THE OPTIMISATION OF THE ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) FOR THYROID PEROXIDASE AUTOANTIBODIES (TPO) AUTOANTIBODIES (aAbs)

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The Optimisation of the Enzyme Linked Immunosorbant Assay (ELISA) for Thyroid Peroxidase (TPO) Autoantibodies (aAbs)

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Chapter 1

1. Introduction

1.1 Autoimmune Diseases of the Thyroid and Thyroid Peroxidase

Autoimmune diseases of the thyroid gland affect between 2-4% of women and 1% of men worldwide[1-4]. They are generally characterized by hyperthyroidism (Graves disease) or hypothyroidism (Hashimoto's thyroiditis) along with some form of enlargement (goitre) of the thyroid gland, resulting in hormone disruption which can lead to many adverse symptoms including tachycardia, tremors and glucose intolerance[1,5]. Both Graves disease and Hashimoto's thyroiditis are propagated by the body producing antibodies that attack the naturally occurring thyroid products, Thyroid Stimulating Hormone (THS) and Thyroid Peroxidase (TPO), a surface bound enzyme[6-8]. In a healthy patient TPO is produced by the thyroid in order to catalyse certain enzymic functions such as the halogenation of thyroglobulin and the reaction between iodotyrosine residues to form thyroid hormone[1,8]. However, in patients with autoimmune thyroid diseases TPO becomes an autoantigen that stimulates a humoral immune response from anti-TPO antibodies (TPO aAbs), either caused by, or leading to, the aforementioned conditions[7, 8]. TPO aAbs are found in patients with both Graves' Disease and Hashimoto's Thyroiditis, with their presence detected in 75% and 90% of patients, respectively[1,4].

Therefore, to aid in diagnosis, it has become important for there to exist a clinical method that can ascertain the concentration of TPO aAbs in human blood sera. This technique is usually a biological assay, which exist in many forms. When studying antibodies, assay types include,

(i) the simple western blot technique[9]

(ii) immunoprecipitation[10]

(iii) agglutination[11]

(iv) immunofluorescence [12]

(v) immunoassay[13, 14]

In this thesis, immunoassays are considered, more specifically, the Enzyme-Linked Immunosorbent Assay or ELISA.

The concept of the immunoassay has been around since the 1960's. The idea was first developed based on the need for a highly explicit, highly responsive assay for insulin. The methods that resulted were the original immunoassays, the radioimmunoassay (RIA)[15] and the immunofluorescence assay (IMF)[16]. However, despite being effective in their own ways they both had serious shortcomings[15, 16]. It would be a decade until the superior ELISA technique would first be reported.

1.2 The History of Enzyme-Linked Immunosorbant Assays

The ELISA technique was first introduced between 1971 and 1972. It is based on the practical theories and experiments of Engvall and Perlmann who described a method for the analysis of the concentration of Immunoglobulin G in human sera[17]. The method involves a solid phase where the proposed antigen (or antibody) could be bound followed by the addition of sera for examination[18]. If the sera sample contained antibodies associated with the immobilized antigen or antibody, then they would bind the solid phase. This antigen/antibody surface binding is the basis of all immunoassays and occurs due to the 'lock and key' system, where the antibody contains a chemical 'lock' and the

antigen contains a specific chemical group or 'key' that fits this lock. In an organism the attached antibody is a marker indicating to the body that the antigen it is bound to should be destroyed *i.e.* it is the immune trigger[17,18]. In an immunoassay antibodies are bound in a 'well' to the antigen, allowing their concentration to be read. However, what makes ELISA so superior to other techniques is the method by which the bound analyte is read. In RIA for instance, the antigen of interest is measured indirectly by radio-labeling a known concentration of the same antigen and binding it on to a solid phase-bound antibody. When a sera, positive for the desired antigen, is introduced to the solid phase the antigen present will displace the radio-labeled versions. The concentration of displaced antigen is calculated by measuring the free radioactive signal. This process was extremely time consuming and tedious. In ELISA the concentration of the desired analyte is measured by adding a generic monoclonal antibody that has been conjugated to an enzyme. The enzyme will undergo a colour change with the addition of a simple substrate, allowing the concentration of bound analytes to be read. This more accessible read process makes ELISA a cheap, simple and direct process, whilst only losing negligible amounts of specificity and sensitivity.

This marked improvement in read methods meant that the ELISA technique was recognized quickly as superior to previously used tests, such as the aforementioned RIA and IMF. Both RIA and IMF did achieve the same goal as ELISA techniques, that being the detection and quantification of antibodies or antigens within human sera. However, IMF is time consuming and small scale with high sensitivity, whereas RIA can handle large scale tests, but the instruments involved were too technical and the involvement of isotopic reagents gave the test a poor shelf life. In short, neither test was sufficient as a viable, dependable and consistent clinical test[19]. The ELISA method combined the most

advantageous elements of RIA and IMF, by being responsive and reliable, as well as being relatively quick and cheap to produce and perform. In addition, ELISA is a highly adaptable test, being possible to modify the test procedure in order to detect almost any antigen, antibody or other protein contained within human sera. The most common types of ELISA technique are,

(i) Direct ELISA

(ii) Indirect ELISA

(iii) Sandwich ELSIA

(iv) Competitive ELISA.

The latter two variants follow the same principle as direct and indirect ELISA, but methods are changed slightly to accommodate different types of antigen, or take different observations for experimental work. The direct and indirect methods only differ slightly in technique. Namely, when using direct ELISA the antibodies are bound to the solid phase and antigens are detected within sera, whereas indirect ELISA binds antigen to the solid phase so that antibody concentrations can be examined.

In the three decades since the discovery of the ELISA technique, it has been adapted and tested as a viable bioassay for many hundreds of antigens and antibodies. Such analytes include,

(i) Cytomegalo-virus[20-22]

(ii) Toxoplasmosis[23-25]

(iii) Hepatitis-A[26, 27] and Hepatitis-B[28-30]

(iv) Rheumatoid Factor[31-33]

More recently, due to various experimental and statistical analyses, the ELISA test for Antinuclear Antibodies (ANA) is being accepted as an increasingly valuable tool in the

diagnosis of many autoimmune diseases, including systemic lupus erythematosus[34-36]. However, due to the much needed specificity in ANA testing, immunofluorescence has remained the preferred ANA diagnostic for most clinicians. Additionally, ELISA has been employed in the detection of the Human Immunodeficiency Virus (HIV)[37-40]. It is this use of ELISA in HIV testing that represents the cutting edge of ELISA use. With techniques being developed and incorporated that use such innovation as two separate immunosorbent stages in order to detect all the sub-types of the HIV virus in sera[37], combined with new ways of making recombinant proteins that the HIV virus will respond to on a solid phase[38, 40], ELISA tests continue to make improvements to the way HIV is diagnosed.

Despite the multitude of changes to the uses for which ELISA has been utilized, the general methodology has remained largely unchanged since it was first introduced. In its current form the ELISA technique uses a polystyrene microtitre well as a solid phase, arranged in to a 96-well plate, allowing for multiple assays to be carried out under the same conditions in a short space of time. It is difficult to pinpoint at which point the ELISA method was first conducted within microtitre wells rather than the tubes used by Engvall and Perlmann, but it can be safely assumed that most researchers had switched to the more efficient wells by 1978[20, 27]. Other than this, the main element of the ELISA procedure which has changed since its introduction is the source of the material bound to the solid phase. In the early days of ELISA antigens and antibodies to be applied would have to be harvested from naturally occurring tissues, a costly and infecund process which restricted the development of ELISA techniques quite significantly. However, during the 1980's came the full-scale introduction of recombinant biological materials[41,42]. The use of recombinant materials (rather than those harvested from living

tissue) involves producing many antigens and antibodies in a laboratory environment. Production is achieved by taking the relevant cDNA's for that antigen or antibody and transplanting them into bacterium, or other suitable cells (many prokaryotic and eukaryotic systems have been tried[14]), and purifying the expressed material[41, 42].

1.3 ELISA as a Tool for the Quantification of TPO aAbs in Human Sera

The ELISA for TPO aAbs uses a typical indirect ELISA technique: recombinant TPO is bound inside microtitre wells and human sera from patients with suspected thyroid conditions are introduced. If TPO aAbs are present in the sera binding to the TPO will occur. A read step using anti-human antibody that has been enzyme linked allows the amount of binding that has occurred to be read. Reading occurs *via* a substrate that changes colour in the presence of the linked enzyme. The intensity of this colour is representative of the amount of TPO aAbs present in the sample. Intensity is then read by colorimitry and each well is assigned an absorbance value in optical density. In addition, solutions containing known concentrations of TPO aAbs are tested and a simple calibration curve is constructed using optical density values. The calibration curve is then used to calculate the relative concentration of TPO aAbs in the patient sera. The TPO ELISA is well established as a diagnostic process, being cited as both an experimental and clinical technique since the first published example of a TPO ELISA in 1992[43]. The publication by Lauberg *et al.,* demonstrated a stable ELISA for TPO aAbs with a good dose response and good reproducibility[43]. Since then the test has been used in diagnosis[1, 2] as well as in experimental work involving TPO or TPO aAbs[6, 44].

1.4 Problems with TPO as an Analyte

TPO ELISA is still an imperfect system due to the fact that information regarding several aspects of the test is still obscure[19]. Research in this area concerns two areas: role of the target antibody during the antigen/antibody interaction and the nature and structure of the TPO molecule. TPO is a large complex enzyme (approximately 104000amu) that consists of 933 amino acid residues in combination with a complement control protein, epidermal growth factor protein and a protein comparable to myeloperoxidase[44, 45]. Due to this formidable complexity and several structural idiosyncrasies its full three-dimensional arrangement is yet to be fully elucidated. To further complicate matters there is also evidence to suggest that TPO exists in two isoforms, named TPO-1 and TPO-2. TPO-1 being the full length naturally occurring enzyme and TPO-2 being an alternatively spliced shorter version (833 residues)[46]. It is still unclear whether TPO-2 has any role in thyroid function, but is expressed in the thyroid tissue of Graves' disease patients.

1.5 TPO, the Current State of Research

However, there are numerous studies that reveal aspects of the TPO enzyme's true nature. These mainly look at, amongst other things,

(i) The isolation of the TPO from its native tissues[45, 47]. This area of research is largely outdated now where ELISA is concerned, as recombinant methods are far superior for obtaining TPO. However, harvesting native TPO still occurs, mainly as a source of material for research looking at TPO's natural functions.

(ii) Analysis of TPO activity and role during natural enzyme reactions[47, 48]. The analysis of TPO's natural functions is generally an *in vitro* examination of its iodinating properties or its role in catalyzing enzymatic functions.

(iii) The region of the TPO molecule that binds antigenically (the epitope or immunodominant region (IDR))[8, 44, 49, 50]. The search for the TPO IDR and its activity is an area of considerable interest to modern science, it is hoped that analysis of this aspect of the TPO molecule will shed light on the causes of Graves' disease and Hashimoto's thyroiditis, eventually leading to causes and treatments.

(iv) The gene sequence that codes for TPO production[14, 41, 42, 51-53]. These sequences concerns are essential for the production of recombinant TPO.

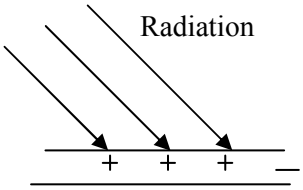
In addition, there is also some data on the activity of TPO when binding with it's associated aAbs within the ELISA well[44]. Work carried out by Bresson *et al.* showed that TPO's IDR is split into two regions (IDR/A and IDR/B)[44]. Each region has aAbs that bind with more affinity to one particular region, but in more advanced stages of autoimmune thyroid disease the surface of the TPO molcules become increasingly more antigenic. It is also shown in the same paper that the varying specificity of TPO aAbs corresponds to a varying binding activity on the surface of the plate. It is also indicated that, generally, when increasing the concentration of TPO aAbs in a *antibody calibrator* by one order of magnitude should result in an increase in one unit of absorbance, giving sigmoidal calibration curve shapes, flattening at around three orders of magnitude. Bresson's work aside, very few studies exist that observe TPO within an ELISA environment, making it difficult to predict its behavior in such situations.

To summarise, it can be observed from current literature that the ELISA method for antibody detection is a much tested and useful technique. However, when used to detect the antibodies associated with autoimmune thyroid disease (*i.e.* TPO aAbs) then there are still many areas of knowledge that are unclear and require further research.

1.6 Indirect ELISA Process

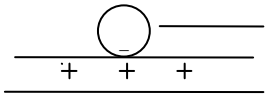
The indirect ELISA method is described. Like all ELISA techniques the indirect version relies on the specific binding of antibodies in human sera to the antigen bound to the solid phase of the test, in the case of this project, the surface of a microtitre well. The following set of steps describe the current indirect ELISA process (experimentally, the specifics of each step will be described in Chapter 2) A pictorial presentation of indirect ELISA Steps 1-7 can be found in figure 1 with details of each step below. Steps 1-3 concern the preparation for the assay (*i.e.* production of assay plates) and Steps 4-8 concern the assay proper.

Step 1. **Irradiation**



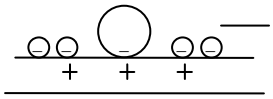
Surface of microtitre well is irradiated to produce positive charge.

Step 2. ***Coating buffer*** **addition**



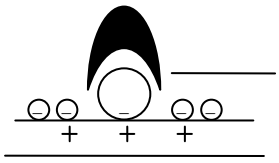
TPO is bound to the surface of the well.

Step 3. ***Blocking buffer*** **addition**



BSA molecules are bound to the surface to block non-specific binding.

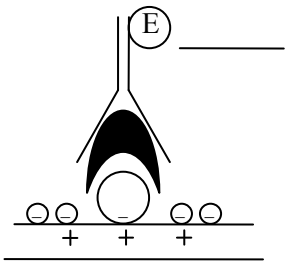
Step 4. **Samples and *antibody calibrator*s addition**



TPO aAb binds to TPO molecule when sera is added.

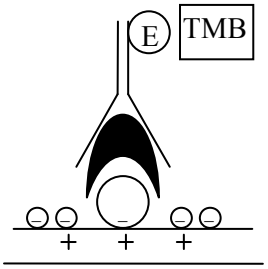
Step 5. **Incubation and wash**. The well is washed to remove non-bound material.

Step 6. ***Conjugate antibody*** **addition**



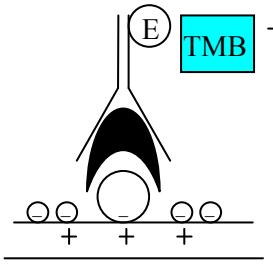
Enzyme-conjugated anti-human IgG binds to TPO aAb. (Step 5 is repeated).

Step 7. **Substrate addition**



TMB substrate is added.

Step 8. **Read and Results**



TMB changes colour in the presence of the enzyme allowing the TPO aAb concentration to be read.

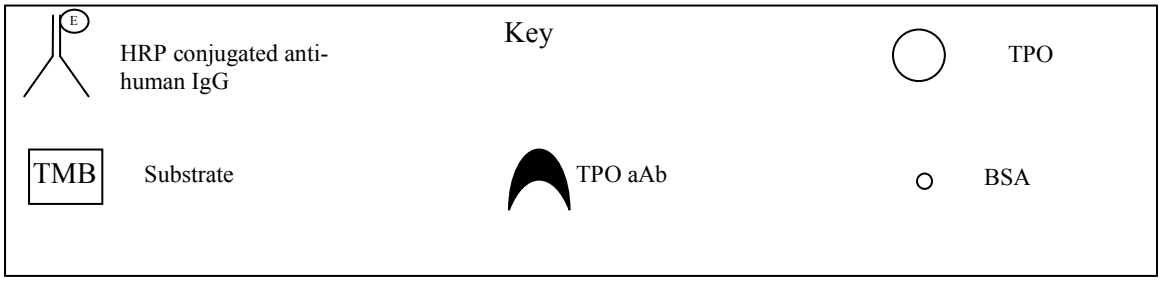


Figure 1. A schematic representation of the Indirect ELISA process.

