Detection of Human Transferrin Protein Using Sandwich ELISA Assay

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Abstract

Sandwich ELISA is a powerful diagnostic tool that is widely used in bioanalytical immunoassay for protein detection and quantification due to its high sensitivity and specificity. However, several factors are contributed for determining the limit of detection and the dynamic range of this technique such as the optimum concentration of immobilized antibodies, the maximum reduction of background noise by blocking the non-specific site and preventing the cross reactivity. This project has evaluated a sandwich ELISA technique for the detection of human transferrin in a buffer sample. Human transferrin is serum glycoprotein responsible for transporting iron in human body. The majority of the assay trials was conducted using goat polyclonal anti-transferrin as captured and detecting antibodies, in addition to HRP anti goat IgGs as secondary antibodies. However, the results were not satisfactory due to the high background noise resulting from the non-specific binding between the HRP anti-goat IgG and both the captured immobilized anti-transferrin and the surface of the well. Furthermore, several contribution factors were verified such as the washing solution by which PBST with concentration of 1% was high enough to wash all the antibodies out the well and produce negative results, the BSA blocking was sufficient to block the none specific site, the immobilisation of antibodies by physical adsorption was verified successfully and finally the efficiency of HRP enzyme and the recommendation for optimum ABTS concentration were examined. However, another trial using monoclonal anti-transferrin and its conjugate HRP monoclonal anti transferrin. The results of this trial were positive and correlated with the captured human transferrin in the well. The assay was more effective than using polyclonal antibodies since less step required to finish the assay. However, more optimisation of the protocol is required to evaluate the detection limit and the dynamic range of this technique. In addition, the use of new technologies such as microfluidic and gold nanoparticle have a great promises for improving this assay toward a point of care device.
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Chapter 1

1. Introduction and background theory

The need for sophisticated bioanalytical tools for the detection of the biomolecules has driven the researches toward exploiting new technologies in developing high sensitive, reliable and high throughput devices. The enzyme linked immunosorbent assay (ELISA) is one of the most common analytical biosensors that used for the detection and quantification of proteins. It has been considered a powerful diagnostic tool for the detection of immunological problems such as infectious diseases, the hormonal disorders and the cancer due to its high sensitivity and specificity.

This project aims to evaluate one of ELISA techniques called sandwich ELISA for the detection of human transferrin protein and optimising the assay for better limits of detection and sensitivity. In addition, to study the different parameters and factors that influence the results of the assay such as the reagents types and concentrations and the assay protocols. This project will clarify the fundamental concepts of ELISA colorimetric detection of human transferrin and the future work to develop high sensitive protocol using new technologies such as gold nanoparticles and microfluidics.

1.1 Thesis Structure

This project is divided into four chapters by which the first chapter is the introduction and the theoretical background of ELISA that includes the antibodies and immune response, ELISA principles and review of human transferrin detection. The second chapter will introduce the material and methodology used in this project. The third chapter provides the results and discussion of experimental work. Finally the fourth chapter includes the conclusion, project limitation and future work.

1.2 History of ELISA

Recently, Enzyme linked immunosorbent assay (ELISA) has become an important analytical technique in medical laboratory for the detection and the quantification of protein or peptide based samples. In 1960, Solomon Berson and Rosalyn Yalow have introduced the application of radioimmunoassay (RIA) to measure plasma insulin based on labelling the antigen with a radioactive isotope [1]. A new method of solid-phase radioimmunoassay has been developed by Catt and Tregear in 1967 to coat the antibodies over a polymeric surfaces by adsorption and it drove the development of the microtiter plates that has been used for different types of immunoassays [2]. However, Miles and Halse had successfully labelled the antibodies with a radioactive material instead of labelling the antigen itself in 1968 [3].
Engvall and Perlmann in 1971 have demonstrated the first quantitative enzyme linked immunoassay by conjugating rabbit Immunoglobulin IgG with alkaline phosphatase enzyme, and in the same year Weemen and Schuurs have successfully developed another immunoassay to detect human chorionic gonadotropin (HCG) hormone by tagging the antibodies with horseradish peroxidase (HRP) enzyme [4][5]. Furthermore, between the late 1970s and the early 1980, ELISA has become commercially available in the market when the first assay for hepatitis B has been developed by Organon Teknika and marketed in 1976 [6]. Hence, ELISA has been considered as the gold standard and the powerful tool for diagnosing great number of illnesses such as infectious diseases, cancer and hormonal disorders [6].

However, the classical microtiter plate ELISA suffered from several challenges such as long incubation time, the high cost of the reagents, the complexity of the assay, the narrow dynamic range and the low sensitivity [7], in addition, other fundamental problems such as antibodies tagging and immobilising, antibodies-antigen binding and the lack of portability have been also reported. Therefore, several advancements have been occurred to overcome these limitations, for example, the development of microfluidic ELISA that minimised the volume of the reagents and the time needed to perform the assay [8], the use of magnetic microbead to improve sensitivity [9], silver enhancement with gold nanoparticles to amplify the generated signal [10][11] and the use of Surface Plasmon Resonance (SPR) detection method [12].

1.3 Antibody and immune response

Antibodies have been introduced in 1890 by Behring and Kitasato who reported the presence of substance that capable to neutralize the toxin of diphtheria in the blood [13]. Antibody or it is also referred as immunoglobulin (Ig) is a glycoprotein molecule that produced by immune system (plasma cell) in response to the presence of an antigen or foreign body.

1.3.1 The structure of antibody

The basic structure of the immunoglobulin is illustrated in figure 1 and it consists of:

![Antibody structure](image_url)  
*Fig. 1: Antibody structure [14].*
1- Two identical heavy chains- H with molecular weight of 55 kDa and two identical light chains- L with molecular weight of 25 kDa to form a Y shape structure.

2- The two heavy chains are held together at the hinge flexible region by disulphide bonds.

3- The heavy and light chains are also joined together by disulphide bonds.

4- Each chain has one NH₂-terminal variable domain (Vₕ and Vₗ) and another constant COO-terminal domain (Cₕ and Cₗ).

5- The light chain (L) structure can contain either ʎ or κ chain based on the sequence and the type of amino acids formed the constant domain Cₗ.

6- Oligosaccharide molecules are also attached to the constant domain of heavy chain Cₕ.

7- Immunoglobulin can be fragmented by proteolytic enzymes into:
   
a- Fab fragment that is produced by cleaving the hinge region with papain enzyme resulting in the formation of two identical Fab parts and one Fc as shown in figure 2(a).

b- F(ab')₂ fragment can be created by pepsin enzyme treatment that cleave the bond between Fc and Fab fragments as shown in figure 2(b).

Furthermore, immunoglobulin can be classified into five classes according to the different types and sequences of amino acids in the heavy chain. These classes are IgA, IgM, IgD, IgE and IgG by which it is the most dominant immunoglobulin in the blood serum, it forms about 75% of the total amount of immunoglobulin and it has a molecular weight of 150 kDa [15].

1.3.2 Antibody – Antigen interaction

Each Fab fragment of the antibody consists of two variable domains (Vₕ and Vₗ) and two constant domains (Cₕ and Cₗ). The two variable domains are also divided into three variable segments that folded into loop shape. When the two variable domains are paired together within the Fab fragment, these segments create a specific site at the tip of the fragment which is called paratope and it is considered the binding site for the antigen. Hence, these segments form a complementary region to the antigen binding site that gives the specificity of the antibody. These regions are called complementary...
determining regions or CDRs and they vary by producing different sequences of the variable segments [16].

The antibody can identify the antigen by recognising a small part of its surface called epitope, then the antigen and the antibody bind together by different type of forces such as electrostatic interaction, hydrophobic interaction, van der Waals forces and hydrogen bonds. The strength of binding between the antibody and the antigen is called affinity by which the higher affinity will increase the sensitivity, decrease the possibility of the antibody to lose the bound antigen and enable the antibody to bind to low antigen concentrations. However, there are several factors that may interfere with this binding when running an immunoassay experiment, for instance, the high concentrations of ions in the solution, the use of detergents in washing steps, the pH, the purity of the antibodies and the temperature [16].

Moreover, the number of antibody – antigen complex formation in the immunoassay where the antibody has been immobilized on the surface of the microtiter plate is inversely proportional to the size of the antigen and it depends on the density of immobilised antibodies and the method of immobilisation [17].

1.3.3 Monoclonal and Polyclonal Antibodies

Polyclonal antibodies are mixture of heterogeneous antibodies that can bind to multiple epitopes in the same antigen. The process of producing polyclonal antibodies is illustrated in figure 3.

