# Development and Evaluation of Novel Methods for the Detection of Trypsinogen Activation Peptide

A thesis submitted for the award of Master of Science

By

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## Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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## Acknowledgements

To my wonderful parents and family, for their immeasurable love, support and friendship, all of which I would be lost without. I am truly lucky to know all of you.

To my supervisor, Richard, for his guidance, support, caring nature and kind words of encouragement. Thank you for allowing me this opportunity and welcoming me into the Applied Biochemistry Group family.

To Sharon, from whom I have learned so much working together these past years, facing many ups and downs. Your application and positive approach has left a lasting impression on me as a scientist. Thank you for your continued support and kindness in helping me with this undertaking.

To Justine, for lighting up my life.

# Table of Contents

## Abbreviations

| | |
|---|---|
| AAT | Accelerated aging time |
| AMNCH | Adelaide and Meath National Children's Hospital |
| AP | Acute pancreatitis |
| BSA | Bovine serum albumin |
| CARS | Compensatory anti-inflammatory response syndrome |
| CLSI | Clinical Laboratory Standards Institute |
| COOH | Carboxylic acid |
| Conc | Concentration |
| CRP | C-reactive protein |
| cTnI | Cardiac Troponin I |
| CV | Coefficient of variation |
| $dH_2O$ | Deionised water |
| ED | Emergency department |
| EDC | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide |
| EDTA | Ethylene-diamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EU | European Union |
| FDA | Food and Drug Administration |
| FT | Freeze-thaw |
| GAC | Goat anti-chicken |
| GAR | Goat anti-rabbit |
| HRP | Horse radish peroxidase |
| IgG | Immunoglobulin class G |
| IgY | Immunoglobulin class Y |
| IL-6 | Interleukin-6 |
| ISO | International Organisation for Standardisation |
| LFIA | Lateral flow Immunoassay |
| LoB | Limit of blank |
| LoD | Limit of detection |
| LoQ | Limit of quantification |

| | |
|---|---|
| MARS | Mixed antagonist response syndrome |
| Max | Maximum |
| MICT® | Magnetic immunochromatographic test |
| Min | Minimum |
| MWCO | Molecular weight 'cut-off' |
| NC | Nitrocellulose membrane |
| NHS | *N*-hydroxysuccinimide |
| NPV | Negative predictive value |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline, Tween20® |
| PC | Positive control |
| POC | Point-of-care |
| PPV | Positive predictive value |
| QC | Quality Control |
| ROC | Receiver operator characteristic curve |
| RT | Real time |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SIRS | Systemic inflammatory response syndrome |
| SPMP | Superparamagnetic particle |
| TAP | Trypsinogen activation peptide |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline, Tween20® |
| TBSTB | Tris buffered saline, Tween20®, BSA |
| TEM | Transmission electron microscopy |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TNF-α | Tumour necrosis factor-α |
| USG | Urine specific gravity |
| UTI | Urinary tract infection |
| WHO | World Health Organisation |

## Units

| | |
|---|---|
| µg | microgram |
| Da | daltons |
| kDa | (kilo) Daltons |
| kHz | (kilo) Hertz |
| kg | kilogram |
| µL | microlitre |
| °C | degrees Celsius |
| h | hours |
| L | litre |
| Ln | natural logarithm |
| M | molar |
| MAR | magnetic assay reading |
| mEq/L | milliequivalents per liter |
| mg | milligram |
| mL | millilitres |
| mm | millimetres |
| ng | nanograms |
| nM | nanomolar |
| nm | nanometers |
| nmol | nanomoles |
| rpm | revolutions per minute |
| U | units |
| v/v | volume per unit volume |
| w/v | weight per unit volume |

## Thesis Outline and Project Background

This project is a joint collaboration between academic (Dublin City University) and industrial (EKF Diagnostics Limited) partners, with the financial backing of the Irish Research Council. EKF Diagnostics is a global medical diagnostics company which specialises in the development, production and distribution of a range of IVD products for Point of Care and Central Laboratory testing, with a focus on detection and management of diabetes, anemia, lactate and organ-related diseases.

Acute pancreatitis (AP) is a complex inflammatory disease with varying etiology and presentation of severity. It has a clinical course that is difficult to predict and is associated with local and systemic complications, organ failure and death. The intra-pancreatic activation of trypsinogen to trypsin is recognised as one of the earliest events in the onset of AP. The simultaneous and proportional release of trypsinogen activation peptide (TAP) into the blood and urine therefore offers a potential biomarker of this disease. The initial development of a TAP radioimmunoassay by the Hermon-Taylor group at St. George's Hospital, London (Hurley *et al*., 1988) was swiftly followed by the licencing and commercialisation of a promising polyclonal enzyme-linked immunosorbent assay (ELISA) format developed by Biotrin International Limited. Significant literature (Huang *et al*., 2013) was published exploring and suggesting the utility of TAP as a biomarker for prediction of disease severity in AP. However, failure to achieve reproducibility in the performance of this product due to the nature of polyclonal antibody-based technology led to significantly reduced interest in the TAP biomarker. Recently, a monoclonal anti-TAP antibody was produced for evaluation as a solution to this issue.

This thesis entails the development of a competitive ELISA and rapid lateral flow immunoassay (LFIA) for the detection of TAP. It was anticipated that these prototype devices would provide the basis for a clinical feasibility study of TAP as a predictor of severity in AP. The findings of such a study represent the opportunity to re-open the dialogue on the clinical utility of TAP at a time where there is greater understanding of AP through pathological re-classification, the incidence of the disease appears to be increasing, and the advent of effective point-of-care (POC) technologies make the approach a legitimate prospect for application in patient management.

## Aims of the Research

The specific undertakings of this research can be divided into the following sections:

Chapter 2 describes the materials and methods used within this research.

Chapter 3 focuses on the development and optimisation of a reproducible monoclonal anti-TAP ELISA with clearly defined performance characteristics and the ability to function as a "gold standard" reference tool for the subsequent LFIA work.

Chapter 4 describes the development of an anti-TAP LFIA prototype capable of quantifying and distinguishing between varying urinary TAP concentrations through the use of superparamagnetic particle labels and a commercially established point-of-care instrument.

Chapter 5 investigates the clinical utility of the TAP biomarker for the stratification of patients with acute pancreatitis (AP) based on disease severity as defined by the Adelaide and Meath National Children's Hospital clinical treatment criteria and, the internationally recognised Atlanta classification. The ability of TAP to predict disease severity is also explored. This analysis was achieved using an exploratory cohort of patients with AP and the anti-TAP ELISA from chapter 3. The feasibility of the anti-TAP LFIA prototype to distinguish between TAP positive and negative urinary samples is also discussed.

Chapter 6 covers the overall conclusions of this project and the future work that is envisaged.

## Publications

**Connolly, R.** and O'Kennedy, R. (2017), Magnetic lateral flow immunoassay test strip development – considerations for proof of concept evaluation. **Manuscript in preparation.**

## Title: Development and Evaluation of Novel Methods for the Detection of Trypsinogen Activation Peptide

## Author: Rory Connolly

## Abstract

Acute Pancreatitis (AP) is an inflammatory condition of the pancreas, presenting initially as severe upper abdominal pain. This disease can range from mild AP to moderately severe or severe AP, with development of local and systemic complications and a diverse range of outcomes. Consequently, it is imperative to diagnose and predict disease severity at the earliest juncture. Numerous guidelines exist for the management of AP, including the revised Atlanta classification system. This guideline does not have the capacity to predict the degree of disease severity from hospital admission. Thus, the availability of a predictive biomarker, working in conjunction with the established classification system, would be advantageous. Several markers such as amylase, lipase and C-reactive protein are used for the diagnosis of AP by hospitals at present. However, it is now fundamentally agreed that the early intra-pancreatic activation of normal protein digestion enzymes is associated with development of AP. Trypsinogen Activation Peptide (TAP) is a potential predictive biomarker of AP that is proportionally produced and rapidly excreted into the urine following cleavage of the zymogen, trypsinogen, to trypsin. Herein, the establishment of a gold standard ELISA (with an intra- and inter-assay coefficient of variation of 1.7 – 10.1% and 4.8 – 10.4%, respectively) and the development of a lateral flow test strip for urinary TAP are described. An exploratory study (n = 15) using the ELISA prototype to determine the clinical utility of TAP for early prediction of AP severity demonstrated that TAP is superior to CRP for the stratification of patients with mild and moderate AP. The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of TAP to predict the development of moderate AP at 48h after admission was 85.7%, 100.0%, 90.0% and 100%, respectively. The sensitivity, specificity, NPV and PPV of CRP to predict the development of moderate AP at 48h after admission was 85.7%, 69.2%, 90.0% and 60.0%, respectively. The lateral flow device also demonstrated the ability to distinguish between TAP positive and TAP negative clinical urine samples. In conclusion, TAP is a promising biomarker for the early prediction of severity in AP.

# Chapter 1

Introduction

## 1.1 The pancreas – function and malfunction

The pancreas is the second largest gland connected to the digestive tract after the liver, and is found in the upper abdominal cavity, posterior to the stomach and anterior to the kidneys. This complex organ is capable of both exocrine and endocrine functions within the human body (see Figure 1.1.1). The larger exocrine portion of the pancreas (up to 98% of pancreatic tissue) consists of lobed ducts called acinar cells that produce digestive enzymes for transport from the main pancreatic duct to the duodenum of the small intestine in response to food intake. The different endocrine cells of the pancreas exist as islets of Langerhans grouped in the surrounding tissue and are responsible for the secretion of hormones into the blood for delivery throughout the body (Sargent, 2006). These hormones include the blood-sugar regulators glucagon and insulin, the multi-tasking inhibitor somatostatin and, pancreatic polypeptide, an inhibitor of gastrointestinal emptying and exocrine secretion (Koska, 2004; Sargent, 2006).

The complexity and functional demands placed on the pancreas require that this organ is highly innervated and vascularised. The involuntary parasympathetic and sympathetic nerves stimulate and inhibit the secretion of digestive enzymes from the pancreas, respectively (van Orshoven, 2008). The release of digestive enzymes aids in the degradation of food products such as fats, proteins and carbohydrates into absorbable nutrients for the body (Sargent, 2006). These enzymes are stored as inactive or "zymogen" pro-enzymes inside intracellular vesicles of acinar cells within the pancreas (Hammer, 2014). The secretion of these zymogens occurs through the process of exocytosis whereby the vesicles fuse with the acinar cell membrane to form a pore or channel for excretion of the zymogens into the pancreatic duct and, subsequently, the gut. The process of exocytosis is generally triggered by a rise in cytosolic calcium ions ($Ca^{2+}$) following extracellular entry through the cell membrane. However, this differs for acinar cells, where the initial $Ca^{2+}$ stimulation is supplied from preexisting intracellular provisions. This finely tuned $Ca^{2+}$ signaling pathway has the potential to become unbalanced, leading to a sustained elevation in $Ca^{2+}$ stimulation and subsequently, excessive intracellular release and extracellular access. In what can be termed a vicious circle, sustained

intracellular stimulation results from the release of $Ca^{2+}$ from the cell into the extracellular matrix through channels formed in the plasma membrane, depleting the intracellular $Ca^{2+}$ stores. Consequently, the cell must replenish these empty stores by activating additional channels to allow entry from the now $Ca^{2+}$ rich extracellular environment. This stimulatory effect can also be coupled with the neurotransmission of acetylcholine to acinar cells and presence of the circulating hormone cholecystokinin. The instability of this process was demonstrated to have the capacity to kill cells, causing necrosis of the pancreatic tissue (Beger *et al*., 2008). While this mechanism is still not fully understood and additional activation pathways have been studied involving for example, damage by reactive oxygen species (Lankisch *et al*., 2015), it is widely agreed that the inappropriate activation of zymogens within acinar cells leads to the auto-digestion of pancreatic tissue and the release of digestive enzymes into the blood.

