Design and Optimisation of the Resonant Mirror, an Optical Biosensor, for the Analysis of Microalbuminuria in Trauma Patients.

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Thesis Summary

Microalbuminuria is characterised by elevated levels of albumin in urine. It reflects localised increases in vascular permeability. Increases in vascular permeability occur as a result of inflammatory response to trauma or infection.

Resonant mirror biosensors permit real time analysis of molecular interaction without the need for labelling either the receptor or the ligand. A resonant mirror-based assay was developed to detect albumin using anti human serum albumin antibody 0220-0704. Albumin standards were measured from 1-100 μg/ml to produce reproducible, linear calibration curves in a one-well assay format and from 2-32 μg/ml in a four-well format, which allows for the assay of control samples. The latter assay was used to detect albumin in human urine. Results were comparable with conventional methods and were reproducible.

Secondly, a novel assay for creatinine was developed using the resonant mirror. Creatinine has a molecular weight below the detection limit of the device and so a response based on localised pH change, caused by the deamination of creatinine by creatinine deaminase was exploited. This novel application of the resonant mirror successfully detected creatinine at concentrations as low as 270μM. The inclusion of creatinine should permit correction for urine flow variation.

The concept of using a resonant mirror for monitoring microalbuminuria has applications for analysis in trauma severity of patients. The exciting potential for this technology is the rapid, non-invasive, measurement of multiple medical markers in urine for use in clinical diagnosis.

Keywords: Microalbuminuria, creatinine, trauma, inflammation, biological assay, association phase, coupling chemistry.

Dedication

This thesis is devoted to Zoulikha, Mohamed, Dr. Megdad, Saadia, Dr. Mokhtaria, Yazid and my beloved mother Khadra. I am indeed very fortunate to have your unlimited love. Your teachings and wisdom have made it possible for me to be where I am today. Mere words cannot express how much you all mean to me.

Acknowledgements

I would like to thank my supervisor Dean Payne, Debbie for her help with the resonant mirror and Martin for his ideas on creatinine detection.

I wish to show my utmost gratitude to both my external supervisor Ann Vernallis and my external advisor Anna Hine for making me realize the importance of thorough data analysis and for also making it possible for me to complete my thesis. Their patience and encouragement has been a great asset.

My special thanks goes to Khadra and friends. Thank you for being there for me every step of the way. Your strength and determination has been an inspiration.

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Abbreviations

SD	standard deviation of the mean
SEM	standard error of the mean
MAU	microalbuminuria
RM	resonant mirror
HPLC	high performance liquid chromatography
Cat. no.	catalogue number
NHS	N-hydroxysuccinimide
EDC	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
PBST	phosphate buffered saline and tween
MOF	multiple organ failure
ARDS	adult respiratory distress syndrome
SIRS	systemic inflammatory response syndrome
TNF	tumour necrosis factor
IL	interleukin
ICU	intensive care unit
CMD	carboxymethyl dextran
UAE	urinary albumin excretion
IDDM	insulin dependent diabetes mellitus
[ ]	concentration
CH	channel(s)
ADP	adenosine diphosphate
ATP	adenosine triphosphate

Chapter 3
RESULTS - ANALYSIS OF ALBUMIN

1.1 Trauma and its impact on society

In the Western world, trauma is the principle cause of death between the ages of 1 and 40. In Great Britain injured patients occupy more hospital beds than patients with cancer and heart disease combined (Westaby *et al.*, 1989). Adolescent and young adult males are the most vulnerable to major trauma, though deaths are more likely in the elderly and in those with critical head injury (Oakley *et al.*, 2001). Experiencing trauma is an essential part of human history, touching all our lives to a lesser or greater extent.

Trauma, defined as a wound or injury resulting in cellular damage and tissue disruption, is caused by physical forces or chemical agents. In clinical practice, trauma appears in many forms, blunt contusion (e.g. falls, car accidents) perforations (e.g. stab wounds), blast injury, ischemic necrosis, crush injury, burns, radiation and many other forms (Girolami *et al.*, 1999). Whether blunt or penetrating, trauma tends to affect more than one body region and often involves several physiological organ systems. Identification and treatment of trauma, regardless of type, must be as effective as possible in order to increase the chances of survival. Studies by Luk *et al.*, (1999) suggest that with efficient detection systems and effective treatments few patients are left disabled. In 1999 nearly 11million people were left temporarily disabled and 450,000 permanently disabled. The economic impact from this is significant (Luk *et al.*, 1999).

A significant problem in providing effective care to victims of major trauma is the inherent difficulty in determining accurately, the severity of the injuries the patient has suffered. Multiple injuries to various body organs can cover the spectrum of severity. Determining which patients are at risk involves first identifying a threshold of injury severity, which has been shown to result in significant mortality and then assessing whether the patient has reached that threshold (Westaby 1989).

Severity scores can be used to define specific points at which significant risk to the patient exists and to determine the level of care needed to treat the patient's injuries adequately. Such scores characterise and quantify severity of injury by mathematically combining weighted variables that reflect the patients condition. Many severity-scoring systems have been devised, however most of them adhere to the requirements outlined by Gibson in 1981.

The costs associated with trauma victims are considerable. In the UK the department of transport alone estimated the cost of road accidents to be £13.3 billion in 1995 (Girolami *et al.*, 1999). Therefore the economics of improving the care of seriously injured patients makes sense. Enhancement of detection methods for patients undergoing trauma not only improves care but also increases survival, reduces future complications as well as reduces costs.

1.2 The effects of trauma on the human body

In a physiological sense, trauma is not a single insult but a combination of haemorrhage, tissue injury, pain and fear. Such characteristics were identified by Celsus in 100AD as 'tumor' (swelling), 'rubor' (redness), 'calor' (heat) and 'dolor' (pain). The body's response, on insult, can be demonstrated as circulatory, metabolic and inflammatory (Foex 1999) (figure 1.1).

1.2.1 Circulatory response

Haemorrhage is an inevitable consequence of injury. If the severed vessel is small, the efficient haemostatic mechanism is available to stop the bleeding. If a large vessel is severed quite extensively, large volumes of blood loss is inherent and the body may undergo shock and later death (Glauser *et al.*, 1994; Westaby 1989). The haemostatic mechanism is concerned with platelets, vasoconstriction, the endothelial cell, blood clotting and fibrinolysis.

Direct trauma to the vessel wall results in haemorrhage, the injury to tissues such as muscle, can lead to the initiation of a microcirculatory thrombotic process shown by disseminated intravascular coagulation (DIC) (Foex 1999). DIC is a complication of injury whereby the haemostatic balance being overwhelmed causes fibrin and platelet deposits within the intravascular compartment.

Following vascular injury, there is a brief period of vasoconstriction, especially of arterioles. This helps in the reduction of blood loss in the short term. The damage to the

endothelial cells attracts the adherence of platelets in the blood issuing from the severed vessels. The platelets adhere, become contact activated, change shape and undergo a release reaction (Marshall 1998). This encourages additional platelets to aggregate on those already adherent to the subendothelium. Accumulation of platelets is accompanied by their granulation whereby dense granules (serotonin and adenosine diphosphate) including alpha granules (fibrinogen, von Willebrand factor, coagulation factor V) are secreted (Clowes 1998).

Thrombin formation via a prothrombinase complex occurs causing enzymatic conversion of fibrinogen to fibrin. The lumen of the arteriole is sealed and stabilised by fibrin allowing for sealage of the wound.

On completion of haemostasis, anti-haemostatic (anti-thrombotic forces) takes place. The process is then reversed by coagulation inhibitors and unwanted thrombotic haemostatic material is removed by fibrinolysis, leading to healing of the wound and repair.

It is important to note that the above process is not without complications. The most obvious is thrombosis in areas remote form the site of injury leading to blood clot formation and deep vein thrombosis. Pulmonary embolism represents one of the most serious complications of trauma, which could lead to death (Maxwell *et al.*, 1999).

1.2.2 Metabolic response

Immediately following injury, there occurs a relative loss of circulating blood volume leading to hypovolemia. Various metabolic and biochemical processes take place to enhance the healing process and restore homeostasis. Metabolic responses can be divided into three overlapping stages: acute phase, catabolic phase and anabolic phase. The first phase is sometimes called the ebb or shock phase and is an immediate defensive response primarily to protect the circulation. The latter two stages can be thought of as one phase sometimes referred to as the flow phase.

Acute phase

Overall oxygen utilisation and heat production decrease in this stage. Energy is derived mainly from fat via free fatty acids, the plasma concentration of which increases. There may be an increase in hepatic glycogenolysis and impaired glucose intolerance. The primary hormonal change is the increase in plasma catecholamines. Plasma insulin concentration is usually low and plasma glycogen is increased. Plasma adrenocorticotropic hormone (ACTH) and cortisol are both increased. Increased antidiuretic hormone (ADH) secretion induces oligouria which may persist for 24 hours. The whole of the acute phase lasts about 24 hours.

Catabolic phase

The catabolic phase is primarily one of overall rapid mobilisation and turnover of substrates in order to match the increased demand for energy. Oxygen consumption and heat production are increased and the resting energy expenditure may be more than doubled. Healthy muscle protein is increasingly broken down to provide amino acids for synthesis of new proteins and for gluconeogenesis. Glycogen and triglycerides from the fat stores are also utilised (Scannell *et al.*, 2001). These processes are stimulated primarily by catecholamines as well as cytokines. It is the surge of catecholamines secretion that is responsible for the 'fight or flight' response.

Damaged muscle is degraded. Inhibition of muscle protein synthesis contributes to a negative nitrogen balance. Although net proteins synthesis in the liver increases during this period, the healing wound does require synthesis of new proteins. These processes do not however counterbalance the nitrogen loss from muscle. These changes are accompanied by loss of weight mainly derived from the muscle protein where depletion of both actin and myosin leads to muscle weakness.

Catabolism of exogenous substrates e.g. administered nutrients, tend to also increase during this phase. Intracellular ATP is depleted, which reduces many cell functions however its significance is not fully understood. This phase lasts around 5 days (Mason 1993).

Anabolic phase

This third phase involves the restoration of nitrogen balance followed by a gain in weight. By this stage the glycogen stores will have been depleted and major utilisation for energy exists. Water, potassium and nitrogen move back into the cells. The duration of this phase may be several weeks, until full recovery of steady state levels are established including muscle mass and fat stores.

1.2.3 Inflammatory response

Inflammation is a complicated process of dilating vessels, causing them to leak protein and allowing for the transfer of white cells from intravascular to extravascular sites. Inflammatory mediators provoke changes in vascular flow and endothelial permeability. They cause mobilisation and chemotaxis of cell types to the site of injury where promotion of phagocytosis of foreign material takes place as well as tissue repair (Baynes *et al.*, 1999).

The acute inflammatory response is the initial response obtained after injury. As discussed previously, damage to vessels leads to vasoconstriction later followed by vasodilation. As a consequence escape of leucocytes (e.g. neutrophils) from the blood into extravascular tissue spaces occur. Local vascular permeability increases allowing for extravasation of antibodies to reach the site of tissue providing the host with rapid innate immune protection, while the adaptive immune response develops (Davies *et al.*, 1997). Increased capillary and venular permeability cause the passage of proteins (e.g. albumin) with water from the vascular to the interstitual spaces. Edema continues to increase until equilibrium is restored with an increase of tissue pressure. Edema is the term given to the passage of plasma to the extravascular space due, for example, to a reduction in plasma oncotic pressure. Histamine, serotonine and bradykinin release all contribute to increased permeability by enlarging the intracellular spaces due to shortening of intracellular actinomyosin fibrils.

Other acute phase proteins are released to aid wound repair. Cytokines are messenger molecules which influence chemotaxis of neutrophils and synthesis of interferons. Cytokines also attract leucocytes to the site of injury removing any invading organisms

and damaged tissue (Baynes *et al.*, 1999). Two important proinflammatory cytokine mediators, which act synergistically, are interleukin 1 (IL-1) and tumour necrosis factor (TNF). Together they cause adhesion of white cells to endothelium and induce the synthesis and release of prostacyclin. Prostacyclin antagonises the platelet haemostatic response. TNFα has been involved in haemorrhage and traumatic shock where it has been shown by Ayala *et al.*, (1991) to be produced after haemorrhage. Studies of elective surgery have shown that there seems to be some correlation between the degree of tissue injury and the production of IL-6 (Hoch *et al.*, 1993). Release of IL-6 can be stimulated by tissue injury, even before any haemorrhage has taken place (Ayala *et al.*, 1991). IL-10 has been characterised as an anti-inflammatory cytokine. Plasma IL-10 was associated with hypotension and development of sepsis but not related to the severity of injury (Neidhart *et al.*, 1996).

Localised inflammation is a physiological protective response which is usually highly controlled at the site of injury. Loss of this control or an overly activated response due to severe trauma, results in an exaggerated systemic response known as SIRS (systemic inflammatory response syndrome). SIRS develops due to a failure of homeostasis in such areas as cytokine production (Davies *et al.*, 1997). The flood of inflammatory mediators triggers numerous humoral cascades, resulting in sustained activation of the reticular endothelium system with loss of microcirculatory integrity and insults to various remote organs. Examples of remote damaged organs are kidneys, liver and lungs. Acute renal failure occurs as a consequence of damage to the glomerular capillaries, systemic hypertension and tubular overload. Liver dysfunction is common in association with acute renal failure (Oakley *et al.*, 2000). Respiratory failure has also been said to relate to trauma severity. Loss of osmotic pressure diminishes the lungs ability to maintain fluid balance. This occurs due to an increase of diffuse leakage of protein and haemorrhagic oedema fluid across the alveolar capillary membrane. Respiratory rate increases leading to adult respiratory distress syndrome (ARDS). Failure to reverse such cellular damage inflicted at the intracellular level leads to multiple organ failure (MOF). This is almost always followed by death.

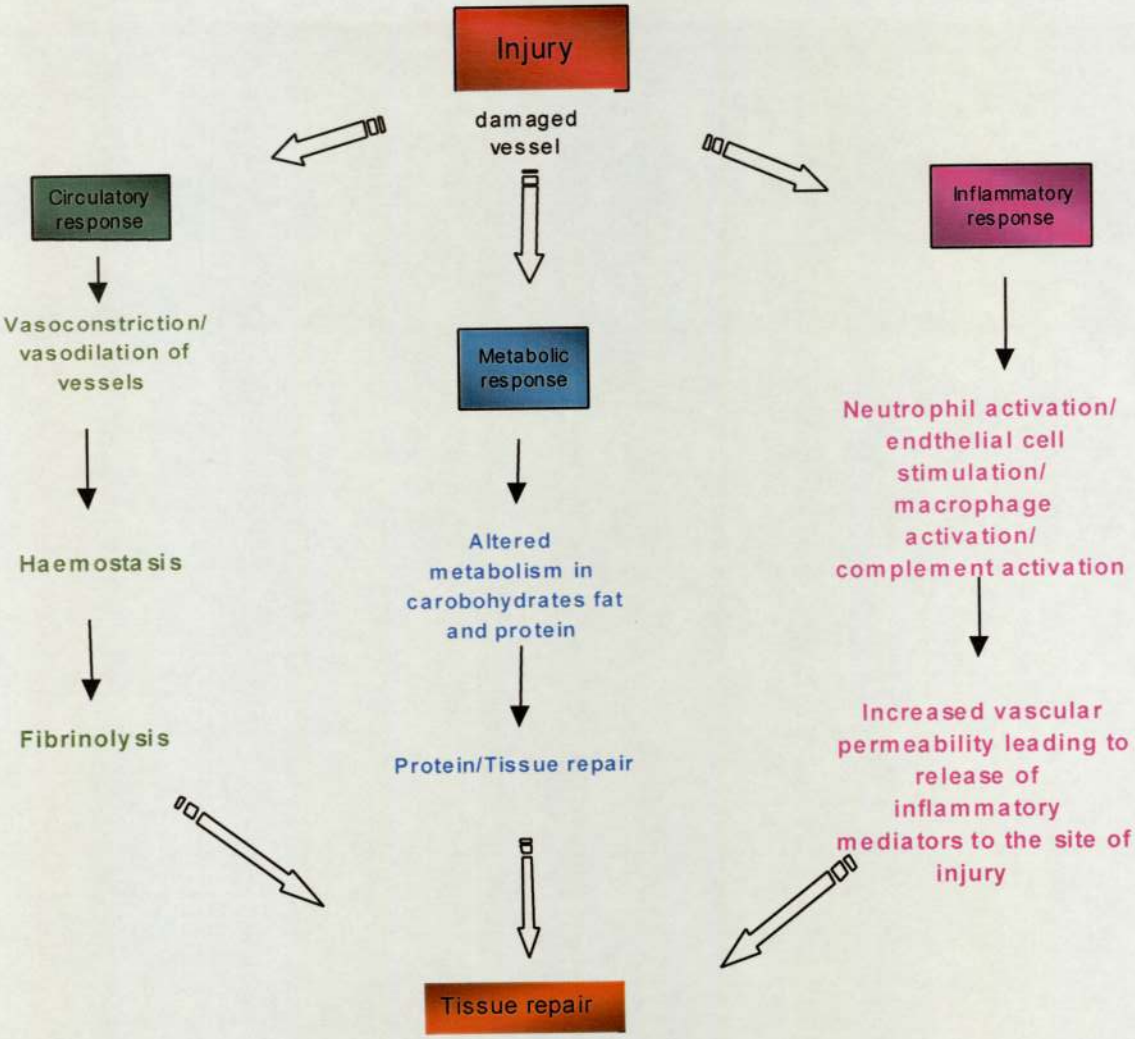


Figure 1.1. Overview of the body's response to injury. The body's response, on insult, can be demonstrated as circulatory, metabolic an inflammatory all of which play a role in tissue repair.

1.3 Microalbuminuria- a marker of trauma

1.3.1 Trauma and capillary permeability

With severe trauma, inflammation can escalate, leading to prolonged complications such as adult respiratory distress syndrome (ARDS) and in some instances death. Monitoring the severity of trauma in a patient at its early stage may reduce or even prevent such complications.

In trauma it was realised that capillary permeability could correlate with severity of insult upon the body. Increased vascular permeability follows major inflammatory insults (Doty *et al.*, 1999). Leakage of protein molecules and water from capillary to interstitual space increases the gap between the capillary and the cell, across which oxygen and other substrates have to travel. In uncomplicated cases, excess interstitual fluid is lost within a few hours and vascular permeability returns to normal. However patients suffering from mild to severe trauma would suffer from a prolonged and excessive capillary leak and hence increasing the amounts of water and protein molecules (Gosling 1999) in the interstitual fluid.

Direct measurement of increased capillary permeability cannot be easily performed. It has been proposed that albumin excretion serves to reflect a generalised systemic vascular permeability (Gosling *et al.*, 1988). Studies looking at urine albumin excretion and glomerular permeability show a relationship between small levels of albumin excretion and glomerular disease in 84 IDDM patients (insulin dependent diabetes mellitus) (Marre *et al.*, 1994). The term mircoalbuminuria (MAU) was used by Viberti *et al.*, (1982) for the study of small elevations of urinary albumin excretion (UAE) which existed in patients suffering from insulin dependent diabetes mellitus (IDDM). Since then microalbuin excretion has been associated with many inflammatory processes such as bacterial meningitis (Roine 1993), rheumatoid arthritis (Pederson 1993), inflammatory bowel syndrome (Muhmood *et al.*, 1993), trauma (Gosling *et al.*, 1994) and surgery (Smith *et al.*, 1994). In each condition microalbumin excretion was said to be proportional to the severity of the condition, occurring within minutes of acute insults and able to reflect minute changes on vascular endothelial permeability. Microalbumin excretion was also valued in predicting later complications associated with interstitual oedema such as ARDS (Gosling *et al.*, 1994).

Another study performed by Fleck *et al.*, (1985) showed that the rate of loss of albumin in the tissue spaces (measured as transcapillary rate) rose by more than 300% in patients with septic shock. The average increase within 7 hours of cardiac surgery was 100%. The transcapillary escape rate in cachetic cancer patients was twice that of a group of healthy individuals indicating that increased vascular permeability is an important cause of small concentrations of albumin commonly seen in acute and chronic disease.

1.3.2 Capillary permeability and microalbuminuria (MAU)

Microalbuminuria is defined as a sub-clinical increase in urinary albumin from 20-300mg/l (De Gaudio *et al.*, 1995). Small amounts of albumin pass through the glomerular barrier of the kidney, which under normal conditions would be reabsorbed by proximal tubular cells. The passage of molecules through the glomerular basement membrane is dependent upon size and electrical charge. Damage to the endothelial membrane filtration barrier of the glomerulus can substantially alter its function. Similarly increase of capillary permeability allows for a much larger influx of protein molecules entering the glomerulus. About 1g of protein leaks through the glomeruli of a healthy individual, every 24 hours where around 97% is reabsorbed by the proximal tubule by pinocytosis. Because the reabsorptive process is working at near maximal capacity, any moderate increases in filtered protein such as albumin are reflected by an increase in albumin excretion rate (Jones 1987).

MAU is said to be a good marker of trauma because the response occurs far earlier than for example the rise in serum C reactive protein concentration which takes 2-3 days to reach maximum (Gosling 1994). C-reactive protein is a major component of the acute phase response and is synthesized in the liver. Increased synthesis of C-reactive protein usually occurs in tissue damage. MAU is directly related to capillary permeability and hence precise in its determination. Excretion of albumin even at small levels can be detected in the urine due to the amplification mechanism of the kidneys. Small increases in glomerular permeability are amplified by the renal concentrating mechanism to produce large changes in albumin excretion.

1.3.3 Measurements of MAU

Small increases in urinary albumin excretion (UAE) cannot be measured by routine laboratory tests (De Gaudio 1999), as the levels are too low. Radioimmunoassay was proposed (Keen *et al.*, 1963) and is still used as a routine laboratory test, however, it is time consuming and very expensive. Most radioimmunoassay methods label human albumin with the isotope ^{125}I and using a saturation type assay detect the presence of albumin molecules in the urine. An immunochemical method was then proposed by Watts *et al.*, (1986) as an alternative, where levels of albumin were detected by an antigen-antibody reaction in wells containing antibody in excess. The antigen-antibody complex was stained whereby the intensity of the stain was measured (Watts *et al.*, 1986). This technique is considered to be labour intensive with a low degree of sensitivity (Wincour *et al.*, 1998). Several immunological antigen-antibody complex based assays have since been developed however they usually involve several steps, long incubation times and dilution steps; all of which are time consuming and allow for error (Mohamed *et al.*, 1984; Chachati *et al.*, 1987; Llyod *et al.*, 1987). Enzyme linked immunosorbent assays (ELISA) have also been established with the use of a microtitre plate (Fielding *et al.*, 1983). Such assays are usually 'two-site' whereby bound complexes are detected and quantified by addition of a second antibody conjugated to an enzyme label. Most conjugates contain horseradish peroxidase, which on addition of o-phenylenediamine and hydrogen peroxidase generates a quantifiable colour reaction (Watts *et al.*, 1986).

Recently a dye-binding assay has been developed (Pugia *et al.*, 1999) as an alternative method of albumin detection. In this assay a protein solution is added to the dye, monitored by one or more wavelengths, to assess binding and estimate the equilibrium-binding constant. The difficulties with this technique are the changes in absorptivity of the dye as protein is added. Many dyes have been placed on a dipstick e.g. 2-(4'-hydroxyazobenzene) and bromocresol green, which are said to bind to both strong and weak binding sites of albumin. However dyes can also change colour with other macromolecules that may be present in the sample therefore it is not a specific enough technique for clinical use.

Commercial dipstick kits are now available; Bayer CLINITEK 50, Boerhinger Mannheim Micra® (Rumley 2000; Pugia 1998) being the most popular. They are cheap

and simple to use, however at best they are semi-quantitative. For clinical use the instrument must indicate the exact degree of trauma severity in a patient in order to establish suitable therapy. As these dipsticks are semi quantitative their use is not practical.

Of the current wide range of available techniques in detecting albumin, most are either expensive, time consuming, involve numerous stages or are semi quantitative in their analysis. In this project having already established MAU as a prognostic marker of trauma, the use of an optical based method will be explored.

1.4 Use of creatinine as an index for urine flow

Albumin levels in MAU fluctuate quite radically for reasons not associated with any form of injury or illness of the body. Examples of factors affecting levels of albumin excretion are alcohol intake, patients posture, exercise, dietary protein and protein metabolism (Marshall 1998). The introduction of creatinine as an index against non specific albumin fluctuations were reviewed and shown to be effective (Newman *et al., 2000*; Ng *et al.,* 2000; Cundy *et al.,* 2000). Creatinine is thought to be an ideal index against urine flow due to its constant excretion levels through out the day, endogenous nature being neither reabsorbed nor secreted and creatinine is not affected by posture, exercise, alcohol consumption or any other non-specific factor.

Creatine is synthesized in the liver, kidneys and pancreas and released into the blood. It is transported to the sites of usage; mainly the muscle and brain. Creatinine is a waste product derived form creatine and creatine phosphate (Marshall 1998). About 2% of total muscle creatine is converted daily to creatinine through spontaneous non-enzymatic loss of water and is removed from the plasma by glomerular filtration (Baynes *et al.,* 1999). Normal creatinine concentration in plasma is about 1mg/dl or 60-120μmol/l and in urine is around 25-400mg/dl.

Creatinine can be detected using the Jaffe reaction. This assay was one of the first assays, established in 1970s, where creatinine was said to react with alkaline picric acid to form a coloured species that absorbs at 500nm. This assay is still used and conveniently available as a kit. However the procedure is prone to error due to interference from other non-specific proteins/molecules and hence lacks specificity (Winquist *et al.*, 1986). An enzymatic assay incorporating electrode probes is an alternative to the Jaffe reaction. The assay does require large sample volumes and can be expensive. Much more sophisticated analysis can be performed using ion-chromatography (Palmisano *et al.*, 1995) however this method has been found to be laborious and time consuming.

1.5 Detection of MAU and creatinine using the resonant mirror

Optical evanescent wave biosensors have become readily available and increasingly popular as a tool for the quantitative and qualitative characterisation of reversible interactions. Figure 1.2 shows a schematic representation of the resonant mirror and its components.

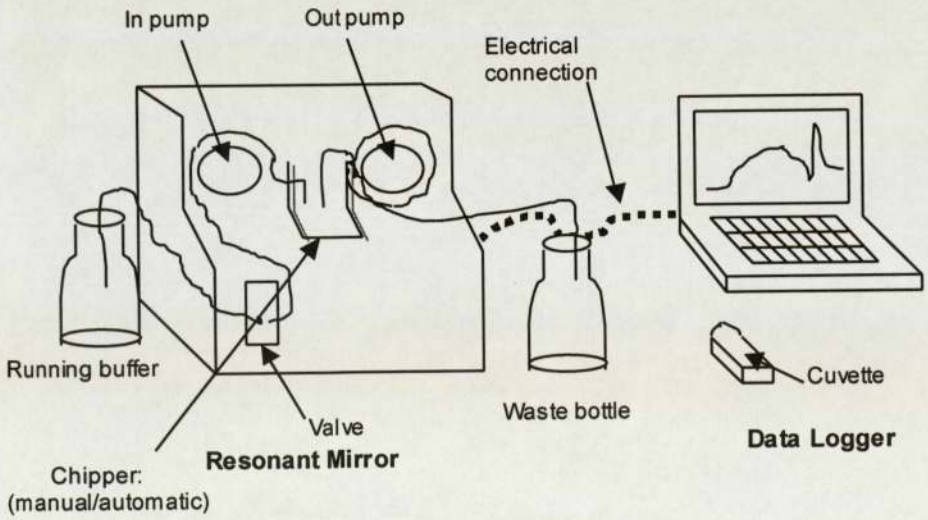


Figure 1.2. Diagrammatic representation of the RM showing its main features as well as the data logger used to record any responses obtained from the RM. The cuvette is inserted into the chipper and samples passed over via the valve system. Any changes in response are visualised by the data logger.

1.5.1 Detection principles of the resonant mirror

The basic optical configuration of an RM biosensor consists of a glass prism coated with a dielectric resonant layer of high refractive index (e.g. titania). This is separated from the prism by a low refractive index layer (e.g. silica) (Cush *et al.*, 1993). In this configuration, the total internal reflection of light excites the evanescent field within the resonant structure (figure 1.3). The evanescent field is a type of electromagnetic wave that exists at the surface of many forms of optical waveguides (Schuck 1996). The electromagnetic wave excites flurophores which decay exponentially with increasing distance to the sensor surface. The electromagnetic wave can be resonantly excited by light only at a well defined angle of incidence with phase matching between the incident beam and the resonant modes of the high index layer (Ramsden *et al.*, 1997). The resonance causes an energy loss in the reflected light, which is visible as a sharp change in angle reflectance. The resonance angle is dependent upon the angle of reflectance producing a shift in the resonant angle. For any given refractive index, the signal is approximately proportional to the mass that is bound to the sensor surface.

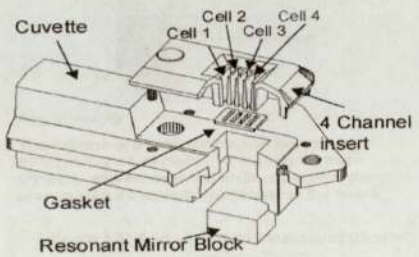


Figure 1.3A. Schematic drawing, adopted from IAsys manual, showing the structure of a standard carboxylmethyl dextran cuvette. This also shows the positioning the gasket insert would take upon the cuvette. The cells shown here are referred throughout this report as wells.

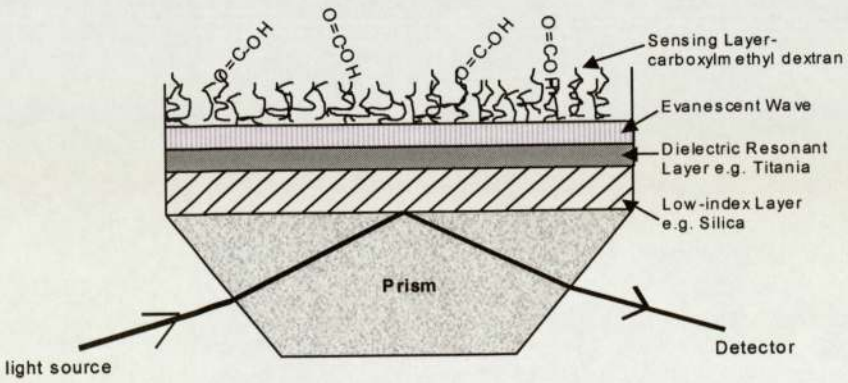


Figure 1.3B. Schematic diagram of the cuvette showing how total internal reflection of light occurs within the evanescent field.

1.5.2 Sensor surface and immobilisation chemistry

For molecules to be detected using the RM, one of the binding partners has to be immobilised on the sensor surface. For the purpose of this project the term given to the immobilised binding partner is 'ligand'. The word 'analyte' will be used to denote the compound of interest present in the sample solution. It is important that the measured binding reflects the interaction of both reactants and so non-specific surface binding should be negligible and that immobilisation of one of the binding partners, does not affect the confirmation of the binding site. The sensor surface and immobilisation techniques are therefore very important.

The RM cuvette consists of a layer of modified dextran. The dextran is chemically modified so that it has an overall negative charge allowing for non-specific electrostatic adsorption at high immobilisation densities. Activation of the dextran layer is done using NHS/EDC, which react with carboxyl groups in the dextran to form an amine-reactive intermediate (figure 1.4). This intermediate (o-acylisourea) is unstable in aqueous solutions. NHS is present to stabilise the reaction. Once the dextran has been activated, binding of one of the molecules takes place via a displacement reaction. Any remaining exposed activated carboxyl groups are deactivated by the addition of ethanolamine. This leads to the successful immobilisation of one of the reactants to the sensor surface for the study of reversible interactions.

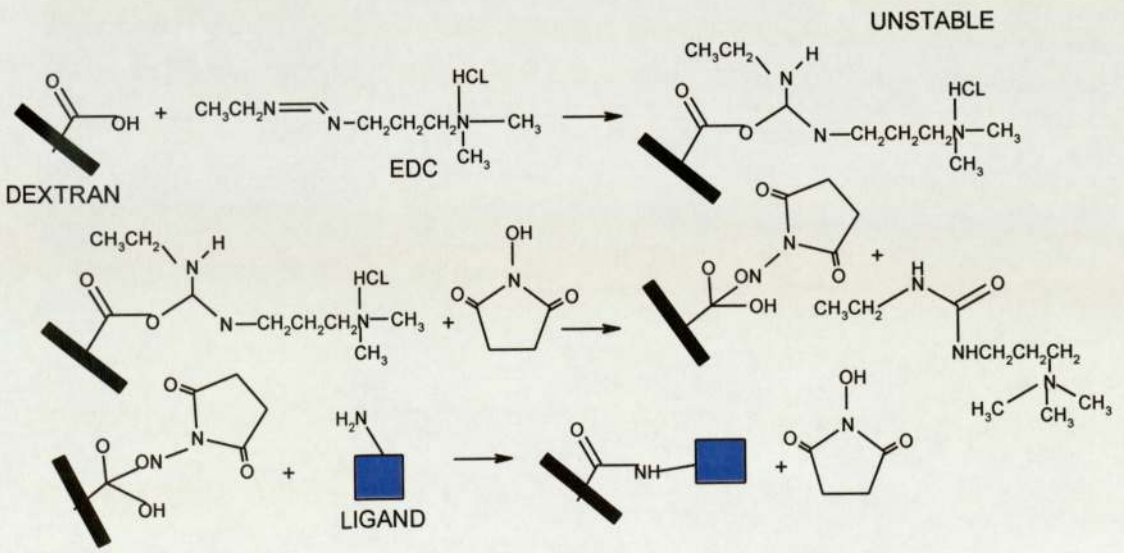


Figure 1.4. Amine coupling chemistry is used to activate the surface of the cuvette. The various chemical steps involved in this process are outlined with the use of NHS/EDC and ethanolamine. The black rectangles represent the surface of the cuvette.

1.5.3 Data analysis

The time-dependent change of the refractive index in the vicinity of the binding surface upon binding is commonly measured in arcsec units where 1 arcsec is equivalent to $5x10^{-3}ng/mm^2$ protein. A binding-progress curve commonly referred to as a response curve, measures arcsec units against time (figure 1.5). The simplest model for the interaction of an immobilised species A (e.g. antibody) and a mobile molecule (e.g. antigen) is a reversible 1:1 complex (AB) at a chemical on-rate constant (K_{on}) and a chemical off-rate constant (K_{off}) for dissociation. The thermodynamic equilibrium constant is then said to follow:

$$K_D = K_{on}/K_{off} \qquad \text{Langmuir reaction}$$

At equilibrium the occupancy of a ligand is related to analyte concentration by the langmuir equation. This model however does not take into consideration mass transport and assumes that the concentration of the mobile reactant is held constant. For the purpose of this project use of the linear equation, y=mx+c is sufficient for data analysis. The initial rate or gradient of the association phase response may also be calculated and used to construct a calibration curve (figure 1.6).

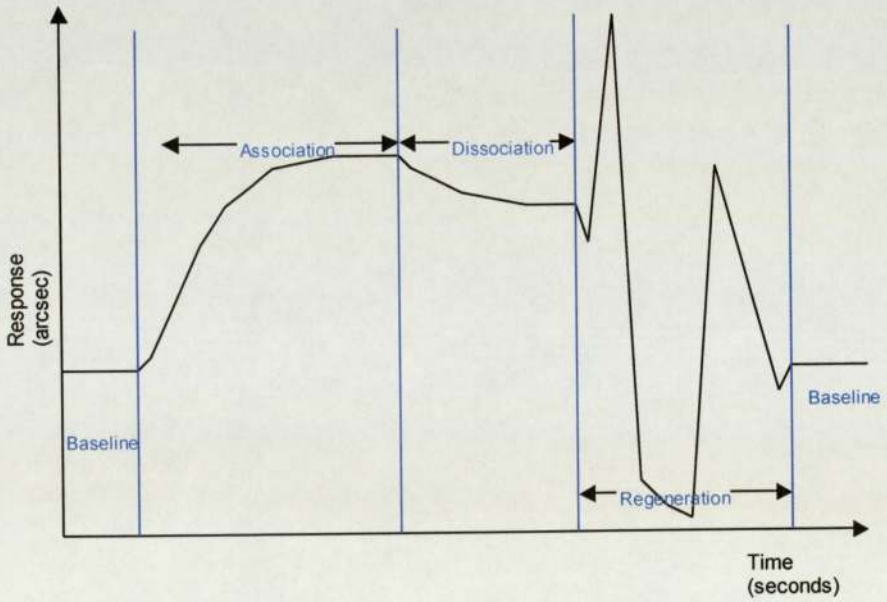


Figure 1.5. Illustration showing the various stages involved in binding assays when human albumin is added to anti-human albumin antibody on the RM. The binding event is shown by a gradual increase in response (association). Dissociation occurs on addition of PBST (running buffer). Regeneration of the cuvette surface occurs by addition of acid, which removes bound albumin but keeps the antibody intact for further use. Since the anti-human serum albumin antibody is unaffected by the regeneration process, the cuvette can be re-used in further binding experiments.

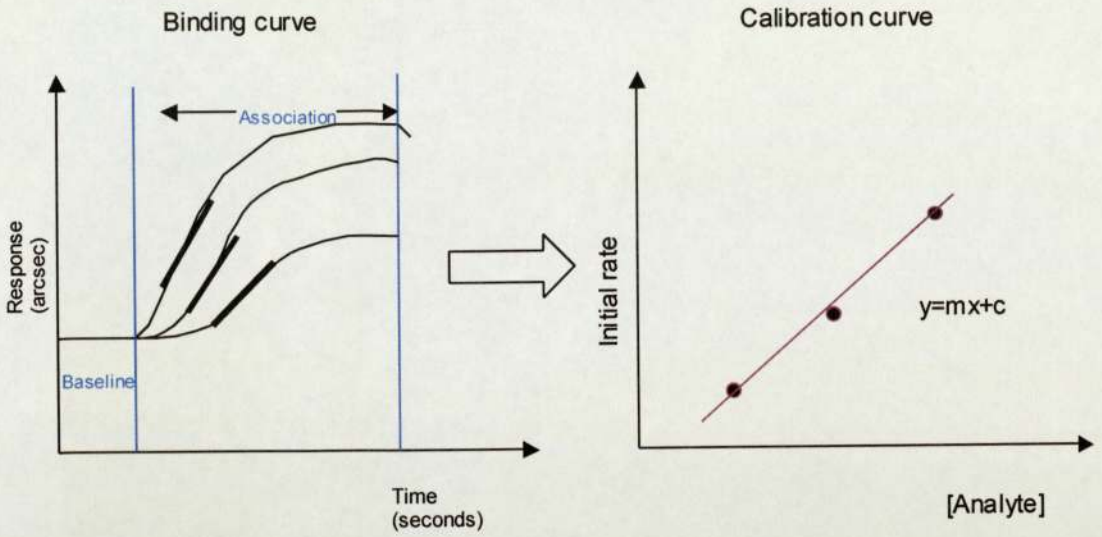


Figure 1.6. A calibration curve can be derived by calculating the gradient of the slope from the association phase. The gradient, stressed by the heavy black line is automatically calculated by the software BIAevaluation to give the initial rate, which can then be plotted against analyte concentration. Each data point in the calibration curve represents the gradient of the bold black line in the binding assay

1.5.4 Binding assays

The strategy used in a typical RM biosensor experiment follows three stages. In the first stage, covalent attachment of the ligand to the sensor surface occurs. In the association phase (figure 1.5) of the experiment, a mobile second reactant (analyte), at constant concentration, is introduced into the buffer flow above the sensor surface and the progress of complex formation at the sensor surface is monitored. This stage is followed by the dissociation phase in which the free mobile reactant is absent from the buffer and the time course of the complex dissociation is recorded. Finally, the sensor surface is regenerated by a short exposure to a buffer at low pH. This removes the remaining complex. The cycle of association, dissociation and regeneration can be repeated several times on the same surface. The obtained sequence of binding-progress curves contains information on the chemical rate constants and on the thermodynamic equilibrium constant interaction (section 1.5.3).