Assay Preparation:

Step 1. **Irradiation:** The first stage of the ELISA process requires the solid phase. In most cases this is a microtitre plate (figure 2) that consists of 96 polystyrene wells (figure 3). The polystyrene wells are then irradiated with a source of beta radiation. The radiation creates positive surface charge within the well, caused by the reaction shown in figure 4.

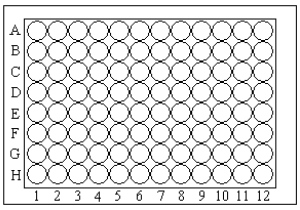


Figure 2. 96 well microtitre plate

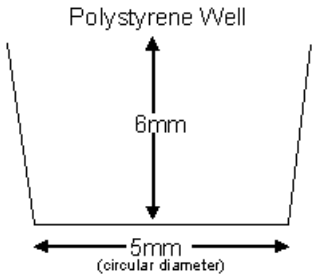


Figure 3. 1 microtitre well

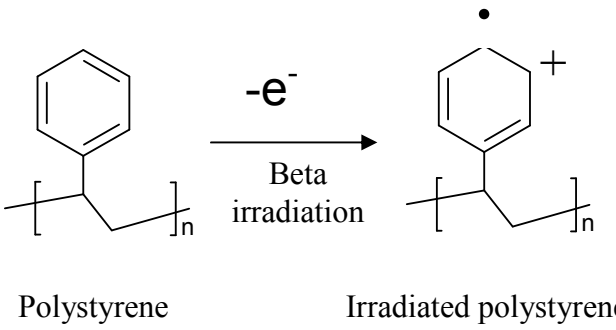


Figure 4. Irradiated polystyrene on the surface of the microtitre well containing positive charge to allow adsorption of antigen.

Step 2. ***Coating buffer*** **addition:** The target antigen is diluted in an appropriate chemical buffer (known as the *coating buffer)* and added to one of the irradiated plates. The antigen will undergo adsorption on to the surface of the well. Adsorption occurs due to negatively charged functional groups on parts of the TPO protein strand which allows electrostatic binding between TPO molecules and the well surface.

Step 3. ***Blocking buffer*** **addition:** Following the coating stage is the blocking stage. The blocking stage is where a protein (usually Bovine Serum Albumin, BSA) is absorbed on to the well surface that is not covered by the antigen. The BSA is added in a second chemical buffer known as the *blocking buffer*. The process of blocking blocks the areas of the well surface where TPO is not bound prevents non-specific antibody binding from occurring when human sera is added.

Assay:

Step 4. **Samples and *antibody calibrator*s addition:** Samples of human sera for testing are diluted into a chemical buffer. *Antibody concentrates* containing known amounts of antibody are diluted in to a range of concentrations to create a set of *antibody antibody calibrators*. Each sample is added to a separate well on the microtitre plate in duplicate. For the purpose of the TPO ELISA *antibody calibrator* concentrations are calculated in U/ml. The unit "U/ml" is a standardized unit indicating the relative amount of the desired antibodies that are present in a solution, it should be read as "Antibody Units/ml". *Antibody*

calibrator concentrations are usually 2700 U/ml, 900 U/ml, 300 U/ml, 100 U/ml and 33 U/ml.

Step 5. **Incubation and wash:** The plate is incubated for 30 minutes at room temperature. Incubating the plate allows time for antibodies specific to the target antigen present the well to bind accordingly. Once this process is complete each well is thoroughly washed, this is to ensure that all unbound antibodies and other biological materials are removed from the well. If unbound antibodies were to remain in the well (sera samples are likely to contain antibodies not specific to the target antigen) then they would be read in the following phases of the test, giving a falsely elevated result.

Step 6. ***Conjugate Antibody*** **addition:** Following the wash, the conjugate is added. The conjugate is anti-human Immunoglobulin G (IgG) that will bind to analyte antibodies (TPO aAbs in a TPO ELISA). The IgG has been conjugated with an enzyme that will allow the read process to take place (in this ELISA the enzyme is horseradish peroxidise (HRP)). This step is followed by a further incubation and wash as in step 5 (above). In the case of a positive ELISA test conjugated antibodies will bind to any TPO aAbs bound inside the well. In the case of a negative test the conjugated antibody will be removed with the wash. This discrimination means that wells containing sera that are positive for TPO aAbs will contain conjugated antibody.

Step 7. **Substrate addition:** Next, a substrate is added to the well. This substrate will undergo a redox reaction with the enzyme conjugate. The redox reaction between the conjugate enzyme and the substrate induces a colour change within the well, the intensity of which will be related to the amount of TPO aAbs. The substrate that is added is 3,3',5,5'-tetramethylbenzidine (TMB)[54, 55]. The TMB, upon addition to the well, is oxidized by the H_2O_2 in the horseradish peroxidase giving the liquid in the well a blue colour. The reaction is then quenched using phosphoric acid.

Step 8. **Read and Results:** Each well is read by colorimetry at 450nm. The result for each well is an intensity given in optical density. The relative optical intensity given by a well is directly proportional to the amount of TPO/TPO aAb binding that occurred within the well. The optical density values for each of the *antibody calibrator*s is taken and used to plot a calibration curve (figure 5).

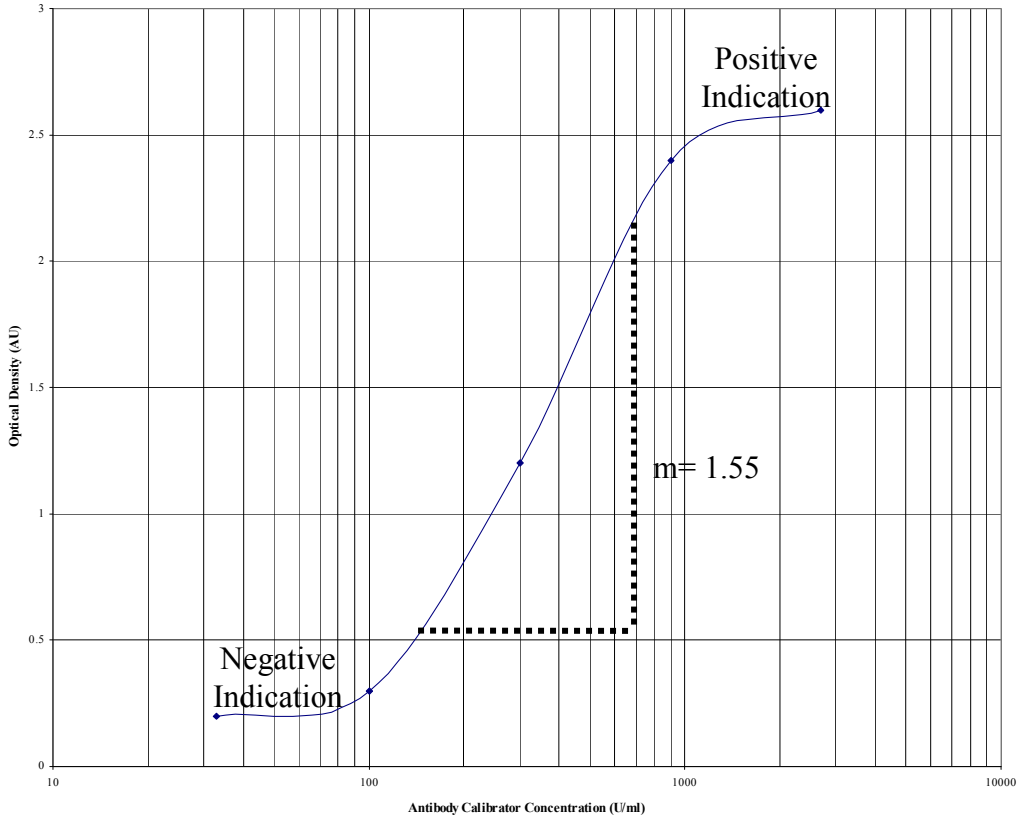


Figure 5. ELISA calibration curve for typical assay. Result taken from The Binding Site Ltd. ELISA for Tetanus toxoid.

ELISA calibration curves are plotted logarithmically and favourably have a steep gradient as shown in figure 5. A steep gradient provides a strong positive/negative indication i.e. patients sera test against this curve could be clearly defined as positive or negative.

1.7 The Drawbacks of TPO ELISA

The graph in figure 5 shows a typical ELISA results graph for Tetanus toxoid, the graph in figure 6 shows a typical ELISA result for TPO.

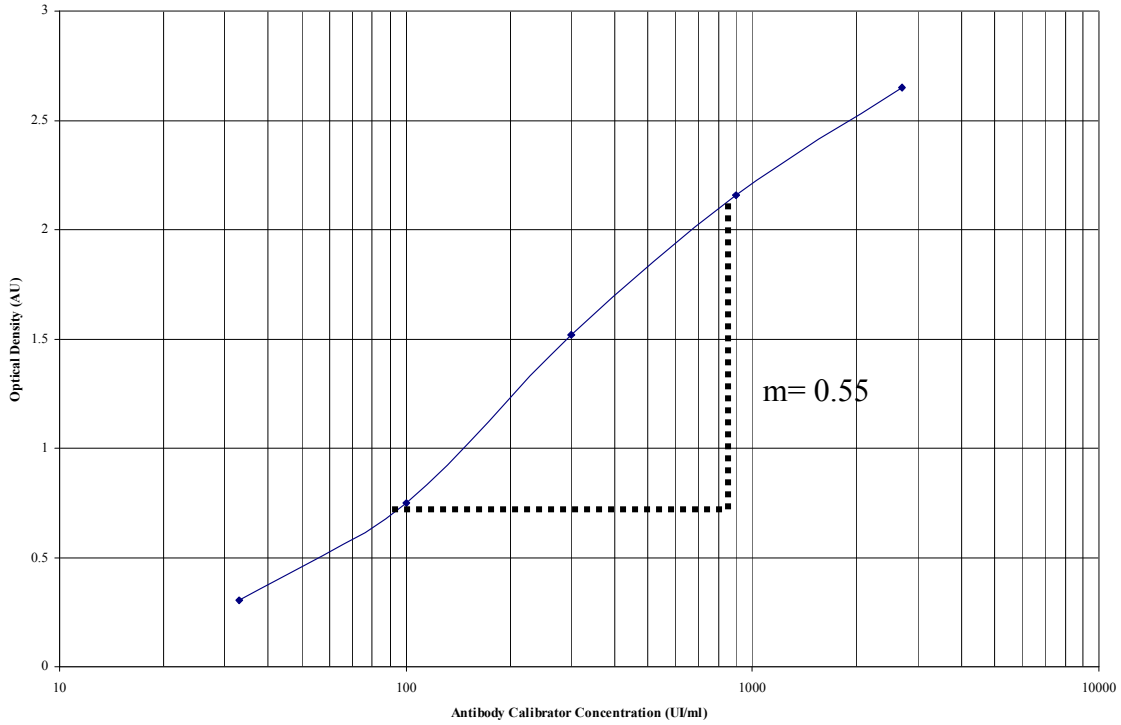


Figure 6. ELISA calibration curve for typical TPO assay.

It is clear from the comparison between figures 5 and 6 that the TPO ELISA has a much less steep gradient overall (m= 0.55). A lower value for m and flattening of the curve makes the test less clinically useful as there are not clearly defined positive/negative indicators.

1.8 Objectives of Study

The objective of this study, primarily, was to rectify the problem of the TPO ELISA calibration curve flattening shown in figure 6 in order to obtain more normal boundaries between positive samples and negative samples (as shown in figure 5). The second objective of this study was to test and analyse the reproducibility of the assay over time and, if necessary modify the test to improve it.

This gives two objectives:

Objective 1: To modify either the way in which the assay is produced or the way in which the assay is run in order for it to produce a stable and reproducible calibration curve with a steeper gradient similar to the one shown in figure 5.

Objective 2: To analyse the reproducibility of the assay over time and, if necessary, modify either the way in which the assay is produced or the way in which the assay is run in order to improve the reproducibility of the assay.

Objective 1 was tackled by modifying different areas of the test production and procedure. The areas studied correspond to steps outlined in section 1.6, these steps being,

(i) Step 2. Coating conditions were examined. The first stage was to look at how changing the buffers within which TPO is bound to the solid phase affects the assay.

(ii) Step 4. Samples and *Antibody calibrators*. The chemical buffers that contain samples and *antibody calibrator*s during the assay process were studied for their effect on assay performance.

(iii) Step 6. *Conjugate antibody* addition. The read chemical, the *conjugate antibody*, was studied for its affect on assay performance.

Objective 2 was tackled almost exclusively by looking at the *antibody calibrators* and *antibody concentrates* used within the test.

Any modifications to the assay production or procedure made throughout the study in order to reach *Objectives 1* or *2* must not change either of the following,

(i) The economic feasibility of the test. Any modification made to the test, its production or its procedure must not increase the cost by a large margin. This mainly concerns increasing concentrations of expensive reagents.

(ii) The practical feasibility of the test. For the test to sell as a product its procedure must adhere to a standard model. For example, changing the dilutions of *antibody calibrators* and samples or prolonging incubation times cannot be deemed acceptable as part of a solution to either objective.

Chapter 2

2. Experimental

This section will describe, firstly the traditional steps taken when producing and assaying a TPO ELISA test and secondly, the various modifications made to this procedure during the experiments conducted for this study.

2.1 Basic Method for Production of TPO Indirect ELISA Plates

2.1.1 Method for Coating TPO ELISA Wells (Step 2, Figure 1)

A solution of 2-amino-2-(hydroxymethyl)-1,3-propanediol (trizma base) (0.975g), of sodium chloride (NaCl) (4.5g) and sodium deoxycholate (NaDC) (0.125g) was adjusted to pH 8.00 by adding 5M HCl dropwise. Recombinant TPO (5µl, 1mg/ml) (Diarect AG, Germany) was added to the solution to give a final concentration of antigen within the solution of 0.2µg/ml. The solution is mixed and is known as *coating buffer*. The *coating buffer* (110µl) is added to each of the 96 wells of the microtitre plate. Plates are then placed in an air-tight container lined with paper towels that have been pre-saturated in ultra-pure water. Plates are then incubated for 24 hours at 5ºC. Following incubation the plates are removed from the air-tight container and the *coating buffer* is aspirated by hand.

2.1.2 Method for Blocking TPO ELISA Wells (Step 3, Figure 1)

Stabilguard is diluted by 50% in ultra-pure water. This solution is known as the *blocking buffer*. Stabilguard is phosphate-buffered saline containing 1% BSA, as well as other sugars (including lactose) used in stabilizing large solid phase bound proteins (the exact constituents are proprietary to Diarect AG). The *blocking buffer* solution (100µl) is added to each microtitre well and left for 30 minutes. Following this the *blocking buffer* is removed and each plate is dried by placing the plates in a 37ºC incubator for 2 hours.

Once dry, each plate assay plate is sealed in a sterile foil bag and stored at 5ºC before use.

