http://waikato.researchgateway.ac.nz/

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

The Development of ELISA and SPR-Based Immunoassays for the Detection of Heat Shock Proteins

By

Bei Gong

A thesis submitted in partial fulfilment of the requirements for the degree

of

Master of Science in Biological Sciences

at the

University of Waikato

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2009

Abstract

Heat shock proteins (Hsps) are highly conserved molecules in all eukaryotes and prokaryotes. They are named on the basis of their molecular weight and their synthesis is up-regulated by stress conditions such as inflammation, oxidative stress and exposure to high temperature. They function by assisting the folding of newly synthesized proteins and thus prevent aggregation or damage to other cellular components. Beside the role in intracellular protein folding, heat shock proteins can particularly act as intercellular signals with a wide variety of biological effects, such as to stimulate cells to produce proinflammatory cytokines, like TNFα, and other proteins involved in immunity and inflammation, or they are also thought to be involved in the pathogenesis of a wide range of diseases such as diabetes mellitus and cardiovascular disease. Thus, screening for aberrant expression of this protein could be an easy and useful tool to detect at risk individuals for developing and those more susceptible towards developing these diseases.

Currently, the Enzyme-Linked ImmunoSorbent Assay (ELISA) is the main immunoassay method used for the measurement of heat shock proteins levels in both clinical and research laboratories. It is relatively more rapid and sensitive than RadioImmunoassay (RIA), and most importantly, it is safer because enzymes are used as labels instead of harmful radioactive substances. However, a newly developed biosensor method, the Surface Plasmon Resonance (SPR), offers a number of

advantageous over ELISA. For example, it is much more simple and rapid because a lot of steps can be set up automatically and no labels are required for SPR immunoassays.

In this study, a comparison of different types of ELISA assays was made. The results showed that the Sandwich format of ELISA is much more sensitive than Indirect ELISA for detection of the concentrations of Heat Shock Protein 70 (Hsp70), and this sensitivity can be further improved by applying an Avidin-Biotin system together with Sandwich ELISA under certain conditions.

To develop the SPR protocol for the detection of heat shock protein concentrations, two Hsp70 binding curves using different assay formats, the Sandwich SPR immunoassay and the Competitive SPR immunoassay, were set up by using CM 5 sensor chip. Another sensor chip, the mixed Self-Assembled Monolayer (mSAM), was also examined using Sandwich SPR format. No subsequent experimental steps were carried on for SPR studies since the regeneration conditions of these tests for both sensor chips need to be well studied. Thus, this study suggests that in order to set up a SPR immunoassay protocol that is a better choice for the detection of heat shock protein levels in complex matrixes than ELISA, experimental conditions, such as the choice of regeneration buffer and the duration of regeneration cycle, need to be well optimized.

Acknowledgements

I would like first and foremostly to express my thanks to my direct masters supervisor, Dr. Ryan Martinus. Thank you for supporting and helping me throughout the development of this research project, and especially for checking through many drafts of this thesis in the last couple of months.

My gratitude also goes to Jing Yuan from HortResearch, Ruakura, Hamilton, for guiding me and providing me with all the helpful information and materials for the development of immunoassay techniques, and for her great help when things were not going right.

To everyone that I have worked with in the Cellular and Molecular Biology Laboratory over the last two years, Julie, Anchi and Joy, thanks for your encouragement, friendship and support. I would like to express my appreciation to Kerry, thanks for taking care of me when Julie was away. I am especially grateful to Jonathan, thank you for all your help and encouragement throughout my two years study, and it has been a real pleasure working with you.

To my lovely family, Dad and Mum, thank you for being there with me all the way. I would not have been able to make it through without your understanding and support.

Table of Contents

Chapter Two Materials and Methods

Chapter Three ELISAs' Results

List of Figures

List of Tables

List of Abbreviations

µ	Micro (10^{-6})
11-MOH	11-Mercapto-1-Undecanol
16-MUA	16- Mercaptohexa-decanoic acid
ABC	ATP-Binding Cassette
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic Acid
BIA	Bimolecular Interaction Analysis
BSA	Bovine Serum Albumin
CD14	Cluster of Differentiation 14
Cpn60	Chaperonin 60
CV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
EAH	Ethanolamine-HCl
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked ImmumoSorbent Assay
ER	Endoplasmic Reticulum
EtOH	Ethanol

FC	Flow Cell
g	Gram
Grp	Glucose Regulated Protein
HBS	HEPES Buffer Saline
HIV	Human Immunodeficiency Virus
Hsc70	Heat Shock Cognate Protein 70
Hsp70	Heat Shock Protein 70
HHSP70	Heat Shock Protein 70 Family
HSPs	Heat Shock Proteins
IgG	Immunoglobulin G
IL	InterLeukins
kDa	kilo Dalton
L	Liter (s)
LOD	Limit of Detection
LPS	Lipopolysaccharide
M	Moles per liter
mAb70	Monoclonal Anti-Hsp70 Antibody
mSAM	Mixed Self-Assembled Monolayer
n	Nano (10^{-9})
NAvidin	NeutrAvidin
NHS	N-hydroxysuccinimide

NTA	Nitrilotriacetic Acid
ºC	Degree Celsius
ONPG	*o*-nitrophenyl-β-D-Galactoside
OPD	*o*-Phenylenediamine Dihydrochloride
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pg	picogram
pI	Isoelectric Point
pNPP	*p*-Nitrophenyl Phosphate
rHsp70	Recombinant Human Heat Shock Protein 70
RIA	Radioimmunoassay
RU	Response Unit
SD	Standard Deviation
SPR	Surface Plasmon Resonance
TLR2	Toll-Like Receptors 2
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor Necrosis Factor α
Tween 20	polyoxyethylene 20 sorbitan monolaurate
WR	Working Reagent

Chapter One
Introduction and Literature Review

1.1 Heat Shock Proteins (HSPs)

Heat Shock Proteins (HSPs) are a group of ubiquitous and highly conserved proteins found in all living cells. These proteins were firstly discovered and reported as a pattern of "puffing" in the chromosomes of *Drosophila* in 1962 (Ritossa, F., 1962). In 1974, Tissier, A. and his colleagues reported these proteins as heat shock proteins (Tissier, A., *et al.*, 1974). In 1978, a new term "molecular chaperones" was first used by Laskey (Laskey, R. A., *et al.*, 1978) and redefined about a decade later by R. J. Ellis as a group of unrelated proteins that assist the correct assembly of other proteins without being components of the final structures (Ellis, R. J., and Hammingsen, S. M., 1989). It is now believed that many HSPs function as "molecular chaperones" by mediating the protein homeostasis of both prokaryotic and eukaryotic cells. To date, HSPs are well studied not only in their chaperoning property, but also in their physiological, pathological and clinical roles in almost all cellular processes and many human diseases, including Ageing, Heart Diseases, Infections, Inflammatory Diseases, Autoimmune Diseases, and even Cancer (Macario, A. J. L., 1995).

1.1.1 *Locations of HSPs*

HSPs are present not only in the cytosol, but nearly all cellular compartments of both eukaryotes and prokaryotes, including mitochondria, Endoplasmic Reticulum (ER), lysosome, nucleus and even membranes (Parsell, D. A., and Lindquist, S., 1993). For example, members of the HSP70 family are present in a number of cellular sites, including mitochondria, plasma membranes, nucleus and chloroplast in plant cells.

1.1.2 *Classification of HSPs*

According to their apparent molecular weights, kiloDalton (kDa), HSPs are classified into several families: HSP 110, HSP 90, HSP 70, HSP 60, HSP 40 families, which are high molecular weight HSP families and represent HSPs with molecular weights around 100kDa, 90kDa, 70kDa, 60kDa, and 40kDa respectively, plus the small HSPs (sHSPs) family, which includes HSPs with molecular weight range from 18kDa to 30kDa (Lindquist, S., and Craig, E. A., 1988). **Table 1.1** shows a brief summary of the names and locations of some major Heat Shock Proteins (Macario, A. J. L., 1995) (Ellis, R. J., and van der Vies, S. M., 1991) (Parsell, D. A., and Lindquist, S., 1993) (Pockley, A. G., *et al*., 2008).

Table 1.1 A brief summary of the name and location of some major heat shock proteins.

HSPS Family	Molecular Weight	Organism	Proteins	Compartment
Small HSPs	18kDa-30kDa	*E.coli*	IbpA and IbpB	Cytosol
		Mammals	Hsp27	Cytosol
			α A and α B-crystallin	Eye Lens
HSP 40	~40kDa	*E.coli*	DnaJ	Cytosol
		Mammals	Hsp40	Cytosol/Nucleus
HSP 60	~60kDa	*E.coli*	GroEL	Cytosol
		Plants	Cpn60	Chloroplast
		Mammals	Hsp60	Mitochondria
HSP 70	~70kDa	*E.coli*	DnaK	Cytosol
		Mammals	Hsp70	Cytosol/Nucleus
			Hsc70	Cytosol/Nucleus
			Grp78 (Bip)	Endoplasmic Reticulum (ER)
			mHsp70	Mitochondria
HSP 90	~90kDa	*E.coli*	HtpG	Cytosol
		Mammals	Hsp90	Cytosol
			Grp94	Endoplasmic Reticulum (ER)
HSP 100	~100kDa	*E.coli*	ClpA,B,C	Cytosol
		Mammals	Hsp110	Cytosol/Nucleus

1.1.2.1 Heat Shock Protein 70s (HSP70)

Among all of these families, HSP70 family, which contains a number of HSPs ranging in size from 66kDa to 78kDa, is the best conserved throughout the evolution, and also the best studied class. In 1984, Bardwell and Craig found that the sequences of the Hsp70 gene of *Drosophila* and *Escherichia Coli* heat-inducible DnaK gene, which is similar to Hsp70 gene, are homologous by sharing 48% identical DNA sequences (Bardwell, J. C. A., and Craig, E. A., 1984). Few years later, Gupta supported this hypothesis by finding that the amino acids of prokaryotic Hsp70 and eukaryotic Hsp70 were 50% identical (Gupta, R. S., and Singh, B., 1992). More recently, some research showed that the sequence identity of Hsp70 can be up to 78% among eukaryotes (Caplan, A. J., *et al.*, 1993). All of these data illustrated that these proteins are significantly conserved in evolution.

Several members of this family have been found in human cells, including stress-inducible Hsp70, constitutively expressed Hsc70 (heat shock cognate protein 70), mitochondrial Hsp75 and Glucose Regulated Protein Grp78 (Bip), which localized in the Endoplasmic Reticulum (Tavaria, M., *et al*., 1996). This family of proteins is comprised of multi-domain proteins that all consist of a 44kDa NH_2-terminal domain that is most conserved and contains a high affinity ATP-binding site, a 15kDa substrate-binding domain and a 10kDa COOH-terminal region that is less conserved (Kiang, J. G., and Tsokos, G. C., 1998).

1.1.2.2 Heat Shock Protein 60s (HSP60)

HSP60 family, or Chaperonin 60 (Cpn60s), is another well studied family of HSPs. They can be found in the cytosol of bacteria (where they were referred as GroEL proteins), in the mitochondrial matrix and in the stroma compartment of chloroplasts (Hartl, F. U., *et al*., 1992) (Lorimer, G. H., 1992). As similar as HSP70s, this family of proteins can be found under both normal conditions and stresses.

It has been shown that the amino acid sequence homology of these proteins can be up to 54% and most of them share a common oligomeric structure (Hemmingsen, S. M., *et al*., 1988). They consist of two seven-membered rings of approximately 60kDa that stack back to back with each other. This structure allows the formation of two central cavities that are essential for protein folding function of HSPs (Maguire, M., *et al*., 2002), which will be discussed in details in later section.

1.1.3 *Expression of HSPs*

As the name implies, HSPs are expressed under various stressful conditions. Increased expression of HSPs under stresses allow the cell to adapt to gradual changes of the environment by modulating the unfolded and misfolded proteins which are the results of exposure to stresses, thus to survive under lethal conditions. However, some HSPs are also constitutively synthesized in the absence of stresses (Welch, W. J., 1992), and those constitutive proteins are frequently very abundant.

For example, under normal conditions, HSP70s function as molecular chaperones by assisting the folding and assembly of newly synthesized polypeptide chains. When under stressful conditions, those synthesized HSP70s, which are inducible, ensure the cells are able to deal with increased concentrations of denatured and unfolded proteins (Nollen, E. A. A., *et al*., 1999).

As mentioned earlier, heat shock is not the only stress that will induce the expression of heat shock proteins. In addition to extreme temperatures, exposure to heavy metals, oxidative stresses, ultraviolet irradiation, microbial infections, nutritional deprivation, ethanol, nitric oxide, ischemia injury, drugs, amino acid analogues and some antibiotics can also stimulate and increase the synthesis of Heat Shock Proteins (Gehrmann, M., *et al*., 2008).

1.1.4 *Functions of HSPs*

1.1.4.1 The Chaperone Functions of HSPs

The primary function of HSPs is to act as molecular chaperones, modulating the folding and unfolding of other proteins and facilitating assembly and disassembly of complexes with several subunits.

Protein folding is a very important cellular process. Newly synthesized polypeptide chains must fold to the correct conformations to be activated and become functional. However, not all the polypeptides can fold efficiently. *In vitro*, under the proper conditions, small proteins, those with a single domain and soluble, can spontaneously and efficiently refold to their functional structures (Dobson, C. M., and Karplus, M., 1999). By contrast, *in vivo*, the spontaneous folding of a protein is much faster than the synthesis of its polypeptide chains. In this case, the folding of nascent chains is restricted (Rothman, J. E., 1989). In the crowded and stressed cellular environment, those large proteins with more than one domain, such as eukaryotic proteins, are more likely to be partially folded, or misfolded, thus are easily aggregate. These misfolded and aggregated proteins are non-functional, and when accumulate in cells, can lead to several deleterious consequences. For example, a certain level of protein aggregation in cells may lead to the formation of protein clusters, known as amyloid (Dobson, C. M., 1999), which are thought to associate with some human diseases, like Huntington's Diseases and Type II Diabetes Mellitus (Chiti, F., *et al*., 1999), probably by disrupting the normal cell function. In some cases, even though the proteins are folded to their native states and localized, denaturation of those proteins can happen easily under extreme temperature or other forms of stresses. Thus, those heat shock proteins that also known as molecular chaperones, which function to assist the correct assemblies of polypeptide chains, are responsible of protecting those polypeptide chains against misfolding and aggregation during their synthesis.

Their models of actions are described as “assisted self-assembly” because molecular chaperones prevent incorrect interactions in order to allow the protein to fold to their proper conformations without being parts of the final structures (Ellis, R. J., and van der Vies, S. M., 1991). They promote the protein folding process by binding and releasing of the substrates in an ATP-dependent manner (Morimoto, R. I., *et al*., 1997), sometimes with the assistance of cofactor proteins, co-chaperones.

1.1.4.1.1 *Hsp70 and Protein Folding*

As we mentioned earlier, these 70kDa heat shock proteins are central components of both molecular chaperones and cellular networks. They are responsible for assisting of a wide range of protein folding processes, including the correct folding and assembly of newly synthesized polypeptide chains, refolding of misfolded and aggregated proteins, translocation of proteins through membranes (Bukau, B., *et al*., 2000) (Neupert, W., and Brunner, M., 2002) (Ryan, M. T., and Pfanner, M., 2002) (Young, J. C., *et al*., 2003). However, their activities require the co-operation of ATP and co-chaperones, such as DnaJ and GrpE (Parsell, D. A., and Lindquist, S., 1993) (Liberek, K., *et al*., 1991), to fulfill their specific cellular functions. These HSP70 proteins with their co-chaperones constitute a complex network of proteins folding.

Typically HSP70 proteins, Hsp70 as an example, has a 44kDa ATPase domain, a 15kDa substrate-binding domain and a 10kDa less conserved C-terminal domain as

mentioned previously. One of the hypotheses of the role they play in proper protein folding it that this heat shock protein interacts with the unfolded polypeptide chains by binding the 15kDa substrate-binding domain to hydrophobic residues of the polypeptide chains (Rudiger, S., *et al*., 1997A), to stabilize the proteins in a folded or partially folded state (Pelham, H. R., 1986), this binding will then stimulate the hydrolysis of ATP, which promotes the release of substrates and also provides the energy for subsequent folding (Gething M. J., and Sambrook, J., 1992). When ATP bound to the NH_2-terminal region of Hsp70 and is hydrolysed to ADP, the substrate binding cycle, which is driven by the switching of low-affinity ATP bound state and the high-affinity ADP bound state for Hsp70, starts (Rudiger, S., *et al*., 1997B). Thus, the ATP binding-domain and substrate-binding domain of Hsp70 are inter-dependent.

1.1.4.1.2 *Hsp60 and Protein Folding*

As mentioned previously, Hsp60 is generally consisted of two seven-membered rings. It promotes the folding of newly synthesized and newly transported proteins to their native forms by isolating these proteins and then facilitating their folding in the central cavities formed by these two rings. Similar as Hsp70, this system is also ATP regulated. Generally, the non-native proteins are captured by hydrophobic interaction with the substrate binding region on the Hsp60 subunit, thus will be isolated from other non-native proteins and aggregation, and then folded to their native state by the energy provided from ATP hydrolysis (Bukau, B., and Horwich, A. L., 1998). This

binding of ATP to one ring is also required for the substrate release from the other ring (Rye, H. S., *et al*., 1997).

1.1.4.2 The Cytokine Functions of HSPs

Despite their role in protein folding, heat shock proteins, such as Hsp60 and Hsp70, have been shown to be potent activators of the immune system (Wallin, R. P., *et al.*, 2002). They were shown to induce the production of many proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukins (IL) and C-C Chemokines (Galdiero, M., *et al.*, 1997), which may contribute to the pathogenesis of many autoimmune diseases and chronic inflammation, such as Diabetes Mellitus and Atherosclerosis. This cytokine effect of HSPs are shown to be mediated through the Cluster of Differentiation 14 (CD14) and Toll-Like Receptors 2 and 4 (TLR2 and TLR4) signaling pathways (Kol, A., *et al*., 2000) (Ohashi, K., *et al*., 2000) (Asea, A., *et al.*, 2000 & 2002). They can also stimulate the adaptive immune response by binding to antigenic peptides during antigen processing (Noessner, E., *et al*., 2002). Thus, they are thought to serve as "danger signals" to the immune system (Chen, W., *et al.*, 1999). Compare with the molecular chaperone function of HSPs, cytokine function does not require ATP hydrolysis, co-factors and protein assembly, thus is unique. However, some recent studies suggest that this cytokine function of HSPs may due to the contamination of LPS (lipopolysaccharide) during preparation. Gao and Tsan showed that the LPS-free human recombinant Hsp70 was failed to induce

the activation of TNFα while its molecular chaperone function was entirely retained (Gao, B., and Tsan, M. F., 2003).

1.1.5 *Secretion Pathways of Hsp70*

As mentioned previously, Hsp70 is found in intracellular compartments. However, recent study demonstrated its presence in human circulation (Pockley, A. G., *et al*., 1998), indicating that it can also be extracellular secreted. Based on the cytokine function of Heat Shock Proteins, a lot of studies have been done on the secretions of these stress proteins. Hsp70s are reported to be released from neuronal cells (Hightower, L. E., and Guidon, P. T., 1989), glial cells (Guzhova, I., *et al*., 2001) and human carcinoma cells (Evdonin, A. L., *et al*., 2004). The mechanisms of Hsp70 secretion are also well investigated and three different secretion pathways of Hsp70 are reported.

Generally, the secretory pathway for a protein starts in the rough Endoplasmic Reticulum (ER), followed by many compartments of the Golgi apparatus, then ended up at plasma membrane, where the protein will be released to the surroundings. However, the secretion of Hsp70 can be non-classical because this protein lacks a secretory signal sequence. This non-classical pathway may involve a number of pathological conditions that will cause necrosis or the widespread cell death (Pockley, A. G., 2002), which is not depended on signal transduction and will result in cell

swelling and the destruction of plasma membrane, thus, release the Hsp70 to the environment. The Hsp70 is proved to be secreted by both active secretion and passive release from necrotic cells when these cells were exposed to heat shock (Mambula, S. S., and Calderwood, S. K., 2006A).

The second Hsp70 secretion pathway involves the alternation of molecular composition of exosomes. Exosomes are vesicles secreted from a wide range of cell types (Keller, S., *et al*., 2006), including B-cells, T-cells and dendritic cells (DC) (Blanchard, N., *et al*., 2002) (Raposo, G., *et al*., 1996) (Zitvogel, L., *et al*., 1998). They can deliver proteins from one cell to another because these exosomes can be easily fused with the membranes of neighboring cells. When the cell is exposed to heat shock, Hsp70 which is located within the exosome lumen will be released and the composition of exosomes under this stressed condition is demonstrated to be different from that of steady-state exosomes (Clayton, A., *et al*., 2005). This was further demonstrated by Lancaster and Febbraio, whose work also indicated that the Hsp70 level in the exosomes from heat shocked Peripheral Blood Mononuclear Cells (PBMCs) was much higher than those under normal conditions (Lancaster, G. I., and Febbraio, M. A., 2005), implying a possible secretory pathway for the active release of Hsp70 from cells under stresses.

A third Hsp70 secretion pathway is thought to be similar with the possible secretory pathway of other leaderless proteins, such as Interleukin 1β (IL-1β), where the protein first entered into the intracellular endolysosomes that was fused by late endosomes

and early lysosomes, followed by transportation of these organelle to the cell surface and then released the proteins by fusing the endosomes to the plasma membrane (Andrei, C., *et al*., 1999) (Andrei, C., *et al*., 2004). Later research showed that Hsp70 can be released from tumor cells by entering lysosomal endosomes, followed by releasing of the contents of the endolysosome into the extracellular space (Mambula, S. S., and Calderwood, S. K., 2006B). More recently, it was demonstrated that the entering of Hsp70 into the endolysosome compartment and the transportation of this compartment to the cell surface may involve the ATP-Binding Cassette (ABC) family transporter proteins, and the release of Hsp70 is suggested to be regulated by the binding of extracellular ATP to the cell surface (Mambula, S. S., *et al*., 2007) (Calderwood, S. K., *et al*., 2007).

1.1.6 *Measurements of Hsps Concentrations*

Since heat shock proteins are becoming very important mediators of intercellular signaling and may be associated with many human immunological diseases, recently, a lot of studies have reported that the heat shock proteins, such as Hsp60 (Pockley, A. G., *et al*., 1999) and Hsp70 (ranging 0-18550ng/ml) (Pockley, A. G., *et al*., 1998), and their specific antibodies, anti-Hsp60 and anti-Hsp70, have been found in the serum of normal individuals. Later on, many research reported that the circulating concentrations of Hsp60 (1197ng/ml under normal condition VS 3640ng/ml in patients with borderline hypertension) and Hsp70 (0.43 relative units of control group

VS 0.78 relative units of Type I Diabetic patients) were elevated in subjects associated with some diseases (Xu, Q., *et al*., 2000) (Pockley, A. G., *et al*., 2000) (Zhang, X. M., *et al*., 2008) (Yabunaka, N., *et al*., 1995) or under stresses (Walsh, R. C., *et al.*, 2001).

The most commonly used technique to determine the concentrations of these heat shock proteins in human body fluids, such as serum in these studies, is reported to be the Enzyme-Linked ImmunoSorbent Assays (ELISA). It is the most popular immunoassay used during last couple of decades for investigating the concentrations of proteins or other biological substances contained in complex matrixes. However, recently, a new technique named Surface Plasmon Resonance (SPR) working together with a biological detection system is also well studied. This method is predicted to be a better replacement of ELISA to measure biological interactions.