RM biosensors are attractive alternative assays currently available. Since it is based on changes in refractive index no labelling of molecules is required. The biosensor provides real-time, in situ, information on the course of the binding, it can be applied to interactions within a broad range of affinities, and uses small sample volumes. The laser used means it is very sensitive to detection of low weight molecules of 551MW and because it measures localised change, it is not subject to noise. RM as every other device suffers from drawbacks such as steric hindrance and mass transport (Schuck *et al.*, 1996). It has been noted that high local concentrations of immobilised ligand at the sensor surface can lead to steric hindrance in binding to neighbouring binding sites. This in turn would affect the association phase response. To prevent such hindrance occurring it is important that the determined concentration of immobilised ligand is at optimal levels without causing steric hindrance. Mass transport is when the rate of transport of the mobile analyte to the sensor surface is either too slow or too fast to allow for any meaningful data in terms of the kinetic analysis (K_{on}, K_{off}). Increasing the flow rate at which the mobile analyte passes over the sensor surface will avoid occurrence of steric hindrince. As the RM will not be used for kinetic analysis the aforementioned drawbacks will not be relevant in the analysis of MAU.

Currently several hundred studies on molecular interactions using the RM have been published in a variety of fields. These include antibody-antigen interactions (Dmitriev *et al.*, 2002; Charalambous *et al.*, 1999; Gorgani *et al.*, 1999), protein-protein interactions (Muhonen *et al.*, 2002; Rubio *et al.*, 1997) and inflammatory mediators (Griffith *et al.*, 2001; Sheng *et al.*, 2000). No publications using the resonant mirror for albumin and creatinine detection exist, however analysis of albumin has been made using a surface plasmon resonance biosensor (SPR) whereby the interaction between human serum albumin and drug compounds were explored (Frostell-Karlsson *et al.*, 2000). SPR is another form of optical biosensor based on exciting plasmons found on the surface of the cuvette (chip), which contains a gold layer of metal. Stocklein *et al.*, (2000) describe of a procedure whereby binding creatinine antibody onto the chip allows for analysis of creatinine deaminase.

1.7 Experimental aims

The overall aim is to design a method of detecting MAU, using creatinine as an index of urine flow. The experiments will concentrate on the use of the resonant mirror and will be addressed by:

- Testing various anti-human serum antibodies in order to optimise binding and detection of albumin in human urine. Detection limits of the assay as well as the reproducibility of the data will be explored.

- Establishing whether multi-well cuvettes offer any advantages over a single well cuvette.

- Designing an assay for the detection of creatinine in human urine. As there is no commercial anti-creatinine antibody available, it is not feasible to employ the strategy that will be used for albumin detection. Therefore an alternative approach using an enzyme, which breaks down creatinine (creatinine deaminase) will be investigated.

Chapter 2
MATERIALS AND METHODS

2.1 Materials

All reagents were supplied from Sigma-Aldrich unless otherwise indicated. Anti-human serum albumin antibody, catalogue number 0220-0704, was ordered from Biogenesis, as was antibody 0219-9970. Anti-human serum albumin antibody, catalogue number A1151, was ordered from Sigma. Anti-ovalbumin antibody, catalogue number 126705, was ordered from CN Biosciences. The carboxymethyl dextran cuvettes, the activation kit containing NHS/EDC and ethanolamine were supplied by BIAcore.

2.2 Method

2.2.1 One Well Immobilisation

1. The carboxymethyl dextran cuvette (catalogue number FCD 3111) was washed in phosphate buffered saline containing 0.05% Tween 20 (pH 7.4) and dried using dry nitrogen. The exact constituents of PBST are shown in appendix A. This was repeated twice. The cuvette was then placed into the manual chipper of the resonant mirror and 170µl of PBST added. The manual chipper allowed for samples to be placed over the cuvette manually while still in the RM (figure 1.2).

2. Once an established baseline was seen using IAsys software, the cuvette was washed with NHS/EDC mixture twice before a final volume of 170µl was added. Stock volumes of 10ml for both NHS (catalogue number BR-1000-50) and EDC (catalogue number BR-1000-50) were frozen and when needed were defrosted. See appendix A for preparation procedures of NHS/EDC. The system was left for two minutes before being washed with PBST.

3. Stage 2 was repeated.

4. The antibody of interest was added to the cuvette after being dissolved in acetate buffer pH 4.5 (catalogue number BR-1003-50) to give a total concentration of 50µg/ml. On addition of the antibody the cuvette was left for ten minutes before being washed three times with PBST ready for the next stage.

5. When a constant baseline had been established, the cuvette was deactivated by the addition of 170µl of 1M ethanolamine (catalogue number BR-1000-50). This was left for ten minutes. Again the cuvette was washed three times in PBST and a final baseline was established. This baseline should be the overall response and hence antibody bound upon the cuvette. The sensitivity of a carboxymethyl dextran (CMD) cuvette is around 200arcsec per ng/mm^2. This number was used to calculate the density of protein immobilised.

2.2.2 Four well immobilisation

1. The carboxymethyl dextran cuvette was washed in PBST and dried using dry nitrogen. This was repeated twice. The cuvette was then placed into the manual chipper of the resonant mirror and 170µl of PBST added.

2. The cuvette was removed from the manual chipper. The multi channel cuvette was assembled as shown in figure 1.3A. The first step was to assemble and align the gasket with the four channel insert using the alignment jig. It was important to ensure that the gasket was aligned with the smooth side against the insert to ensure that liquid did not leak between wells. This was then inserted into the cuvette and the alignment jig removed. This then added to the cuvette clamp, was left for ten minutes before being washed three times in PBST.

3. The antibody of interest was added to the cuvette after being dissolved in acetate buffer pH 4.5 (catalogue number BR-1003-50) to give a total concentration of 50µg/ml. On addition of the antibody the cuvette was left for ten minutes before being washed three times with PBST ready for the next stage.

4. 1M ethanolamine was added and left for ten minutes. It was washed in PBST three times and when all liquid was removed the cuvette was removed from the clamp and gasket.

5. Ethanolamine was again added to the whole of the surface and left for a further couple of minutes to ensure all areas were deactivated. The cuvette was then washed three times in PBST.

In both immobilisations the cuvettes were either used immediately or else sealed and stored in fridge at 4°C until needed for albumin assays or else the cuvette could be used straight away.

2.2.3 Albumin assays

1. Human serum albumin (catalogue number A8763) was diluted in PBST to obtain the desired concentrations. The use of PBST as a diluent avoided the occurrence of bulk refractive change that occurs with the use of different buffers that may mask any binding event which may occur.

2. The manual chipper was removed and the automatic one inserted into the RM. The automatic chipper allowed for samples to be added over the cuvette in a continuous flow. The cuvettte was then inserted and the IAsys software started. An established baseline was ensured before adding any standards onto the cuvette.

3. Albumin standards were added and allowed to run for two minutes. PBST was then added and left for a two minutes. PBST washed any loosely bound albumin as well as non-specific molecules.

4. Regeneration of the cuvette was needed in order for the cuvette to be used again and so 50 mM HCl (supplied by Merck, catalogue number 101252F) was added to the cuvette and left for 1.5 minutes to 2 minutes. This was then immediately washed off with PBST to allow for equilibrium.

5. Another albumin standard was then added and performed in the same procedures as steps 1-4 or else the cuvette was stored for further use by removing the cuvette from the RM, and covered with nescofilm. PBST was added to the cuvette well to ensure the surface would remain active.

2.2.4 Immobilisation of Creatinine Deaminase

1. The carboxymethyl dextran cuvette was washed in PBST and dried using dry nitrogen. This was repeated twice. The cuvette was then placed into the manual chipper of the resonant mirror and 170µl of PBST added. The manual chipper allowed for samples to be placed over the cuvette manually while still in the RM.

2. Once an established baseline was seen using IAsys software, the cuvette was washed with NHS/EDC mixture twice before a final volume of 170ul was added. Stock volumes of 10ml for both NHS and EDC were frozen and when needed were defrosted. See appendix A for preparation procedures of NHS/EDC. The system was left for two minutes before being washed with PBST.

3. 200µg of creatinine deaminase (catalogue number C9409) was dissolved in 800µl of distilled water since the enzyme was in powder form. This stock contained 10 units of enzymatic activity. 150µl of acetate buffer pH 4.5 was added to the cuvette followed by immediate addition of 50µl of creatinine deaminase. This was left for ten minutes before being washed with PBST.

4. 1M ethyldiamine pH 8.5 was added to the cuvette, deactivating any remaining sites not taken up by the enzyme. Ethyldiamine was used because of the presence of an additional amine group compared with ethanolamine. The additional amine group is thought to optimise changes in hydrogen ions when creatinine is broken down by the enzyme. The cuvette was left for ten minutes, washed in PBST three times and when all liquid was removed, the cuvette was removed from the clamp and gasket.

6. Ethyldiamine was again added to the whole of the surface and left for a further two minutes to ensure all areas were deactivated. Finally the cuvette was washed three times in PBST before being used. Storage of the cuvette was at 4°C.

2.2.5 Creatinine assays

1. Creatinine (catalogue number 859702) was diluted in PBST to obtain the desired concentrations. The use of PBST as a diluent avoided the occurrence of bulk refractive change that occurs with the use of different buffers that may mask any binding event which may occur.

2. The manual chipper was removed and the automatic one inserted into the RM. The automatic chipper allowed for samples to be added over the cuvette in a continuous flow. The cuvettte was then inserted and the IAsys software started. An established baseline was ensured before adding any standards onto the cuvette.

3. Creatinine standards were added and left for two minutes. PBST was then added and left for a two minutes. PBST washed any creatinine which had not been broken down by the enzyme and restored the baseline back to zero.

4. Regeneration of the cuvette by HCl was not necessary. Another standard was then added and performed in the same procedures as steps 1-3 or else the cuvette was stored for further use by removing the cuvette from the RM, and covering with nescofilm. PBST was added to the cuvette well to ensure the surface would remain active.

2.2.6 Equipment

The RM was specially modified from the commercial instrument by Affinity Sensors in collaboration with dstl, for the use of developing a detection device in this project. The modifications included the ability to change the chipper from automatic to manual. Valves were inserted to allow for a continuous flow system to be set up as well as the introduction of running buffer. The software used with the device- IAsys, was adapted for the use of the modified RM. It allowed for 16 individual channels to be monitored instead of an average response being shown across the whole of the cuvette. The software also allowed for the data to be imported into BIAevaluation for the statistical analysis of initial rate to be determined as well as the construction of standard curves. BIAevaluation is a commercial package available form BIAcore. While the cuvette was of standard design the gasket was especially adapted for the introduction of four wells.

Chapter 3
RESULTS - ANALYSIS OF ALBUMIN

The main focus behind the experiments conducted was to determine the feasibility of the resonant mirror (RM) as a tool for the detection of albumin and creatinine in urine. Various developmental stages were investigated where both advantages and disadvantages of the device were explored.

3.1 Albumin detection in urine samples using the RM biosensor

3.1.1 Optimisation of antibody binding conditions

For the RM to pose any great benefits, the capturing ligand attached to the sensor surface of the cuvette, must be pure, bind in such a configuration so as to not obstruct the binding site, have a high enough affinity towards the analyte of interest and remain unaffected after several regenerations. Regeneration of the cuvette surface, allows for the re use of the cuvette. A range of anti-human serum albumin antibodies were investigated from a range of suppliers. Preliminary experiments using anti-human serum albumin (Biogenesis cat. number 0220-0704) had shown this antibody to be a viable capturing ligand for human serum albumin (Fisher 1998-data not published). Attachments of the antibody over the cuvette (one well immobilisation) as well as striping of the cuvette (four well immobilisation) were performed.

A. One well immobilisation

Antibody 0220-0704 was attached to the whole cuvette surface with the use of an automatic chipper. The automatic chipper allowed for the monitoring, using the RM, of the activation, binding and deactivation stages involved in the cuvette preparation. Each stage is described in further detail below.

ACTIVATION-The cuvette was washed, inserted into the RM and a baseline was established using PBST (Figure 3.1:A). Addition of NHS/EDC mixture caused a change in refractive index and a sharp inflection in response was noted which then later equilibrated to around 1000-2000 arcsec (Figure 3.1:B). It is here that carboxymethyl groups present on the cuvette were converted to active NHS-esters i.e. activation occurred. This stage was repeated, indicated by a slight increase in response (Figure

3.1:C) and the surface was then washed in PBST allowing a second baseline to establish (Figure 3.1:D). The difference in response between the original baseline and the latter was due to the change of the surface of the cuvette by the amine coupling procedure.

BINDING-Here the antibody 0220-0704, which was dissolved in acetate buffer (pH4.5), was added to the activated surface of the cuvette. Subsequently, the primary amine groups of the antibody displaced the NHS, resulting in the formation of peptide bonds between the protein and the carboxymethyl dextran surface of the cuvette (Figure 3.1: E). The low buffer pH ensured the increased electrostatic attraction between the antibody and the surface of the cuvette. Again a wash using PBST was performed removing any loosely bound antibody or unwanted compounds (Figure 3.1: F).

DEACTIVATION-In this last stage, 1M ethanolamine was added to block any remaining activated sites by attaching NH_2^+ groups present in the ethanolamine mixture to COO^- via displacement of NHS. The addition of ethanolamine was characterised by a sudden rise in refractive index, which plateaued at about 5000arcsec (Figure 3.1: G). The cuvette was later washed with PBST and a baseline of around 2724arcsec (Figure 3.1: H) was seen. The baseline corresponds to the protein density or total antibody bound onto the cuvette, which in this experiment was 13.62ng/mm^2. This was calculated according to the manufacturer's guidelines that stated 200arcsec response to be equivalent to 1ng of bound protein/mm^2.

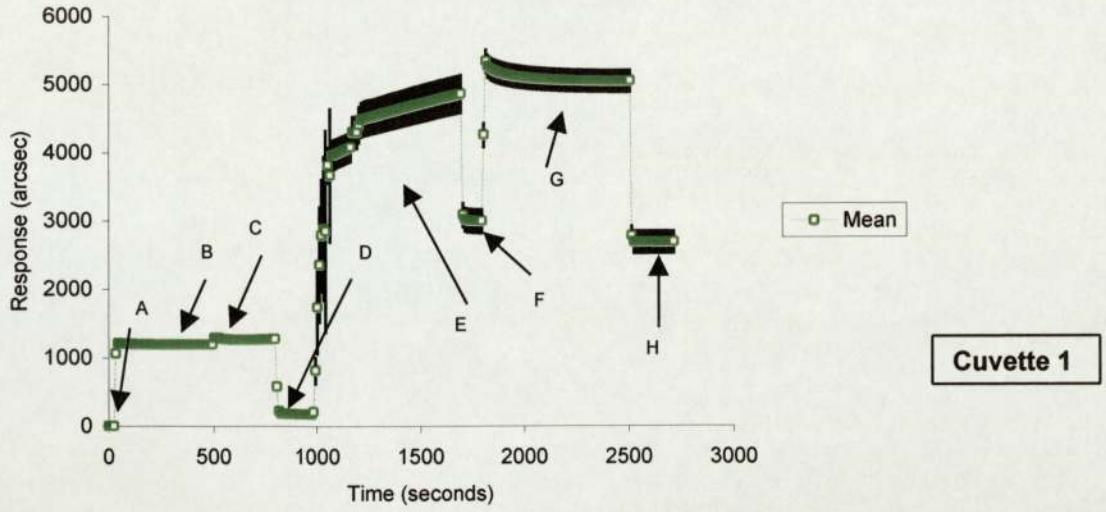


Figure 3.1. The mean response obtained when attaching antibody 0220-0704 onto the cuvette surface. All three stages are highlighted in section 4.1.1 A. A shows the initial baseline, B shows the response obtained when the cuvette is activated, C shows a second activation. D shows the baseline obtained when the cuvette is washed with PBST, E refers to the response obtained when antibody 0220-0704 is placed over the cuvette well, F denotes the third baseline where excess unbound antibody is washed of the cuvette surface, G shows the deactivation of the cuvette and H shows the final baseline where everything is removed from the surface cuvette leaving the bound antibody 0220-0704 covalently attached. Error bars, shown in black, represent the standard deviation of the mean.

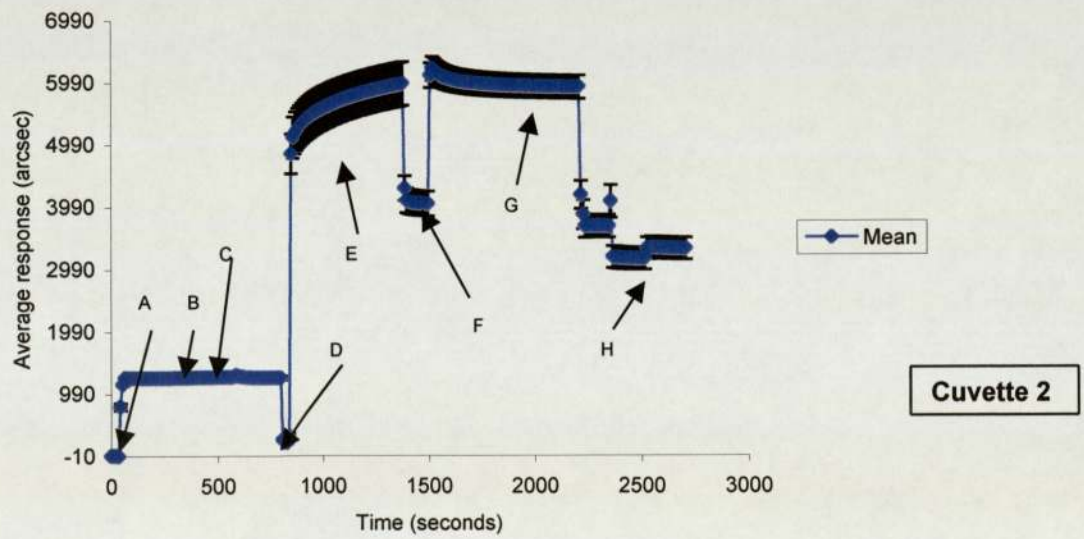


Figure 3.2. The mean response obtained when antibody 0220-0704 is immobilised onto a cuvette. A shows the initial baseline, B shows the response obtained when the cuvette is activated, C shows a second activation. D shows the baseline obtained when the cuvette is washed with PBST, E refers to the response obtained when antibody 0220-0704 is placed over the cuvette well, F denotes the third baseline where excess unbound antibody is washed of the cuvette surface, G shows the deactivation of the cuvette and H shows the final baseline response equating to bound antibody.

Binding of antibody 0220-0704 was repeated on another cuvette from a different batch (figure 3.2). Figures 3.1 and 3.2 demonstrate that both cuvettes show very little variation between 16 channels across the sensor surface. Table 3.1 shows the standard deviation (SD) and standard error of the mean (SEM) response for both cuvettes.

	Mean response (arcsec)	SD (arcsec)	SEM (arcsec)	T	Probability of T
Cuvette 1 16 channels	2695	161	40		
Cuvette 2 16 channels	2987	169	42		
				-4.28	0.22

Table 3.1. Final response of total bound antibody 0220-0704 using one well immobilisation.

An independent two way T-Test was performed to compare the differences between the responses seen in cuvette 1 and 2. The results are shown in table 3.1. T represents the t value i.e. the standard difference between each measurement and P shows the probability of there being a significant difference. T is –4.28 however the probability of this occurring by chance is 0.22 suggesting there to be no real significant difference between the two cuvettes.

A. Four well immobilisation

The gasket divided the cuvette surface into four individual wells. Each of the four wells contained four channels. With the introduction of a gasket it was possible to incorporate a blank (PBST) and a negative control (anti-ovalbumin antibody). Figure 3.3 shows an outline of the wells with respect to the cuvette, highlighting the contents of each well.

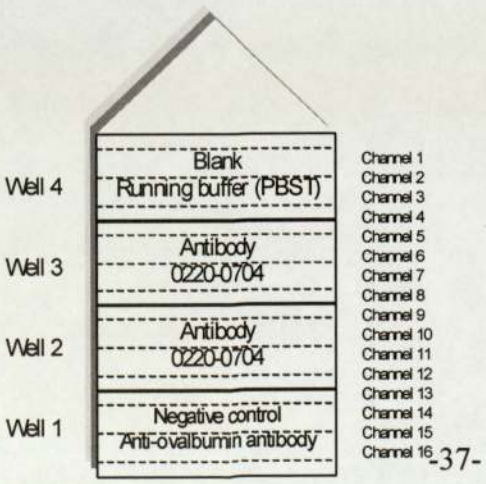


Figure 3.3. Schematic diagram, showing the positions of wells on the cuvette surface. The positioning of the antibody with respect to the wells and channels are also shown.

Using the gasket to divide the cuvette surface into four wells meant that the cuvette could not fit within the chipper of the RM. This prevented the monitoring of the various ligands being bound onto the cuvette surface. The only stage that did not require the use of the gasket was the activation stage. Figure 3.4 outlines the activation stage performed on a cuvette.

The inability to monitor the final response of bound ligand in each channel made it difficult to determine or even compare the amounts of bound molecules. The experiment was repeated upon another cuvette where figure 3.5 shows the mean response for the activation stage. Table 3.2 compares the mean, SD and SEM of the two cuvettes for this stage. The mean activation response between the two cuvettes varies by about 100 arcsec even though the same amount of reagents was exposed to the cuvette.

	Mean response (arcsec)	SD (arcsec)	SEM (arcsec)
Cuvette 1	153	18	5
Cuvette 2	253	30	8

Table 3.2. The average activation responses of two separate cuvettes are compared for variability.

In summary immobilisation of antibody 0220-0704 over a one well cuvette showed sufficient binding with use of the amine-coupling chemistry used to activate the well surface. Consistent amounts of antibody 0220-0704 were bound with the use of a one well cuvette however binding of antibody 0220-0704 over a four well cuvette could not be monitored visually because of the arrangement of the gasket upon the cuvette. Monitoring of the activation stage could be performed on a four well cuvette and ensured that the surface was activated, however a difference was observed in the activation response between the two different cuvettes.

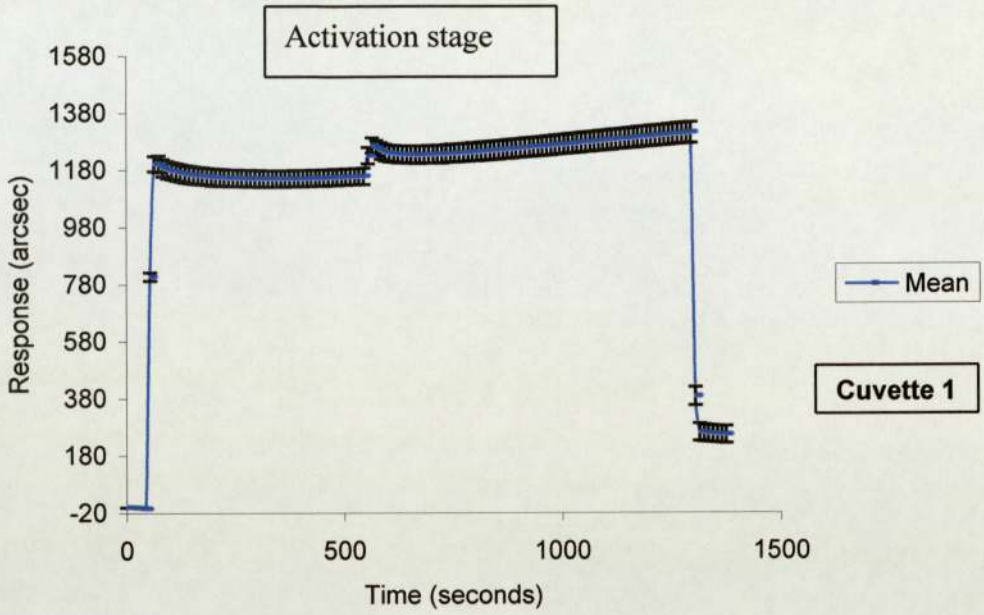


Figure 3.4. The mean response obtained when activating the cuvette surface with NHS/EDC. Error bars, shown in black, represent the standard deviation of the mean response.

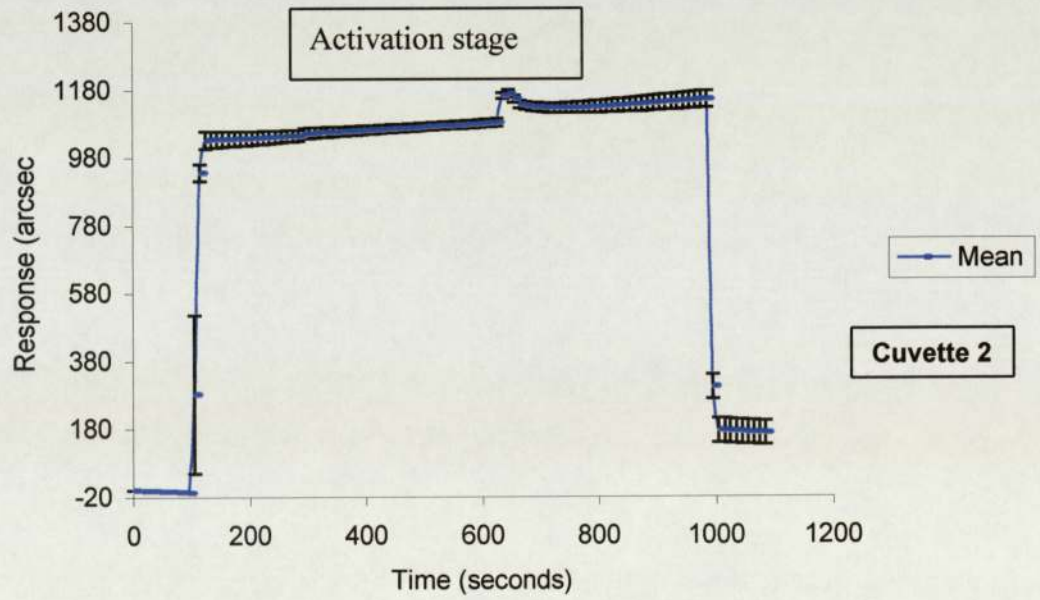


Figure 3.5. The mean response obtained when activating the cuvette surface withNHS/EDC. Error bars, shown in black, represent the standard deviation of the mean response.

3.1.2 Validation of the RM using albumin standards

A. One well cuvette

Seven albumin standards ranging from 1-100µg/ml [HSA] were passed over the cuvette and the responses recorded using the RM. A wide range in [HSA] was used in order to determine the detection limits of antibody 0220-0704. Figure 3.6A-N shows the responses of each albumin standard for channels 1-8 and 9-16. The responses have been divided into the upper (channels 1-8) and lower regions of the cuvette to emphasize the difference in response seen. Figure 3.7A-G shows the average response for each albumin standard. Only the association phase is shown in both figures, as it is the gradient of the initial slope that is later calculated in order to determine the calibration curve (section 1.5.3). While the SD and SEM are low in comparison to the mean (table 3.3), variations in responses were seen between channels especially towards the lower half of the cuvette (channels 9-16). Each standard was repeated and the mean response was averaged. It is the average response which is shown on table 3.3. Appendix C outlines the repeated responses obtained.

A paired t-test was performed to determine if any differences were observed between the two repeats for each albumin standard. A t-value of –3.59 was obtained with a probability of 0.5 suggesting there to be no significant difference between the two sets of repeats. This would suggest reliability of data with the use of the RM in concentration determination. The initial rate determined for each response was expressed as a calibration curve (figure 3.8). The initial rate was calculated by dividing time by the gradient of response obtained during that time frame. The assay is reproducible and a linear relationship is observed between [HSA] and initial rate.

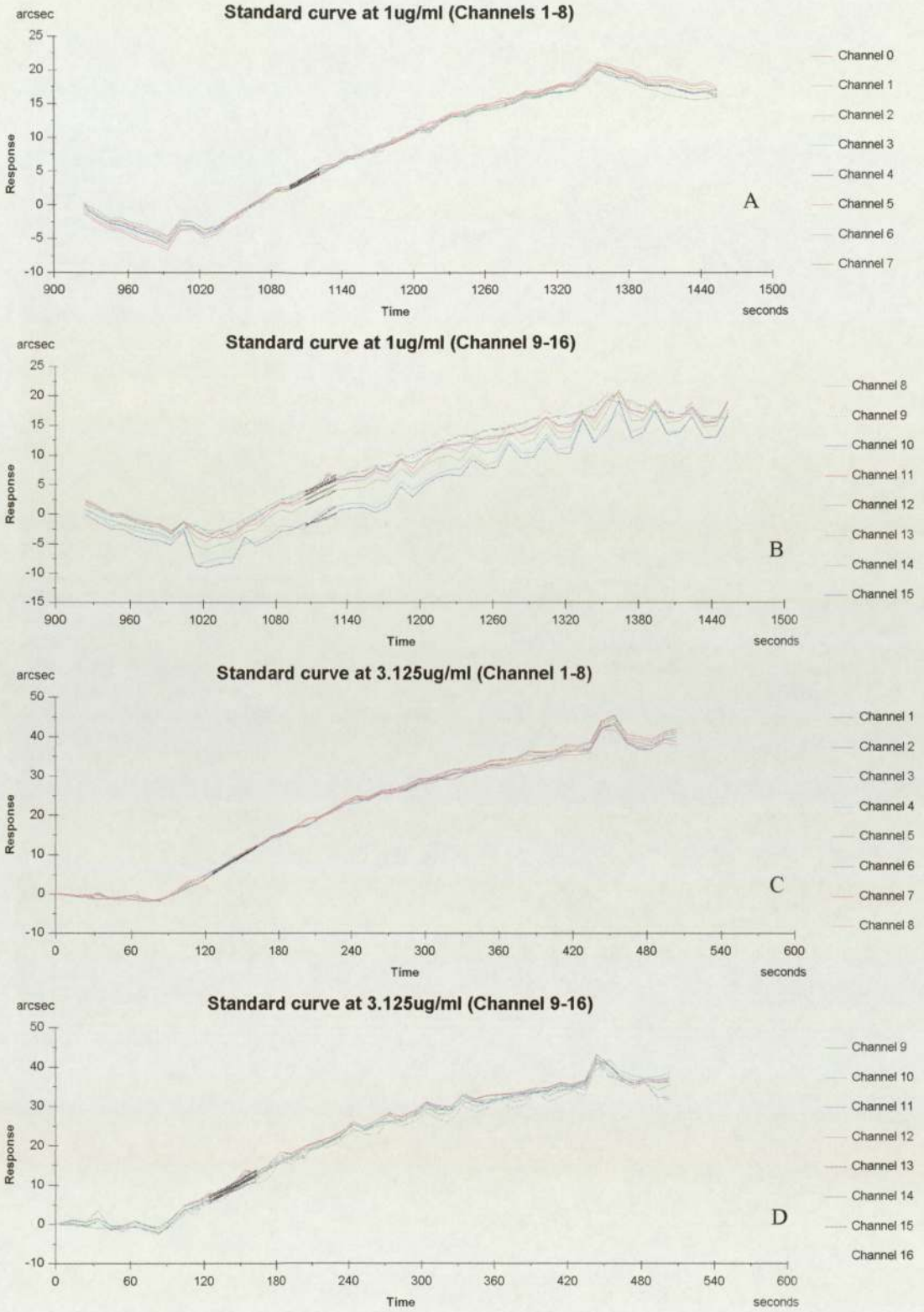


Figure 3.6 A-D. The association stage of each albumin standard is shown. The channels are shown in two groups of eight to explain the difference in response along the cuvette. The black lines indicate the segment where the gradient was taken using BIAevaluation software.

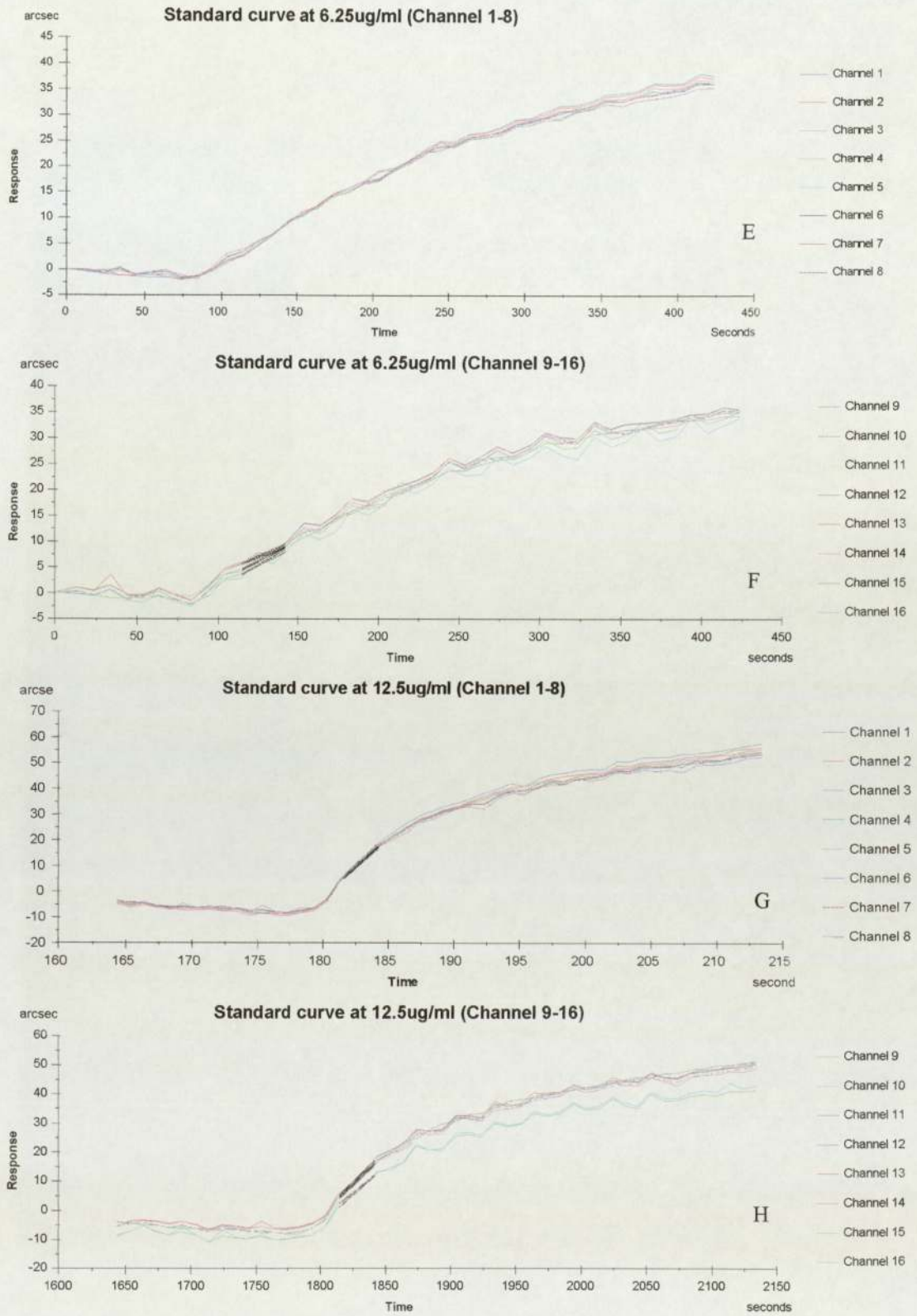


Figure 3.6 E-H. The association stage of each albumin standard is shown. The channels are shown in two groups of eight to explain the difference in response along the cuvette. The black lines indicate the segment where the gradient was taken using BIAevaluation software.

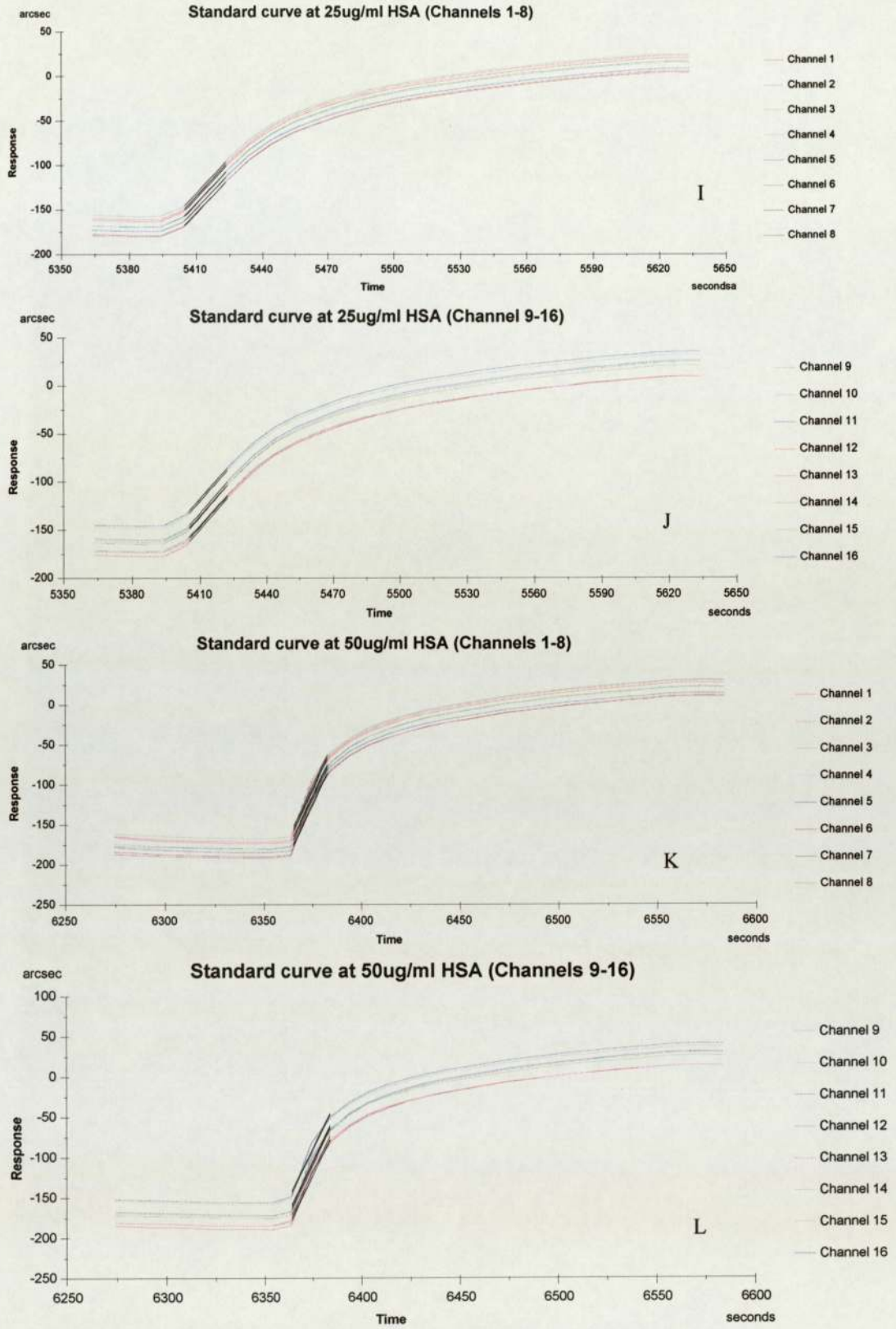


Figure 3.6 I-L. The association stage of each albumin standard is shown. The channels are shown in two groups of eight to explain the difference in response along the cuvette. The black lines indicate the segment where the gradient was taken using BIAevaluation software.