Cleavage of the zymogen known as trypsinogen by enterokinase, an enzyme located in the epithelial cells of the small intestine, to form trypsin is a crucial step in normal protein digestion. Trypsin is capable of self-activation as well as activation of all other zymogens simultaneously (see Figure 1.1.1). Several mechanisms exist for the regulation of trypsinogen activation. The process of regulation by serine protease inhibitor, Kazal type 1 (SPINK1), which can prevent auto-activation of trypsin, is the most understood (Figure 1.1.2).

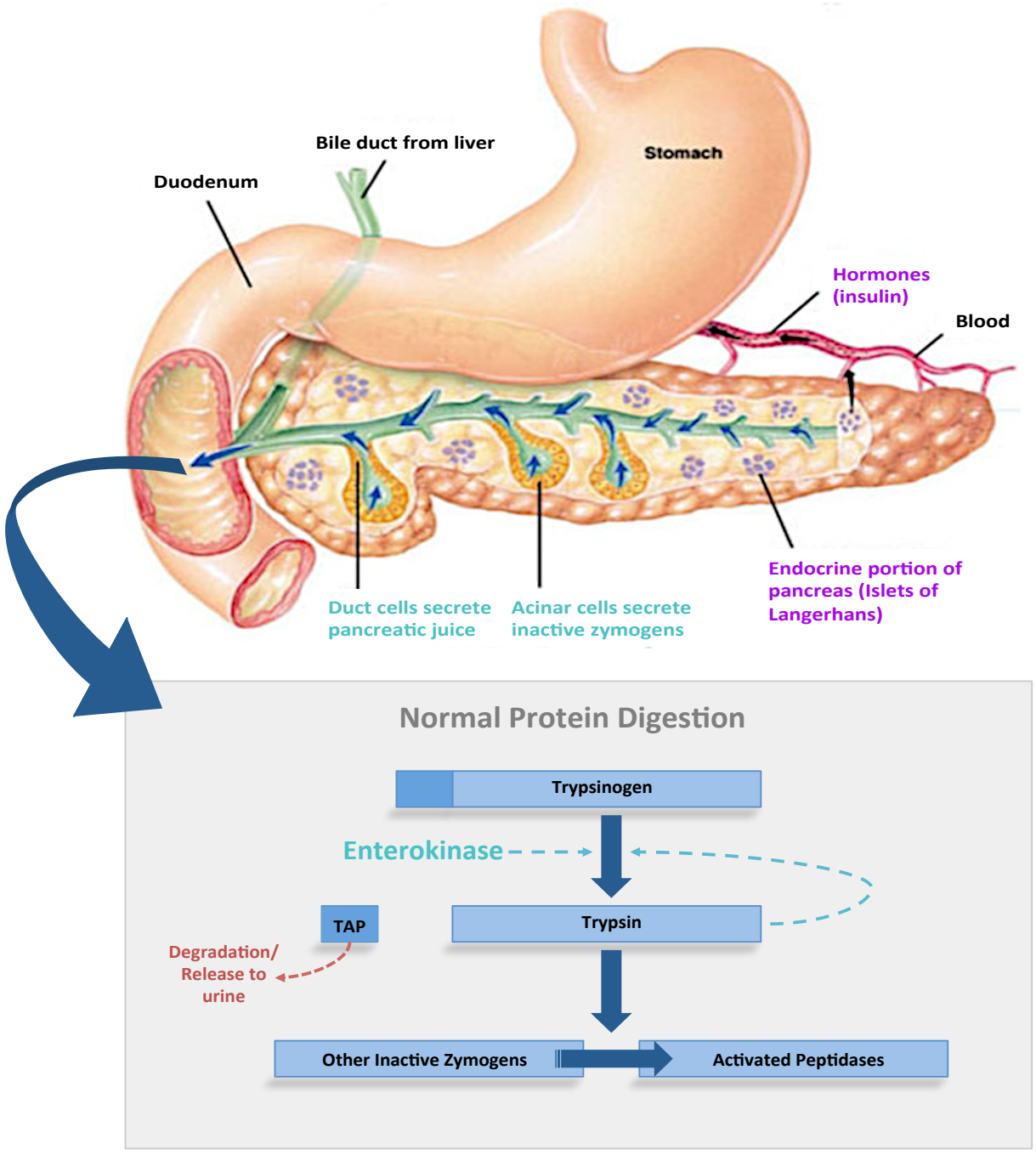



**Figure 1.1.1** The pancreas and normal protein digestion; adapted from olivelab.org. Trypsinogen cleavage and activation by enterokinase or trypsin autocatalysis and the subsequent zymogen activation cascade are shown. During the activation of trypsinogen, the conserved peptide by-product of trypsinogen activation peptide (TAP) is formed and rapidly released into the blood and urine due to its small size. This peptide will be discussed at length later in the chapter (section 1.5.4).

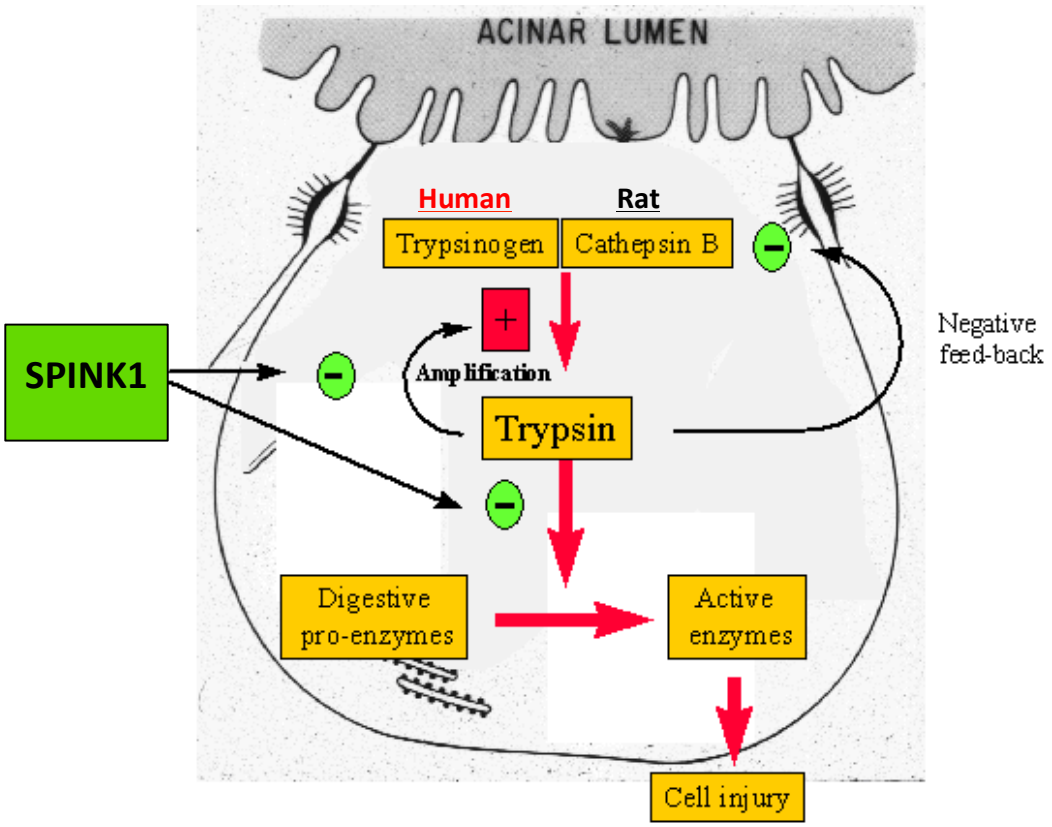



**Figure 1.1.2** Trypsinogen activation and inhibition pathways in human and rodent acinar cells; adapted from Frossard, 2001. SPINK1 inhibits the activation of trypsinogen by trypsin (-). In rats, the inhibitory capacity can also prevent activation by the cathepsin B enzyme, however, this has not yet been extensively proven in human studies (Lerch & Halangk, 2006). Trypsin can also inactivate itself through hydrolysis of the globular domains of the trypsin enzyme. If this negative feedback mechanism should be overcome, the zymogens (pro-enzymes) can become activated inside the acinar cell, leading to cell injury, death and tissue necrosis.

The capacity of SPINK1 to inhibit trypsin activity can become uncontrolled, leading to excessive activation of trypsinogen and a cascade of inappropriate zymogen activation and auto-digestion of the pancreas. This is ultimately followed by the induction of leukocytes to release pro- (C-reactive protein (CRP), interleukin (IL)-1, 6, 8 and tumour necrosis factor (TNF)-α) and anti-inflammatory (IL-10) cytokines to the immediate area (Jain *et al*., 2011; Hammer, 2014). This process is believed to be one of the earliest occurrences leading to the development of the inflammatory condition of acute pancreatitis (AP). AP is associated with intense upper abdominal pain, nausea, vomiting, jaundice and abdominal distension (Kingsnorth & O'Reilly, 2006). The level of pain experienced by patients is not unusual due to the highly innervated nature of the pancreas. These nerves are sensitive to inflammatory attack and increases in mechanical pressure from ductal obstruction (Vardanyan & Rilo, 2010). Acute pancreatitis is an extremely aggressive condition that can lead to systemic inflammation, multiple organ failure and death. Initial localised inflammation of the pancreas can rapidly spread to surrounding tissues and organs due to its numerous vascular connections and proximity to vital organs such as the lungs (Zhou *et al*., 2010) and kidneys (Petejova & Martinek, 2013). Persistent organ failure accounts for one-third to half the deaths in AP occurring in the first week, a large proportion of which (50%) is associated with lung injury (Pastor *et al*., 2003). A second wave of late deaths occurs more than one week after hospital admission and are usually associated with local complications of the pancreas, such as infected necrosis and sepsis (Johnson *et al*., 2004).