1.2 Immunoassays

1.2.1 *Introduction*

The term "immunoassay" describes a group of analytical techniques that are extensively used in both clinical and research laboratories. It could be a biological or a chemical test that utilizes the specific interactions between antigen and its antibody, to measure the concentration of a substance (the analyte) in a biological liquid, typically body fluids such as serum, urine and saliva.

The antibody molecules are typically "Y" shaped molecules that consist of two identical heavy chains and two identical light chains which are connected by disulfide bonds (Woof, J. M., and Burton, D. R., 2004). The structures of the antibody molecules are very similar; however, a small region at the tips of both the heavy and light chains is highly variable in the amino acid sequence. This huge diversity of antibodies allows the antibody to non-covalently bind to its unique antigen at a part on the antigen molecule called the antigenic determinant, or epitope in a high specific manner. This binding nature between antibody and antigen allows the directly use of both proteins in immunoassays with complex biological matrixes.

Detecting the quantity of an analyte, for example the concentration of an antigen or an antibody in the sample, can be achieved by a variety of methods. However, labeling of the analytes is a pre-requisite for the development of these immunoassays.

The types of labels used for determination of the assay sensitivity, could be various, such as a radioisotope (radioimmunoassay (RIA)), or an enzyme (enzyme-linked immunosorbent assay (ELISA)). The combination of specificity of the antigen-antibody interactions and appropriate labels enable that these immunoassays have a very important role in diagnosis and monitoring of various diseases, such as HIV, on a regular basis.

1.2.2 *Radioimmunoassay (RIA)*

In 1960, about a decade prior to the development of the enzyme immunoassays (EIA), Berson and Yallow firstly introduced radioimmunoassay (RIA) (Berson, S. A., and Yalow, R. S., 1960). The paper described an immunoassay in detail for the detection of insulin concentration in plasma in men, by using a radioactive label to the insulin in plasma. Yallow received the Nobel Prize in Medicine in 1977 for this achievement. Since then, a lot of RIA protocols have been studied and developed for the detection of different materials. These radioimmunoassays were widely used because by using antibodies with high affinity, the sensitivity of these RIA assays could be as sensitive as picogram (10^{-12} g). Even though the RIA offers a very good sensitivity, there are several major disadvantages with this method. The use of radioactive materials in the protocol requires special equipments and laboratory facilities for well trained personnel to work with these materials safely. The disposal of these radioactive materials may also present a significant environmental problem

since they are quite harmful. On the other hand, the radio-labeled compounds used in the assays are relatively expensive. During late 1960s and early 1970s, many researchers have focused on finding labels that can be used in immunological reactions without interfere the binding affinities of antibody and antigen, but would offer high specificity and sensitivity as the same as RIA.

1.2.3 *Enzyme-Linked ImmunoSorbent Assay (ELISA)*

Enzyme-linked immunosorbent assay (ELISA), also called enzyme immunoassay (EIA), is now a widely used solid-phase immunological assay for detecting the presence of a substance in complex matrixes. Compared with RIA, which has a severe health threat, ELISA is a much safer alternative which utilizes non-toxic enzymes to label either the antigen or the antibody molecules.

In 1971, Perlmann, P. and his graduate student Engvall, E. published a paper of the quantitative measurement of IgG in serum samples by using an enzyme, the alkaline phosphatase, for labeling (Perlmann, P., and Engvall, E., 1971). This is the first paper published about the development of ELISA for quantitative detection of an antigen or antibody. Since then, ELISA has been widely investigated and the number of publications on ELISA increased year by year. Compared with RIA, which used to be the only choice of immunoassays, ELISA possesses a number of advantages. The major advantage of ELISA over RIA is the absence of harmful or even deadly

radioactive materials in the experimental process. Thus, there is no special facilities and equipments needed and the reagents for ELISA are relatively cheap, easy to prepare and can last for long time. With relatively less money required, ELISA can provide even higher sensitivity, reproducibility and specificity than RIA.

Ideally, the enzymes used to label the antigen or antibody should be stable, safe and inexpensive. These enzymes, when react with substrate molecules, act as the catalysts and can convert a colorless substrate to a colored product which can be easily detected using a plate reader at a specific wavelength for this substrate. Compared with the limited range of labels for RIA, many enzymes, such as alkaline phosphatase and peroxidase, are used for ELISA labeling. **Table 1.2** listed some examples of most commonly used enzymes and their corresponded substrates.

Table 1.2 The most commonly used enzymes and their substrates in immunoassays.

Enzymes	Substrates
Horse Radish Peroxidase	3,3',5,5'-Tetramethylbenzidine (TMB)
Alkaline Phosphatase	*p*-Nitrophenyl Phosphate (pNPP)
Beta-Galactosidase	*o*-nitrophenyl-β-D-Galactoside (ONPG)

However, ELISA has its own limitations. It only provides information on the quantity of the analyte present in the sample but no information about the biochemical properties of this analyte can be obtained. On the other hand, the control of experimental temperature is essential for high sensitivity of the assay because the enzymes used can be easily denatured.

Several different types of ELISA that mostly different in the sequence of addition of antigens or antibodies to the solid plate have been developed and studied:

- Direct ELISA
- Indirect ELISA
- Competitive ELISA
- Sandwich ELISA

The whole ELISA experimental process includes several steps, the immobilization of an analyte (for example an antibody if its specific antigen is going to be detected) on the plate, the binding between the immobilized antibody and its antigen, the blocking of binding sites, the detection of binding signals and of course, many washing steps involved in the whole process.

The surfaces of most plates used in immunoassays are made of polystyrene or its derivatives because polystyrene can bind to a wide range of substances, such as proteins, peptides, small molecules, carbohydrates, lipids and DNA. Generally,

polystyrene surface will provide a very hydrophobic environment for protein binding. However, to enhance the adsorption of materials on the plate surfaces, some of the benzene rings of the polystyrene molecules are broken and carboxyl and hydroxyl groups which can provide hydrophilic interactions are released. The immobilization of one analyte can be achieved by a number of different modifications, such as amine coupling, maleimide coupling, hydrazine coupling and N-oxysuccinimide coupling. These different types of covalent linkages of analyte on the polystyrene plate surface will eliminate the risk of use of detergent in the washing buffer that may disrupt the binding of the analyte. Overnight incubation of the analyte at 4ºC is generally required for efficient coating of the analyte molecules on the surfaces of the plate.

The washing step that is applied between each binding of the ELISA assay aimed to remove any antigen or antibody molecules that are not bound. The choice of the washing buffer is very important since this buffer should be effective enough to disrupt non-specific bindings but will neither interfere with the bindings between antibody and antigen molecules nor the enzyme activity.

The blocking step is applied after the immobilization of analyte on the solid plate surface. It is a crucial step for the whole assay procedure because the immobilization of antibody molecules will leave many un-occupied hydrophobic sites on the plate surface, which will be responsible for most of the non-specific bindings of subsequent reactions. The most commonly used blocking agents in the ELISA blocking buffer are Bovine Serum Albumin (BSA) and Skim Milk. These proteins

can block the un-occupied hydrophobic sites on the solid plate surface; on the other hand, they can also prevent the adsorption of other proteins on the plate. The efficient blocking of the non-specific bindings will provide reasonable background and high specificity and sensitivity of the assay.

Two different antibodies are used in this study, the biotin monoclonal anti-Hsp70 which acts as a primary antibody, and the polyclonal anti-Hsp70 which acts as the capture antibody. Monoclonal antibodies are the same antibodies that are specific for a single epitope of an antigen (Cole, S. P., *et al.*, 1984), while the polyclonal antibodies are multiple antibodies that capable to bind to different epitopes on the antigen (Tini, M., *et al*., 2002). The bindings between these two antibodies to their antigen, Recombinant Human Hsp70, are performed in a highly specific manner as mentioned previously. These two antibodies will bind with antigen at different epitopes, thus will capture more antigen molecules than using just one antibody. This will allow the detection of low concentration of the antigen in the sample.

After holding the antigen molecules tightly with two different antibody molecules, the binding between antibodies and antigen is enhanced by the further binding with a secondary enzyme-linked antibody. This enzyme acts as an amplifier that allows the detection of even few binding signals generated by the antibody-antigen complexes. In order to produce a response that can be easily measured, a substrate is added and is converted to the detectable form by the enzyme. This visible signal is then spectrometrically measured by a plate reader at a specific wavelength according to

different substrates used. A standard curve can be then generated from the data obtained and is used later for determining the sensitivity of the assay, which is defined as the concentration of the analyte that gives the absorbance of zero standard plus two standard deviations.

However, before the actual samples can be tested, several standard curves should be set up to ensure the high sensitivity and reproducibility of the assays. Experimental conditions, such as the incubation time for the binding between antigen and immobilized antibody and the incubation time for substrate reaction, need to be optimized if the standards set up are not as sensitive as expected.

1.2.3.1 Direct ELISA

This type of ELISA is usually considered to be the simplest form of ELISAs since it requires only one labeled antibody for its specific antigen detection. Generally, the sample contains an unknown concentration of target antigen is coated on the surface of 96-well plate, followed by the binding between the immobilized antigen molecules and an enzyme-linked antibody. This binding signal is then converted to a colored detectable product by a substrate specific for that enzyme and is then measured (**Figure 1.1**). Although the use of only one antibody could make the assay less complex and relatively rapid, the sensitivity and specificity of the assay are reduced.

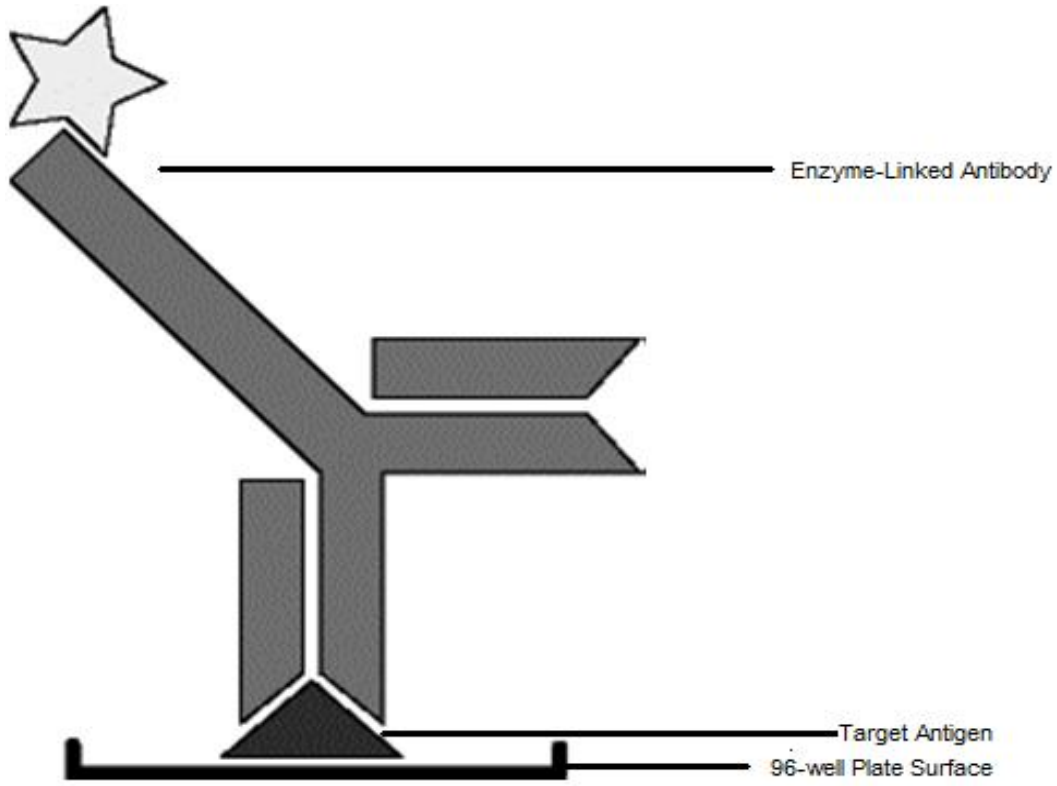

Figure1.1 Direct ELISA

1.2.3.2 Indirect ELISA

The basic theory of indirect ELISA is the same as direct ELISA. However, compared with direct ELISA, the indirect ELISA assay requires an addition of a primary antibody before the labeled antibody will bind to antigen molecules. In brief, the 96-well plate is coated with the antigen molecules, the same as direct ELISA. Then a known concentration of primary antibody is added and these antigen molecules will

bind tightly to those immobilized antigen molecules. The bindings between these two molecules can be then recognized by an enzyme-labeled secondary antibody, which will then convert a specific substrate to a colored product to be measured (**Figure 1.2**). The addition of the primary antibody will provide more binding sites for the enzyme-linked secondary antibody compared with Direct ELISA. Thus, more enzyme molecules can be attached and reacted with its substrate, then converted to detectable signals. However, the use of the primary antibody may also be responsible for some non-specific bindings occurring during the assay process.

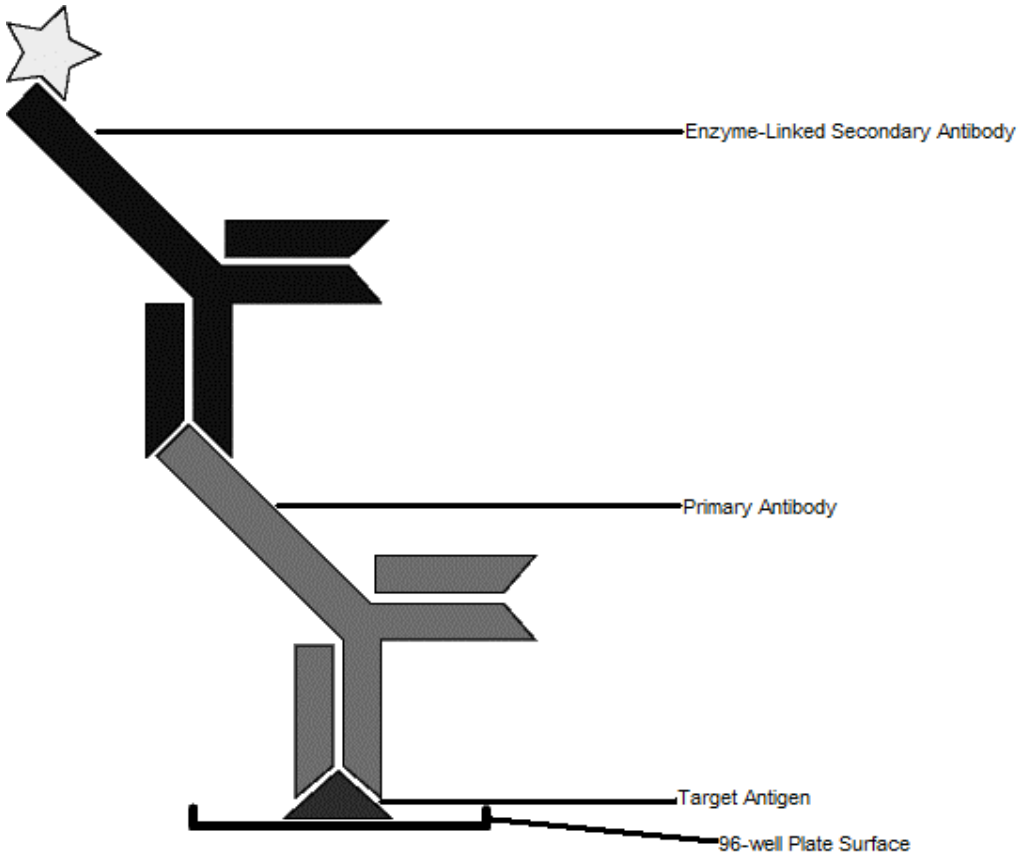

Figure 1.2 Indirect ELISA

1.2.3.3 Competitive ELISA

Compared with the standard curves of other types of ELISA, for competitive ELISA, the higher the detected antigen concentration, the weaker the detectable signal. Thus, this type of ELISA assay is mainly used to detect the concentration of an antigen or antibody molecule with small molecular weight in a sample since there are limited binding sites available on the surface of these molecules. Or it can also be used when two matched antibodies for one antigen are not available because only one antibody is required for the assay procedure.

Compared with two different ELISA types described above, the experimental steps in the competitive ELISA assay are somewhat different. The 96-well plate is coated by a known concentration of target antigen. Then the primary antibody and the sample containing unknown concentrations of its specific antigen is incubated together and allow the bindings between them to occur as many as possible. This mixture is then added into the antigen-coated wells and incubated. During incubation, those primary antibody molecules that are not occupied will bind to the antigen molecules that are immobilized on the plate surface. After the washing step that is responsible for removing all the unbounded antigen and antibody molecules in the wells, an enzyme-conjugated secondary antibody is added and will convert a specific substrate to a detectable product that can be spectrometrically measured at a particular wavelength (**Figure 1.3**).

It has been called "Competitive ELISA" because the antigen molecules immobilized on the plate surface will compete with the antigen molecules in the sample that is going to be detected for binding sites on the primary antibody molecules. The more antigen molecules present in the unknown sample, the less free primary antibody molecules will be left to bind with the immobilized antigen molecules. Hence the "competition". As a result, for those competitive ELISA assays, the stronger the detectable signal, the lower the antigen concentration in the unknown sample.

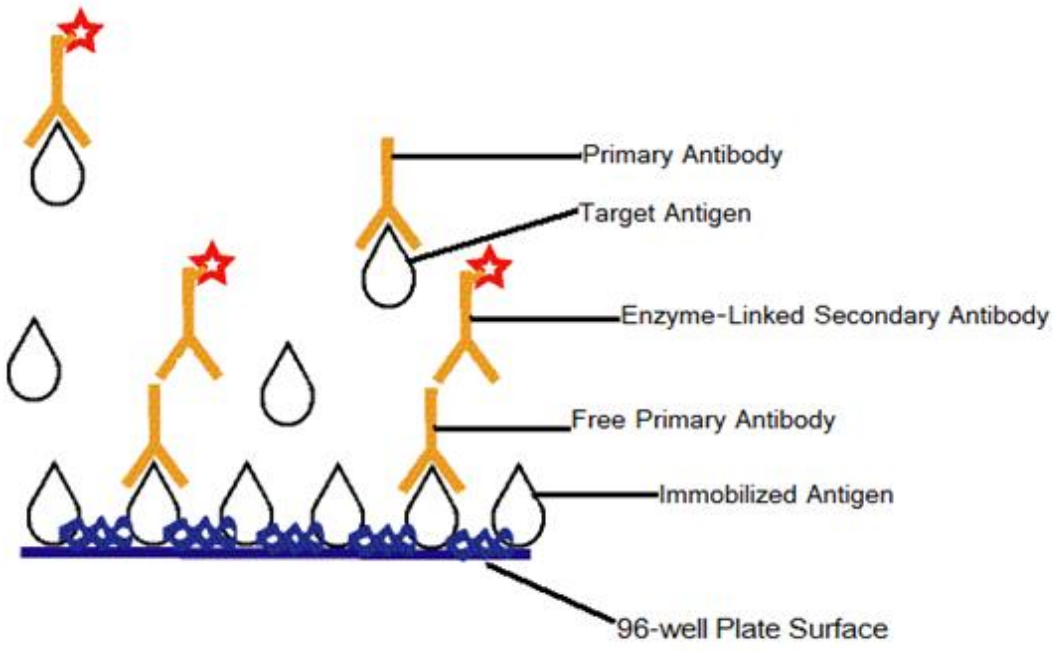

Figure 1.3 Competitive ELISA (modified from KPL, 2006)

1.2.3.4 Sandwich ELISA

This type of ELISA assay is considered to be the most accurate and sensitive enzyme immunoassay compared with all other types of ELISA described previously. It measures the amount of an antigen between two layers of antibodies. Hence, the antigen to be detected must contain multiple epitopes that two different antibodies can react with, and these two antibodies could not bind to the same epitope on the antigen molecule. Generally, the first layer of antibody, called the primary antibody, is immobilized on the 96-well plate surface by different covalent linkages mentioned previously. After blocking the un-occupied hydrophobic sites on the plate surface, the samples containing unknown concentrations of antigens are added and incubated to ensure as many primary antibody molecules will bind to antigen molecules. These antibody-antigen complexes are then recognized by the addition of a detecting antibody, which will bind to the antigen molecules at their free epitopes tightly. The binding signal is then amplified by an enzyme-labeled secondary antibody and the reaction between this enzyme and its colorimetric substrate will produce a visible signal that will then be measured spectrometrically (**Figure 1.4**).

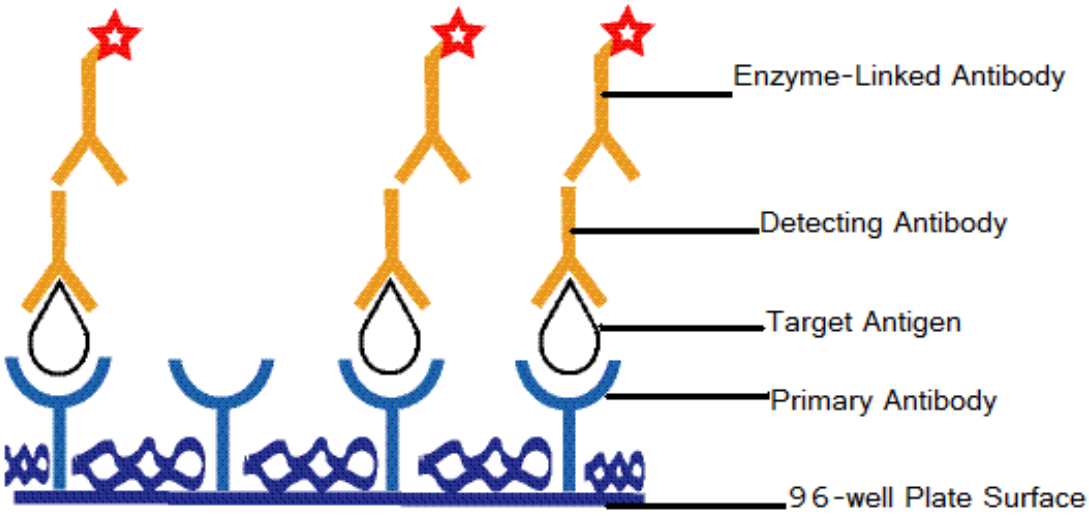

Figure 1.4 Sandwich ELISA (modified from KPL, 2006)

Compared with other types of ELISA assays, Sandwich ELISA offers a number of advantages. Since it requires two matched antibodies for a particular antigen, the sample to be detected does not need to be purified, thus, allows the examination of complex matrixes, such as serum and urine, which may contain a lot of other proteins. This indicates that the Sandwich ELISA assay is highly specific. Also, the use of two different antibodies to bind to the antigen molecules will allow the detection of a low concentration antigen in a sample to be possible. Thus, this type of ELISA assay is considered to be the most sensitive.

1.2.4 *ELISA with an Avidin-Biotin System*

1.2.4.1 Introduction to Avidin

In 1942, Woolley, D. M. and Longsworth, L. G. proved that avidin, which was isolated from egg white, is a basic glycoprotein having an isoelectric point at pH 10 (Woolley, D. M. and Longsworth, L. G. 1942). In 1969, O'Malley and his colleagues proved that the synthesis of avidin was taken place in the goblet cells of the oviduct epithelium which was different from the synthesis of other egg white major proteins (O'Malley, B. W., *et al*., 1969). Since then, avidin has been well studied and found to be a tetrameric protein, with four essential subunits of identical amino acid composition and sequence (Delang, R. J., and Huang, T. S., 1971). The activity of avidin was observed in the eggs or oviducts of many species, such as birds, reptiles and amphibians, at a maximum concentration of approximately 0.05% of the total protein in chicken egg white (Green, N., 1975). In its tetrameric form, avidin was estimated to have a molecular weight between 66,000 Da and 69,000 Da (Korpela, J., 1984).