![Fig. 3: Polyclonal antibodies production process][19].

The following steps demonstrate the method of generating these antibodies:

1. A host animal such as rabbit or goat is immunized with an antigen or immunogen. This will stimulate the immune system to generate antibodies against this antigen.

2. The host animal is boosted every two weeks till the end of immunization period that may last for 3-8 months.

3. The blood is collected from the host animal and the cells are removed by centrifuging.

4. The remaining serum that contains the polyclonal antibodies produced from different B-cells is undergoing into further purification to remove the other proteins from the serum [18].
There are several advantages of using polyclonal antibodies in immunoassay such as:

1- The low cost and the short production time.
2- They are stable and can be stored easily.
3- Their overall affinity is high as they bind with various antigen epitopes.
4- They are capable to capture the targeted antigen quickly.
5- They are suitable for robust frequent assays
6- They are frequently used as captured antibodies in sandwich assay [20].

On the other hand, the disadvantages of polyclonal antibodies include the variations in the antibodies for different production lots as a result of varying the response of the immune system for different host animals. Although the assay could be highly sensitive but it may not be very specific due to cross reactivity. However, the specificity can be increased by affinity purification that should be balanced with the decrease of antibody activity due to the multiple purification process. [21]

In contrast, monoclonal antibodies are homogenous immunoglobulin because they are produced from a single B cell clone of the same animal and they can only bind to one unique epitope in the antigen. Monoclonal antibodies are most likely produced in mice or rabbit as illustrated in figure 4.

![Fig. 4: Monoclonal antibodies production process [22].](image)

The following steps describe the method of generating the monoclonal antibodies:

1- The host animal is immunized with an antigen usually through peritoneal injection.
2- Immunization boost is given every two weeks till two months in mice and 4 months in rabbit.
3- Blood sample is taken frequently to test the generation of the antibodies.
4- The B cells are removed from the spleen and fused in myeloma cell line to form hybridoma.
5- The hybridoma cell line is incubated for culture and proliferation purpose.
6- The clone immunoglobulins are collected for purification [18].
Monoclonal antibodies have also several advantages in compared with polyclonal antibodies such as:

1- They have sustained and homogenous production for different batches.
2- There is no concern about cross reactivity as they bind only to one epitope.
3- They are suitable for quantification assay [20].

However, the disadvantages of monoclonal antibodies can be summarized in the following points:

1- More expensive and they need longer time for production.
2- They required more attention in storage.
3- They become unable to detect the antigen if any small deformation occurs to the epitope.
4- They are more sensitive to pH and buffer solution [20].

1.3.4 Antibody conjugating

The concept of ELISA detection method is based on enzyme mediated reaction to generate a coloured signal in a solution where the targeted analyte is present. Therefore, it is essential to conjugate the detected antibody with a suitable enzyme to produce the colour change. However, the first enzymes that have been used for antibody labelling were horseradish peroxidase (HRP) and alkaline phosphatase (ALP) [4][5].

Horseradish peroxidase (HRP) is the most common enzyme that have been used to conjugate the immunoglobulins because it generates an intense coloured solution that can be detected with various methods such as absorption, luminescence and fluorescence [23]. The abundant form of HRP isoenzymes is HRP C among more than 30 isoenzymes and it consists of a single polypeptide monomeric glycoprotein of 308 amino acids residues with pyroglutamate blocked the N-terminal residue while C-terminal is heterogeneous. The three dimensional structure of the enzyme has shown a largely α – helical structure with β-sheet region, in addition, there is a heme group that located between the distal and proximal domains as shown in figure. 5 [24][25].

[Image: HRP 3D structure [25].]
HRP mechanism of action includes the catalysis of a redox reaction as following:

\[ \text{H}_2\text{O}_2 + 2\text{XH}_2 \xrightarrow{\text{HRP}} 2\text{H}_2\text{O} + 2\text{XH}^+ \]

The catalytic cycle is initiated by a reaction between hydrogen peroxide and the Ferritin (Fe III) in the HRP heme to produce a high oxidative compound oxoferryl (Fe IV^{+2}). Then the reducing substrate \( \text{XH}_2 \) will be oxidised by losing one electron to oxoferryl (Fe IV^{+1}) and produce \( \text{XH}^+ \), therefore, two compounds of the substrate \( \text{XH}_2 \) is needed to return Ferritin into resting state [25].

Thus, this reaction is relatively slow because it has intermediate reactions with different rate constants, therefore, the time of the detection should be considered when reading the results of the immunoassay. Aromatic phenols, phenolic acids, indoles, amines and sulfonates are the typical substrates that have been used with HRP enzyme [25].

Furthermore, HRP can be conjugated with immunoglobulin IgG by producing aldehyde group through oxidizing the monosaccharide residues of the enzyme with sodium periodate. The aldehyde group is highly reactive and it binds directly to the amino groups in the immunoglobulin, however, glutaraldehyde could be used to conjugate HRP with the IgG but their activity will be low since more antibodies will bind together other than with HRP. The conjugates have a range of molecular weights between 500-1000kDa since more than one HRP enzyme could bind to one IgG [23].

Finally, the turnover number of HRP is about 66000 min\(^{-1}\) that means the maximum number of the substrate that can be oxidized by HRP per second is 300 per catalytic site. Therefore, a proper time should be considered for the reaction to get a significant signal for low concentration analyte and it is most likely not less than 30 minutes [36].

1.4 ELISA principles

Enzyme linked immunosorbent assay (ELISA) and enzyme immunoassay are biochemical techniques that widely used in immunological laboratories to detect either the antibody or the antigen that present in a sample. ELISA has been used as diagnostic tool in medicine and other biological applications, in addition to various industries, for example, food industry as quality control check. It also has been considered in the field of biomedical researches that required detection and quantification of specific protein in a given sample. Recently, ELISA has become the most popular assay for high throughput screening and single molecule analysis [26][27].

The basic principle of ELISA exploits the immunological response that occurs as a result of antibody-antigen reaction by which one of these reactors is immobilized to a solid surface such as polystyrene
microplate. Therefore, as the antibody and the antigen come together in the microplate, they will bind and form antibody-antigen complex that attach to the surface of the microplate, however, the unbound antigens and antibodies will be washed out. Thus, the antibody-antigen complex will only be formed if the sample contains the specific targeted analyte. The purpose of the enzyme conjugated with the antibody is to amplify and visualize the formation of this complex in the well by catalysing a reaction of a chromogenic substrate that produces the coloured signal intensity proportional to the concentration of these complexes [27][28].

1.4.1 Types of ELISA

There are four types of ELISA based on the method of antigen capturing and detection.

- **Direct ELISA:**
  - This assay is used for evaluating the specificity of the antigen and eliminating cross reactivity between antibodies.
  - The process can be summarized as following, 1- specific antigen is immobilised to the well of the microtiter plate. 2- a predetermined concentration of labelled antibodies is added followed by enzyme substrate. This process also is illustrated in figure 6.
  - The disadvantages of this assay are the less sensitivity and the weakness of generated signal [27].

![Fig. 6: Direct ELISA](image)

- **Competitive ELISA:**
  - The concept of this type of ELISA is based on the competition between the antibody-antigen complex that is coated in the microwells and the antigen in the sample. The advantage of this assay is the high sensitivity, in addition, it is useful to detect smaller molecules [29].
  - The principle of this assay include the incubation of the primary antibodies and the antigen together in the microwells to form the antibody-antigen complex. Then, the sample that contains the antigen is added by which it will compete with the bound antigen resulting in releasing the antibodies from the complex and bind to the soluble antigen in the solution.
Next, these soluble antibodies-antigen complexes will be washed out and secondary labelled antibodies are added followed by the substrate. Thus, the presence of the antigen in the sample will result in washing the primary antibodies out the wells and consequently the higher antigen concentration in the sample the less colour intensity produced [27][28].

- **Indirect ELISA:**
  - It is usually used to detect specific antibody in the given sample.
  - The process to perform this assay beginning with immobilization of the specific antigen is to the wells of the microtiter plate. Then, the primary antibodies are added and bind to the immobilized antigen to form antigen-antibody complex. Next, the secondary labelled antibodies are added by which the primary antibodies are the antigens for the secondary antibodies and they will bind together. This process is illustrated in figure 7.