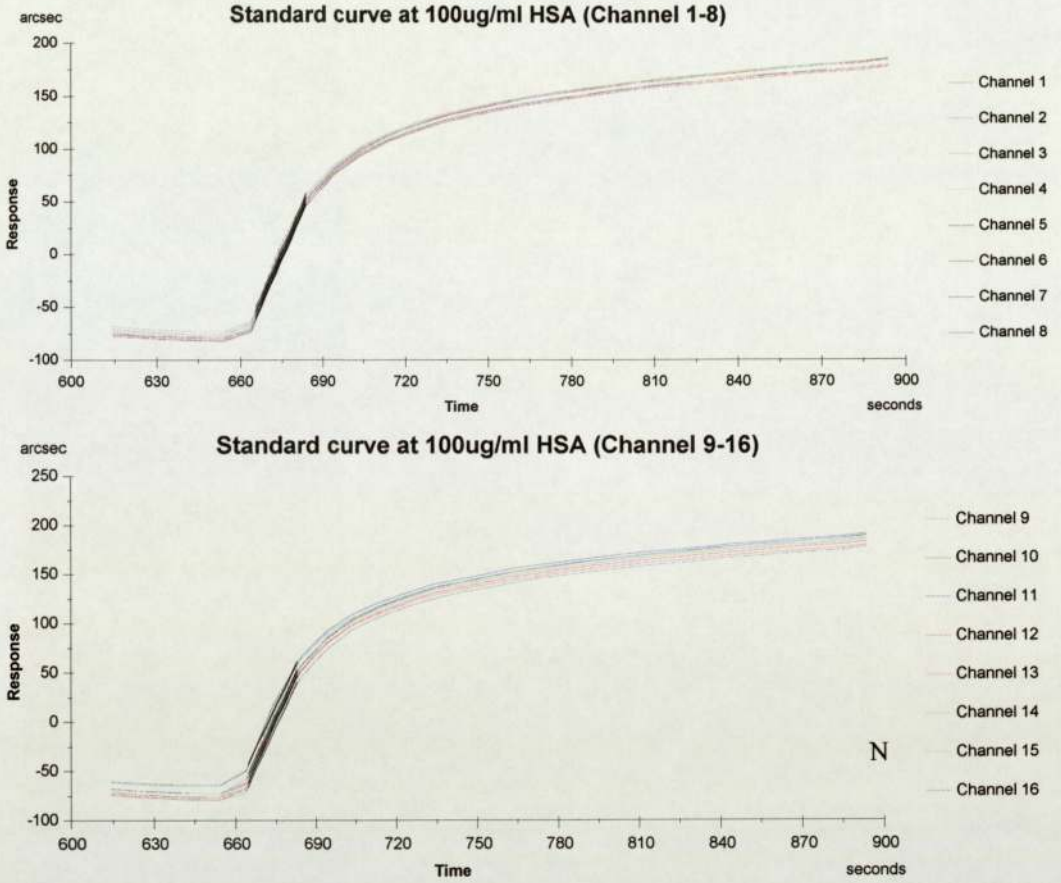


Figure 3.6 M-N. The association stage of each albumin standard is shown. The channels are shown in two groups of eight to explain the difference in response along the cuvette. The black lines indicate the segment where the gradient was taken using BIAevaluation software.

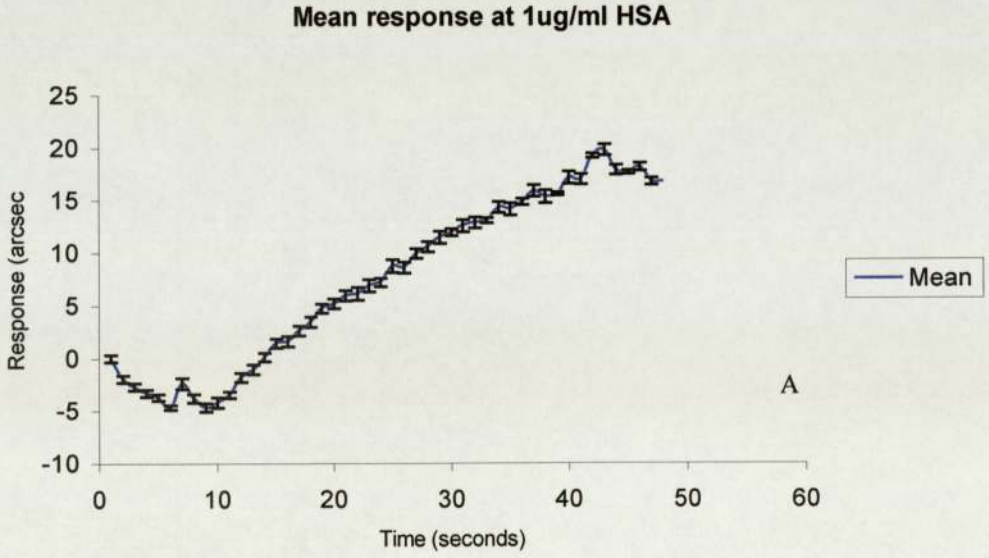
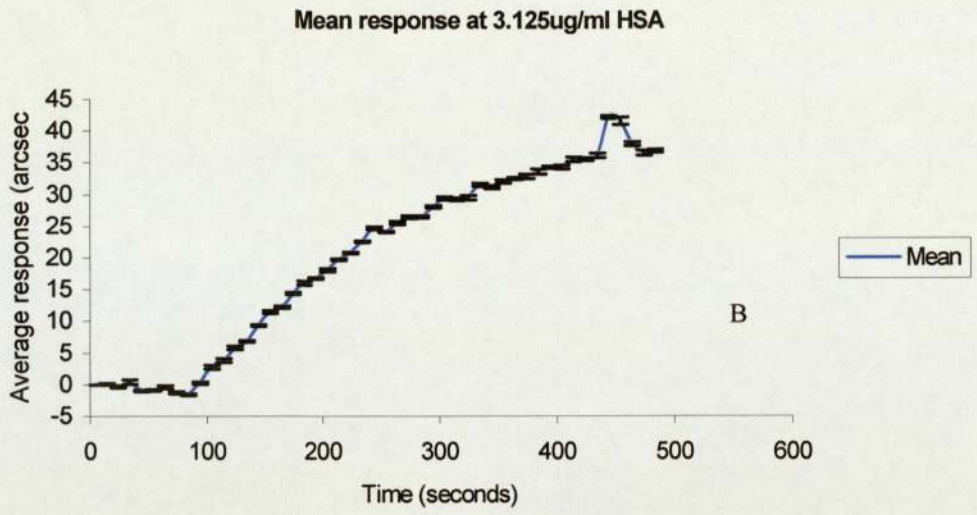
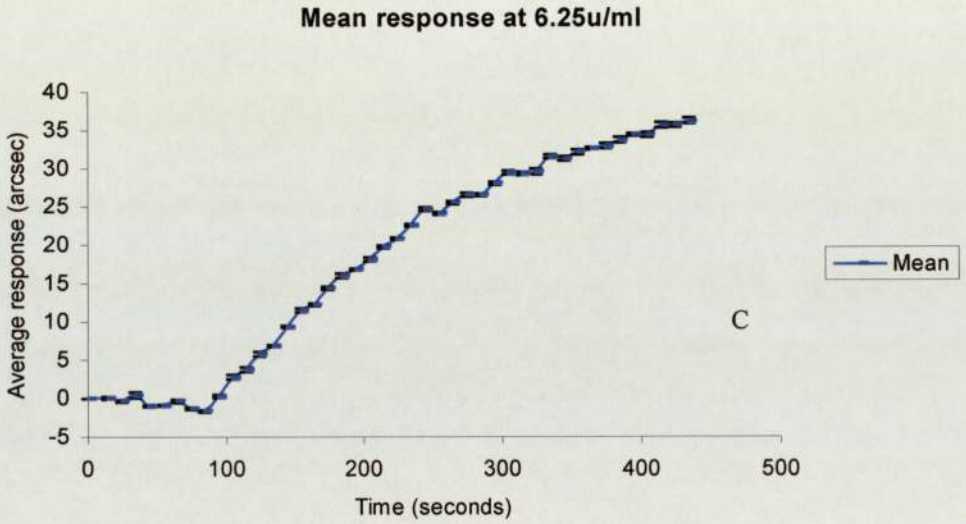


Figure 3.7A. A mean response was taken of all 16 channels obtained for each albumin standard. The black arrows denote the standard error of the mean.





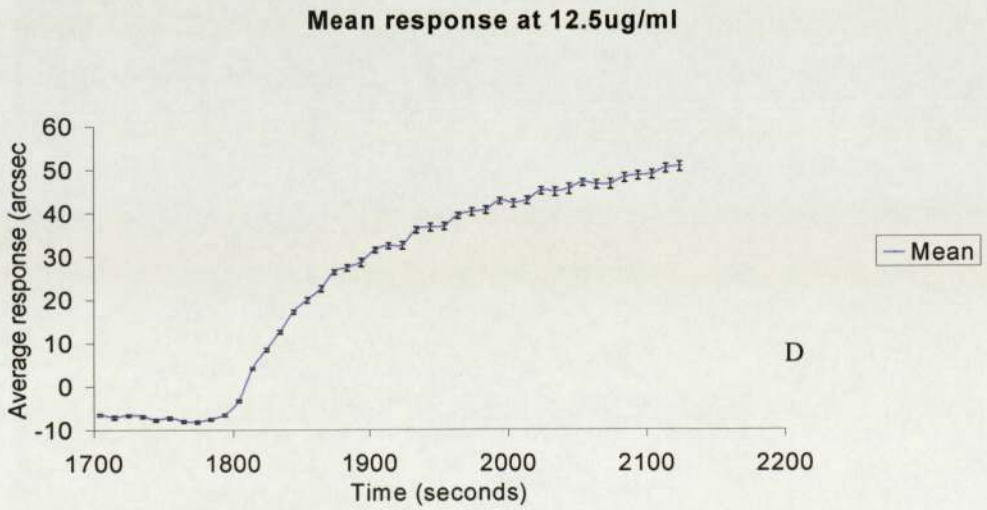
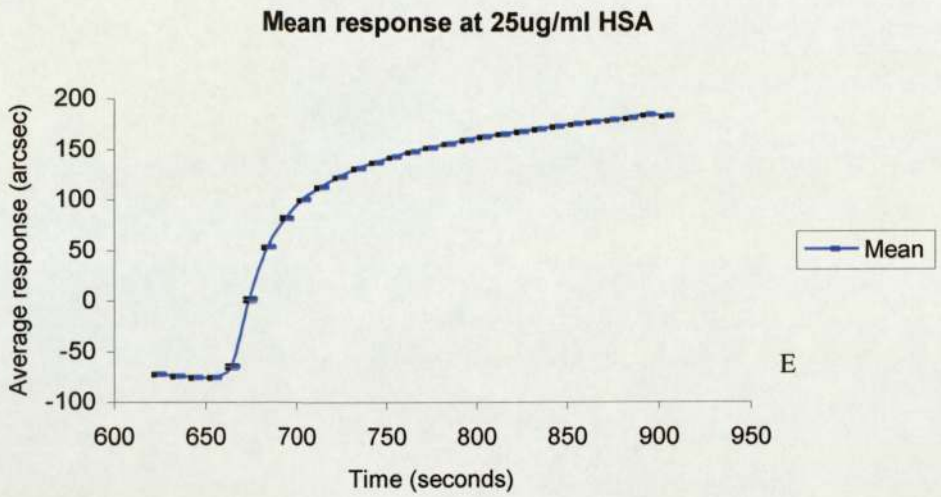
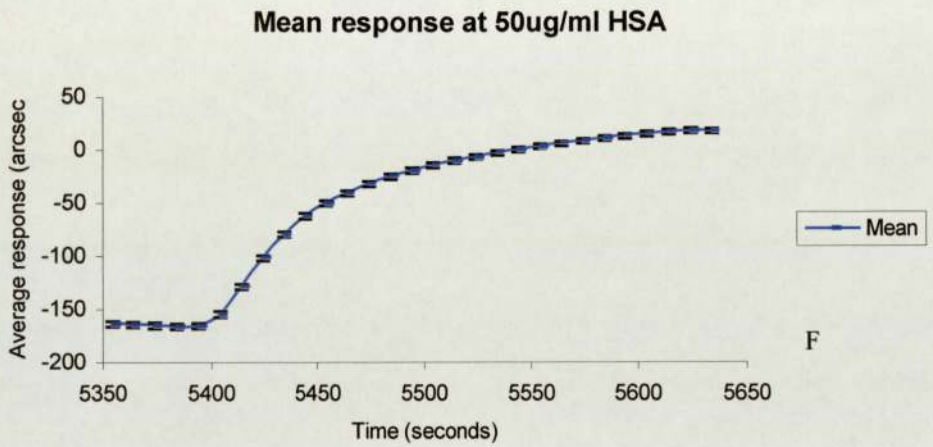


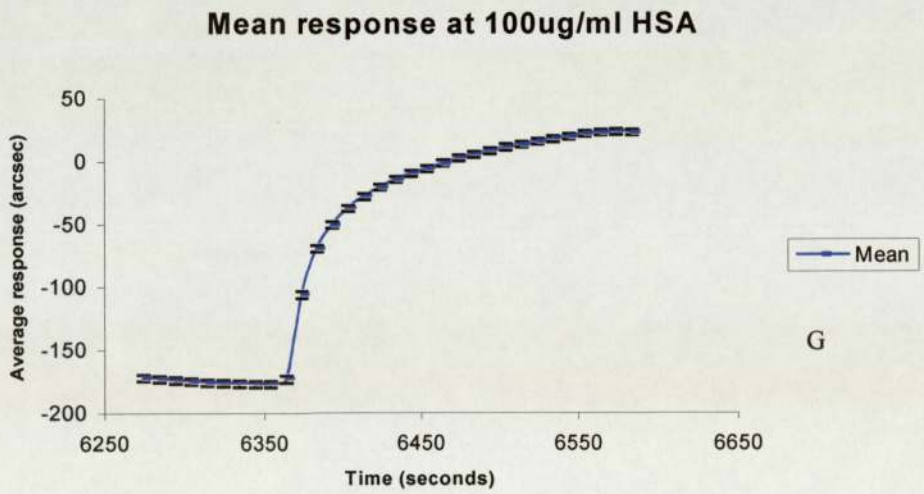
Figure 3.7 B-D. A mean response was taken of all 16 channels obtained for each albumin standard. The black arrows denote the standard error of the mean.



E



F



G

Figure 3.7. A mean response was taken of all 16 channels obtained for each albumin standard. The black arrows denote the standard error of the mean.

Initial Rate	[HSA] (µg/ml)					
(arcsec/sec)	**1.5625**	**3.125**	**6.25**	**12.5**	**50**	**100**
CH1	0.085	0.199	0.22	0.485	0.974	2.180
CH2	0.083	0.200	0.214	0.467	0.963	2.170
CH3	0.075	0.190	0.212	0.459	0.930	2.140
CH4	0.097	0.182	0.211	0.452	0.920	2.070
CH5	0.096	0.181	0.209	0.446	0.927	2.060
CH6	0.100	0.176	0.196	0.449	0.923	2.040
CH7	0.106	0.179	0.188	0.460	0.937	2.050
CH8	0.091	0.172	0.173	0.445	0.908	1.950
CH9	0.071	0.175	0.179	0.437	0.935	2.060
CH10	0.111	0.176	0.165	0.427	0.899	1.860
CH11	0.107	0.174	0.157	0.423	0.884	1.830
CH12	0.112	0.166	0.136	0.439	0.869	1.830
CH13	0.098	0.178	0.134	0.443	0.854	1.820
CH14	0.094	0.149	0.127	0.422	0.816	1.750
CH15	0.131	0.134	0.155	0.395	0.863	1.60
CH16	0.073	0.13	0.161	0.365	0.884	1.690
Mean response	0.096	0.173	0.177	0.438	0.905	1.940
SD	0.016	0.019	0.030	0.029	0.042	0.178
SEM	0.004	0.005	0.008	0.007	0.010	0.045

Table 3.3. The initial rate for each albumin standard is shown above for all 16 channels. The values are averaged from the experiment repeated twice. SD defines the standard error of the mean while SEM denotes the standard error of the mean.

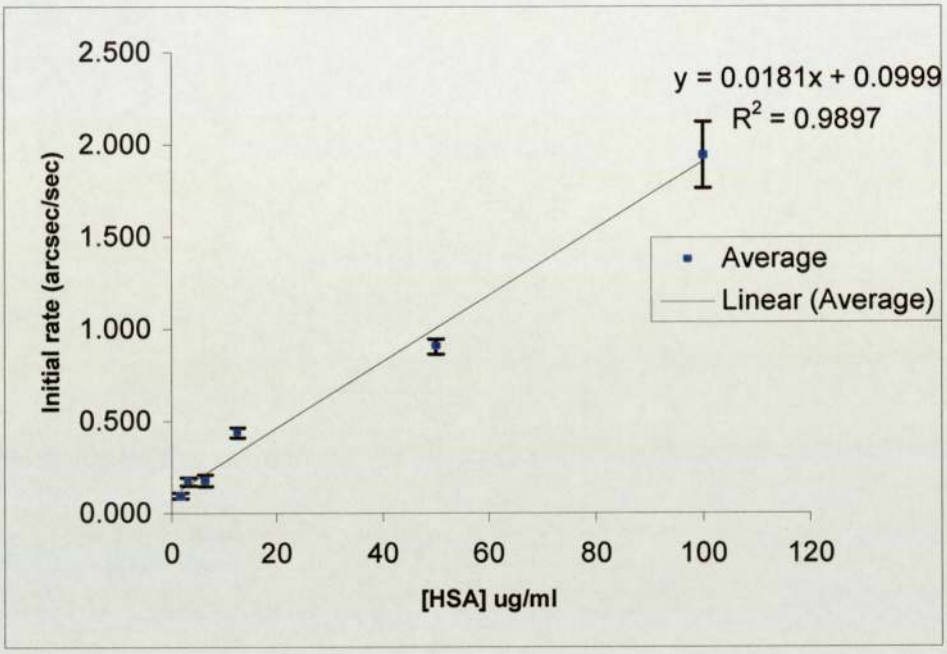


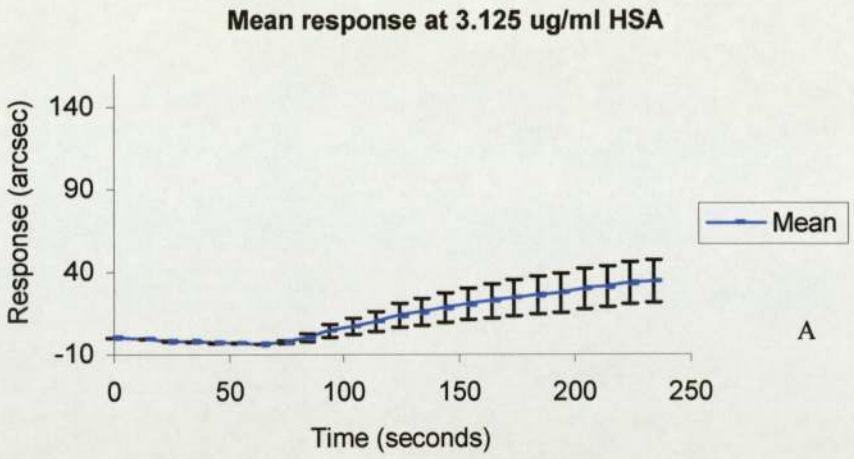
Figure 3.8. Calibration curve showing a linear response between the initial rate and [HSA] derived from table 3.3. The initial rate is calculated by calculating gradient of slope of association phase. The equation represents the linear relationship of y=mx+c where m (gradient) is 0.0181 and c (intercept) is 0.0999. R^2 is the correlation of line of best fit.

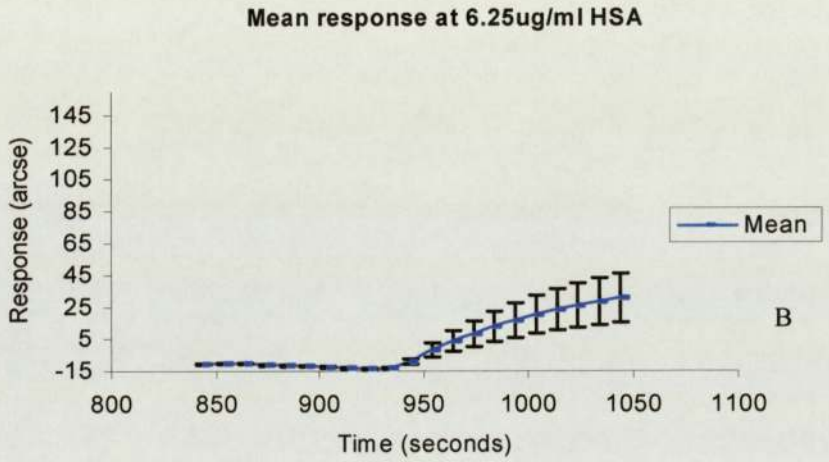
B. Four well cuvette

Albumin standards used in section 3.1.2 were also used to determine the response using a four well cuvette. For all albumin standards, apparent inconsistencies were observed between channels in separate wells. As each well contained one capturing ligand it would seem appropriate for all channels in that well to act in concert. For example, no antibodies were immobilised in well 4 and so no responses should be seen in channels 1 to 4. Nevertheless responses were observed in channels 3 and 4. Similarly in well 3, channel 5 and 6 showed a response corresponding to the [HSA]. Channel 8 showed no response and channel 7 showed an intermediate response. Well 3 contained bound 0220-0704 and thus channel 5 through to 8 should all show similar response values. In well 2, which also contained bound antibody 0220-0704, responses were only seen in channels 9-11, where channel 12 showed no response. A response occurred in channel 13 but not in 14-16, in well 1. Well 1 contained the negative control anti-ovalbumin antibody. The raw data illustrating the observed differences between channels is seen in appendix D.

Previous experiments (figure 3.6) using a one well cuvette have shown all 16 channels to respond to varying albumin standards with little disparity. The use of a four well cuvette nevertheless caused inconsistent readings of the channels in the same well. Only selected channels were used for further analysis from wells 2 and 3 to represent the response using 0220-0704. These were channels 5,6,10, 11, 12. Channels 14, 15 and 16 were used as controls. These channels were favoured based on the maximal response obtained or with the case of control, no response.

Averages were taken of the channels within the wells and the response of the control was then subtracted from the response with antibody 0220-0704 using BIAevaluation. Figure 3.9 shows mean response. The initial slope of the association phase was then calculated and shown in table 3.4. The data is represented by a calibration curve (figure 3.10) where the error bars signify the SEM. The error bars in figure 3.10 are masked by the data points. To show that a significant difference existed between the response values in wells 2/3 to well 1 (control), an ANOVA test was performed. The test was used to to verify that the responses generated were true values and not similar to control values. The F ratio (variance) obtained was 44.74 where the probability was below 0.05 showing there to be a significant difference between the response and the control.





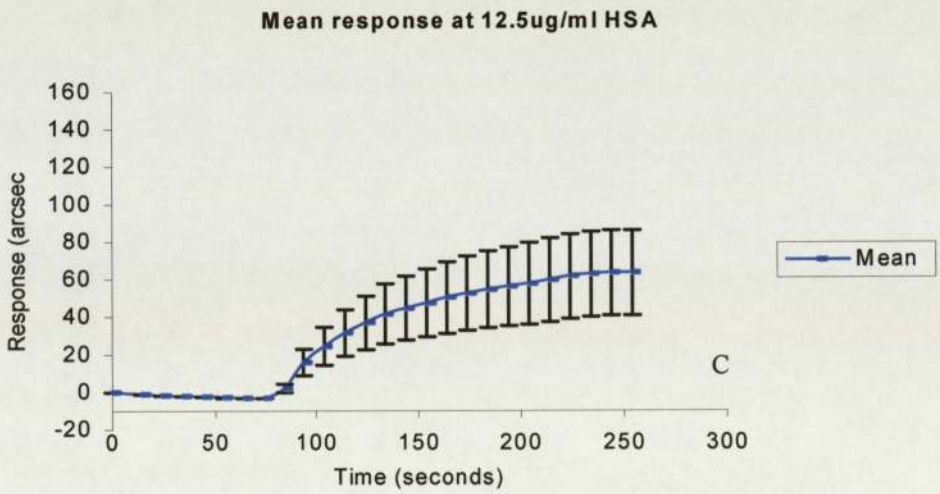
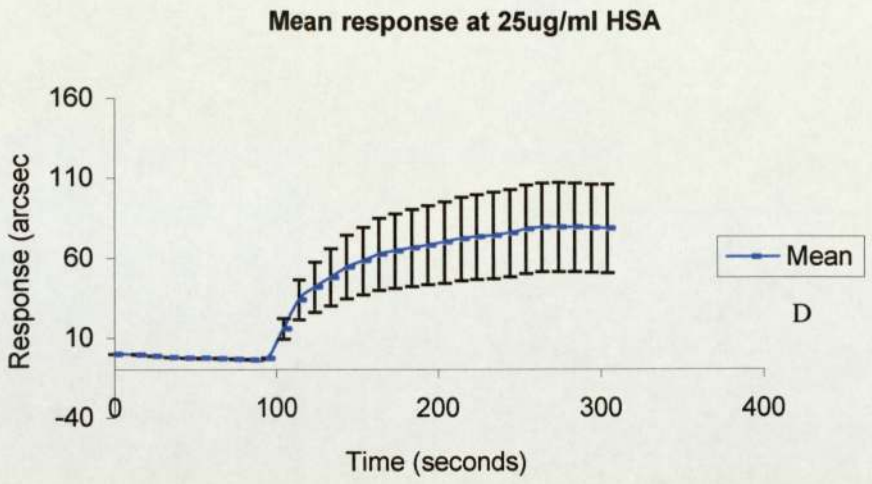
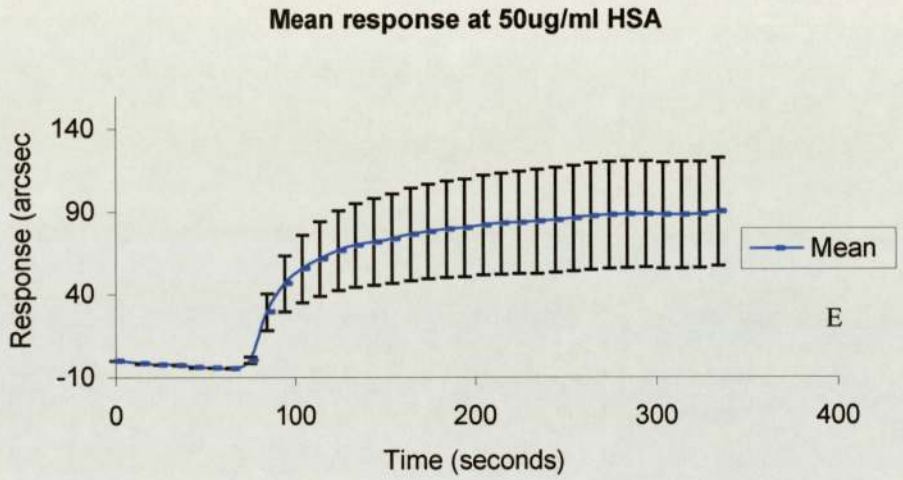


Figure 3.9 A-C. A mean response was taken of channels 5,6,10,11 and 12 obtained for each albumin standard. The black arrows denote the standard error of the mean.





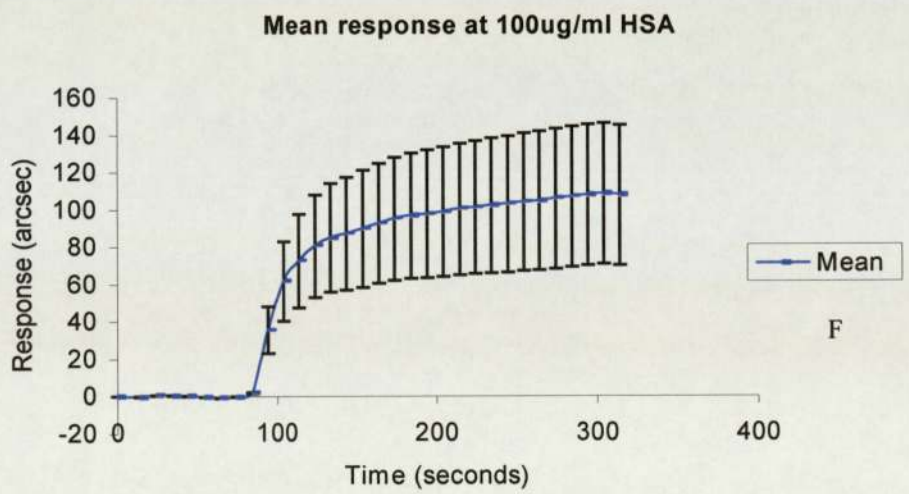


Figure 3.9 D-F. A mean response was taken of channels 5,6,10,11 and 12 obtained for each albumin standard. The black arrows denote the standard error of the mean.

[HSA] ug/ml	Response (arcsec)					Mean Response (arcsec)	SD (arcsec)	SEM (arcsec)
	CH5	CH6	CH10	CH11	CH12			
3.125	0.52	0.60	0.58	0.764	0.64	0.62	0.09	0.04
6.25	0.89	0.75	0.705	0.887	1.04	0.85	0.13	0.06
12.5	1.30	1.69	1.58	1.88	2.19	1.73	0.33	0.15
25	2.33	2.86	2.58	2.33	3.33	2.69	0.42	0.19
50	2.73	3.59	3.26	3.05	4.27	3.38	0.59	0.26
100	4.48	5.92	4.18	4.42	5.10	4.82	0.70	0.31

Table 3.4. The gradient of the association phase is shown for each albumin standard. CH refers to the word channel. The responses represent the average of two experiments.

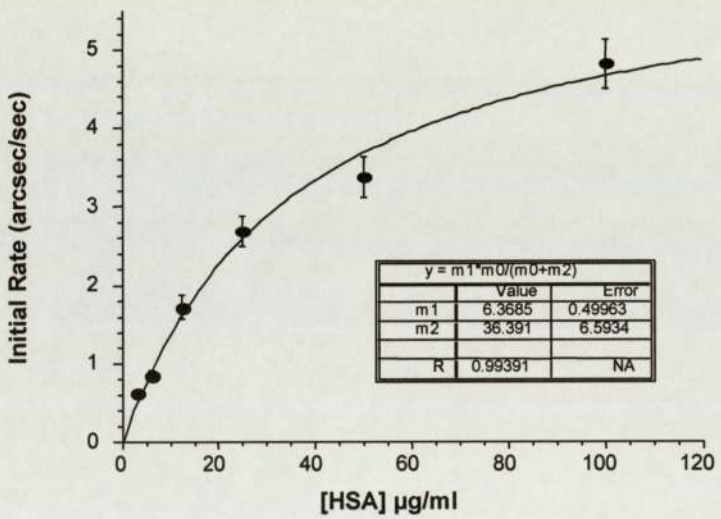


Figure 3.10. The calibration curve shows a saturation response between the initial rate and albumin concentration using a four well cuvette. The equation represents a saturation relationship where the equation y=m1 x mo / (mo + m2) defines the model. M1 denotes Bmax, mo refers to the free [HSA] and m2 is the Kd. R denotes the correlation of line of best fit.

3.1.3 Test samples and quality controls

Having established that antibody 0220-0704 was sensitive enough to detect albumin between 1-100µg/ml, further studies were conducted concentrating on [HSA] within the diagnostic range for MAU. MAU in human urine is said to range from 20-300µg/ml (Feldt-Rasmussen *et al.*, 1994; De Gaudio *et al.*, 1999). It was thought advisable to measure human urine samples in a 1:10 dilution with buffer (PBST). The dilution would buffer against any pH changes as well as dilute any interfering compounds that may be present. Therefore for the purpose of this experiment, the diagnostic range needed was 2-30µg/ml. Although a one well set up clearly gave consistent readings between channels, a four well cuvette was employed as it incorporated both the control and blank.

The cuvette was prepared in a similar fashion as described in section 3.1.1.B. Figure 3.11 shows the response for the activation stage. The layout of the cuvette with respect to the immobilised antibody is shown in figure 3.12.

Inconsistencies existed between channels of the same well throughout each albumin concentration. In well 4, channel 1 and 2 showed no response as expected, since this was a blank well. Channel 3 and 4 however showed an elevated response. Inconsistent responses also existed in well 2 which had contained antibody 0220-0704. In this well, only channels 9 and 10 showed a response and channels 11 and 12 showed no response. No inconsistencies were seen in either well 1 (negative control) or well 3 (antibody 0220-0704). The raw data, showing the inconsistencies between channels of well 2 and 4 are illustrated in appendix E.

Again only selected channels were used to represent responses seen with the use of antibody 0220-0704. While channels 9 and 10 showed a response, channels 5 to 8 only were used for further analysis. This was because channel 9 and 10 showed intermediate responses. Channel 11 and 12 were not chosen due to their lack of response. The average response for channels 5 to 8 is shown in figure 3.13. The association phase relating to each albumin concentration is underlined in black. Figure 3.14 shows the calibration curve where table 3.5 outlines the data for the initial rate. Two calibration curves were plotted, based on the same data in table 3.5, to consider the fitting of both a curved and linear model.

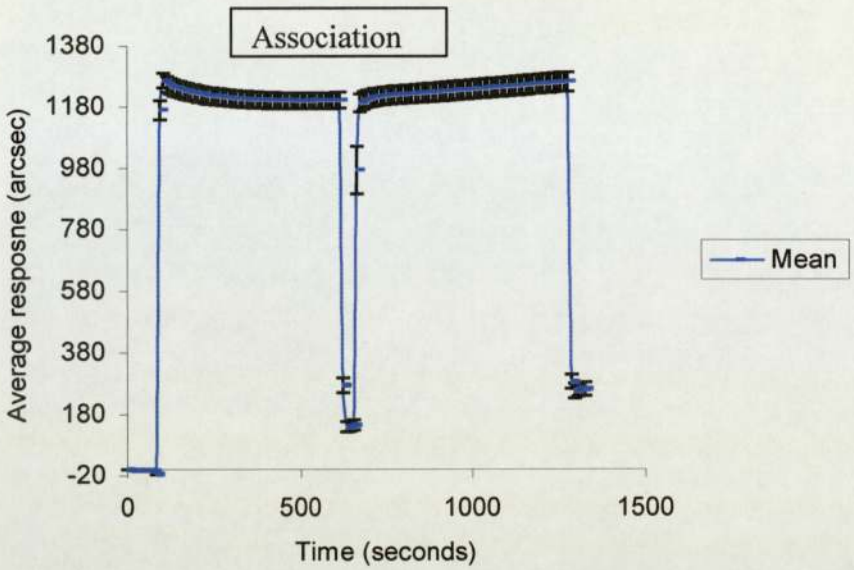


Figure 3.11. The mean response obtained when activating the cuvette surface with NHS/EDC. Error bars, shown in black, represent the standard deviation of the mean.

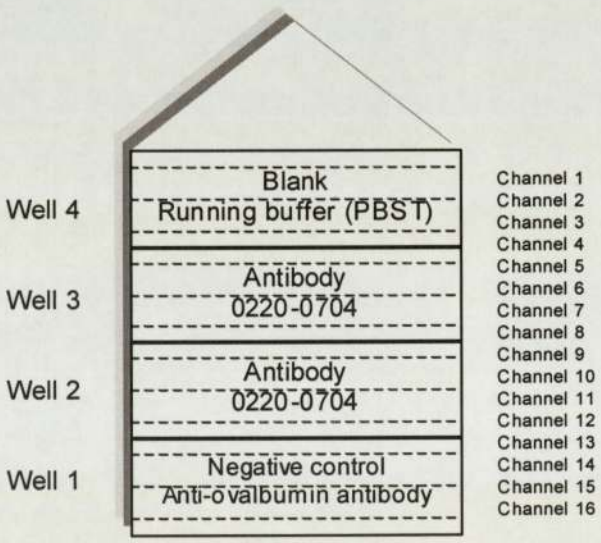


Figure 3.12. Schematic diagram of the cuvette showing the layout of the wells and channels. The positioning of the antibody with respect to the wells and channels are also shown.

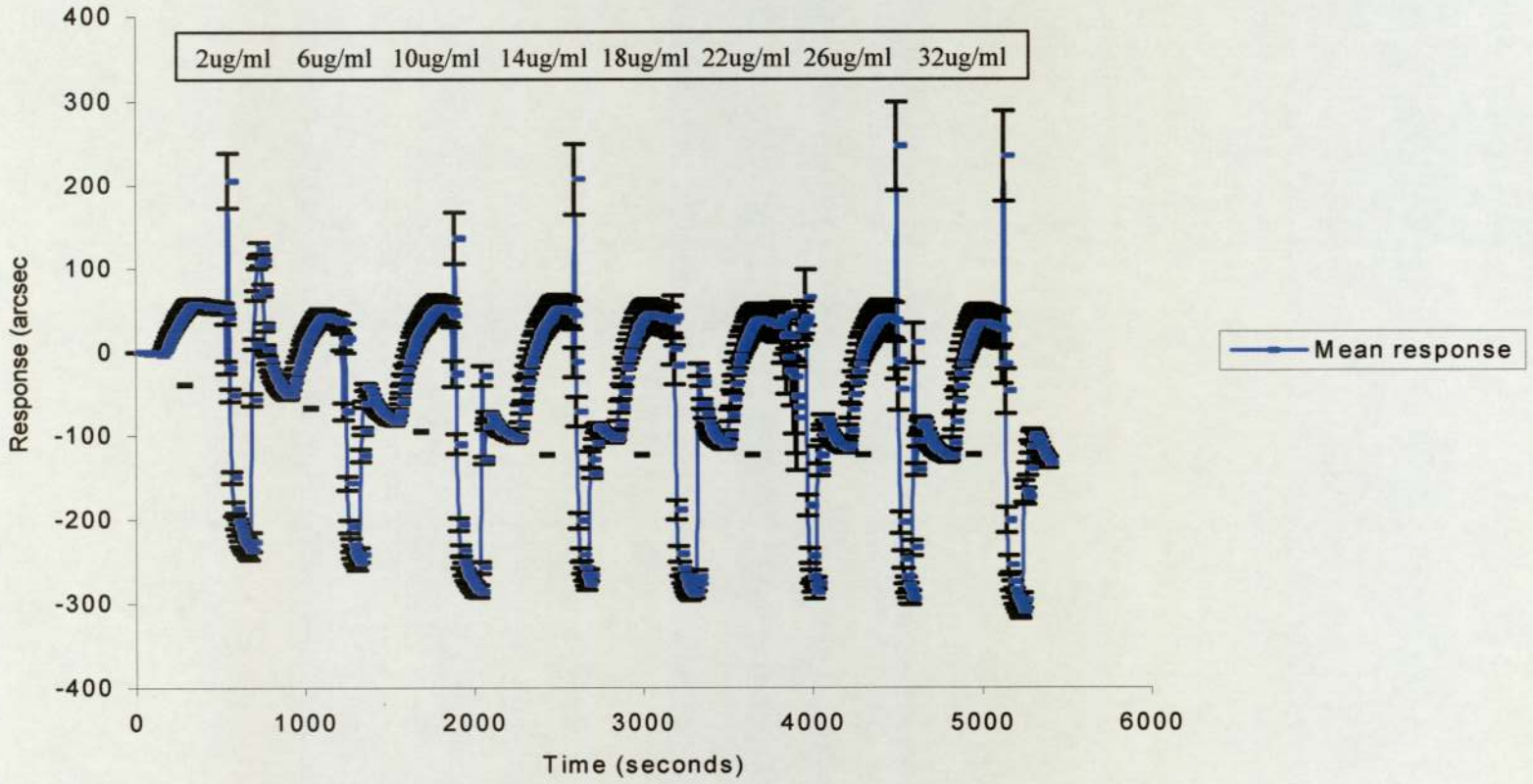
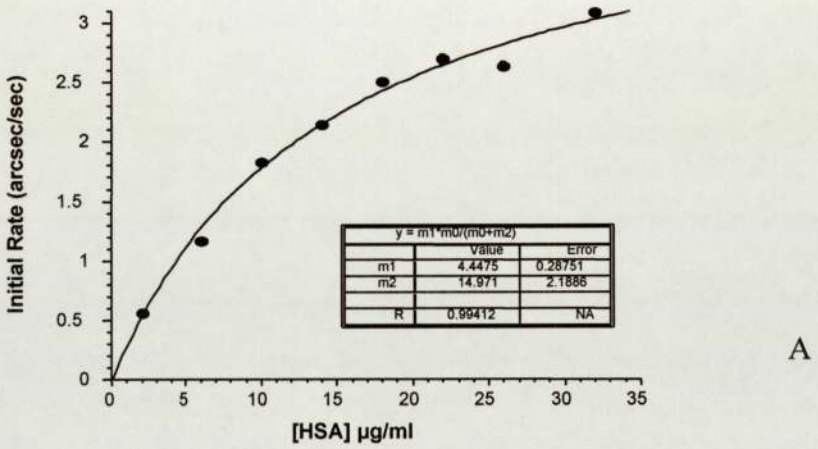


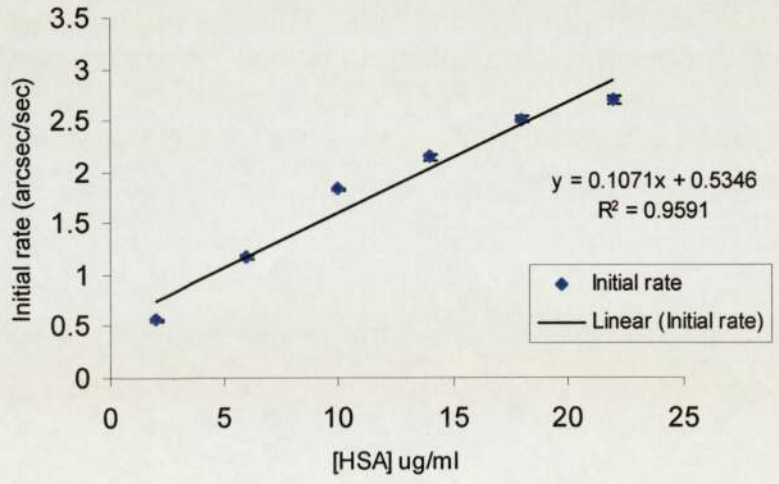
Figure 3.13. Various standards were passed over the cuvette containing antibody 0220-0704. The corresponding concentrations are shown above each response curve. The association phase is highlighted by a black underline for each albumin concentration.