1.2.4.2 Avidin Binds to Biotin

Avidin was named based on its affinity for Biotin, also known as Vitamin H, and was thought to be an anti-biotin factor at early time (Kresge, N., *et al.*, 2004). It is

investigated that the avidin molecule can bind to the biotin molecule with a very high degree of affinity and specificity. The bindings between Avidin and Biotin molecules are thought to be the strongest biochemical (non-covalent) bonds so far (Green, N., 1963). Actually, the first report about the stability of Avidin-Biotin complex was based on Launer and Fraenkel-Conrat's work (Launer, H. F., and Fraenkel-Conrat, H., 1951). They measured the affinity constant for Avidin to Biotin to be $2.4 \times 10^{10}\ M^{-1}$ and determined that the dissociation of Avidin-Biotin complex to be negligible, which was not the case in later studies. The work done by Green and Toms in early 1970s showed an affinity constant of $7 \times 10^{14}\ M^{-1}$, and a dissociation rate of $10^{-7}\ S^{-1}$ (Green, N., and Toms, E. J., 1973).

Since then, much research about the binding between Avidin and Biotin has been investigated. Their high binding affinity allows the Avidin-Biotin system to become a very powerful tool in a wide range of biochemical or immunological assays, such as ELISA.

1.2.4.3 NeutrAvidin

NeutrAvidin (NAvidin) is a deglycosylated Avidin derivative with a molecular mass of approximately 60,000 Da. Just like Avidin molecules, NAvidin is a tetrameric protein with four identical units that allow four biotin molecules to bind to it with a strong binding affinity. However, compared with Avidin, the carbohydrate moiety of

the NAvidin molecule, which possesses about 10% of the total molecular weight, is removed. This feature is thought to provide fewer non-specific bindings when bind NAvidin molecules to Biotin molecules, compared with the binding between Avidin and Biotin molecules. This is because the biotin binding activity of the avidin molecules is mainly dependent on the protein part of the molecule, that the importance of carbohydrate in the binding reaction has been minimized (Green, N., 1963). At the mean time, the near-neutral nature of NAvidin, which is shown by an isoelectric point of 6.3, may also reduce the non-specific binding property of NAvidin molecules.

1.2.4.4 Applications of Avidin-Biotin System

Since one Avidin molecule has four binding sites that each allow one Biotin molecule to bind to it with high affinity, the sensitivity of immunoassays can be amplified. The Avidin-Biotin system has been widely used and become a general tool for laboratory and clinical assays.

Generally, this Avidin-Biotin system can be used in two ways.

- The Avidin molecules can be coated on the plate, followed by the immobilization of biotinylated antibody or antigen molecules (Pesce, M. A., *et al.*, 1992).
- Or they can be used as part of the detection system (Kakabakos, S. E., and Khosravi, M. J., 1992).

To use the combination of this Avidin-Biotin system and standard ELISA assay, many researchers reported improved sensitivity of assay by comparing with the sensitivity of the standard ELISA assay (Tsai, H. J., and Saif, Y. M., 1991) (Nara, S., *et al*., 2008). However, not much work has been done for the detection of Heat Shock Proteins by using this Avidin-Biotin ELISA system.

1.3 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical technique that has been developed about couple of decades ago. Now, it is widely used for detection and monitoring the interactions between molecules. By working together with an optical biosensor, SPR based system, such as BIAcore-SPR, can provide a rapid and highly sensitive assay in real-time without the need of labeling. Recently, this technique is being more and more used by both research and commercial purposes.

1.3.1 *The Principle of BIAcore SPR*

Generally, a standard SPR optical unit contains a light source, a prism which the light will pass through and a detector which measures the refractive index near a sensor surface (**Figure 1.5**). At certain conditions, a photon of light can transfer its energy to the electron on the gold surface to generate a plasmon, which is a group of excited electrons that behave like a single electrical entity (Pattnaik, P., and Srivastav, A., 2006). This transfer can be observed by measuring the reflection of light. The wavelength at this transfer can occur depends on the chemical environment of the metal surface. Any change in the sensor surface environment will produce a change in the light reflected.

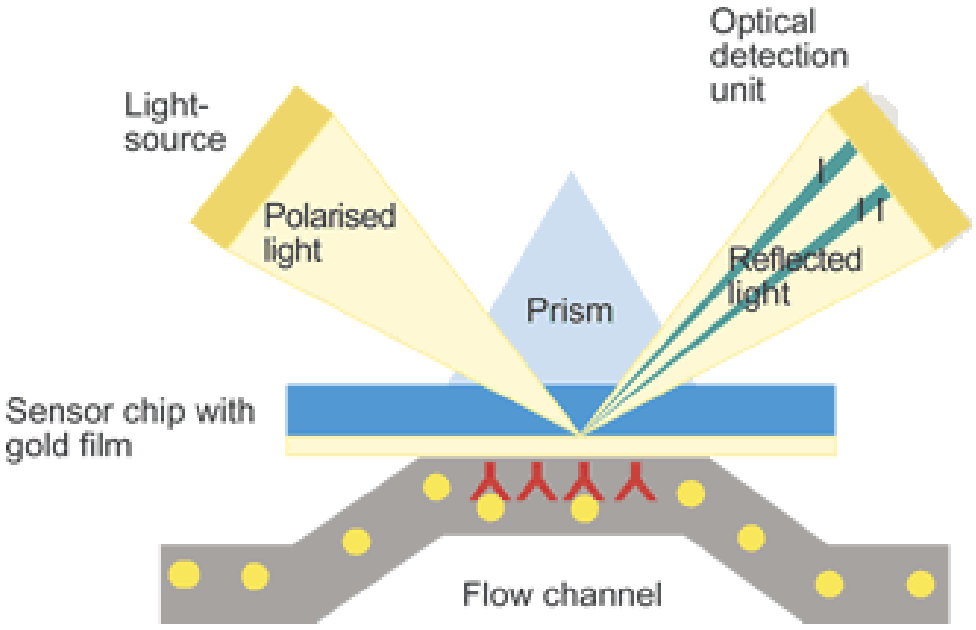

Figure 1.5 SPR with a sensor chip (Canziani, G., *et al*., 1999)

In brief, in order to detect an interaction, the analyte is continuously running through the ligand immobilized sensor chip surface, generally at 20µl per minute for assay. Protein will accumulate on the chip surface as the analyte will bind to the ligand and this accumulation can result in the increased refractive index that can be measured and presented in the form of a sensorgram, which is a plot of response units (RU) versus time (T) (**Figure 1.6**). The change in RU will indicate the amount of analyte bound to the immobilized ligand.

1.3.2 *The Sensor Chip*

In BIAcore technology, the bio-specific sensor chip where the interaction between the immobilized ligand and the analyte occurring is generally made by applying a thin layer of gold to a glass surface. To ensure as many different types of ligands can be immobilized on it, several different surface chemistries are applied. Rich and Myszka listed many different types of SPR surfaces and their applications in 2000 (Rich, R. L., and Myszka, D. G., 2000), examples are:

- Sensor Chip CM 5 (carboxymethylated dextran matrix) that is the most widely used sensor chip for most applications.
- Sensor Chip SA, which has streptavidin pre-coupled and is suitable for the binding with biotin conjugations. However, this type of sensor chip is quite expensive and cannot be re-used, thus would cost more than coating streptavidin on CM 5 Sensor Chip.
- Sensor Chip NTA, as similar as Sensor Chip SA, this type of sensor chip has nitrilotriacetic acid (NTA) coupled and is designed to capture proteins with oligo-histidine tags.
- Sensor Chip HPA, which is flat hydrophobic surface and can be used for immobilizing lipid monolayers.
- Sensor Chip B1, which has low level of carboxymethylation, thus showed reduced non-specific bindings compared with other types of sensor chips.

- Sensor Chip F1, this kind of sensor chip has a thin layer of dextran matrix, and is used for binding with large analytes.
- Sensor Chip J1, the surface of this sensor chip is unmodified gold and thus can be used to design different coupling chemistries.
- Sensor Chip L1, this type of sensor chip is mainly used for immobilization of liposomes, so the surface of this sensor chip is a layer of dextran with lipophillic compounds.

Two different types of sensor chips were used in this study, the CM 5 Sensor Chip and the mixed self-assembled monolayer sensor chip (mSAM).

As mentioned above, the most commonly used sensor chip is to covalently couple a layer of carboxymethylated dextran to the gold surface (CM5 Sensor Chip) (Löfås, S., and Johnsson, B., 1990). A layer, which serves to prevent the proteins and other ligands to contact with the gold surface, and a hydrogel-modified surface, that would reduce the risk of undesired bindings, are formed and this linkage will provide an interaction layer for one of the interacting molecules (the ligand), such as antibodies, antigens and cells, to bind to the carboxyl groups that present in this matrix. In 1991, Johnsson, B., *et al*., described a method that proteins can be covalently immobilized on a carboxymethyl dextran modified gold surface in a fast and simple way (Johnsson, B., *et al*., 1991). The binding between the interaction molecules, such as a protein, and the sensor chip surface is generally achieved by amine coupling. Before the protein can be injected, the carboxymethyl groups on the surface of the sensor

chip is activated by reacting with N-hydroxysuccinimide (NHS), to form a succinimide ester layer which is highly reactive and can react with amine groups on the protein. Then the protein is injected and coupled to this activated surface. The remaining activated carboxymethyl groups are deactivated, or blocked, by a high concentration of ethanolamine. This deactivation could also wash away those non-covalent bound materials, which is the major purpose of the regeneration process. The regeneration allows many times of the re-use of the sensor chip surface since the activity of the ligand should not be disrupted. However, this regeneration cycle could be time-consuming, and can be interfered by a lot of factors, such as the pH of the regeneration buffer and the numbers of the regeneration cycles.

The other type of sensor chip used in this study is called Mixed Self-Assembled Monolayer (mSAM). The most studied type of this sensor chip is generated by the adsorption of thiols from mixtures of thiolated compounds on a gold surface (Prime, K. L., and Whitesides, G. M., 1993). This reaction is thought to be the oxidation of the abundant S-H bonds of thiolated compounds on to gold, followed by the reduction reaction of hydrogen (Heeg, J., *et al*., 1999). Compared to the carboxymethylated dextran layer of the CM 5 Sensor Chip, this type of reaction surface is demonstrated that it could be more sensitive than CM 5 Sensor Chip for the detection of low concentrations of the analytes present in the samples with significantly reduced amount of non-specific bindings occurring during the experimental process (Frederix, F., *et al.*, 2003). It is because this monolayer on the gold surface provides shorter

attachment intermediates compared with CM 5 sensor chip with dextran layer of 100nm, thus the signal of SPR assay could be improved.

1.3.3 *The sensorgram of BIAcore SPR*

In a typical sensorgram, a baseline signal with no RU change should be stabled over time before the sample can be injected. After injection, an association phase occurs as the RU increases as the result of analyte binding to immobilized ligand. As buffer keeps running through the sensor surface, the ligand-analyte complex starts to dissociate, followed by the regeneration of sensor surface before any new experimental cycles can take place.

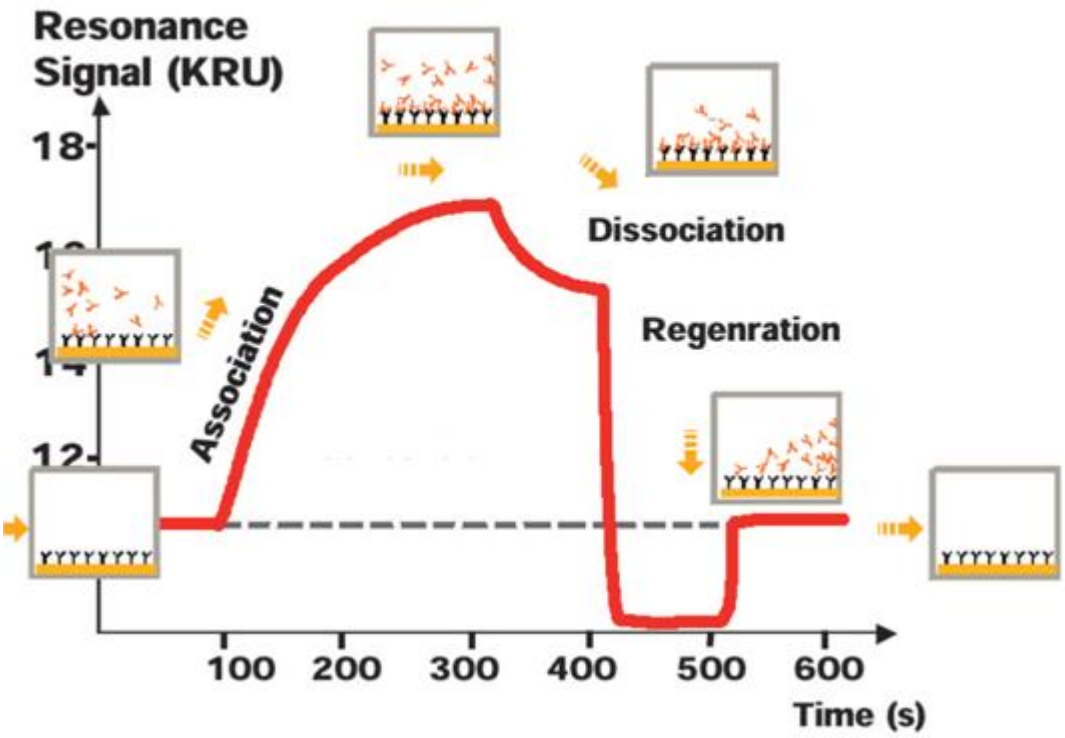

Figure 1.6 A sensorgram for BIAcore-SPR (modified from Biacore, 2009)

Comparing with ELISA, which can only measure the amount of an analyte in the sample, BIAcore SPR is such a powerful technique since it not only measures the quantity of a complex formed (Thillaivinayagalingam, P., *et al.*, 2007) but also monitors the thermodynamic, kinetic (Karlsson, R., and Falt, A., 1997) and even binding affinity of reactant molecules (Malmqvist, M., and Karlsson, R., 1997) (Zhang, X. S., and Oglesbee, M., 2003). These features make the BIAcore-SPR technique more suitable than ELISA assay for a wide range of applications.

1.4 Outline of this study

So far, since the heat shock proteins are more and more important in the understanding of the pathogenesis of many human diseases, such as Diabetes Mellitus and its main complication, Cardiovascular Disease (Xu, Q., *et al*., 2000), the measurement of these proteins in the circulation is thought to be crucial in order to monitor and for early diagnosis of a wide range of diseases.

The aim of this study is to set up an ELISA and BIAcore-SPR based assay system to measure the levels of Heat Shock Proteins from biological fluids, for example, serum and milk.

Chapter Two
Materials and Methods

This chapter covers the materials and methods that were utilized during this study, including all reagents, antibodies & antigen, chemical solutions, overview of the methods used, as well as the details of methods optimizations.

2.1 Materials

2.1.1 *Antibodies*

The antibodies used in this study and their sources are listed in **Table 2.1**.

Table 2.1 The antibodies used in this study

Antibodies	**Product #**	**Source**
Mouse Anti-Hsp70: Biotin Conjugate Monoclonal Antibody	SPA- 810B	Stressgen
Rabbit Anti-Hsp70 (Hsp72) Polyclonal Antibody	SPA- 812	Stressgen
Anti-Rabbit IgG (whole molecule) - Peroxidase antibody produced in goat	A 0545	Sigma-Aldrich

2.1.2 *Antigen*

The antigen used and its source is listed in **Table 2.2**.

Table 2.2 The antigen used in this study:

Antigen	**Product #**	**Source**
Recombinant Human Hsp70	NSP- 555	Stressgen

2.1.3 *Substrate*

The substrate used and its source is listed in **Table 2.3**.

Table 2.3 The substrate used in this study:

Substrate		**Product #**	**Source**
O-Phenylenediamine (OPD)	Dihydrochloride	P 8287	Sigma-Aldrich

2.1.4 *Kits*

Two kits were used in this study, the BCA Protein Estimation Assay Kit and the Amine Coupling Kit.

2.1.4.1 The BCA Protein Estimation Assay Kit

The bicinchoninic acid protein estimation assay kit, also called the BCA protein estimation assay kit, was used to determine the concentration of NeutrAvidin, which was used to coat the 96-wells plate. It was purchased from Pierce, Rockford, Illinois, USA. The reagents of this kit are listed in **Table 2.4**, including their contents.

Table 2.4 The BCA kit reagents and their contents

Reagents	Contents
Bovine Serum Albumin (BSA) Standards	Flame sealed ampules each containing 1 ml of 2,000 µg/ml BSA in 0.9% saline and 0.05% sodium azide.
Reagent A	Sodium Carbonate, Sodium Bicarbonate, Bicinchoninic Acid, and Sodium Tartrate in 0.1 M Sodium Hydroxide.
Reagent B	4% Cupric Sulfate

2.1.4.2 The Amine Coupling Kit

This kit was used for activation and deactivation of the biosensor chip surface in SPR immunoassays. It was purchased from Biacore AB, Sweden (BR-1000-50). Its reagents and contents are listed in **Table 2.5**.

Table 2.5 The Amine Coupling Kit reagents and their contents:

Reagents	**Contents**
Activation Reagent I	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)
Activation Reagent II	N-Hydroxysuccinimide (NHS)
Deactivation Reagent	1.0M Ethanolamine-HCl (EAH) pH 8.5; 10.5ml 1M solution

2.1.5 *Common laboratory chemicals*

All common laboratory chemicals used and their sources are listed in **Table 2.6**.

Table 2.6 Common Laboratory Chemicals used in this study

Chemicals	**Product #**	**Source**
Albumin from Bovine Serum (BSA)	A 7030	Sigma-Aldrich
Tween 20 [polyoxyethylene 20 sorbitan monolaurate]	437082Q	BDH Laboratory Supplies
Goat Serum	G9023	Sigma-Aldrich
NeutrAvidin (NAvidin)		HortResearch
HBS running buffer for BIAcore-SPR		Biacore AB
Skim Milk		Local Supermarket

2.1.6 *Common Solutions*

All common solutions were made on a regular basis (weekly). Common solutions and their compositions are listed in **Table 2.7**.

Table 2.7 Common solutions

Solution	Volume	Composition
0.1 M Carbonate buffer at pH 9.6	50ml	8ml of 0.2M Sodium Carbonate; 17ml of 0.2M Sodium Bicarbonate; 25ml of MilliQ H_2O; Adjust pH to 9.6 if necessary Store at 4°C.
Phosphate-buffered Saline (PBS)	1000ml	135mM NaCl; 1mM KH_2PO_4; 40mM $Na_2HPO_4 \cdot 12H_2O$; 3mM KCl; Dissolve in 800ml of MilliQ H_2O; Adjust pH to 7 and then make up to 1000ml; Store at 4°C.
Phosphate-buffered Saline with 0.1% Tween 20 (PBS/ 0.1% T)	1000ml	135mM NaCl; 1mM KH_2PO_4; 40mM $Na_2HPO_4 \cdot 12H_2O$; 3mM KCl; Dissolve in 800ml of MilliQ H_2O; Adjust pH to 7 and then make up to 1000ml; Add 1ml of Tween 20 and mix well; Store at 4°C.
Blocking buffer I (PBS/ 0.1% Tween 20/ 0.2% BSA)	100ml	Make up 100ml of PBS, pH 7.0; Add 0.1ml of Tween 20; Add 0.2g of BSA and mix well; Store at 4°C.
Blocking buffer II	100ml	Make up 100ml of PBS, pH 7.0;

(PBS/ 0.1% Tween 20/ 1% BSA/ 5% Skim Milk)		Add 0.1ml of Tween 20; Add 1g of BSA; Add 5g of Skim Milk and mix well; Store at 4°C.
PBS/ 0.1 Tween 20/ 5% Goat Serum	100ml	Make up 100ml of PBS, pH 7.0; Add 0.1ml of Tween 20; Add 5ml of Goat Serum and mix well; Store at 4°C.
Phosphate-Citric buffer at pH 5.0	100ml	25.7ml of 0.2M Dibasic Sodium Phosphate; 24.3ml of 0.1M Citric Acid; 50ml of MilliQ H_2O and mix well; Adjust pH to 5.0 if necessary; Store at 4°C.
1 N H_2SO_4	250ml	6.9ml of Concentrated H_2SO_4; 243.1ml of MilliQ H_2O; Add H_2SO_4 into water to avoid explosion; Store at 4°C.
10mM Sodium Acetate at pH 4.5	30ml	Dissolve 1.3608g Sodium Acetate in 10ml MilliQ H_2O to make 1M stock solution; 0.3ml of Stock solution; 25ml of MilliQ H_2O ; Adjust pH to 4.5 and make up to 30ml; Filter before Store at 4°C.
10mM Glycine buffer at pH 2.0	30ml	Dissolve 0.7507g Glycine in 10ml MilliQ H_2O to make 1M stock solution; 0.3ml of Stock solution; 25ml of MilliQ H_2O; Adjust pH to 2.0 and make up to 30ml; Filter before Store at 4°C.

2.2 Methods

The key method used in this study was Enzyme-Linked ImmunoSorbent Assay, also called ELISA. Two different types of ELISAs, the Sandwich ELISA and the Indirect ELISA, were applied in order to compare and improve the sensitivity of this method. Optimizations of these assays were required to obtain reliable and accurate results. Details of these optimizations were described in relevant sections.

2.2.1 *Protein estimation*

Bicinchoninic Acid (BCA) protein assay kit from Pierce, U.S.A, contains two reagents, Reagent A and Reagent B, plus 10 × 1mL ampules of Bovine Serum Albumin (BSA) at concentration of 2.0 mg/mL in the mixture of 0.9% saline and 0.05% sodium azide. This kit was used to estimate the protein concentration of NeutrAvidin (NAvidin) which was used to form a avidin-biotin system in Sandwich ELISA by comparing against a BSA standard curve set up over a broad working range (20 – 2,000 µg/mL).

According to the manufacturer's instructions, BSA standards were prepared in phosphate-buffered saline (PBS) over the range of 20 – 2,000 µg/mL in sterile eppendorf tubes. The working reagent (WR) was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B prior to use, in this case, 9 mL of Reagent A and

0.18 mL of Reagent B. Sample is diluted 1 / 5 by PBS to ensure it is in the range of standard curve.

To each well of the plate, 200 µl of working reagent was added followed by 25 µl of each standard and sample. This was done in duplicate to ensure the accuracy of the assay. The plate was shaken immediately after adding standards / samples and working reagent for 30 seconds, then covered and incubated at 37°C for 30 minutes. After incubation the plate was cooled to room temperature and the absorbance of each well was read at 570nm by a Microplate Reader, Model 680, from Bio-Rad. A linear standard curve was obtained by the absorbance values of BSA standards, while the concentration of the sample can be then calculated using the formula generated by standard curves and its absorbance readings.

2.2.2 *Sandwich ELISA*

Two different sandwich ELISAs were applied, a) with directly coating of the biotin monoclonal antibody on a 96-wells plate, and b) with an avidin-biotin system which showed improved sensitivity of assays.

All the buffers and solutions were made at room temperature and kept at 4°C for up to two weeks (details described in previous sections), and they were brought to room temperature before use.

2.2.2.1 Preparation of washing buffer (PBS/ 0.1% Tween 20)

The washing buffer for both sandwich ELISAs was the same. Phosphate-Buffered Saline at pH 7 was chosen to be the major component in washing buffer because it would help with washing off the un-bound proteins without degrade the binding partners and also can preserve the enzyme activity. However, a mild detergent was also required in the washing buffer, such as Tween 20. At concentration of 0.05% to 0.1%, it works well to disrupt low affinity non-specific interactions. To make 1 L of washing buffer, add 1 mL of Tween 20 (BDH Laboratory Supplies, England) into 1 L of freshly made PBS at pH 7 (details were described in previous section).