![Fig. 7: Indirect ELISA [26]](image)

- The disadvantage of this method is the lack of antigen specificity because when the antigen is coated to the well and the serum sample is added, the other proteins in the serum will also adhere to the wells and compete with the specific binding site of the antibodies that could bind none-specifically with those proteins [27][29].

- **Sandwich ELISA:**
  Sandwich ELISA assay has been frequently used to detect analyte samples due to its high sensitivity, high specificity, in addition, it has wider dynamic range and low background noise compared with other methods.

This assay exploits two antibodies, the first one is the captured antibody that used to coat the surface of the microplate wells and the other one is the detecting antibody. Therefore, the antigen will be sandwiched between these two antibodies. However, a secondary antibody that conjugated with an enzyme such as HRP is used to amplify the signal by catalysing a suitable substrate reaction. The process of this assay is illustrated in figure 8 [35].
However, this method has some limitations such as the antigen should have at least two epitopes for binding since two antibodies would bind to the antigen. Furthermore, if the captured antibody that immobilized to the well surface is significantly large, it will prevent the secondary antibody from binding to the antigen due to steric hindrance effect. However, studies suggested that the minimum distance between two epitopes in an antigen should be at least 11.7 Å to have high potential to be detected by sandwich ELISA technique [35].

1.4.2 The sensitivity of commercial ELISA and new technologies

Recently, great demand on optimising different aspects of assay technique has become the most challenging to increase the dynamic range of ELISA assay, improve its detection limits and subsequently increase the sensitivity. The substantial development of nanotechnology leads to more sophisticated and sensitive assays. For example, the use of silicon-based photonic crystals with laser scanning platforms, nanoplasmonic label free biosensing and the use of nanoparticle such as gold nanoparticle probes. However, table 1 shows the detection limits claims of these technologies in comparison with the commercial available kit in the market.

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Product/ technology</th>
<th>Limit of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorimeter (HRP/TMB)</td>
<td>Commercial kits</td>
<td>~2 pg mL⁻¹</td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td>Commercial kits</td>
<td>~0.25 pg mL⁻¹</td>
</tr>
<tr>
<td>Nanotechnology based detection methods</td>
<td>Photonic crystal</td>
<td>0.1 pM</td>
</tr>
<tr>
<td></td>
<td>Nanostructured microfluidic array</td>
<td>5-50 fg mL⁻¹</td>
</tr>
<tr>
<td></td>
<td>Plasmonic nanoparticle</td>
<td>Single protein</td>
</tr>
<tr>
<td></td>
<td>sELISA with gold nanoparticle</td>
<td>0.05 ng mL⁻¹</td>
</tr>
<tr>
<td></td>
<td>Nano-ELISA</td>
<td>5 pg mL⁻¹</td>
</tr>
</tbody>
</table>

The detection of limits claimed in table 1 require more research validation to check the reliability of these assay since based on absorbance calculations of HRP/TMB reaction and the limitations of current
UV-VIS spectrophotometers, the limit of detection using HRP/TMB should be around 100 pg mL⁻¹ [37].

However, the detection limit in conventional ELISA assay depends on 1- the amount of immobilized antibodies to the surface of the microwell, 2- the amount of antigen that binds to these antibodies, 3- the amount of oxidized substrate of HRP enzyme conjugated with detecting antibodies. Therefore, based on the calculations of microwell dimensions (diameter of 0.6 cm), the volume of the solution pipetted in the well (100 ul) and the size of the antibody (hydrodynamic radius 5 nm), then the maximum number of antibodies that can be adsorb to the surface of the well is 1.3 x 10¹², however, for binding and affinity purposes the amount should be half of the maximum number.

In addition, other aspects that could be considered to improve the detection limits include: 1 - increase the affinity of antibody binding to antigen as the binding affinity of antibody-antigen would vary in the range of 10⁵ – 10¹² M⁻¹ and 2- decrease the number of HRP conjugated to the immunoglobulin to produce more efficient antibodies since the detection limit is inversely proportional with the number of HRP attached to the antibody [37].

1.5 Human transferrin detection limits

Human transferrin is a monomer glycoprotein with two metal binding sites existing in N-terminal and C-terminal and it has a molecular weight of 80 kDa. These domains are the location where iron ions can bind and being transported between different sites of human body. Therefore, human transferrin belongs to none-haem iron binding family that includes serum transferrin, lactoferrin and ovotransferrin. The main function of serum transferrin is carrying iron among the sites that utilised iron such as liver, spleen and bone marrow [38].

Serum transferrin or it is referred as total iron bind capacity (TIBC) has been considered an important indicator for many disorders such as iron deficiency anaemia and other inflammatory diseases [39]. Therefore, many immunoassay has been developed for the detection of serum transferrin. For example in 1986, F. Ca at, el. developed an assay to detect serum transferrin by using monoclonal antibodies to improve the sensitivity and the limit of detection by which the working range of the assay was 1 -100 ng /ml with detection limit of 0.5 ng/ml [40].

Furthermore, in 1988, M. Guindi at, el. developed a sandwich ELISA to detect serum transferrin in controlled samples with working range of 10 – 1000 ng/ml by using a combination of polyclonal antibodies as captured antibodies and monoclonal antibodies conjugated with HRP for detection[39]. Another immunoassay that exploited a piezoelectric crystal for the detection of human transferrin was developed by Z, Yang at, el. in 1999 with a satisfactory limit of detection within a range of 0.24 - 8.36 ug/ml [41].
Chapter 2

2. Materials and Methodology

2.1 Materials

Human Transferrin (H.T 98%)

A human origin transferrin protein with purity ≥ 98% was ordered from Sigma-Aldrich; product number T3309-100 mg as a lyophilized powder that is partially iron-saturated. It has a homologous N-terminal and C-terminal iron-binding domains. These domains are globular moieties of about 330 amino acids. It has an approximate molecular weight of 76-81 kDa and it is soluble in water 2mg/ml. The aqueous stock solution was prepared by adding the required amount of transferrin into PBS x1 solution and it has been left in refrigerator at 4°C for not more than 10 days [44].

Human Transferrin (H.T 95%)

However, a similar product of human transferrin, product number 90190 with purity ≥ 95% was also purchased from Sigma-Aldrich [52].

Bovine Serum Albumin; BSA

BSA lyophilized powder ≥ 96% was obtained from Sigma-Aldrich; product number A2153. It has a molecular weight of approx. 66kDa and it consists of a single polypeptide chain with about 583 amino acids. BSA is usually used as a blocking agent in immunoassays [45].

Casein

A technical grade Casein powder produced from bovine milk was purchased from Sigma-Aldrich; product number C7078. It has more protein impurities present and a higher fat content compared to partially purified casein product. However, Casein is a phosphor-protein found in milk and it has four main subdivisions that form about 80% of the total protein in bovine milk, these types are α-s1 Casein, α-s2 Casein, β-Casein, and κ-Casein. Casein has many experimental applications and it is used as a blocking agent in immunochemistry [46].

Horseradish Peroxidase (HRP) enzyme

HRP is a single chain polypeptide glycoprotein that used to oxidize different kind of substrates such as ABTS. It has a molecular weight of 44kDa and extinction coefficient of 100 at 403nm. This enzyme is relatively stable and has a smaller size compared with other enzymes, therefore, it has been used as a label for the antibodies by conjugating it with immunoglobulins in immunoassay [47].
**Polyclonal Anti-Transferrin (PAb)**

Polyclonal anti-transferrin antibodies (P Abs), product number T2027 were ordered from Sigma-Aldrich. These antibodies had been produced by injecting a goat with an isolated transferrin from human plasma and the whole antiserum was treated to remove the lipids continents, then 15mM sodium azide was added as preservative. The antiserum contains about 3.2 - 4.8 mg/ml of the antibodies. However, the repeated freezing of the antibodies is not recommended, therefore, 20 aliquots with a volume of 100 ul and a concentration of 3.3mg/ml were prepared. These antibodies will be used as primary antibodies that have different binding sites (epitopes) to bind directly to human transferrin [43].

**HRP conjugated anti-Goat IgG**

A whole molecule anti-goat IgG conjugated with HRP enzyme was purchased from Sigma-Aldrich; product number A5420. The anti-goat IgG has been produced in Rabbit by using anti-transferrin antibodies (antiserum) as an antigen after removing all rabbit serum proteins and immunoglobulins that don’t bind to anti-goat IgG. The peroxidase enzyme was conjugated with anti-goat IgG by protein cross linking with a molar ratio (IgG: Peroxidase) of 0.6 – 1.5. These antibodies also have different epitopes to bind with anti-transferrin antibodies and generate the positive signal or colour change [48].

