while the rest remained at the baseline. This may be due to the small molecular size of the IFN-γ (17 kDa) but also the concentration used for the assay (1 µg/ml), which was too small to saturate the sensor surface. Because IFN-γ was the limiting reagent in this assay, the signal after IFN-γ incubation remained at the base line and the only measurable signal is observed with the TMB amplification. TMB signal, however, was not what one would normally observe; dipping of the signal was always apparent with all positive assays but not with negative assays without IFN-γ.

**Figure 3.6** The dotLab® measurement with the biotin-GAM antibody for IFN-γ assay
The TMB signal dip shown in figure 3.6 might be due to the non-specific binding of mab to IFN-γ to the surface between avidin patterns as well as on the avidin pattern, causing accumulation of TMB precipitate on both areas. The relative height differences of the pattern might have been decreased due to the deposition of the precipitate on both the pattern and the polystyrene plate, and as a result, the TMB signal decreased. Such a dip signal was not shown in the negative control assays (Figure 3.7), but a small TMB signal was observed, and might be due to the non-specific binding of the GAR-HRP antibody to biotin-GAM antibody and/or mab to IFN-γ.
Even if a slight increase after the signal dip was a real signal, the sensitivity of the assay would be too low to be a diagnostic test; the assay was performed with 1 µg/ml of IFN-γ, which is about 100 times higher than the clinically relevant concentration (10 ng/ml).\textsuperscript{13}

3.3.2.2 Second Approach: Biotin-mab to IFN-γ

3.3.2.2.1 Characterization

After purifying the reaction mixture with a MWCO spin column, the protein was characterized with UV/Vis absorption at 280 nm and the dotLab® measurement.

![Absorbance vs [IFN-γ] plot](image)

**Figure 3.8** Calibration curve of the mab to IFN-γ

The concentration of the biotin-mab to IFN-γ was calculated based on the best fit equation shown in Figure 3.8. The percent of recovery was 138%; recovered concentration (2.84 mg/ml) was higher than the initial concentration started with (2.05 mg/ml). Such difference in concentrations might be due to different protein assay technique used by the manufacturer.
Figure 3.9 The dotLab® characterization of the biotin-mab to IFN-γ with GAM-HRP antibody

Figure 3.9 shows that the purified biotin-mab to IFN-γ was able to bind to the avidin surface and capture GAM-HRP antibody, indicating that the mab IFN-γ was biotinylated and biologically functional.
3.3.2.2.2 The dotLab® measurement

IFN-γ was successfully detected with the biotin-mab to IFN-γ (Figure 3.10). Signal amplification was required due to the lack of diffraction intensity change observed after the addition of the biotin-mab to IFN-γ. A negative control assay (Figure 3.11) showed small background signal, indicating that there might be still some non-specific binding happening between proteins. The assays were done with many different concentrations of IFN-γ, all lower than 1 µg/ml. The TMB signal was normalized to the signal increase of the biotinylated antibody because each sensor may contain a different number of available avidin molecules; the TMB signal was divided by the biotin-mab to IFN-γ signal. The normalized signals were plotted against concentration of IFN-γ used. (Figure 3.12)
Figure 3.11  The dotLab® measurement with the biotin-mab to IFN-γ for IFN-γ assay, negative control

Figure 3.12  Calibration curve of IFN-γ assay: [IFN-γ] vs. normalized signal. Error bars represent standard errors (n=3).
Overall, the biotin-mab to IFN-γ was usable for detection of IFN-γ. However, assays had low sensitivity and failed to have consistency in measurements as indicated by large error bars. This suggests that it cannot be recommended as a diagnostic test for TB.

3.3.2.3 Third Approach: HRP conjugation

3.3.2.3.1 Characterization: gel electrophoresis

First, the HRP conjugated protein was checked by SDS-PAGE. A typical antibody breaks down into light and heavy chains in reducing conditions, which would result in the two lower bands shown in well 5. The two bands at the top of well 5 were non-reduced and partially reduced antibodies. The molecular weight of the HRP is 44 kDa and this corresponds to the band shown in well 3. Bands of the HRP conjugated antibody in well 4 were shifted higher compared to well 5, approximately 40 kDa heavier than the two bottom bands in well 5. The rabbit
polyclonal antibody to IFN-γ HRP in well 2 had two prominent bands and they appeared at similar heights as the two bands observed in the well 4 and none of the heavy and light chain bands appeared in well 5 were shown in well 2. All such evidence showed that the rabbit polyclonal antibody was HRP conjugated.

3.3.2.3.2 Characterization: ELISA

ELISA with rabbit polyclonal antibody to IFN-γ and GAR-HRP antibody successfully detected IFN-γ. (Figure 3.14) The negative control was 0.029 ± 0.001, indicating low background signal. However, the ELISA experiment with rabbit polyclonal antibody to IFN-γ (Figure 3.15) was not successful as there is no linearity shown in the graph. The high absorbance for the negative controls (0.060 ± 0.006) suggested that there is non-specific binding of the rabbit polyclonal antibody to IFN-γ HRP to the polystyrene plate.