2.2 Basic ELISA Test Procedure

2.2.1 Assay Preparation (Step 4, Figure 1)

The assay plate must be removed from storage and left to stand (in its packaging) until the plate is at room temperature. During this time the *antibody calibrator*s are produced. Production of *antibody calibrator*s is achieved by taking an *antibody concentrate* and diluting it to five known concentrations (33U/ml, 100U/ml, 300U/ml, 900U/ml and 2700U/ml of TPO aAbs). The *antibody concentrate* is a solution of TPO aAbs of known concentration refined from the blood sera of a Graves disease patient. For standard diluent (trizma-buffered saline (TBS) with 0.1% BSA, 0.25% sodium azide and 1% 4,5-dichloro-2-n-octyl-isothiazolin-3-one (Kathon) and 1% polyethylene glycol (Triton X-100)) is used to dilute all *antibody calibrators*. Then each sera sample (10µl) (and in-house standard if necessary) is diluted in to sample diluent (1000µl) to give 1:100

solutions. Sample diluent is a similar mixture to standard diluent. Horseradish peroxidase (HRP) conjugated anti-human rabbit Immunoglobulin G (IgG) (10µl) is added to HRP conjugate diluent (200µl ultra-pure water, 10% stabilzyme, 0.45% Proclin 300 and 8% NaCl, 200µl) to create a 1:20 solution. This solutution is then diluted again in the same diluent to create a 1:20000 solution of HRP-conjugated anti-human rabbit IgG (referred to as *conjugate antibody*).

2.2.2 Basic ELISA Assay Method (Steps 4-6, Figure 1)

The assay plate is removed from its packaging each and *antibody calibrator* and diluted sample (100µl) is added to two separate wells in order to examine each *antibody calibrator* and sample in duplicate *(Step 4, Figure 1)*. The loaded assay plates are placed in an incubator for 30 minutes at 25^{o}C. The plate is removed and the liquid aspirated from each well. The wash process is performed by an automatic aspirator/dispenser. This device is used to wash each well with a 1% solution of tween-20. This process, known as the 'wash' process, is then repeated twice more *(Step 5, Figure 1)*. The wash process serves to remove any unbound material from the well. *Conjugate antibody* (100µl, 1:20000) solution is added to each well *(Step 6, Figure 1)* and the plate is placed in the incubator for a further 30 minutes at 25^{o}C. The wash process is then repeated to remove any unbound *conjugate antibody*. After this 3,3',5,5'-tetramethylbenzidine (TMB, 100µl) is added to each well *(Step 7, Figure 1)* and the plate is again placed in the incubator for 30 minutes at 25^{o}C. This stage of the process is referred to as 'substrate addition'. When the plate is removed from the incubator for the final time the wells that have a positive response for TPO aAbs will have developed so that the liquid in the well is now blue. The

intensity of this colour responds to the relative concentration of TPO aAbs in the original sample added to that well. Phosphoric acid (100µl, 0.5M) is added to each well in order to stop the reaction. The plate is then read by an absorbance colorimeter at 450nm. Each well is assigned a value in optical density which is then averaged for each sample. The calibration curve is then constructed from the known five concentrations of the *antibody calibrator*s and each sample is read from this curve.

2.3 Methods for Modifications made to the TPO ELISA and Assays run to Assess TPO ELISA

2.3.1 Variation of Sodium Deoxycholate Concentration (Step 2, Figure 1)

Sodium deoxycholate (NaDC) (figure 7) is added in the *coating buffer* stage as an agent that unfolds the TPO protein strand, theoretically making the antigen/antibody interaction more likely. Protein unfolding of this nature occurs due to the amphiphilic nature of NaDC. In the first stage of the project, plates were made using *coating buffers* with a range of NaDC concentration to test its affect upon assay performance and calibration curve shape. The concentration range consisted of 0%, 0.005%, 0.010%, 0.020%, 0.025%, 0.030%, 0.035% and 0.050% NaDC in *coating buffers* (with 0.025% being standard). *Note: concentrations are expressed in percentage by weight.*

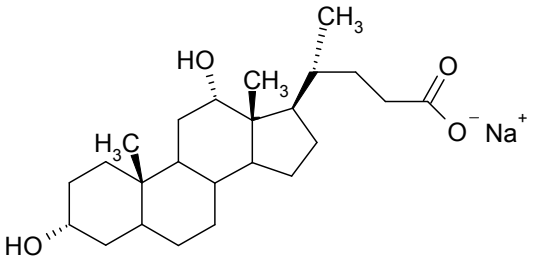


Figure 7. Sodium Deoxycholate

Production

Eight *coating buffers* were made by the method in section 2.1 (250ml each). An amount of NaDC was added to each to make the desired concentration (i.e. to one buffer solution (250ml) 0.00625g of NaDC was added to create a 0.025% solution). Each *coating buffer* coated 2 ELISA plates. No other changes were made to the procedures stated in section 2.1.

Assay

The 8 concentrations of NaDC (8 plates in total) were then assayed using standard procedures as stated in section 2.2. On each plate a *antibody calibrator* set of 33U/ml, 100U/ml, 300U/ml, 900U/ml and 2700U/ml was used. *Antibody calibrator*s were diluted into standard diluent. Six in-house standards (panels) were also tested to confirm the accuracy of the test. Panels were diluted in sample diluent.

2.3.2 Modification of Coating buffer from trizma-buffered saline to sodium carbonate (Step 2, Figure 1)

To ascertain whether the nature of the *coating buffer* was affecting the performance of the assay in any way the *coating buffer* was changed from the normal TBS to a $NaHCO_3$ solution as reported in a method by Rebuffat[56].

Production

To ultra-pure water (500ml) was added sodium carbonate (0.785g), sodium hydrogen carbonate (1.456g) and NaDC (0.125g). The solution was then adjusted to a pH of 9.0 (as recommended by Rebuffat[56]) using 5M HCl. All other procedures for coating and blocking stated in section 2.1 were followed.

Assay

The plate was then assayed according to standard procedures as stated in section 2.2. As a comparison, a plate using a standard TBS *coating buffer* was assayed in parallel, using exactly the same test materials and *antibody calibrator*s. On each plate a *antibody calibrator* set of 33U/ml, 100U/ml, 300U/ml, 900U/ml and 2700U/ml was used. *Antibody calibrator*s were diluted into standard diluent. Six in-house standards were also tested to confirm the accuracy of the test. Panels were diluted in sample diluent.

2.3.3 Variation of Bound Antigen Concentration (Step 2, Figure 1)

Testing the elevation of the concentration of antigen bound to the surface of the well was the next variation made to the TPO ELISA test. It was thought that by increasing the amount of binding occurring between the antigen and antibody the gradient of the calibration curve could be increased. However, this meant that the optical densities of the wells with higher concentrations of binding would exceed the operational limit of the colorimeter. Therefore, it was decided that lowering the concentration of the *conjugate antibody* solution (effectively reading less of the plate) would solve this problem. In this experiment plates were coated with 0.2, 0.3, 0.4 and 0.5µg/ml recombinant TPO (with 0.2µg/ml being the standard concentration).

Production

Coating buffer solution was made according to procedure stated in section 2.1. Following pH adjustment the solution was split into four equal parts of 25ml. Recombinant TPO (1mg/ml. 5µl, 7.5µl, 10µl and 12.5µl) was added to each solution, respectively. Final antigen concentrations in each well were therefore 0.2, 0.3, 0.4 and 0.5µg/ml. Four blank microtitre plates were then coated, each with a different concentration of antigen. Incubation, blocking and drying then occurred according to normal procedure.

Assay I

Each plate was tested according to normal procedure with a standard *antibody calibrator* set made from *antibody concentrate* EQ1601 and a *conjugate antibody* solution of

1:20000. In-house standards were also tested on each plate. All other standard procedures were followed from section 2.2.

Assay II

In the second test, each plate was tested again, but this time to each plate the same *antibody calibrator* sets was assayed three times. On each was applied a different concentration of *conjugate antibody* solution. The *conjugate antibody* solutions used were 1:25000 and 1:30000 in concentration using the standard HRP-conjugated anti-human rabbit IgG. The third *conjugate antibody* solution was a 1:50000 solution of the same type of IgG, but from a different manufacturer (Jackson Immunoresearch, U.S.A), who produce a more refined and responsive version of the same conjugated antibody. All other standard procedures were followed from section 2.2.

2.3.4 Modification of Sample Diluent from trizma buffered saline to phosphate buffered saline (Step 4, Figure 1)

To determine if the contents of the sample diluent was restricting the interaction between TPO and TPO aAbs, assays were run to compare the traditional TBS with phosphate-buffered saline (PBS). This test was run on plates made in sections 2.3.1 and 2.3.2 as to observe the effect of different diluents on plates with varying NaDC concentration and *coating buffer*.

Production

Plates from sections 2.3.1 and 2.3.2 were used.

Assay

A plate using the standard TBS *coating buffer* (section 2.3.1) and a plate using the experimental $NaHCO_3$ *coating buffer* (section 2.3.2) was assayed. On each plate two *antibody calibrator* sets of 33U/ml, 100U/ml, 300U/ml, 900U/ml and 2700U/ml were used; one set diluted in standard diluent (TBS) and one set diluted in PBS. The PBS used was made from ultra-pure water with 10% NaCl, buffered with 0.25% KCl, 1% disodium hydrogen orthophosphate and 0.2% sodium dihydrogen orthophosphate. The solution also contains the same stabilizing agents and preservatives as standard diluent; namely BSA, Kathon and sodium azide. Six in-house standards (panels) were also tested on each plate to confirm the accuracy of the test. The rest of the procedures stated in section 2.2 were followed.

2.3.5 Examination of Antibody Concentrates from Varying Patients (Step 4, Figure 1)

Various *antibody concentrates* used to produce the *antibody calibrator*s were tested for differing effects on assay performance. Several different *antibody concentrates* were obtained and *antibody calibrator* sets made from each of them, each *antibody concentrate* coming from a different patient. The various *antibody concentrates* were coded as EQ1227 (the standard *antibody concentrate*), EQ1207, EQ1600, EQ1601 and Ha28790. All the calibration solutions were then tested on mimic plates from section 2.3.4 as well as plates obtained from Diarect AG and plates manufactured according to the methods from section 2.1.

Production

Plates were manufactured according to methods in section 2.1 and section 2.3. Plates obtained from Diarect AG were also used.

Assay

The *antibody calibrator* set 33U/ml, 100U/ml, 300U/ml, 900U/ml, 2700U/ml was produced from each *antibody concentrate* (EQ1227, EQ1207, EQ1600, EQ1601 and Ha28790). One of each plate type (mimic, Diarect AG, standard) was then taken and the *antibody calibrator* sets made from all six *antibody concentrates* were tested on each plate. The six in-house standards (panels) were also tested on each plate. All other assay procedures from section 2.2 were followed.

2.3.6 Variation of Conjugate antibody Dilution (Step 6, Figure 1)

To examine the effect of *conjugate antibody* solution upon assay performance, several assays were carried out using various antibody concentrations. A single *antibody concentrate* was used in this experiment (EQ1601). This experiment was carried out on plates manufactured in the standard manner.

Production

Plates were produced according to the methods in section 2.1.

Assay

A standard *antibody calibrator* set of 33U/ml, 100U/ml, 300U/ml, 900U/ml, 2700U/ml was produced from *antibody concentrate* EQ1601. Then four different solutions of *conjugate antibody* were produced from a standard 1:20 solution: concentrations of 1:5000, 1:10000, 1:15000 and 1:20000 were made. Then the *antibody calibrator* set was tested four separate times on a single plate along with the in-house standards. During the assay process each *antibody calibrator* set was subjected to a different concentration of *conjugate antibody*. All other assay procedures stated in section 2.2 were followed.

2.3.7 Variation of Antigen Batch and Concentration (with Variation of Conjugate antibody Concentration) (Step 2 and 6, Figure 1)

The concentration of antigen coated per well was increased again, this time to 1 and 1.5µg/ml (five and seven times higher than normal, respectively). However, the concentration of the *conjugate antibody* solution was modified from 1:20000 to 1:100000 to account for the increased activity; keeping optical density readings within the operational parameters of the colorimeter.

Production

Two plates were produced according to the method shown in section 2.3.2 one with 1µg/ml of TPO in its *coating buffer* and one with 1.5µg/ml. All other standard procedures from section 2.1 were followed.

Assay

Each plate was tested with a standard *antibody calibrator* set made from *antibody concentrate* EQ1601. In-house standards were also tested on each plate. A *conjugate antibody* solution was made from IgG from Jackson Immunoresearch (U.S.A) at a concentration of 1:100000. All other assay procedures from section 2.2 were followed.

2.3.8 Production and Testing of Mimic Plates

Production methods for TPO ELISA plates were acquired from Diarect AG and tested. Supposedly, the method suggested by Diarect AG would give a more favourable curve shape. In parallel, plates manufactured by Diarect AG were obtained and results were compared to determine whether the Diarect AG plate did have more favourable curve shape and whether or not it could be replicated with available materials.

Production of Mimic Plates

Plates were manufactured according to the method suggested by Diarect AG.

-Coating

To ultra pure water (250ml) was added sodium chloride (2.19g), trizma base (0.304g) and NaDC (0.135g). The pH of the solution was then adjusted to pH 8.00 by adding 5M HCl dropwise. Then recombinant TPO (5µl, 1mg/ml) was added to the solution to give a final antigen concentration in the solution of 0.1µg/ml. The solution was thoroughly mixed throughout. Next, two blank microtitre plates were taken and 120µl of the *coating buffer* was added to each well. The plates were then incubated for 14 hours at 15ºC before the *coating buffer* was removed.

-Blocking

A blocking solution of 0.5% lactose and 1% BSA in PBS (ultra-pure water with 9% NaCl, 3% disodium hydrogen orthophosphate and 1% sodium dihydrogen orthophosphate) was adjusted to pH 7.4 was prepared for blocking. To each well *blocking*

buffer (150 µl) was added and left for 1 hour. Following this the *blocking buffer* was removed and the plates were dried in a 37ºC incubator for 2 hours.

Assay I

The mimic plate was then tested using standard procedures described in 2.2. On the plate a *antibody calibrator* set of 33U/ml, 100U/ml, 300U/ml, 900U/ml and 2700U/ml was used. *Antibody calibrator*s were diluted into standard diluent. Six in-house standards (panels) were also tested to confirm the accuracy of the test. Panels were diluted in sample diluent.

Assay II

Plates manufactured by Diarect AG were then obtained and the same assay described above was repeated. Alongside this assay, the mimic plates from above were tested again. This repeat was performed because the concentration of the *conjugate antibody* was increased from 1:20000 to 1:10000 in this second test and for the purposes of fair comparison both plates were assayed under the same conditions. All other condition from the above assay and procedures stated in section 2.2 were followed.

Assay III

In addition *Assay II* was repeated using the *conjugate antibody* solution supplied by Diarect AG.

2.3.9 Analysis and Comparison of Competitors Plate

To rule out the possibility that the problems observed in the TPO ELISA caused by something localised to the laboratory (e.g. a contaminant) several ELISA tests for TPO aAbs were obtained from an external source. A plate was then assayed following all of the manufacturer's instructions as well as substituting all the standard diluents, *antibody calibrator*s, *conjugate antibody* solution and other test materials for those supplied by the manufacturer. Further tests were then conducted to compare the performance of the ELISA test under study and that of the test obtained externally.

Production

Anti-TPO ELISA plates were obtained from Phadia AB (Sweden). Plates for comparison were manufactured according to the methods in section 2.1.