**Monoclonal Anti-Transferrin antibody (MAb)**

Monoclonal anti-transferrin antibodies with a concentration of 2.0 mg/ml and product number ab10212 were ordered from Abcam. These antibodies were produced in mouse using human transferrin as antigen and preserved in 0.1% sodium azide solution. They have only one binding site (epitope) to bind with human transferrin and they will be used as primary antibodies in this assay [49].

**Monoclonal HRP conjugated anti-Transferrin antibody (HRP-MAb)**

Mouse monoclonal HRP conjugated anti-transferrin antibodies with a concentration of 0.8 mg/ml and product number ab10208 were purchased from Abcam. These antibodies were also produced in mouse by using human transferrin as antigen and preserved in 0.05% Proclin. They have one binding site (epitope) to bind with human transferrin and generate the positive signal or colour change in the assay [50].

**ABTS**

2, 2’-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) substrate (ABTS) was obtained from Sigma-Aldrich. ABTS is HRP substrate that produces green colour end product solution to indicate the presence of HRP conjugated antibodies [51].

**Phosphate buffered Saline (PBS)**

PBS x1 was prepared as buffer solution for dilution and washing steps.
**Tween 20**

Tween 20 with product number P9416 is produced by Sigma-Aldrich for molecular biology applications. The main structure of Tween 20 composed of polyoxyethylene sorbitol ester and it is used as non-ionic detergent in immunoassay.

**Micro titre plate**

Fisher brand micro titre plate sterilin with 96 (8x12) flat bottom non-sterile wells, product number FB56426 was purchased from fisher scientific as seen in figure 9. The surface of the well is hydrophobic to facilitate antibody immobilisation by passive adsorption.

![Fig. 9: Micro titer plate](image)

**Multichannel Pipette**

Fisher brand elite multichannel pipette was purchased from fisher scientific. It was used for washing the wells efficiently since it has 8 channels pipetters with volume range of 1 up to 300µl as seen in figure 10.

![Fig. 10: Multichannel Pipette](image)
**Eppendorf Pipetters**

Eppendorf pipettes with various volume ranges were used to fill the wells with different reagents and solution during the assay as seen in figure 11.

![Eppendorf Pipetters](image)

**Fig. 11: Eppendorf Pipetters**

**Stereo microscopic camera**

The images of the microplate wells during the development of the signal or the colour change in the wells for the assays were captured by stereo microscopic camera with back lighting as seen in figure 12.

![Stereo microscopic camera](image)

**Fig. 12: Stereo microscopic camera**

**2.2 Methodology**

The following procedure demonstrates the general steps that carried out for this ELISA assay. However, the actual protocol that used for each experiment will be discussed in details in the results and discussion section:

1- Coating the wells with primary capturing antibodies:
   
a- Preparing the antibodies by diluting one aliquot from the stock in PBS x1 solution to get the required concentration for the assay. There are two types of antibodies in the aliquots; the anti-transferrin and the anti-goat IgG as shown in table 2:
Table 2: Antibodies ( aliquot ) Stock

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Aliquot Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-transferrin</td>
<td>3.3 mg/ml</td>
<td>100 µl</td>
</tr>
<tr>
<td>Anti-goat IgG HRP tagged</td>
<td>9.0 mg/ml</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The final antibody concentration that used as stock in the experiments was 40 µg/ml. This concentration has been achieved by diluting one aliquot of anti-transferrin antibodies in 8.15 ml PBS x1 and one aliquot anti-goat antibodies in 11.20 ml PBS x1 as shown in table 3.

Table 3: Antibodies Stock for the experiment

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Stock Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-transferrin</td>
<td>40 µg/ml</td>
<td>8.25 ml</td>
</tr>
<tr>
<td>Anti-goat IgG HRP tagged</td>
<td>40 µg/ml</td>
<td>11.25 µl</td>
</tr>
</tbody>
</table>

b- Pipetting 200 µl of the diluted antibodies into the microplate wells.

c- Place the wells in the refrigerator 4 ºC for overnight incubation.

2- Washing the wells with PBS x1 solution or PBS-Tween 20 by using the multichannel pipette and flip the wells in the sink. This process was repeated three times per wash step. PBS-T 0.03% solution was prepared by 3 µl of Tween 20 to 10 ml PBS x1.

3- Blocking the nonspecific sites by adding 200 µl of BSA 5% or Casein into the wells after washing them with PBS x1 and cover them for 1-2 hours at room temperature. BSA 5% was prepared by adding 0.5 gm of BSA powder in 10 ml PBS x1 and Casein solution 1% was prepared by adding 100 mg of casein powder in 10 ml PBS x1.

4- Preparing different concentrations of human transferrin protein in PBS x1 solution and add 200 µl of the final diluted solution into the wells after washing step and cover them for 1-2 hours at room temperature.

5- Adding 200 µl of specific concentration of anti-transferrin antibodies from the same solution prepared for coating to the wells after another washing step and cover them for 1-2 hours at room temperature.

6- Adding 200 µl of specific concentration of detecting anti-goat antibodies that labelled with HRP into the wells after washing step and cover them for 1-2 hours at room temperature.

7- Adding 200 µl of specific concentration of ABT solution into the wells. ABTS 10mM solution was prepared by adding 54.8 mg of ABTS powder in 10 ml PBS x1 and 5 µl hydrogen peroxide.

8- Images for colour changes in the wells were captured using mobile camera and microscopic camera.
Chapter 3

3. Results and Discussion

Several ELISA protocols have been conducted and optimised for the detection of human transferrin diluted in buffer solution sample. However, the followings are the common steps for all assays unless stated elsewhere:

- PBS buffer solution has been used as a dilution solution for all reagents, in addition it also has been used for washing the wells three times after the incubation of the reagents.
- The incubation time for the coating step was overnight in the refrigerator at 4 ºC, while for all other steps it was one hour at room temperature.

3.1 Preliminary ELISA

A preliminary ELISA has been conducted as an initial ELISA trial to figure out the limitations and the potential modifications of the protocol that can be suggested to optimise the assay. Table 4 shows the protocol that has been used for each well. However, the wells from A1 to A10 received similar reagents in all steps whereas the wells A11 and A12 were prepared as negative controls for the assay since they received only a blank sample that contains PBS x1 buffer solution instead of human transferrin 98%.

Initially all the wells have been coated with captured antibodies by pipetting 10 µg/ml of polyclonal anti-transferrin antibodies (PAbs). The microtiter plate has been covered and placed in refrigerator at 4 ºC for incubation overnight. Then, all the wells have been washed three times with PBST 1% (PBS-Tween 20) instead of PBS buffer solution after each step of the protocol.

Next, BSA 5% was added as blocking buffer to block the nonspecific sites of the wells followed by adding 10 µg/ml of human transferrin 98% to the wells A1 – A10, while PBS buffer solution was added to the wells A11 and A12.

Then, 10 µg/ml of detecting polyclonal anti-transferrin antibodies (PAbs) was added to all wells followed by adding HRP anti-goat IgG 10 µg/ml. Finally, ABTS solution 1 mM was pipetted in all wells.

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coating with Anti-transferrin antibodies 10 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocking BSA 5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adding H. Transferrin 10 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adding Anti-transferrin 10 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adding HRP-anti-goat IgG 10 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Adding ABTS 1mM and image captures after 15 minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

Fig. 13: Preliminary ELISA
Figure 13 shows the image captured for the wells after 15 minutes from adding the ABTS solution. It can be noticed that the result of the preliminary ELISA shown in fig. 11 exhibits no colour change occurred after adding the ABTS solution to the wells. This indicates the absence of HRP-anti goat antibodies from the wells. However, it is suggested that the possible reasons could be:-

1- Either the captured PAbs didn't adsorb to the surface of the wells and they washed out or they became not active after immobilisation [29].
2- Or the captured PAbs were adsorbed but they didn’t bind to the human transferrin protein.
3- Or the captured PAbs bound to human transferrin and to the detecting PAbs but HRP anti-goat antibodies didn’t bind to the detecting PAbs.
4- But, there was no colour change in the negative control wells, however, it is suggested that blocking efficiency of BSA cannot be 100% [33], therefore, the none-specific binding cannot be eliminated and most likely all the antibodies have been washed out the wells.

Thus, it is suggested that the use of Tween 20 in the washing solution has removed all the antibodies from the wells. Therefore, the effect of both PBST and PBS solutions was evaluated in the next trial.