2.2.2.2 Preparation of Blocking buffers (PBS/ 0.1% Tween 20/ 0.2% BSA and PBS/ 0.1% Tween 20/ 1% BSA/ 5% Skim Milk)

Two blocking buffers, PBS/ 0.1% Tween 20/ 0.2% BSA and PBS/ 0.1% Tween 20/ 1% BSA/ 5% Skim Milk, were used for Sandwich ELISAs with a) directly coating of Biotin mAb70 on 96-wells plate and b) avidin-biotin system, respectively. The former buffer was also used as the diluents for two antibodies, polyclonal Anti-Hsp70 (Stressgen Bioreagents) and Anti-Rabbit IgG Peroxidase produced in goat (Sigma-Aldrich, USA).

Bovine Serum Albumin (Sigma-Aldrich, USA) was the most common blocking agent used in blocking buffer and was responsible for blocking the unoccupied active sites of the binding partners. Generally, a range of 1% to 5% of BSA in PBS was acceptable. The detergent Tween 20 in the blocking buffer was thought to disrupt those undesired protein-protein interactions rather than just block hydrophobic sites, and the addition of skim milk (General Distributors Ltd., New Zealand) would also help to improve the blocking activity. However, skim milk is prone to deteriorate rapidly, so it was suggested that the blocking buffer with skim milk should be made prior to use and may only be kept for a maximum of two days.

To make 100 mL of PBS/ 0.1% Tween 20/ 0.2% BSA, add 0.1 mL of Tween 20 and 0.2 g of BSA into 100 mL of newly made PBS. Stir the mixture well to make sure all the powders were dissolved.

To make 100 mL of PBS/ 0.1% Tween 20/ 1% BSA/ 5% Skim Milk, increase the amount of BSA to 1 g and additionally, add 5 g of Skim Milk. This mixture may need to be stirred overnight if necessary to ensure all powders were dissolved.

2.2.2.3 Preparation of substrate buffer

The substrate used in this study was *o*-Phenylenediamine Dihydrochloride (OPD) from Sigma-Aldrich, USA. This substrate produces a yellow-orange coloured end product that can be read spectrophotometrically at 450 nm. With the addition of stop

solution 1 N H_2SO_4, the colour of solution turned orange-brown and can be read at 490 nm. It can be stored at 2 - 8°C but needs to be brought to room temperature before use.

According to the manufacturer's information, the buffer used to dissolve this substrate was 0.05M Phosphate-Citric Buffer at pH 5.0. This buffer was made by mixing 25.7 mL of 0.2 M dibasic sodium phosphate and 24.3 mL of 0.1 M citric acid with 50 mL of MilliQ water. pH needs to be adjusted to 5.0 if necessary. Typically, an OPD tablet of 10 mg was dissolved in 25 mL of 0.05 M phosphate-citric buffer without touching the tablet with fingers or use of metallic forceps. This would give an OPD solution at concentration of 0.4 mg/mL, which was widely used in ELISA assays. However, the OPD solution is well known to be very unstable in the air or under the light, so 0.05 M phosphate-citric buffer needs to be degassed for about 40 minutes just before use and the tablet should be dissolved in dark. This can be stored for about 6 hours. Immediately prior to use, 40 µl of 30% hydrogen peroxide (Andrew Industrial Ltd., New Zealand) per 100 mL of 0.05 M phosphate-citric buffer solution was added. For best results, the substrate solution with hydrogen peroxide should be used within one hour.

2.2.2.4 Preparation of stop solution 1 N H_2SO_4

The OPD reaction can be stopped with 1 N H_2SO_4. To make 250 mL of 1 N H_2SO_4, 6.9 mL of concentrated H_2SO_4 was mixed with 243.1 mL of MilliQ water. Extra attention needs to be paid that the acid should always be added into water to avoid any explosion that may happen. This stop solution can be stored at 4°C for up to four weeks.

2.2.2.5 Preparation of diluents for antibodies

For coating the monoclonal antibody to the 96-wells plate, the optimum buffer should have pH ranges from 7 to 9 since it helps to maintain the native conformation and the solubility of the protein. 0.1 M Carbonate Buffer at pH 9.6 is one of the widely used coating buffers for antibodies. To make 50 mL of this buffer, 8 mL of 0.2 M Sodium Carbonate, 17 mL of 0.2 M Sodium Bicarbonate were mixed with 25 mL of MilliQ water. Adjust pH to 9.6 if necessary.

The buffer used to dilute polyclonal anti-Hsp70 and Anti-Rabbit IgG peroxidase was PBS/ 0.1% Tween 20/ 0.2% BSA. The addition of BSA in the diluent would help to block the undesired bindings. To make up 100 mL of this buffer, 0.1 mL of Tween 20 and 0.2 g of BSA was added to 100 mL of PBS, followed by 3 hours stirring to ensure all the BSA were well dissolved.

2.2.2.6 Preparation of Biotin Conjugate Monoclonal Mouse Anti-Hsp70

According to the manufacturer's instruction, this Biotinylated Monoclonal Antibody (Biotin mAb 70, concentration 1.0 mg/mL), from Stressgen Bioreagents USA, can detect inducible Hsp70 but will not react with the constitutive Hsc70 and has a cross reactivity with a wide range of species, human, mouse, rat, rabbit and bovine, etc.

Prior to use, this Biotin mAb70 was aliquot to 10µl each vial and kept at -20°C to prevent from degradation. Each vial can be thawed just before use and the remaining mAb70 in the vial can be left at 4°C for one to two weeks.

The buffer used to dilute the biotin mAb70 was 0.1 M Carbonate buffer, at pH 9.6. The starting concentration used for direct coating of monoclonal antibody molecules on to the polystyrene 96-wells plate (NUNC, Denmark) was 5µg/mL based on the work done by Njemini, R., et al. in 2003 (Njemini, R., *et al.*, 2003). However, this concentration was reduced to 1µg/mL when coating on a layer of NeutrAvidin molecules, which was pre-coated on the 96-wells plate before biotin mAb70 was added, because the avidin-biotin system increases the sensitivity of the ELISA assays.

2.2.2.7 Preparation of Human Recombinant Hsp70 at different concentrations

Human Recombinant Hsp70, rHsp70 (Stressgen Bioreagents), was five-fold serial diluted in phosphate-buffered saline (PBS) to 8 different concentrations, ranging from 0 ng/mL to 2000 ng/mL. In order to reduce the errors that may occur during the dilution process, a stock at the concentration of 4000 ng/mL was made before the serial dilution by adding 1 µl of rHsp70, which has a concentration of 1.85 mg/mL, into 461.5 µl of PBS in an eppendorf tube.

In order to make enough 2000 ng/mL rHsp70 for the following dilutions, 230 µl of 4000 ng/mL rHsp70 was added to the same amount of PBS buffer. The five folded serial dilution was carried on by transfer 100 µl of each mixture to the next eppendorf tube that contained 400 µl of PBS buffer. Make sure that sufficient diluted rHsp70s was left in the eppendorf tubes for adding to each well after blocking step.

2.2.2.8 Preparation of Polyclonal Anti-Hsp70

This polyclonal antibody was produced from whole rabbit serum and has a cross reactivity with various species, e.g. human, mouse, bovine and sheep, but was only specific for inducible form of Hsp70.

This polyclonal anti-Hsp70 was diluted 1:400 in PBS/ 0.1% Tween 20/ 0.2% BSA. For optimal storage, this product was aliquot down to 10 µl per vial and stored at -

20°C. Each vial was thawed prior to use and may be stored at 4°C for two to four weeks. For best results, each vial was centrifuged after thawing.

2.2.2.9 Preparation of Anti-Rabbit IgG peroxidase antibody produced in goat

This secondary antibody was shipped in dry ice in order to prevent from denaturation, thus, aliquot a vial of 50 µl and stored the remaining antibody at -20°C to avoid repetitive freeze-thaw cycles.

Specificity of this peroxidase showed bindings with all rabbit Igs but has no reaction with human IgG. Aliquot vial was thawed on ice prior to use to avoid denaturation of the enzyme and then diluted 1:10,000 in PBS/ 0.1% Tween 20/ 0.2% BSA.

2.2.2.10 Sandwich ELISA procedure

2.2.2.10.1 *The protocol of sandwich ELISA with Biotin mAb70 only*

This protocol was based on the work done by Njemini, R., *et al*. in 2003 (Njemini, R., *et al*., 2003). It is the first method used in this study to exam the sensitivities of Hsp70 protein detections and required two days approximately. The 96-wells plate was coated with 100 µl per well of 5 µg/mL diluted biotin monoclonal anti-Hsp70 in

0.1 M Carbonate Buffer at pH 9.6. Before incubated the plate at 4°C overnight, it was shaken at room temperature for 3 hours with cover at the speed of 400 rev/ minute. On the second day, each well was washed with washing buffer for six times by using a Multi-Channels Auto-Pipette (Hamilton, USA). The wells were blotted dried on paper towels after each washing step. Then 300 µl of blocking buffer, PBS/ 0.1% Tween 20/ 0.2% BSA, was added to each well and shaken at 400 rev/ minute in a 37°C incubator with cover for four hours followed by six times washing with PBS/ 0.1% Tween 20. The antigen, human recombinant Hsp70 was diluted in PBS to 8 different concentrations, ranging from 0 ng/ mL to 2000 ng/ mL (details were described previously). 100 µl per well of antigen with different concentrations was added to wells A to H in an ascending order and then triplicate for each concentration. For better binding between coated monoclonal antibody and antigen, the plate was incubated and shaken at 37°C with cover for two hours. After washing step, 100 µl of 1:400 diluted polyclonal anti-Hsp70 in PBS/ 0.1% Tween 20/ 0.2% BSA was added to each well and incubated with shaking at 37°C for one hour. While incubation, the Anti-Rabbit IgG peroxidase was thawed on ice and then diluted 1:10,000 in PBS/ 0.1% Tween 20/ 0.2% BSA. The plate was washed with PBS/ 0.1% Tween 20 after incubation as before and 100 µl of this diluted IgG peroxidase was added to each well, followed by another hour incubation with shaking at 37°C. One OPD tablet was dissolved in 25 mL of degassed phosphate-citric buffer in dark with vortex only. 10 µl of 30% H_2O_2 was added to substrate solution immediately prior to use. After washing, 200 µl of OPD solution with H_2O_2 was added to each well and incubated at 37°C in dark for 30 minutes. After incubation, 50 µl of 1 N H_2SO_4 was added directly to each well, followed by 60 seconds shaking at room temperature, then read at 490

nm. A standard curve can be generated according to these absorbance readings by using Microsoft Office Excel 2007. The sensitivity of the assay was defined as the concentration that gave the absorbance of the zero standard plus two standard deviations and can be calculated by using the equation obtained from the standard curve. The Coefficient of Variation (CV) of the assay was also calculated as the standard deviation divided by the mean absorbance value.

2.2.2.10.2 *The protocol of sandwich ELISA with an avidin-biotin system*

This protocol was developed from the previous sandwich ELISA assay and was expected to show an improved limit of detection (LOD). However, the total time needed for this protocol was elongated to four days compared with two days required previously. The 96-wells plate was firstly overnight coated with 100 µl per well of 5 µg/ mL NeutrAvidin diluted in MilliQ water. Compared with the previous protocol, the plate was incubate at 37°C overnight without cover to allow dryness instead of chilled at 4°C. On the second day, the washing step was applied between each incubation process as previous. After washing, 100 µl of 1 µg/ mL biotin monoclonal anti-Hsp70 diluted in 0.1 M Carbonate Buffer at pH 9.6 was added to each well followed by shaking at room temperature for three hours with cover before left at 4°C overnight. The first thing on day three was 6 times washing with PBS/ 0.1% Tween 20. After washing and blotted dry, 300 µl of blocking buffer, PBS/ 0.1% Tween 20/ 1% BSA/ 5% Skim Milk, was added to each well and the plate was shaken at room

temperature for three hours with cover before incubation overnight at 4°C. The next day, eight different concentrations of human recombinant Hsp70 diluted in PBS (the same dilution procedure as previous protocol) was added to wells in triplicate, 100 µl per well, followed by three hours incubation at 37°C with shaking at the same time. Plate was then washed 6 times with PBS/ 0.1% Tween 20 as before. Polyclonal anti-Hsp70 antibody was diluted 1:400 in PBS/ 0.1% Tween 20/ 0.2% BSA and 100 µl of this was added to each well. Plate was incubated again at 37°C for one hour with shaking. After incubation, washing step was applied and followed by addition of 100 µl per well of 1:10,000 Anti-Rabbit IgG peroxidase diluted in PBS/ 0.1% Tween 20/ 0.2% BSA. Wells was incubated at 37°C for an hour with shaking. During incubation, 25 mL of phosphate-citric buffer was degassed as previously described for 40 minutes and one OPD tablet was dissolved in this degassed buffer in dark. Prior to use, 10 µl of 30% H_2O_2 was added to OPD solution. After washing, 200 µl per well of OPD solution with H_2O_2 was added to each well and five minutes incubation at 37°C with shaking was applied. To stop the substrate reaction, 50 µl of 1 N H_2SO_4 was added to each well and the absorbances were read immediately at 490 nm by a plate reader. A standard curve would be generated as the same as previously described and the sensitivity and Coefficient of Variation of the assay was also calculated.

2.2.2.11 The Indirect ELISA

This method requires a coating of antigens with different concentrations on the surface of the plate, followed by the blocking and then binding of primary antibody to the antigen. This binding was then labelled by a secondary antibody so the signal could be detected after the addition of substrate.

First of all, wells were incubated with 100 µl per well of different concentrations of antigen, ranging from 0 to 2000 ng/ml, overnight at 4°C. Before blocking on the next day, the wells were washed six times with PBS/ 0.1% Tween 20. The blocking buffer was made of PBS/ 0.1% Tween 20, 1% of BSA and 5% of Skim Milk. The blocking process was applied at 4°C with overnight incubation to make sure that non-specific bindings were disrupted as many as possible. On the third day, polyclonal anti-Hsp70 was diluted 1:400 in PBS/ 0.1 Tween 20/ 5% Goat Serum and 100 µl of these was added to each well after the washing step. The wells were incubated at 37°C for three hours with shaking at the same time. The secondary antibody, Anti-Rabbit IgG Peroxidase produced in Goat, was diluted in PBS/ 0.1% Tween 20/ 0.2% BSA at the ratio of 1:10,000. After incubation, wells were washed six times by PBS/ 0.1% Tween 20, followed by addtion of 100µl of diluted IgG Peroxidase to each well. The wells were again incubated at 37°C for one hour with shaking. As the same as the NAvidin-Biotin system, the phophate-citric buffer, which was used as the diluent of OPD tablet, was degassed for 40 minutes and the tablet was dissolved in dark with the addition of 10 µl H_2O_2 just prior to use. 200 µl of this OPD solution was added to

each well and the plate was incubated at 37°C for five minutes and this reaction was stopped by the addition of 50 µl of 1 N H_2SO_4 to each well. The absorbances were read at 490 nm by a Plate Reader and these values were used not only for the generation of standard curve, but also for calculation of the assay's sensitivity and Coefficient of Variation.

2.2.3 *Surface Plasma Resonance (SPR) with the use of biosensors*

The basis of this method is to use an optical instrument to measure the refractive index on a sensor surface. In this study, two different sensor chips, the CM5 chip and the mixed self-assemble monolayer (mSAM), and two different types of SPR, competitive and sandwich SPR, were investigated respectively. The protocol used in this study was based on Yuan, J's unpublished data.

2.2.3.1 Preparation of Immobilization and Regeneration buffer

The immobilisation buffer used for SPR immunoassays was 10 mM Sodium Acetate buffer, at pH 4.5. First to make 10 ml of 1 M sodium acetate, 1.3608 g of sodium acetate was dissolved in 10 ml of distilled water before it could be diluted down to 10 mM. 30 ml of 10 mM sodium Acetate was diluted by adding 0.3 ml of 1 M stock

solution to 29.7 ml of distilled water and then the pH was adjusted by 5 M and 1 M HCl. This solution needed to be filtered by a 0.2 µm filter to get rid of the small particles, such as dust, that contained in the buffer which may block the solution injection needle. The filtered solution can be stored at 4ºC for up to 2 weeks.

To make 10 mM of regeneration buffer, glycine buffer pH 2.0, 1 M of stock was made first by dissolving 0.7507 g of glycine powder in 10 ml of distilled water. Then 0.3 ml of this stock was added to 29.7 ml of distilled water to make 30 ml of 10 mM glycine buffer. The pH was adjusted after dilution, followed by filtering of this diluted buffer before chilled it at 4°C.

2.2.3.2 Preparation of mSAM surface buffer

The cleaned bare gold film was immersed in this buffer to generate a binding surface for the following reactions. It was consisted of two chemicals, 10 mM 11-Mercapto-1-Undecanol (11-MOH) and 10 mM 16- Mercaptohexa-decanoic acid (16-MUA). To make 10 mM 11-MOH and 10 mM 16-MUA, 21.5 mg and 6.6 mg of the chemicals were dissolved in 10.520 ml and 2.288 ml of EtOH respectively. Extra care should be taken as the former is irritant and the latter is toxic. 9 parts of 10 mM 11-MOH and 1 part of 10 mM 16-MUA was mixed in a beaker that was pre-cleaned by H_2SO_4, H_2O_2 and Ammonia.

2.2.3.3 Sandwich SPR immunoassay with CM 5 sensor chip

There were four flow cells (FC) on this chip. The FC 2 was used for immobilization of biotin monoclonal anti-Hsp70. The chip needed to be primed for three times before the first time of use, or primed once when it was undocked from the machine. Generally, before any antibodies can be immobilized on the CM 5 chip, the sensor chip surface was activated by a mixture of N-ethy-N'-dimethylaminopropyl carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in the ratio of 1:1 at a flow rate of 5µl/minute for 7 minutes. After activation, 25 µl of 50 µg/ml Biotin monoclonal anti-Hsp70 diluted in 10 mM Sodium Acetate at pH 4.5 was injected to FC2 at a flow rate of 5µl/minute for 5 minutes, in order to covalently immobilize this antibody on the sensor chip surface. The sensor chip was then deactivated by injection of 35 µl of Ethanolamine-HCl (EAH) at a flow rate of 5µl/minute for 5 minutes. To allow better sensitivity of the assay, the sensor chip was blocked after deactivation by injection of 100 µl of HBS/ 1% BSA to FC 2 at a flow rate of 5µl/minute for 20 minutes. The final step involved in immobilization of monoclonal antibody on the sensor chip surface was the regeneration of the chip surface. This was done by injecting 10 µl of 10 mM glycine buffer at pH 2.0 at a flow rate of 20µl/minute for 30 seconds. After immobilization, 60µl of 8 different concentrations of human recombinant Hsp70 (rHsp70) (0ng/ml – 2000ng/ml) diluted in HBS buffer was injected in duplicate at a flow rate of 20µl/minute for 3 minutes, followed by running 10µl of 10mM Glycine buffer at pH 2.0 at a flow rate of 20µl/minute for 30 seconds to regenerate the sensor surface. The standard deviations

and the Coefficient of Variation for each concentration were calculated and the Hsp70 Binding Curve was generated from these data. After obtained the standard curve of this trial, we tried another mode of SPR, the competitive SPR immunoassay.

2.2.3.4 Competitive SPR immunoassay with CM 5 sensor chip

Compare with Sandwich SPR, which immobilized monoclonal anti-Hsp70 on the sensor chip surface, this competitive assay started by binding of antigen on the sensor chip surface. Therefore, after activation of the sensor chip by the mixture of EDC/NHS on FC 3, 50 µl of 80 µg/ml Human Recombinant Hsp70 (rHsp70) diluted in 10 mM Sodium Acetate at pH 4.5 was injected at a flow rate of 5µl/minute for 10 minutes, followed by another 7 minutes injection of EAH at a flow rate of 5µl/minute to deactivate the sensor surface. Then the regeneration buffer was injected at a flow rate of 20µl/minute for 1 minute to remove any unbound ligand from the sensor surface.

After immobilization of antigen on the surface, the assay was carried on by injecting a mixture of monoclonal anti-Hsp70 (100µl) with Hsp70 (100µl) at eight different concentrations (0-1000 ng/ml) added to it at the ratio of 1:1 and then followed by 30 minutes incubation at room temperature with shaking at the same time to ensure maximum binding. A wizard was set up with 60 µl injection of each concentration at flow rate of 20µl/minute, followed a 180 seconds stabilization waiting time before the

sensor surface was washed by 10 mM glycine buffer at pH2.0 for 30 seconds at a flow rate of 20µl/minute. A competitive standard binding curve was generated after injections of all different concentrations were finished.

2.2.3.5 Sandwich SPR immunoassay with a mixed self-assemble monolayer sensor chip (mSAM)

Compare with the CM 5 sensor chip, mSAM was less stable and can be easily oxidised because of its abundant –SH groups. The sensor surface was made on a gold film which was cleaned by immersing into a mixture of concentrated H_2SO_4 and H_2O_2 at the ratio of 3:1 in a beaker, which was pre-cleaned by H_2SO_4, H_2O_2 and Ammonia. The whole beaker was then incubated at 50°C for 30 minutes followed by washing with distilled water and then EtOH. The film was dried by N_2 to make sure no H_2O or O_2 was left on the film. The mSAM surface was made by immersing the cleaned gold film into a mixture of 10 mM 11-Mercapto-1-Undecanol (11-MOH) and 10 mM 16- Mercaptohexa-decanoic acid (16-MUA), which were all dissolved in EtOH, at a ratio of 9:1 in a pre-cleaned beaker for 24 hours at room temperature.

As the same as the CM 5 chip, there were also four flow cells on mSAM sensor chip. The sensor chip was primed three times before use. FC 1 was set up as a blank flow cell, which only has NeutrAvidin (NAvidin) immobilized to the surface after activation. FC 2 was activated by 100 µl of a mixture of EDC/NHS at ratio of 1:1

after prime. 150 µl of 500 µg/ml NAvidin diluted in 10mM Sodium Acetate pH 4.5, was injected into FC 2, at a flow rate of 5µl/minute for 30 minutes, before the biotin monoclonal anti-Hsp70 was bound onto the surface. The same as the CM 5 chip, the sensor surface was then deactivated by 100 µl of EAH for 20 minutes. The washing buffer used in this assay was a mixture of 1 M NaCl and 50 mM NaOH. This mixture was injected 3 times at a flow rate of 20µl/minute, 1 minute each time. Then 200 µl of 100 µg/ml biotin monoclonal anti-Hsp70 diluted in HBS was injected, followed by washing with 10 mM glycine buffer at pH 2.0. The flow cell was then blocked by 1 mg/ml of D-Biotin diluted in HBS for 20 minutes before regenerated by 30 µl of 10 mM glycine buffer, pH 2.0.

Chapter Three
ELISA Results

3.1 Introduction

Enzyme-Linked ImmunoSorbent Assay (ELISA) became extraordinary useful since last couple of decades because it allows rapid screening of the quantitation of an analyte present in a sample and it also allows examination of a large number of samples at the same time. The accuracy of the ELISA assays is generally determined by:

- The sensitivity level of the assay, also expressed as the Limit of Detection (LOD) in some paper. This value was calculated as the concentration of analyte that gives the absorbance of zero standard plus two standard deviations. It indicated the lowest concentration of the analyte that can be detected in the samples or standards. Thus, the lower the value, the better the sensitivity of the assay.

- The Inter- / Intra- assay(s) Coefficient of Variation (CV), which indicated the level of reproducibility within / between assay(s). It is expressed as the percentage of the standard deviation (SD) of the data set divided by the mean

of the data set. For both inter- and intra- assay(s), a CV value which is in the range of 1% - 10% indicated that the wells of the same concentration, in our study the triplicates, of one assay / between different assays are well repeated.