HSA (µg/ml)	Initial rate (arcsec/sec)	SD (arcsec/sec)	SEM (arcsec/sec)
2	0.564	0.023	0.011
6	1.173	0.030	0.015
10	1.830	0.032	0.016
14	2.148	0.057	0.028
18	2.510	0.059	0.030
22	2.698	0.087	0.044
26	2.643	0.057	0.029
32	3.095	0.083	0.041

Table 3.5 The initial rate was calculated from figure 3.13, for each albumin standard and used to construct a calibration cure in figure 3.13. The albumin concentrations were passed over the cuvette once (n=1).



A



B

Figure 3.14. A shows the calibration curve obtained form the initial rate of each albumin standard fitted with the equation. B shows a line of best fit placed on the linear region of the calibration curve. R^2 represents the correlation of fit.

A Test samples and quality controls

Four test samples and two quality controls were placed over the cuvette, calibrated in section 3.13, and the gradient of the association slope calculated. Figure 3.15 and figure 3.16 show the actual responses obtained for test samples and quality controls respectively. Initial rates for each sample were calculated using both the saturation and linear equation established from the calibration curve in figure 3.14. Table 3.6 compares the values for each sample calculated and the known values. As the test samples and quality controls were made in a similar fashion to the albumin standards, the actual concentration was already pre-determined. The test samples contained random concentrations of albumin with the MAU range where the two quality controls were run at the very end to demarcate accurate reproduction of results after several regenerations. It was important to determine whether the antibody had been affected by regeneration and to see if recalibration of the cuvette was required.

Correlation analysis was performed to compare the known and calculated values of both test samples and quality controls. A correlation coefficient of 0.994 was determined with statistical significance for the saturation model and a correlation of 0.995 with a similar significance was obtained for the linear model.

B Urine sample analysis

Ten urine samples were passed over the cuvette and the responses recorded, as with all other samples. The initial response was much higher in the association phase, where a lot of non-specific binding was washed off in the dissociation phase. Figure 3.17 shows an example of such binding characteristics using urine sample 3. To obtain specific binding from total binding, the control was subtracted from the response (figure 3.17).

The slope of the association phase was calculated (figure 3.18 A-J) for each urine sample and placed in the calibration equation. Table 3.7 shows the results obtained. Most responses generated negative values using the langmiur equation and so these were placed as void. There was very little correlation between the calculated values,

based on the calibration curves in figure 3.14, and the values predetermined by a DAKO assay (table 3.8). The DAKO assay is an immunoturbumetric assay using rabbit anti-human serum albumin antibody for the detection of albumin based upon a colourimetric reaction. However a comparison of the rank order, including the negative values, showed a relative correlation between the two assays (figure 3.19).

In summary a four well immobilisation was repeated using antibody 0220-0704. A series of albumin standards were used based upon the range for MAU. From the response a calibration curve was calculated where both a curved fit and a linear fit were used to calculate the urine samples, test samples and quality controls. Good correlation was determined when comparing calculated concentrations with the known concentration values. Further analysis with the use of urine samples nevertheless showed poor similarities between calculated values based upon the calibration curve and those estimated with the DAKO assay. A good correlation was seen with the rank order of the samples between the two assays.

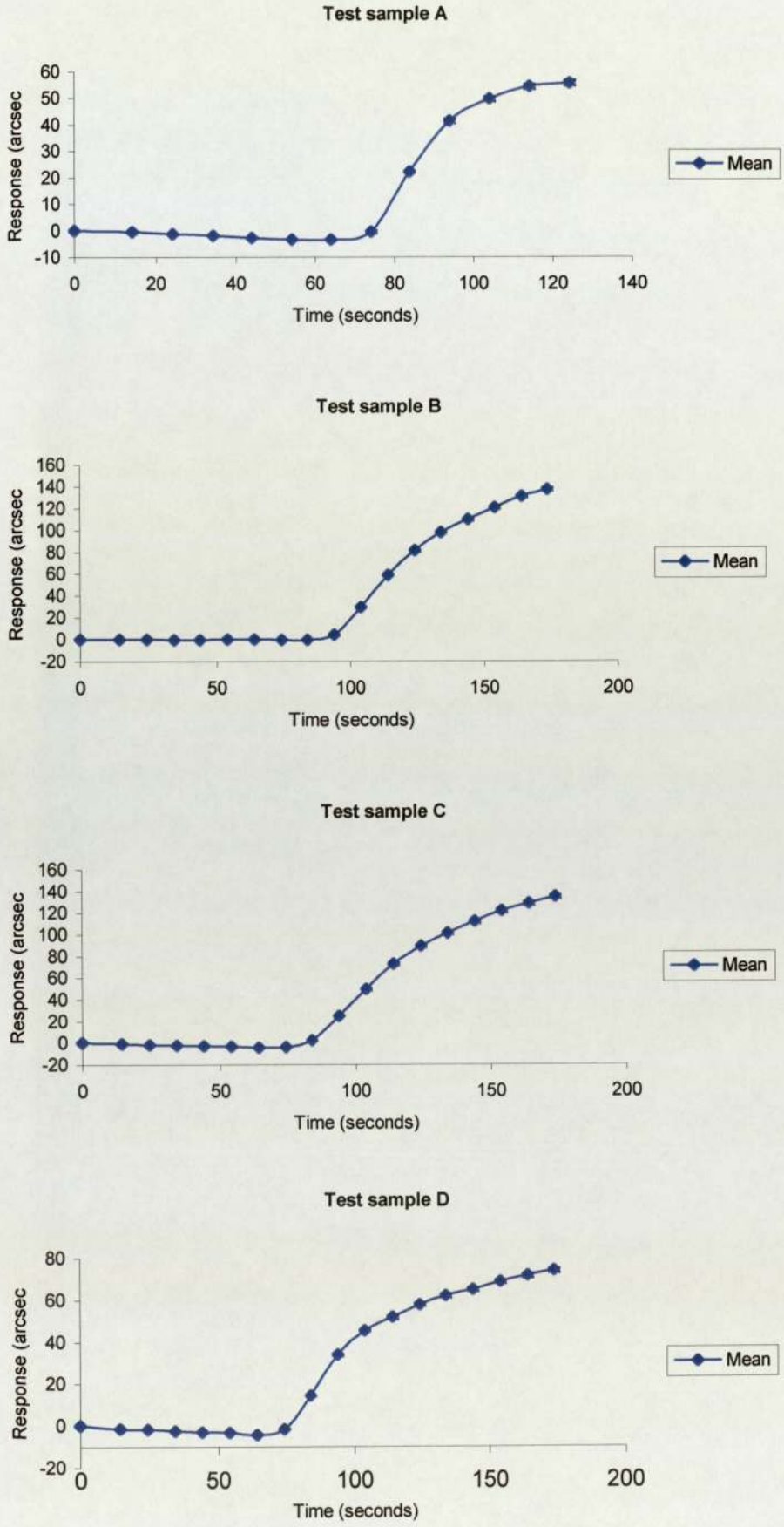


Figure 3.15. Responses obtained when test samples were passed over the cuvette.

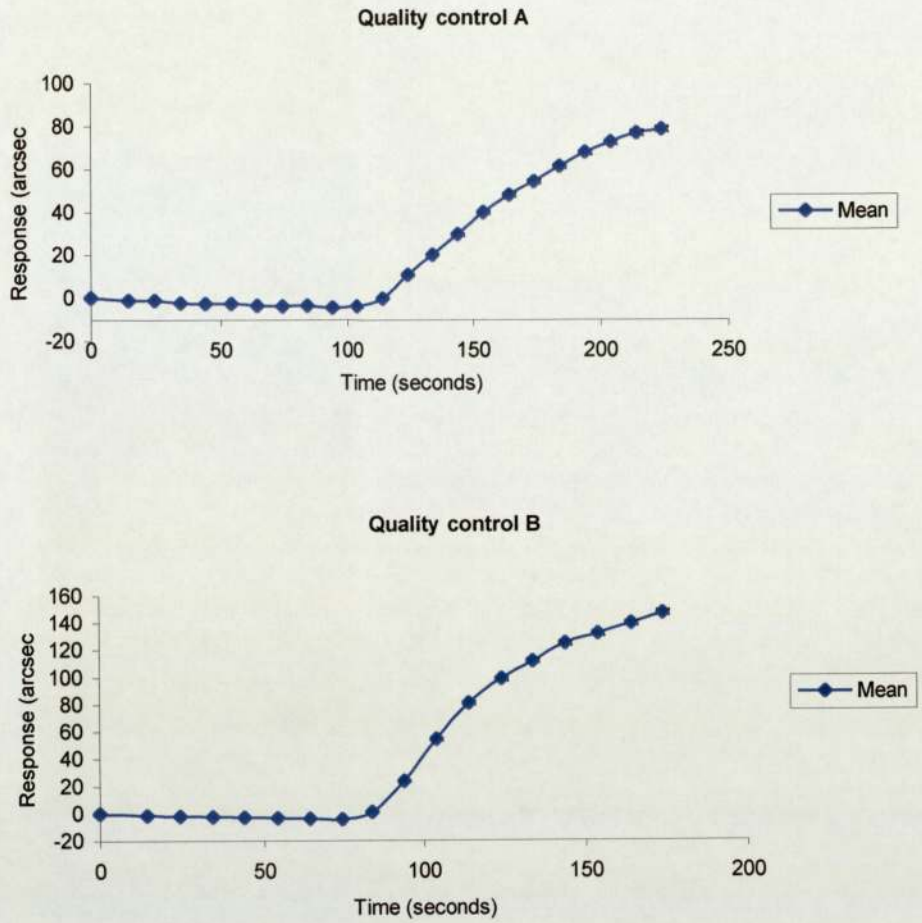
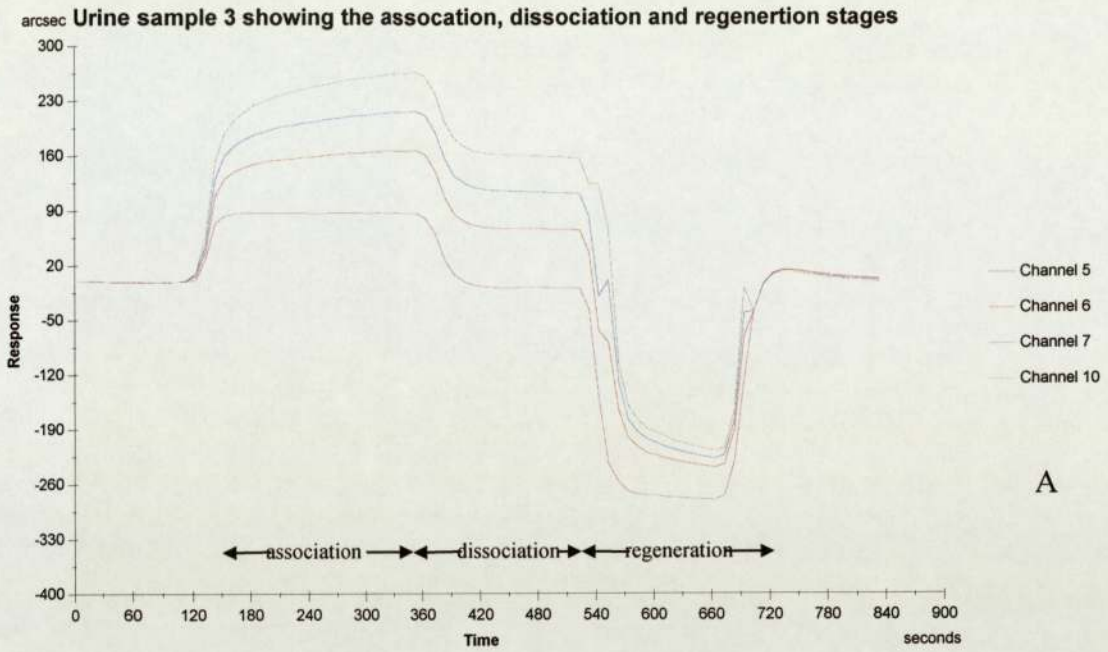


Figure 3.16. The responses obtained when passing two quality controls over the cuvette.

	Initial Rate (arcsec/sec)						Average response	[HSA] ug/ml based on saturation	[HSA] ug/ml based on linear	Actual
	CH 5	CH 6	CH 7	CH 8	CH 9	CH 10	CH 5 to 8			[HSA]ug/ml
Test sample A	1.86	1.92	1.92	1.82	0.98	1.04	1.88	10.96	12.56	12
Test sample B	1.23	1.23	1.26	1.19	0.64	0.67	1.23	5.72	6.46	7
Test sample C	2.62	2.74	2.8	2.69	1.41	1.45	2.71	23.35	20.34	22
Test sample D	2.18	2.28	2.28	2.21	1.11	1.19	2.24	15.19	15.9	16
Quality Control A	1.14	1.17	1.16	1.13	0.58	0.63	1.15	5.22	5.75	6
Quality Control B	2.36	2.47	2.55	2.47	1.28	1.39	2.46	18.5	18	18

Table 3.6. Initial rate determined for each test sample and quality control. The table shows the difference obtained form the known concentration values and those calculated from the saturation equation (curve fit) and linear equation established in figure 3.14.



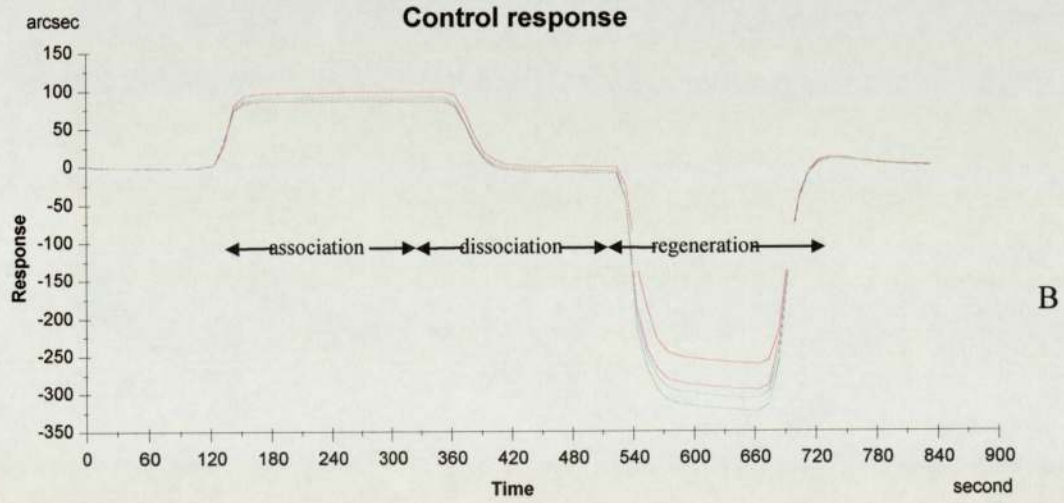


Figure 3.17. A shows the raw response observed when passing a urine sample over the cuvette. B shows the response the control gives. Only when subtracting both graphs can a real response be determined.

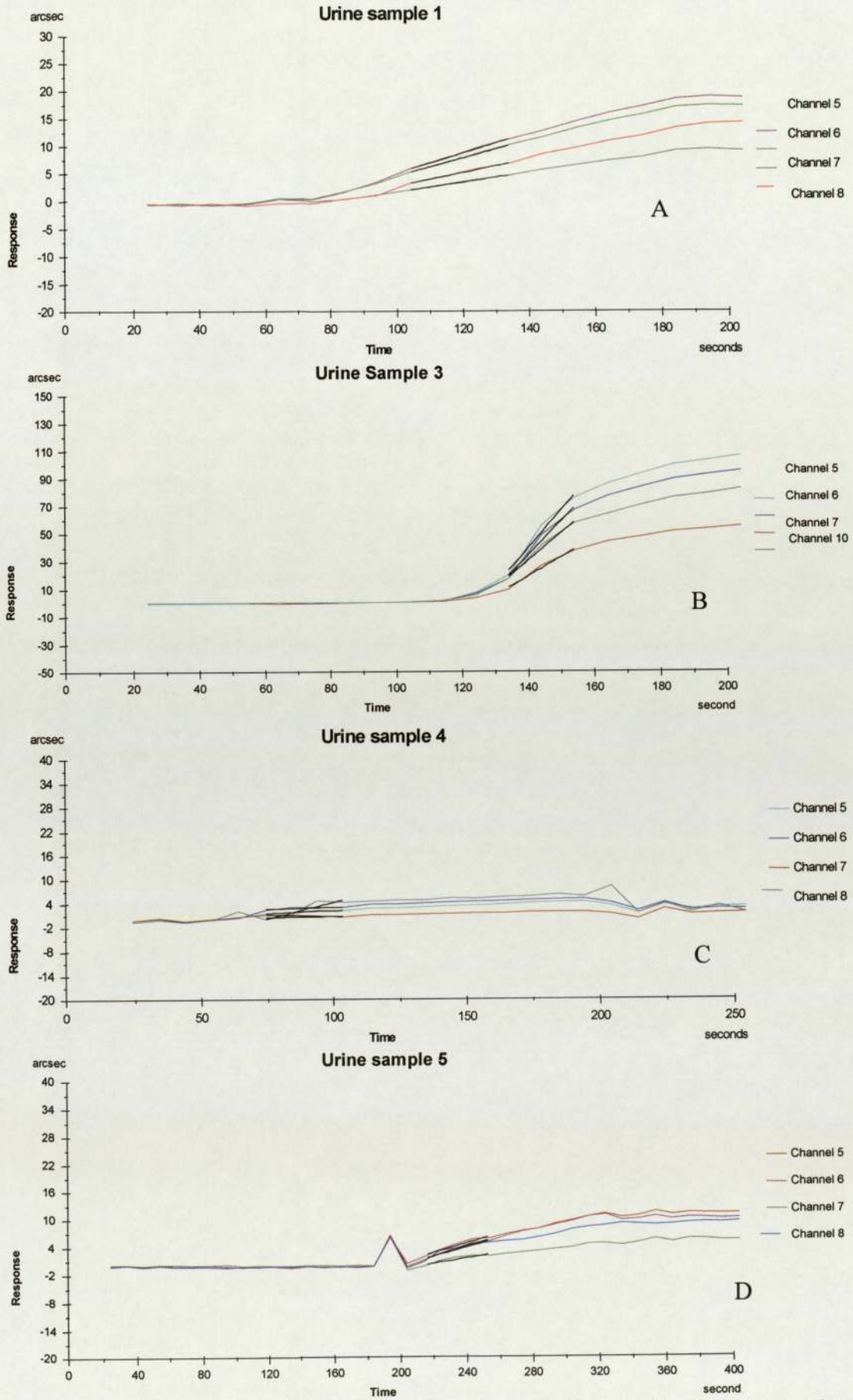


Figure 3.18 A-D. Responses shown after adding urine over the cuvette. Each response has been subtracted from the control.

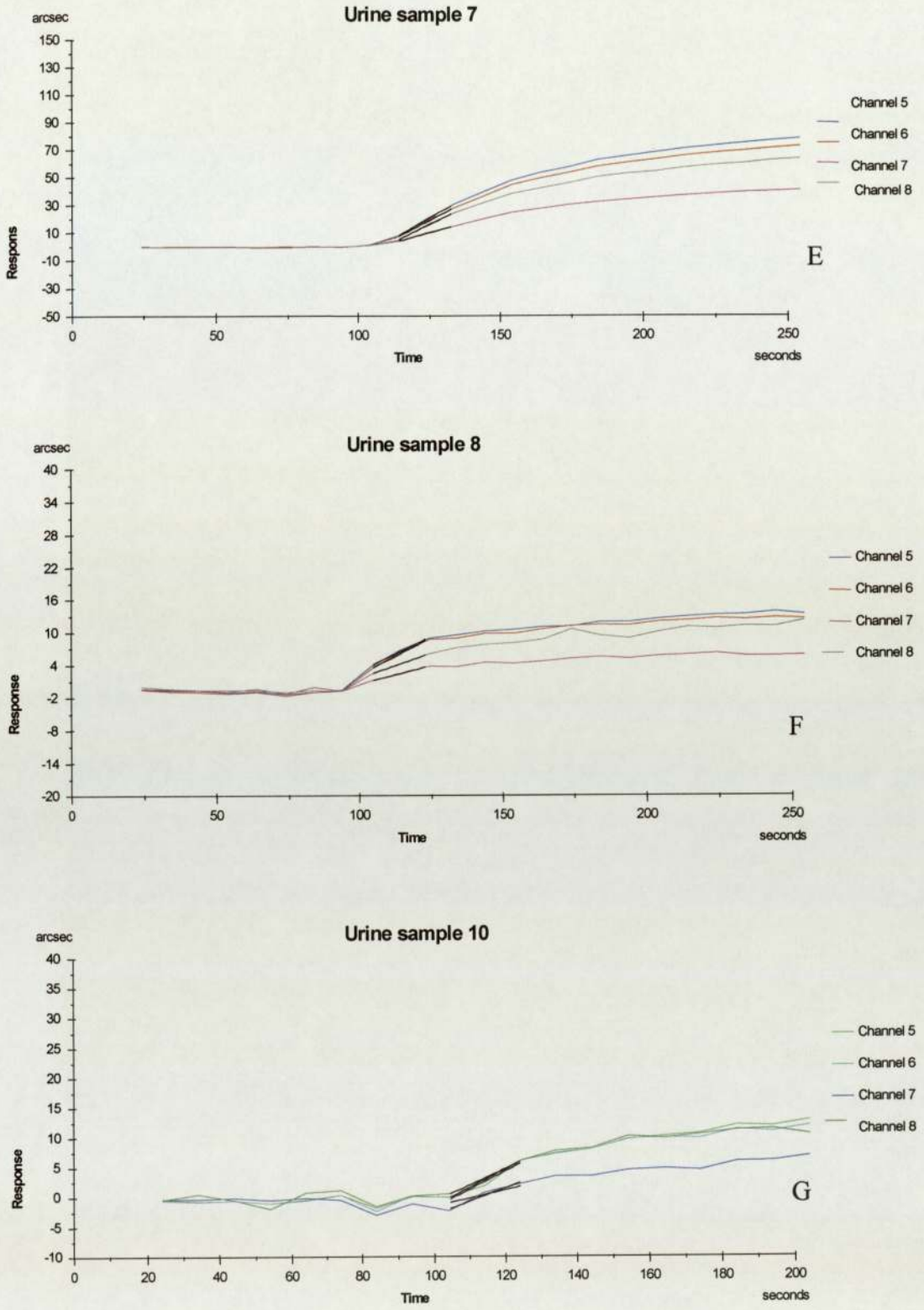
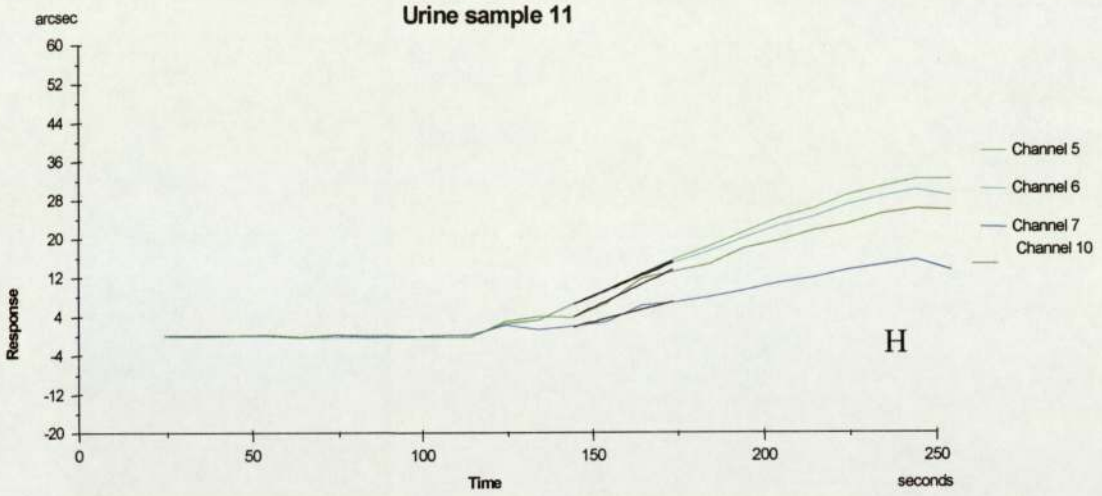
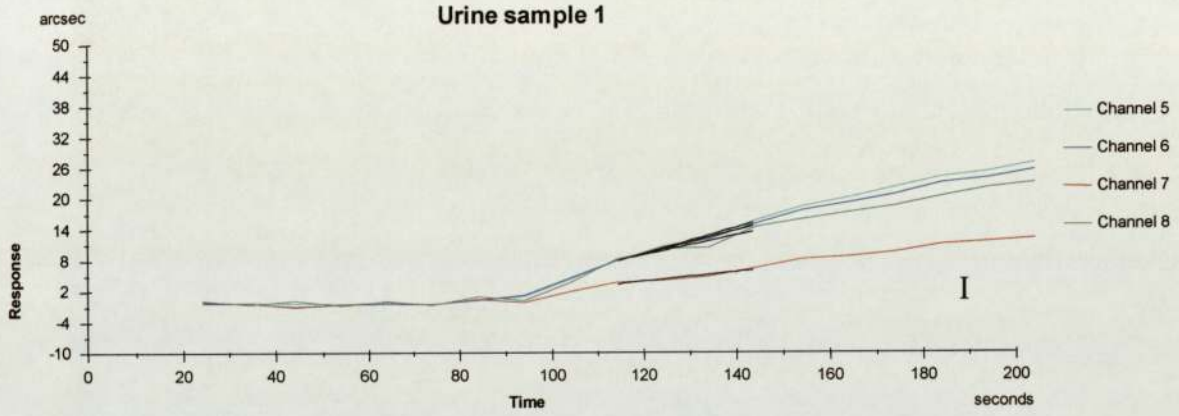


Figure 3.18 E-G. Responses shown after adding urine over the cuvette. Each response has been subtracted from the control.





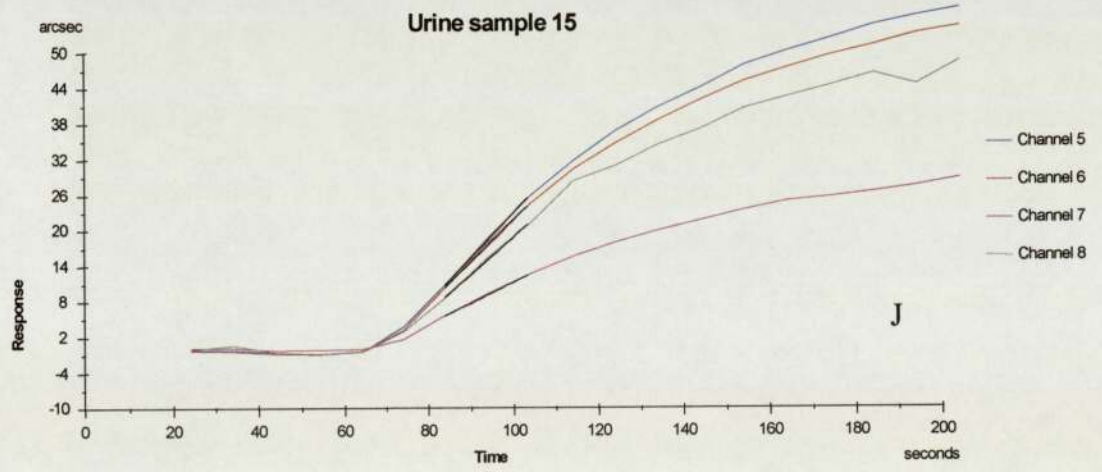


Figure 3.18 H-J. Responses shown after adding urine over the cuvette. Each response has been subtracted from the control.

Urine Sample	Initial Rate (arcsec/sec) CH 5	CH 6	CH 7	CH8	Average CH response	[HSA] µg/ml {2} 1:10 dilution	[HSA] µg/ml {2} Neat	[HSA] µg/ml {1} 1:10 dilution	[HSA] µg/ml {1} Neat
1	0.162	0.149	0.101	0.125	0.134	0.47	4.7	-3.74	VOID
3	1.850	1.660	1.880	1.380	1.693	9.2	92	10.8	108
4	0.025	0.016	0.017	0.016	0.055	0.19	1.9	-4.48	VOID
5	0.107	0.103	0.099	0.098	0.102	0.35	3.5	-4.04	VOID
7	0.827	0.771	0.876	0.681	0.789	3.23	32.3	2.37	24
8	0.252	0.231	0.201	0.191	0.219	0.78	7.8	-2.95	VOID
10	0.261	0.259	0.260	0.249	0.257	0.92	9.2	-2.59	VOID
11	0.285	0.258	0.281	0.249	0.268	0.96	9.6	-2.49	VOID
13	0.195	0.194	0.177	0.162	0.580	2.25	22.5	0.43	4
15	0.755	0.706	0.754	0.629	0.711	2.85	28.5	1.65	17

Equation {1} $y=mx+c$ where m(gradient) = 0.1071
c(intercept) = 0.5346

Equation {2} $mo=\frac{m2 \times y}{m1 - y}$ where m2(Kd) = 14.971
m1 (Bmax) = 4.4475

Table 3.7. Shows the response for each urine sample passed over the cuvette once. Using the above equations the initial rate was used to calculate the [HSA] present. The equation originates from the standard curve in figure 3.14. All urine samples were diluted 1:10 in order to buffer the samples from any pH changes which may affect the response.

Urine Sample	Albumin (µg/ml) DAKO Kit	Albumin (µg/ml) {1} RM analysis	Albumin (µg/ml) {2} RM analysis	Rank Order DAKO Kit	Rank Order RM {1} analysis	Rank Order RM {2} analysis
1	22	VOID	4.7	4	-	2
3	904	108	92	10	4	10
4	3	VOID	1.9	1	-	6
5	9	VOID	3.5	2	-	1
7	251	24	32.3	9	3	9
8	12	VOID	7.8	3	-	3
10	30	VOID	9.2	6	-	4
11	24	VOID	9.6	5	-	5
13	55	4	22.5	7	1	7
15	213	17	28.5	8	2	8

Table 3.8. Rank order of urine samples based upon table 3.7. As most of the values were void only those values based on positive values were compared. {1} refers to the calculated values using equation 1 based on the linear equation (3.14 B).{2} are the values calculated using equation 2 which is based on the saturation model (figure 3.14A).

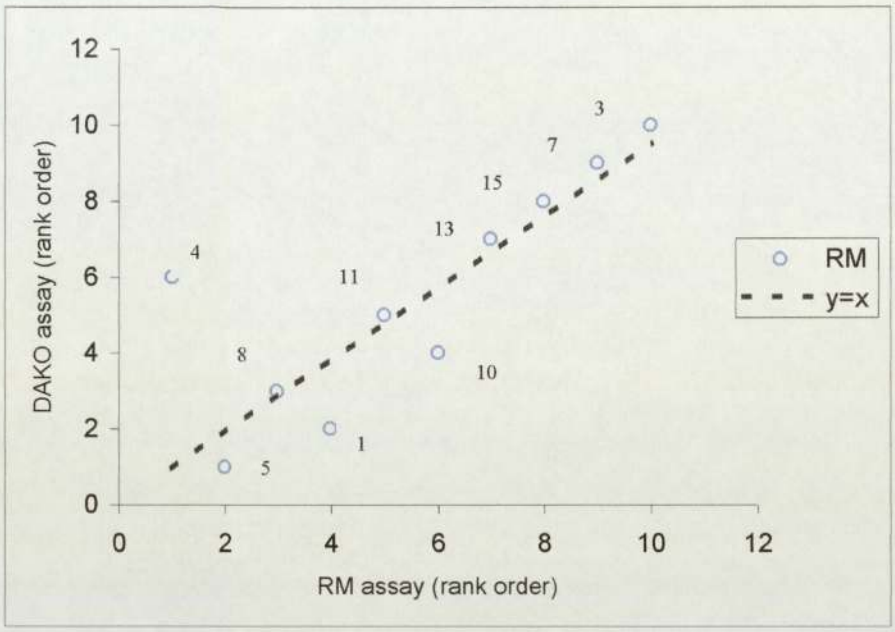


Figure 3.19. A scatter plot represents the correlation between the rank order of the urine samples using both the DAKO and RM assays. The dashed line represents a correlation of 1 and hence all samples which lie on the line have a correlation of 1. The numbers adjacent to each data point shows the urine sample used.

3.1.4 Use of other capturing ligands

For comparative reasons as well as potential optimisation of the assay, other capturing ligands (antibodies) were investigated. These were a polyclonal anti-human serum antibody (Biogenesis cat. number 0219-9970) and a monoclonal antibody (Sigma cat. number A1151).

A. Antibody 0219-9970

A four well cuvette was set up where antibody 0219-9970 was immobilised to wells 2 and 3. Well 1 contained the control and well 4 contained no antibody. Activation of the cuvette surface was performed as standard (figure 3.20) and the standard deviation of all 16 channels was calculated. The binding and deactivation stages were then done according to the standard methodology. Figure 3.21 shows the exact layout of the cuvette with respect to the various ligands.

No response was observed, on application of a range of albumin standards to the cuvette (figure 3.22). A dip was seen at 2µg/ml, which is frequent of some assays however the dip is usually masked by the response being much higher in proportion.

Due to the lack of response seen with albumin assays, it was decided that no further analysis would be conducted with this antibody.

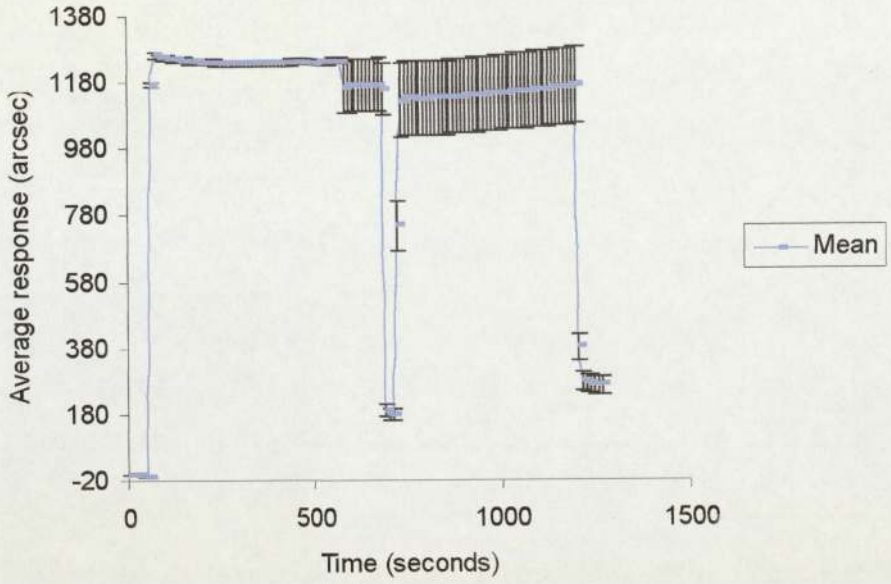


Figure 3.20. The mean response obtained when activating the cuvette surface with NHS/EDC. Error bars, shown in black, represent the standard deviation of the mean.

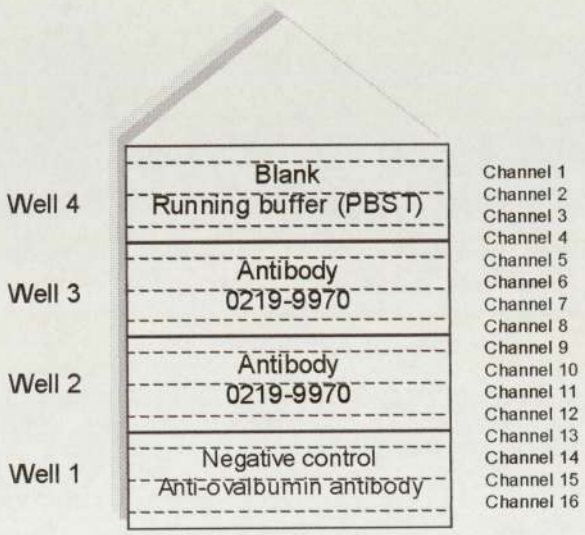


Figure 3.21. Schematic diagram of the cuvette showing the layout of the wells and channels. The positioning of the antibody with respect to the wells and channels are also shown.

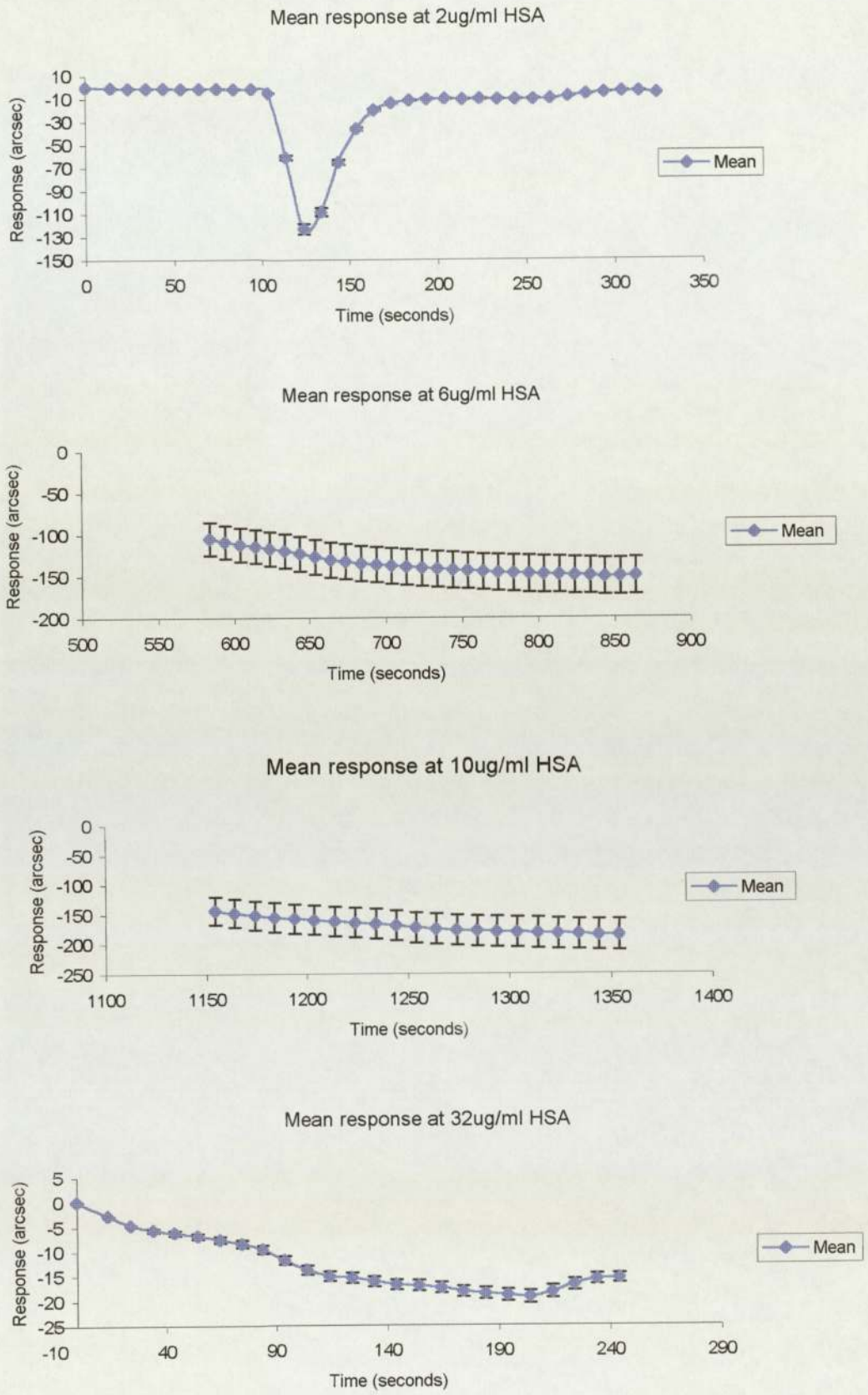


Figure 3.22. Average response obtained for [HSA] ranging from 2-32ug/ml using antibody 0219-9970. SEM bars.