3.2 The effect of PBST and PBS as washing solution

The use of PBST 1% buffer as washing solution has been evaluated against PBS x1 without Tween 20. Table 5 shows the ELISA protocol that has been used to study the effect of Tween 20 as washing solution by which it is the same protocol used in preliminary ELISA.

The wells A and B were coated with 10 ug/ml of captured PAbs antibodies and placed in the refrigerator for incubation overnight. PBS x1 buffer solution has been used in all washing steps of well A and PBST 1% was used for well B. Then, the none-specific sites in both wells were blocked using BSA 5% followed by adding 10 ug/ml of human transferrin 98% to the wells and incubated for one hour at room temperature. Next, PAbs antibodies 10 ug/ml were pipetted and followed by adding HRP anti-goat antibodies 10 ug/ml. Finally, ABTS solution1mM has been added to the wells. All the washing steps have been performed three time after reagents incubation.
Table 5: ELISA protocol for PBS vs PBS washing solutions

<table>
<thead>
<tr>
<th></th>
<th>A washing with PBS</th>
<th>B washing with PBST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coating Anti-transferrin 10 µg/ml</td>
<td>Coating Anti-transferrin 10 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
</tr>
<tr>
<td>3</td>
<td>Adding HT 10 µg/ml</td>
<td>Adding HT 10 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>Adding Anti-transferrin 10 µg/ml</td>
<td>Adding Anti-transferrin 10 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Adding anti-goat HRP 10 µg/ml</td>
<td>Adding anti-goat HRP 10 µg/ml</td>
</tr>
<tr>
<td>6</td>
<td>Adding ABTS 1 mM</td>
<td>Adding ABTS 1 mM</td>
</tr>
</tbody>
</table>

Fig. 14. The effect of washing solutions (A) PBS, (B) PBST.

The image in figure 14 (A) shows the result of ELISA by which PBS was used as washing solution whereas figure 14 (B) shows the result of ELISA that PBST was used for washing the wells in all steps.

It can be recognized that there was a colour change or positive signal in well A indicating the presence of HRP anti-goat IgG in the well and it wasn’t washed out with PBS solution. On the other hand, there was no colour change in well B similar to the findings in preliminary ELISA trial by which it means no HRP anti-goat IgG remained in the well.

Thus, using Tween 20 with PBS together as washing buffer solution is most likely resulting in washing the antibodies out the wells. Therefore, PBS x1 buffer solution will be used as washing solution for the rest trials of this project.

3.3 Complete ELISA setup:

The relationship between colour intensity variations of the generated signal in ELISA and various concentrations of both human transferrin 98% and ABTS solution was evaluated by setup a complete ELISA protocol as shown in table 6.

The total number of wells required to conduct this trial was 9 wells by which the wells in column G (G1, G2 and G3) were coated with 20 ug/ml of HRP anti-goat IgG instead of PAbs and all other wells were coated with 20 ug/ml of captured PAbs antibodies. Then, BSA 5% was used to block the none-specific sites for all wells.
Different concentrations of human transferrin 98% have been added to the wells by which the wells in column C (C1, C2 and C3) received 100 μg/ml of human transferrin, the wells in column D (D1, D2 and D3) received 10 μg/ml and the wells in column E (E1, E2 and E3) received 1 μg/ml. On the other hand, the wells in column F (F1, F2, and F3) and the wells in column G (G1, G2 and G3) received only PBS (blank samples). Next, the detecting PAbs antibodies 20 μg/ml were added to all wells followed by adding 20 μg/ml of HRP anti-goat IgG.

Finally, different concentrations of ABTS solution were added to the wells by which ABTS 10 mM was pipetted to all wells in the first row (C1, D1, E1, F1 and G1). The wells in second row ((C2, D2, E2, F2 and G2) received 1 mM of ABTS solution and the wells in the third row (C3, D3, E3, F3 and G3) received ABTS 0.1mM. An image for the microwells has been captured after 30 minutes from adding the ABTS solution.

Table 6: The protocol of complete ELISA setup with different concentrations of H.T 98% and ABTS

<table>
<thead>
<tr>
<th>Protocol for all wells except G1, G2, G3</th>
<th>Protocol for G1, G2, G3 wells</th>
<th>Details of transferrin and ABTS concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating with Anti transferrin 20 μg/ml</td>
<td>Coating with HRP anti goat IgG 20 μg/ml</td>
<td>BA1 5% blocking</td>
</tr>
<tr>
<td>BSA 5% blocking</td>
<td>BSA 5% blocking</td>
<td>ABTS 10 mM C1 C2 C3</td>
</tr>
<tr>
<td>Adding H. transferrin</td>
<td>PBS</td>
<td>10 μg/ml D1 D2 D3</td>
</tr>
<tr>
<td>Adding of Anti-transferrin 20 μg/ml</td>
<td>Adding of Anti-transferrin 20 μg/ml</td>
<td>1 μg/ml E1 E2 E3</td>
</tr>
<tr>
<td>Adding of HRP anti-goat IgG 20 μg/ml</td>
<td>Adding of HRP anti-goat IgG 20 μg/ml</td>
<td>Blank sample F1 F2 F3</td>
</tr>
<tr>
<td>ABTS</td>
<td>ABTS</td>
<td>Transferin</td>
</tr>
</tbody>
</table>

Figure 15 shows the colour signals that generated of the complete ELISA trial an they indicate positive results in all wells. However, this colour change has taken place due to the presence of HRP anti goat IgG in these wells. The following findings can be identified from these results: First, The darkest colour
Intensity was obtained in well G1 that suggested a successful adsorption or coating of HRP anti-goat IgG to the well. In addition, it received high concentration of ABTS solution (10mM) in compared with other wells in G column where HRP anti-goat IgG was also coated to these wells. Second, by comparing the wells in the three different rows, the variations in colour intensity between the three rows can be clearly identified. This suggests that the higher concentration of ABTS added to the well produces more intense or darker colour.

Third, by looking at the wells in different columns, it can be noticed that there was no significant variations in the colour intensity between the columns C, D and E where they received different concentrations of human transferrin. In addition, the wells in column F were prepared as negative controls since no transferrin was added to these wells, however, there was a colour change comparable with the colour change in other wells in column C, D and E. It is suggested that the followings are possible reasons for this case:

- Since all the colour changes were almost similar in all wells of column C, D, E and the negative control wells, then there was no significant effect of various human transferrin concentrations. Hence, human transferrin likely didn’t bind to the captured PAbs antibodies.

- The background noise was too high (very low signal-to-noise ratio) due to the none-specific bindings of HRP–anti-goat IgG that could bind either to the unblocked areas of the microwell or to the captured (PAbs) antibodies or both.

Thus, the following suggested trials were conducted to verify the possible reasons of the false ELISA results; PAbs antibodies binding to human transferrin, HRP anti-goat IgG binding to PAbs and BSA blocking efficiency.

### 3.4 BSA blocking

The ELISA protocol shown in table 7 was conducted to evaluate the efficiency of BSA blocking. Two wells were used to execute the protocol, well B was coated with BSA 5% and well C was coated with PAbs antibodies 10 ug/ml and placed in refrigerator at 4°C for incubation overnight. Then, 10 ug/ml of PAbs antibodies were added to well B while BSA 5% was added to well C. Next, 10 ug/ml of human transferrin 98% was only added to well C while PBS buffer solution was added to well B followed by adding 10 ug/ml of detecting PAbs antibodies to well C and PBS solution to well B. Finally, HRP anti-goat IgG 10 ug/ml was added to both wells followed by adding ABTS 1 mM solution. However, ABTS solution was also added to well A as a colour reference for other wells. Hence, the protocol used for well C is similar to the general protocol of sandwich ELISA while the steps performed in well B referred to suggested modified protocol to study BSA blocking properties.
Table 7: The protocol for evaluating BSA blocking efficiency

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coating with BSA 5%</td>
<td>Coating Anti-transferrin 10 µg/ml</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Adding Anti-transferrin 10 µg/ml</td>
<td>Blocking with BSA 5%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Adding PBS</td>
<td>Adding H.T 10 µg/ml</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Adding PBS</td>
<td>Adding Anti-transferrin 10 µg/ml</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Adding anti-goat HRP 10 µg/ml</td>
<td>Adding anti-goat HRP 10 µg/ml</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Adding ABTS 1 mM</td>
<td>Adding ABTS 1 mM</td>
<td></td>
</tr>
</tbody>
</table>

Figure 16: Evaluation of BSA blocking, (A) reference colour, (B) Coated with BSA, (C) Coated with PAbs

Figure 16 shows the images that captured for the three wells after 30 minutes from adding ABTS solution. However, figure 16 (A) is referred as the original reference colour of ABTS solutions added to the wells. Therefore, by comparing the colour intensity of well B with the colour intensity of the reference well A, it can be noticed that there was no significant colour change occurred although both PAbS antibodies and HRP anti-goat IgG were added to well B after BSA blocking. Thus, coating well B with BSA is suggested to be efficient enough to block the none-specific bindings for both PAbS antibodies and HRP anti-goat IgG.