3.2 Sandwich ELISA with antigen concentration 0-2,000ng/ml

Generally, if a protein which molecule has more than one epitopes is being detected, Sandwich ELISA would be a better choice compared with other ELISA formats, such as Indirect ELISA. Three different antibodies are used in this study, a primary antibody, a capture antibody and an enzyme-linked secondary antibody (**Figure 3.1**). The bindings of the primary and capture antibodies to different epitopes on the surfaces of the antigen molecules allow the formation of as many antigen-antibody complexes as possible. Thus, allows the capture of antigen molecules even under low concentrations. This may also enhance the signal generated since the use of a capture antibody may increase the spatial length for signal detection. In brief, to set up a Sandwich ELISA standard, a primary antibody, usually a monoclonal antibody, will be covalently immobilised on the solid surface of the 96-well plate. Those primary antibody molecules that are not immobilised on the plate surface are washed away by washing buffer and then blocked by blocking agent, in our study the Bovine Serum Albumin (BSA). After this, antigen at different concentrations will be added and incubated at optimal temperatures to allow better interactions between the monoclonal antibody and antigen molecules. Since the monoclonal molecule can only

occupy one epitope on the antigen molecule, another antibody, the polyclonal antibody in this study, will be required to bind with other epitopes left on the antigen molecule. The binding signal is then amplified by the addition of a secondary antibody, generally an enzyme, which is then visualised by a specific substrate of that enzyme. The reaction between the enzyme and the substrate is generally stopped before the final signal can be spectrometrically measured by a plate reader at specific wavelengths.

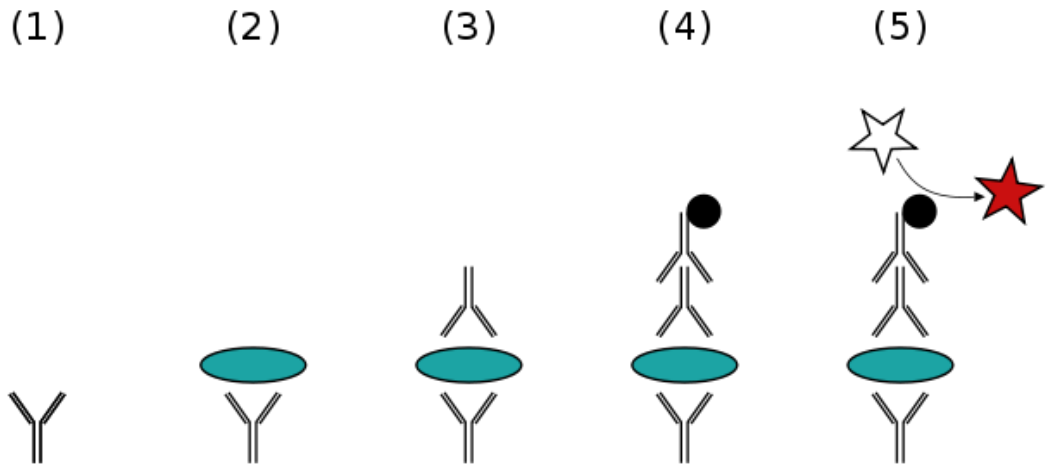

Figure 3.1 A Sandwich ELISA

1. The primary antibody is immobilised on the surface of the 96-wells plate
2. Standards or samples containing that antigen are added and these antigen molecules will be captured by immobilised antibody molecules.
3. A detecting antibody is added and will bind to primary antibody-antigen complex.
4. It also allows the enzyme-linked secondary antibody to bind to the complex.
5. Substrate is added and is converted to detectable form.

3.2.1 *Sandwich ELISA with Biotin Monoclonal Anti-Hsp70 directly coated on plate*

The experimental protocol in this study was set up following the published work done by Njemini, R. and co-workers in 2003 (Njemini, R., *et al.*, 2003). The detailed procedure is described in Chapter 2. The antigen (Hsp70) concentration used for this standard curve was five-fold serial diluted, in the range of 0 – 2,000ng/ml.

The result of the first standard set up showed extremely high level of background (absorbances at antigen concentration of 0ng/ml), which has an average value of 1.04 (**Figure 3.2**). Theoretically, there should not be any bindings occurring in the absence of added antigen, so the expected background for the study should be no higher than 0.2. These abnormally high background absorbances indicated that there was a huge number of non-specific bindings occurred during the experimental process. On the other hand, since the backgrounds were too high, the absorbances for those wells at antigen concentration of 2,000ng/ml could not be read.

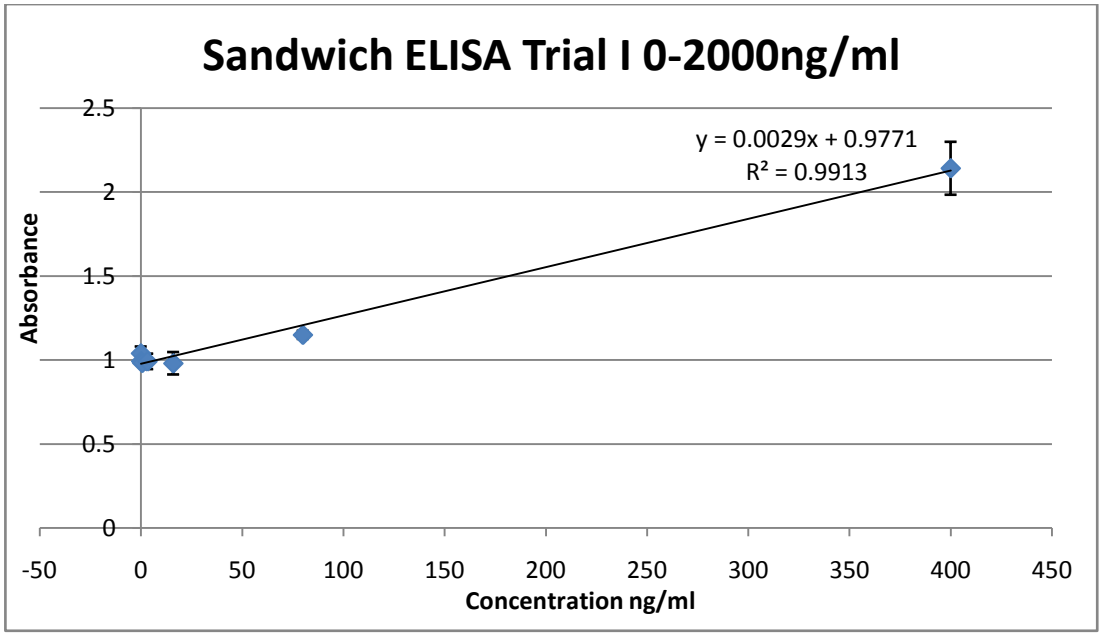

Figure 3.2 Standard Curve of Sandwich ELISA with Biotin mAb70 coating

The sensitivity of the assay was calculated to be 50ng/ml, compared with Njemini's work, which has a sensitivity of 4ng/ml. This indicated that the experimental protocol needed to be optimised in order to get rid of those non-specific bindings. However, the inter-assay CVs for each concentration of this assay were all in the range of 1% - 7% (**Figure 3.3**), indicated that the assay was well repeated and the experimental errors were acceptable.

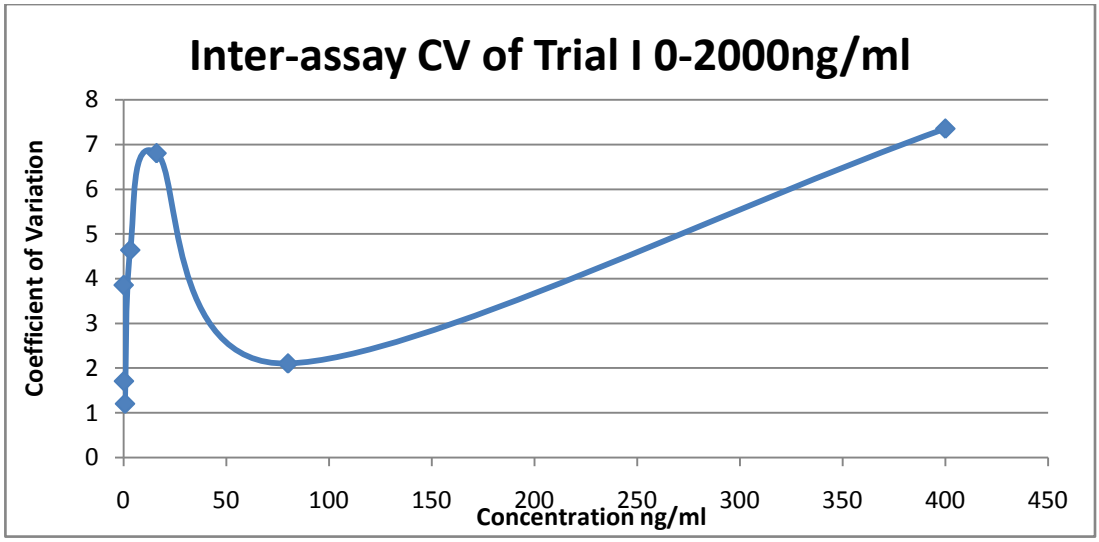

Figure 3.3 The Inter-assay CVs for Standard Curve with Biotin mAb70 coating

3.2.2 *Optimisations*

3.2.2.1 Blocking Conditions

The blocking step of the experimental process is mainly responsible for eliminating those unwanted non-specific bindings. Two factors were examined: the blocking time and the concentration of the blocking agent Bovine Serum Albumin (BSA).

Without changing any other conditions, two sets of wells were firstly set up to check the effect of blocking time. The ELISA protocol used in the previous standard was followed with antigen concentration of 0ng/ml only. The first set of 4 wells were

blocked by PBS/ 0.1% Tween 20/ 0.1% BSA for 1 hour, with the second set of another 4 wells were blocked by the same blocking buffer for 2 hours.

The result showed significant decrease of the background absorbances for those wells with 2 hours blocking (Average of 0.79 VS average of 0.48) (**Figure 3.4**). However, the average background of 0.48 was still high, indicating that there was still non-specific binding occurring in the assay.

In the next experiment, blocking time was kept at 2 hours constant and another two sets of wells were set up for antigen concentration of 0ng/ml only, to check the effect of the concentration of the blocking agent. The first set of 4 wells were blocked by PBS/ 0.1% Tween 20/ 0.2% BSA for 2 hours, with another set of 4 wells were blocked by PBS/ 0.1% Tween 20/ 1% BSA for 2 hours.

This time, the result did not show much difference between BSA concentration of 0.2% and 1% in the blocking buffer respectively (Average of 0.41 VS Average of 0.45) (**Figure 3.4**). However, compared to the 2 hours blocking by 0.2% BSA with 2 hours blocking by 0.1% BSA, the background reading was reduced (Average of 0.41 VS Average of 0.48). But the result was still much higher than expected, indicating that there was still non-specific binding in the experimental process. Next, the blocking time was increased from 2 hours to 4 hours at BSA concentration of 0.2%, in order to get rid of as many non-specific bindings as possible. The comparison between those two sets of wells showed the background was significantly lowered with 4 hours blocking of 0.2% BSA in PBS/ 0.1% Tween 20 (Average of 0.25 VS Average of

0.41). Thus, 4 hours blocking by PBS/ 0.1% Tween 20/ 0.2% BSA would be adopted for the subsequent work.

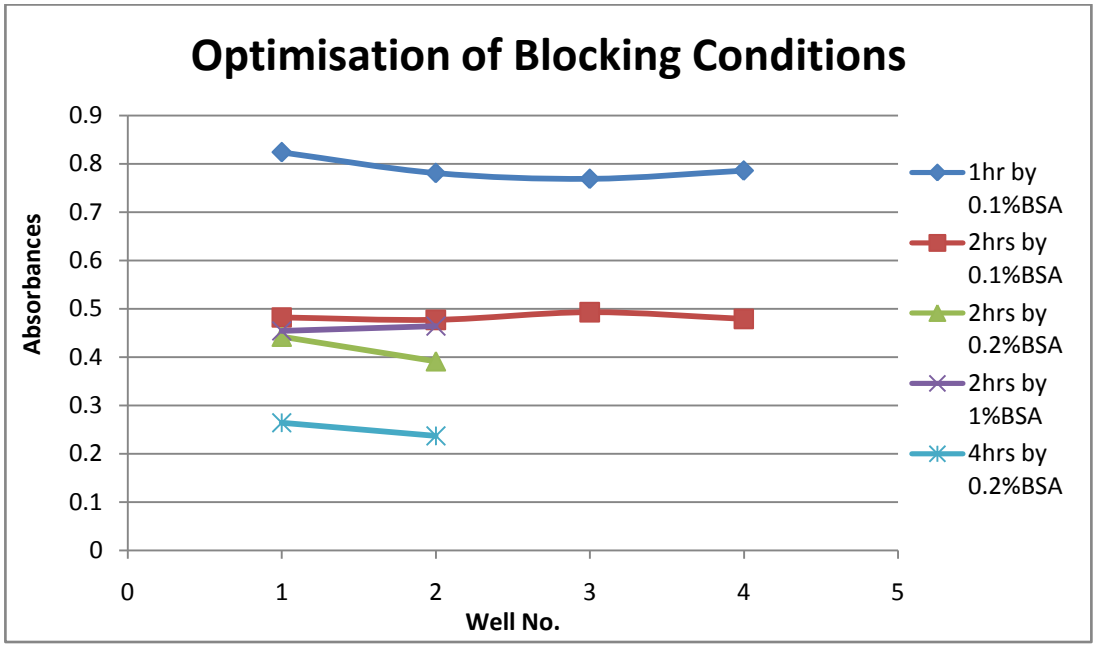

Figure 3.4 Optimisation of blocking time and blocking agent concentrations.

3.2.2.2. Substrate Conditions

According to Sun, W., *et al*., 2001, the substrate used in our study, the Ortho-Phenylenediamine (OPD), was very unstable in the air or under the light, and can be easily oxidized after the addition of H_2O_2 (Sun, W., *et al*., 2001), turning to light orange in less than 1.5 hours. These may also contribute to the abnormally high results of the first standard.

To check the effect of air for the OPD substrate, two sets of wells, 4 wells each, were set up. Both of these two sets of wells were incubated 1 hour with IgG Peroxidase, the enzyme used in this study, and then one set of wells were incubated with degassed-OPD solution, which was made by dissolving the OPD tablet in 40 minutes-degassed phosphate-citric buffer, while the other set of wells were incubated with undegassed OPD solution. After 30 minutes incubation, the reactions of both sets of wells were stopped by 1 N H_2SO_4.

The absorbances of these two sets of wells showed significantly reduced background levels with degassed substrate buffer (Average of 0.0465 VS Average of 0.142) (**Figure 3.5**), indicated that this substrate is very sensitive to air.

A third set of 4 wells were set up 1.5 hours after adding H_2O_2 to OPD solution. The same process was applied as previous two sets. After stopped the reaction, the absorbances of these 4 wells were read. Compared with those wells with degassed and freshly made OPD solution, these 4 wells showed a dramatic increase of the background noise (Average of 0.0465 VS Average of 0.202) (**Figure 3.5**). On the other hand, these absorbances were even higher than those wells with un-degassed OPD solution. All of these indicated that for the following experiments, the substrate solution needed to be freshly made, degassed and used within 1.5 hours.

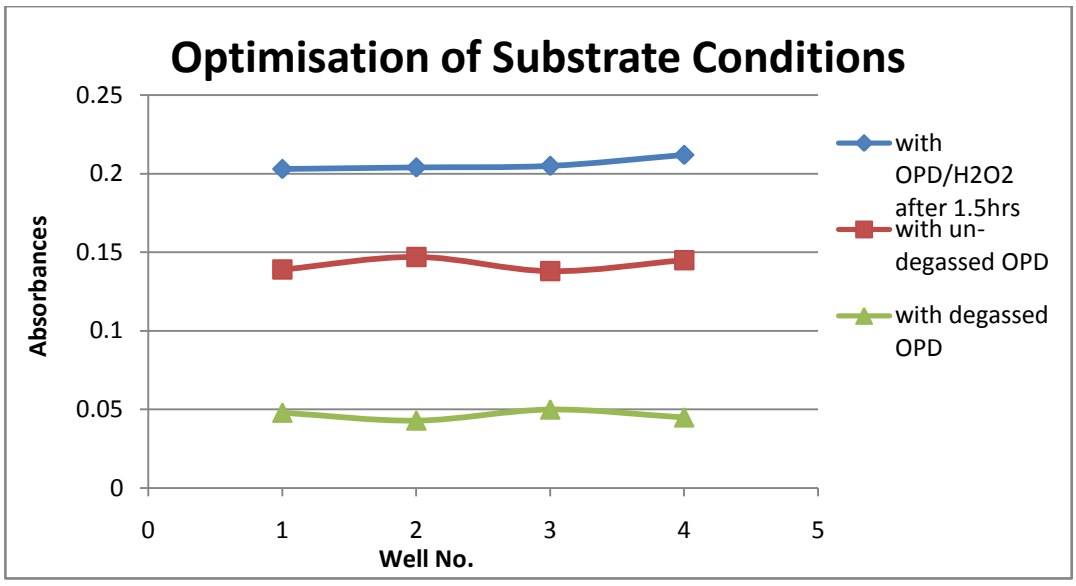

Figure 3.5 The effects of air and time on substrate OPD.

3.2.3. *Sandwich ELISA standards after optimisation*

After optimising both the blocking and substrate conditions, 3 Sandwich ELISA standards, with 8 different antigen concentrations (which were five-fold serial diluted as previous experiment) were set up using improved method, i.e. increasing the blocking time to 4 hours instead of 2 hours, increasing blocking agent (BSA) concentration in the blocking buffer from 0.1% to 0.2% and making degassed OPD solution every time prior to use.

The sensitivities of these 3 standards were calculated. Compared with the first set of standard, the average assay's sensitivity of these 3 sets standards was significantly improved (Average of 39ng/ml VS Average of 50ng/ml) (**Table 3.4**), indicated that

the previous optimisations of both blocking and substrate conditions did eliminate some non-specific bindings of the assay. However, our protocol needed to be further optimised compared with the published data, which has an assay sensitivity of 4ng/ml.

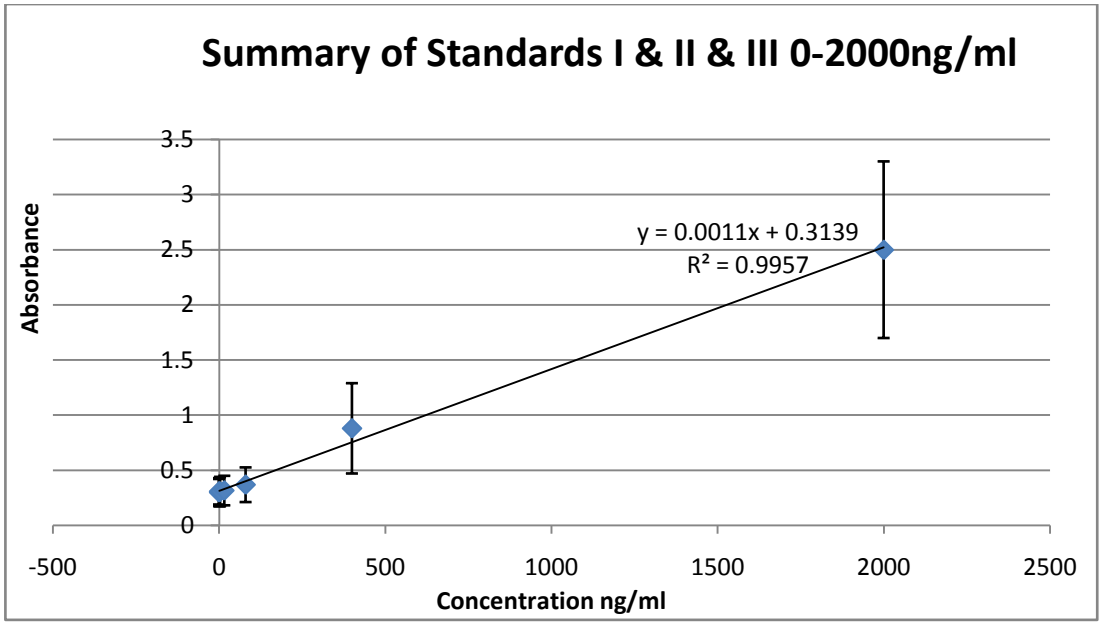

Figure 3.6 Summary of 3 Standards with antigen concentration 0-2,000ng/ml.

The background absorbances of these standards were also dramatically lowered compared with previous work (Average of 0.30 VS Average of 1.04). However, they were very unstable, ranging from 0.14 to 0.42 (**Figure 3.6**), thus the intra-assays CV of these 3 standards was up to 40% in average (**Figure 3.7**), indicated very poor experimental reproducibility. These experimental errors might contribute to the poor sensitivities of the assays. Compared with the intra-assays CVs, most of the inter-

assay CVs were all in the range of 1% - 10% (**Table 3.1**), indicated that within each assay, the experimental errors between wells of the same concentration were acceptable.

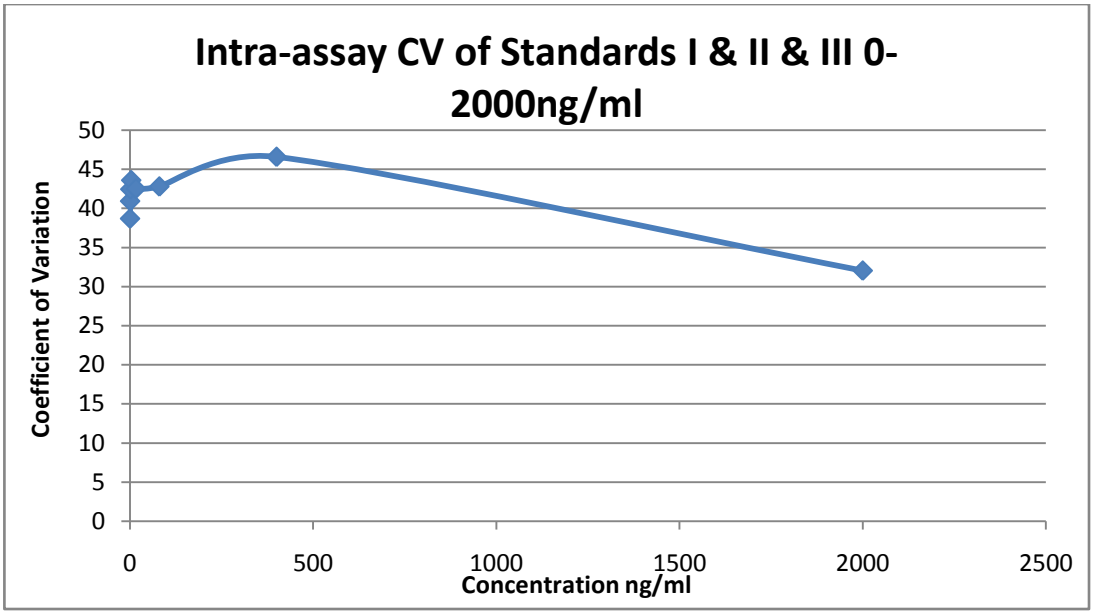

Figure 3.7 Intra-assays CVs of 3 standards with antigen concentration 0-2,000ng/ml.