The protein was conjugated with HRP but failure in the ELISA experiment implies that the biological activity of the protein had been compromised during the HRP conjugation reaction. It is also possible that HRP was over conjugated; excess amount of HRP had been added and this might caused blocking of the binding site of the antibody.
Figure 3.14  Result of ELISA for IFN-\(\gamma\) assay with rabbit polyclonal antibody to IFN-\(\gamma\) and GAR-HRP antibody. Error bars represent standard errors (n=6).

Figure 3.15  Result of ELISA for IFN-\(\gamma\) assay with the rabbit polyclonal antibody to IFN-\(\gamma\) HRP. Error bars represent standard errors (n=6).
3.3.2.3.3 The dotLab® measurement

The initial intent of HRP conjugation was to improve the sensitivity of the assay. However, as shown in Figure 3.16, sensitive detection of IFN-$\gamma$ could not be achieved. Even with the same IFN-$\gamma$ concentration, 1 $\mu$g/ml, the assay did not produce a similar degree of amplification in comparison to the one performed with the GAR-HRP antibody. This also suggests that the protein may no longer be active and its biological function had been lost during the conjugation process.

![Figure 3.16](image)

**Figure 3.16** The dotLab® measurement with the biotin-mab to IFN-$\gamma$ and rabbit polyclonal antibody to IFN-$\gamma$ HRP for IFN-$\gamma$ assay
3.3.2.4 Fourth Approach: biotinylation using a commercial kit

A more efficient biotinylation reaction could be achieved with the ChromaLink™ Biotin Protein Labeling kit.

3.3.2.4.1 The dotLab® measurement

As a consequence of using the biotin-mab to IFN-γ, a more sensitive detection of IFN-γ was established. Negative control measurements without IFN-γ proved that the cross reactivity was minimal. Figure 3.17 shows the TMB signal obtained with 10 ng/ml of the IFN-γ. Different IFN-γ concentrations were examined using the same experimental conditions and a calibration curve was constructed using normalized TMB signals (Figure 3.18). Overall, a sensitive detection of IFN-γ was achieved with the detection limit below 1 ng/ml of IFN-γ.

Figure 3.17  The dotLab® measurement with the biotin-mab to IFN-γ, produced by commercial kit, for IFN-γ assay.
Figure 3.18  Calibration curve of IFN-γ assay: [IFN-γ] vs. normalized signal. Error bars represent standard errors where n=2.

As shown in the Figure 3.17, a higher DI change was exhibited by the biotin-mab to IFN-γ than before when EDC and NHS reaction scheme was used.

Figure 3.19  Diffraction intensity change with different concentrations of the biotin-mab to IFN-γ. Concentrations were (1) 25 µg/ml, (2) 50 µg/ml, and (3) 100 µg/ml of the biotin-mab to IFN-γ and they were conjugated with the commercial kit. The curve (4) was with 100 µg/ml of the mab to IFN-γ produced by EDC mediated reaction.
Curve (1), (2), and (3) in the Figure 3.19 were the signal obtained by biotin-mab to IFN-γ which was conjugated with the commercial kit. Even though the concentrations used were different, all reached about the same DI change at the end. The only difference was the time required to saturate the surface, where higher concentration solutions saturated the sensor spot more rapidly. Curve (4) is the amount of signal increased by biotin-mab to IFN-γ produced by EDC mediated conjugation reaction and the amount of signal was about 7 times lower than the signal caused by the biotin-mab to IFN-γ produced by the commercial kit. A higher signal indicates more proteins bound to the surface, thus providing a greater density of the capturing antibody available for IFN-γ.

3.4 Discussion

A number of experiments were performed in order to improve the sensitivity of IFN-γ assays on the dotLat® system. Sensitivity could be increased with fewer number of steps when biotin-GAM antibody and GAR-HRP antibody were eliminated by conjugating biotin to the mab to IFN-γ and HRP to the rabbit polyclonal antibody to IFN-γ. Biotin conjugation using the ChromaLink™ Biotin Protein Labeling kit was successful but HRP conjugation was not successful, thus rabbit polyclonal antibody to IFN-γ HRP could not be used for the IFN-γ assay. SDS-PAGE gel suggested that the rabbit polyclonal antibody to IFN-γ was HRP conjugated but ELISA experiments suggested that the HRP was no longer active. The dotLab® measurement further confirmed its inability to detect INF-γ. The most likely explanation of the loss in activity was that HRP was over conjugated to the antibody, especially to its binding sites. Reducing the amount of the activated HRP used for the conjugation reaction may help to retain the biological activity of the antibody. Also, a purification process, such as a MWCO spin column or a
chromatographic method, needs to be incorporated after the reaction in order to remove unreacted HRP molecules. The ELISA characterization with the rabbit polyclonal antibody to IFN-γ HRP resulted in a high background signal (0.060 ± 0.006) and large error bars, which might be a result of non-conjugated HRP present. (Figure 3.15)

Rapid saturation of the sensor surface is expected due to the robust avidin and biotin interaction \( (K_a \approx 10^{15} \text{ L/mol}) \), especially with high biotin-antibody concentration (i.e. 50 μg/ml). The typical DI change by 50 μg/ml of biotinylate antibody was 0.35 to 0.4. Although the calculated concentration of the mab to IFN-γ based on the UV/Vis measurement was similar to the initial protein concentration, the biotin conjugation reaction was not as efficient because the amount of signal increase was only ~0.05; this was 7 times lower in magnitude than signal produced by the commercial biotinylated antibody.