On the other hand, there was a significant colour change in well C that followed the general protocol of sandwich ELISA compared with both well A and well B indicating the presence of HRP anti-goat IgG. Thus, either HRP anti-goat IgG has bound to detecting PAbS or to captured (immobilized) antibodies or both, but most likely there is no concern about binding HRP anti-goat IgG with none-specific sites of the microwell.

### 3.5 Comparing BSA and Casein blocking efficiency

The blocking efficiency of BSA has been verified by comparing it with Casein protein. Therefore, the general protocol of sandwich ELISA has been used to conduct this trial as shown in table 8. Two wells A and B were coated with 15 µg/ml of captured PAbS antibodies and they placed in refrigerator at 4°C for incubation overnight. Then the none-specific site in well A was blocked by casein whereas BSA was used to block the none-specific site in well B.

Next, 20 µg/ml of human transferrin 98% was added to both wells followed by adding 15 µg/ml of detecting PAbS antibodies. Finally, 15 µg/ml of HRP anti goat IgG was added in both wells followed by adding ABTS 1mM solution.
Table 8: the protocol for BSA and casein blocking

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coating Anti-transferrin 15 µg/ml</td>
<td>Coating Anti-transferrin 15 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Blocking with Casein 1%</td>
<td>Blocking with BSA 5%</td>
</tr>
<tr>
<td>3</td>
<td>Adding H.T 20 µg/ml</td>
<td>Adding H.T 20 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>Adding Anti-transferrin 15 µg/ml</td>
<td>Adding Anti-transferrin 15 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Adding anti-goat HRP 15 µg/ml</td>
<td>Adding anti-goat HRP 15 µg/ml</td>
</tr>
<tr>
<td>6</td>
<td>Adding ABTS 1 mM</td>
<td>Adding ABTS 1 mM</td>
</tr>
</tbody>
</table>

Fig. 17: comparison between BSA and Casein blocking. (A) Blocked with Casein, (B) Blocked with BSA

Figure 17 shows the images captured for both well A and B after 30 minutes from adding the ABTS solution. By comparing the colour intensity of well A that blocked with casein and well B that blocked with BSA as shown in figure 17 (A) and (B) respectively, it was recognised no significant difference in colour intensity in both wells indicating that both casein and BSA are likely having same efficiency of blocking the none-specific site.

Similarly, the colour change in both wells was indication of the presence of HRP anti-goat IgG in both wells by which it is most likely to be related to background noise. Thus, it is suggested that both casein and BSA have almost similar efficiency as blocking agent to block the none-specific site, therefore, BSA will be used in the rest of the trials in this project as the blocking agent.

3.6 Evaluating PAbs – transferrin 98% binding

Indirect ELISA protocol was considered for evaluating the binding between immobilized human transferrin and PAbs antibodies as shown in table 9. The first step of indirect ELISA assay was coating the well with 10 µg/ml of human transferrin instead of immobilizing captured PAbs antibodies in sandwich ELISA assay. Then, the microplate was placed in refrigerator at 4°C for incubation overnight followed by blocking the none-specific site with BSA 5%. Next, 10 µg/ml of PAbs antibodies was added to the well followed by adding 10 µg/ml of HRP anti-goat IgG. Finally, ABTS was added into two wells, the assay well B and well A as reference colour.
Table 9: Indirect ELISA protocol for evaluating PAbs – transferrin binding

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ABTS 1 mM reference colour</td>
<td>Coating with human transferrin 10 ug/ml</td>
</tr>
<tr>
<td>2</td>
<td>Blocking BSA 5%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Adding PBS (sample)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Adding Anti-transferrin 10 ug/ml</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Adding anti-goat HRP 10 ug/ml</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Adding ABTS 1mM</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 18: Indirect ELISA for evaluating PAbs – transferrin binding
(A) ABTS reference colour, (B) Assay produced colour

Figure 18 shows the images of the colour produced after 30 minutes of adding ABTS to the wells. Figure 18 (B) shows a significant colour change in the assay’s well compared with the ABTS reference’s well indicating the presence of HRP anti-goat IgG in the well. However, it is suggested that human transferrin has successfully adsorb to the well surface in the coating process. In addition, the positive signal generated is resulting from HRP binding to PAbs antibodies since the none-specific site was blocked by BSA 5%. Therefore, PAbs antibodies were binding to the immobilized human transferrin. Thus, the PAbs antibodies are most likely binding to human transferrin and to HRP anti-goat IgG.

3.7 Verifying PAbs – HRP anti-goat IgG binding

A simple modified protocol was used to evaluate the binding between PAbs antibodies and HRP anti-goat IgG as shown in table 10. Two wells were coated with PAbs antibodies 20 ug/ml and placed in refrigerator for incubation overnight. Then, the none specific sites were blocked using BSA 5% followed by adding 20 ug/ml of HRP anti-goat IgG. Finally, ABTS 10mM solution was added to the assay’s wells, in addition to an extra well for colour reference.
Table 10: Modified protocol to verify PAbs antibodies and HRP anti-goat IgG

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ABTS 10 mM reference colour</td>
<td>Coating with anti-transferrin 20 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Blocking with BSA 5%</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Adding anti-goat HRP 20 µg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adding ABTS 10 mM</td>
</tr>
</tbody>
</table>

![Image of protocol steps]

Fig. 19: Verifying PAbs – HRP anti-goat IgG binding. (A) ABTS reference colour, (B) Assay wells

Figure 19 shows the images captured for the assay’s wells and the reference well after 30 minutes of adding the ABTS solution. However, figure 19 (B) shows a significant colour change in the assay wells in compared with the reference ABTS colour indicating the presence of HRP anti-goat IgG in the assay’s wells. Therefore, it is suggested that the HRP anti-goat IgG has bound to the captured PAbs antibodies, in addition, the remarkable intense colour change that noticed in compared with the reference colour and the previous trials was most likely due to the higher concentration of both HRP anti-goat and PAbs antibodies, in addition to the high concentration of ABTS. Thus, it is suggested that the background noise in the previous experiments is likely resulting from the none-specific binding of HRP anti goat IgG with the captured PAbs antibodies immobilized to the well surface.

3.8 Evaluating time vs colour intensity

The development of ELISA signal or the colour change has been evaluated against the time by considering a simplified protocol as shown in table 11. Twelve wells were coated with 20 µg/ml of HRP anti goat IgG since the HRP enzyme conjugated with the immunoglobulin is responsible for mediating the interaction of ABTS substrate to generate the colour signal. Then, the wells placed in refrigerator at 4°C for incubation overnight followed by blocking the none-specific site with BSA.

Then, different concentrations of ABTS solution was added as seen in in table 8 and figure 18 by which:
- The wells in Row (A) received 10 mM of ABTS solution.
- The wells in Row (B) received 1.0 mM of ABTS solution.
- The wells Row (C) received 0.1 mM of ABTS solution.

However, various images for these wells were captured during the development of the colour at 1, 15, 30 and 60 minutes from adding the ABTS solution to the wells as shown in fig 18 by which:
- The image of the wells in Column (I) has been captured after 1 minute.
- The image of the wells in Column (II) has been captured after 15 minute.
- The image of the wells in Column (III) has been captured after 30 minute.
- The image of the wells in Column (IV) has been captured after 60 minute.

Table 11: Protocol for time vs colour intensity evaluation

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coating with HRP anti-</td>
<td>Coating with HRP anti-</td>
<td>Coating with HRP anti-</td>
</tr>
<tr>
<td></td>
<td>goat IgG 20 µg/ml</td>
<td>goat IgG 20 µg/ml</td>
<td>goat IgG 20 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
</tr>
<tr>
<td>3</td>
<td>Adding ABTS 10 mM</td>
<td>Adding ABTS 1 mM</td>
<td>Adding ABTS 0.1 mM</td>
</tr>
</tbody>
</table>

Fig. 20: Colour signals produced in various time and ABTS conc.