The causes of pancreatitis are as complex and diverse as the organ itself. The two most common causes of AP are alcohol and biliary obstructions such as gallstones (Lankisch *et al*., 2015). Excessive alcohol or, more specifically, ethanol intake has been demonstrated to increase and destabilise the zymogen content of acinar cells, sensitizing them to premature activation and, in the presence of additional factors, this can manifest as AP (Gorelick, 2003; Lerch *et al*., 2003). Alcohol sensitisation can also aid in the progression of AP to become a recurring issue with long-term inflammatory episodes leading to permanent structural changes such as fibrosis, and are described as chronic pancreatitis (Clemens *et al*., 2014). The formation of

gallstones and subsequent blockage of bile ducts leads to a reduction in pancreatic juice secretion and in turn also prevents exocytosis of zymogens from acinar cells. Additional blockages can also arise from a pancreatic abscess, cyst or tumour (Wilmer, 2004). Hundreds of drugs have been included as potential causative agents of AP in the World Health Organisation (WHO) database; however, the definitive list currently includes drugs such as acetaminophen, cisplatin, enalapril, erythromycin, oestrogens, opiates, and steroids (Nitsche *et al*., 2012; Lankisch *et al*., 2015). Numerous inherited or genetic conditions can also cause AP, such as mutation of the trypsinogen gene (Whitcomb *et al*., 1996) or the metabolic abnormalities of hypertriglyceridemia and hypercalcemia (Siva & Pereira, 2006). In addition, accidental blunt abdominal trauma or post-operative trauma such as endoscopic retrograde cholangio-pancreatography (ERCP) for bile duct surgery and parasitic, viral or bacterial infection are also known causes of disease (Steinberg & Tenner, 1994). More recently, there is increasing evidence for smoking as an independent risk factor in AP, despite its regular association with alcohol (Schurmann *et al*., 2009; Sadr-Azodi *et al*., 2012). Additional co-morbidities or inherited conditions such as diabetes mellitus, cystic fibrosis, Crohn's disease, pancreas divisum and Sphincter of Oddi dysfunction are also increased risk factors for AP (Lankisch *et al*., 2015). Finally, AP can also be caused by idiopathic complications (Van Brummelen *et al*., 2003).

### 1.2 Acute pancreatitis – incidence and impact

Reginald Huber Fitz (1889) compiled the first modern assessment and description of acute pancreatitis (AP), with the first hypothesis of pancreatic auto-digestion coming several years later (Chiari, 1896). Since then, this condition has been studied in a diverse range of animals, from the zebrafish (Lerch & Gorelick, 2013) to dogs (Mansfield *et al*., 2003). This exceptionally varied and often unpredictable disease can range from mild and non-threatening to severely aggressive clinical courses resulting in high morbidity and mortality. While the majority of mild cases are self-resolving and have a mortality rate of less than 1%, requiring only conservative medical supervision (Swaroop *et al*., 2004); up to 30% of cases can develop serious

life-threatening complications with a subsequent mortality rate as high as 25-35% (Swaroop *et al*., 2004; Schepers *et al*., 2013). The increasing incidence of AP has been reported internationally, in countries such as the United States (U.S.) (Frey *et al*., 2006; Fagenholz *et al*., 2007), Sweden (Appelros & Borgstrom, 1999), Finland (Jaakkola & Nordback, 1993) and the Netherlands (Spanier *et al*., 2013). The number of cases of AP has also doubled in the UK by the turn of the 21st century (McKay *et al*., 1999; Goldacre & Roberts, 2004). Increasing incidence of AP has also been reported for children in multiple countries with a 64% increase by the year 2000 (Bai *et al*., 2011). In Ireland, increasing incidence of AP has largely been linked to alcohol abuse, particularly in the younger population (O'Farrell *et al*., 2007). This trend persists worldwide with rises in per capita alcohol consumption, especially amongst women (Williams *et al*., 2007). Despite a number of studies demonstrating that gender can be an independent risk factor for gastrointestinal diseases (Zarling *et al*., 1997), this does not appear to be the same for AP, even with alcohol-induced AP appearing to occur more frequently in males and biliary AP showing prevalence in females (Lankisch *et al*., 2001). There are simply many other risk factors, such as age, which must be taken into account (Fagenholz *et al*., 2007). While increased awareness and improvements in diagnostic methods of AP may have somewhat contributed to the augmented incidence, there is no denying the enormity of this disease's impact on hospital resources and the subsequent demand for effective treatment and patient management (Mofidi *et al*., 2007). The median length of hospital stay for patients of AP is 7 days (Fagenholz *et al*., 2007; O'Farrell *et al*., 2007), rising to months in patients with severe disease who will require intensive multidisciplinary and specialised care (Schepers *et al*., 2013). Consequently, the treatment costs associated with these patients is significantly higher than those of other intensive care patients (Neoptolemos *et al*., 1998) and has been estimated to be as high as US$ 2.6 billion (Peery *et al*., 2012). To summarise this healthcare burden, a recent U.S. study by Peery *et al*., (2012) found AP to be "the leading cause of admission to hospital for gastrointestinal disorders, the second highest cause of hospital stays, the largest contributor to aggregate costs and the fifth leading cause of in-hospital deaths".

## 1.3 Classification and staging of acute pancreatitis

### 1.3.1 Physiological disease scoring systems

Diagnosis of AP is only a small step in the battle against a lethal disease that can manifest as a myriad of complications. The greatest challenge facing the treatment and management of AP is the ability to predict the severity of disease that a patient will develop. Numerous classifications and guidelines have evolved to aid the clinician's definition and prognosis of this disease in recent decades. This approach has directly improved patient management and outcome with underlying principles of simplified treatment methods and avoidance of unnecessary surgery, which can often prove fatal (Uhl *et al*., 2002). Physiology-based scoring systems such as the Ranson criteria, Glasgow Imrie and Acute Physiology and Chronic Health Evaluation (APACHE)-II have previously been developed and validated for use with patients of AP (Ranson *et al*., 1974; Blamey *et al*., 1984; Larvin & McMahon, 1989). These multifactorial criteria include measurements of various parameters such as age, blood glucose, urea and hematocrit levels and, patient heart and respiratory rate (see Table 1.3.1). Organ-dysfunction criteria systems such as the Marshall score (Marshall *et al*., 1995), sequential organ failure assessment (SOFA) (Vincent *et al*., 1996) and multiple organ dysfunction score (MODS) (Marshall *et al*., 1995) have also been applied to patients of AP due to the disease propensity to spread systemically and attack other vital organs. However, these scoring systems are often complex, time-consuming in their application and can take several days before a clear conclusion can be made (Mofidi *et al*., 2007). Furthermore, they are intended to assess the current severity of illness and likelihood of in-hospital mortality. They do not have the ability to predict outcome or complications in patients who may survive (Bouch & Thompson, 2008).

**Table 1.3.1** Physiology-based scoring systems for acute pancreatitis**:**

| **Ranson score** | | | | | | |
|---|---|---|---|---|---|---|
| Time | Analysed Criteria (Units) | Threshold Level | Total Criteria | Severity Score | Advantages | Disadvantages |
| At admission | Age (years) | >55 | 11 | ≥3 = severe AP<br>0-2 = 2% mortality<br>3-4 = 15% mortality<br>5-6 = 40% mortality<br>7-8 = 100% mortality | Long established | Complex, time-consuming |
| | WBC count ($mL^{-1}$) | >16,000 | | | | |
| | Blood glucose (mg/dL) | >200 | | | | |
| | AST (U/mL) | >250 | | | | |
| | LDH (U/mL) | >350 | | | | |
| At 48h | Hematocrit (%) | >10 ↓ | | | | |
| | BUN (mg/dL) | >5 ↑ | | | | |
| | Serum $Ca^{2+}$ (mg/dL) | <8 | | | | |
| | $PaO_2$ (mm/Hg) | >60 | | | | |
| | Base deficit (mEq/L) | >4 | | | | |
| | Fluid sequestration (L) | >6 | | | | |
| **Glasgow Imrie score** | | | | | | |
| Time | Analysed Criteria (Units) | Threshold Level | Total Criteria | Severity Score | Advantages | Disadvantages |
| At admission and 48h | WBC count ($mL^{-1}$) | >15,000 | 8 | ≥3 = severe AP | Reduced number of criteria, simplified calculation | 48h required to complete |
| | Blood glucose (mg/dL) | >180 | | | | |
| | AST (U/mL) | >200 | | | | |
| | LDH (U/mL) | >600 | | | | |
| | BUN (mg/dL) | >45 | | | | |
| | Serum $Ca^{2+}$ (mg/dL) | <8 | | | | |
| | Serum albumin (g/dL) | <3.2 | | | | |
| | $PaO_2$ (mm/Hg) | <60 | | | | |
| **APACHE-II score** | | | | | | |
| Time | Analysed Criteria | Threshold Level | Total Criteria | Severity Score | Advantages | Disadvantages |
| At admission and daily up to 48h | Temperature | + | 14 | ≥8 = severe AP | Validated for numerous diseases, can be calculated daily | Very complex |
| | Mean arterial pressure | + | | | | |
| | Heart rate | + | | | | |
| | Respiration rate | + | | | | |
| | $PaO_2$ | + | | | | |
| | Arterial pH | + | | | | |
| | Serum sodium | + | | | | |
| | Serum potassium | + | | | | |
| | Serum creatinine | + | | | | |
| | Hematocrit | + | | | | |
| | WBC count | + | | | | |
| | Glasgow coma scale | + | | | | |
| | Age | + | | | | |
| | Pre-existing illnesses | + | | | | |

WBC = white blood cell; AST = aspartate transaminase; LDH = lactate dehydrogenase; BUN = blood urea nitrogen; Ca = calcium, $PaO_2$ = blood gas/partial pressure of arterial oxygen; ↓ = decrease by; ↑ = increase by. Multiple point scores exist for each individual criteria of the APACHE-II score and are therefore denoted by "+" for their inclusion. Adapted from Forsmark & Gardner (2015).

### 1.3.2 The Atlanta classification

During a 1992 symposium of international clinical experts, the Atlanta classification of AP was described to provide a consistent and universal definition of disease severity. This guideline was widely accepted and has subsequently been revised to reflect further comprehensive consultation of key opinion leaders. UK guidelines have aligned with this definition (NHS Trust, 2016). It is now understood that there are two phases of the AP disease process (Figure 1.3.2.1). In the early phase (lasting approximately one week), localised pancreatic inflammation can trigger a systemic inflammatory response syndrome (SIRS) through the mass release of cytokines. If this action is continuous it can drive organ failure, becoming transient (<48 hours) or persistent (>48 hours) and with the ability to affect multiple organs. In the majority of cases, the initial inflammation remains localised to the pancreas and reasonably resolves. During the late phase, the disease progresses with the development of local complications due to peripancreatic fluid and necrotic tissue collections. The systemic inflammatory response is still prevalent with organ failure remaining transient or persistent and the body entering into a compensatory anti-inflammatory response syndrome (CARS) to offset the initial inflammatory events (Banks *et al*., 2012). The presence of CARS leads to deactivation of the immune system in an attempt to restore homeostasis. However, this can facilitate infection of the pancreatic and peripancreatic tissue by bacteria trans-located from the gut and is thought to be the major cause of late morbidity and mortality in acute pancreatitis (Phillip *et al*., 2014). The co-existence of both SIRS and CARS can also develop and is referred to as a mixed antagonist response syndrome (MARS), which can be associated with the development of sepsis in the case of SIRS, or infection in the case of CARS while the body struggles to balance these pro- and anti-inflammatory surges (Ward *et al*., 2008; Novotny *et al*., 2012).