Table 3.1 Comparison of inter-assay CVs and intra-assays CVs of 3 Standards with antigen concentration 0-2,000ng/ml

Hsp70 Concentration (ng/ml)	Inter-Assay CVs (%)			Intra-Assay CVs (%)
	Standard I	Standard II	Standard III	
0	6.93	0.74	4.29	38.69
0.128	3.88	4.38	7.47	40.92
0.64	6.89	7.37	7.40	42.44
3.2	3.35	1.83	10.26	43.58
16	1.68	4.52	5.57	42.51
80	5.04	13.22	5.04	42.78
400	12.80	2.61	6.11	46.58
2000	15.62	4.39	3.33	32.04

The main problem for further study was how to eliminate as many non-specific bindings as possible. These unwanted bindings were the major contributions of the high backgrounds and they might be related to a lot of factors, such as the substrate stability and the choice of antibodies. In this study, the main reason of those abnormally high background noises was the incorrect use of the biotin conjugated monoclonal anti-Hsp70 without binding with avidin molecules. It has been known that the biotinylation of monoclonal antibodies would result in cross-reactivities of these monoclonal antibodies with other irrelevant peptides or proteins. However, it is

also proved that the specificity of the biotinylated monoclonal antibody will not be altered (Panyutich, A. V., *et al*., 1993). Thus, a system, which the biotin conjugated monoclonal anti-Hsp70 molecules bind to avidin molecules before it can react with antigen molecules, is required for solving this main problem. This is because each avidin molecule can bind four biotins with high affinity and selectivity, thus would help with blocking the unwanted activated binding sites. This binding is also very stable and requires very harsh conditions to break.

3.3 Sandwich ELISA with NAvidin-Biotin System

This Avidin-Biotin system is known to be more sensitive than general sandwich ELISA which uses normal monoclonal antibody to coat the plate surface. The Avidin used in our study is called NeutrAvidin (NAvidin).

Compared with the previous method, which has biotin monoclonal anti-Hsp70 molecules directly immobilised on the plate surface, this NAvidin-Biotin system requires a layer of NAvidin molecules on the plate surface before biotin molecules can be coated. This binding between the NAvidin and Biotin molecules occupied most of the unwanted active binding sites on biotin molecules, thus, would allow a more sensitive detection of antigen concentration. Also, this binding system requires much less biotin monoclonal anti-Hsp70 compared with our previous work.

3.3.1. *BCA Assay for determination of NAvidin concentration*

Before setting up the new Sandwich ELISA standards, the concentration of NAvidin solution needed to be determined. NAvidin was diluted 1:4 in PBS to make sure that its concentration would be within the detection limit of the assay. Two BCA assays were performed as described under Section 2.2.1 and standard curves were generated respectively according to their absorbance readings (**Figure 3.8 A** and **B**). The concentration of NAvidin of each assay was calculated using corresponded equations of its standard curve. The final concentration of NAvidin was determined as the average concentration of these two assays and it was used as the original concentration for further dilution.

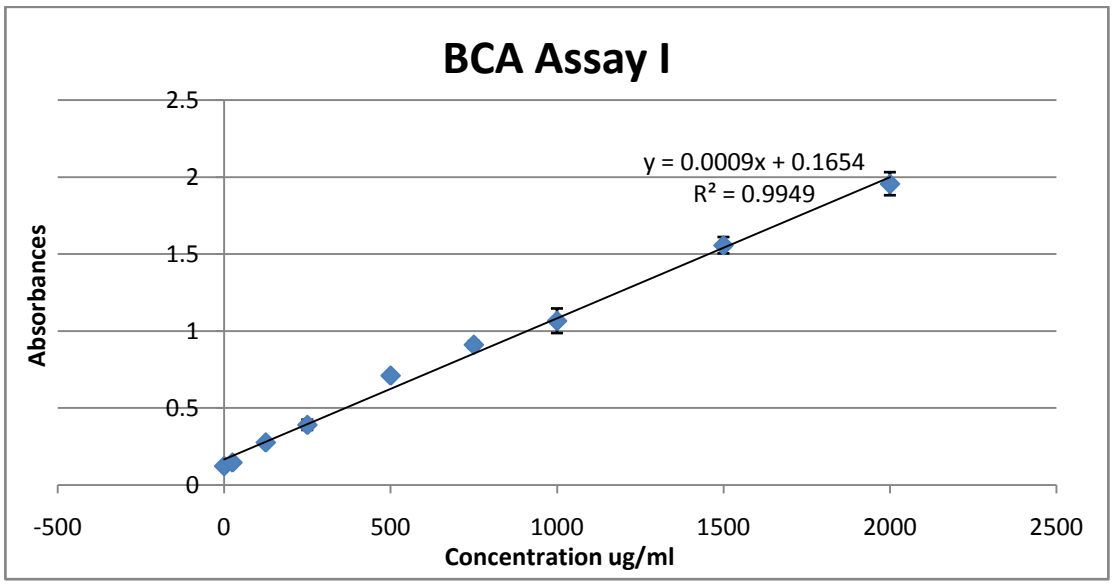

Figure 3.8 A The standard curve of first BCA assay

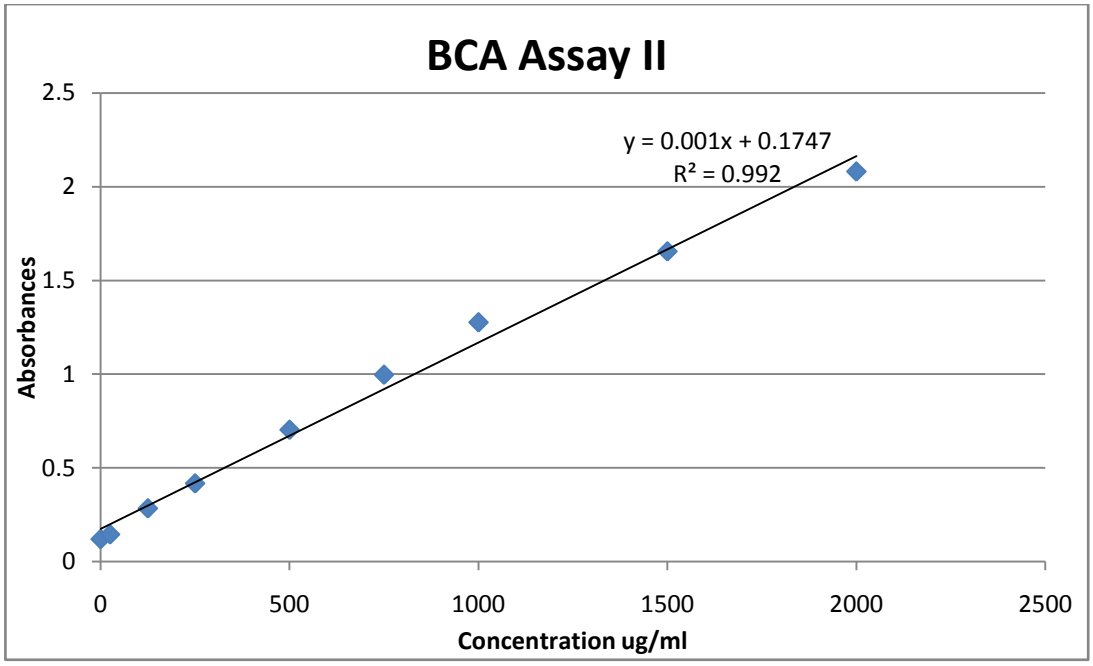

Figure 3.8 B The standard curve of second BCA assay.

3.3.2. *NAvidin Coating Conditions*

NeutrAvidin is a deglycosylated version of Avidin with a molecular weight of approximately 60,000 Daltons. Generally, native avidin has an isoelectric point (pI) of 10.5. However, the NeutrAvidin is much closer to neutrality than avidin and has a near-neutral pI of 6.3. Thus to ensure the NAvidin molecules would immobilised on the plate surface with the best binding capacity, two diluents, 0.05M Sodium Carbonate at pH 9.7 which is close to the pI of Avidin, and MilliQ water at pH 7.0 which is close to the pI of NeutrAvidin, were examined and compared respectively.

Two sets of wells were set up with 5 different Hsp70 concentrations: 0ng/ml, 2.5ng/ml, 25ng/ml, 250ng/ml and 1,000ng/ml. The first set of wells were coated by 5µg/ml of NAvidin diluted in 0.05M Sodium Carbonate at pH 9.7 and the other set was coated by 5µg/ml of NAvidin diluted in MilliQ water. The following experiment was performed using the same ELISA protocol described under Section **2.2.2.10.1**.

Standard curves were generated respectively with their equations (**Figure 3.9**). The background and the overall absorbance readings for the wells with NAvidin diluted in MilliQ water were much lower than those with NAvidin diluted in 0.05M Sodium Carbonate at pH 9.7, indicated better interaction between immobilised NAvidin and biotin monoclonal anti-Hsp70 molecules. Thus, for any further experiment, MilliQ water would be used as the diluents for NAvidin.

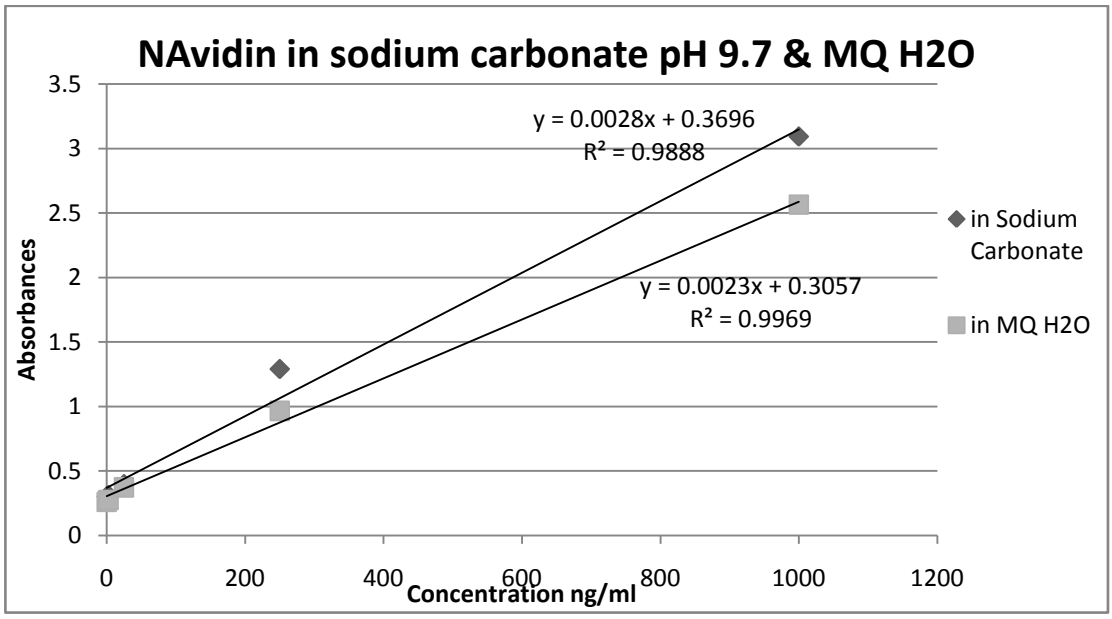

Figure 3.9 Comparison of different diluents for NAvidin.

3.3.3. *NAvidin-Biotin Sandwich ELISA 6 Standards*

Based on our previous method and optimisations, some changes were made for this NAvidin-Biotin system:

- Since the binding between NAvidin and Biotin monoclonal anti-Hsp70 molecules should increase the sensitivity of Sandwich ELISA assay, the concentration of biotin antibody used was reduced from 5µg/ml to 1µg/ml. To allow the NAvidin molecules bind to biotin molecules as many as possible, overnight incubation of biotin mAb70 at 4°C was applied.

- In 1984, Johnson, D. A., *et al*. reported that non-fat dry milk was a better blocking agent than BSA and it was showed a 5% non-fat dry milk solution acted as efficient as BSA to block non-specific bindings in ELISA processes (Johnson, D. A., *et al*., 1984). About twenty years later, Kaur, R. reported that compared with traditionally used blocking agent BSA, skim milk could block more non-specific bindings of the ELISA process by showing significantly lowered background absorbance readings (Kaur, R., *et al*., 2002). Thus, in this study, although the assay with NAvidin diluted in MilliQ water at pH 7 showed less background noises, a 5% skim milk was added to blocking buffer with 1% BSA, in order to get rid of as many non-specific bindings in the

process as possible. At the mean time, the blocking time was increase to overnight, to ensure the most effective blocking.

- Each measuring instrument is thought to be accurate in a limited range. For absorbance reading of a sample, which is proportional to the thickness of the sample and the concentration of the absorbing substance in the sample, the theoretical best accuracy of most instruments is in the range from 0 to 1, with the highest possibility of 2. Within the detectable range, the results will follow the Beers-Lambert Law to be linear shaped. Once over the detection limit, it will become non-linear and the readings are less accurate. In this study, since the substrate OPD used is very sensitive, the appropriate incubation time of the substrate is crucial for reducing the non-specific binding occurred in the process. Before setting up the new standards, wells with substrate reacting with enzyme were simply checked in a five-minute interval for any visual ascending colour change which would change from colourless to light yellow and/ or orange. This substrate incubation time was finally determined to be 5 minutes.

By using the improved NAvidin-Biotin protocol described under Section **2.2.2.10.2.**, 6 standards were set up at 8 different antigen concentrations ranging from 0-2,000ng/ml, which were also five-fold serial diluted. Each standard curve was generated after each assay (**Figure 3.10**), sensitivity and inter-assay CVs were also calculated (**Table 3.2**). After finishing all 6 standards, the intra-assays CVs were calculated (**Figure 3.11**).

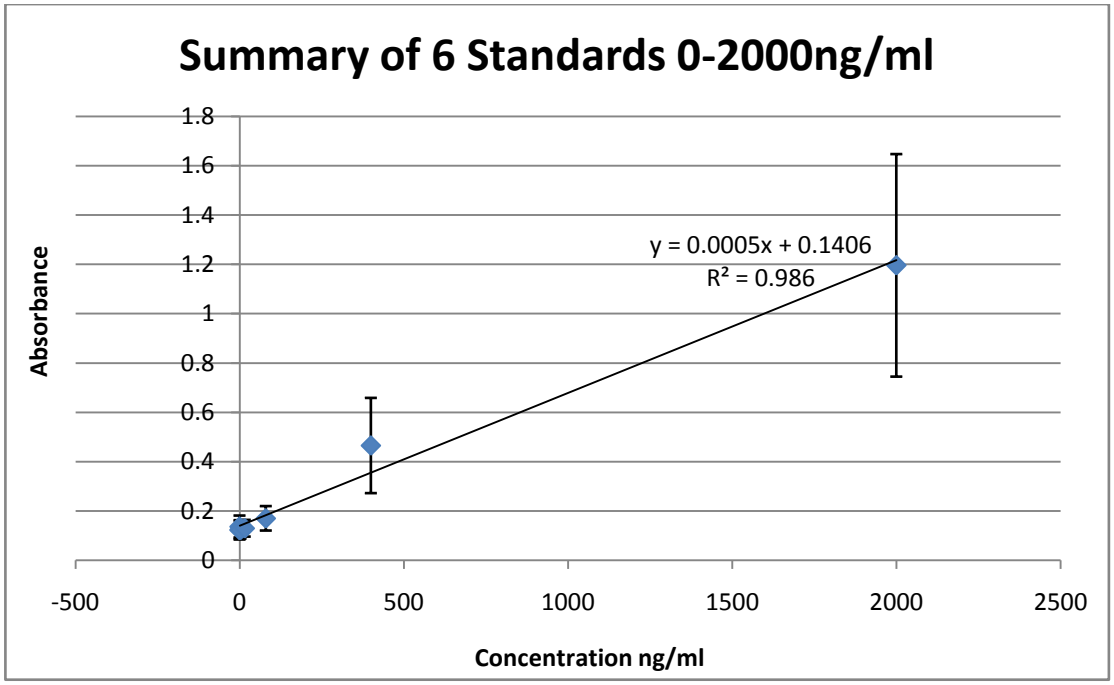

Figure 3.10 The standard curve of 6 NAvidin-Biotin Sandwich ELISA standards

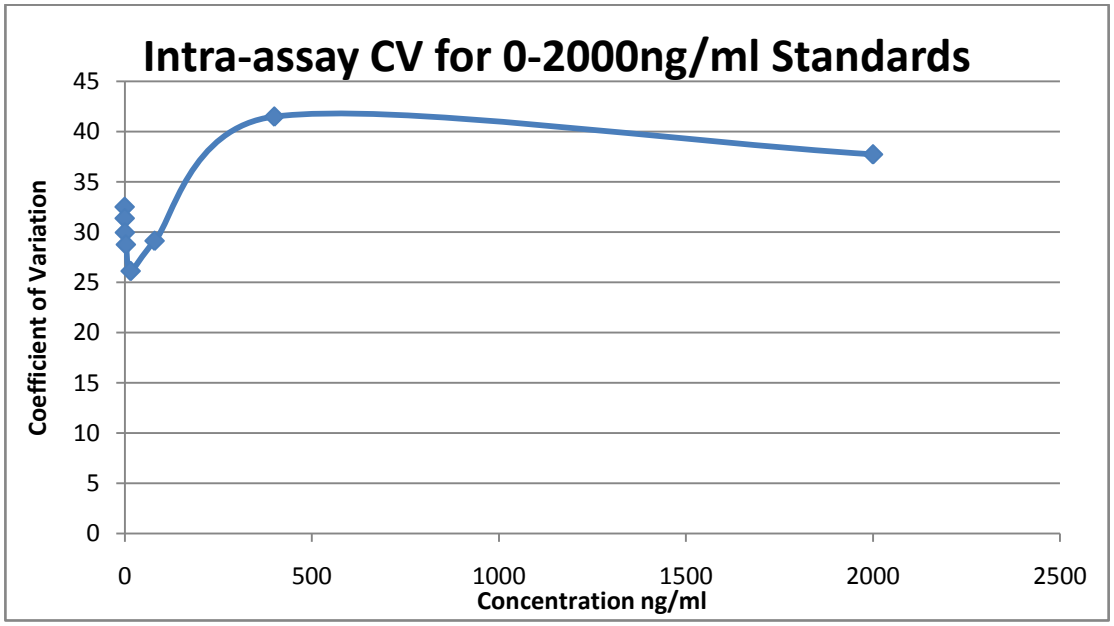

Figure 3.11 The Intra-Assays CVs of 6 NAvidin-Biotin Sandwich ELISA standards

Table 3.2 Comparison of the inter-assay CVs and the intra-assays CVs of 6 NAvidin-Biotin Sandwich ELISA Standards

Hsp70 Concentration (ng/ml)	Inter-Assay CVs (%)						Intra-Assays CVs (%)
	Std I	Std II	Std III	Std IV	Std V	Std VI	
0	4.36	10.75	7.20	4.03	18.63	3.65	32.50
0.128	5.79	3.94	8.62	7.81	11.22	3.87	31.37
0.64	5.72	6.83	8.43	8.19	7.07	4.20	29.95
3.2	7.36	10.05	7.48	6.08	6.34	1.96	28.75
16	5.83	10.80	7.37	6.60	7.74	2.74	26.12
80	0.92	3.83	5.16	9.59	14.75	9.46	29.12
400	2.22	5.00	6.23	9.90	7.56	3.18	41.48
2000	8.14	2.42	19.16	3.34	2.08	2.82	37.72

The sensitivity of NAvidin-Biotin system was significantly improved compared with our previous work as expected (the lowest of 2ng/ml VS the lowest of 32ng/ml). This was even more sensitive than Njemini's published data (2ng/ml VS 4ng/ml). However, the sensitivities of all 6 standards varied in a wide range from 2ng/ml to 129ng/ml. This indicated that the Sandwich ELISA with an Avidin-Biotin system can be much more sensitive than those normal Sandwich ELISAs, but to get consistent results, experimental conditions needed to be further optimised.

Most of the inter-assay CVs for each standard was below 10% but the intra-assays CVs for all standards were very high, with an average of 31% (**Table 3.2**), indicated there were large differences between each assay.

3.4 Indirect ELISA with antigen concentrations 0-2,000ng/ml

In order to compare the sensitivities of different ELISA formats for the detection of Hsp70 concentration, 5 Indirect ELISA standards were set up with 10 different Hsp70 concentrations, 0ng/ml, 1ng/ml, 5ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, 250ng/ml, 500ng/ml, 1,000ng/ml and 2,000ng/ml. Unlike Sandwich ELISA or Sandwich ELISA with an Avidin-Biotin system, these different concentrations of antigen were diluted in 0.1M Carbonate buffer at pH 9.6 and then directly immobilised on the plate surface, followed by binding with only one antibody, the polyclonal anti-Hsp70 antibody in our study. Instead of using 0.2% BSA as the blocking agent, 5% of Goat Serum was added into PBS/ 0.1% Tween 20 because the enzyme used, the IgG Peroxidase, was produced from goat, thus, PBS/ 0.1% Tween 20/ 5% Goat Serum would be more specific for eliminating the non-specific bindings.

The sensitivity of each standard was calculated by using the standard curve and equation generated from the absorbance readings (**Figure 3.12**). These 6 sensitivities were varied in a wide range as the same as NAvidin-Biotin Sandwich ELISA

standards, with 10.74ng/ml the lowest and 106ng/ml the highest. As expected, this Indirect ELISA was neither as sensitive as NAvidin-Biotin Sandwich ELISAs (10ng/ml VS 2ng/ml), nor as sensitive as the published data (10ng/ml VS 4ng/ml). However, it was more sensitive compared with our first Sandwich ELISA standards, which incorrectly used biotin monoclonal anti-Hsp70 for direct immobilisation on the plate surface (10ng/ml VS 34ng/ml).

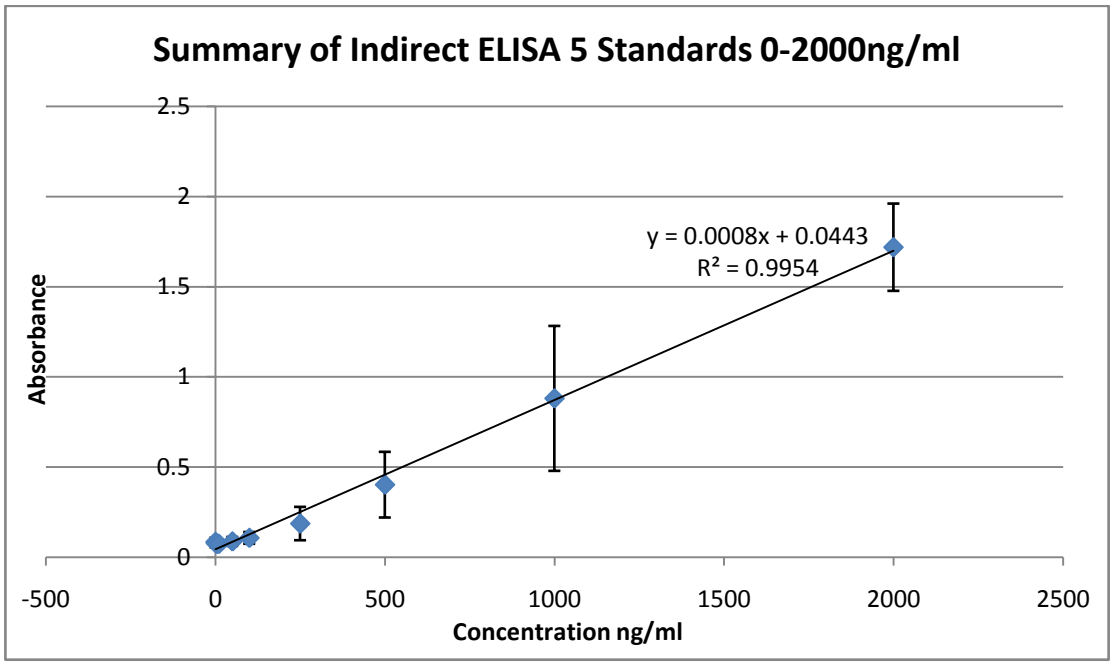

Figure 3.12 The standard curve of 5 Indirect ELISA standards

As the same as previous results, most of the inter-assay CVs were below 10% (**Table 3.3**), with much higher intra-assays CVs, an average of 31% (**Figure 3.13**).