As seen in figure 20, the signals produced in each row have showed significant colour intensity variations along with adding different ABTS concentrations. However, it is suggested that the colour intensity of wells in row C that received 0.1 mM of ABTS are likely to be not significant enough to distinguish the colour change although the image has been captured at 60 minutes of adding the ABTS that is considered long time to read the signal from ELISA assay colour.

On the other hand, the change in colour intensity occurred in the wells of both row A and B was more significant and can be easily distinguished in the time period. However, it is suggested that the development of colour intensity in wells of row A that received 10 mM of ABTS solution was remarkable and it took less time to be significant and recognized in compared with the colour intensity of other wells at the same time. Furthermore, the colour intensity became more uniform and homogenous in all wells after 30 minutes of adding the ABTS solution.

Thus, it is suggested that both reading the colour intensity in the microwells after 30 minutes of adding ABTS solution and the use of ABTS concentration 10 mM are likely to be more efficient in recognizing
the colour signal generated in the wells when conducting a colorimeter ELISA. In addition, this could be more convenient for quantification purposes of the analyte.

3.9 **Comprehensive ELISA assay with monoclonal Antibodies (MAbs) and Human transferrin 95%**

A comprehensive ELISA assay using polyclonal anti-transferrin antibodies (PAbs), monoclonal anti-transferrin antibodies (MAbs), HRP anti goat IgG, HRP monoclonal anti transferrin antibodies (HRP-MAbs) and transferrin 95% in the same microtiter plate and at the same time was conducted for complete comparison between these reagents with different protocols as shown in table 12. However, 20 wells in the microplate were used to conduct this experiment by which:

- Row 1 has the wells C1, D1, E1, F1.
- Row 2 has the wells C2, D2, E2, F2.
- Row 3 has the wells C3, D3, E3, F3.
- Row 4 has the wells C4, D4, E4, F4.
- Column G has the reference wells (colour intensity for positive control and original ABT solution colour)

All the wells in Row 1 and Row 3 were coated with MAbs 20 ug/ml while the wells in Row 2 and Row 4 were coated with PAbs 20 ug/ml. In addition, the well G1 coated with HRP-MAbs and well G3 was coated with HRP anti-goat IgG. Then, the microplate was placed in refrigerator at 4 C for incubation overnight.

Then, BSA 5% was used to block the none-specific site for all wells followed by adding different concentrations of human transferrin 95% by which C–wells received 100 ug/ml, D–wells received 10 ug/ml, E – wells received 1 ug/ml and blank sample (PBS buffer solution) was added to F–wells and control wells.

Next, 20 ug/ml of HRP MAbs was added to the wells in Row 1 and Row 2 followed by PBS solution to keep them wet for the further steps. On the other hand, 20 ug/ml of PAbs was added to the wells in Row 3 and Row 4 followed by adding 20 ug/ml of HRP anti-goat IgG. Finally, ABTS 10 mM solution was added to all wells.
Table 12: Comprehensive ELISA Protocols

<table>
<thead>
<tr>
<th>C1, D1, E1, F1</th>
<th>C2, D2, E2, F2</th>
<th>C3, D3, E3, F3</th>
<th>C4, D4, E4, F4</th>
<th>G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Coating with MAbs 20 ug/ml</td>
<td>Coating with PAbs 20 ug/ml</td>
<td>Coating with MAbs 20 ug/ml</td>
<td>Coating with PAbs 20 ug/ml</td>
<td>Coating with HRP-MAbs 20 ug/ml</td>
</tr>
<tr>
<td>2 Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
<td>Blocking with BSA 5%</td>
<td>BSA 5% Blocking</td>
</tr>
<tr>
<td>3 Adding of transferrin, 100, 10, 1, 0ug/ml</td>
<td>Adding of transferrin, 100, 10, 1, 0ug/ml</td>
<td>Adding of transferrin, 100, 10, 1, 0ug/ml</td>
<td>Adding of transferrin, 100, 10, 1, 0ug/ml</td>
<td>ABTS 10 mM</td>
</tr>
<tr>
<td>4 Adding of HRP – MAbs 20 ug/ml</td>
<td>Adding of HRP – MAbs 20 ug/ml</td>
<td>Adding of PAbs 20 ug/ml</td>
<td>Adding of PAbs 20 ug/ml</td>
<td>G3</td>
</tr>
<tr>
<td>5 Adding of PBS</td>
<td>Adding of PBS</td>
<td>Adding of HRP-anti goat 20 ug/ml</td>
<td>Adding of HRP-anti goat 20 ug/ml</td>
<td>Coating with HRP anti-goat 20 ug/ml</td>
</tr>
<tr>
<td>6 Adding of ABTS 10 mM</td>
<td>Adding of ABTS 10 mM</td>
<td>Adding of ABTS 10 mM</td>
<td>Adding of ABTS 10 mM</td>
<td>BSA 5% Blocking</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ABTS 10 mM</td>
</tr>
</tbody>
</table>

Fig. 21: Comprehensive ELISA assay.

Figure 21 shows the signals generated for the comprehensive ELISA assay. The image was captured after 60 minutes from adding the ABTS solution to the wells.

The signals produced in Row 1 are related to the presence of HRP MAbs that bound to human transferrin. However, it is suggested that:

- MAbs has successfully adsorbed to the surface of the wells.
- Human transferrin has bound to the captured MAbs.
- HRP MAbs has also bound to human transferrin and catalysed the ABTS reaction.
- The negative control F1-well didn’t show a significant colour change indicating that BSA has blocked the none-specific and HRP MAbs didn’t bind to the well surface.
- The colour produced in the wells showed variations in intensity proportional to the concentration of the human transferrin. Furthermore, high concentration of human transferrin 100 ug/ml didn’t produce significant high colour intensity compared with lower concentration.
of human transferrin 10 μg/ml since the amount of human transferrin bind is limited to the amount of immobilized captured antibodies.

The signals generated in Row 4 are related to the presence of HRP –anti goat IgG that most likely bound none-specifically to the captured antibodies since all the colour signals have same intensity regardless of human transferrin concentrations. Thus, it is suggested that monoclonal assay has been successfully able to detect human transferrin in the sample, in contrast, the polyclonal assay generated high background noise and the signal-to-noise ratio was very low.

The signal generated in Row 2 were produced as a result of the presence of HRP MAbs in the well. However, the colour intensity of these wells are not significant to human transferrin. Therefore, it is suggested that there is a problem in binding between human transferrin 95% with the immobilized PAbs.

Finally, the signals generated in Row 3 are resulting from the presence of HRP anti goat IgG in the wells indicating that human transferrin has bound to the coated MAbs and to the detecting PAbs, in addition HRP anti-goat has also bound to PAbs. However, there are some evidences that colour intensity of these wells are correlated to the concentration of human transferrin since the negative control showed a background noise and there is a significant colour change in the other wells compared with colour intensity of the negative control. Thus, the combination of PAbs and MAbs are most likely not reliable for the detection of human transferrin and it required more evaluation and validation.

3.10 Summary of the results

The following points are summary for the results obtained in all previous experiments:

1- Washing the wells with PBST has resulted in false negative signals while washing the wells with PBS alone has resulted in producing positive signals in all wells.
2- All the wells showed a positive signal including the negative control.
3- BSA exhibits an efficient blocking for none-specific site.
4- BSA and casein have same effect as blocking agents.
5- All the antibodies and the human transferrin were successfully immobilized to the surface of the well.
6- ABTS 10 mM with 30 minutes incubation time exhibit better signal differentiation.
7- Polyclonal antibodies has high level of background noise and low signal –to- noise ratio. Human transferrin detection with polyclonal antibodies was unsatisfactory.
8- Monoclonal antibodies exhibits better results in detection human transferrin with low background noise.
Chapter 4

4. Conclusion, limitations & Future work

1.1 Conclusion

This project has evaluated different challenging aspects in the detection of human transferrin protein by sandwich ELISA. Human transferrin is a glycoprotein present in the blood serum and it is responsible for transporting iron in human body. It has been considered as strong indicator for several diseases that related to iron deficiency such as anaemia in addition to other inflammatory disorders.

Sandwich ELISA was used for detection of human transferrin due to its high sensitivity and specificity. However, two types of anti-transferrin antibodies were used in this assay, polyclonal anti-transferrin ABs from Sigma Aldrich and monoclonal anti transferrin ABs from Abcam. In addition, HRP anti-goat IgG was used with the polyclonal anti-transferrin as secondary antibodies while HRP monoclonal anti-transferrin conjugate used with monoclonal anti-transferrin antibodies.