Taking all of this into account, the revised Atlanta classification defines disease severity based on the persistence of organ failure and the presence of local or systemic complications. Three main categories of disease severity are defined for AP: mild, moderately severe and severe AP. Mild AP is characterised by the absence of organ failure and local or systemic complications. Inflammation is usually localised to the

pancreas and very low rates of mortality are recorded. Transient organ failure and the presence of local or systemic complications, with persistent inflammation and an increased risk of mortality are observed in patients with moderately severe AP (Banks *et al*., 2012). However, moderately severe AP cannot be accurately distinguished from severe pancreatitis in the first 48h until the extent of pancreatic necrosis is also determined (Kwong *et al*., 2016). Persistent failure of multiple organs from an early stage lasting >48h and the presence of one or more local complications are typical of severe AP. Extended duration of organ failure lasting three or more days is strongly associated with the risk of death or development of local complications whereas the resolution of organ failure within 48h suggests a lower (25%) risk of local complications and death (Johnson *et al*., 2004). This is where the associations between severe and moderately severe AP can be made with the modified Marshall scoring system defining organ failure (Banks *et al*., 2012). Any arising local complications must be monitored closely in the preceding weeks as the development of acute necrotic collections can impact long-term patient survival and be a significant risk factor for death (Umapathy *et al*., 2016). Collections of necrotic tissue enclosed inside an inflammatory wall (walled-off necrosis) can form, remaining sterile and thus manageable or potentially becoming infected and life-threatening, requiring further specialised care. Through these definitions, the Atlanta classification condenses the significant stages and complications of AP. While alternative classification systems such as the determinant-based classification where an additional (critically severe) category have subsequently also been suggested, no significant improvement over the revised Atlanta classification have emerged to date (Acevedo-Piedra *et al*., 2014).

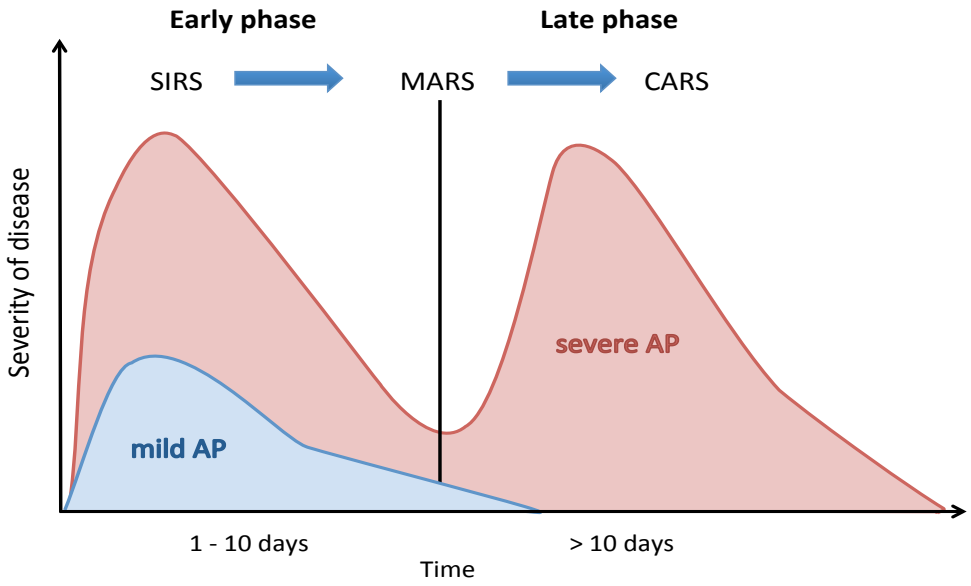



**Figure 1.3.2.1** The progression of mild and severe acute pancreatitis (adapted from Phillip *et al*., 2014). The disease phases and various host inflammatory responses are also displayed.

## 1.4 Management of acute pancreatitis

### 1.4.1 Diagnosis and detection

At present, diagnosis of AP is usually made upon admission to hospital and based on physical examination of symptoms determined at presentation such as acute abdominal pain, radiating to the back in some cases (Meher *et al*., 2015). Abdominal pain could also be due to a diverse number of other conditions such as gastritis, appendicitis and peptic ulcer. The measurement of pancreatic enzymes such as serum amylase or lipase is therefore also recorded, with an elevation to three times the upper limit of normal signifying a diagnosis of AP (Neoptolemos *et al*., 2001). If the levels of these enzymes are below the threshold value and diagnosis is still uncertain, abdominal imaging can be preformed using contrast-enhanced computed tomography (CECT), abdominal ultrasonography or magnetic resonance imaging (MRI) (Arvanitakis *et al*., 2007). However, these scans are not without their disadvantages with expense, exposure to ionizing radiation and risk of nephrotoxicity from iodinated contrast medium (Arvanitakis *et al*., 2007) listed as concerns (Frossard, 2001). Furthermore,

with a lack of sensitivity and specificity in the early stage of disease (<48h) and a significant number of self-resolving mild cases that do not require routine scans, the prognostic value of utilising such costly equipment needs to be evaluated. However, the value of imaging techniques to the assessment of severe AP must be recognised. Indeed, CECT performed 48-72h after the onset of symptoms is the gold standard (Arvanitakis *et al*., 2007) for detection of necrotizing pancreatitis, with sensitivity close to 100% (Wilmer, 2004). Severity indexes have been compiled for characterisation of morphological changes to the pancreas that can correlate to patient outcome (Balthazar, 2002; Mortele *et al*., 2004). However, the consensus that CECT should not be performed any earlier than 48-72h after the onset of symptoms means that this tool is better equipped for severity detection rather than prediction (Schütte & Malfertheiner, 2008). MRI, or more specifically, magnetic resonance cholangio-pancreatography can provide detailed assessment of the biliary system and has been shown to be a comparable to CECT in diagnosis and staging of AP, but is not applicable to patients with metallic implants such as pacemakers or patients connected to supportive systems (Lippi *et al.,* 2012). It is also more expensive and time-consuming than CECT (Schütte & Malfertheiner, 2008). While the utility of abdominal ultrasonography for diagnosis and severity assessment of AP is questioned, this low-cost technique is widely available and considered as the gold standard for detection of gallstones (Lippi *et al*., 2012). Despite the relative proficiency of these approaches for the assessment and prediction of disease severity, the search for a single analytical method or biomarker capable of earlier prediction of severity has remained of great interest.

### 1.4.2 Treatment regimen

Establishment of early aggressive treatment is essential to decreasing the likelihood of developing severe AP and also minimising potential complications. In general, patients with predicted mild AP should have basic vital signs monitored and intravenous fluids administered. Nasogastric drainage is only necessary for patients with persistent vomiting (Grace *et al.,* 2003). These patients can resume oral feeding as soon as possible, with this shown to be safe and leading to shorter hospital stays (Schepers *et*

*al*., 2013). Suspected cases of gallstones or biliary obstruction should undergo endoscopic retrograde cholangio-pancreatography (ERCP) prior to hospital discharge (Mayor, 2016). Patients with predicted severe AP should be admitted to the intensive care unit (ICU) where central venous fluids are administered. Hourly monitoring of vital signs is required with close assessment of temperature, central venous pressure, arterial blood gases and respiratory rate. A urinary catheter should be used for monitoring urine output in case of kidney failure. Once again, nasogastric drainage may be necessary with persistent vomiting (Grace *et al.,* 2003; Sargent, 2006). Early fluid resuscitation or hydration for the prevention of hypo-perfusion and organ failure has been demonstrated to reduce complications and mortality (Wall *et al*., 2011), with an infusion rate of 5-10 mL/kg/h deemed appropriate (Schepers *et al*., 2013. The use of broad-spectrum prophylactic antibiotics is recommended for the prevention of bacterial translocation from the gut, which has been linked with the infection of pancreatic necrosis. Pain management is prioritised through the administration of morphine (Sargent, 2006) with paracetamol and non-steroidal anti-inflammatory drugs often proving insufficient (Wilmer, 2004). Early enteral nutrition, preferably via oral intake, is favoured to maintain gut function and to limit gut permeability, thus potentially reducing the opportunity for bacterial translocation and infection. Probiotics are not recommended for patients with AP due to possibility of bowel ischemia and potential increased risk of death (Besselink *et al*., 2008). The recommended nutritional requirements for severe AP include protein at 1.2 – 1.5 g/kg/day, carbohydrates at 3-6 g/kg/day and lipids at 2 g/kg/day, along with strict blood glucose control through administration of insulin (Kingsnorth & O'Reilly, 2006). Previous preferences for surgical intervention of AP have been replaced and it is now generally only applied in the presence of infected necrosis. Deferral is maintained while patients respond to more conservative treatment methods (Uhl *et al*., 2002). While strides have been made with pancreatic transplantation, this intervention is not favoured (Demartinesa *et al*., 2005).

## 1.5 Biomarkers of acute pancreatitis

Several definitions have been proposed in relation to the term “biomarker”. The most applicable interpretation with respect to AP is “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (Strimbu & Tavel, 2010). The two main challenges facing prospective biomarkers of AP is the length of time between the onset of a patient’s symptoms and admission to hospital, and the small window period (approximately 48h following the onset of symptoms) in which differentiation of disease severity can be made. Consequently, the idea of a single biomarker capable of diagnosis of AP and early prediction of severity is appealing. Many diagnostic and prognostic biomarkers of AP have been reported, from inflammatory markers such as CRP and IL-6, to digestive enzymes such as amylase, lipase and trypsinogen-2, and the by-product of trypsinogen activation - trypsinogen activation peptide (TAP). All of these biomarkers have enjoyed some recognition; with amylase and lipase routinely employed internationally in a diagnostic capacity (Hofmeyr *et al*., 2014), and CRP the most frequently used predictor of severity (Staubli *et al*., 2015). Despite this, novel biomarkers of AP continue to be reported in the literature with specific focus areas, namely prediction of severity (cytokeratin 18, copeptin, neutrophil gelatinase-associated lipocalin (NGAL)-1); prediction of pancreatic necrosis (adipocytokines, matrix metalloproteinase (MM)-9, fibrogen-like protein (fgl)-2); and prediction of persistent organ failure (angiopoietin 2, D-dimer, soluble CD73) (Meher *et al*., 2015). However, the performance of these biomarkers has yet to be fully evaluated in large clinical studies. While there is obviously an underlying dissatisfaction driving continued exploration for novel biomarkers of AP, the recent reclassification of the disease and the advent of point-of-care (POC) technology provide ample scope to revisit and reassess some of the more promising biomarkers, such as TAP.

### 1.5.1 Amylase and lipase

The pancreas is one of two major sources of amylase in humans, the salivary glands being the other. Pancreatic amylase degrades starch into smaller carbohydrate molecules as part of normal digestion. Amylase has also been found in other body tissues such as the lung, ovary and testis (Vissers *et al*., 1999). Consequently, amylase is not pancreas-specific. Serum amylase levels also quickly revert to normal. Furthermore, normal amylase levels have frequently been reported in patients with AP (Vissers *et al*., 1999) and most importantly in patients with severe AP (Lankisch *et al.,* 1999). Amylase is also ineffective with measurement of alcohol-induced pancreatitis (Hofmeyr *et al.*, 2014). Despite these facts, amylase is still the most commonly measured biomarker of AP and a cut-off of three times the upper limit of normal (>3 ULN) is generally recommended for the diagnosis of AP by amylase (Hofmeyr *et al.*, 2014).