Assay I

The plate obtained from Phadia AB was tested according to the manufacturer's instructions. Two supplied controls were also tested. The method for this assay was fundamentally the same as the procedures stated in section 2.2 with some minor changes. Mainly, the *antibody calibrator* set supplied contained six solutions of 0U/ml, 30U/ml, 100U/ml, 300U/ml, 1000U/ml and 3000U/ml. For full details of these instructions see Appendix A.

Assay II

To compare the two tests one plate made using standard method and one from Phadia were taken and tested identically. Applied to each plate were three separate *antibody calibrator* sets: one made from the standard EQ1227, one made from EQ1601 and the *antibody calibrator*s supplied by Phadia. On both plates all assay materials including wash buffers, diluents and *conjugate antibody* solutions were standard as in section 2.2. The assay of both plates was then repeated, but in this second instance assay materials used were those supplied by Phadia.

2.3.10 Method for Assessment of TPO ELISA In-House Standards

The in-house standards, which are coded P1.2, P2.2, P3.2, P4.2, P5.2 and P6.2 are fluids taken from a stock solution containing an exhaustively tested concentration of TPO aAbs. They are then tested as normal samples (i.e. diluted in sample diluent to a concentration of 1:100) within a TPO ELISA and are used to regulate the calibration curve. These standards are prepared and stored separately to other fluids used in assay procedure to avoid contamination. Details of each standard can be found below in Table 1. The coefficient of variation (CV) is a number derived by looking at the concentration value returned for a particular standard by several assays of the same type. Over time, this value should not fluctuate by more than 10%. Standards were tested over a period of 5 days.

Standard	Dilution in assay	Mean Concentration (U/ml)	Acceptable Range (U/ml)
P1.2	1:100	87	74-100
P2.2	1:100	168	143-193
P3.2	1:100	223	189-256
P4.2	1:100	280	238-322
P5.2	1:100	376	320-432
P6.2	1:100	700	595-804

Table 1. Concentration values for each in-house standard and the range of values an assay may return to be deemed acceptable.

Production

Ten assay plates were made identically according to the methods in section 2.1.

Assay

One plate was assayed morning and afternoon for five days. All methods (indicated in section 2.2) were followed and all assay conditions, additives and procedures were followed in exactly the same manner for every assay. A new sample of each standard was taken for use each day (as they are stored in 100µl batches). A standard *antibody calibrator* set was produced from *antibody concentrate* EQ1227. In addition a 400U/ml solution of EQ1207 was tested on each plate. *Note: a solution of 400U/ml is a positive control supplied with each test.*

2.3.11 Method for Assessment of TPO ELISA Antibody concentrate

A recovery test was performed on each standard listed in Table 1. In a recovery test serial dilutions are made from standards. For example P1.2, before an assay it is diluted just like a sample to make a solution of 1:100: this solution is expected to give a value of 87U/ml. In this test however, a solution of 1:10 is made and then serially diluted to give a set of solutions with 1:10, 1:20, 1:40, 1:80 and 1:160 (with expected values of approximately 800, 400, 200, 100 and 50U/ml). A serial dilution is range is created in this manner for all in house standards. However, some top concentrations are adjusted to keep the serial within the calibration range (see Table 2). All solutions are then tested against the same *antibody calibrator* set.

Production

Plates produced according to the methods in section 2.1 were used for this test.

Assay I

In this first assay standard *antibody concentrate* EQ1227 was tested. All serial dilutions were tested on one plate with a *antibody calibrator* set made from EQ1227. Dilution series shown in Table 2 were made. In addition, a positive control was tested as in the assay from section 2.3.10.

Standard	P1.2		P2.2		P3.2		P4.2		P5.2		P6.2	
Expected conc. 1:100	80		200		240		300		400		1000	
	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.
Serial Dilutions	800	1:10	2000	1:10	2400	1:10	2000	1:15	2000	1:20	2000	1:50
	400	1:20	1000	1:20	1200	1:20	1000	1:30	1000	1:40	1000	1:100
	200	1:40	500	1:40	600	1:40	500	1:60	500	1:80	500	1:200
	100	1:80	250	1:80	300	1:80	250	1:120	250	1:160	250	1:400
	50	1:160	125	1:160	150	1:160	125	1:240	125	1:320	125	1:800

Table 2. Showing serial dilutions made from standards in assessment of *antibody concentrate* EQ1227. Concentrations given in U/ml.

Assay II

In a second assay the test was repeated in a similar fashion, but this time only using a cross section of the standard serial dilutions (those tested are shown in Table 3). The test was carried out in this manner in order to fit more sets of *antibody calibrator*s on the same plate. On this plate *antibody calibrator* sets made from EQ1600, EQ1601 and EQ1227 were tested along with two new *antibody concentrates* coded Cal5078 and Cal28793. All *antibody calibrator* sets were made to the standard concentrations. Performing the test in this way meant that several *antibody concentrates* could be efficiently tested for recovery. The plate was assayed according to all procedures stated in section 2.1.

Standard	P1.2		P3.2		P6.2	
Expected conc. 1:100	80		240		1000	
	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.
Serial Dilutions	800	1:10	2400	1:10	2000	1:100
	400	1:20	1200	1:20	1000	1:200
	200	1:40	600	1:40	500	1:400
	100	1:80	300	1:80	250	1:800
	50	1:160	150	1:160	125	1:1600

Table 3. Showing serial dilutions made from standards in assessment of *antibody concentrates* EQ1600, EQ1601, EQ1227, Cal5078 and Cal28793. Concentrations given in U/ml.

2.3.12 Analysis of National Institute for Biological Standards and Control (NIBSC) Reference

A nationally recognised standard was used to examine TPO *antibody concentrates*. A national standard for TPO aAbs was obtained from National Institute for Biological Standards and Control (NIBSC, U.K.) and tested on standard plates. A *antibody calibrator* set was made from NIBSC reference material was tested against *antibody calibrator sets* made from *antibody concentrates* EQ1600 and EQ1601.

Production

Plates were produced with the new TPO batch according to the methods in section 2.1.

Assay

NIBSC reference was taken and diluted (into sample diluent) to make a *antibody calibrator* set consisting of 5 solutions with concentrations 30.86U/ml, 92.59U/ml, 277.77U/ml, 833.33U/ml and 2500U/ml. *Antibody calibrator* sets using *antibody concentrates* EQ1600 and EQ1601 were also made to standard concentrations. The three sets were then tested on a single plate along with the six in-house standards according to procedures from section 2.2. In parallel, the exact same *antibody calibrator* sets and standards were tested on a plate coated with the original TPO batch for added comparison.

2.3.13 Method for Fixing Antibody calibrators against NIBSC Reference

A method to use the NIBSC reference material to align *antibody calibrator* sets with their correct values. Assays were run using in-house standards with an *antibody calibrator* set made from NIBSC reference. The concentrations for the in-house standards returned by the NIBSC reference calibration curve were considered to be taken as correct. Comparing the concentration values for each standard returned by the NIBSC curve with those returned by other *antibody calibrators* sets would allow an adjustment value to be derived. This value would then be used to adjust the concentration of the *antibody concentrates* so that the calibration curves obtained from their *antibody calibrator* sets matched the curves obtained from the NIBSC.

Example

The results in table 4 show the concentrations of all six standards by two different *antibody calibrator* sets.

Antibody Concentrate	NIBSC Reference	EQ1600
Standard	Concentration (U/ml)	
P1.2	85.73	71.18
P2.2	141.42	122.4
P3.2	203.97	177.94
P4.2	268.305	229.24
P5.2	423.49	333.6
P6.2	666.36	471.06

Table 4. Concentration results for each standard returned by the NIBSC reference calibration and a calibration curve given by *antibody concentrate* EQ1600

It is clear from the values in Table 4 that EQ1600 is returning incorrect values. Therefore the results are processed according to table 5.

		NIBSC Reference	EQ1600
Standard	Concentration (U/ml)	Adjustment Value (NIBSC concentration value/ Returned concentration value)	
P1.2	85.73	1.00	1.22
P2.2	141.42	1.00	1.37
P3.2	203.97	1.00	1.25
P4.2	268.305	1.00	1.22
P5.2	423.49	1.00	1.13
P6.2	666.36	1.00	1.49
	Mean	1.00	1.24

Table 5. Deriving adjustment value from results in Table 4. The concentrations of standards given by the NIBSC calibration curve are translated as the correct values.

In this scenario then, when making an *antibody calibrator* set from EQ1600 the initial dilution would be adjusted by 1.24. This change would mean that if in the assay these results came from EQ1600 was diluted to 1:60 to get a concentration of 2700U/ml then in the next assay it would be diluted to 1:75 to adjust.

Production

All plates in assays from section 2.3.14 were made according to methods stated in section 2.1.

Assay

A NIBSC *antibody calibrator* set was produced and assayed with concentrations 30.86U/ml, 92.59U/ml, 277.77U/ml, 833.33U/ml and 2500U/ml. The top NIBSC *antibody calibrator* (2500U/ml) was made by diluting the concentrated NIBSC solution by 1:200. *Antibody calibrator* sets of standard concentration were made from EQ1600 and EQ1601 were also assayed. EQ1600 was diluted to a 1:60 (20.5µl in 1250µl) solution to create the 2700U/ml *antibody calibrator* and EQ1601 was diluted to 1:33 (25µl in 840µl). NIBSC reference material and the six in-house standards were diluted into

sample diluent and other *antibody calibrator* sets were diluted into standard diluent. All assay procedures from section 2.2 were followed.

Assay II

Antibody calibrator sets of standard concentration were made from EQ1600, EQ1601. The 2700U/ml *antibody calibrator* for each set was made according to the following dilutions: EQ1600 was diluted to 1:75 (14.5µl in 1100µl) and EQ1601 was diluted to 1:58 (15.5µl in 900µl). NIBSC reference material and the six in-house standards were diluted into sample diluent and other *antibody calibrator* sets were diluted into standard diluent. All assay procedures from section 2.2 were followed.

Assay III

The assay as above was repeated with the following adjustments: EQ1600 adjusted by 1.14 to a dilution of 1:86 and EQ1601 adjusted by 1.22 to a dilution of 1:73

Assay IV and V

Assay II and then Assay III were repeated to confirm results. *Antibody calibrator* sets from EQ1600 and EQ1601 were tested twice during each repeat, once at 1:83 and once at 1:50. The final concentration for EQ1600 was 1:75 and the final result for EQ1601 was 1:58.

2.4 Materials

2-Amino-2-(hydroxymethyl)-1,3-propanediol (trizma base) (Sigma Aldritch, Germany)

3,3',5,5'-Tetramethylbenzidine

4,5-Dichloro-2-n-octyl-isothiazolin-3-one (Kathon) (Diarect AG, Germany)

Blank 96-well Microtitre Plates (Thermo Fisher Scientific, UK)

Bovine Serum Albumin BSA (Sigma Aldritch, Germany)

Disodium Hydrogen Orthophosphate (Sigma Aldritch, Germany)

Horseradish peroxidase (HRP) conjugated anti-human Immunoglobulin G (IgG) (Dako, Denmark)

Horseradish peroxidase (HRP) conjugated anti-human Immunoglobulin G (IgG) (Jackson Immunoresearch, U.S.A)

Hydrochloric Acid (HCl) (Sigma Aldritch, Germany)

Phosphoric Acid (Sigma Aldritch, Germany)

Polyethylene Glycol (Triton X-100)

Pottasium Chloride (KCl) (Sigma Aldritch, Germany)

Proclin 300 (Sigma Aldritch, Germany)

Recombinant TPO (Diarect AG, Germany)

Sodium Chloride (NaCl (Analar)) (Sigma Aldritch, Germany)

Sodium Deoxycholate (NaDC) (Sigma Aldritch, Germany)

Sodium Dihydrogen Orthophosphate (Sigma Aldritch, Germany)

Stabilguard (Diarect AG, Germany)

Stabilzyme (Diarect AG, Germany)

Sodium Azide (Sigma Aldritch, Germany)

Thyroid Peroxidase (TPO) (Diarect AG, Germany)

TPO ELISA Kit (Diarect AG, Germany)

TPO ELISA Kit (The Binding Site, U.K.)

TPO ELISA Kit (Varelisa, Denmark)

Tween-20 (Sigma Aldritch, Germany)

Chapter 3

3. Results and Discussion: Objective 1

3.1 The Effect of Coating Conditions

3.1.1 The Effect of NaDC Concentration in the Coating buffer (Step 2, Figure 1)

The first experiment to examine the effect different *coating buffers* had on assay performance looked at sodium deoxycholate (NaDC, figure 7). In this experiment several plates were assayed with identical *antibody calibrator*s, each plate having a different concentration of NaDC in its *coating buffer,* results are shown in figure 8.

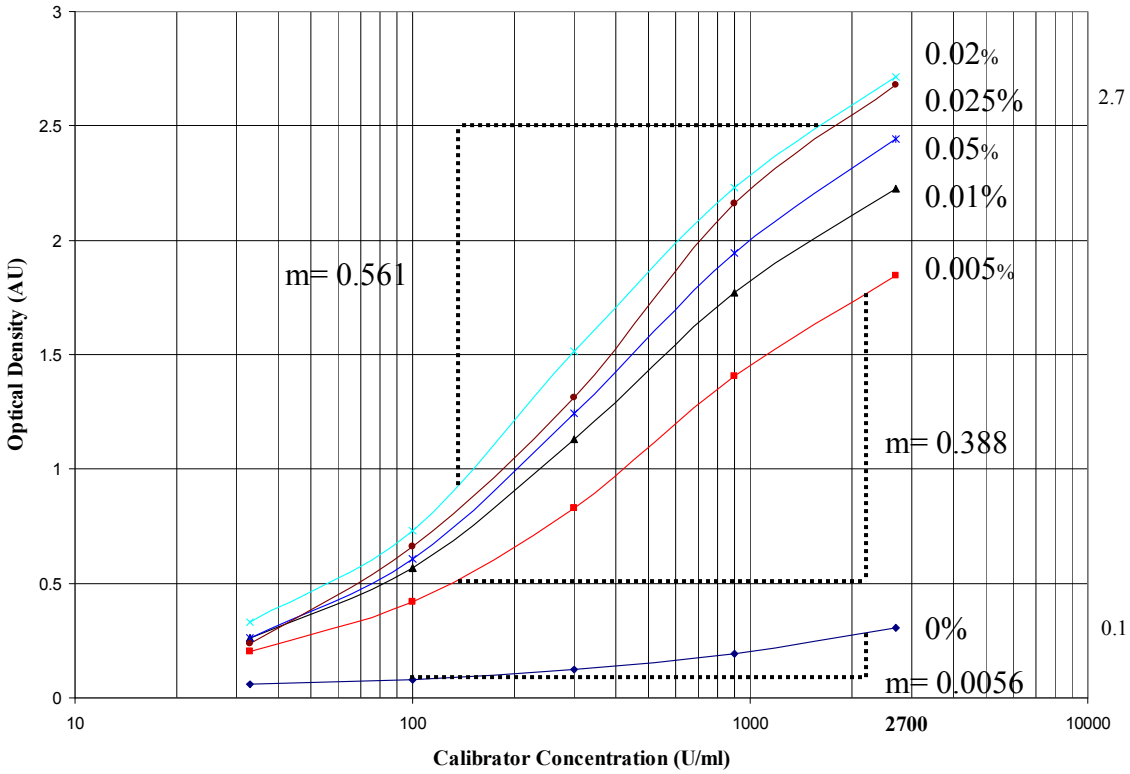


Figure 8. Graph showing how NaDC concentration to *coating buffers* effects assay response. Attached percentages show the amount of NaDC (% by weight) added to the *coating buffer* for each assay plate during production.