B. Antibody A1151

Immobilisation of antibody A1151 was placed over a four well cuvette in a similar fashion to antibody 0220-0704 and antibody 0219-9970. Figure 3.23 shows the mean response for the activation stage giving a final response of 183 arcsec. Figure 3.24 shows the positioning of antibody A1151 on the cuvette.

A series of albumin standards from 2-34μg/ml [HSA] was passed over the cuvette containing antibody A1151 and the response recorded for all instances. Inconsistencies were noticed between channels of the same well. In well 2, containing antibody 0220-0704, all channels showed an elevated response where channels 7 and 8 were intermediary. An elevated response was also seen at channel 10 in well 3. Channel 9 and 11 showed an intermediate response. Channel 12 showed no response. No responses were seen in well 4 except for channel 4, which showed an elevated response. No inconsistencies were observed in well 1. Due to the discrepancies between channels in wells 2 and 3, only channels, which showed maximal response, were chosen and their mean response calculated. These channels were 5, 6 and 10. All of the channels in well 4 except channel 4 were averaged to give a mean control value from which the responses were subtracted. Appendix F illustrates the raw data for individual channels in each well. Figure 3.24 shows the mean response of association phase for channels 5,6 and 10. The slope for each response was calculated and the initial rate is shown in figure 3.26 and table 3.9.

From table 3.9, it is apparent that at 2μg/ml a proportionally high initial rate and hence response was observed. This response was out of phase with regards to the rest of the data suggesting it to be a false elevation. On repetition of the albumin standard a much smaller response was seen in comparison; allowing for a curved shape calibration (figure 3.27). Table 3.10 outlines the actual values obtained.

A comparison was made between the performances of antibody A1151 with antibody 0220-0704 by contrasting the individual responses in each albumin standard obtained for each antibody. The reason for using the t-test was to try and establish which if the two antibodies were optimal for this particular assay. An independent sample t-test gave

a t value of 4.493 with a probability of less than 0.05. In conclusion, a significant difference in response was observed between antibody 0220-0704 and A1151 where antibody 0220-0704 had higher responses in comparison to A1151 for each albumin standard, suggesting antibody 0220-0704 to be a more sensitive ligand in this assay.

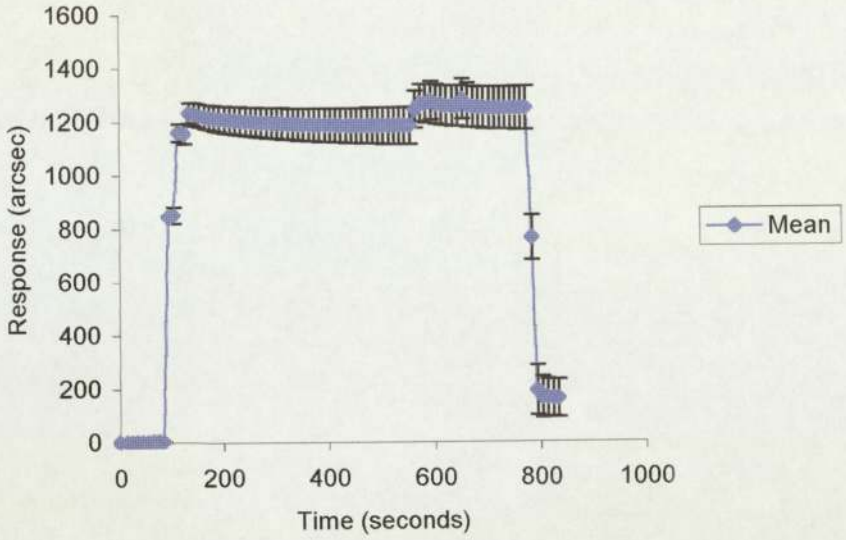


Figure 3.23. Mean response for the activation of the cuvette to allow for the immobilisation of antibody A1151.

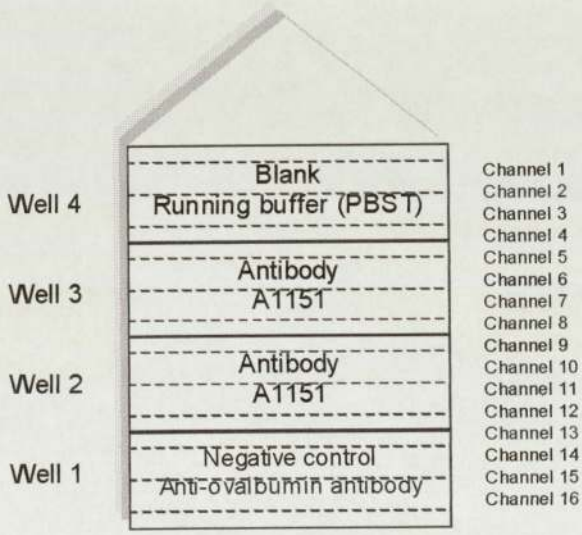


Figure 3.24. Schematic diagram showing the position of antibody A1151 within the cuvette.

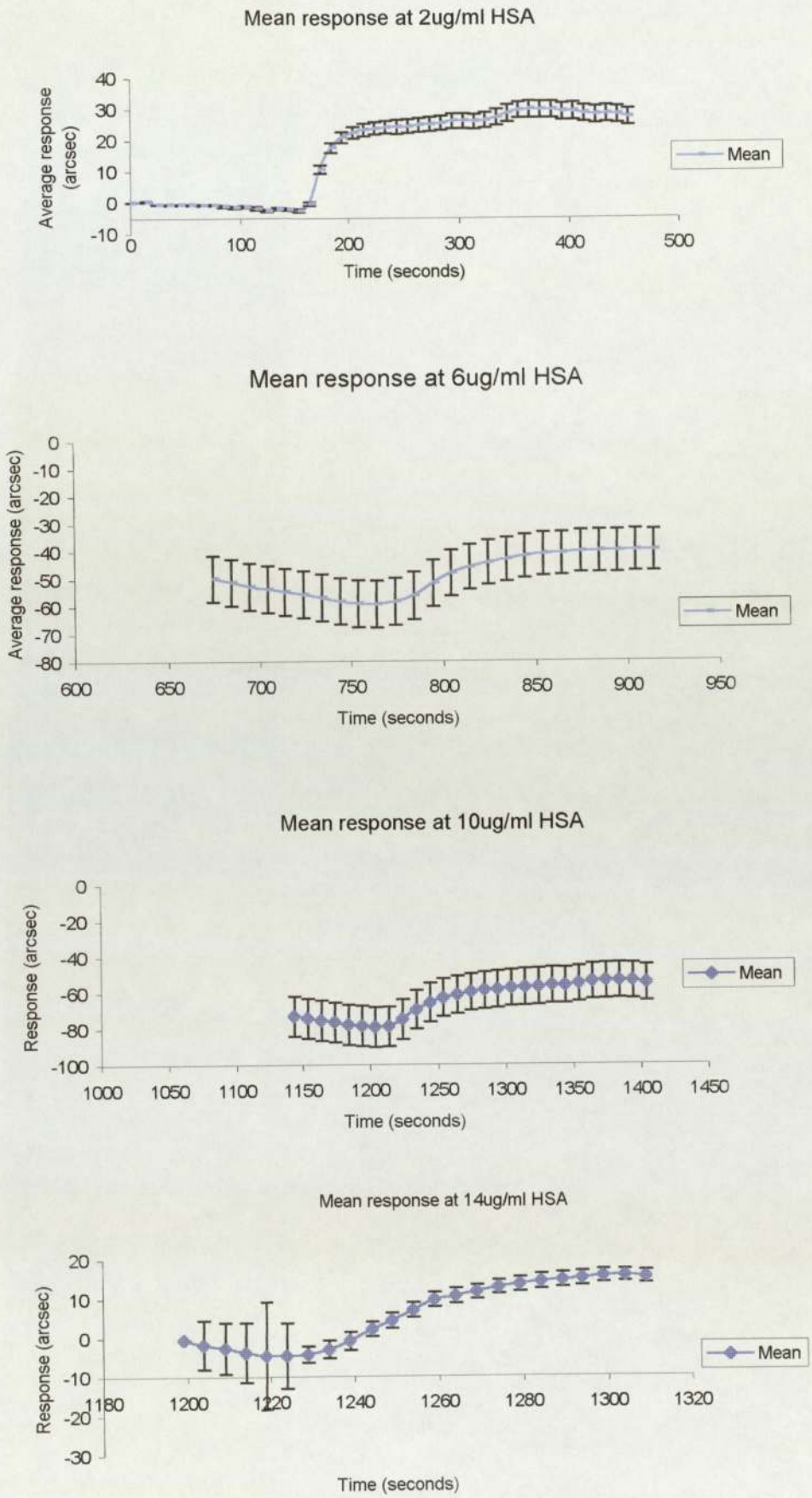


Figure 3.25. Mean response obtained when passing albumin standards over the cuvette.

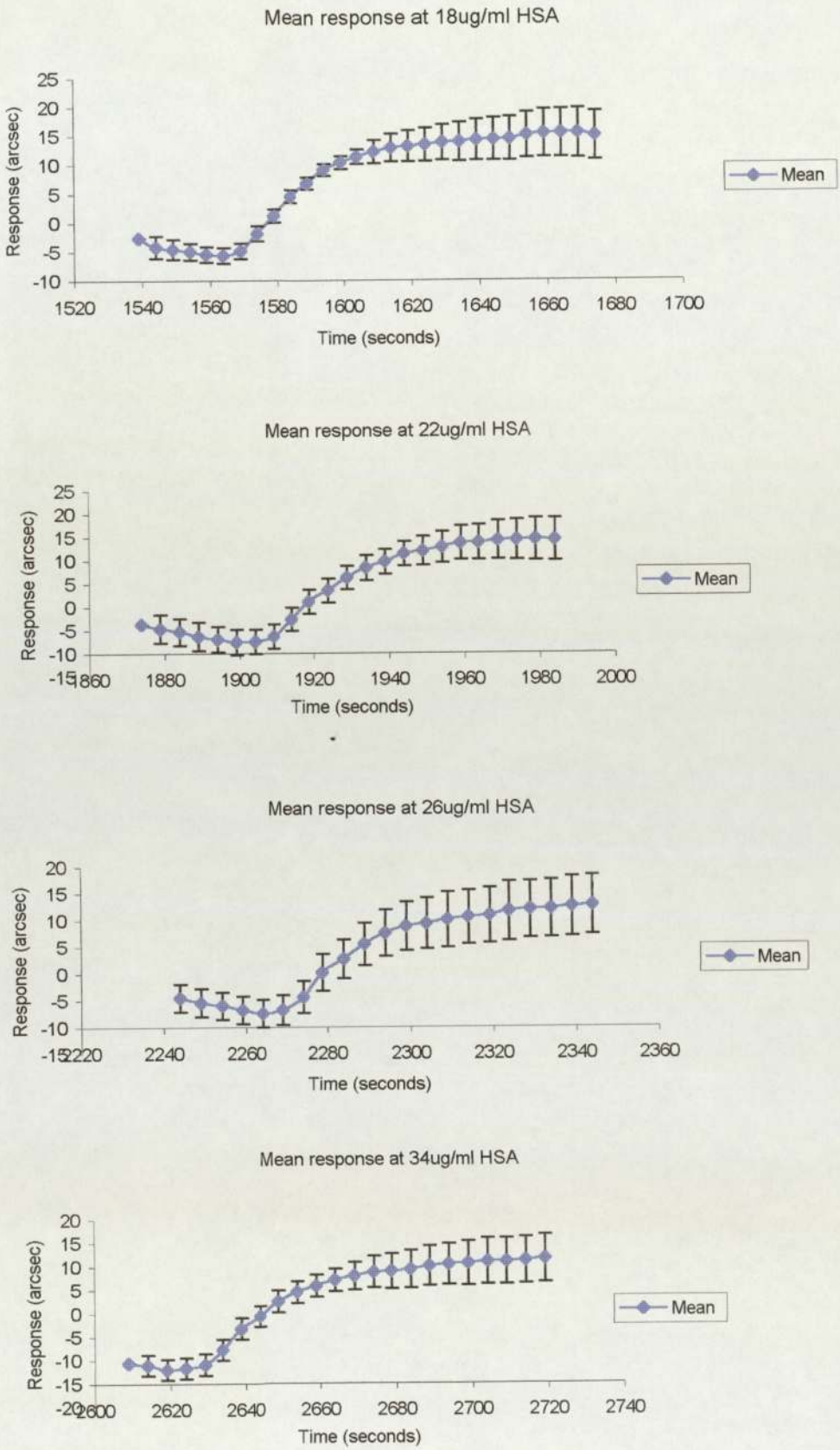


Figure 3.25. Mean response obtained when passing albumin standards over the cuvette.

[HSA] (µg/ml)	Initial rate (arcsec/sec)			Average Initial rate (arcsec/sec)	SD (arcsec/sec)	SEM (arcsec/sec)
	CH 5	CH 6	CH 10			
2	1.030	1.040	1.190	1.087	0.090	0.052
6	0.523	0.478	0.587	0.529	0.055	0.032
10	0.651	0.601	0.760	0.671	0.081	0.050
14	0.560	0.491	0.705	0.585	0.109	0.063
18	0.776	0.707	0.920	0.801	0.109	0.063
22	0.741	0.656	0.909	0.769	0.129	0.074
26	0.781	0.706	0.925	0.804	0.111	0.064
30	0.820	0.745	0.999	0.855	0.130	0.075
32	0.699	0.623	0.848	0.723	0.115	0.066

Table 3.9. Mean initial rates obtained from each albumin standard passed over the cuvette containing A1151. CH is an abbreviation for channels.

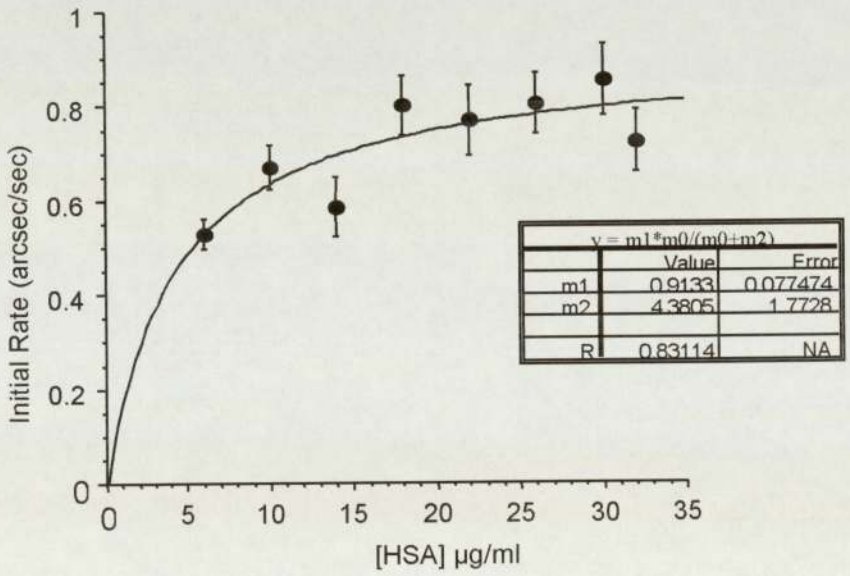


Figure 3.26. Calibration curve was established from the initial rate of albumin standards passed over the cuvette containing antibody A1151.

[HSA] (µg/ml)	Initial rate (arcsec/sec)			Average Initial rate (arcsec/sec)	SD (arcsec/sec)	SEM (arcse/sec)
	CH 5	CH 6	CH 10			
2	0.265	0.209	0.359	0.278	0.076	0.044
6	0.458	0.386	0.569	0.471	0.092	0.053
10	0.471	0.45	0.651	0.524	0.110	0.064
14	0.534	0.472	0.703	0.570	0.120	0.069
18	0.597	0.548	0.769	0.638	0.116	0.067
22	0.702	0.629	0.851	0.727	0.113	0.065
26	0.673	0.603	0.849	0.708	0.127	0.073
30	0.645	0.618	0.872	0.712	0.140	0.081
34	0.651	0.629	0.894	0.725	0.147	0.085

Table 310. Mean initial rates obtained from each repeated albumin standard passed over the cuvette containing A1151. CH is an abbreviation for channels.

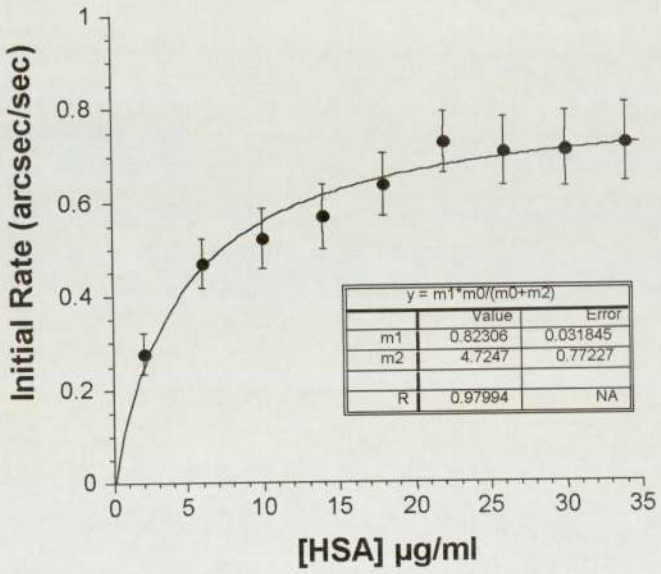


Figure 3.27. Calibration curve was established form the initial rate of repeated albumin standards passed over the cuvette containing antibody A1151.

Chapter 4
RESULTS - ANALYSIS OF CREATININE

4.1 Creatinine detection with the use of the RM

The molecular weight (MW) detection limit of the RM is around 551. Creatinine has a molecular weight of 131 and so the RM is not sensitive enough to detect its presence in solution. To overcome this handicap, an alternative approach involving the immobilisation of creatinine deaminase was investigated. Creatinine deaminase enzymatically degrades creatinine with the release of ammonium ions. The change in H^+ affects the charged ions present upon the dextran surface. Any change in the configuration of the sensor surface elicits a change in refractive index and hence a response is generated.

4.1.1 Optimisation of creatinine deaminase binding conditions

Immobilisation of creatinine deaminase was performed using the one well method, similar to that used for antibody 0220-0704 (section 3.1.1). Figure 4.1 shows the response obtained when the cuvette surface was activated and deactivated including the immobilisation of the enzyme. A final response of 325 arcsec (equating to 1.63 ng/mm^2 of protein) was bound to the cuvette. This is very poor and in fact nine tenths lower than the amount of antibody 0220-0704 (section 3.1.1 A). The MW of creatinine deaminase is 260 000 in comparison to a MW of 150 000 for most antibodies.

To optimise the attraction and binding of creatinine deaminase onto the cuvette surface, an experiment was set up to investigate the optimal pH conditions needed upon the cuvette.

A series of pH from 3 to 6, using acetate buffer, were placed over the cuvette initially. Once the buffer was added, creatinine deaminase was then placed over the cuvette (figure 4.2). The amount of creatinine deaminase attracted to the surface was determined by the increase in arcsec where the higher the response, the more creatinine deaminase attracted and hence bound to the cuvette. Figure 4.2 shows a maximal response at pH 4.5 and a minimum at pH 6. Table 4.1 shows the final average response for each pH.

Based on the data shown in figure 4.2 immobilisation of creatinine deaminase was repeated except that acetate buffer (pH 4.5) was added to the cuvette first and later followed by the addition of creatinine deaminase. This made a difference to the final amount of bound enzyme. A mean response of 933 arcsec (table 4.2) was observed in figure 4.3 which outlines the activation, binding and deactivation stages. This was a vast improvement from the previous experiments.

4.1.2 Validation of the resonant mirror using creatinine standards

Six creatinine standards ranging from 31µg/ml to 1mg/ml were placed over the cuvette (figure 4.4) and the response recorded using the RM. A wide range of creatinine standards were initially used in order to determine the detection limit of the enzyme. A different pattern of response occurred with this type of reaction. An association between the ligand was not observed but instead a sharp inflection in response later followed by a plateau existed. Once the cuvette was washed a sharp inflection of response back to baseline occurred. Table 4.3 shows the mean response. The raw data showing the response of all 16 channels for each creatinine concentration can be seen in appendix G.

The standards were repeated and the data used to construct a calibration curve (figure 4.5) using an average of the two readings. The repeated data is shown in appendix G. Figures 4.4 and 4.5 show reproducibility of data, both of which demonstrated a linear relationship between creatinine concentration and maximal response.

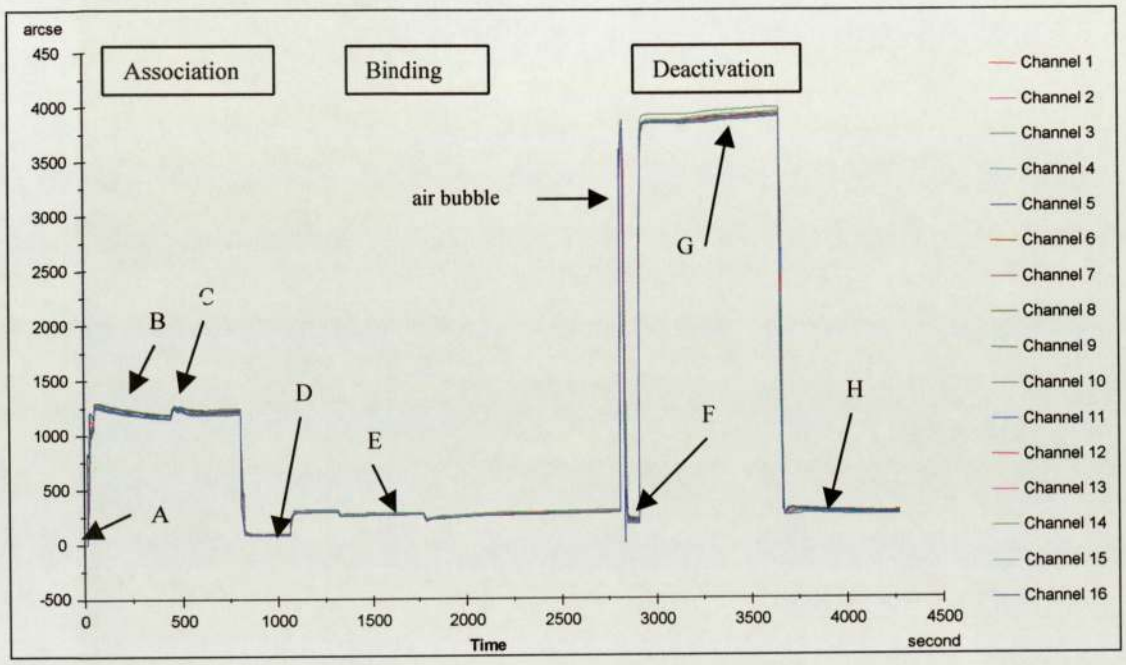


Figure 4.1. The response obtained when creatinine deaminase is bound onto the cuvette via amine coupling chemistry. A shows the initial baseline, B shows the response obtained when the cuvette is activated, C shows a second activation. D shows the baseline obtained when the cuvette is washed with PBST, E refers to the response obtained when creatinine deaminase is placed over the cuvette well, F denotes the third baseline where excess unbound antibody is washed of the cuvette surface,G shows the deactivation of the cuvette and H shows the final baseline where everything is removed from the surface cuvette leaving the bound enzyme covalently attached. Error bars, shown in black, represent the standard deviation of the mean.

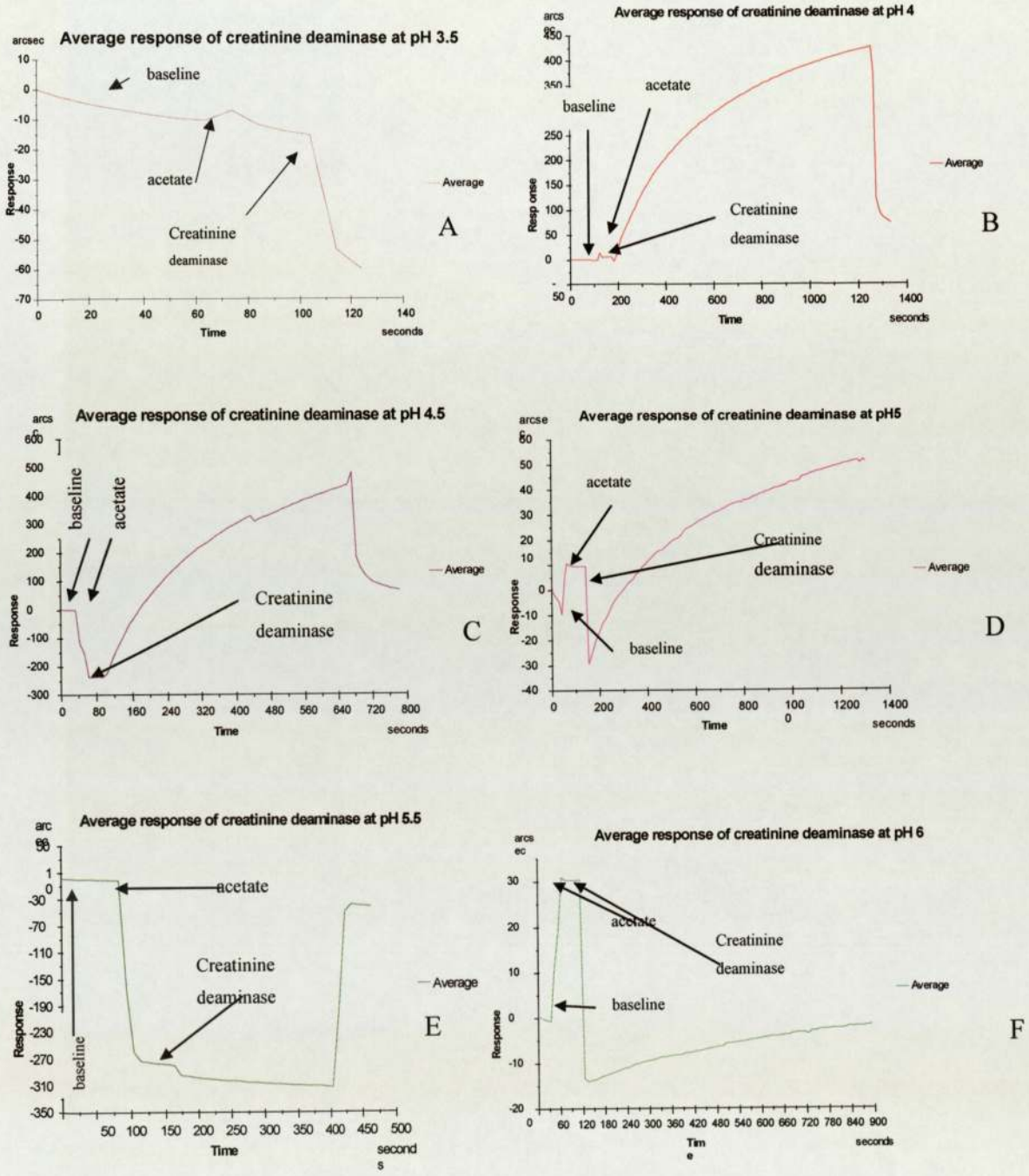


Figure 4.2. A-F. The effect of creatinine deaminase dissolved in buffer at different pH with regards to binding to the surface, which in this case is represented by the level of response.

pH	Response (arcsec)
3.5	-60
4.0	425
4.5	680
5.0	80
5.5	-40
6.0	15

Table 4.1. An outline of the average response observed at different pH with creatinine from figure 4.2.

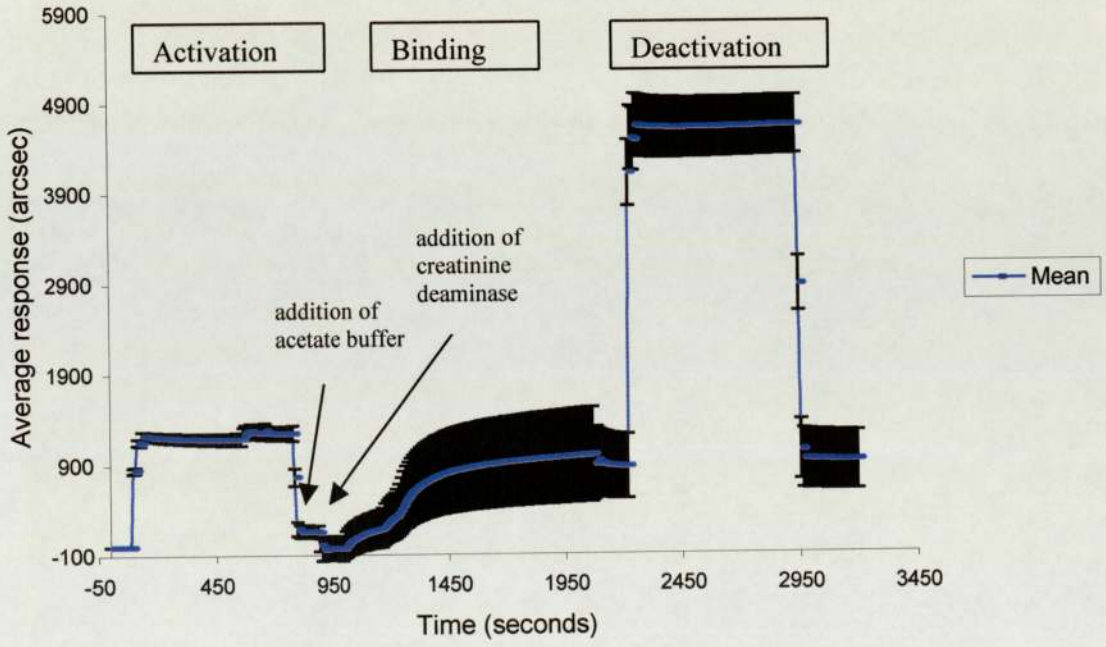


Figure 4.3. The immobilisation of creatinine was repeated giving a final response of 933 arcsec.

Mean response (arcsec)	SD (arcsec)	SEM (arcsec)
933	17.8	4.5

Table 4.2 Final response of total bound enzyme, creatinine deaminase, using a one well cuvette.

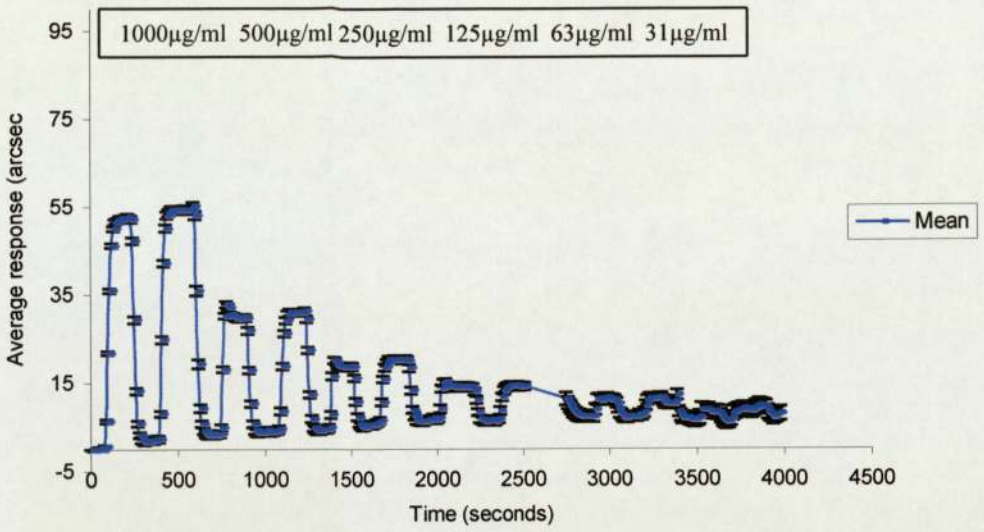


Figure 4.4. The mean response when creatinine standards from 100-32µg/ml were passed over the cuvette.

[Creatinine] µg/ml	[Creatinine] mM	Average Response arcsec
31	0.27	7.5
63	0.54	11.0
125	1.1	14.5
250	2.2	19.0
500	4.3	30.5
1000	8.6	53.0

Table 4.3. Mean responses obtained from passing varying concentrations of creatinine over the cuvette. The experiment has been repeated twice and shown above.

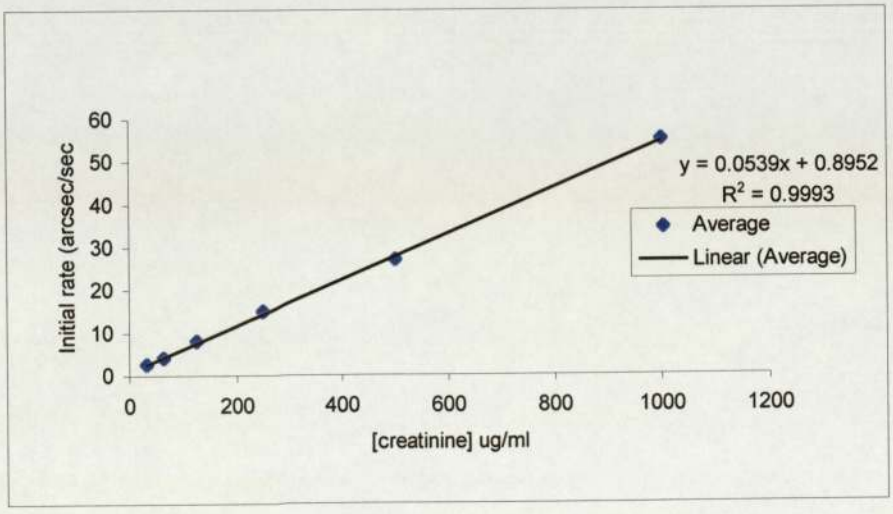


Figure 4.5. The cuvette containing creatinine deaminase was calibrated using a range of creatinine concentrations.

4.1.3. Verification of creatinine assay

Due to the design of the experiment, the direct binding event between creatinine and the enzyme cannot be seen. Nevertheless to verify that there is true linear relationship between initial rate and creatinine concentration two experiments were performed. The first consisted of a control assay where albumin protein was bound to the cuvette and 100μg/ml and 1000μg/ml of creatinine was passed over the cuvette. At 100μg no response was observed. At 1000μg, a mean response of 20arcsec (figure 4.6) was observed. This is lower than the response obtained when the same creatinine standard was passed over the cuvette containing creatinine deaminase.

A second experiment looked at the response of dextran to varying pH. If the changes in response were due to increases in H^+ ions release, it would be a fair assumption that the changes in pH would cause differing responses. A range of pH from 3 to 7.6 was used with citrate buffer for acidic pH and phosphate buffer for alkaline. Each buffer was added twice and the responses are shown in figure 4.7. Positive and negative responses were seen for different pH where figure 4.8 shows the overall pattern of response. Figure 4.7 and 4.8 demonstrate the pH effects upon dextran, illustrating that the dextran itself can be affected eliciting a response, as well as the observation that the response obtained does not coincide with the responses obtained with creatinine standards; further verifying that the assays using creatinine is response specific.

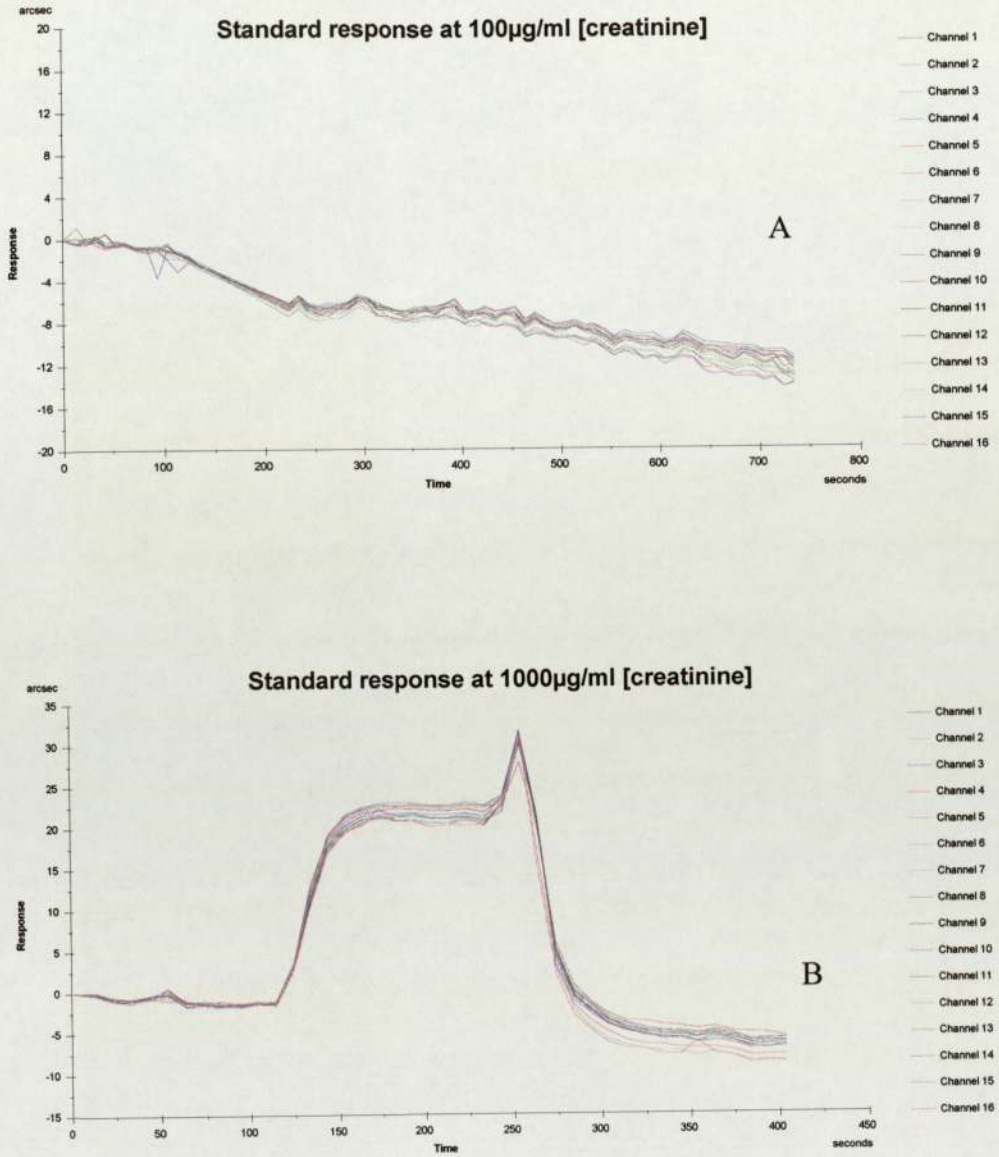


Figure 4.6 A-B. Response obtained when creatinine is passed over a cuvette containing immobilised albumin in all 16 channels. A shows the response obtained when 100µg/ml of creatinine is added to a cuvette containing bound albumin. B shows the response obtained when 1000µg/ml of creatinine is passed over the same cuvette.