Lipase is synthesised by pancreatic acinar cells and is involved in digestion of fats. Elevated levels of lipase can be observed from 4-8h after the onset of AP, with a subsequent peak at 24h and a return to normal levels over a period of up to 14 days (Yadav *et al.,* 2002). Therefore, lipase is beneficial for diagnosis of AP in late patient presentation (Hofmeyr *et al.*, 2014). Initial laboratory methods for lipase measurement were labour-intensive and cumbersome due to insolubility issues in aqueous solutions (Vissers *et al*., 1999). Consequently, adoption of this biomarker for clinical application was slow until the arrival of more reproducible assay methods, as with the Roche COBAS INTEGRA (Moridani & Bromberg, 2003) or Siemens Advia 1800 system (Hofmeyr *et al.*, 2014). Since then, lipase was shown to be a superior to amylase for diagnosis of AP, particularly in alcohol-induced pancreatitis (Hofmeyr *et al.*, 2014). However, lipase can also be elevated in other conditions such as peptic ulcer, intestinal obstruction, liver disease, kidney failure, inflammatory bowel disease, acute cholecystitis and mumps (Yadav *et al*., 2002; Hofmeyr *et al.*, 2014). A 'cut-off' of (>3 ULN) is also recommended for the diagnosis of AP by lipase (Hofmeyr *et al.*, 2014). However, while normal lipase values are rare in patients with AP (Lippi *et al*., 2012), similar lipase increases (>3 ULN) have also been reported in patients with kidney failure (Vissers *et al*., 1999) and there is no ability to predict severity of AP. Lipase and amylase are clearly biomarkers for diagnostic purposes only.

### 1.5.2 C-reactive protein

An acute phase protein that is produced by the liver in response to cytokine stimulation (Jain *et al*., 2011), CRP has gained popularity as the biomarker of choice for the prediction of AP severity (Staubli *et al*., 2015). It has been demonstrated to predict pancreatic necrosis (Schütte & Malfertheiner, 2008). However, it cannot predict infected necrosis (Staubli *et al*., 2015). A 'cut-off' value of 150 mg/L by 48h after hospital admission is generally applied for the prediction of severe AP. However, CRP can take at least 48-72h before a clear level peak can be distinguished (Staubli *et al*., 2015). It was also demonstrated to be superior to other potential inflammatory biomarkers such as IL-8, and TNF-α (Yadav *et al*., 2002). Numerous cytokines have been studied with respect to prediction of severity in AP. However, most of them have proven to be of pathophysiological interest rather than of clinical utility. While some studies of IL-6 have shown equivalent ability to stratify severity of AP (Yadav *et al*., 2002), a simple, cheap and widely available method of determination does not currently exist (Meher *et al*., 2015). It is important to note that CRP is not 'disease-specific' but is instead a general inflammatory biomarker. For instance, other inflammatory conditions such as cholangitis and pneumonia must be eliminated when using CRP for diagnosis of AP. Tellingly; CRP is not included in any of the current guidelines for the management of AP (Meher *et al*., 2015).

### 1.5.3 Trypsinogen-2

The inactive zymogen trypsinogen is stored in the pancreatic acinar cells prior to release and activation by enterokinase in the small intestine, as discussed previously in section (1.1). In addition to inappropriate zymogen cleavage and activation within the acinar cells during the early stages of AP, it was demonstrated that a large amount of uncleaved trypsinogen leaves the acinar cell and moves from the interstitium through the lymphatic and portal circulation. Activation of this extracellular trypsinogen can cause necrosis, even in mild AP, is believed to be lead to the progression from mild to severe AP, and possibly contributes to extra-pancreatic injury such as in the lungs (Hartwig *et al*., 1999). Trypsinogen exists in two major isoforms, namely trypsinogen-1 and -2. Both have been found at increased levels in the blood and urine of patients with AP, however, trypsinogen-2 may auto-activate faster than trypsinogen-1 due to $Ca^{2+}$ and pH changes during AP (Paju & Stenman, 2006) and thus 50- versus 15-fold increases in serum concentration have been observed (Kylänpää *et al*., 2002). Urinary tyrpsinogen-2 concentration is also elevated at the onset of AP, with trypsinogen-1 either reabsorbed by the kidneys or degraded prior to excretion in urine. Like any urinary biomarker, its levels can be affected by dehydration or reduction in kidney function (Hedström *et al*., 1996). Urinary trypsinogen-2 levels are more increased in alcohol-induced pancreatitis than in gallstone-related disease (Lempinen *et al*., 2003).

A commercially available (Medix Biochemica, Finland) dipstick test for the rapid determination of urinary trypsinogen-2 has been studied in great detail. This dipstick is immersed into the urine sample where blue latex particles labeled with monoclonal antibody bind to trypsinogen-2 present in the sample and migrate across a nitrocellulose membrane to a region containing another antibody specific for a separate distinct epitope of trypsinogen-2. A trypsinogen-2 concentration greater than 50 µg/L results in the development of a blue line at this region, and the result is considered positive if this clear blue line is observed within 5 minutes (Abraham, 2011). Antibodies and this test approach are discussed further in sections 1.6 and 1.8. Measurement of trypsinogen-2 was shown to compare favourably with serum amylase and lipase for the diagnosis of AP, albeit at conflicting time measurement intervals (Jang *et al*., 2007). Still, there are other studies

showing that its diagnostic ability is secondary to lipase (Jin *et al*., 2013). Moreover, while trypsinogen-2 is useful for the diagnosis of ERCP-induced pancreatitis (Meher *et al*., 2015), lipase is still superior (El-Garem *et al*., 2013). With trypsinogen-2 levels peaking by 24h, diagnostic ability of this biomarker declines rapidly and is therefore less accurate in late hospital admissions (Çevik *et al*., 2010). All of this is indicative that trypsinogen-2 would require the support of additional tests for the successful diagnosis of AP. Conflicting results exist on the assessment of trypsinogen-2 as a biomarker for prediction of disease severity, with superior results observed at 24h after hospital admission compared with CRP (Lempinen *et al*., 2003) but not in the case of work reported by Kamer *et al*. (2007). Accordingly, interest in this function has diminished. The short test time, low cost and ease-of-use provided by this dipstick technology still means that trypsinogen-2 could be used to eliminate a diagnosis of AP, with an emphasis on differentiation between acute abdominal diseases of pancreatic and extra-pancreatic origin (Çevik *et al*., 2010). Furthermore, these virtues highlight the applicability of such a technology in this sphere.

### 1.5.4 Trypsinogen activation peptide

The cleavage of trypsinogen by enterokinase results in the activation of trypsin and the release of the carboxy (C)-terminal peptide known as trypsinogen activation peptide (TAP) in the small intestine (Mayer *et al*., 1999). As this activation peptide is proportionally produced to trypsinogen activation (Lempinen *et al*., 2003) and is highly conserved in vertebrates (Hurley *et al*., 1988), antibodies directed against TAP have been widely used in experimental model studies relating to trypsinogen activation (Frick *et al*., 1997; Grady *et al*., 1998; Matsukura *et al*., 2006). During normal digestion (see Figure 1.1.1), the release of TAP should be difficult to detect as the peptide is likely degraded by intestinal oligopeptidases, however, during the onset of AP, the intra-pancreatic activation of trypsinogen results in the rapid release of TAP into the peritoneal fluid, blood and urine (Tenner *et al*., 1997). Characterisation of TAP has shown that this peptide is largely detected as a penta-peptide molecule with a tetra-L-aspartyl-L-lysine structure. The existence and detection of both smaller (Hurley *et al*., 1988) and larger (Petersson & Borgström, 2006) activation peptide molecules has also been shown, but it is believed that the penta-peptide is the more stable molecule due to rapid degradation of larger molecules in pancreatic juice and in blood. The serum concentrations of TAP have been shown to decrease after 6-24h (Petersson & Borgström, 2006) and plasma concentrations can show no significant difference between normal and diseased patients (Vogel *et al*., 1997). Contrastingly, urinary concentrations of TAP are observed at elevated concentrations up to 48h after the onset of AP/admission. Higher urinary concentrations of TAP have been found in patients with alcohol-induced pancreatitis than in gallstone-related disease (Lempinen *et al*., 2003). However, no particular etiology is associated with this biomarker (Huang *et al*., 2013). Consequently, the penta-peptide is detected in large amounts in urine (Petersson & Borgström, 2006). Antibodies directed against TAP are C-terminal binding and do not bind trypsinogen (Hurley *et al.,* 1988). Trypsinogen activation peptide has been shown to bind calcium in some instances, leading to the discovery of calcium-dependent and independent antibodies that may bind two individual epitopes. It is also suggested that calcium may directly bind to anti-TAP antibodies, leading to a conformation change and enhancing the binding affinity for TAP (Hurley *et al.,* 1988). Antibodies are discussed further in section 1.6.

Numerous studies have detailed the ability of TAP as a biomarker for the early prediction of disease severity in AP. In addition, significantly elevated urinary TAP levels for patients with severe AP when compared to those with mild AP have been demonstrated from early onset of AP, clearly demonstrating the stratification potential of this biomarker (Huang *et al*., 2013). Studies have shown that TAP is superior to CRP for the prediction of severe AP when the measurement is performed within 48h after the onset of symptoms of AP (Tenner *et al*., 1997; Neoptolemos *et al*., 2001; Liu *et al*., 2002). Additionally, the superiority of TAP to scoring systems such as APACHE-II (Neoptolemos *et al*., 2001; Liu *et al*., 2002), Ranson (Khan *et al.,* 2002) and Glasgow-Imrie (Gudgeon *et al.,* 1990) has also been proven. The particularly high negative predictive value of TAP, i.e. the ability to distinguish those patients who do not have severe AP, allows for the exclusion of patients from admission to the ICU, requirement of CECT analysis and specialist care resulting in a reduction of patient management costs (Al-Bahrani & Ammori, 2005). The outcomes from these studies initially lead to some making the claim that TAP should be used in routine clinical practice (Neoptolemos *et al.,* 2001). However, there is an obvious disadvantage in that TAP levels decrease after two days and thus cannot be used for late hospital admissions (Gudgeon *et al*., 1990). Despite the positive perception of TAP as a biomarker, it has all but disappeared from the literature in the last decade. This has largely been due to reproducibility issues experienced with the polyclonal antibody technology employed by the enzyme-linked immunosorbent assay (ELISA) produced by EKF Diagnostics. ELISAs are further discussed in chapter 3. Even so, TAP has also been shown to surpass one of the more promising biomarkers of recent years, namely trypsinogen-2, for the prediction of severe AP (Lempinen *et al*., 2003). Despite the shortcomings of trypsinogen-2 as a prognostic biomarker, great interest was shown due to its application as a point-of-care (POC) device in a rapid dipstick test format. The recent evolution of POC testing methods has the potential to translate into improvements for clinical assessment by re-evaluating biomarkers that were studied previously (Staubli *et al*., 2015). Therefore, overcoming the antibody reproducibility issues for the TAP ELISA through the use of a monoclonal antibody, and investigating the application of the TAP biomarker in a rapid test format could potentially renew the interest in this once promising biomarker. Moreover, with the inclusion of an additional moderately severe category in the revised Atlanta reclassification (Banks *et al*., 2012) and the ability of TAP to clearly distinguish between

mild and severe patients, there is also scope to evaluate the potential of the TAP biomarker to stratify patients across these three categories.