As it can be seen from figure 8 the addition of NaDC to the assay has a dramatic effect on response. Increasing the amount of NaDC in the coating matrix of the TPO increases the antigenic activity during the assay procedure and therefore the calibration gradient (by 10 fold between 0% and 0.02% NaDC). Even at *antibody calibrator* concentrations of 2700U/ml TPO aAbs the plate coated with 0% NaDC was returning values 7-8 times lower than the standard plate coated with 0.25%. The plate which had the standard 0.025% NaDC returned an expected curve, similar to the one in figure 5. The effect of NaDC on antigen/antibody interaction is likely to occur because of the amphiphilic nature of NaDC. Figure 7 (page 26) shows that NaDC has a hydrophilic end, which would attach itself to parts of TPO and a hydrophobic end which would, at the same time, be dissolved in the aqueous phase, in turn unfolding the protein. To illustrate how this is so particularly requisite to the TPO/TPO aAb interaction a depiction of a TPO enzyme is shown in figure 9.

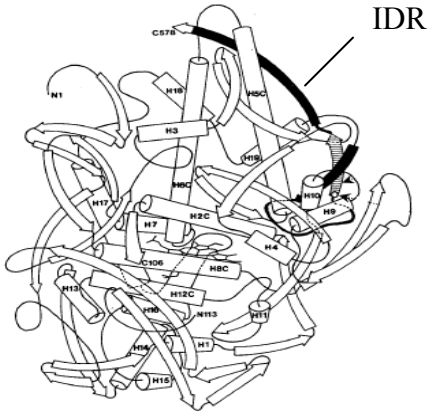


Figure 9. Depiction of TPO protein.

This picture, although no definite conclusions can be drawn from it, shows there is the possibility that the immunodominant region (IDR) of the TPO molecule (the regions recognized by the TPO aAb) could be inaccessible or at least hindered if the protein is not

unfolded in some fashion; a theory borne out by the results is figure 8. It is also observed that increasing the amount of NaDC in the matrix too much starts to null the desirable effect that NaDC has on assay interaction. In figure 8, this seems to begin to happen after 0.02%, with 0.025% and 0.05% giving lower responses. This effect can be seen more clearly in figure 10 (which includes data for plates with 0.03% and 0.035% NaDC).

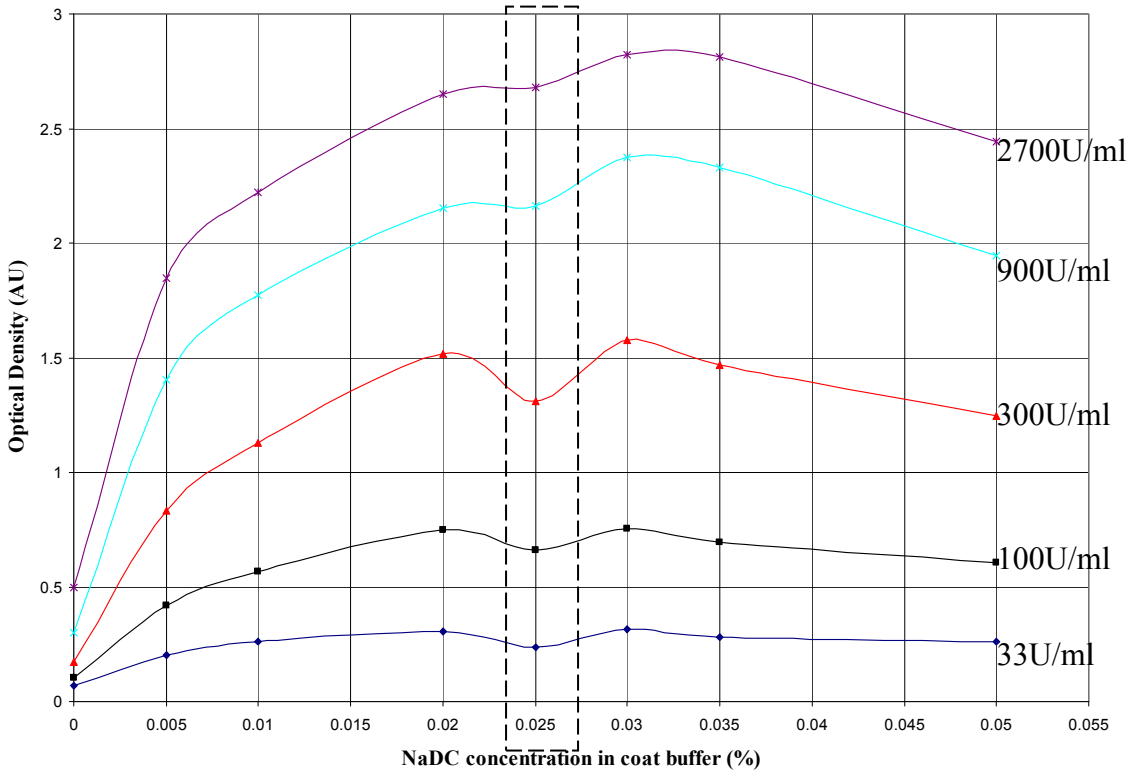


Figure 10. Graph showing how NaDC concentration affects the response value of each *antibody calibrator*. Labels indicate the concentration of the *antibody calibrator*.

From the graph in figure 10 it is visible that the effect of NaDC on assay activity peaks and then tails of after around 0.03% NaDC in the *coating buffer*. The decline in activity could be explained by the high concentration of NaDC unfolding the protein to a point where it is denatured. However, the effect of NaDC also dips at around 0.025% unexpectedly (the results boxed on figure 10). This dip may indicate that the NaDC may form micelles at this concentration as reported[57,62].

NaDC is a compound known to aggregate under certain conditions and form micelles[57-62]. In aqueous solutions these conditions seem to be optimal at pH 7.8 but no more than pH 8 (pH 8 being the approximate pH of *coating buffer*s in most cases). In addition, it has been shown by Robinson and Tanford that amphipathic compounds tested (including Triton X-100, used in both standard and sample diluent) will bind to proteins in a desirable manner at concentrations surrounding the critical micelle concentration (CMC)[57]. Therefore, it would follow naturally that if the concentration of NaDC added to the *coating buffer* was exactly the CMC for NaDC then the unfolding effect would be decreased by the formation of micelles. In addition, if the work by Robinson and Tanford translates for TPO molecules then the optimal effect would be observed at concentrations slightly above and below the CMC. As shown in the work by Masuoka and Moroi the CMC for NaDC is dependant on the temperature of the solution[62]. In general the temperature of *coating buffer* solutions can be assumed to be at room temperature (approx. 295K). It can be seen from the graph in figure 11 that the CMC for NaDC at this temperature would be around 6mmol/dm^3.

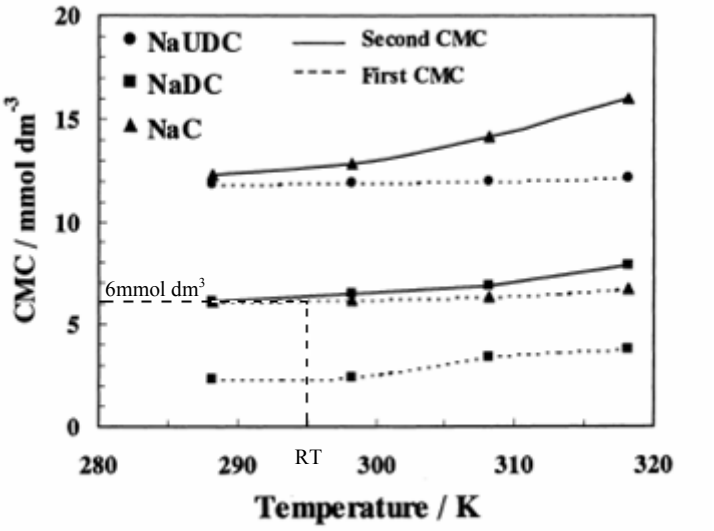


Figure 11. Graph from study by Matsuoka and Moroi[62] indicating the Critical Micelle Concentration (CMC) for NaDC at varying temperature. The dotted line indicates the CMC at temperature of 295°K.

Therefore concentrations either side of the CMC should produce an optimal effect. From figure 10, it can be seen that an optimal effect is observed at 0.02% and 0.03% NaDC in *coating buffers*, concentrations which correspond to 4.83mmol/dm^3 and 7.24mmol/dm^3 respectively. The concentration 0.025% seen to experience a deviation in the unfolding effect on figure 10 corresponds to concentration of 6.038mmol/dm^3 and hence the CMC of NaDC.

3.1.2 The effect of increasing Concentration of TPO in the Coating buffer (Step 2, Figure 1)

To assess whether or not the flattening in the curve could be due to the concentration of TPO being coated to the inside of each well (0.2µg/ml) an experiment was conducted where plates were coated with increasing amounts of the antigen. The hypothesis was that if more antigen/antibody activity could be encouraged at higher concentrations (e.g.

900U/ml, 2700U/ml *antibody calibrator*s) whilst keeping the same activity at the lower concentrations then a favourable steepening of the curve may be observed. Figure 12 shows the results.

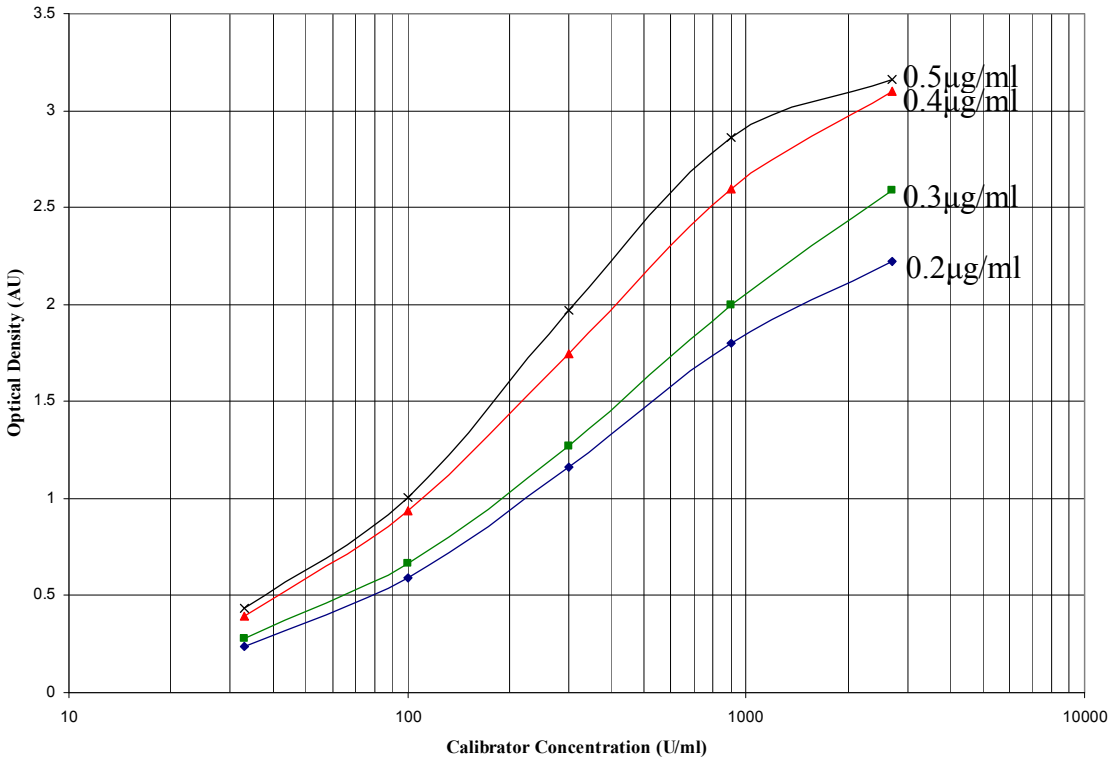


Figure 12. Graph showing 4 assay calibration curves, each with a different concentration of TPO coated in its wells (attached values). The same *antibody calibrator* sets were used for each assay. The concentration of the *conjugate antibody* was 1:20000

It can be seen from figure 12 that the hypothesised increase in gradient is observed. Table 6 shows the increasing gradient of the calibration curves shown in figure 12.

Amount of TPO in *Coating buffer* (µg/ml)	Calibration curve gradient (m)
0.2	0.599
0.3	0.717
0.4	0.945
0.5	1.016

Table 6. Gradient (m) values for curves shown in figure 12.

Therefore, in the following experiment the concentration was pushed up again, to 1µg/ml and 1.5µg/ml.

When preparing for the test using 1µg/ml and 1.5µg/ml coats, it was realised that assays coated with these higher concentrations of TPO would experience an increase in activity that would return optical density values likely be to be too high to be read by the colorimeters. Therefore, the concentration of the *conjugate antibody* solution used would be significantly reduced, lowering the overall assay response as far fewer TPO/TPO aAb binding situations would be read. Lowering the amount of TPO aAbs read would reduce overall assay response, meaning that optical density values would be in range of the colorimeter. Consequently, it was decided that the concentration of the *conjugate antibody* solution should be reduced from 1:20000 to 1:100000 for the assays with 1 and 1.5µg/ml of TPO. Results for this assay are shown in figure 13 and table 7.

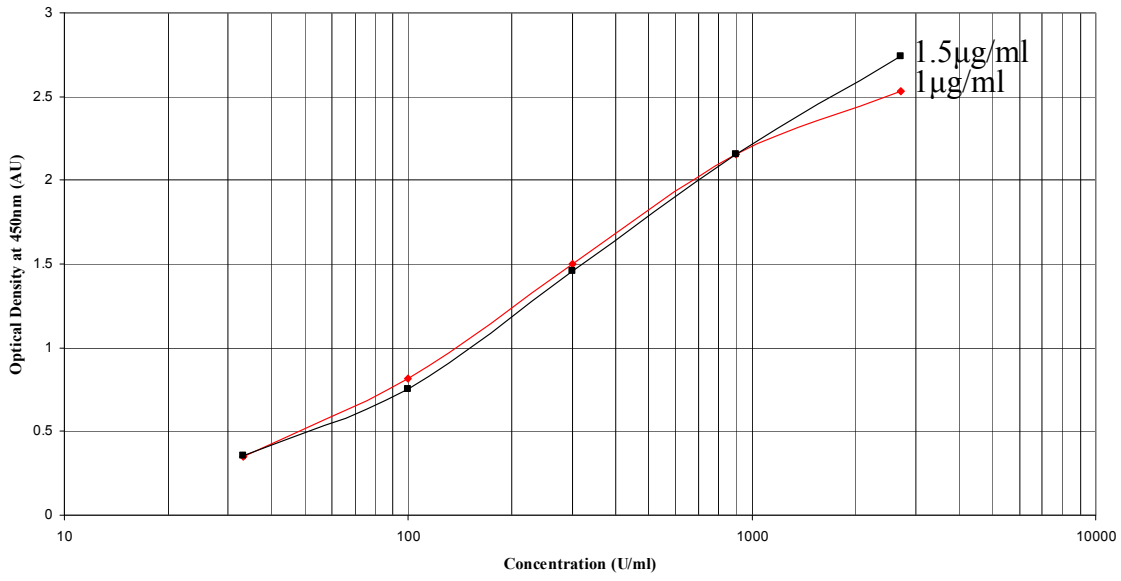


Figure 13 Graph showing calibration curves from two assays, one with 1µg/ml TPO coated and one with 1.5µg/ml. The same *antibody calibrator* sets were used for both assays. The concentration of the *conjugate antibody* solution was 1:100000.