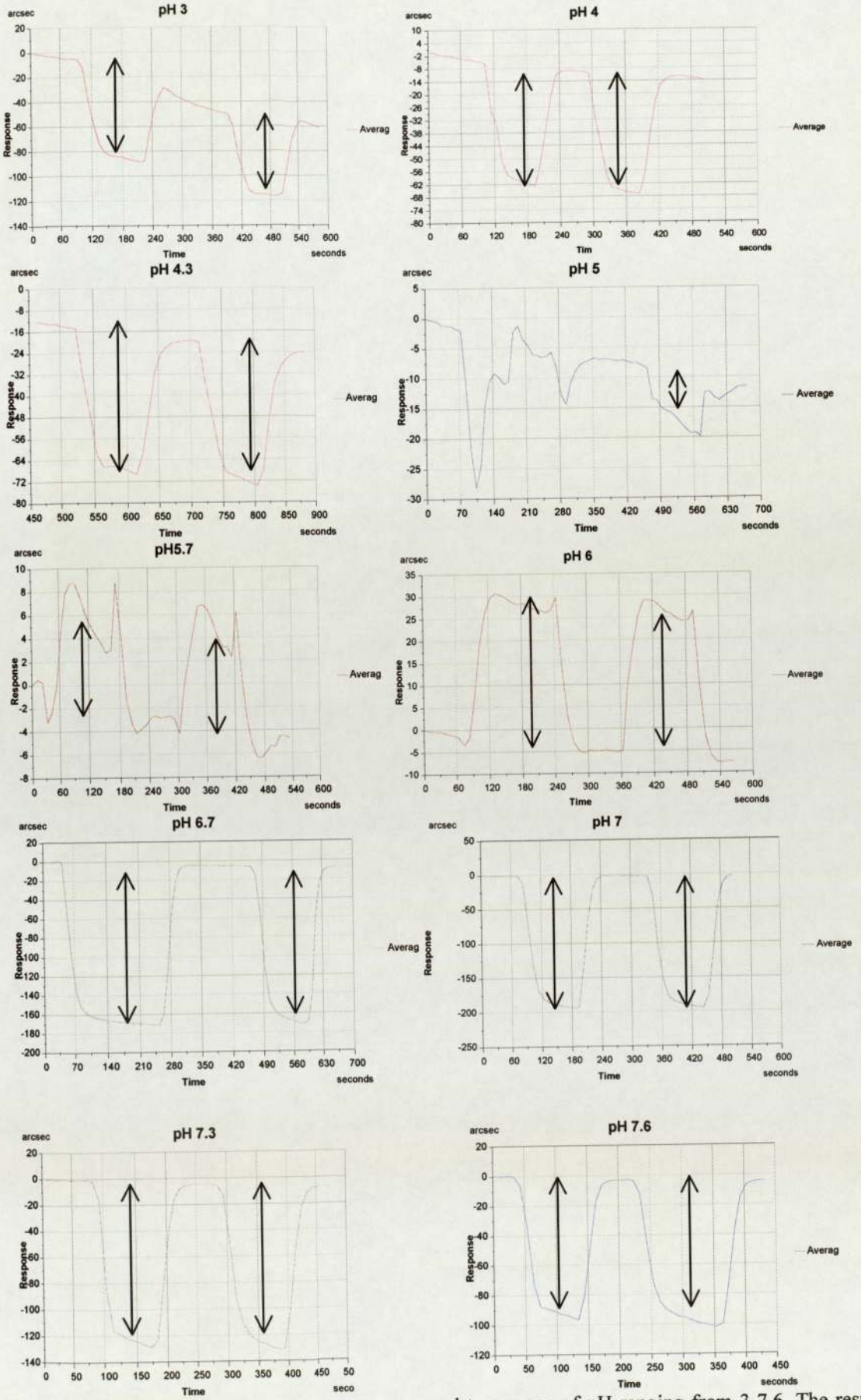


Figure 4.7. The surface of the cuvette was exposed to a range of pH ranging from 3-7.6. The response obtained at each pH is shown in duplicate. The arrows indicate the change in response observed.

pH	Average response (arcsec)
3	-78
4	-57
4.3	-53
5	-10
5.7	6
6	29
6.7	-180
7	-190
7.3	-127
7.6	-95

Table 4.4. The mean responses obtained for each pH plotted in figure 4.8 are outlined in the above table. Each pH was repeated twice over the cuvette

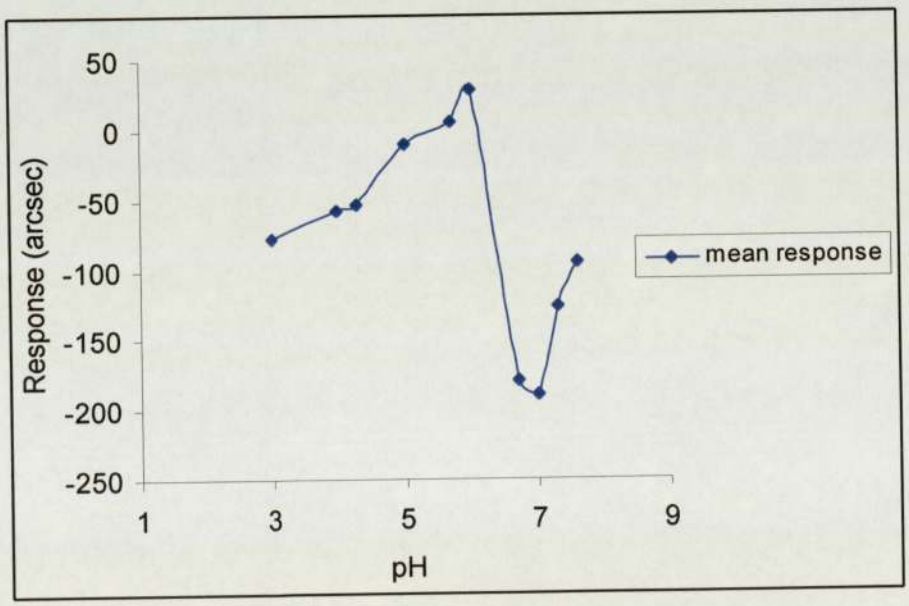


Figure 4.8. The plot shows the pattern of response when different pH are passed over a blank cuvette.

Chapter 5
DISCUSSION

5.1 Use of the resonant mirror for the detection of albumin

The use of the RM biosensor was successful in the detection of albumin in buffer solution as well as albumin detection in urine samples. The first approach required optimal conditions for the immobilisation of anti-human serum albumin onto the cuvette surface. Experiments using antibody 0220-0704 showed successful binding using amine coupling chemistry (method 2.1). Two approaches in the number of wells of the cuvette; a one well immobilisation and four well immobilisation, were analysed using antibody 0220-0704.

Using a one well immobilisation, it was possible to monitor the binding of antibody 0220-0704. During the experiments conducted a response ranging from 2500-3500 arcsec was typical of any immobilisation. This was within the manufacturers recommended range of response. Results from two cuvettes showed average bound responses of 2695 and 2987arcsec. In both cuvettes, all of the 16 channels showed little variation in response having a SD from the mean of 161 and 169 arcsec respectively. This indicated that while there was not an even distribution of bound antibody, antibody did bind effectively, throughout the cuvette in all 16 channels, allowing all 16 channels to act as a replicate of one another. Comparison of the responses obtained in both cuvette 1 and 2 (figure 3.1 and 3.2) showed similarities in response throughout. Statistical analysis using the independent t-test, which compared the responses obtained in both cuvettes, showed there to be no significant difference between the cuvettes from two different batches. Use therefore of cuvettes from different batches should not alter greatly in the total amount of bound antibody.

A different approach was needed with the division of the cuvette into four wells. A gasket along with an alignment jig was fitted to the cuvette (section 1.5) and acted as dividers splitting the sensor surface into four distinct wells. It was designed so that four channels could be detected in each well. The attachment of the gasket and alignment jig to the cuvette made it impossible for the cuvette to fit into the chipper of the RM. This meant that the RM could not be used to monitor antibody binding, as was the case for one well immobilisation. The only stage that could be monitored was the activation stage as this was performed over the whole of the cuvette surface without the use of a gasket. Looking at the response obtained in the activation stage (figure 3.4 and 3.5) a

similar pattern of response was seen as with other activation stages on other cuvettes. Nevertheless verification of bound antibody was not possible. Determining the concentration of bound antibody was not feasible and hence comparison of total amounts was not realistic. One way of overcoming such uncertainty would be to place a secondary antibody against anti-human serum antibody over the cuvette. The antibody recognising antibody 0220-0704 would bind and give a response. The greater the response, the more bound antibody 0220-0704 present on the cuvette. During the four well immobilisation, 200µg/ml of antibody was added to each well instead of 50µg/ml used in one well immobilisation. McDonald *et al.*, (personal communication) had shown 50µg/ml to be too low an antibody concentration to allow for reasonable binding events between the antibody and analyte. Instead increasing the antibody concentration four times was high enough a concentration to compensate for reduction in immobilised ligand.

The reason for the introduction of a gasket was to allow for the addition of controls. The control used in this experiment was anti-ovalbumin antibody. The reason for its suitability as a control was the inability of the antibody to bind to albumin. Anti-ovalbumin antibody is a monoclonal antibody of similar size and structure to antibody 0220-0704. However previous SPR analysis has shown the antibody to not bind varying concentration of human serum albumin. The lack of binding event between human albumin and ovalbumin could be attributed to the difference in epitope recognition of the antibody structure and hence is an ideal control. Anti-ovalbumin detects chick egg albumin but human albumin contains different binding types and so is not recognised by the antibody. The blank well did not contain any antibody but was still exposed to PBST and all other solutions as were the other wells. Any response in the blank well would suggest PBST and other reagents to have a bias effect upon the results.

Comparisons were made between two activation stages of two cuvettes which were prepared by four well immobilisation. Similar mean responses were seen and both the SD and SEM were very low in comparison to the mean suggesting very little variation between channels. No further analysis could be made besides verification of surface activation because correlation cannot be made from activation stage to the binding stage.

Calibration of a one well cuvette showed a linear relationship between initial rate and [HSA]. The initial rate is the calculation of the gradient of slope for the association response generated when albumin passes over the cuvette and binds to anti-human serum albumin antibody already immobilised onto the cuvette. Antibody 0220-0704 was sensitive enough to detect albumin from 1µg-100µg/ml where 1µg/ml generated a maximum response of 25 arcsec in the association phase and 100µg/ml a maximum response of 250 arcsec. There is no reason to suggest that antibody 0220-0704 cannot detect below or above the range used.

Whilst assaying albumin standards over the cuvette, more noise was observed in the lower part of the well (channel 9-16) than in the upper region of the well (channel 1-8) (figure 4.6). This was particularly obvious at lower albumin concentrations (< 12.5µg/ml). It is unclear why this might occur however it is possible that the strirrers present in the RM were not effective in distributing albumin over the cuvette, or another unknown attribute of the device may account for the difference in response across the well. Analysis of antibody binding over the cuvette has shown very little variation to occur throughout the surface and does not explain why one half of the cuvette has a difference in response.

For each albumin standard used, a response generated in all 16 channels meant that each channel represented a replicate. In addition each albumin standard was passed over the cuvette twice and comparisons were made in order to determine the reproducibility of the results. A paired t-test analysis suggested there to be no significant difference between the replicates. The device was reliable enough to give reproducible data on the same assay. Replication is important when calibrating a cuvette however a compromise between the time spent on calibration and on analysing patients samples must be considered in the clinical setting. To improve the experiment, it is better if the number of replicates were over 3 or 4 however in a system where there are 16 replicates for every assay, very little repeats are needed proving an advantage in the clinical setting.

Unexpected differences were observed between channels of the same well. Looking at the responses obtained using a four well cuvette, responses occurred in wells which contained no antibody. One reason for this occurrence is possible leakage of antibody between the gasket dividers for each well. Comparing channels to their position on the

cuvette, those channels who are affected appear to lie on the border of the gasket divide (figure 5.1).

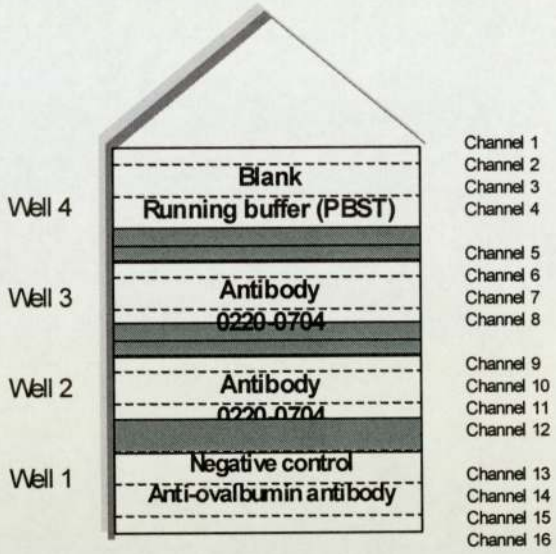


Figure 5.1. The blue/grey areas represent where the gasket divides lie and also shows the channels which have shown leakage throughout all four well immobilisations.

The leakage problem could be addressed by a better design of the gasket and alignment jig. Alternative plastic polymers may allow for a more efficient seal. Introduction of a microfluidic system would separate the cuvette such that addition of the antibody of interest could be done in a more specific and accurate manner, however this alternative is expensive. A third alternative would be to activate the surface of the cuvette within the well instead of all over, as was done here. If leakage were to occur without activation of the cuvette, the ligand should not be able to permanently bind to the cuvette and so once the cuvette was washed, any due to leak should also be removed. The obvious disadvantage is that it could not be ensured the surface was activated.

Antibody 0220-0704 is favoured for its ability to detect albumin over a wide range (1-100μg/ml). It has high enough affinity and avidity to bind low albumin concentration yet still be able to dissociate from the antigen and can be reused. It can withstand several regenerations without affecting the binding capability (data not shown).

The responses of those channels selected were averaged and the initial rate calculated. The calibration curves showed there to be a curve relationship between initial rate and

[HSA]. This does not compare to the calibration curve for a one well immobilisation which showed a linear plot (figure 3.8). One reason for the difference is the increased concentration of antibody exposed to the cuvette. Whilst normally an increase in antibody concentration would increase the number of binding sites, possible saturation of antibody on the sensor surface may lead to hindering of the binding site.

Although possible leakage occurred between wells with the use of a four well cuvette, this layout was used for further analysis of urine samples. The reason primarily, for the use of the four well cuvette was because of the control and blank wells. While they made very little difference with albumin standards, a control is important to include in order to ensure the validity of experimental result.

A separate cuvette was immobilised using a four well immobilisation protocol and a range of albumin standards, more specific to the MAU range, were placed over the cuvette in order to calibrate it. As there were inconsistencies in channel response only selected channels were further used to calibrate the cuvette. These channels were chosen based on their ability to generate a response at maximal levels. Calibration of the cuvette produced a rectangular hyperbola standard curve showing saturation towards 32µg/ml [HSA]. This follows the pattern observed in the calibration curve obtained in section 3.1.2.

It is necessary to optimise conditions to obtain consistent values for every channel in each well. It is also important to recognise that the channels of each well also act as replicates of n=4 of each other. On average, it takes 5minutes for each albumin standard response to reach completion and the cuvette to be regenerated. An initial calibration of the cuvette should thus only last 40 minutes if eight albumin standards were used. This could then allow for up to 100 patient samples to be analysed. According to manufacturers guidelines the cuvette is deemed useful up until 50 regenerations. However experiments using these cuvettes have shown that after 75 regenerations (data not shown), the cuvette is still viable with this antibody. For this particular experiment, the cuvette was regenerated 24 times, including the standards, where no recalibration of the cuvette was necessary. The high correlation (0.995) between the quality controls, which were used at the end of the experiment, demonstrates that the antibody on the cuvette had not deteriorated in its binding capacity.

One way of avoiding a curved calibration would be to linerase the data. Various statistical models may be applied, which would do this however this would complicate further data analysis and a computer package would need to be set up in order to calculate unknown samples into the equation. A simpler alternative would be to reduce the number of bound antibody upon the cuvette to see if this had any effect. A series of trial and error experiments would need to be established in order to determine the maximum level of antibody required without affecting the final response. The antibody concentration is the only difference between a one and four well that could contribute to the difference in calibration relationship. Another possible reason may be due the positioning of the gradient in each assay response. Taking the gradient of the slope from the initial reflection in response may introduce errors because the system is assuming association is occurring at this point and not bulk refraction.

A curve was fitted with a saturation model and a comparison was made between the calculated albumin concentrations using this model to the linear model. It was observed that only a slight difference in correlation of 0.994 was obtained using the saturation equation where a fit of 0.995 existed for the linear model. While figure 3.14 showed the saturation model to fit best, table 3.6 demonstrated the linear fit to be superior. As the entire test samples and quality controls were within the range of the linear region of the standard curve fitting a saturation model would not fit the actual event hence allowing for the linear model to give more precise calculations.

Ten urine samples were investigated using the calibration curve from figure 3.14 A and 3.24 B. All of the samples had previously been analysed by Selly Oak hospital using DAKO assay. When comparing the results obtained using this assay to that of the DAKO assay very little similarities existed between the samples. One possible reason for the difference is the time difference between DAKO analysis and the current study. Although the urine samples had been stored in the fridge with sodium azide to act as a preservative, possible breakdown of albumin could still have occurred due to the long incubation time for which the samples were kept at 4°C. Another reason for the difference is due to the use of different antibodies, hence a difference in epitope recognition for human serum albumin exists making it invalid to compare the results of both assays. Instead comparison of the rank order was performed to allow for a fair

analysis; and indeed a good correlation between the response of the two assays were observed.

Comparing the calculated urine samples using the saturation equation with the linear equation, showed very different values. Negative results were obtained when using the linear equation, where this was not the case for the saturation model (table 3.7 and 3.8). While the values derived from the saturation equation were not comparable to the DAKO assay, it is apparent that it is a better model to use based on the positive values derived. The saturation model is a better fit for urine samples because the samples range well over the range of the assay. A linear model indicates that the plot would be straight for all concentrations where it is obvious that saturation occurs towards the higher concentration of albumin and so fitting a saturation model is more appropriate allowing for better data.

In theory there should be no preference as to which model should be used for future assays. In diagnostic settings the user is not concerned with the line of best fit for example, there concern is with the accuracy and reproducibility of data. As long as the model used describes the binding events correctly and is appropriate than that would be the best model to use.

Four test samples were initially run before the urine samples in order to determine feasibility of the calibration curve. All four tests samples showed very good correlation. The test samples were random albumin concentrations designed to fall within the linear range of the standard curve. They were freshly made up alongside the albumin standard and so their concentrations were already known.

Two further antibodies were investigated in an attempt to optimise albumin detection. Experiments using antibody 0219-9970 showed no response when albumin standards ranging from 2-32μg/ml was passed over the cuvette. From these studies it suggests that antibody 0219-9970 cannot detect albumin concentration below 32μg/ml. Reasons for this could be due to a low affinity between albumin and the antibody; and so a much higher albumin concentration is required in order to drive a response. For the purpose of this assay, antibody 0219-9970 is not suitable as the derived detection range is 2-32μg/ml.

Antibody A1151 was able to detect albumin within the desired diagnostic range. As with antibody 0220-0704, leakage occurred between wells when assaying the various albumin concentrations. An abnormally high response was generated when 2μg/ml [HSA] was added to the cuvette. When this assay was repeated a much lower response was obtained. Previous experiments have always shown the repeats to be consistent with very good reproducibility. The reason for the difference in response on this occasion may be associated with the fact that it was the first standard to be placed over the cuvette. Whilst normally this should not make a difference, in some instances the first assay usually elicits false readings, which was the case in this instance.

The calibration curve obtained using antibody A1151 followed a similar pattern to that obtained by antibody 0220-0704 however the responses obtained using antibody A1151 were much lower suggesting the antibody to be less sensitive and hence has no visible advantage over antibody 0220-0704. Due to this no further analysis using antibody A1151 was performed.

5.2 Creatinine detection using the resonant mirror.

Unlike the immobilisation of anti-human serum albumin antibody, the binding of creatinine deaminase to the surface of the cuvette proved to be difficult. From the response curves obtained it was thought that there was not enough attraction for the enzyme to reach the cuvette surface in order for effective binding. The overall charge of the enzyme was unknown and so establishing optimum pH conditions for the binding stage had to be performed by trial and error. Referring to figure 4.2, pH 4.5 appeared to give optimum conditions for the attraction of the enzyme to the surface of the cuvette. This only occurred when the buffer was inserted into the cuvette first and not when the enzyme was dissolved into the buffer and the mixture then added to the cuvette. Using these conditions, the concentration of bound creatinine deaminase tripled, giving a final response of 933 arcsec. It is not known why the order in which the buffer is added to the cuvette should make a difference to total binding of creatinine deaminase. The hydrogen ions available in the buffer may change or prepare the sensor surface such that when the enzyme is added more attraction and binding occurs.

Further optimisation of creatinine deaminase is still needed to bring the response to recommended binding levels of around 2500 arcsec. This would in turn increase the potential sensitivity of the assay since the more available sites the more creatinine will be able to bind and the higher the response generated. A way of improving the immobilisation of creatinine deaminase may be to change the coupling chemistry of the enzyme to the dextran surface of the cuvette. Other possible coupling chemistries are streptavidin binding, cystamine as well as PDEA (BIA application handbook). Large variation in response throughout all 16 channels of a one well cuvette occurred (figure 4.3) denoted by a large SD. A reason for such differences could be due to inconsistency in charges over the cuvette and hence a difference in attraction of the enzyme to the sensor surface.

Creatinine standards were placed over the cuvette containing creatinine deaminase. As the standards increased in concentration, an increase in response was observed. An indirect detection of the reaction occurred, showing there to be a linear relationship between creatinine concentration and maximum response generated. Creatinine

concentration was determined by the release of hydrogen ions. The more creatinine present in solution, the more will be broken down by the enzyme, causing an increase in hydrogen ion release. It is thought that the change in localised pH caused by the release of hydrogen ions changed the dextran layer, which in turn elicited a response. Another reason to the relationship of creatinine to response changes may be due the change in enzyme configuration. When creatinine binds to creatinine deaminase, a slight change on configuration may take place in order to make other creatinine binding sites more available. The change in enzyme structure could also elicit a change in refractive index. These effects can be removed by simply washing the cuvette with PBST. Creatinine is broken down and so it is not a case of chemically removing bound ligand as would be the case with an antibody-antigen complex. Washing the cuvette not only restores the baseline but it also means that a regeneration stage as such is not needed because any creatinine not broken down by the enzyme would be automatically washed away.

The current amount of bound creatinine deaminase allowed for a minimum detection of creatinine of no less than 31μg/ml. Concentrations of creatinine below 31μg/ml were analysed (data not shown) however no visual response was generated suggesting 32μg/ml to be the detection limit for this assay. 31μg/ml is equivalent to 270μM. Studies utilising creatinine as an index for variations of in urine flow, use mmol/l ranges together with albumin to give a UAER (urinary albumin excretion rate) which if greater than 30mg/g indicates MAU to be present (Ng *et al.,* 2000; Jenson JS *et al.,* 1997). Conversion of 32-1000μg/ml would give 270mmol/l – 8.6 X 10^3 mmol/l.

Further experiments were conducted to verify that the responses obtained in creatinine assays were due to creatinine breakdown. Two creatinine assays of varying creatinine concentrations were passed over a cuvette containing bound albumin. A slight response was only seen when 1000μg/ml creatinine was added to the cuvette. This suggests that at such a high creatinine concentration bulk effect, not specific to creatinine breakdown, will exist. However running a control alongside each creatinine standard and subtracting the values from the total response would allow for the specific response of creatinine. To show that the pH affects the cuvette, a series of buffer with different pH were run over a blank cuvette. Varying responses were seen with varying pH buffers however a distinct pattern was not observed. In particular negative values occurred at certain pH and positive at others. A reason why this might occur is to do with the swelling or

deflation of the dextran. Dextran is negatively charged and so maintains a certain shape partly aided by the negative charges present. At a certain point positive hydrogen ions will change the overall charge and in some cases causes a deflation in overall structure, whereas at other pH a swelling of the dextran surface may occur.

Chapter 6
CONCLUSION

The resonant mirror has been shown by other groups to be one of the most accurate and precise detection systems for biological solutions. Benefits include such features as rapid data output, direct interaction analysis, real-time responses able to detect turbid and low analyte samples. Clinically the use of such a system may one day become real. Patients can be diagnosed instantaneously, saving lives by reducing later complications associated with trauma and reducing costs.

In this particular study use of MAU as a marker of trauma was investigated. From the data, it is apparent that further analysis is required to optimise all conditions needed for the detection of MAU. Nevertheless, it has been successfully demonstrated that detection of both albumin and creatinine is possible. The data obtained is accurate, reproducible and sensitive enough for diagnosis of MAU. Initially problems were incurred with the use of creatinine deaminase, however there is potential optimisation.

There is the potential to include the detection of other markers for trauma. The introduction of several wells in one a cuvette allows for the inclusion and thus detection of several compounds. It is necessary, however, that the seal used to incorporate wells into the cuvette, be refined to reduce possible leakage of molecules. It has also allowed for a novel application for which compounds below the detection limit of the device can be analysed.

Overall this project has given a better insight into the use of an optical biosensor device for its use in the field of clinical chemistry.

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APPENDICES

Contents

Appendix A

Reagents

A. Constituents of PBST

0.01M Phosphate buffered saline

NaCl- 0.138M

KCl - 0.0027 M

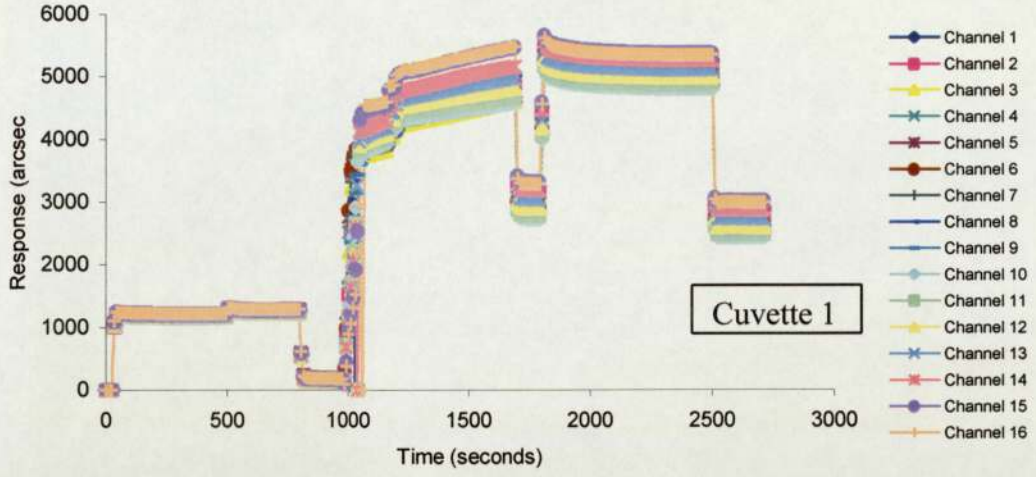
Tween 20 - 0.05%

pH 7.4 at 25° C

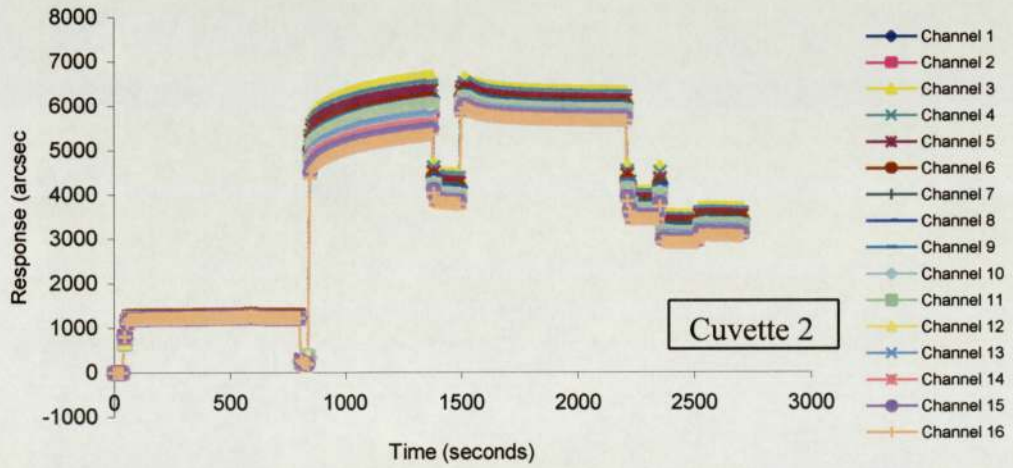
B. Preparation of NHS/EDC mixture

15mg of NHS provided was dissolved in 10ml HPLC grade water to give 0.1M solution. 750mg of EDC provided, was also dissolved in 10ml HPLC grade water to give 4M. These were then mixed in a 1:1 dilution ready for use.

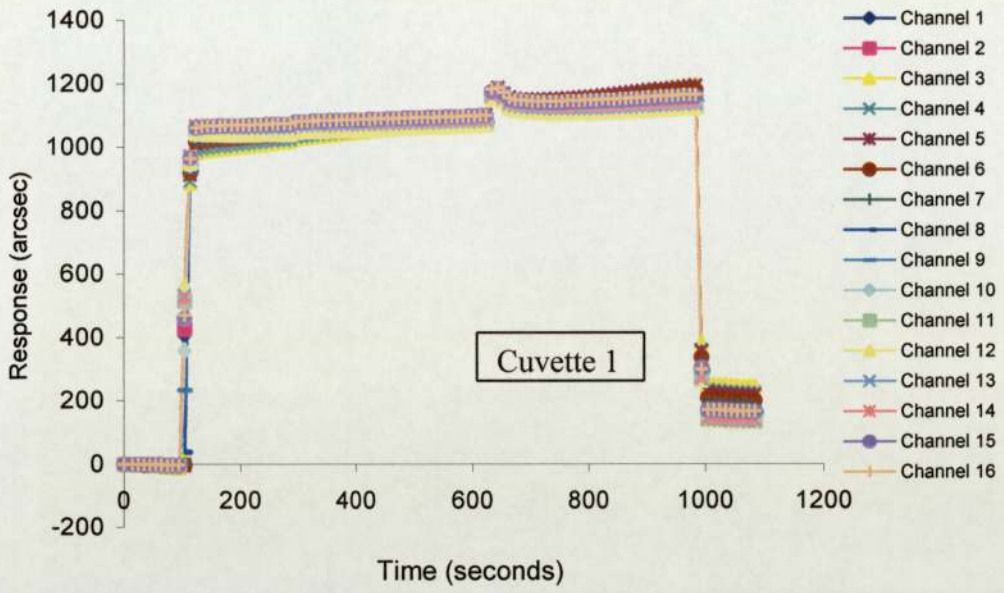
Appendix B



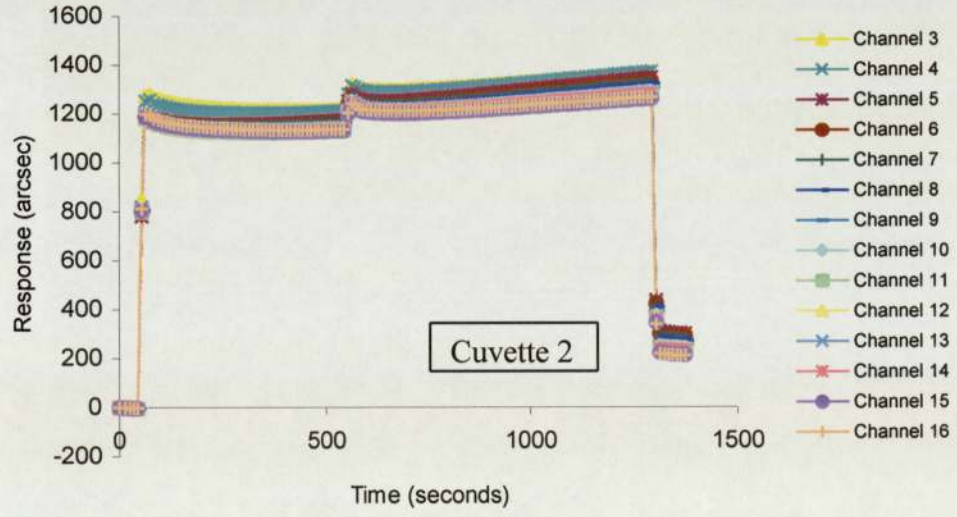
B.1 Raw data showing the association, binding and dissociation stages using antibody 0220-0704 over a one well cuvette.



B.2 Raw data showing the association, binding and dissociation stages using antibody 0220-0704 over a one well cuvette.

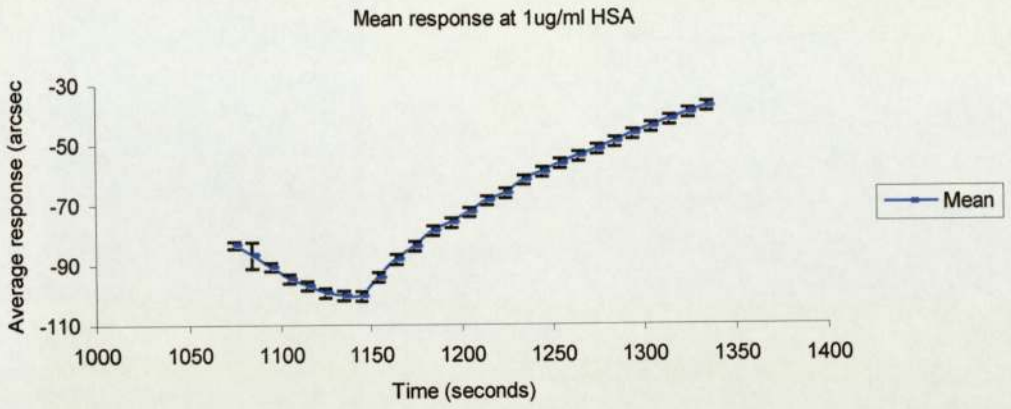


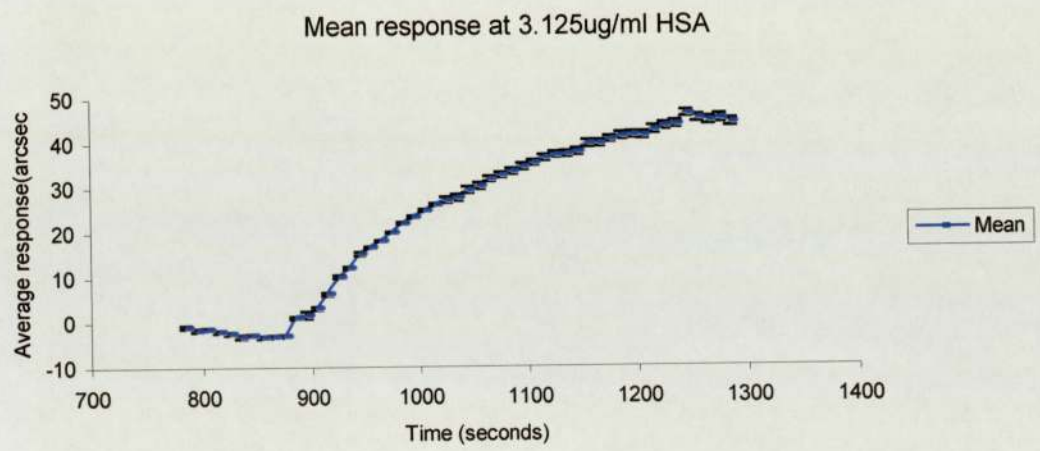
B.3 Activation of a four well cuvette corresponding to figure 3.4. Responses from all 16 channels are shown individually.

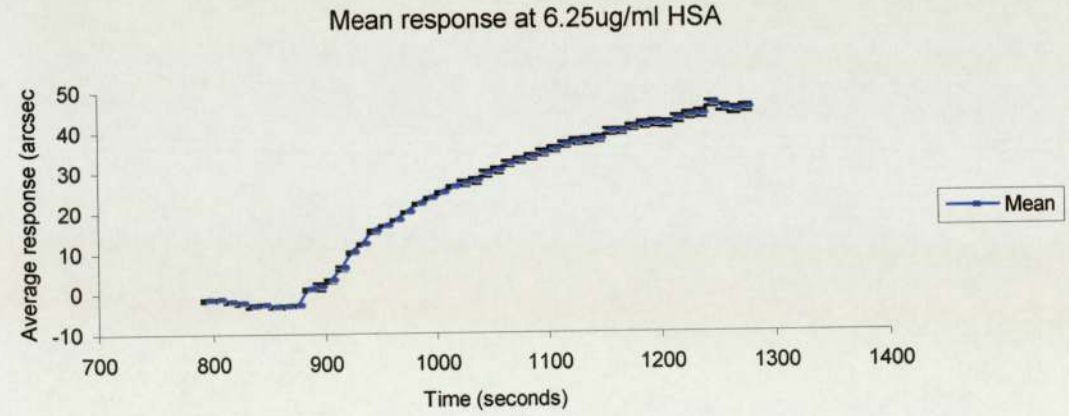


B.4 Activation of a four well cuvette corresponding to figure 3.5. Responses from all 16 channels are shown individually.

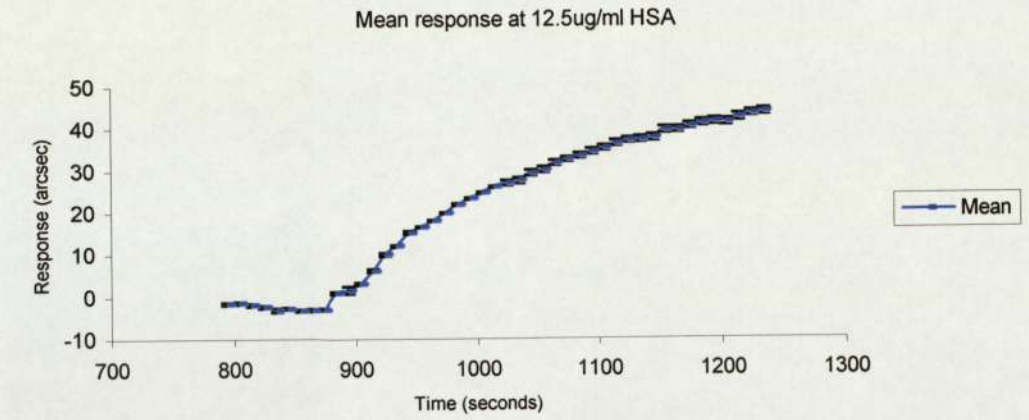
Appendix C

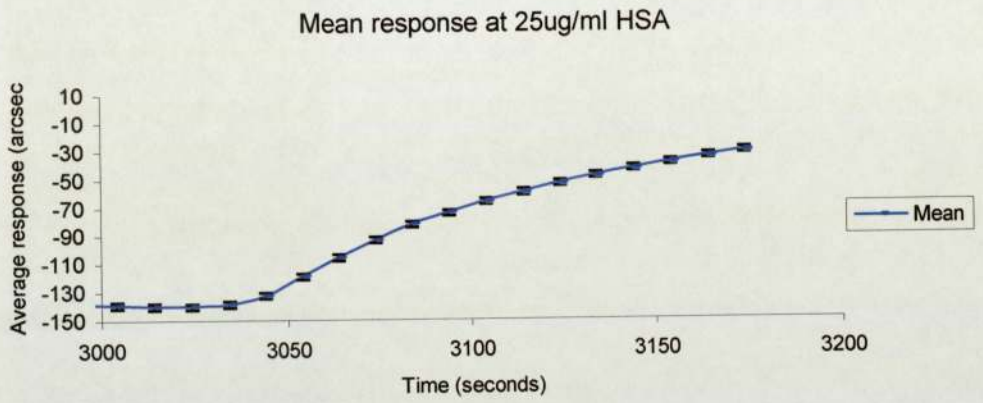


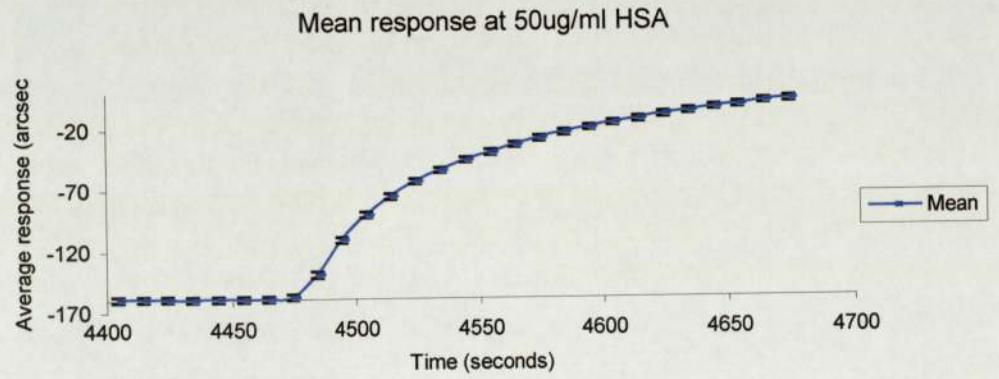


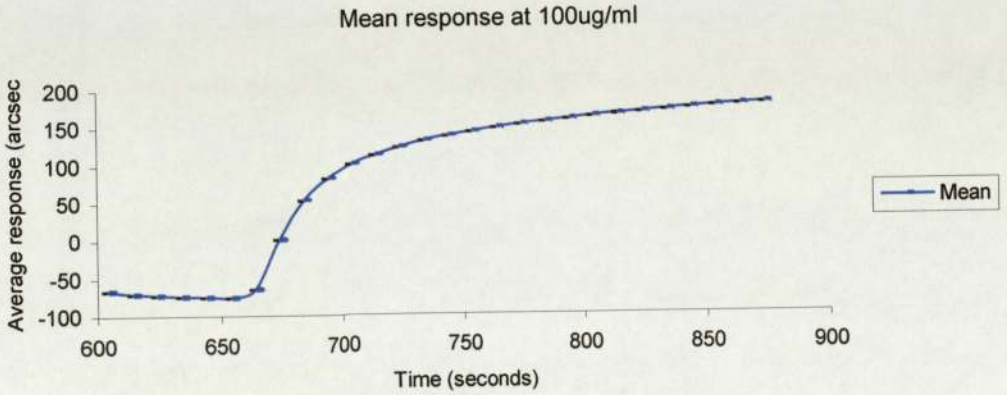


C.1 Mean response taken of all 16 channels for each albumin standard from a one well cuvette. Each standard is a repeat where the black arrows denotes SEM.