### 1.6 Antibodies – polyclonal versus monoclonal

Antibodies or immunoglobulins (Ig) are soluble glycoproteins produced by B-lymphocyte – derived plasma cells in response to foreign/toxic organisms and/or products during the adaptive immune response of a host. Antibodies have two functions – binding and deactivation of a target antigen such as a bacterial toxin or viral receptor, or binding and activation of immune signalling. This basic but powerful utility has resulted in their widespread application as diagnostic reagents. Antibodies are composed of four polypeptides consisting of two identical heavy chains (with a molecular weight of ~50 kDa) and two identical light chains (~25 kDa) that are held together by disuphide bonds. Both of these chains contain constant (C) and variable (V) regions. The fragment antigen-binding (Fab) region of the antibody is composed of two identical fragments with the $V_H$ and $V_L$ regions. The fragment crystallisable (Fc) region is composed of the constant heavy regions ($C_{H1}$, $C_{H2}$ and $C_{H3}$), which are important in immune signaling and effector functions. There are five different classes of Ig found in mammals (IgA, IgD, IgE, IgG and IgM), with IgG and IgA also being further subdivided into different isotypes. IgG is the most abundant class found in serum and the most widely used in diagnostic applications. Its structure is outlined in Figure 1.6.1. The diversity of presenting antigens can lead to the generation of distinctive antibodies. Polyclonal antibodies (pAb) are derived from a collection of different plasma cells whereas monoclonal antibodies are derived from a single clone. PAbs recognise and bind to multiple epitopes of a target antigen, ensuring greater binding of the antigen on a larger scale, but with a lower affinity and specificity. PAbs can also be generated rapidly at lower costs. Monoclonal antibodies (mAb) recognize and bind to a single individual epitope of a target antigen, resulting in a highly specific interaction. In addition, the use of hybridoma technology provides homogenous and reproducible mAb performance. However, structural changes such as antigen denaturation can prevent mAb binding (Lipman *et al*., 2005). Antibodies have played a crucial and successful role in many research applications such as purification and enrichment of antigens and ELISAs, and continue to play an essential role in the growing POC industry for medical diagnostics.

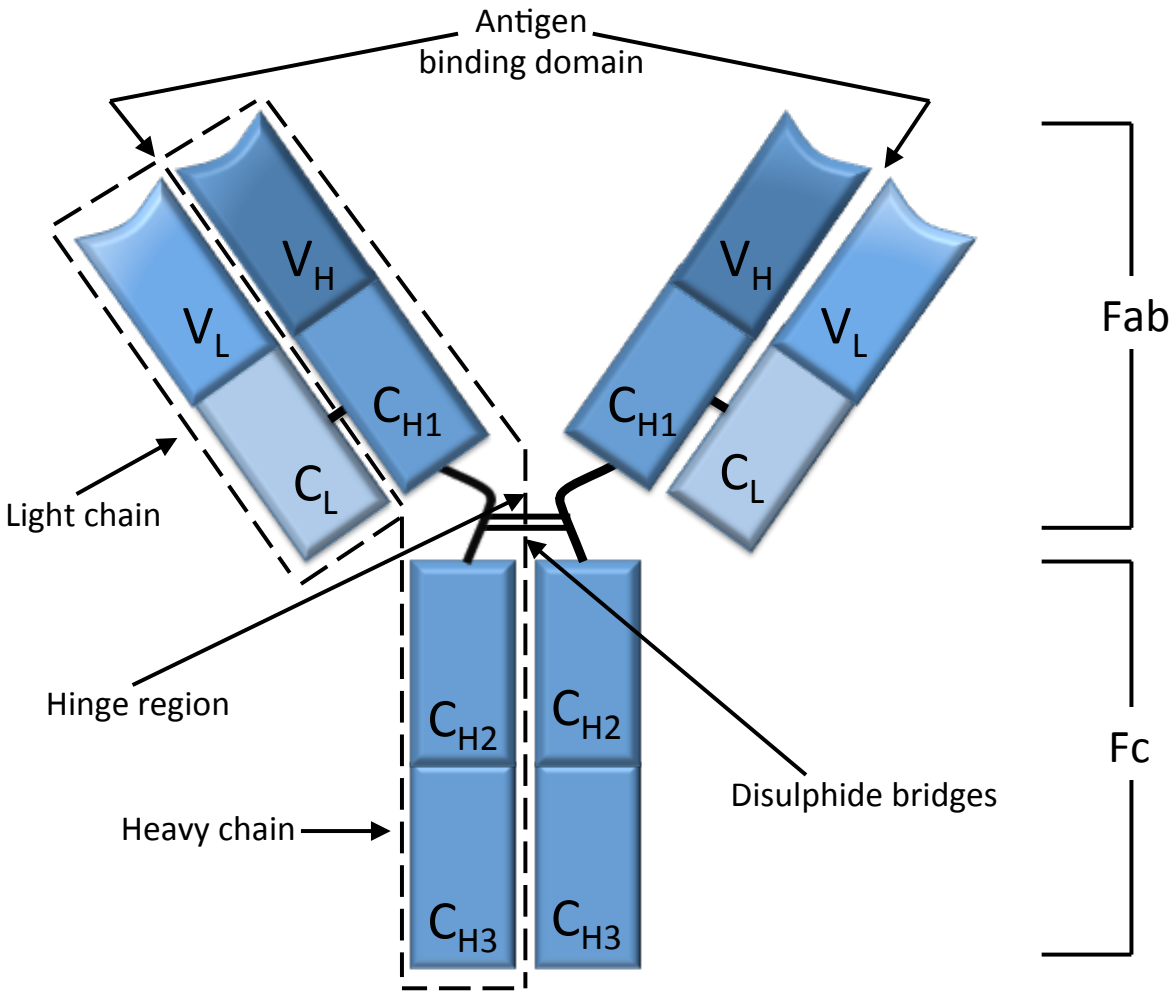



**Figure 1.6.1** Basic structure of an IgG molecule, showing the heavy chain consisting of a variable ($V_H$) region linked to tree constant regions ($C_{H1}$, $C_{H2}$ and $C_{H3}$). The light chain consists of a single variable ($V_L$) and constant region ($C_L$).

## 1.7 Point-of-Care tests

The growing trend of POC diagnostics in the developed world has emerged from the increasing pressure to limit healthcare budgets and the realisation that the current healthcare model is neither sustainable nor fit-for-purpose. With the increasing burden placed on hospital resources due to budgetary constraints and increasing incidence of illnesses such as cardiac diseases, patient care has become less patient-focused and more fragmented with the need for follow-up visits or consultations required after assessment or hospital discharge. This challenge can be met head-on through the use of POC devices

that will offer the opportunity to decentralise patient care to a community level. POC devices can and are playing a front-line role in hospitals worldwide (St John & Price, 2014). Biomarker POC tests, such as the use of cardiac troponin I (cTnI) for the analysis of acute coronary syndrome, have been demonstrated to reduce the length of stay in the hospital Emergency Department (ED) when compared to central laboratory testing (Singer *et al*., 2005; McDonnell *et al*., 2009). The cost of these tests may be higher than central laboratory testing, but this is offset by the reduction in length of stay and the overall impact to patient management costs (St John & Price, 2014). In addition, POC tests provide rapid and time-saving results without the requirement of a skilled test operator and can contribute to avoidance of sample mislabeling and incorrect interpretation of results (Gubala *et al*., 2012; Sharma *et al*., 2015). Taking all of this into account, the POC market was projected to reach a value of US$18 billion by this year (St John & Price, 2014).

The key requirements of POC devices include simplicity of use, robust storage and operation across varying temperatures, an established reference method for monitoring accuracy of results, and safety of use, i.e. no harmful elements or reagents. These principles have been extended through guidelines provided by the WHO under the acronym of ASSURED for the development of POC devices for the detection of sexually transmitted diseases (Table 1.7.1).

**Table 1.7.1** The ASSURED guidelines for features that should be included in POC devices:

- **A**ffordable – for those at risk of infection
- **S**ensitive – minimal false negatives
- **S**pecific – minimal false positives
- **U**ser-friendly – minimal steps to carry out test
- **R**apid & **R**obust – short turnaround time and no need for refrigerated storage
- **E**quipment-free – no complex equipment
- **D**elivered – to end-users

Current POC devices can be divided into “over-the-counter” products such as glucose monitoring devices for diabetes and pregnancy tests that are aimed at the non-professional market, and devices for the analysis of infectious diseases, cardiac biomarkers and haemotology which are utilised in professional testing. The professional market mainly comprises larger bench-top analysers in critical care, representing an evolution away from central laboratory testing whereby operation can be performed by non-laboratory trained staff such as nurses. This is facilitated by ease of use with limited sample handling, simple touchscreen interface integration, barcode scanner cataloging of test and patient information, connection to central information systems for storage and analysis of patient data and room temperature storage of test reagents. Small hand-held POC devices comprise portable tests that can transcend both the professional and non-professional markets due to built in quality control checks that ensure accuracy of results, including at home (St John & Price, 2014; Sharma *et al*., 2015). The single most successful home POC device is the pregnancy test kit measuring the pregnancy hormone human chorionic gonadotropin (hCG) utilising antibody-antigen binding in the format of a lateral flow test strip. In fact, the majority of POC devices use this immunoassay technology (Gubala *et al*., 2012). Lateral flow immunoassay (LFIA) technology has also been used extensively in the modern success of POC cardiac biomarkers, demonstrating its considered value (Chan *et al*., 2013).

### 1.8 Lateral flow immunoassays and superparamagnetic particles in point-of-care

The lateral flow immunoassay (LFIA) has been adapted for use in a multitude of fields including medical, veterinary, food, agricultural and environmental settings. LFIAs are primarily used for the detection of small antigens or analytes such as peptides, proteins, haptens and nucleic acids. In LFIAs, a liquid sample carrying an analyte of interest migrates though a polymeric strip coated with immobilised molecules that interact with the analyte and produce a signal that can be visually detected through coloured nanoparticles, including gold or latex (Koczula & Gallotta, 2016). In addition to this test region where binding and visualisation occurs, an independent control region using non-specific antibodies is also included to ensure correct functionality. The simplicity of LFIA devices

hides the complex assembly of many vital components which must be fully considered from an early stage. Obviously, the biological reagents such as antibodies are of utmost importance, however, the selection of the most appropriate materials will ensure the consistency of the device. LFIAs are generally composed of a nitrocellulose membrane, sample pad, conjugate pad and absorbent pad which are assembled onto a backing card for support before housing in a plastic test cassette. An example of this assembled architecture is shown in Figure 1.8.1. Nitrocellulose (NC) membranes are porous polymers that are available with a variety of pore sizes to facilitate the capillary flow of the sample to the analytical region. The capillary flow rate controls the time taken for the sample to fill and travel across the test strip. The NC membrane material also effects the binding and immobilisation of antibodies/antigens used in the assay. The sample pad is commonly composed of cellulose and is the loading point for the test sample and can act as a filter for undesired materials such as red blood cells from a plasma sample. This pad can also be pretreated for neutralisation of sample pH when testing with urine. The conjugate pad is generally composed of glass fibre and can be pretreated with dried conjugate such as antibody labeled with nanoparticles. The integrity of the conjugate is maintained through the use of a concentrated sugar buffer that protects the conjugate activity until the sample enters the pad. The absorbent pad, often composed of high-density cellulose, is located at the end of the test strip and wicks up any excess sample reaching this point (Sharma *et al*., 2015). Advantages of LFIAs include that it is a simple, versatile, rapid and low-cost test method that can be used for both qualitative and quantitative purposes and without the need for sample pretreatment (Posthuma-Trumpie *et al*., 2009). Disadvantages of LFIAs include that obstruction of membrane pores by sample matrix components, inaccurate loading volumes including over-loading of the sample port can reduce the precision and reliability of the test result, the viscosity of the sample can affect the test development time, inconsistent binding and release of conjugate can occur, protein incompatibility can lead to binding with the NC membrane, and sensitivity can be limited due to a lack of possibility for signal enhancement using enzyme conjugates like ELISAs (Posthuma-Trumpie *et al*., 2009; St John & Price, 2014). LFIAs are discussed again in chapter 4.