Amount of TPO in *Coating buffer (*µg/ml)	Calibration curve gradient (M)
1	0.611
1.5	0.640

Table 7. Gradient (m) values for curves shown in figure 13.

It can be observed from figure 13 and table 7 that increasing the antigen concentration to 1 and 1.5µg/ml will eventually lead to a reduction in activity

The reduction in the concentration of the *conjugate antibody* however, may have depressed the assay response and therefore the gradient. It is possible therefore, that coating concentrations 1µg/ml and 1.5µg/ml provide a gradient that would meet the expectations of Objective 1. However, increasing the antigen concentration so much would increase the cost of the test and modifying the *conjugate antibody* concentration changes the practical nature of the test, thus breaking both constraints listed in section 1.8.

3.2 The Effect Varying Assay Method

3.2.1 The Effect of Different Antibody calibrator Diluents used during the Assay Process (Step 4, Figure 1)

Two identical *antibody calibrator* sets were diluted into two different diluents (PBS and the traditionally used TBS) and assayed on the same plate. This test was conducted on both the standard TBS coated plate and plates with an $NaHCO_3$ *coating buffer*. Results follow in figure 14.

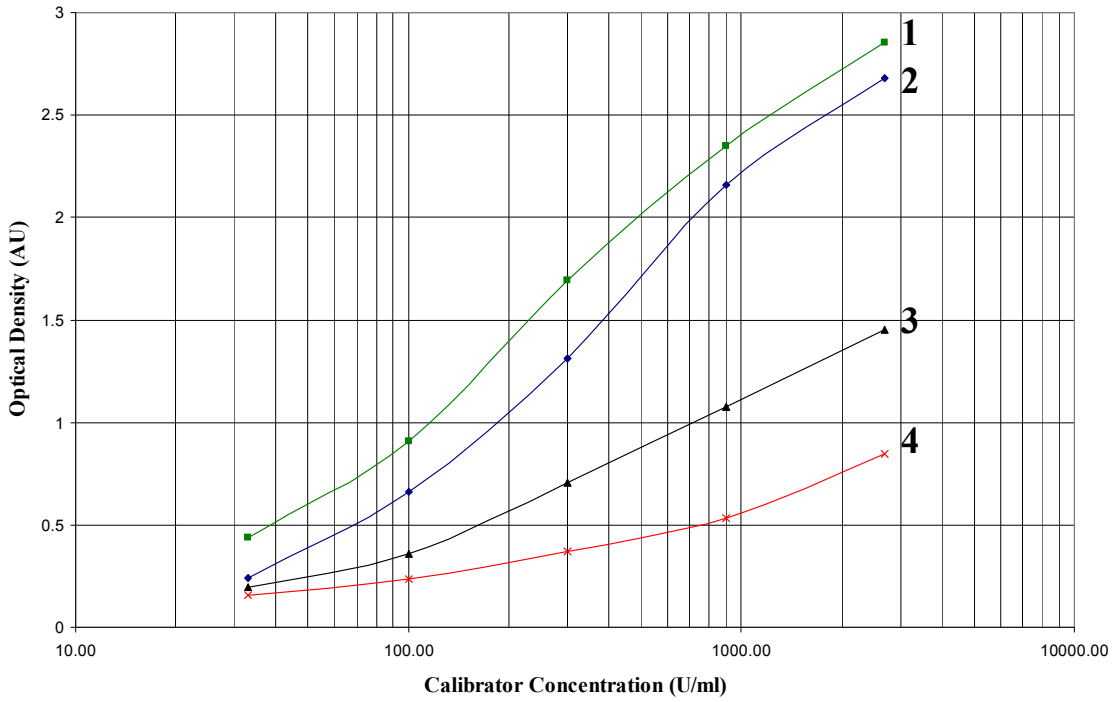


Figure 14. Graph showing four calibration curves: **1**, a TBS coated plate using PBS *antibody calibrator* diluent. **2**, a TBS coated plate using TBS *antibody calibrator* diluent. **3**, an $NaHCO_3$ coated plate using TBS *antibody calibrator* diluent. **4**, an $NaHCO_3$ coated plate using PBS *antibody calibrator* diluent.

The results in figure 14 show several affects. Firstly, that switching the *antibody calibrator* diluent to PBS has little effect when using the normal TBS coated plate, aside increasing optical response by an average of 0.23 units (trends **1** and **2**). Conversely, it can be observed that switching to a PBS diluent actually suppresses activity when using the $NaHCO_3$ *coating buffer*, with that *antibody calibrator* set giving an extremely shallow curve. These results indicate that the trizma base which was completely absent from the PBS diluted/$NaHCO_3$ coated curve is a beneficial additive to the assay matrix, in both the *coating buffer* and the sample diluent, although more effective in the former.

Overall it is observed that a TBS coated plate with a PBS *antibody calibrator* diluent is the most effective combination and therefore part of a solution to Objective 1.

3.2.2 The Effect of Modifying the Concentration of Conjugate Antibody (Step 6, Figure 1)

It was hypothesised at this point that the flattening in the calibration curve at higher concentrations may be because to few of the interactions were being read. It was therefore decided to test this by subjecting a single *antibody calibrator* set to four different concentrations of *conjugate antibody*. Results from this experiment follow in figure 15 and table 8.

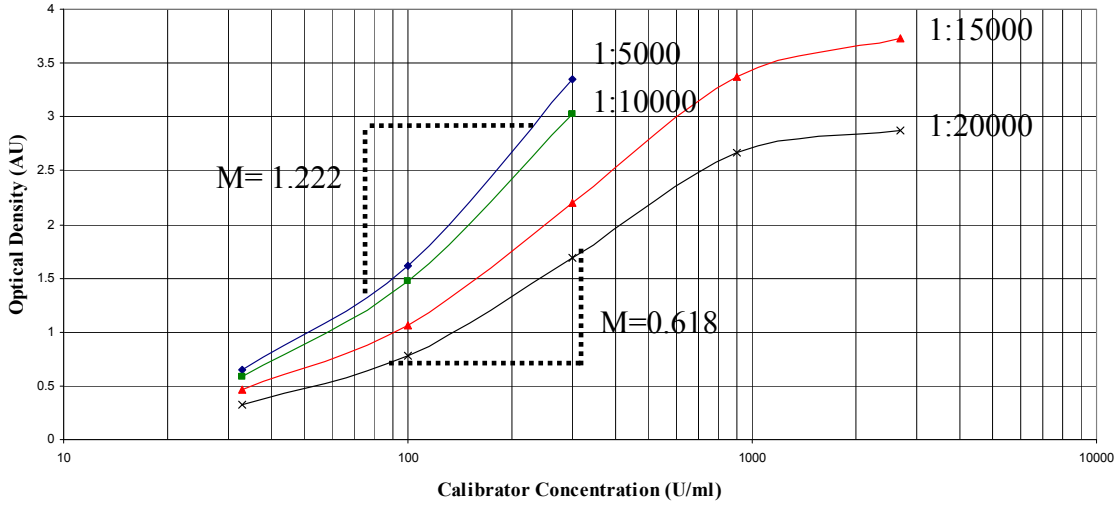


Figure 15. Graph showing four calibration curves from the same *antibody calibrator* set on the same plate but subjected a different concentration of *conjugate antibody.* Labels indicate the concentration of *conjugate antibody used*.

Conjugate Antibody concentration	Calibration curve gradient (M)
1:5000	1.222
1:10000	1.104
1:15000	0.785
1:20000	0.618

Table 8. Gradient (m) values for curves shown in figure 15.

The first thing observed from figure 15 and table 8 is that the two higher concentration solutions of *conjugate antibody* (1:5000, 1:10000) induced such a large portion of the well to be read that the optical density values for the 900 and 2700U/ml *antibody calibrator*s were higher than the colorimeter could read. It can be seen from table 8 however, that these two curves had a much steeper curve up to this point, indicating that increasing *conjugate antibody* solution improves curve gradient, making increased *conjugate antibody* concentration a viable solution to Objective 1.

Increasing *conjugate antibody* concentration as a method for gradient improvement is prevented from being tested more extensively by the fact that this would involve diluting the rest of the test down, and therefore, modifying the test a to too greater degree. Additionally, increasing *conjugate antibody* concentration would disrupt the reliability of the test, *i.e.* trying to detect small numbers of interactions with a large amount of read material would start to increase error.

Having looked at Steps 2, 4 and 6 (figure 1), despite having found many useful premises concerning the TPO ELISA and the improvement of its calibration curve gradient, no completely viable solution objective 1 that meets all the required criteria was found. Therefore, this area of research was abandoned in favour of analysing the tests reliability and reproducibility.

Chapter 4

4. Results and Discussion: Objective 2

The experiments regarding objective 2 are shown here. This section of the study concerns 3 elements.

1. The analysis of the TPO ELISA's poor reproducibility over time, discussed in section 4.1.
2. The discovery of the cause of the TPO ELISA's poor reproducibility, discussed in section 4.2.
3. The fixing of the TPO ELISA's poor reproducibility using the NIBSC reference material discussed in section 4.3.

4.1 Results pertaining to the performance of TPO ELISA In-House Standards

This section of results considers the experiments conducted to test the reliability of the TPO ELISA test as stated in Objective 2.

In order to analyse the stability of the TPO ELISA test over time, in-house standards were tested a total of 10 times over a period of 6 days with each assay being carried out under identical conditions. Results from these assays were then statistically analysed for variation. For each in-house standard a Coefficient of Variation (%CV) was calculated, to be deemed acceptable the %CV must not exceed 10%. Table 9 shows the results to obtained from this analysis.

	Standard	P1.2	P2.2	P3.2	P4.2	P5.2	P6.2	Positive Control
	Time	Concentration U/ml						
Day 1	AM	81.9	198.1	222.0	312.2	439.3	1058.1	524.2
Day 2	AM	Void	Void	Void	Void	Void	Void	Void
	PM	81.5	142.4	260.7	362.8	330.0	1079.6	427.0
Day 3	AM	79.7	261.7	231.6	324.5	480.3	1170.8	530.0
	PM	85.0	260.7	235.4	315.2	453.9	978.3	458.6
Day 4	AM	58.7	227.4	217.8	232.1	305.8	1074.3	523.8
	PM	59.6	226.1	200.2	237.9	290.8	1017.1	512.7
Day 5	AM	79.9	206.3	206.1	249.1	520.6	1025.9	460.1
	PM	88.3	281.6	210.8	257.6	621.3	1222.2	486.9
Day 6	AM	101.7	144.3	210.1	385.7	214.7	1012.7	562.9
	Target Mean	87.0	168.0	223.0	280.0	376.0	700.0	485.0
	Mean	79.6	216.5	221.6	297.4	406.3	1071.0	498.5
	Standard Deviation	13.4	49.5	18.6	56.0	129.5	79.0	43.2
	%CV	**16.9**	**22.9**	**8.4**	**18.8**	**31.9**	**7.4**	**8.7**
	Pass/Fail	F	F	P	F	F	P	P

Table 9. Table showing results returned for each TPO in-house standard over 10 separate assays run under identical conditions, on plates produced by standard procedure and against *antibody calibrator*s made from concentrate EQ1227. The value '%CV' (Coefficient of Variation) is calculated by dividing the standard deviation by the mean and multiplying by 100. To be deemed acceptable (P) a standard must obtain >10% CV over 9 assays.

As the results in Table 9 show, the TPO ELISA does not produce consistent results over time, assuming that the in-house standards are of constant concentration (only 2 out of the 6 standards achieved acceptable levels of variation giving the ELISA for TPO a 66% fail rate). To illustrate the amount that results vary over time the results for P3.2 and P5.2 are represented in figures 16 and 17.

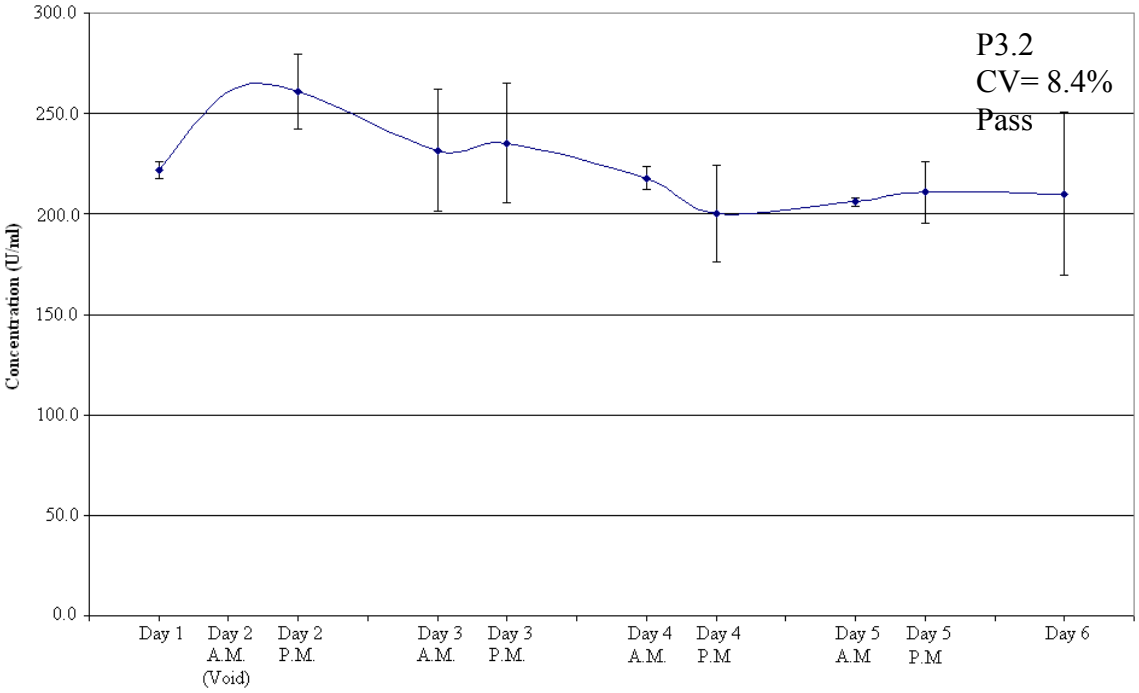


Figure 16. Graph showing how the concentration of standard P3.2 varied over time, P3.2 passed the linearity test. Error taken at 2 standard deviations.