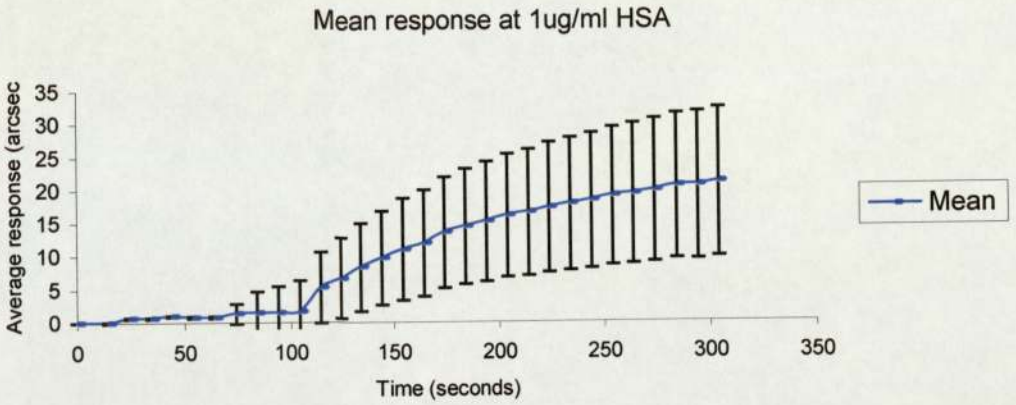


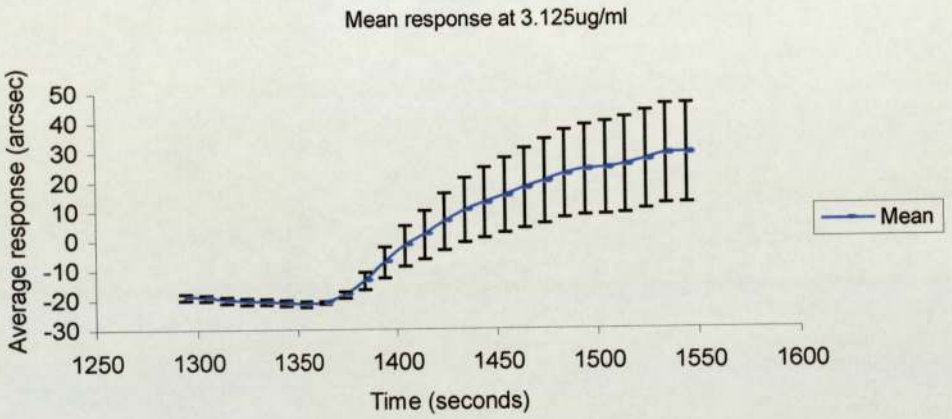


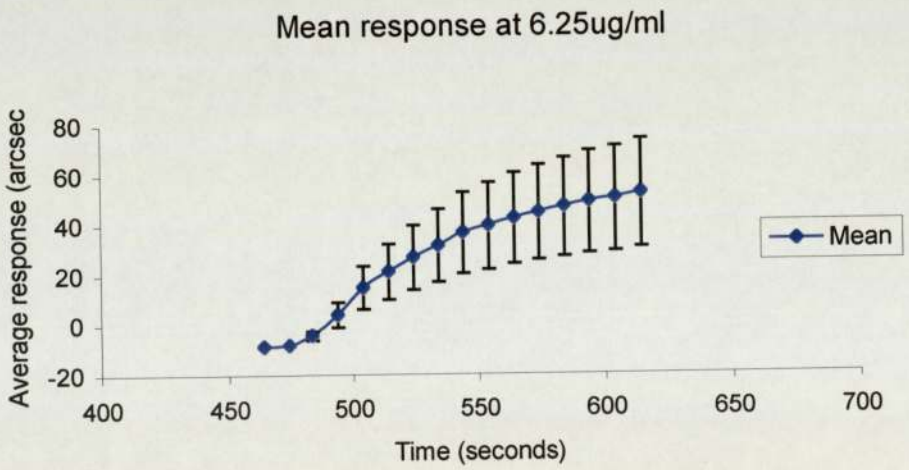




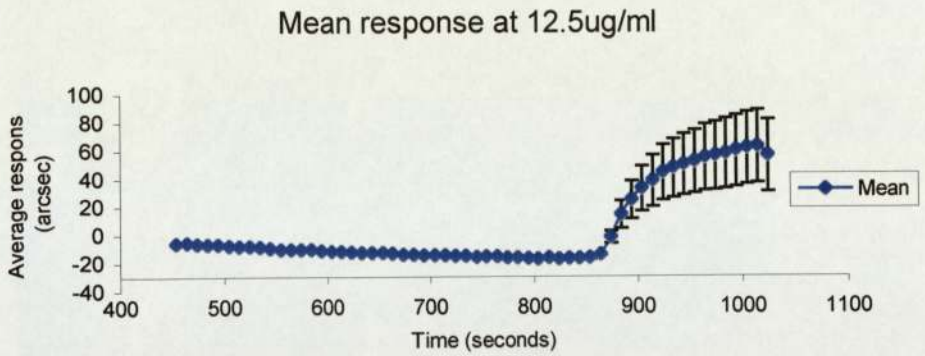
C.1 Mean response taken of all 16 channels for each albumin standard from a one well cuvette. Each standard is a repeat where the black arrows denotes SEM.

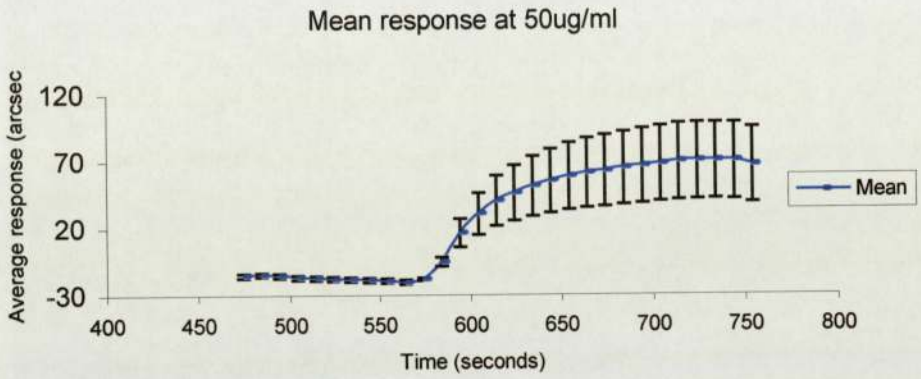


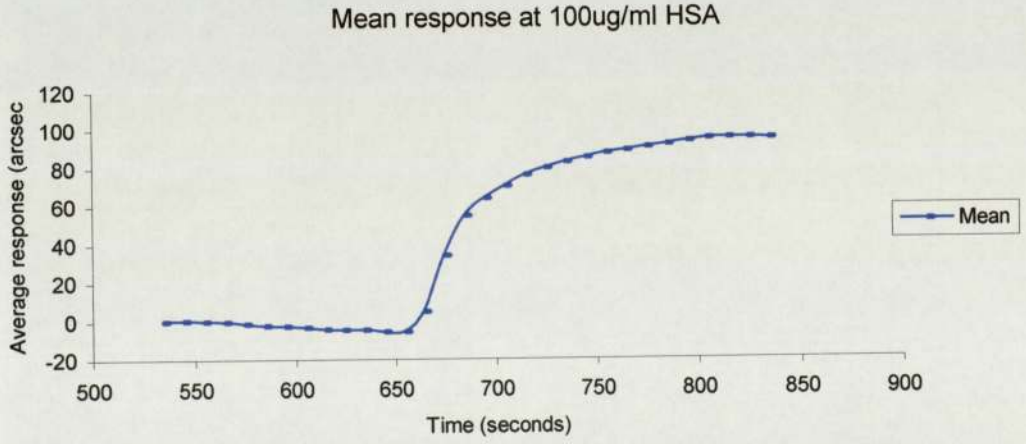




C.2 Repeated data showing the mean response of channels 5,6,10 and 11 from a four well cuvette.

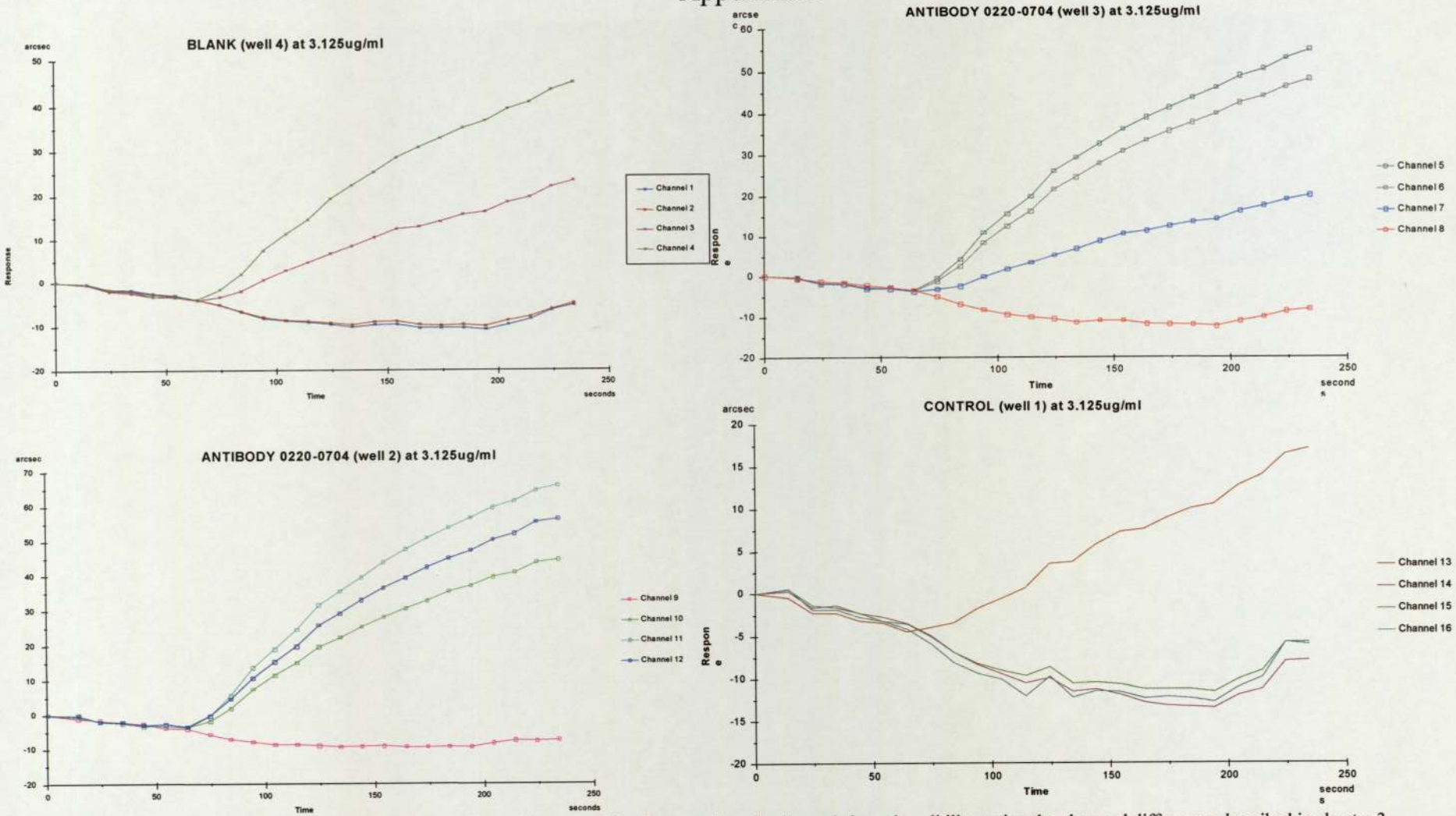




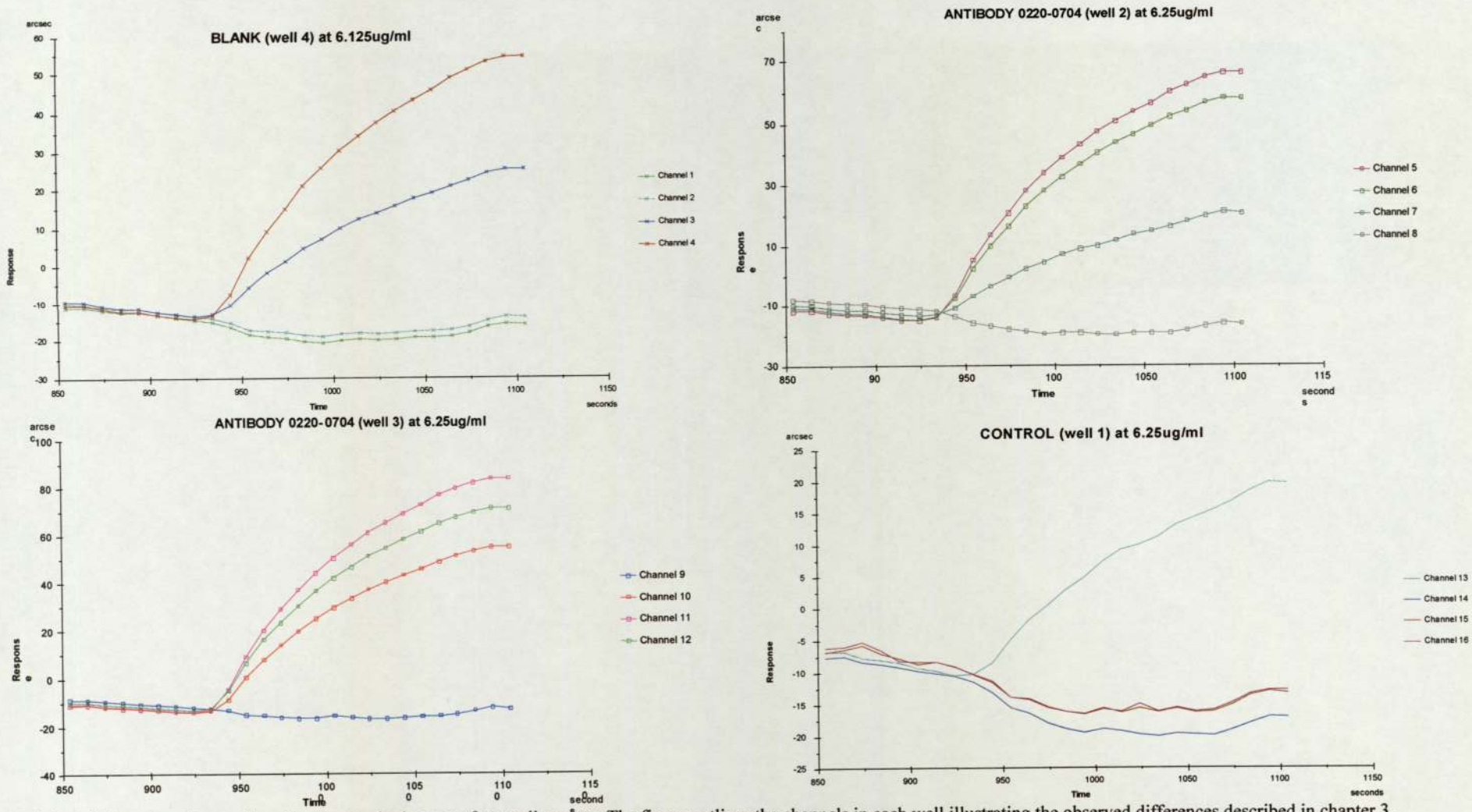


C.2 Repeated data showing the mean response of channels 5,6,10 and 11 from a four well cuvette.

Appendix D



D.1 Association phase for varying albumin standards over a four well cuvette. The figure outlines the channels in each well illustrating the observed differences described in chapter 3.



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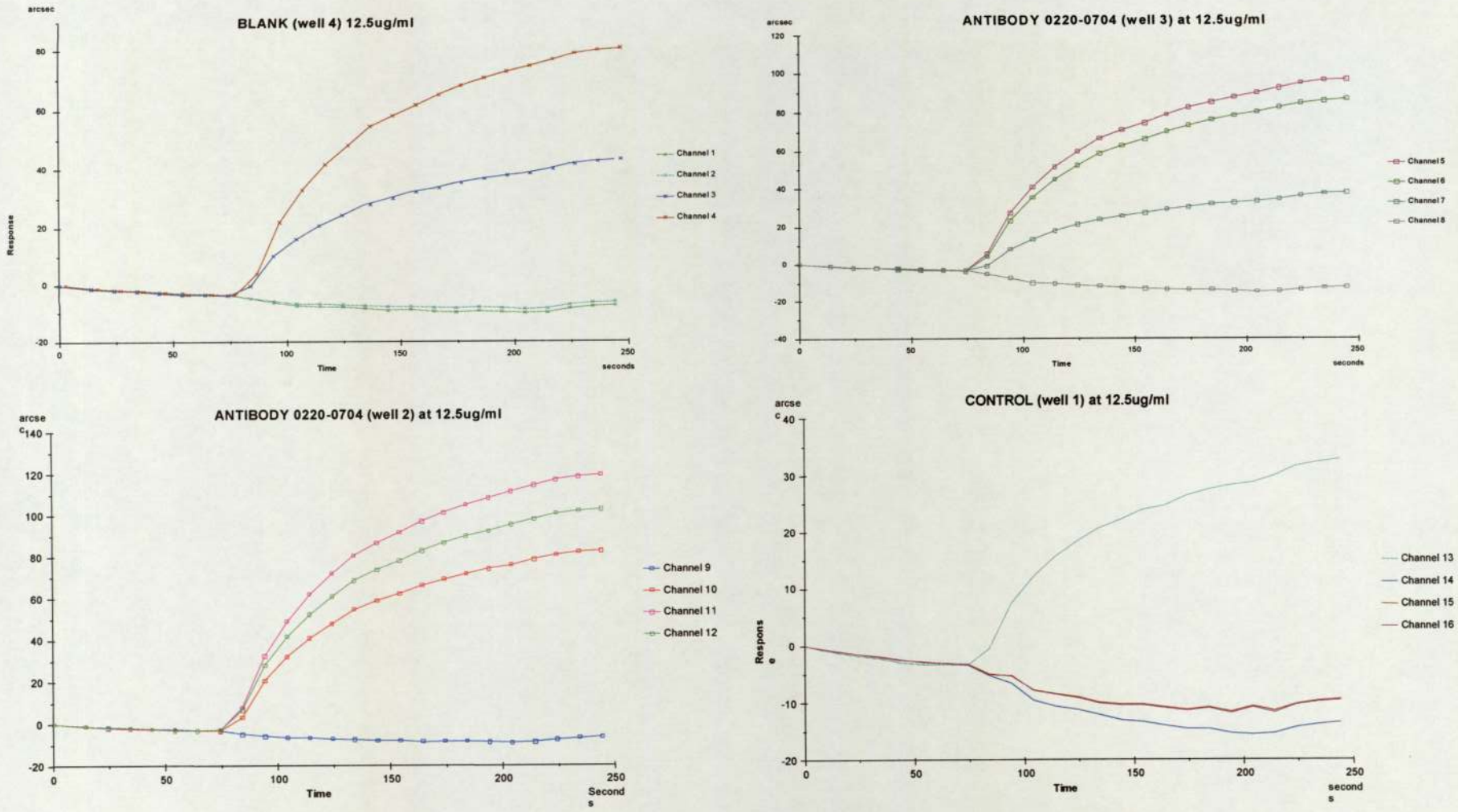
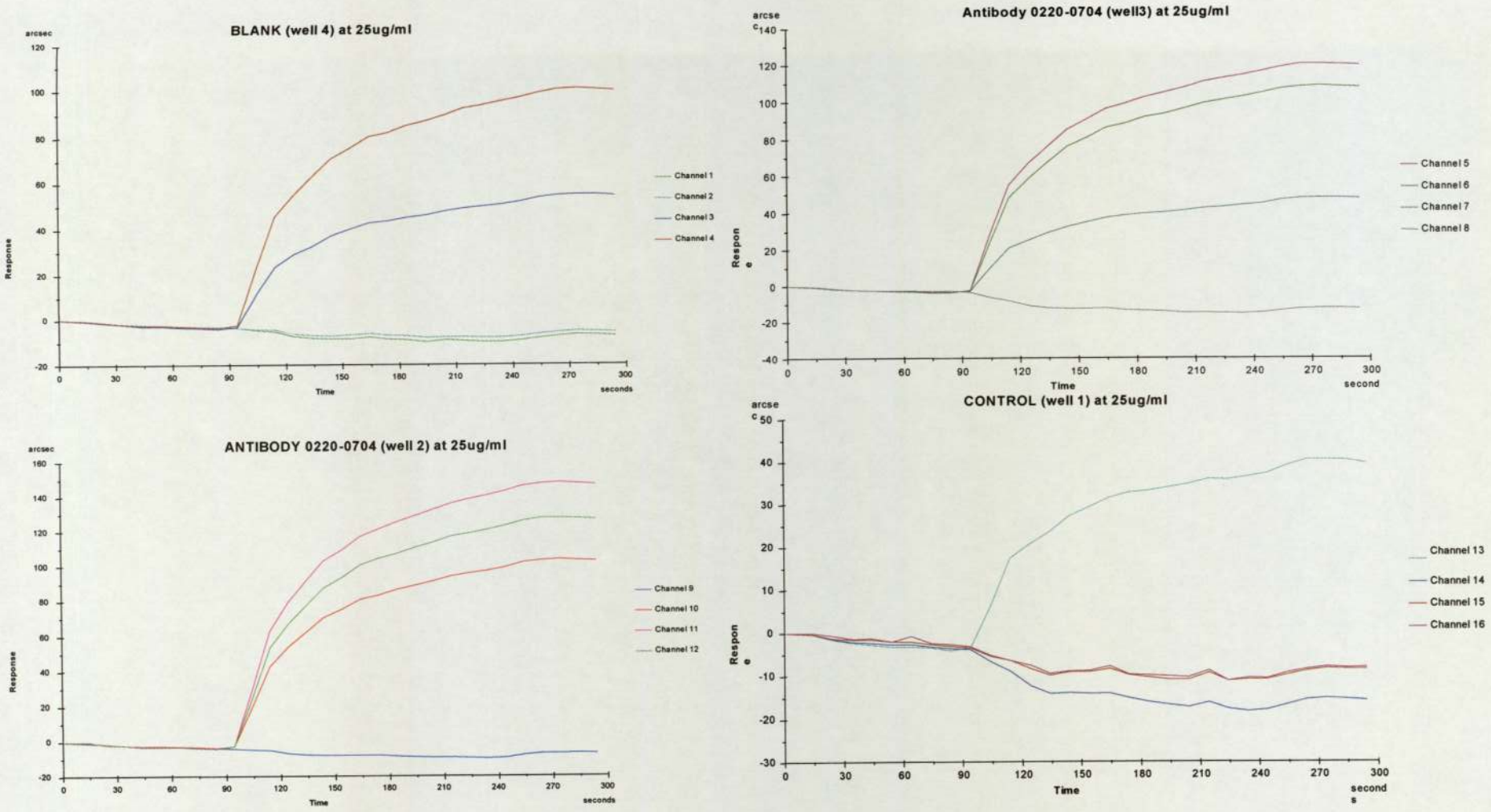
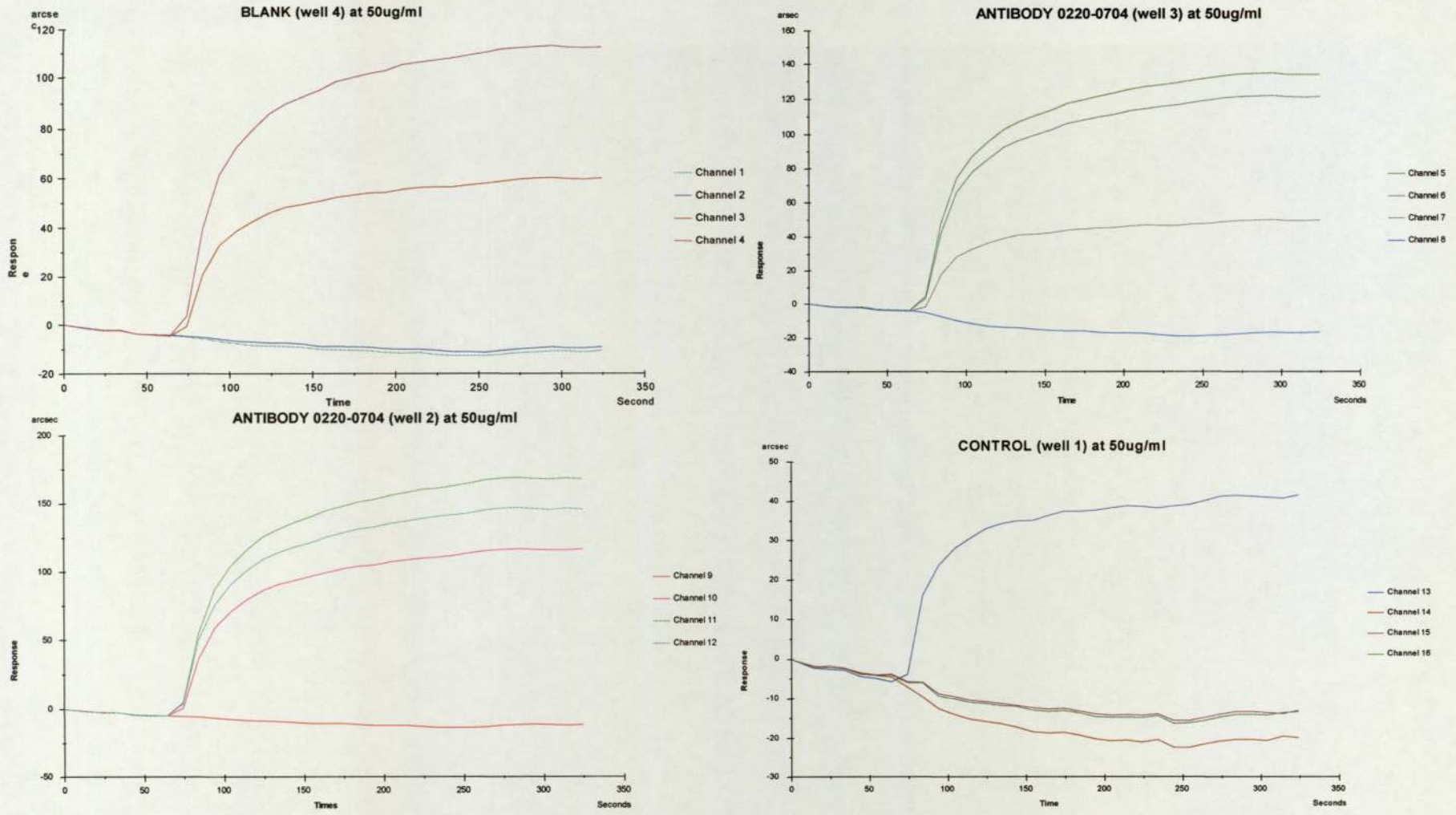


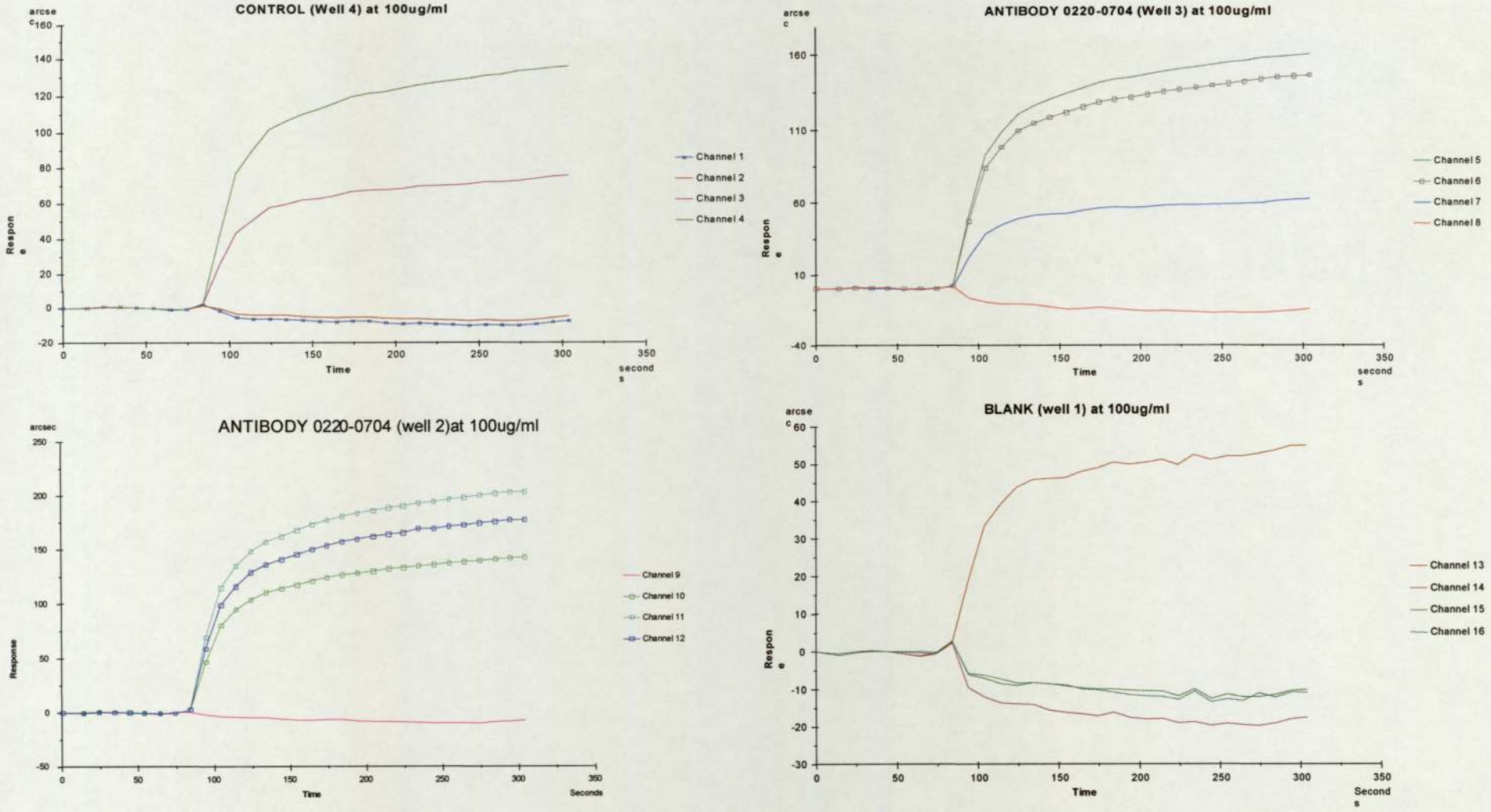
D.1 Association phase for varying albumin standards over a four well cuvette. The figure outlines the channels in each well illustrating the observed differences described in chapter 3.



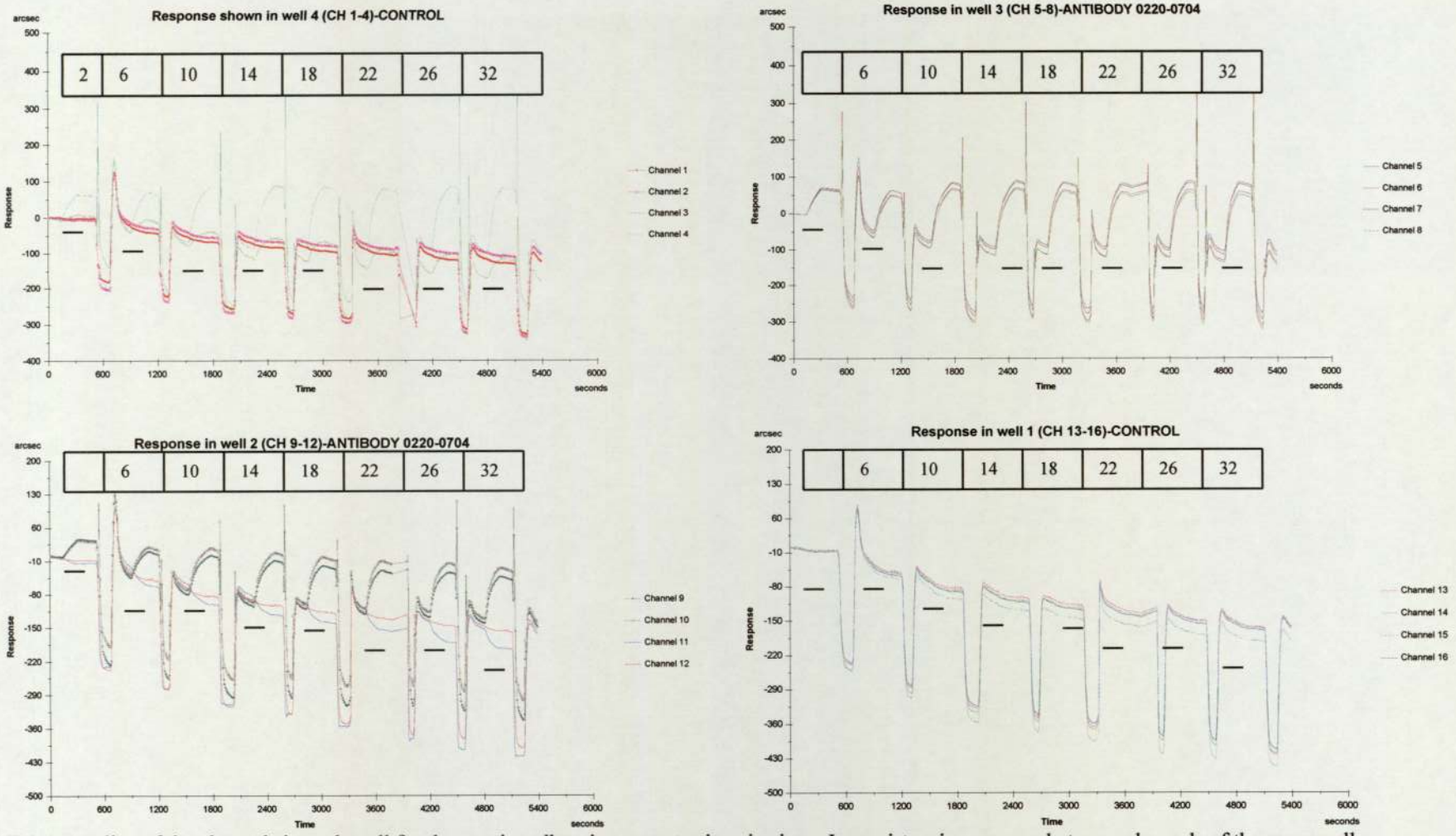
D.1 Association phase for varying albumin standards over a four well cuvette. The figure outlines the channels in each well illustrating the observed differences described in chapter 3.



D.1 Association phase for varying albumin standards over a four well cuvette. The figure outlines the channels in each well illustrating the observed differences described in chapter 3.

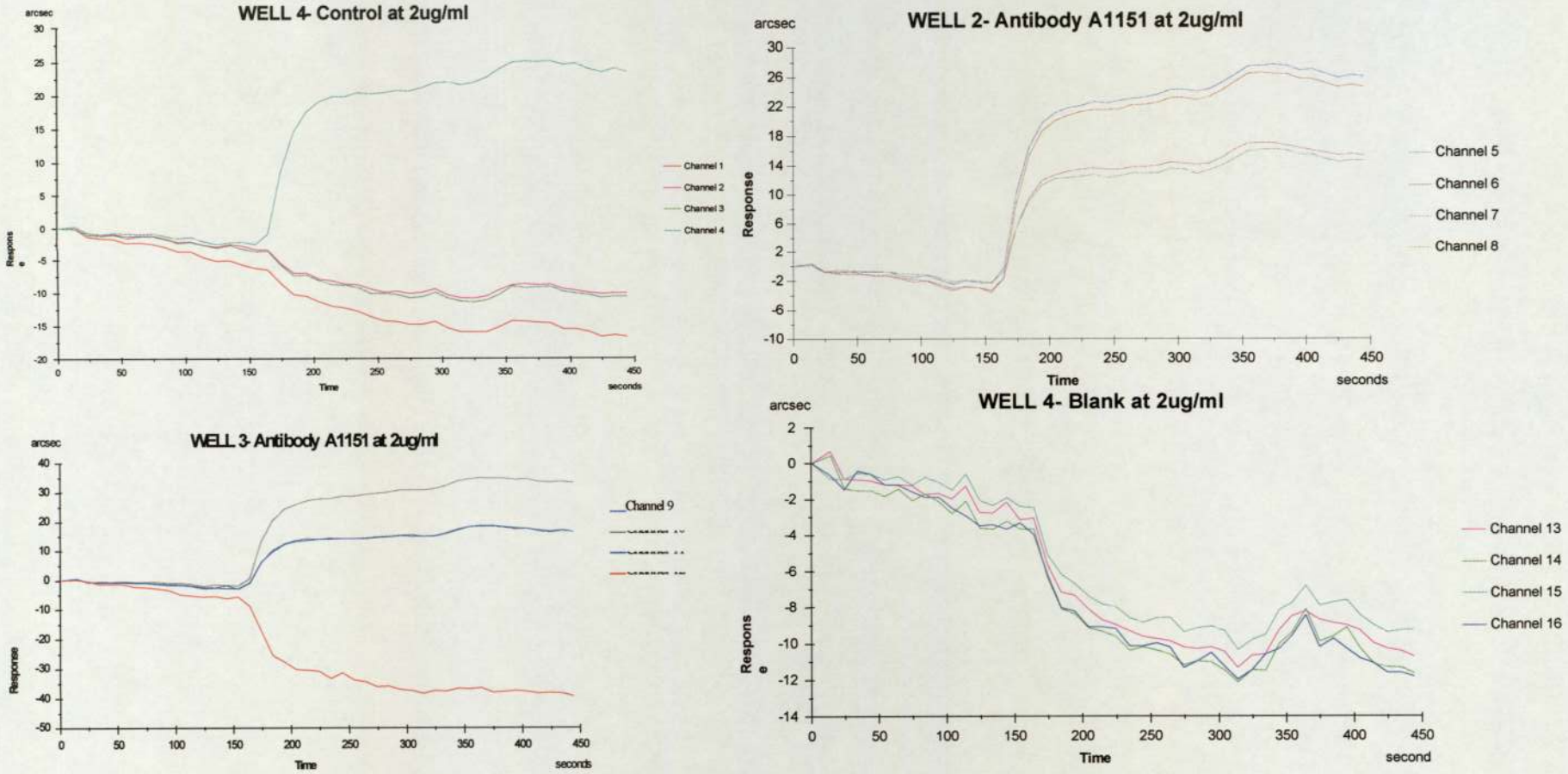


D.1 Association phase for varying albumin standards over a four well cuvette. The figure outlines the channels in each well illustrating the observed differences described in chapter 3.