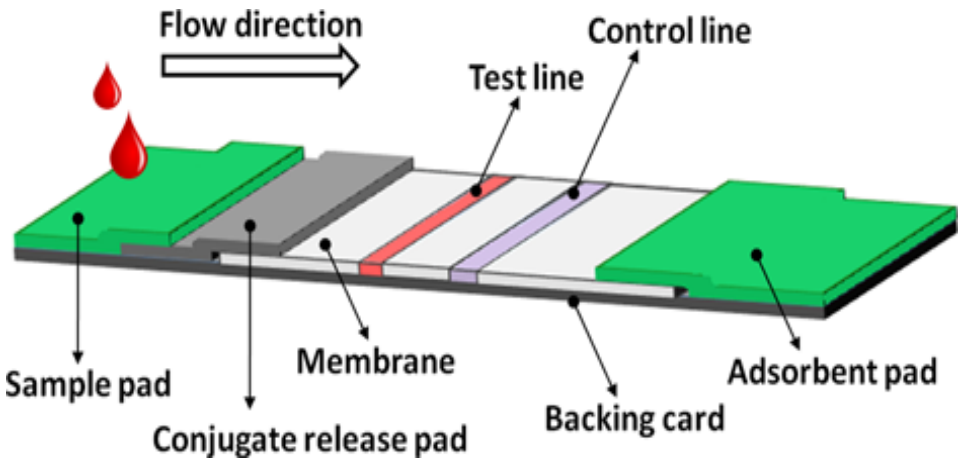



**Figure 1.8.1** Typical LFIA test strip construction (taken from Koczula & Gallotta, 2016), showing backing card, sample pad, conjugate pad, strip membrane where immobilised antibodies are located at test and control regions and absorbent wicking pad.

The use of LFIAs for quantitative applications has been facilitated through the use of instruments and devices that are capable of detection methods such as fluorescence (Posthuma-Trumpie *et al*., 2009) and magnetic excitation (LaBorde & O'Farrell, 2002). The ease of use for gold and latex particles in qualitative LFIAs has influenced the adoption of magnetic particles in this assay format (LaBorde & O'Farrell, 2002). Superparamagnetic particles (SPMPs) are magnetic iron oxide nanoparticles with production techniques that allow for controllable sizes ranging from 10-100 nm (comparable with a cell) to 10-450 nm (comparable with a virus) and beyond. These particles can be coated using biocompatible polymers such as polyethelene glycols (PEG) and poly vinyl-alcohol (PVA) for stabilisation and facilitating conjugation to biological molecules such as antibodies (Nor *et al*., 2012). In addition, magnetic particles can be non-invasively detected by a wide range of methods, are physically and chemically stable and relatively inexpensive to produce (Tamanaha *et al*., 2008). They are also of low toxicity compared to alternative substances such as nickel (Nor *et al*., 2012). The fact that SPMPs are only magnetic when placed in a very strong magnetic field, gives these particles a major advantage as labels for separation or analyte detection (LaBorde & O'Farrell, 2002). The ability of magnetic fields to penetrate into human tissue has resulted in the medical application of these particles in drug delivery and MRI imaging (Pankhurst *et al*., 2003). SPMPs possess a single magnetic domain that can be manipulated by an external magnetic field to produce uniform magnetic excitation

that can be quantified using a magnetic detection sensor (Tartaj *et al*., 2003). Several groups have developed sensors for the detection and quantification of magnetic particles including MagnaBioSciences, LLC, Quantum Design, San Diego, CA, USA, LifeAssays AB, Lund, Sweden, Senova Immunoassay Systems, Jena, Germany, Magnotech, Philips, Amsterdam, The Netherlands, Magnisense, Paris, France and MagArray Inc., Sunnyvale, CA, USA (Barnett *et al*., 2014) and Magnasense, Vantaa, Finland (Magnasense.com). The magnetic immunochromatographic test (MICT) system from MagnaBioSciences, LLC has been evaluated for the development of magnetic LFIAs in an extensive number of fields including parasitic infection (Handali *et al*., 2010), viral infection leading to cervical cancer (Peck *et al*., 2006), bacterial infection (Shi *et al*., 2015), detection of fish allergens (Zheng *et al*., 2012) and acute myocardial infarction (Xu *et al*., 2009). Additionally, the MICT instrument and a commercial thyrotropin-releasing hormone (TSH) test received clearance from the US Food and Drug Administration (FDA) governing body in March 2012, confirming the safety and efficacy of the MICT system (MagnaBioSciences News Release, 2012). With the MICT platform, the LFIA test strip is placed inside a C-shaped electro-magnet that houses several coils between its poles. A voltage is applied from a parallel capacitor to activate the coils and generate a magnetic field. The subsequent magnetisation of the SPMPs is detected by magnetic field sensors and the signal is amplified and processed to quantify the number of magnetic particles at both the test and control line locations only. This signal is quantified as magnetic assay reading (MAR), a unitless number. When using the MICT instrument, the magnetic field excites all of the SPMPs, throughout the NC membrane, allowing for sensitive and quantitative measurement of the test analyte (MagnaBioSciences, 2011). The MICT system has been demonstrated to have almost 10 to 1000-times higher sensitivity over conventional LFIA methods. The magnetic signal does not degrade over a long time period, and thus the MICT LFIA strips can be stored for rechecking of results if necessary (Wang *et al*., 2009). The sensitivity of this platform also allows for the multiplexing of several analyte regions along the NC membrane. Consequently, this platform is capable of measuring a panel of biomarkers, holding major potential for future POC applications. This concept also holds potential in AP assessment and has been alluded to by others (Staubli *et al*., 2015). SPMPs and the MICT instrument are discussed again in chapter 4.

Once again, the aim of this body of work is to develop a competitive enzyme-linked immunosorbent assay (ELISA) and rapid lateral flow immunoassay (LFIA) for the detection of trypsinogen activation peptide (TAP), and to utilise these prototypes to reopen the discussion of the clinical utility of the TAP biomarker at a time when the definition of acute pancreatitis disease severity has been revised under the internationally recognised Atlanta classification.

# Chapter 2

# Materials and Methods

## 2.1 MATERIALS

### 2.1.1 Equipment list

| Equipment | Supplier |
|---|---|
| **XCell SureLock™ Mini-Cell Electrophoresis System**<br>**PowerEase® 500 Power Supply Pack** | Invitrogen Life Technologies, 5791 Van Allen Way, Carlsbad, CA 92008, USA. |
| **Gel Doc EZ Imager** | Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX, UK. |
| **NanoDrop™ ND1000** | NanoDrop Technologies Inc., 3411 Silverside Road, Willmington, DE 19810, USA. |
| **MTS 2/4 Digital Microtitre Plate Shaker** | IKA, Janke & Kunkel-Str. 10, 79219, Staufen, Germany. |
| **Sunrise™ Microplate Absorbance Reader** | TECAN Group Ltd., Seestrasse 103, 8708 Männedorf, Switzerland. |
| **Stuart Gyro-Rocker SSL3**<br>**Stuart SRT6D Tube Rotator**<br>**Stuart SB3 Tube Rotator**<br>**Stuart Magnetic Stirrer CB161**<br>**Techne Dri-Block DB2A Heating Block** | Lennox Laboratory Supplies Ltd., John F. Kennedy Drive, Naas Road, Dublin 12, Ireland. |
| **Sonicator Bath U95** | Ultrawave Ltd., Eastgate Business Park, Wentloog Avenue, Cardiff, South Glamorgan CF3 2EY, UK. |
| **Magnetic Immunochromatographic Test (MICT®) Reader System**<br>**MICT® Cassette Assembly Clamp** | MagnaBioSciences LLC., 6325 Lusk Boulevard, San Diego, CA 92121-3733, USA. |
| **CAMAG Linomat 5 Semi-Automatic Application System** | Mason Technology Ltd., 228 South Circular Road, Dublin 8, Ireland. |
| **Eppendorf Centrifuge 5810R**<br>**Rotors - F45-30-11 (14,000 rpm), A-4-62 (4,000 rpm)** | Unitech Ireland Ltd., Unit 2, Airton Business Park, Airton Road, Dublin 24, Ireland. |
| **OHAUS Enclosed Analytical Balance**<br>**KERN Top Pan Balance 440-53N** | VWR International, LLC., 1310 Goshen Parkway, West Chester, PA 19380, USA. |
| **ThermoOrion model420 pH Meter** | Fisher Thermo Scientific, 47341 Bayside Pkwy., Fremont, CA 94538, USA. |
| **SANYO Incubator MIR-162** | AGB Scientific Ltd., Dublin Industrial Estate, Dublin 11, Ireland. |
| **Autoclave** | Priorclave Ltd., 129-131 Nathan Way, West Thamesmead Business Park, London, SE28 OAB, UK. |
| **Pipettes (Various)** | Eppendorf UK Ltd., Eppendorf House, Gateway 1000 Whittle Way, Arlington Business Park, Stevenage, SG1 2FP, UK. |