On the other hand, two types of human transferrin were used in this assay; human transferrin with purity of >98% and human transferrin with purity >95% from Sigma Aldrich. This difference in purities didn’t show any significant variations in the results, however, more experiments should be considered for better evaluation of both proteins.

The following findings were recognized experimentally by evaluating several modified protocols for optimisation the reagents and the various assay steps.

1- Washing Solution:

The use of PBS-Tween 20 (PBST 1%) washing solution has washed out all the antibodies form the well resulting in generating no signal since the HRP anti-goat IgG were eliminated from the well and it didn’t catalyse the ABTS reaction to produce a positive colour change. However, the literatures suggested that using PBST has saturated the well completely as a blocking agent, in addition, the optimum concentration of PBST in immunoassay applications has been 0.05% [41]. Therefore, using PBST 1% was enough to wash all the antibodies out the well or block the well completely and compete the other antibodies from adsorbing to the well.
2- Antibodies coating.

The coating step for polyclonal anti-transferrin, HRP anti-goat IgG and human transferrin exhibited successful immobilisation by physical adsorption to the wells. This conclusion is based on the positive signal or the colour change occurred when those reagents were coated to the well assuming that antibodies have bound to targeted antigen when needed to produce the signal. However, this requires more investigations and validations using another techniques such as fluorescent tagging or SPR detection method since the negative control wells has showed a positive signal as well[42].

Therefore, it is suggested that the high concentration of the antibodies used for coating the well could cause antibodies agglutination and block the binding site of the antibody, in addition, some of the antibodies became inactive or denatured due to physical adsorption. Thus, the positive signal has been produced by both background noise and some formation of antibodies-antigen complexes where the noise-to-signal ratio was low and it is suggested that coating the well with the optimum amount of the antibodies 5 µg/ml would reduce the effect of agglutination [43].

Efficient immobilisation of primary or captured antibodies on the microtiter plate surface is very crucial step and it determines the sensitivity of the immunoassay. In general, the immobilisation is achieved through the interactions of hydrophobic, electrostatic and ionic forces between the antibodies and the surface of the polystyrene surface. This method could cause denaturing of and random orientation of the antibodies over the surface resulting in a change in the binding site or deactivate the antibody [27][29].

Moreover, N. Tajima, et al. suggested that the affinity of binding of the soluble antibody-antigen complex is 1000 times stronger than the immobilised antibodies by which the dissociation constant of the immobilized antibody to antigen was $10^{-7}$ - $10^{-5}$ mol/L whereas the soluble complexes had a dissociation constant of $10^{-10}$ - $10^{-9}$ mol/L. In addition, the antibodies denaturation and steric hindrance of antigen-recognition sites are responsible for the loss in antibodies activities [31].

On the other hand, coating the wells with monoclonal anti-transferrin and HRP monoclonal anti-transferrin have exhibited a true positive signal since the negative control wells didn’t show significant colour change indicating a very low background noise compared with the wells that received human transferrin.

However, evidences suggested that there is a remarkable advantage of using the monoclonal antibodies to detect human transferrin in very low concentration samples and in reducing the
background noise [40]. In addition, the studies has shown that monoclonal antibodies could not precipitate transferrin in the same efficiency of polyclonal antibodies due to the low molecular weight of human transferrin that required more than one or two antibodies to bind with it for precipitation purpose [39]. This implies inadequate cross linking formation and more human transferrin will dissociate and washed out the well resulting in less correlation between the colour intensity and the actual concentration of human transferrin in the sample, moreover, the assay could show a false negative very low concentration.

Thus, different approaches should be considered to achieve an efficient and stable immobilisation, for instance, covalent bonding such as self-assembled monolayer (SAM) that exhibits a stable covalent bond in various conditions when it is used to immobilise the antibodies. Another method is to immobilize the Fc fragment with Fc-binding receptors such as cell wall protein A and G. In addition, the antibody’s fragments (Fab) can be directly immobilized by self-assembled technique through binding their terminal thiol group with hydrophilic gold surface [30]. Bin Lu, et al. suggested that the activity of the antibodies in the form of oriented Fab fragment was three times higher than the random orientation [32].

3- BSA blocking

Bovine serum albumin in PBS buffer solution was added to the microwells for one hour incubation time to block the none-specific site of the microwell surface. However, it is suggested that BSA was efficient as blocking agent since there was no significant colour change has occurred in the well that coated by BSA as the first step of the assay. This indicates that it is most likely BSA has covered the surface and prevent the adsorption of other antibodies to the well resulting in low background noise.

However, none-specific binding is another challenge that influence the sensitivity and specificity of the immunoassay due to the interaction between the proteins and the remaining surface of the wells after the coating process in addition to the none-specific interaction between the different protein molecules in the well resulting in increase of the background or noise signal [33].

In addition, by comparing the BSA and casein protein as blocking agents, it was suggested that both BSA and casein have exhibited similar efficiency in blocking the none-specific site since the signal generated in both the well coated with BSA and the well coated with casein represented low background noise and they were most likely having same colour intensity.
Furthermore, studies suggested that although several numbers of reagents that have been used as blocking buffer such as bovine serum albumins (BSA), Tween 20, casein and milk proteins, BSA has been considered the most common blocking buffer among all of them due to its low cost and its capability to reduce steric hindrance of the specifically binding antibodies [33].

Finally, Y. Jeyachandran, at el. found that 35% of the surface coverage of adsorbed BSA to a polystyrene surface has been formed when BSA buffer solution with concentration of 1 mg/ml and incubation time 30 minutes, however, the surface coverage increased to 58% when 10 mg/ml was used. Furthermore, the efficiency of blocking the none-specific adsorption of IgG was 95% [33]. This suggests that the background noise cannot be eliminated 100% but it can be reduced to very low level compared with the signal produced from the antibody-antigen binding.

4- HRP - ABTS interaction

ABTS was used as a substrate for HRP enzyme conjugated with antibodies to amplify the signal produced from antibody-antigen interaction by generating a green colour solution with different intensities. However, it was suggested that several factors contribute with colour change such as 1- the availability of both HRP enzyme and ABTS molecules in the well, 2- the concentration of ABTS added to the well, 3- the concentration of HRP conjugating antibodies and 4- the incubation time after adding ABTS solution to the well. However, optimum colour change was obtained by changing the concentrations of ABTS and it is suggested that the range of 1 - 10 mM would be enough to generate a range of significant colour intensities correlated with the amount of HRP remained in the well after washing the unbound antibodies. Furthermore, the typical incubation time for HRP and ABTS to produce a true signal was suggested to be at least 30 minutes since over long time of indefinite mediated reaction of ABTS, the colour intensity will exhibit a false positive signal [29].

However, the studies suggest that up to 6 HRPs molecules can bind or conjugate to a single immunoglobulin IgG [37]. Therefore, calibration curve for positive and negative controls can be used to determine the relative concentration of the antibody-antigen complexes.

In conclusion, the use of goat polyclonal anti-transferrin as captured and detecting antibodies with HRP anti-goat IgGs as secondary antibodies in detecting and quantifying of human transferrin using sandwich ELISA was unsatisfactory due to the high level of back ground noise resulting from the none specific binding of the HRP anti-goat IgG to both the surface of the well and the immobilized antibodies. On the other hand, monoclonal anti-transferrin and HRP monoclonal anti-transferrin antibodies exhibit an interesting results in detecting human transferrin in a buffer
sample, in addition, the time needed to perform the assay was less because HRP monoclonal anti-transferrin antibody was used for detecting and amplifying the generated signal at the same time.

1.2 Project Limitations
This project is lacking of quantitative data that validate the findings and the proposed suggestions for the underline causes due to the time restriction and the availability of the required materials. For example, the use of fluorescent tagged reagents, the optical equipment for colorimetric detection such as ELISA plate reader and UV VIS spectrophotometer. In addition, reagent concentrations, incubation time and conditions need be more optimised.

1.3 Future work
The new development in advanced nanotechnology techniques can be exploiting to develop high sensitive ELISA assay to detect human transferrin protein for medical use. The silver enhanced gold nanoparticles have been proved to generate strong signal for very low analyte concentration, therefore, anti-transferrin antibodies can be conjugated with gold nanoparticle to enhance the sensitivity. Microfluidic ELISA can also be used to develop a point of care device that detect human transferrin easily and in a short time manner with less reagent consumption and less cost.
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