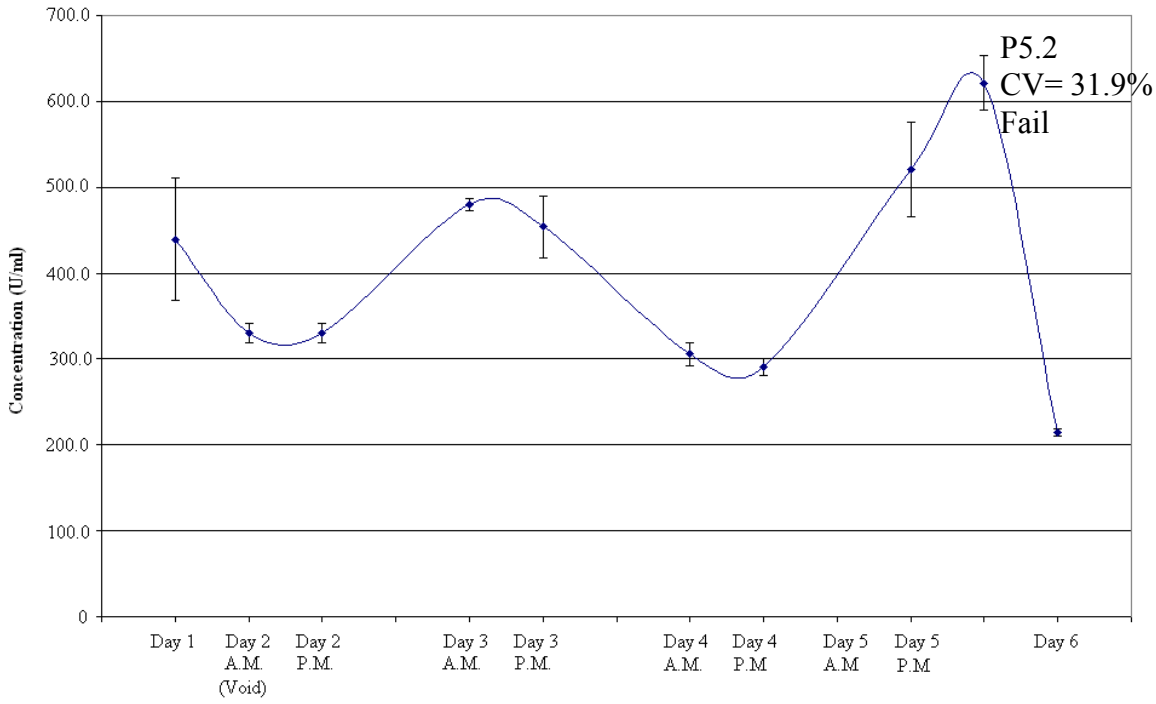


Figure 17. Graph showing how the concentration of standard P5.2 varied over time, P5.2 failed the linearity test. Error taken at 2 standard deviations.

The graphs shown in figures 16 and 17 indicate just how much the results returned over time can vary. It is even shown that the results vary on a day-by-day basis, but that could be purely coincidental and would have to be tested exhaustively to be proven. However, it is certain that at least one aspect of the assay was causing considerable inconsistency in results over time. In a bid to ascertain the nature of the cause and find a solution to this problem, tests were carried out on the *antibody concentrates*. These tests would discern whether or not it was the standards themselves that were the source of the inconsistency (for example, if aggregation was occurring in the standard fluid) or whether it was the *antibody calibrator*s returning inconsistent curves, leading to inconsistent results.

4.2 Results pertaining to the performance of Antibody concentrates

In order to test whether the *antibody calibrator*s were responsible for the obvious inconsistency inherent within the TPO ELISA shown in section 3.5, a recovery test was conducted. A recovery test involves testing a *antibody concentrates* ability to return consistent curves. In the recovery test performed, several *antibody calibrator* sets were tested against serially diluted standards. If the *antibody concentrates* return concentration values that correctly reflect their serial dilutions then it indicates that the concentrate is giving consistent curves.

To illustrate how the values from the following results are calculated, and therefore, how they relate back to actual ELISA results and to a demonstrate how varying

antibody calibrator curve shapes can dramatically skew results, an example set of results and calculations are shown in figure 18 and table 10, below.

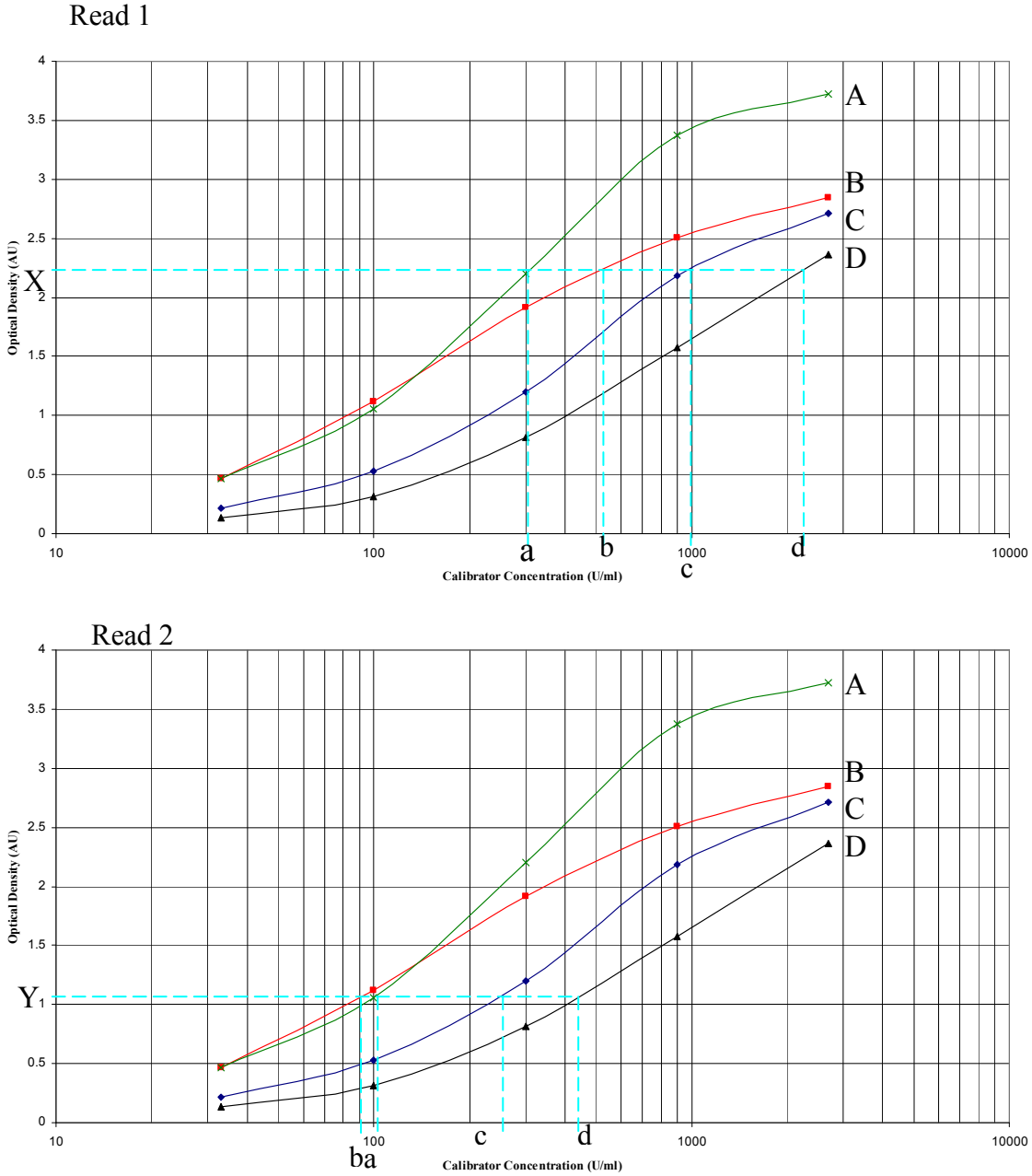


Figure 18. Two identical graphs with 4 calibration curves on each. The curves A, B, C and D were returned by four separate *antibody calibrator* sets, each made from a different *antibody concentrate*. All four sets were tested on the same plate. Also tested on the plate, one in-house standard, diluted to dilution X and dilution Y (denoted), dilution Y is half the of dilution X. Results from read 1 and read 2 are shown in table 10 (read shown as dashed lines, visual approximation only).

Curve	A	B	C	D
	Concentration (U/ml)			
Read X	160	210	515	1410
Expected Y (X/2)	80	105	275	705
Read Y	90	105	210	315
Recovery	112.5%	100%	81.5%	44.6%

Table 10. Table showing results taken from figure 18, illustrating how recovery values are calculated (values taken approximated).

The hypothetical example shown in figure 18 and table 10 demonstrate how ineffective calibration curves can entirely misrepresent the concentration of a sample. Tables 11, 12 and 13, below, show the results from the examination of three *antibody concentrates.* The *antibody concentrates* are coded EQ1227, EQ1600 and EQ1601 and each represent sera taken from a different Graves disease patient.

P3.2				**P6.2**			
Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**	Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**
1:10	903.5	903.5	100.00	1:100	845.0	845.0	100.00
1:20	374.9	451.7	83.00	1:200	363.5	422.5	86.05
1:40	154.5	225.8	68.39	1:400	160.3	211.2	75.89
1:80	75.11	112.9	66.50	1:800	87.55	105.6	82.88
1:160	35.23	56.47	62.39	1:1600	39.40	52.81	74.60
		Mean Recovery (%)	**76.0**			**Mean Recovery (%)**	**83.8**

Table 11. Showing the results of the recovery examination from standards P3.2 and P6.2. Tested against standard *antibody concentrate* EQ1227 on a plate produced by standard procedure.

It can be seen from Table 11, *antibody concentrate* EQ1227 provides an unfavourable curve. The values given for both P6.2 and P3.2 show poor recovery, where halving the concentration in practice delivers less that half concentration from the resulting assay. Falling concentrations indicate that the calibration curve provided by the concentrate becomes unreliable. This inconsistency is inherent in the TPO ELISA, the shallow calibration curve and flattening at higher concentrations are likely to yield these results. Examination of other *antibody concentrates* (i.e. TPO aAbs obtained from the sera of other Graves disease patients) showed some similarly adverse results.

P3.2				**P6.2**			
Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**	Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**
1:10	727.6	727.6	100.00	1:100	694.1	694.1	100.00
1:20	376.2	363.8	103.40	1:200	367.0	347.0	105.7
1:40	173.6	181.9	95.45	1:400	179.7	173.5	103.5
1:80	88.92	90.96	97.76	1:800	102.2	86.76	117.8
1:160	43.93	45.48	96.59	1:1600	48.99	43.38	112.9
		Mean Recovery (%)	**98.64**			**Mean Recovery (%)**	**108.0**

Table 12. Showing the results of the recovery examination from standards P3.2 and P6.2. Tested against standard *antibody concentrate* EQ1600 on a plate produced by standard procedure.

P3.2				**P6.2**			
Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**	Dilution	**Obtained (U/ml)**	**Expected (U/ml)**	**Recovery (%)**
1:10	746.590	746.5	100.00	1:100	716.1	716.1	100.00
1:20	421.370	373.2	112.8	1:200	412.5	358.0	115.2
1:40	212.150	186.6	113.6	1:400	219.1	179.0	122.4
1:80	110.590	93.32	118.5	1:800	126.9	89.51	141.8
1:160	52.831	46.66	113.2	1:1600	59.71	44.75	133.4
		Mean Recovery (%)	**111.6**			**Mean Recovery (%)**	**122.5**

Table 13. Showing the results of the recovery examination from standards P3.2 and P6.2. Tested against standard *antibody concentrate* EQ1601 on a plate produced by standard procedure.

The results in Tables 11, 12 and 13 clearly show that the *antibody concentrates* used in TPO ELISA return poor calibration curves. In order to rectify this problem an outsider standard was obtained in order to fix *antibody calibrator*s and standards to definite concentrations.

4.3 Results pertaining to the use of NIBSC Reference in fixing Calibration

The NIBSC (National Institute for Biological Standards and Controls) reference is a fluid that contains an amount of TPO aAbs that has been exhaustively tested by several external laboratories. It was acquired in order to fix the TPO ELISA test, this was achieved by constructing a *antibody calibrator* set from the NIBSC fluid and assaying against *antibody calibrator* sets from other concentrates as well as the in-house standards. The entire test was conducted on a single standard plate using standard procedures. If the calibration curve given by a concentrate matches the curve of the NIBSC set then that concentrate is functioning properly. The results from the first instance of this experiment are displayed in figure 19.

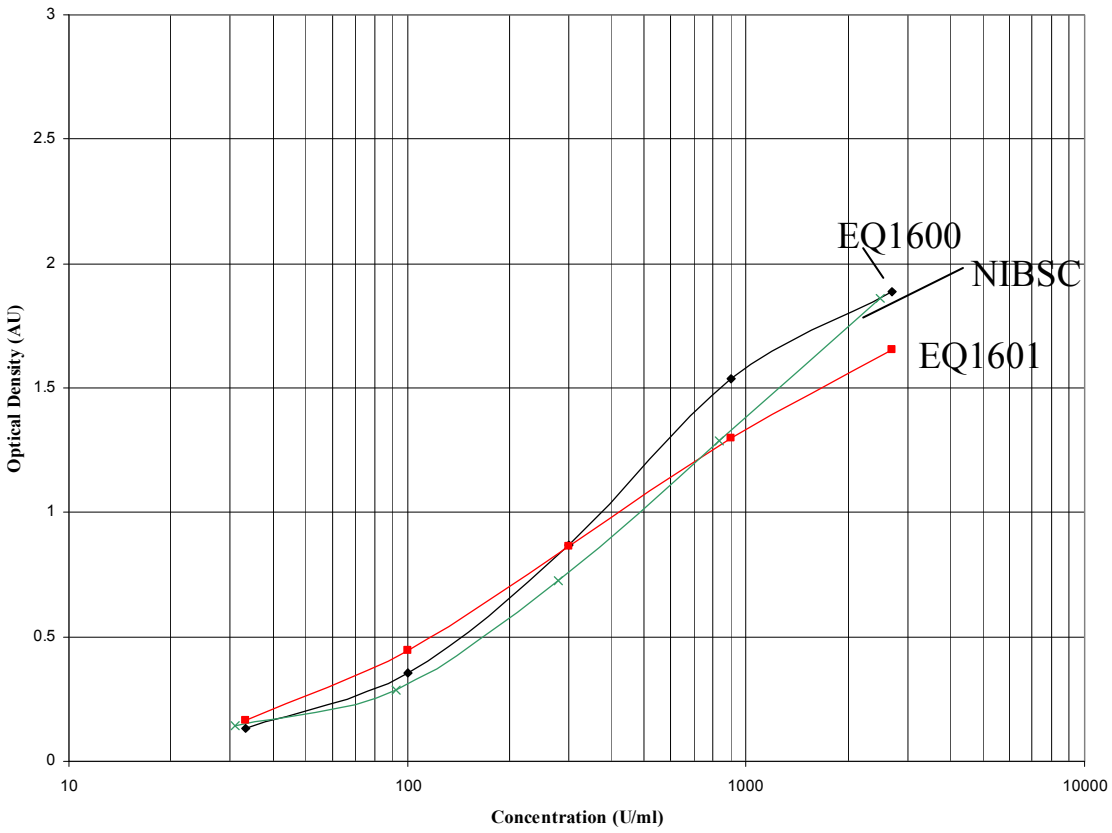


Figure 19. Graph showing three calibration curves returned from the same plate in the first NIBSC experiment. Labels indicate which concentrate each *antibody calibrator* set was constructed from.

Figure 19 shows that the two *antibody concentrates* tested do not behave in the same way as the NIBSC, indicating that they should be adjusted to do so. This adjustment is numerated by observing the value returned for each standard by the NIBSC reference calibration curve. The values for the standards are then compared against the values from the other two curves and an adjustment value is calculated by the method shown in Table 14.