![Figure 3.20](image)

**Figure 3.20** Comparison of biotinylated antibody signals.

As shown in the Figure 3.20, the biotin signal produced by 100 μg/ml of biotin-mab to IFN-γ was about 7 times lower than the signal produced by 50 μg/ml of the biotin-GAM antibody, even with its higher protein concentration. Such a difference indicates that the EDC mediated biotinylated reaction was not efficient and only few biotin molecules were conjugated
to the mab. However, the major drawback of the reaction scheme could be a self reaction between the mabs; the active ester intermediate formed with NHS and a mab could react with another mab because some amino acid residues (arginine and lysine) contain primary amine as well. (Figure 2.3)

IFN-γ is not an exclusive signal to TB infection; it was originally discovered as an agent that interferes with viral replication. It is a cytokine molecule that plays a mandatory role in acquired protective immunity to pathogenic mycobacteria and other intracellular pathogens, by orchestrating many distinct cellular programs through transcriptional control over large number of genes. Due to its significance from an immunological view point, measuring IFN-γ might have a broader spectrum of application when concerning infectious diseases other than TB.
Chapter 4 Conclusion

The basic principle of an immunoassay relies on a binding between an antigen and its antibody pair, which has led to the development of various immunoassay techniques that enable detection of particular antigen or antibody in clinical samples. An alternative strategy to ELISA, the most commonly used immunoassay technique, can be diffraction-based sensing, which is one of the emerging methods in the field of protein research that monitors changes in the diffracted light due to the binding activity of the analyte onto a substrate.

This study showed the effectiveness of the dotLab® system in detecting TB antibody and IFN-γ. Rabbit anti-TB antibody could be detected with biotin-38 kDa TB antigen, and possibly with other TB antigens once they are biotinylated. Instead of using one single antigen, a mixture of antigens can partially be used to increase sensitivity of the technique. The dotLab® system also enables sensitive detection of IFN-γ, with its detection limit below the nanogram per milliliter range. Of the two biotin conjugation approaches, the ChromaLink™ biotin protein labeling kit was more efficient and resulted in a higher DI increase. Better sensitivity was expected with HRP conjugated rabbit polyclonal antibody but the HRP conjugated antibody did not improve the assay. Smaller amounts of activated HRP may need to be used and a purification process may need to be incorporated once the reaction is done. An excess amount of activated HRP may cause over conjugation of an antibody even on the binding sites of the antibody, thus the HRP conjugated antibody would not be active anymore. In addition, a large quantity of left over HRP molecules could cause a false positive signal due to non-specific binding; thus, it may
be beneficial if the reaction mixture is purified after the conjugation reaction, before long term storage.

Figure 4.1 Calibration curve of IFN-γ assay showing clinically relevant IFN-γ concentrations. The point (a) represents the median IFN-γ release (3 ng/ml) in the TB patients. The point (b) is the median IFN-γ release from BCG-vaccinated individuals (0.2 ng/ml). Overall, the dotLab® system was able to detect a low quantity of IFN-γ (0.625 ng/ml) present in a complex medium, 5 mg/ml of BSA in PBS. Although the two points shown in the calibration curve (Figure 4.1) are well resolved, a better sensitivity is required for differentiating BCG-vaccinated and non-vaccinated individuals because the median of the clinically relevant concentrations for both cases are lower than the detection limit observed (0.625 ng/ml); the median values for IFN-γ release are 0.2 ng/ml and 0.03 ng/ml, respectively. The sensitivity of the assay can be improved with use of the HRP conjugated rabbit polyclonal antibody to IFN-γ, and ultimately, it can be used for TB diagnosis for HIV infected patients whose IFN-γ release has been smaller than non-HIV infected patients.
The dotLab® system provided many advantages over ELISA in terms of the duration of the assay, observation in real time, high sensitivity, and the ease of use and convenience. It is adopted successfully as a method in many other studies; including detection of recombinant protein NT-proBNP using biotinylate mab. Generally, it is a very sensitive method that allows real time observation without using labels; thus, it has a bright future in the field of protein research and has the potential to be one of the primary clinical diagnostic tools for the discovery of many diseases.
### Bibliography


31. ChromaLink™ Biotin Protein Labeling Kit, User Manual