Table 3.3 Comparison of the inter-assay CVs and the intra-assays CVs of 5 Indirect ELISA Standards

Hsp70 concentration (ng/ml)	Inter-Assay CVs (%)					Intra-Assay CVs (%)
	Std I	Std II	Std III	Std IV	Std V	
0	6.96	8.62	10.48	2.32	8.54	26.26
1	11.08	9.32	7.07	8.32	31.70	30.14
5	1.59	12.12	2.77	5.55	11.46	24.06
10	3.70	2.73	12.53	1.22	14.01	22.34
50	8.24	8.17	2.45	4.71	15.96	28.54
100	2.42	3.52	6.22	15.51	14.69	30.72
250	18.21	15.15	6.19	8.39	5.66	49.83
500	8.38	12.68	18.54	2.85	5.25	45.38
1000	7.34	6.18	8.75	12.31	20.75	45.67
2000	24.46	7.31	13.87	0.14	10.38	14.08

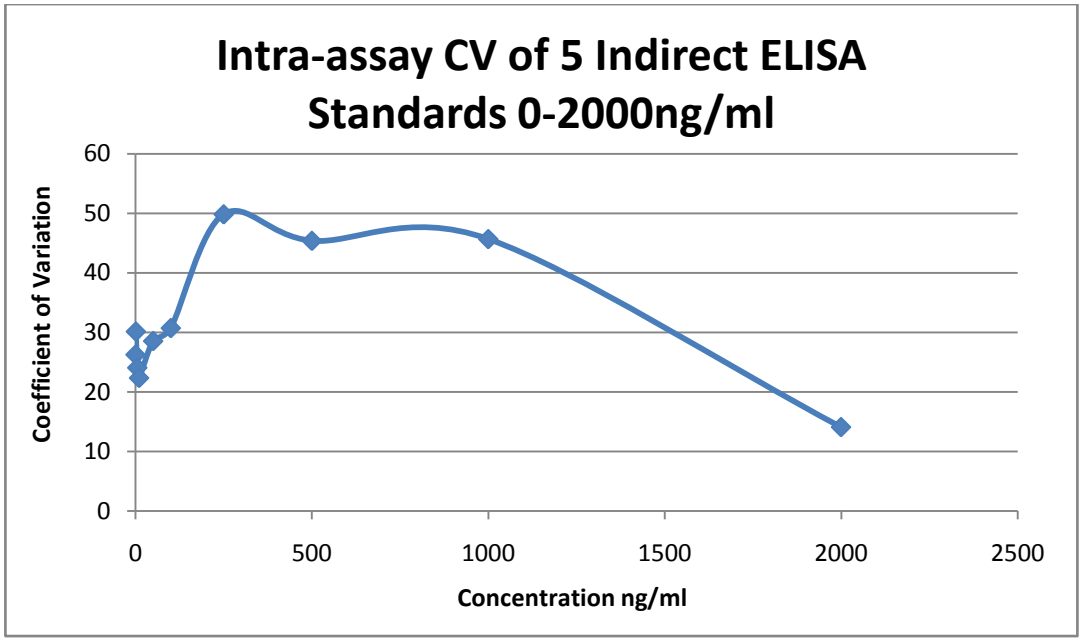

Figure 3.13 The intra-assays CVs of 5 Indirect ELISA standards

3.5 Summary of ELISAs

Among all of these ELISA standards, the NAvidin-Biotin Sandwich ELISA, which is not commonly used, was proved to be the most sensitive format of ELISA for the detection of Hsp70 concentrations. Since the biotin monoclonal anti-Hsp70 was incorrectly used to coat the plate surface, this Sandwich ELISA format was the least sensitive one in these 3 types of ELISAs (**Table 3.4**). But compared the average sensitivity of NAvidin-Biotin Sandwich ELISA with the average sensitivities of other two ELISA formats; it was not as sensitive as Sandwich ELISA. This was due to the wide range of sensitivities of 6 standards, which was also indicated by high intra-assays CV values. However, the experimental protocol of NAvidin-Biotin Sandwich ELISA still needs to be further optimised in order to obtain more consistent results.

Table 3.4 Summary of the sensitivities of different ELISA types

ELISA Types	**Sensitivities (ng/ml)**						**Average Sensitivity (ng/ml)**
	Std I	**Std II**	**Std III**	**Std IV**	**Std V**	**Std VI**	
Sandwich ELISA	52.53	32.02	34.24				39.60
NAvidin-Biotin Sandwich ELISA	32.46	129.0	77.38	39.64	63.54	2.23	57.38
Indirect ELISA	10.74	34.94	97.46	79.47	105.7		65.65
Published Data							4.00

Chapter Four
BIAcore SPR Results

4.1 Introduction

The SPR technology which permits the biomolecular interaction analysis (BIAcore-SPR) provides all kinds of binding information between molecules, such as reaction kinetics, binding affinity constants and the active concentration of biomolecules in a solution, in real-time without the need of labeling the analytes. It is now becoming a more and more popular immunological tool in both research and commercial laboratories.

Generally, to perform a BIAcore-SPR assay, one interaction partner (the ligand) is immobilized on the surface of a sensor chip, and the other interaction molecule (the analyte) is continuously running through the surface at a constant flow rate. This allows the analyte molecules to bind to the ligand molecules as many as possible. The accumulation of the ligand-analyte complex formed on the surface of the sensor chip will result in the increased refractive index that can be measured by the detection unit and then presented in the form of a sensorgram.

Two different types of SPR immunoassays were performed: the Sandwich SPR immunoassay and the Competitive SPR immunoassay. There were two types of sensor chips used for the Sandwich BIAcore-SPR, the most commonly used CM 5 sensor chip and the mixed self-assembled monolayer (mSAM) sensor chip, which can be prepared by mixing two differently terminated thiols in the preparation solutions, 10 mM 11-Mercapto-1-Undecanol (11-MOH) and 10 mM 16- Mercaptohexa-decanoic acid (16-MUA) in this study.

4.2 Sandwich SPR immunoassay with CM 5 Sensor Chip

Flow Cell 2 (FC2) on the CM 5 Sensor Chip surface was used to immobilize biotin monoclonal anti-Hsp70 while the first Flow Cell (FC1) was immobilized by rat anti-progestrone to act as a control cell. After activating the sensor chip surface, 50µg/ml biotin monoclonal anti-Hsp70 diluted in Sodium Acetate at pH 4.5 was immobilized on the surface, followed by the deactivation of the sensor chip surface. Before running rHsp70 through the deactivated surface, the immobilized biotin monoclonal anti-Hsp70 was blocked by the injection of 100µl of HBS/ 1% BSA. After this, 8 different concentrations of rHsp70 were injected in duplicate followed by 30 seconds of regeneration.

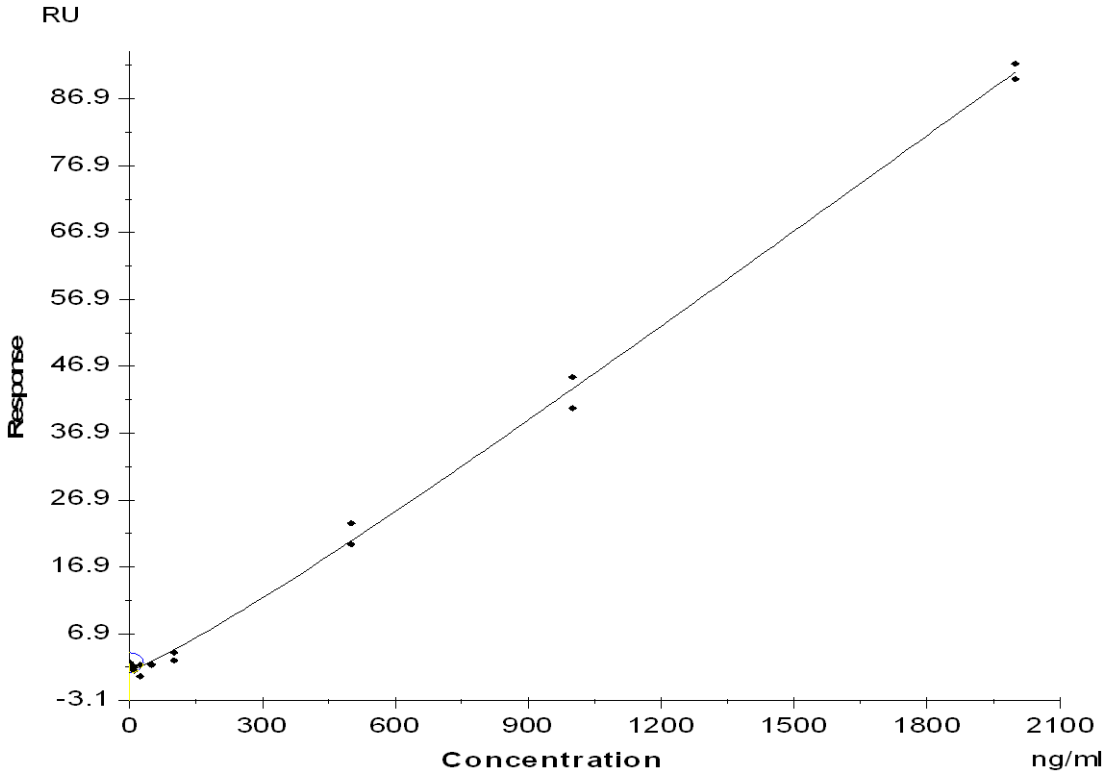

Figure 4.1 Hsp70 Binding Curve (0-2000ng/ml)

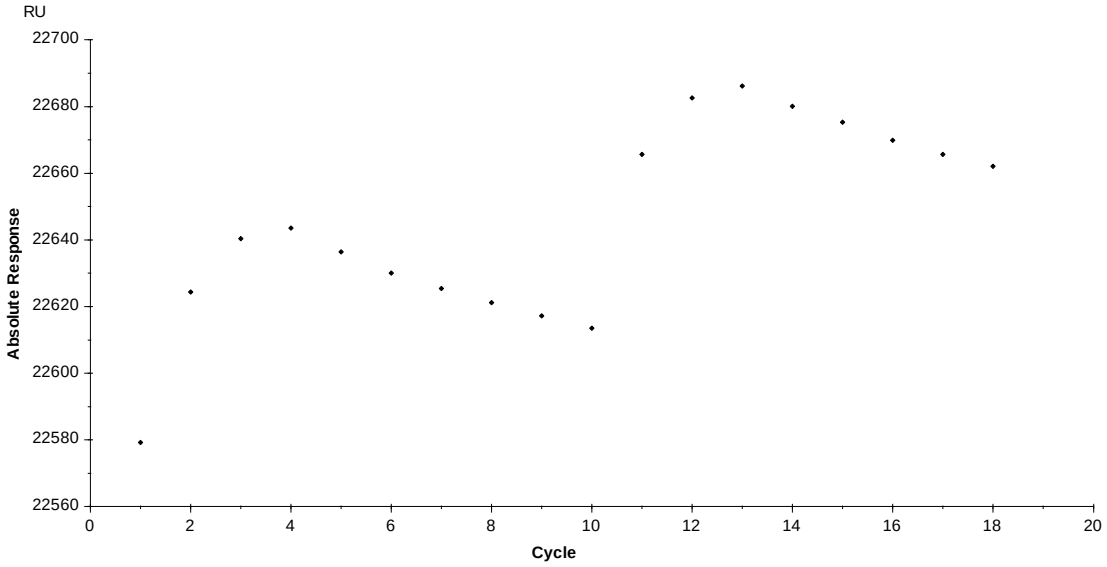

Figure 4.2 The Trend Plot of Hsp70 binding cycles.

The standard curve was generated based on these data (**Figure 4.1**). The bindings between immobilized biotin monoclonal anti-Hsp70 and Hsp70 at concentrations below 500ng/ml could hardly be detected. This might due to the damage of sensor chip surface for binding during regeneration process which disrupted the binding activity of immobilized biotin monoclonal anti-Hsp70. Both the standard deviations and the Coefficient of Variations (CV) were calculated for each concentration. The CVs varied in a wide range, from 2% to highest of 83%. This indicated that the assay was not well repeated. This was also shown by the Trend Plot of each binding cycle (from the lowest Hsp70 concentration to the highest) (**Figure 4.2**). The absolute responses of the duplicate of each concentration were varied in a wide range. Thus, the regeneration conditions, such as the choice of the regeneration buffer, as well as the pH of the regeneration buffer, need to be further optimized before any following steps can be carried on. In this study, this optimization was not studied and instead, another type of SPR was performed and compared.

4.3 Competitive SPR immunoassay with CM 5 Sensor Chip

The working theory of Competitive SPR was the same as the Competitive ELISA. After activation of Flow Cell 3 (FC3) of the CM 5 sensor chip surface, 50µl of 80µg/ml biotin monoclonal anti-Hsp70 was immobilized followed by deactivation and regeneration of the sensor surface. A mixture of biotin monoclonal anti-Hsp70

and Hsp70 at 8 different concentrations (0-1000ng/ml) was incubated before injected to FC3. After each injection, the sensor surface was washed by glycine buffer at pH2.0 for 30 seconds.

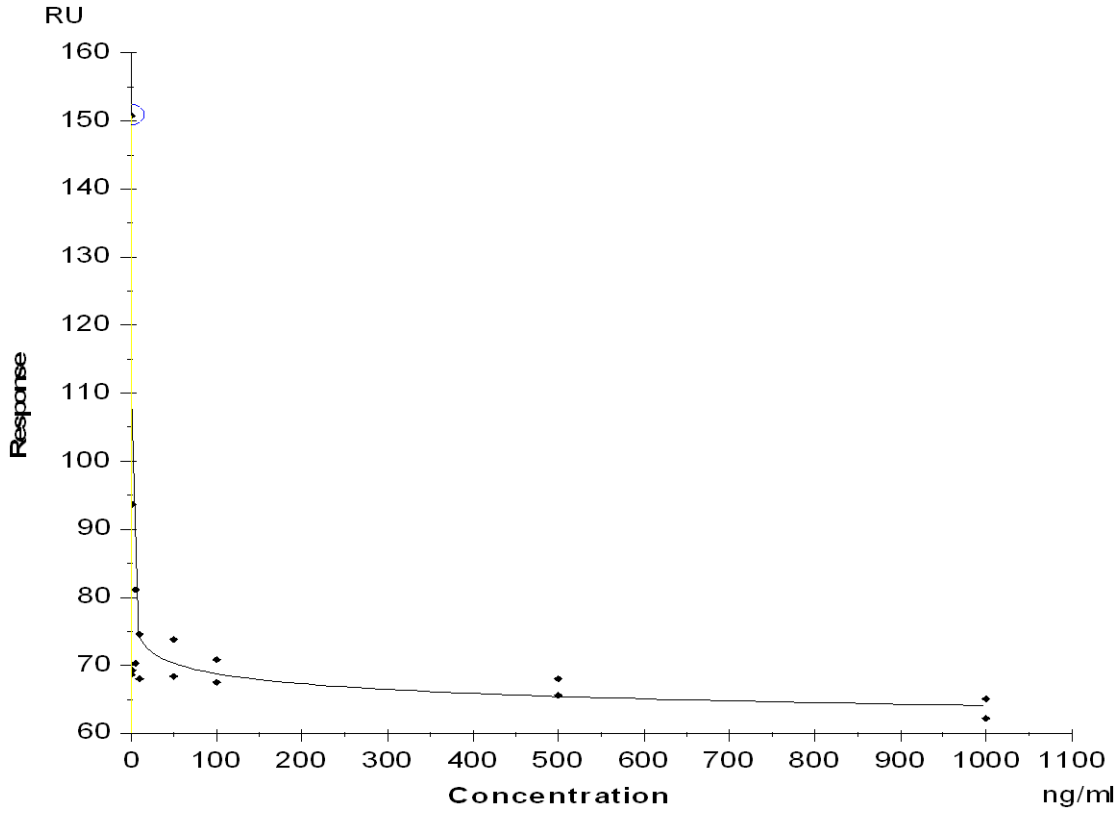

Figure 4.3 The Competitive standard binding curve for Hsp70

The standard binding curve was generated (**Figure 4.3**). Results showed that between each duplicate of the same concentration, the differences of Response change were much bigger than expected. At some Hsp70 concentrations, such as 1ng/ml, 5ng/ml, 10ng/ml and 50ng/ml, the CV values were even over 100%. All of these indicated

that the regeneration buffer might cause the binding sites on the sensor surface to be destroyed. Thus, as the same as Sandwich SPR immunoassay described above, this format also needs to be optimized before further actions to be taken.

4.4 Sandwich SPR immunoassay with mSAM Sensor Chip

The surface of this kind of sensor chip was not as stable as CM 5 sensor chip and can be easily oxidized. Thus, after incubating the gold film in the mixture of two different sources of thiols, this film needed to be 100% dried by N_2 to avoid any possible oxidations.

Since the conjugation of biotin molecules to monoclonal anti-Hsp70 will provide much more active binding sites, as the same as Sandwich ELISA assay, a layer of NAvidin molecules was coated on the sensor surface instead of directly coating of biotin monoclonal anti-Hsp70. After deactivation, the sensor surface was washed followed by the injection of biotin monoclonal anti-Hsp70 diluted in HBS. The last step involved was the regeneration of sensor surface by glycine buffer at pH2.0.

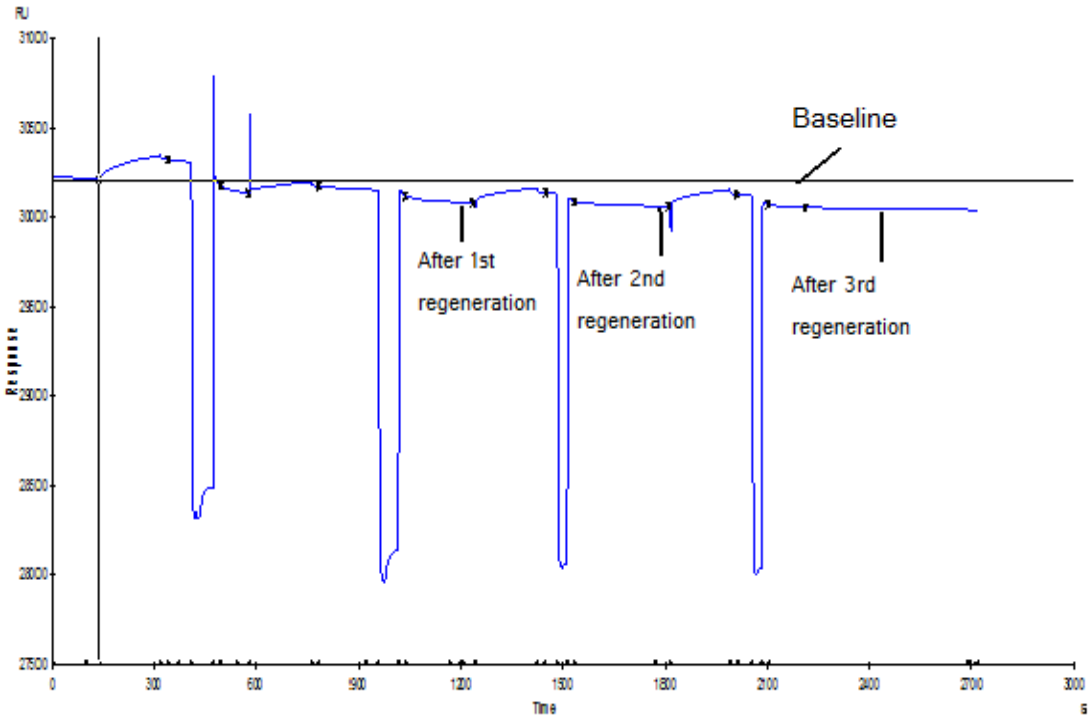

Figure 4.4 Sandwich SPR with mSAM sensor chip

The results obtained from this trial (**Figure 4.4**) indicated that the regeneration of the sensor surface was not successful since the baseline Response reading could not be reached after each regeneration cycle. The same as previous SPR work, before any subsequent experimental step can be carried on, the regeneration conditions needed to be well studied.

Chapter 5
Final Discussion and Future Studies

5.1 Final Discussion

This study compared the assay sensitivities of two different techniques that are now mainly used in immunology in both commercial and research laboratories. The most popular Enzyme-Linked ImmunoSorbent Assay (ELISA) and Surface Plasmon Resonance (SPR) attached with a biosensor chip, which is recently proved to have many advantages over ELISA. In order to compare the assay sensitivities of different types of ELISA, two ELISA formats were performed, the Sandwich ELISA and the Indirect ELISA, to find out a more sensitive and rapid method that can be used for the detection of Heat Shock Proteins in complex matrixes.

The first standard was set up based on the protocol published by Njemini, R., and co-workers in 2003. However, this trial was much less sensitive than the published data, even though the same experimental process was performed. Beside the sensitivity, the result also indicated an abnormally high level of background noises, which is caused by the unwanted non-specific bindings occurring during the experimental process and is believed to be the major contribution of low sensitivity of the assay. Based on this,

an attempt was made to improve the assay by optimising the blocking and substrate conditions respectively.

The standards set up using the modified experimental protocol showed a significantly improved sensitivity of assay but was not as sensitive as the published method. This was subsequently found to due to the incorrect use of biotin monoclonal anti-Hsp70 antibody during the immobilization step. A lot of work done by other researchers has shown that ELISA assays using a monoclonal antibody with biotin molecules conjugated to it will dramatically improved the sensitivity of the assay, when this biotin conjugated monoclonal antibody is not immobilized directly on the plate surface, but on a layer of Avidin molecules. Thus, the experimental method was further improved by coating Avidin molecules on the plate surface before any biotin monoclonal antibody can bind to it. Several working conditions, such as the diluents and the concentration of Avidin used, were optimized before any new standards set up.

The trials using Avidin-Biotin system combined with Sandwich ELISA showed high level of sensitivity of the assay. Compared with the published work, which has a sensitivity of 4ng/ml, the best sensitivity of this study can be up to 2ng/ml. This proved that by using biotin conjugated monoclonal antibody, the sensitivity of the ELISA assay can be significantly improved. However, the working conditions, such as incubation time and temperature, need to be further determined because among the

six standards in this study, sensitivities varied in a wide range, with the best sensitivity of 2ng/ml and the worst of 129ng/ml. At the mean time, to compare the sensitivities of different types of ELISA assays, five standards for Indirect ELISA format were set up. The results obtained from these standards proved the theory that Indirect ELISA is less sensitive than Sandwich ELISA. However, the results of all these three types of ELISA assays showed good Inter-assay Coefficient of Variations (CVs) (1%-10%) while the Intra-assay CVs were at least three times higher, indicating very poor reproducibility of each ELISA type assay.

Two types of biosensor chips were used in this study, the CM5 sensor chip and the mSAM sensor chip. By using these chips, two formats of SPR assay, the competitive SPR and the Sandwich SPR were investigated. The binding curves obtained from these SPR assays indicated the regeneration step of the sensor chip surface after each binding needs to be further studied to find out an appropriate regeneration buffer that will not destroy the surface of the sensor chip. The binding conditions and the concentrations of each reactant, the antibody and the antigen, also need to be optimised. Since no standards were fully set up for these assays, the sensitivities of two different immunological methods, the ELISA and the BIAcore-SPR, cannot be directly compared.

5.2 Future Studies

Although this study showed the Avidin-Biotin system together with normal Sandwich ELISA has many advantages over normal Sandwich ELISA, such as it is much more sensitive and requires much less of monoclonal antibody for the assay, this combination has a number of limitations.