	NIBSC Reference			EQ1600			EQ1601		
Standard	Concentration (U/ml)		Average	Concentration (U/ml)		Average	Concentration (U/ml)		Average
P1.2	84.26	87.19	85.73	69.92	72.45	71.18	51.11	52.8	51.98
P2.2	120.7	162.0	141.4	103.4	141.4	122.4	76.74	111.1	93.95
P3.2	203.0	204.8	203.9	177.1	178.7	177.9	147.0	148.6	147.8
P4.2	254.2	282.3	268.3	218.6	239.8	229.2	193.6	219.8	206.7
P5.2	423.4	423.4	423.4	333.6	333.6	333.6	360.0	360.0	360.0
P6.2	683.6	649.0	666.3	480.5	461.6	471.0	664.1	619.7	641.9
NIBSC Standard Value			Divergence			Divergence			Divergence
85.73			1.00	Adjustment Values		1.22			1.67
141.4			1.00			1.37			1.79
203.9			1.00			1.25			1.51
268.3			1.00			1.22			1.35
423.4			1.00			1.13			1.04
666.3			1.00			1.49			1.09
Standard Dev.			0.00			0.13			0.30
Mean			1.00	Mean Adjustment Value		1.24			1.47

Table 14. Table showing the calculation of adjustment values from the first NIBSC experiment. Adjustment values are calculated by dividing the value for the a standard given by the NIBSC reference by the standard value given by the *antibody calibrator* being assessed.

A divergence of 1.00 indicates that the value returned by that particular calibration curve is 100% correct. The concentration values for each standard returned by the NIBSC curve is assumed to be 100% correct. A divergence value of greater than 1.00 indicates that the values being returned by the *antibody calibrator*s are too low. The mean adjustment value are an average divergence that each *antibody calibrator* set has from the NIBSC curve.

The mean adjustment value gleaned from this test indicated that the *antibody calibrator* sets made from concentrates EQ1600 and EQ1601 would have to be adjusted down in concentration by 1.24 and 1.47, respectively. This meant reducing the concentration of the 2700U/ml *antibody calibrator* from EQ1600 from a concentration of 1:60 to 1:75 (1.e 60 multiplied by 1.24 gives the new dilution factor of 74.4 which is

rounded to 1:75). EQ1601 was also adjusted accordingly. The same assay was then conducted again with the adjusted *antibody calibrator* sets, results from this assay found below in figure 20 and table 15

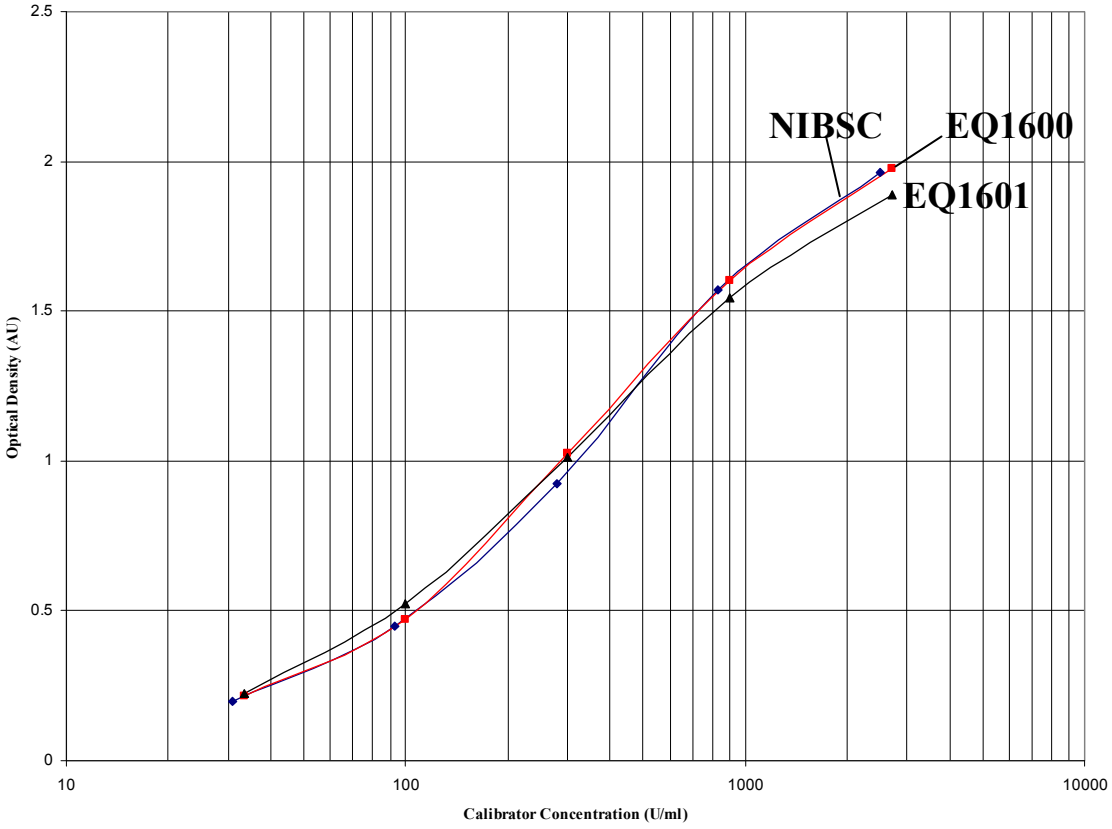


Figure 20. Graph showing four calibration curves returned from the same plate in the second NIBSC experiment. Labels indicate which concentrate each *antibody calibrator* set was constructed from.

	NIBSC Reference			EQ1600			EQ1601		
Standard	Concentration (U/ml)		Average	Concentration (U/ml)		Average	Concentration (U/ml)		Average
P1.2	78.634	79.23	78.93	75.82	76.36	76.09	63.25	63.71	63.48
P2.2	134.2	140.0	137.1	121.5	126.0	123.8	107.7	112.4	110.1
P3.2	186.6	195.0	190.8	161.4	167.8	164.6	150.8	157.8	154.3
P4.2	266.8	276.9	271.8	223.9	232.0	227.9	221.1	230.4	225.8
P5.2	358.0	370.0	364.0	300.7	311.4	306.1	309.8	322.2	316.0
P6.2	482.6	481.9	482.3	415.9	415.1	415.5	443.6	442.7	443.2
NIBSC Standard Value			Divergence			Divergence			Divergence
78.9			1.00			1.04			1.24
137.2			1.00			1.11			1.25
190.8			1.00	Adjustment Values		1.16			1.24
271.9			1.00			1.19			1.20
364.1			1.00			1.19			1.15
482.3			1.00			1.16			1.09
Standard Dev.			0.00			0.06			0.06
Mean			1.00	Mean Adjustment Value		1.14			1.22

Table 15. Table showing the calculation of adjustment values from the second NIBSC experiment. Adjustment values are calculated by dividing the value for the a standard given by the NIBSC reference by the standard value given by the *antibody calibrator* being assessed.

The results in table 15 show that after the first adjustment, although aligning the *antibody calibrator*s sets made from *antibody concentrates* EQ1600 and EQ1601 with the NIBSC calibrator set, they are still not commensurate. This finding is not portrayed in figure 22, which shows the NIBSC curve and the curve from EQ1600 being almost in accord with one another. However, the key difference between the curves is in the lower half of the curve (values below 500 U/ml), where the standard readings are taken, as the curves made from EQ1600 and EQ1601 are aligned.

Using the results from table 15 the process was repeated, adjusting the *antibody calibrator* sets using the mean adjustment values and then running the assay again, in identical conditions. This assay gave the results shown in figure 21 and table 16.

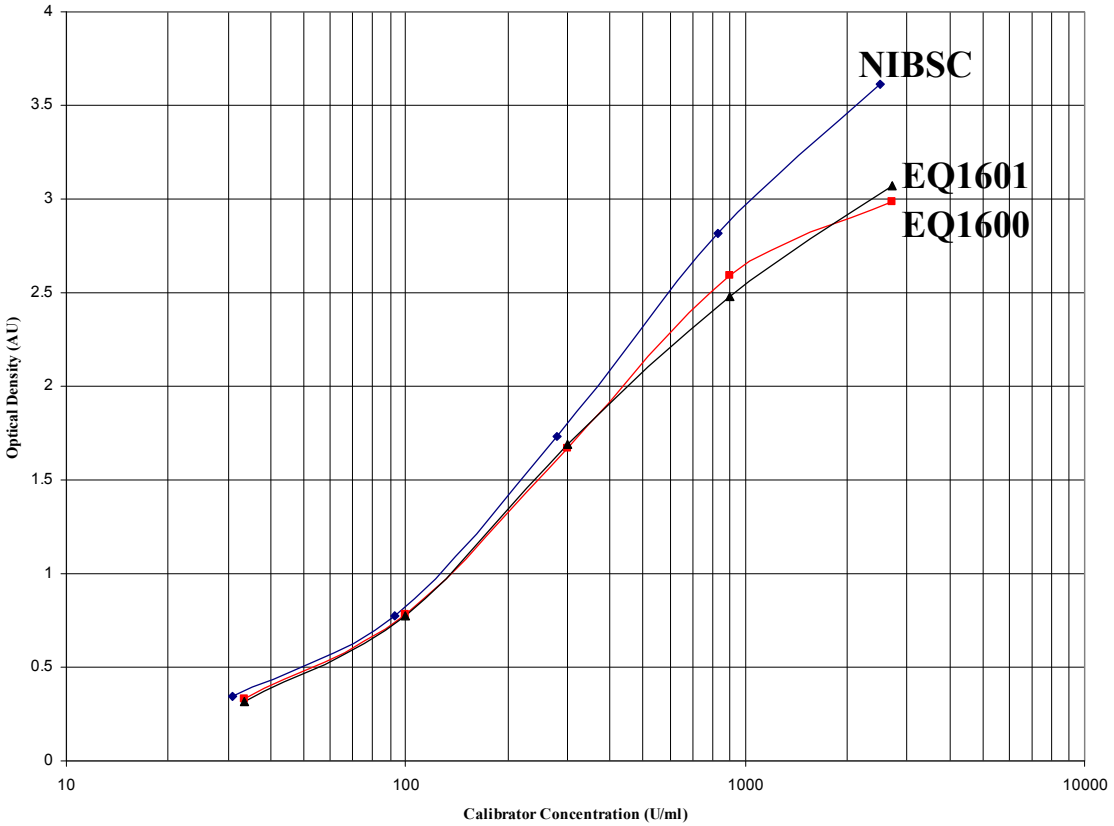


Figure 21. Graph showing four calibration curves returned from the same plate in the third NIBSC experiment. Labels indicate which *antibody concentrate* each *antibody calibrator* set was constructed from.

	NIBSC Reference			EQ1600			EQ1601		
Standard	Concentration (U/ml)		Average	Concentration (U/ml)		Average	Concentration (U/ml)		Average
P1.2	75.50	78.90	77.20	73.722	77.05	75.38	76.06	79.26	77.66
P2.2	182.1	174.9	178.5	187.35	179.2	183.3	181.2	173.6	177.4
P3.2	162.2	161.0	161.6	165.22	164.0	164.6	160.6	159.5	160.1
P4.2	260.3	249.9	255.1	276.13	264.1	270.1	267.7	255.4	261.5
P5.2	235.9	289.7	262.8	248.12	310.1	279.1	239.4	303.5	271.5
P6.2	413.0	406.3	409.7	458.18	449.7	453.9	482.0	471.0	476.5
NIBSC Standard Value			Divergence			Divergence			Divergence
77.20			1.00			1.02			0.99
178.5			1.00			0.97			1.01
161.6			1.00	Adjustment Values		0.98			1.01
255.1			1.00			0.94			0.98
262.8			1.00			0.94			0.97
409.7			1.00			0.90			0.86
Standard Dev.			0.00			0.04			0.06
Mean			1.00	Mean Adjustment Value		0.97			0.99

Table 16. Table showing the calculation of adjustment values from the third NIBSC experiment. Adjustment values are calculated by dividing the value for the a standard given by the NIBSC reference by the standard value given by the *antibody calibrator* being assessed.

Even though the calibration curves do no match exactly in figure 21, mathematically the results in Table 13 show that *antibody calibrator* sets made from EQ1600 and EQ1601 have been adjusted to concentrations commensurate with NIBSC reference. The conclusion that the *antibody calibrator*s had been fixed to an accurate dilution despite the divergence of *antibody calibrator* curves in figure 21 was deemed acceptable because over the range of concentrations values contained within the standards (P1.2-P6.2) both EQ1600 and EQ1601 made accurate returns. This range of concentrations is acceptable because it straddles the limit of diagnosis, above which, sera is deemed 'positive'. The final concentrations for EQ1600 and EQ1601 being 1:86 and 1:73, respectively.

The solution provided by the NIBSC reference material ultimately satisfies Objective 2. After adjusting the *antibody concentrates* to the optimal concentrations described above, the linearity test described in section 4.1, Table 9, in which the TPO ELISA achieved a 33% pass rate, was repeated. In the repeated test the TPO ELISA achieved a 100% pass rate.

Chapter 5

5. Conclusions

The findings in the study have revealed many theories regarding the nature of the TPO ELISA. By assessing assay performance under varying conditions, it have been possible to demonstrate several paradigms concerning the calibration curve and therefore the antigen/antibody interaction of the TPO ELISA.

(i) It was demonstrated in section 3.1.1 that NaDC is essential in the TPO ELISA for the purpose of unfolding the TPO protein. Although unfolding the protein to a greater degree (increasing NaDC concentration) increased curve gradient, the effect only extended up to 0.03% NaDC in the *coating buffer*. Beyong 0.03% NaDC the protein became unfolded to the point where it started to become denatured, causing its antigenic nature to fall.

(ii) It was demonstrated in section 3.1.2 that increasing the coat concentration of TPO between 0 and 0.5µg/ml within the ELISA well greatly increased assay response. Unfortunately above 1µg/ml activity was increased beyond the operational parameters of the colorimeters used to read the ELISA plate. Although increasing antigen concentration was a part-solution to Objective 1, the economic implications meant this avenue of research was discarded.

(iii) In section 3.2 the assay method was examined. Calibrator diluents that optimise assay response were recognised in a combination of TBS as a *coating buffer* and PBS as an *antibody calibrator* diluent. It was also discovered that increasing the concentration of the *conjugate antibody* increased calibrator curve gradient,

making it a possible solution to Objective 1. However, economical and practical implications again meant abandoning the search for a solution to Objective 1.

To summarise these observations it is fair to say that the TPO ELISA's combination of delicate responses and resistance to changing curve shape are likely drawn from the fundamental nature of the relationship between TPO and its antibody. When activity within the ELISA well increased above a certain amount, the assay would return a flattened curve indicating the possibility of a hindering phenomenon between TPO and TPO aAbs not affected by conditions and therefore difficult to investigate within an ELISA setting.

Although these findings hold scientific interest, it is unfortunate that no solutions to Objective 1 of the study were gleaned from them. Changing variables within test conditions and observing the changes in behaviour of the ELISA yielded some incite into the nature of TPO, TPO aAbs and their activity within an ELISA well but the restraints of industrial research restricted any one of the avenues being researched further. The conclusions to this area of the study therefore, are left somewhat unfinished. In closing, further study into the actions and nature of TPO and TPO aAbs, their response to surrounding conditions both *in vivo* and *in vitro* and their responsiveness to other agents such as sodium deoxycholate would be needed to more fully understand the concepts explored here.

The most useful outcome of the study, therefore, extends from the solution provided to Objective 2. Improvements were made to the TPO ELISA by fixing the inability of the TPO ELISA to return consistent results over time. This was achieved by fixing the *antibody concentrates* to their correct concentrations. Fixing the *antibody concentrates* also allowed the in-house standards to be fixed to their correct

concentrations. This solution to Objective 2 improved the TPO ELISA's reproducibility pass rate from 33% to 100%.

6. References

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