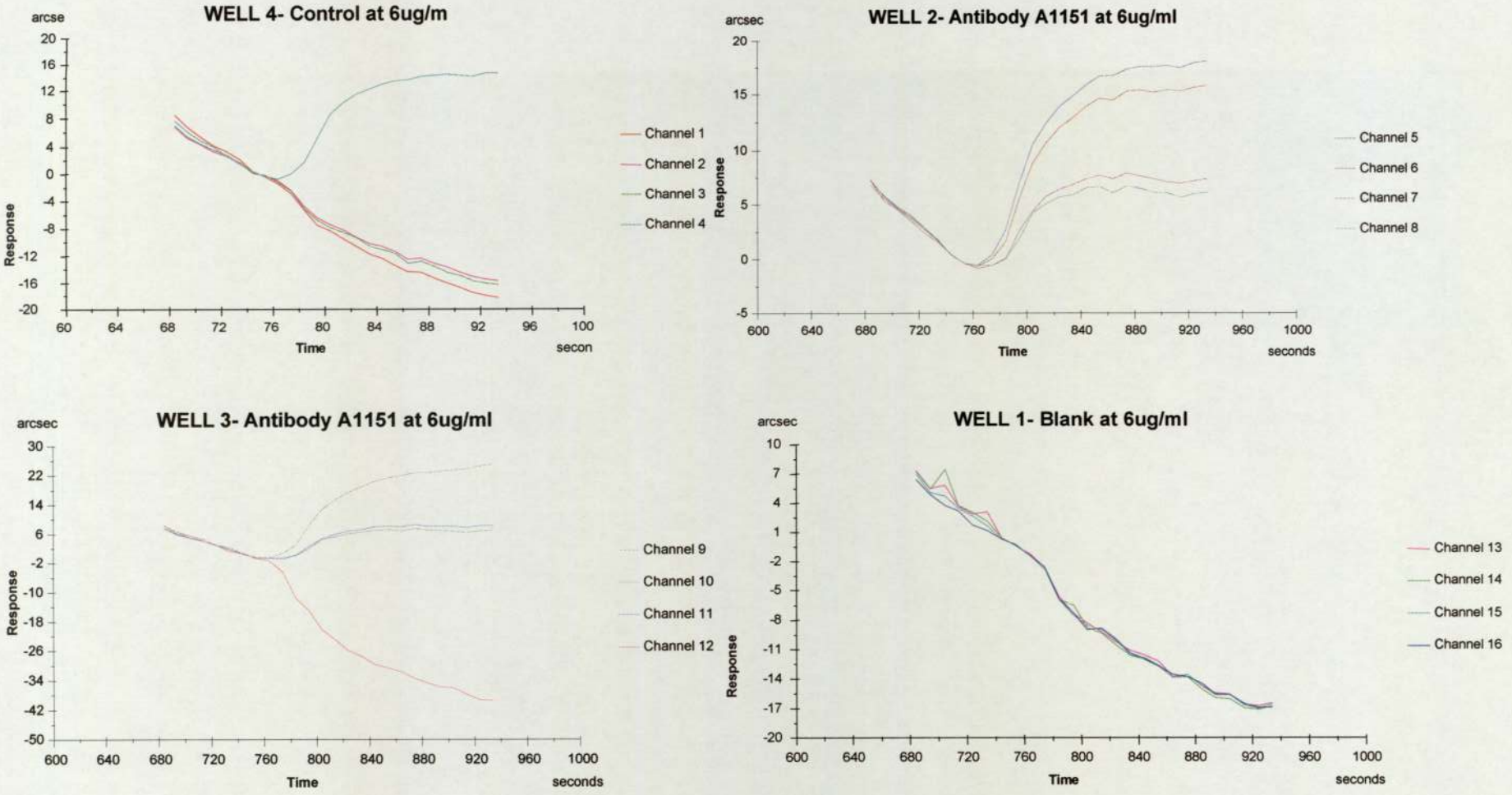


E.1 An outline of the channels in each well for the varying albumin concentrations is given. Inconsistencies are seen between channels of the same well.

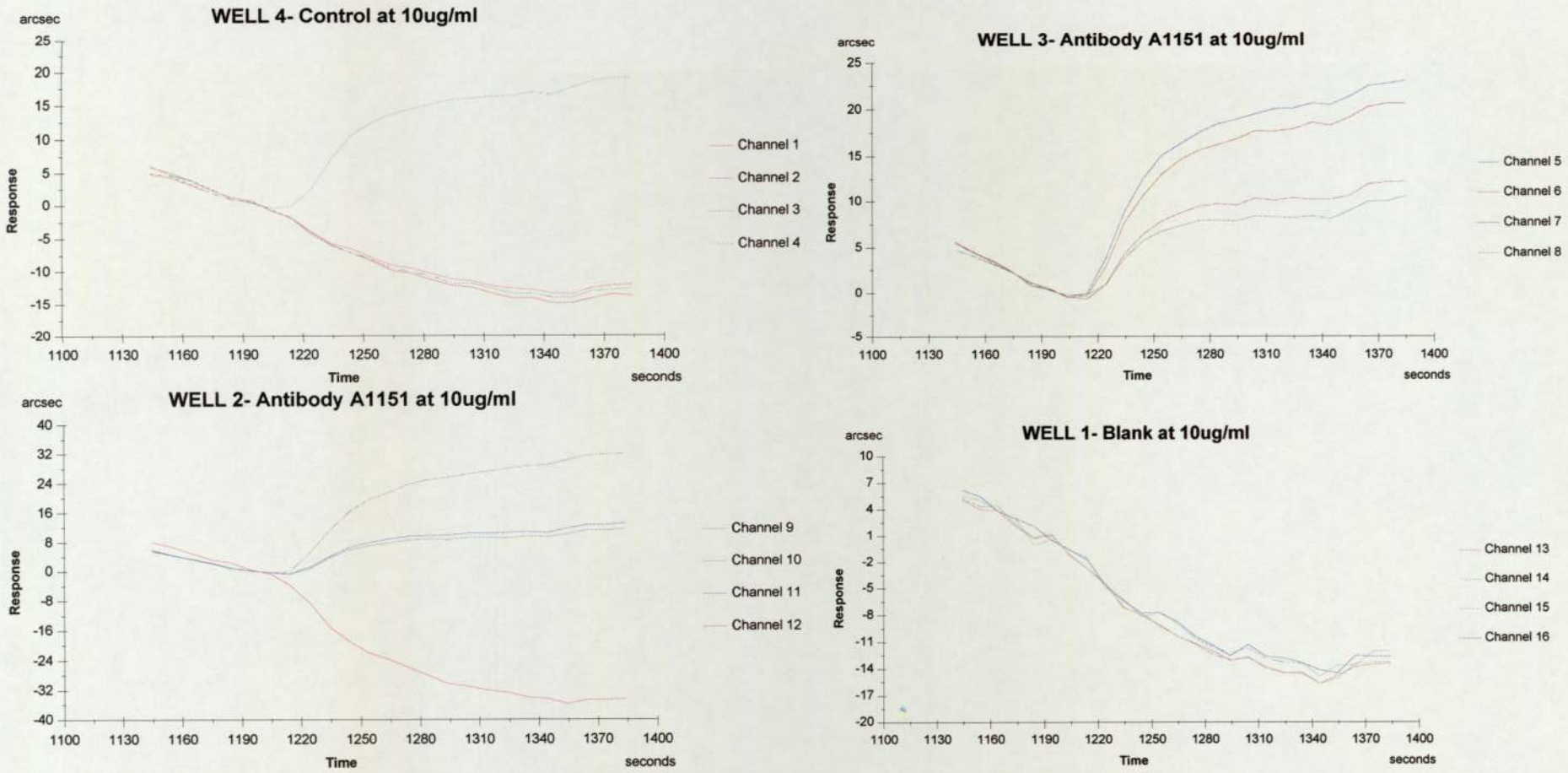
Appendix F



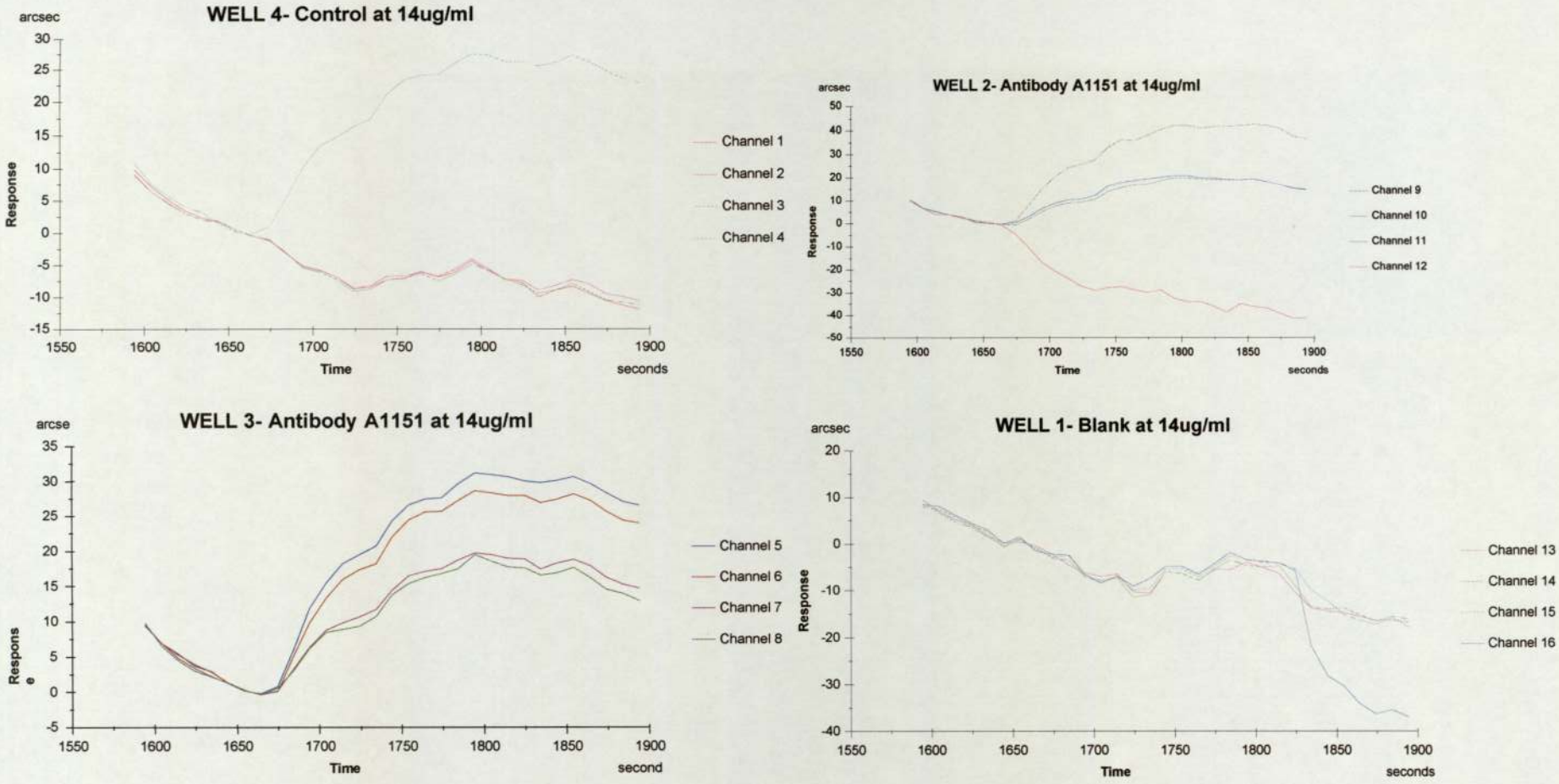
F.1 The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



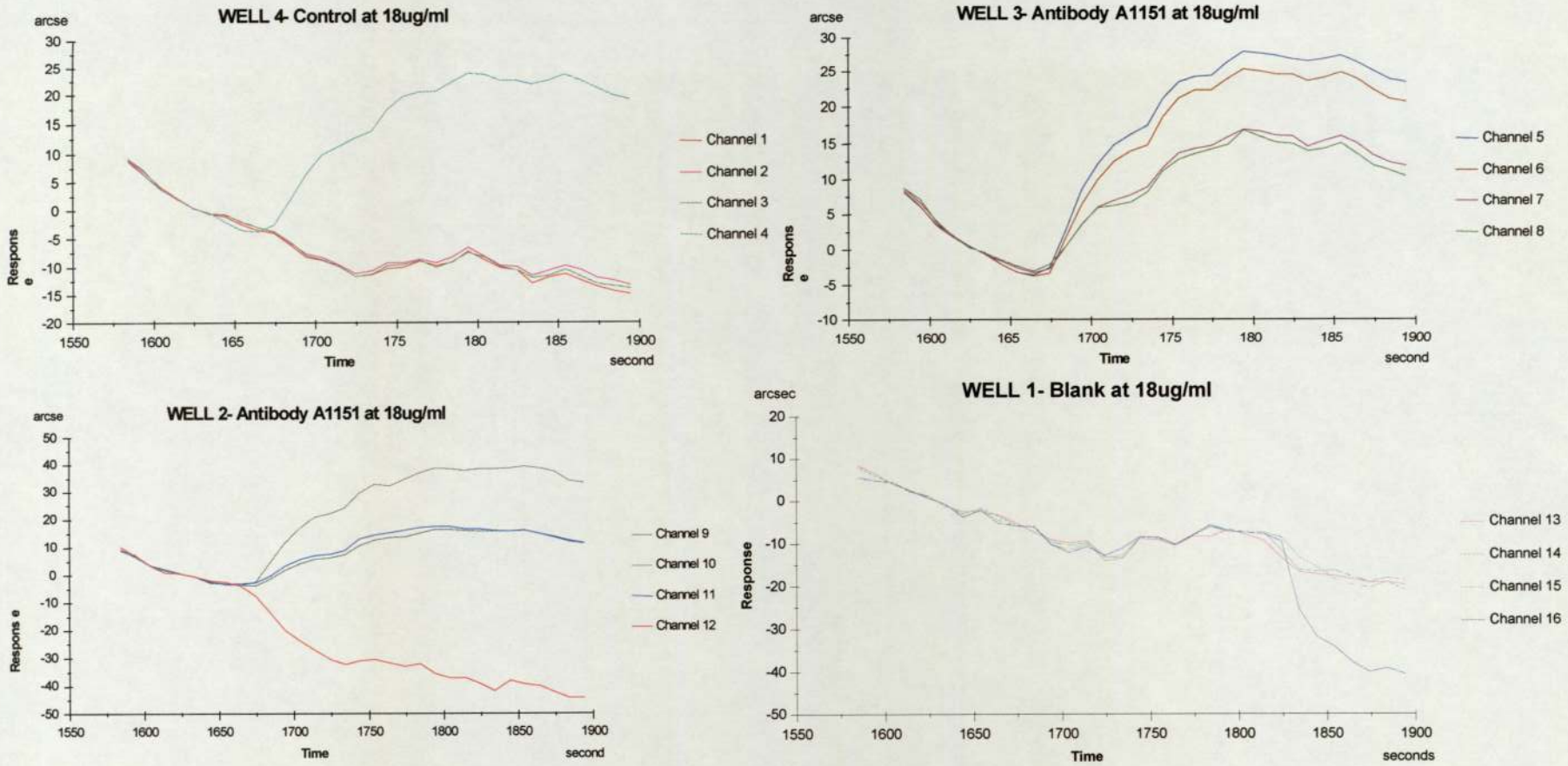
F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



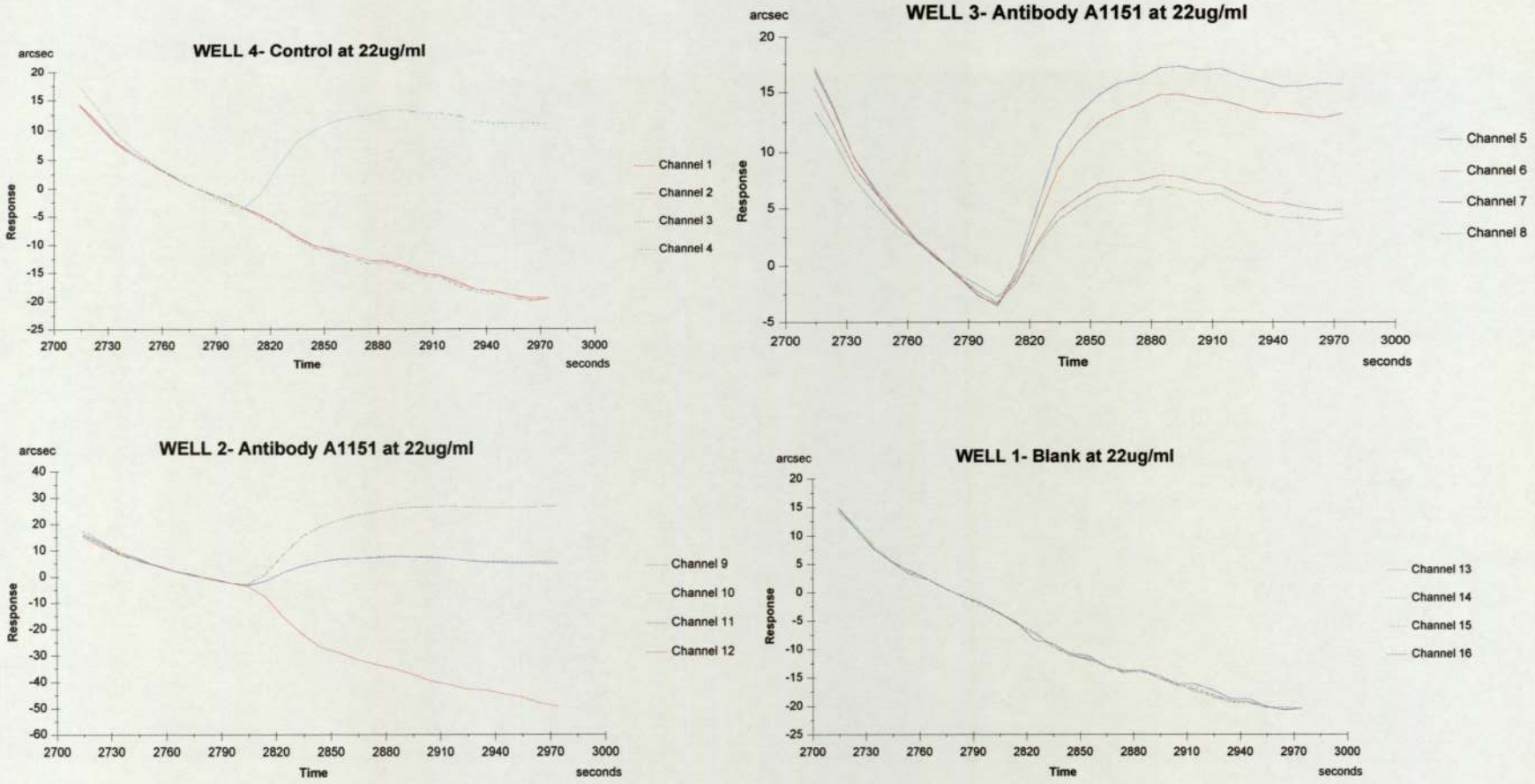
F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



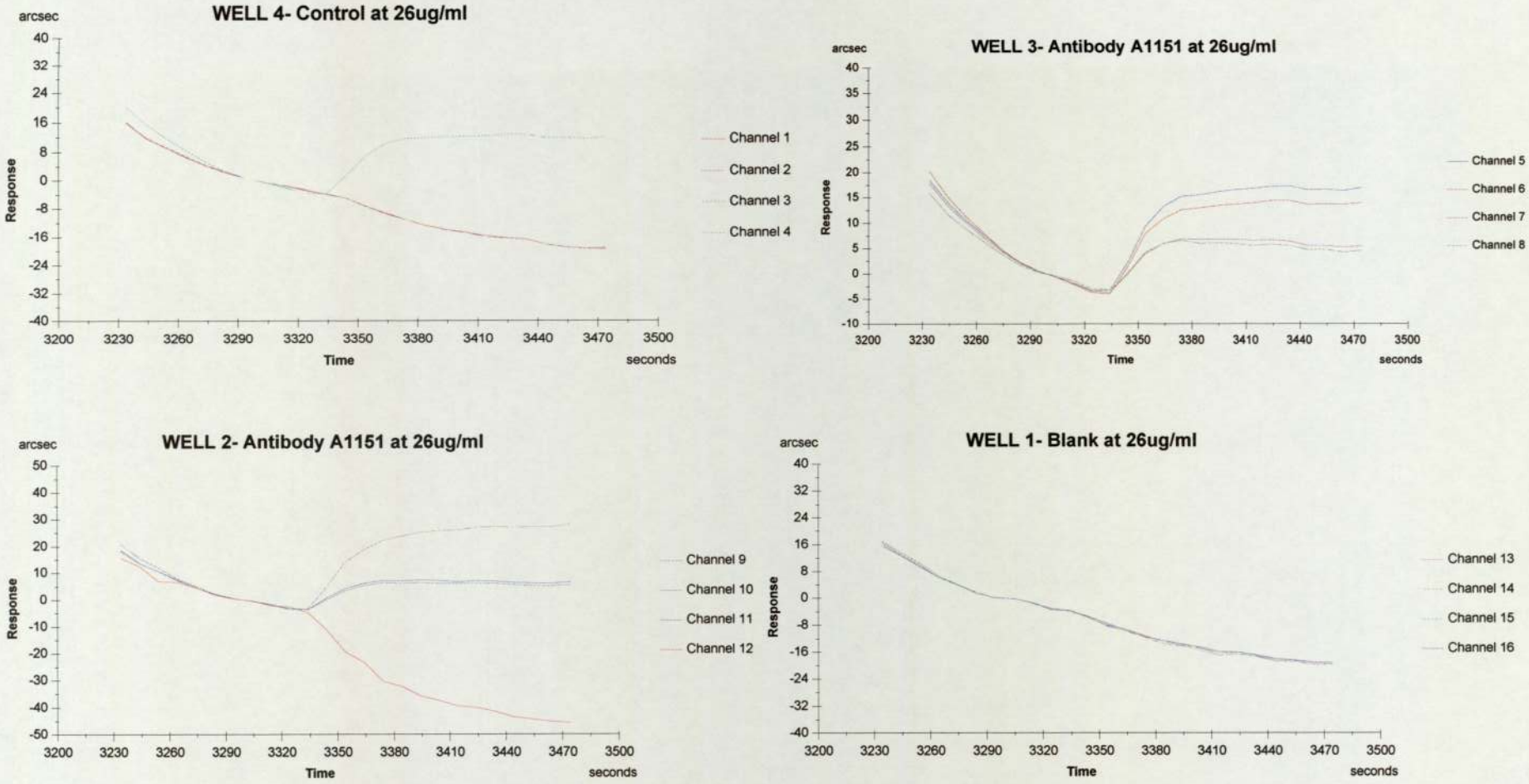
F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



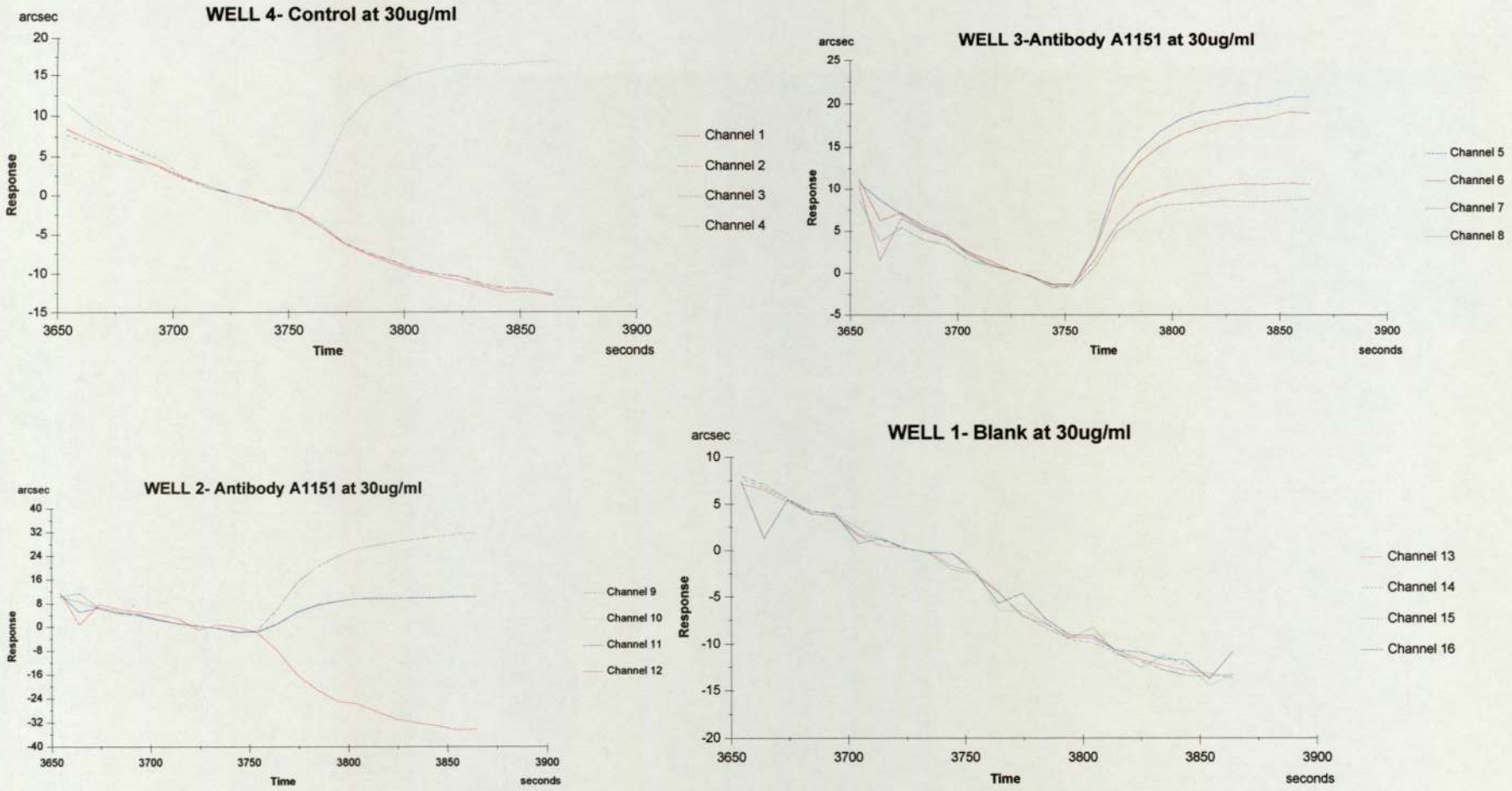
F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



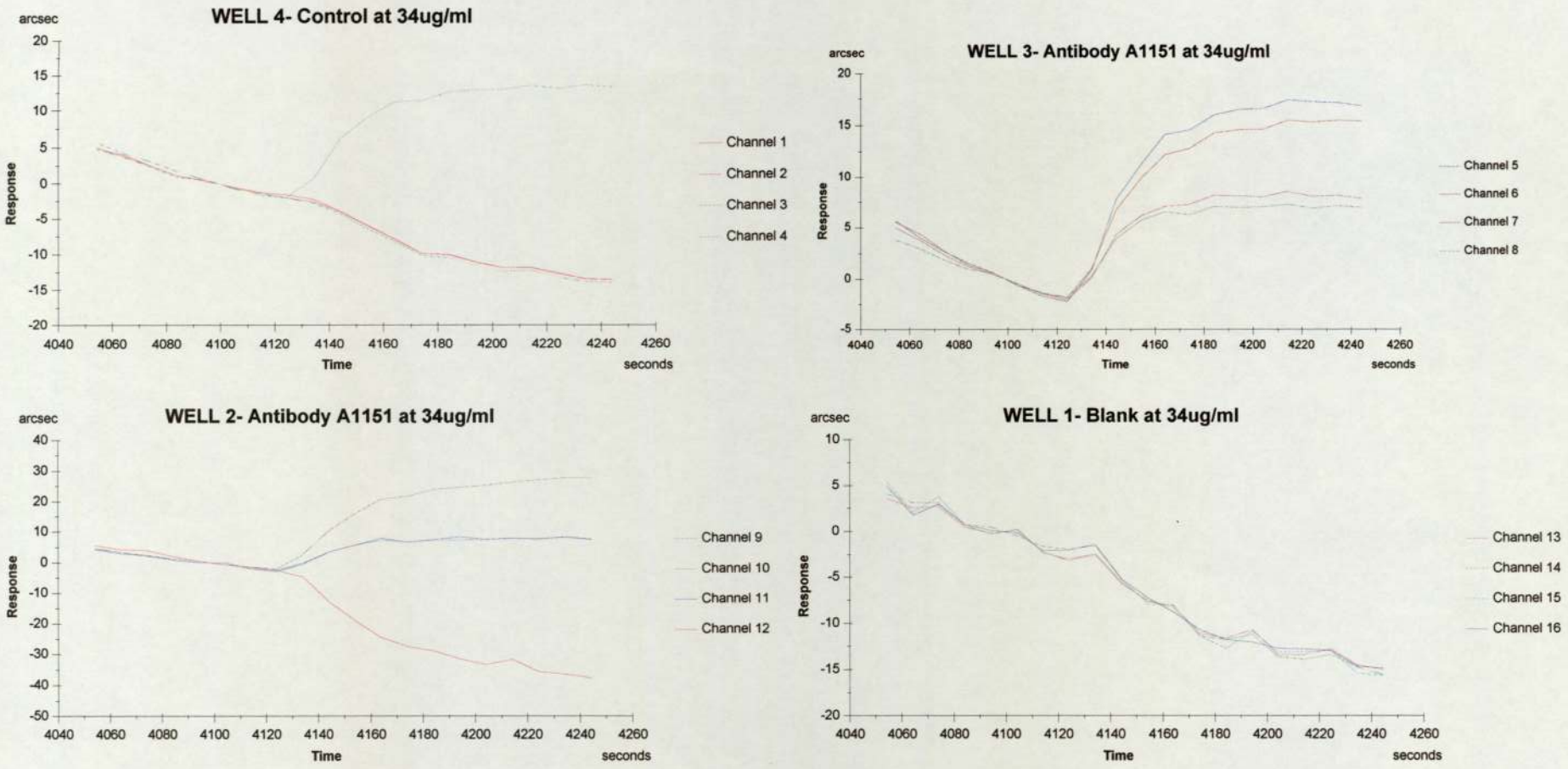
F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.



F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.

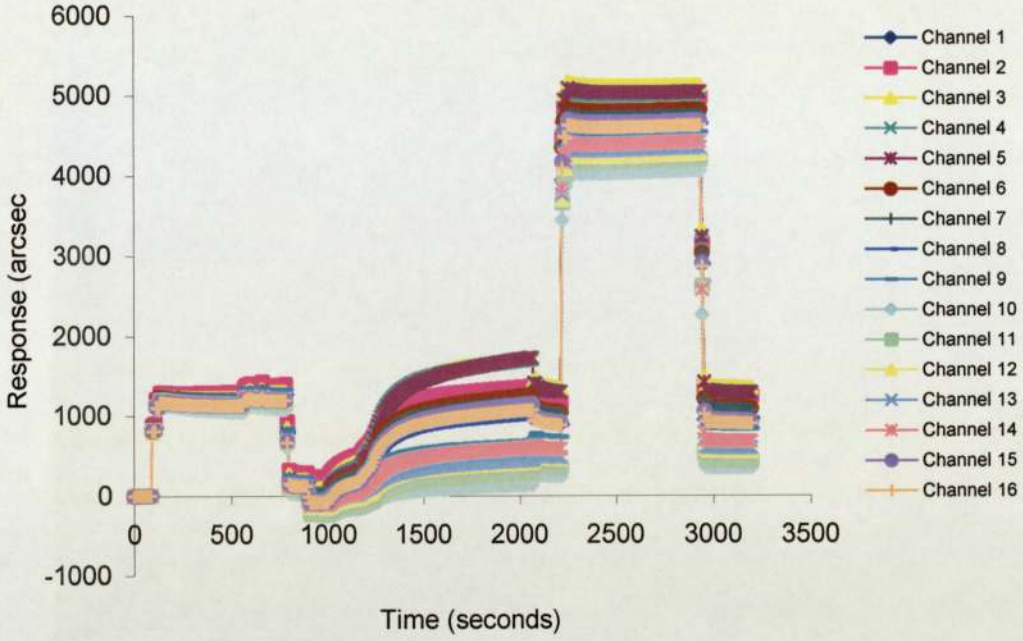


F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.

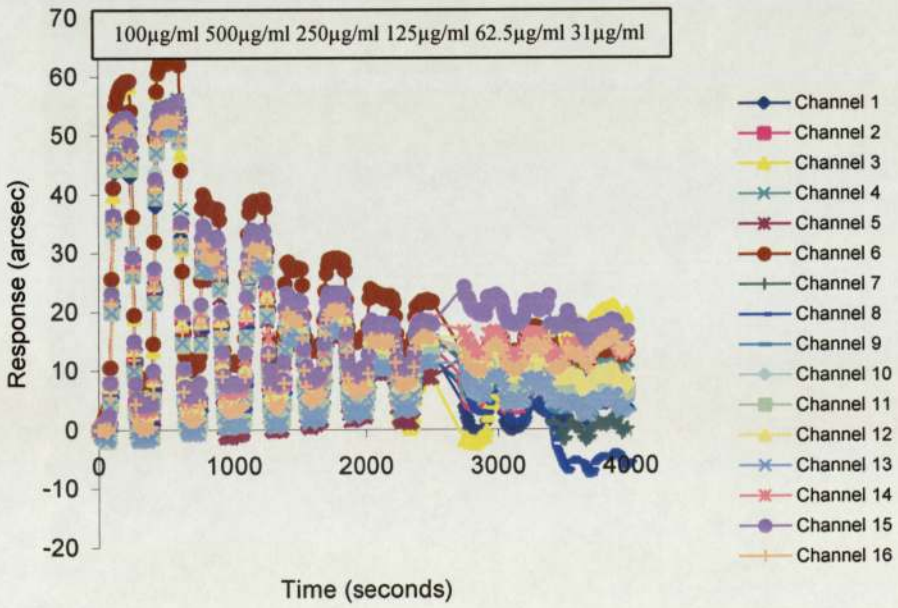


F.1. The response obtained in individual wells is outlined when running consecutive [HSA] over the cuvette containing antibody A1151.

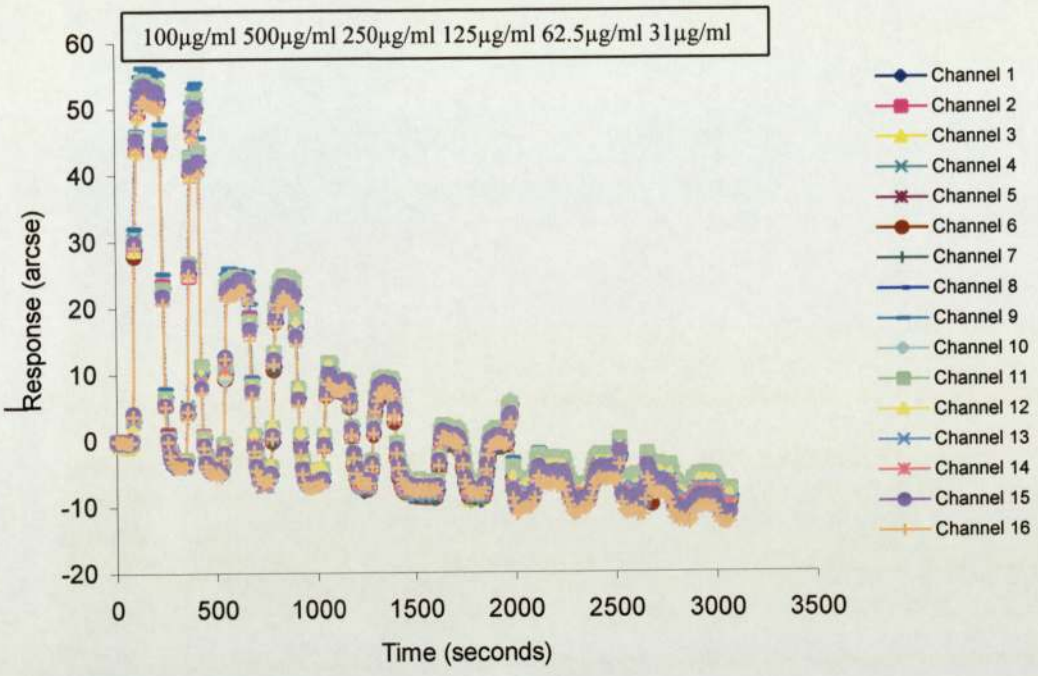
Appendix G



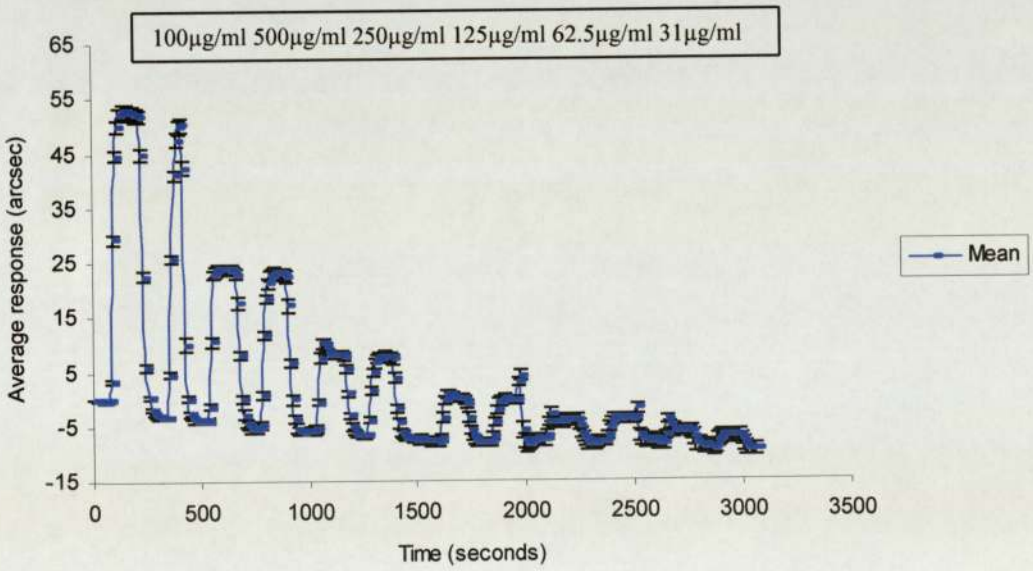
G.1 Immobilisation of creatinine deaminase upon a one well cuvette where a difference can be seen in response between channels.



G.2 Raw response showing all 16 channels responding to varying creatinine concentrations.



G.3 Raw response showing a response when the creatinine assay was repeated upon the same cuvette.



G.4 Average response when varying creatinine concentrations were repeated upon the cuvette. This figure corresponds to G.3.