Compared with the work done by Njemini, R., using the Avidin-Biotin Sandwich ELISA system requires much more time for incubation of each binding, thus, the time needed for the whole experimental process is much longer than general Sandwich ELISA assays (4 days Versus 2 days). On the other hand, the conjugation of biotin molecules to monoclonal antibody molecules provides more active binding sites, which will increase the possibilities for non-specific bindings. This is probably the reason that not many studies have been done for now on detecting the concentration of Hsp70 by using this Avidin-Biotin Sandwich ELISA system. Based on all of these, to set up a better experimental protocol which will be less time-consuming, well reproduced and relatively easy, a lot of reaction conditions need to be studied and compared, such as the choice of Avidin, the binding conditions of Avidin to Biotin monoclonal antibody molecules, the blocking conditions, as well as the substrate conditions.

SPR with a biosensor chip attached has many advantages over any types of ELISA. It can be much more sensitive, and requires less amount of analyte for the process. It is also rapid, and compared with ELISA assays, most of the work can be done automatically; thus, will not require well trained personnel to manually perform each step. When a large number of samples are tested, SPR would be a much cheaper choice rather than Sandwich ELISA. However, the working conditions of SPR method are crucial for obtaining the very accurate and sensitive results.

Over the last many years, SPR has been widely used for the studies of immunology, chaperones, molecular biology, signal transduction, cell adhesion and low-affinity bindings, and screening for new ligands (Szabo, A., *et al*., 1995). However, not much work has been done on the qualitation of heat shock proteins, especially heat shock protein 70 (Hsp70). Thus, the experimental protocol set up in this study was followed the work done on the concentration determination of Hsp60 (Yuan, J., unpublished data). But research have been done on the conformational changes and binding affinity of Hsp70 molecular chaperone, DnaK, with its co-chaperone DnaJ (Suh, W. C., *et al*., 1998) (Mayer, M. P., *et al*., 1999). The interaction between Hsp90 and HSJ1b, which is the human DnaJ homologue, has also been studied (Schnaider, T., *et al*., 2000).

Different sensor chips were used for these studies (CM 5 Sensor Chip and Streptavidin-coated Sensor Chip). Other parameters are also different compare these

studies, for example, the experimental temperatures were different (25ºC VS 30ºC), the flow rates used were 4µl/minute, 20µl/minute and 5µ/minute, respectively. Two types of regeneration cycles were mentioned, with 10 ml of 1 M urea at a flow rate of 5µl/minute, and with two or three times of 2 minutes injection of 20mM HCl at a flow rate of 5µl/minute. Thus, in order to set up an appropriate experimental protocol for the detection of the concentrations of Hsp70 in complex samples, a lot of working conditions need to be well studied and optimized. Such as the experimental temperature, the flow rates for activation, deactivation, assay injection and regeneration. Other parameters, like the concentration of NAvidin used to pre-coat the sensor chip surface, the concentration of Biotin monoclonal anti-Hsp70 used as primary antibody to be bound by the NAvidin molecules, the choice of regeneration buffer, the time of regeneration cycle and the concentrations of subsequent antigen and polyclonal antibody, as well as the presence of enhancement, are plausible at this stage, and need to be examined before any standards can be set up.

References:

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G., and Rubartelli, A., (1999). The secretory route of the leaderless protein interleukin 1 beta involves exocytosis of endolysosome-related vesicles. *Molecular Biology of the Cell*, **10**, 1463-1475.

Andrei, C., Margiocco, P., Poggi, A., Lotti, L. V., Torrisi, M. R., and Rubartelli, A., (2004). Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: implications for inflammatory processes. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 9745-9750.

Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Koo, G. C., and Calderwood, S. K., (2000). Hsp70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature Medicine*, **6**, 435-442.

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Baré, O., Auron, P. E., Stevenson, M. A., and Calderwood, S. K., (2002). Novel signal transduction pathway utilized by extracellular Hsp70. *Journal of Biological Chemistry*, **277**, 15028-15034.

Bardwell, J. C. A., and Craig, E. A., (1984). Major heat shock gene of *Drosophila* and the *Escherichia Coli* heat-shock inducible DnaK gene are homologous. *Proceedings of the National Academy of Sciences of the United States of America*, **81**, 848-852.

Berson, S. A., and Yalow, R. S., (1960). Immunoassay of endogenous plasma insulin in man. *Journal of Clinical Investigation*, **39**, 1157-1175.

Biacore, (2009). Retrieved from http://www.biacore.com.

Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., and Hivroz, C., (2002). TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/{zeta} complex. *The Journal of Immunology*, **168**, 3235-3241.

Bukau, B., Deuerling, E., Pfund, C., and Craig, E. A., (2000). Getting newly synthesized proteins into shape. *Cell*. **101**, 119-122.

Bukau, B., and Horwich, A. L., (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, **92**, 351-366.

Calderwood, S. K., Mambula, S. S., Gray, P. J., and Theriault, J. R., (2007). Extracellular heat shock proteins in cell signaling. *FEBS Letters*, **581**, 3689-3694.

Canziani, G., Zhang, W., Cines, D., Rux, A., Willis, S., Cohen, G., Eisenbrg, R., Chaiken, I., (1999). Exploring biomolecular recognition using optical biosensors. *Methods*, **19**, 253-269.

Caplan, A. J., Cyr, D. M., and Douglas, M. G., (1993). Eukaryotic homologues of *Escherichia Coli* DnaJ: a diverse protein family that functions with Hsp70 stress proteins. *Molecular Biology of The Cell*, **4**, 555-563.

Chen, W., Syldath, U., Bellmann, K., Burkart, V., and Kolb, H., (1999). Human 60kDa heat shock protein: a danger signal to the innate immune system. *Journal of Immonology*, **162**, 3212-3219.

Chiti, F., Webster, P., Tadde, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C. M., (1999). Designing conditions for *in vitro* formation of amyloid protofilaments and fibrils. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 3590-3594.

Clayton, A., Turkes, A., Navabi, H., Mason, M. D., and Tabi, Z., (2005). Induction of heat shock proteins in B-cell exosomes. *Journal of Cell Science*, **118**, 3631-3638.

Cole, S. P., Campling, B. G., Atlaw, T., Kozbor, D., and Roder, J. C., (1984). Human monoclonal antibodies. *Molecular and Cellular Biochemistry*, **62**, 109-120.

Delang, R. J., and Huang, T. S., (1971). Egg White Avidin III: Sequence of the 78-residue middle cyanogens bromide peptide. Complete amino acid sequence of the protein subunit. *The Journal of Biological Chemistry*, **246**, 698-709.

Dobson, C. M., (1999). Protein misfolding, evolution and diseases. *Trends in Biochemical Sciences*, **24**, 329-332.

Dobson, C. M., and Karplus, M., (1999). The fundamental of protein folding: bringing together theory and experiment. *Current Opinion in Structural Biology*, **9**, 92-101.

Ellis, R. J., and Hammingsen, S. M., (1989). Molecular Chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends In Biochemical Sciences*, **14**, 339-342.

Ellis, R. J., and van der Vies, S. M., (1991). Molecular Chaperones. *Annual Review of Biochemistry*, **60**, 321-347.

Evdonin, A. L., Guzhova, I. V., Margulis, B. A., and Medvedeva, N. D., (2004). Phospholipase c inhibitor, u73122, stimulates release of Hsp70 stress protein from A431 human carcinoma cells. *Cancer Cell International*, **4**:2.

Frederix, F., Bonroy, K., Laureyn, W., Reekmans, G., Campitelli, A., Dehaen, W., Maes, G., (2003). Enhanced performance of an affinity biosensor interface based on mixed self-assembled monolayers of thiols on gold. *Langmuir*, **19**, 4351–4357.

Galdiero, M., Delero, G. C., and Marcatili, G. A., (1997). Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. *Infection and Immunity*, **65**, 699-707.

Gao, B., and Tsan, M. F., (2003). Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor α release by murine macrophages. *Journal of Biological Chemsitry*, **278**, 174-179.

Gehrmann, M., Radons, J., Molls, M., and Multhoff, G., (2008). The therapeutic implications of clinically applied modifiers of heat shock protein 70 (Hsp70) expression by tumor cells. *Cell Stress and Chaperones*, **13**, 1-10.

Gething M. J., and Sambrook, J., (1992). Protein folding in the cell. *Nature*, **355**, 33-45.

Green, N., (1963). The use of ^{14}C biotin for kinetic studies and for assay. *Biochemical Journal*, **89**, 585-591.

Green, N., (1975). Avidin. *Advances in Protein Chemistry*, **29**, 85-133.

Green, N., and Toms, E. J., (1973). The properties of subunits of Avidin coupled to sepharose. *Biochemical Journal*, **133**, 687-700.

Gupta, R. S., and Singh, B., (1992). Cloning of the Hsp70 gene from *Halobacterium-Marismortui*: Relatedness of archae-bacterial Hsp70 to its eubacterial homologs and a model for the evolution of HSP70 gene. *Journal of Bacteriology*, **174**, 4594-4605.

Guzhova, I., Kislyakova, O., Moskaliova, O., Fridlandskaya, I., Tytell, M., Cheetham, M., and Margulis, B., (2001). *In vitro* studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. *Brain Research*, **914**, 66-73.

Hartl, F. U., Martin, J., and Neupert, W., (1992). Protein folding in the cell: The role of molecular chaperones Hsp70 and Hsp60. *Annual Review of Biophysics and Biomolecular Structure*, **21**, 293-322.

Heeg, J., Schubert, U., Küchenmeister, F., (1999). Mixed self-assembled monolayers of terminally functionalized thiols at gold surfaces characterized by angle resolved X-ray photoelectron spectroscopy (ARXPS) studies. *Analytical and Bioanalytical Chemistry*, **365**, 272-276.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., and Dennis, D. T., (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature*, **333**, 330-334.

Hightower, L. E., and Guidon, P. T., (1989). Selective release from cultured mammalian cells of heat shock (stress) proteins that resemble gliaaxon transfer proteins. *Journal of Cellular Physiology*, **138**, 257-266.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H., (1984). Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Analysis Techniques*, **1**, 3-8.

Johnsson, B., Löfås, S., and Lindquist, G., (1991). Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Analytical Biochemistry*, **198**, 268-277.

Kakabakos, S. E., and Khosravi, M. J., (1992). Direct time-resolved fluorescence immunoassay of progesterone in serum involving the biotin-streptavidin system and the immobilized antibody approach. *Clinical Chemistry*, **38**, 725-730.

Karlsson, R., and Falt, A., (1997). Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *Journal of Immunological Methods*, **200**, 121-133.

Kaur, R., Dikshit, K. L., and Raje, M., (2002). Optimization of immunogold labeling TEM: An ELISA-based method for evaluation of blocking agents for quantitative detection of antigen. *The Journal of Histochemistry and Cytochemistry*, **50**, 863-873.

Keller, S., Sanderson, M. P., Stoeck, A., and Altevogt P., (2006). Exosomes: from biogenesis and secretion to biological function. *Immunology Letters*, **107**, 102-108.

Kiang, J. G., and Tsokos, G. C., (1998). Heat Shock Protein 70kDa: Molecular Biology, Biochemistry, and Physiology. *Pharmacology & Therapeutics*, **80**, 183-201.

Kol, A., Lichtman, A. H., Finberg, R. W., Libby, P., and Kurt-Jones, E. A., (2000). Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for Hsp60 activation of mononuclear cells. *The Journal of Immunology*, **164**, 13-17.

Korpela, J., (1984). Avidin, a high affinity biotin-binding protein as a tool and subject of biological research. *Medical Biology*, **62**, 5-26.

KPL, (2006). Technical Guide for ELISA. Retrieved from http://www.kpl.com.

Kresge, N., Simoni, R. D., and Hill, R. L., (2004). The discovery of avidin by Esmond E. Snell. *The Journal of Biological Chemistry*, **279**, e5.

Lancaster, G. I., and Febbraio, M. A., (2005). Exosome-dependent trafficking of Hsp70. *The Journal of Biological Chemistry*, **280**, 23349-23355.

Laskey, R. A., Honda, B. M., Mills, A. D., and Finch, J. T., (1978). Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature*, **275**, 416-420.

Launer, H. F., and Fraenkel-Conrat, H., (1951). The Avidin-Biotin Equilibrium. *The Journal of Biological Chemistry*, **193**, 125-132.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M., (1991). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 2874-2878.

Lindquist, S., and Craig, E. A., (1988). The Heat Shock Proteins. *Annual Review of Genetics*, **22**, 631-677.

Löfås, S., and Johnsson, B., (1990). A novel hydrogel matrix on gold surfaces in surface Plasmon resonance sensors for fast and efficient covalent immobilization of ligands. *Journal of the Chemical Society, Chemical Communications*, **21**, 1526-1528.

Lorimer, G. H., (1992). Role of accessory proteins in protein folding. *Current Opinion in Structural Biology*, **2**, 26-34.

Macario, A. J. L., (1995). Heat shock proteins and molecular chaperones: implications for pathogenesis, diagnostics and therapeutics. *International Journal of Clinical and Laboratory Research*, **25**, 59-70.

Maguire, M., Coates, A. R. M., and Henderson, B., (2002). Chaperonin 60 unfolds its secrets of cellular communication. *Cell Stress and Chaperones*, **7**, 317-329.

Malmqvist, M., and Karlsson, R., (1997). Biomolecular interaction analysis: affinity biosensor technologies for functional analysis of proteins. *Current Opinion in Chemical Biology*, **1**, 378-383.

Mambula, S. S., and Calderwood, S. K., (2006A). Heat induced release of Hsp70 from prostate carcinoma cells involves both active secretion and passive release from necrotic cells. *International Journal of Hyperthermia*, **22**, 575-585.

Mambula, S. S., and Calderwood, S. K., (2006B). Heat shock protein 70 is secreted from tumor cells by a non-classical pathway involving lysosomal endosomes. *The Journal of Immunology*, **177**, 7849-7857.

Mambula, S. S., Stevenson, M. A., Ogawa, K., and Calderwood, S. K., (2007). Mechanisms for Hsp70 secretion: Crossing membranes without a leader. *Methods*, **43**, 168-175.

Mayer, M. P., Laufen, T., Paal, K., McCarty, J. S., and Bukau, B., (1999). Investigation of the interaction between DnaK and DnaJ by Surface Plasmon Resonance Spectroscopy. *Journal of Molecular Biology*, **289**, 1131-1144.

Morimoto, R. I., Kline, M. P., Bimston, D. N., and Cotto, J. J., (1997). The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays in Biochemistry,* **32**, 17-29.

Nara, S., Tripathi, V., Chaube, S. K., Rangari, K., Singh, H., Kariya, K. P., and Shrivastav, T. G., (2008). Use of biotin-streptavidin system for developing a visible, sensitive and specific antigen heterologous assay for hapten. *Talanta,* **77**, 210-216.

Neupert, W., and Brunner, M., (2002). The protein import motor of mitochondria. *Nature Reviews of Molecular Cell Biology*, **3**, 555-565.

Njemini, R., Demanet, C., and Mets, T., (2003). Determination of intracellular heat shock protein 70 using a newly developed cell lysate immunometric assay. *Journal of Immunological Methods*, **274**, 271-279.

Noessner, E., Gastpar, R., MilanI, V., Brandl, A., Hutzler, P. J. S., Kuppner, M. C., Roos, M., Kremmer, E., Asea, A., Calderwood, S. K., and Issels, R. D., (2002). Tumor-derived Heat Shock Protein 70 peptide complexes are cross-presented by human dendritic cells. *The Journal of Immunology*, **169**, 5424-5432.

Nollen, E. A. A., Brunsting, J. F., Roelofsen, H., Weber, L. A., and Kampinga, H. H., (1999). *In vivo* chaperone activity of heat shock protein 70 and thermotolerance. *Molecular and Cellular Biology*, **19**, 2069-2079.

O'Malley, B. W., McGuire, W. L., Kohler, P. O., and Korenman, S. G., (1969). Studies on the mechanism of steroid hormone regulation of synthesis of specific proteins. *Recent Progress in Hormone Research*, **25**, 105-160.

Ohashi, K., Burkart, V., Flohe, S., and Kolb, H., (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *The Journal of Immunology*, **164**, 558-561.

Panyutich, A. V., Baturevich, E. A., Kolesnikova, T. S., and Ganz, T., (1993). The effect of biotinylation on the antigenic specificity of anti-defensin monoclonal antibodies. *Journal of Immunological Methods*, **158**, 237-242.

Parsell, D. A., and Lindquist, S., (1993). The function of Heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics*, **27**, 437-496.

Pattnaik, P., and Srivastav, A., (2006). Surface plasmon resonance - applications in food science research: A review. *Journal of Food Science and Technology*, **43**, 329-336.

Pelham, H. R., (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell*, **46**, 959-961.

Perlmann, P., and Engvall, E., (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, **8**, 871-874.

Pesce, M. A., Bodourian, S. H., Sheehan, M., and Henkel, C. F., (1992). An automated biotin-streptavidin procedure for progesterone evaluation. *Clinical Biochemistry*, **25**, 451-455.

Pockley, A. G., (2002). Heat shock proteins, inflammation, and cardiovascular disease. *Circulation*, **105**, 1012-1017.

Pockley, A. G., Bulmer, J., Hanks, B. M., and Wright, B. H., (1999). Identification of human heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies in the peripheral circulation of normal individuals. *Cell Stress and Chaperones*, **4**, 29-35.

Pockley, A. G., Muthana, M., and Calderwood, S. K., (2008). The dual immunoregulatory roles of stress proteins. *Trends in Biochemical Sciences*, **33**, 71-79.

Pockley, A. G., Shepherd, J., and Corton, J. M., (1998). Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunological Investigations*, **27**, 367-377.

Pockley, A. G., Wu, R., Lemne, C., Kiessling, R., de Faire, U., and Frostegard, J., (2000). Circulating heat shock protein 60 is associated with early cardiovascular disease. *Hypertension*, **36**, 303-307.

Prime, K. L., and Whitesides, G. M., (1993). Adsorption of proteins onto surface containing end-attached oligo (ethylene oxide): A model system using self-assembled monolayers. *Journal of the American Chemical Society*, **115**, 10714-10721.

Raposo, G., Nijman, H. W., Stoorvogel, W., Leijendekker, R., Harding, C. V., Melief, C. J. M., and Geuze, H. J., (1996). B lymphocytes secrete antigen-presenting vesicles. *The Journal of Experimental Medicine*, **183**, 1161-1172.

Rich, R. L., Myszka, D. G., (2000). Advances in surface Plasmon resonance biosensor analysis. *Current Opinion in Biotechnology*, **11**. 54-61.

Ritossa, F., (1962). A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, **18**, 571-573.

Rothman, J. E., (1989). Polypeptide chain binding proteins: Catalysts of protein folding and related processes in cells. *Cell*, **59**, 591-601.

Rudiger, S., Buchberger, A., and Bukau, B., (1997A). Interaction of Hsp70 chaperones with substrates. *Nature Structural Biology*, **4**, 342-349.

Rudiger, S., Germeroth, L., Mergener, J., and Bukau, B., (1997B). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *The EMBO Journal*, **16**, 1501-1507.

Ryan, M. T., and Pfanner, N., (2002). Hsp70 proteins in protein translocation. *Advances in Protein Chemistry*, **59**, 223-242.

Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L., (1997). Dsitinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL. *Nature*, **388**, 792-798.

Schnaider, T., Soti, C., Cheetham, M. E., Miyata, Y., Yahara, I., and Csermely, P., (2000). Interaction of human DnaJ homologue, HSJ1b with the 90kDa heat shock protein, Hsp90. *Life Sciences*, **67**, 1455-1465.

Suh, W. C., Burkholder, W. F., Lu, C. Z., Zhao, X., Gottesman, M. E., and Gross, C. A., (1998). Interaction of the Hsp70 molecular chaperone, DnaK, with its cochaperone DnaJ. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 15223-15228.

Sun, W., Jiao, K., and Zhang, S., (2001). Electrochemical ELISA for the detection of cucumber mosaic virus using o-pheneylenediamine as substrate. *Talanta*, **55**, 1211-1218.

Szabo, A., Stolz, L., and Granzow, R., (1995). Surface plasmon resonance and its use in biomolecular interaction analysis (BIA). *Current Opinion in Structural Biology*, **5**, 699-705.

Tavaria, M., Gabriele, T., Kola, I., and Anderson, R. L., (1996). A hitchhiker's guide to human HSP70 family. *Cell Stress Chaperones*, **1**, 23-28.

Thillaivinayagalingam, P., Newcombe, A. R., O'Donovan, K., Francis, R., and Keshavarz-Moore, E., (2007). Detection and quantification of affinity ligand leaching and specific antibody fragment concentration within chromatographic fractions using surface plasmon resonance. *Biotechnology and Applied Biochemistry*, **48**, 179-188.

Tini, M., Jewell, U. R., Camenisch, G., Chilov, D., Gassmann, M., (2002). Generation and application of chicken egg-yolk antibodies. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, **131**, 569-574.

Tissier, A., Mitchell, H. K., and Tracy, U. M., (1974). Protein synthesis in salivary glands of Drosophila Melanogaster: relation to chromosome puffs. *Journal of Molecular Biology*., **85**, 389-398.

Tsai, H. J., and Saif, Y. M., (1991). Detection of antibodies against Bordetella avium in Turkeys by Avidin-Biotin enhancement of the Enzyme-Linked ImmunoSorbent Assay and the Dot-Immunobinding Assay. *Avian Diseases*, **35**, 801-808.

Wallin, R. P., Lundqvist, A., More, S. H., von Bonin, A., Kiessling, R., and Ljunggren, H. G., (2002). Heat-shock proteins as activators of the innate immune system. *Trends in Immunology*, **23**, 130-135.

Walsh, R. C., Koukoulas, I., Garnham, A., Moseley, P. L., Hargreaves, M., and Febbraio, M. A., (2001). Exercise increases serum Hsp72 in humans. *Cell Stress and Chaperones*, **6**, 386-393.

Welch, W. J., (1992). Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiological Reviews*., **72**, 1063-1081.

Woof, J. M., and Burton, D. R., (2004). Human antibody-Fc receptor interactions illuminated by crystal structures. *Nature Reviews Immunology*, **4**, 89-99.

Woolley, D. M., and Longsworth, L. G., (1942). Isolation of an antibiotin factor from egg white. *The Journal of Biological Chemistry*, **142**, 285-290.

Xu, Q., Schett, G., Perschinka, H., Mayr, M., Egger, G., Oberhollenzer, F., Willeit, J., Kiechl, S., and Wick, G., (2000). Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. *Circulation*, **102**, 14-20.

Yabunaka, N., Ohtsukab, Y., Watanabe, I., Noro, H., Fujisawa, H., and Agishi, Y., (1995). Elevated levels of heat-shock protein 70 (HSP70) in the mononuclear cells of patients with non-insulin-dependent diabetes mellitus. *Diabetes Research and Clinical Practice*, **30**, 143-147.

Young, J. C., Barral J. M., and Hartl, F. U., (2003). More than folding: localized functions of cytosolic chaperones. *Trends in Biochemical Sciences*, **28**, 541-547.

Zhang, X. M., He, M. A., Cheng, L. X., Chen, Y., Zhou, L., Zeng, H. S., Pockley, A. G., Hu, F. B., and Wu, T. C., (2008). Elevated Heat Shock Protein 60 levels are associated with high risk of coronary heart disease in Chinese. *Circulation*, **118**, 2687-2693.

Zhang, X. S., and Oglesbee, M., (2003). Use of surface psmon resonane for the measurement of low affinity binding interactions between Hsp72 and measles virus nuclecapsid protein. *Biological Procedures Online,* **5**, 170-181.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Filament, C., Tenza, D., Ricciardi-Castagnoli Raposo, G., and Amigorena, S., (1998). Eradication of established murine tumours using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature Medicine*, **4**, 594-600.