### 2.1.2 Consumables

| Consumable | Supplier |
|---|---|
| **Plastic Labware, including microtubes, syringes, sterilin tubes and pipette tips**<br>**Whatmann PURADISC Disposable Filter Device**<br>**GE Healthcare Disposable PD-10 Desalting and Buffer Exchange Pre-packed Columns**<br>**Pierce® Streptavidin Coated High-Binding Capacity 8-Well Clear Strip Microplates**<br>**NUNC Lockwell C8 Maxisorp™ Clear-Well Microplates**<br>**Scharlau pH Calibration Buffers (pH 4.0, 7.0, 10.0)** | Fisher Thermo Scientific, 47341 Bayside Pkwy., Fremont, CA 94538, USA. |
| **Sterile Screw-Cap Micro tubes (0.5mL)** | Sarstedt AG & Co., Sarstedtstraße 1, 51588 Nümbrecht, Germany. |
| **Novex 4-12% Bis-Tris Gels, 1.0mm** | Bio-Sciences Ireland, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co Dublin, Ireland. |
| **MiniPax® Compact Desiccant Packets** | Multisorb Technologies, 325 Harlem Road, Buffalo, New York 14224, USA. |
| **Vivaspin® 6, 10,000 MWCO PES**<br>**Vivaspin® 500, 30,000 MWCO PES**<br>**UniSart CN 140 Cellulose Nitrate Membrane on 100µm polyester film** | Sartorius Stedim Ireland Ltd., Unit 41, The Business Centre, Stadium Business Park, Ballycoolin Road, Dublin 11, Ireland. |
| **Sample Pad, Grade 205 (27mm width)**<br>**Upper Wick Cellulose Fibre Pad, Grade A222 (21mm width)**<br>**Conjugate Glass Fibre Pad, Grade 8964 (10mm width)** | Ahlstrom Filtration, LLC., 122 West Butler Street, Mt Holly Springs, PA 17065, USA. |
| **ARcare® 7759 Clear Polyester Single-Sided Adhesive Tape** | Adhesives Research Ireland Ltd., Raheen Business Park, Raheen, Limerick, V94 VH22, Ireland. |
| **MICT® Disposable Cleaning Cassettes**<br>**MICT® Test Cassettes for 5.0mm Strips**<br>**Die-cut Base Card for 5.0mm Strips** | MagnaBioSciences LLC., 6325 Lusk Boulevard, San Diego, CA 92121-3733, USA. |

### 2.1.3 Reagents

| Reagent | Supplier |
|---|---|
| **Novex® SeeBlue® Plus2 Prestained Protein Standard**<br>**Invitrogen™ SimplyBlue™ Safe Stain**<br>**Novex® NuPage MES SDS Running Buffer** | Bio-Sciences Ireland, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland. |
| **Trizma® base**<br>**Aprotinin from bovine lung**<br>**Sodium Hydroxide (NaOH)**<br>**Calcium Chloride**<br>**Sodium Dodecyl Sulphate (SDS)**<br>**2-mercaptoethanol**<br>**2-Propanol**<br>**5-bromo-5-nitro-1,3-dioxane (Bronidox-L)** | Sigma Aldrich Ireland Ltd., Vale Rd, Arklow, Co. Wicklow. |
| **Oxoid Phosphate Buffered Saline (PBS) Tablets**<br>**Bromophenol blue**<br>**Glycerol**<br>**Casein**<br>**Hydrochloric Acid, 37% (v/v) in water (HCl)**<br>**EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)**<br>**NHS (*N*-Hydroxysuccinimide)**<br>**BupH™ 0.05M Borate, 0.1M 2-(N-morpholino) ethanesulfonic acid (MES), 0.9% NaCl Buffer Packs, pH 4.7**<br>**BupH™ Borate Buffer Packs, 0.05M Sodium borate, pH 8.5** | Fisher Thermo Scientific, 47341 Bayside Pkwy., Fremont, CA 94538, USA. |
| **Sodium Chloride**<br>**Tween® 20**<br>**Sodium Azide**<br>**Bovine Serum Albumin (BSA)** | Millipore Ireland B.V., Tullagreen, Carrigtwohill, Co. Cork, Ireland. |
| **ProClin 950** | Supelco, 595 North Harrison Road, Bellefonte, PA 16823-0048, USA. |
| **Prestained 3,3',5,5'-Tetramethylbenzidine (TMB) X-tra** | Kem-En-Tec Diagnostics A/S, Kuldyssen 10, 2630 Taastrup, Denmark. |
| **Estapor® Carboxyl-Modified Superparamagnetic Microspheres (MH1-020/50, 200nm)** | Merck Chimie SAS, 201 rue Carnot, F-94126 Fontenay sous Bois Cedex, France. |
| **Ademtech Storage Buffer (10X)** | Ademtech, BioParc Biogalien Bat C 1 étage, 27 allée Charles Darwin, 33600 Pessac, France. |
| **Siemens Multistix® 10 SG, Reagent Strips for Urinalysis** | Promed, Tulligmore, Killorglin, Co. Kerry, Ireland. |

### 2.1.4 Proprietary antibodies used in this research

| Antibody/Peptide | Catalogue Number | Supplier |
|---|---|---|
| **Anti-Trypsinogen Activation Peptide, Rabbit Monoclonal IgG Antibody** | BTR-1-14-8 | AbCam Burlingame, 863 Mitten Road, Suite 103, Burlingame, CA 94010-1303, USA. |
| **Trypsinogen Activation Peptide (Custom Synthesis)** | 4055133 | Bachem AG, Hauptstrasse 144, 4416 Bubendorf, Switzerland. |

### 2.1.5 Commercial antibodies used in this research

| Commercial Antibody/Protein | Catalogue Number | Supplier |
|---|---|---|
| **AffinPure Goat Anti-Rabbit IgG (H+L) (minimal cross-reaction to Human Serum Proteins)** | 111-005-045 | Jackson Immunoresearch Laboratories, Inc., 872 W. Baltimore Pike, West Grove, PA 19390, USA. |
| **AffinPure Goat Anti-Chicken IgY (H+L) (minimal cross-reaction to Human Serum Proteins)** | 111-005-046 | |
| **IgG from Rabbit Serum** | I5006 | Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, USA. |
| **Chicken IgY** | IgY-100 | Gallus Immunotech Inc., 120 Brookbank Hill, Pl Cary, NC 27519, USA. |
| **ImmunoPure Streptavidin (Lyophilised)** | 2122 | Fisher Thermo Scientific, 47341 Bayside Pkwy., Fremont, CA 94538, USA. |

### 2.1.6 Buffers

| Buffer | Composition | |
|---|---|---|
| **Phosphate Buffered Saline (PBS) (0.01M)** | PBS Tablet<br>$dH_2O$<br>Final pH 7.3 | 1 tablet<br>up to 100 mL |
| **Bromophenol Blue Solution (0.5% w/v)** | Bromophenol blue<br>$dH_2O$ | 0.1 g<br>20 mL |
| **Sodium Dodecyl Sulphate (SDS) (10% w/v)** | SDS<br>$dH_2O$ | 1.0 g<br>up to 10 mL |
| **Tris-HCl Buffer (0.5M)** | Tris<br>$dH_2O$<br>Final pH 6.8 | 6.05 g<br>up to 100 mL |
| **5X Solubilisation Buffer** | Glycerol<br>SDS (10% w/v)<br>Tris HCl (0.5M, pH 6.8)<br>2-mercaptoethanol<br>Bromophenol blue<br>$dH_2O$ | 10 g<br>1.6 mL<br>1.0 mL<br>0.4 mL<br>0.2 mL<br>4 mL |
| **Concentrated Coating Buffer**<br>**PBS Tween (PBST)**<br>**Blocking Buffer**<br>**Tris Buffered Saline Tween (TBST)**<br>**Tris Buffered Saline Tween, 1% (w/v) BSA**<br>**Sample Diluent**<br>**Calibrator Diluent**<br>**Conjugate Diluent Buffer**<br>**Positive Control Diluent** | Proprietary composition | |
| **Stop Solution (1M)** | Concentrated $H_2SO_4$<br>$dH_2O$ | 28.0 mL /L<br>972.0 mL /L |
| **Activation Buffer** | BupH™ 0.05M Borate, 0.1M 2-(N-morpholino) ethanesulfonic acid (MES), 0.9% NaCl Buffer Pack, pH 4.7<br>Tween20 (0.55% w/v)<br>$dH_2O$ | 1 pack<br>0.275g<br>up to 500 mL |
| **Cross-Linking Buffer** | BupH™ Borate Buffer Packs, 0.05M Sodium borate, pH 8.5<br>Tween20 (0.55% w/v)<br>NaCl (0.15M)<br>$dH_2O$ | 1 pack<br>0.275g<br>4.383g<br>up to 500 mL |
| **Sample Pad Buffer** | Proprietary composition | |

### 2.1.7 Commercial kits

| Commercial Kit | Catalogue Number | Supplier |
|---|---|---|
| **Lightning-Link™ HRP Conjugation Kit (LL-HRP 3 reactions of 100 µg)** | 701-0000 | Innova Biosciences Ltd., Babraham Research Campus, Cambridge, Cambridgeshire, CB22 3AT, UK. |
| **Lightning-Link™ Biotin Conjugation Kit (Type B - 3 reactions of 100-200 µg)** | 715-0010 | |

### 2.1.8 Software

List of software used for data analysis in this research:

| Software | Supplier |
|---|---|
| **GEN5 MicroPlate Reader Software (Version 1.06)** | Biotek Instruments, Inc., 100 Tigan Street, Winooski, VT05404, USA. |
| **Analyse-it - Statistical Analysis and Data Visualisation Software** | Analyse-it Software Ltd., The Tannery, 91 Kirkstall Road, Leeds, LS3 1HS, UK. |
| **Graphpad Prism Statistical Software (Version 7)** | GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA |

## 2.2 METHODS

### 2.2.1 Characterisation of monoclonal *14-8* anti-TAP antibody

#### 2.2.1.1 SDS-PAGE analysis of monoclonal 14-8 anti-TAP antibody purity

Purified monoclonal antibody (Lot# B_4168, B_4346 and B_37042) was prepared at 250 and 500 µg/mL concentrations with PBS (0.01M). The samples were mixed with 10 µL of 5X solubilisation buffer and heated at 95°C for 10 minutes. The heated samples (20 µL) were loaded into the wells of a 4-12% (w/v) Novex® Bis-Tris gel at final concentrations of 5 and 10 µg/well. The gel was resolved at 250 volts until the prestained protein standard and bromophenol blue dye reached the bottom of the gel (approximately 35 minutes). The gel was removed and washed three times with $dH_20$ before staining with SimplyBlue™ safe stain for one hour. Finally, the stained gel was destained three times, each of 1 hour duration, with $dH_20$.

### 2.2.2 Microtitre plate coating

Purified monoclonal anti-TAP antibody was coated in a 96-well plate (Nunc, Maxisorp™) at various concentrations (2, 3, 4, 6 and 8 µg/mL). The antibody was diluted with 1X coating buffer as required and mixed for 60 minutes by magnetic stirrer prior to commencing plate coating. The coating solution was dispensed at 100 µL/well and the plate was covered with a plate sealer and incubated at 4°C for 16-24 hours. The coated plate was removed and washed four times with 1X PBST (300 µL/well) before firmly tapping on absorbent paper to ensure no residual coating solution remained in the wells. Blocking buffer was dispensed at 200 µL/well and the plate was covered with a plate sealer and incubated at room temperature (20-25°C) for 2 hours. The blocked plate was removed and aspirated before firmly tapping on absorbent paper to ensure no residual blocking solution remained in the wells. The plate was dried for 18-22 hours at 37°C in the presence of 1kg of calcium chloride. The dried plate was sealed in a foil pouch with 2 desiccants and stored at 4°C until required for use.

### 2.2.3 Conjugation of TAP antigen to HRP

Lightning-Link™ HRP conjugation kit (Innova Biosciences) was supplied with 100 µg lyophilised HRP (40 kDa) per vial, equivalent to 2.5 nanomoles of HRP. A 4-fold molar excess of protein to HRP was recommended for use with this kit. The lyophilised TAP antigen (769.72 g/L, 5 mg – as indicated by supplier, Bachem) was reconstituted to 1 mM concentration using 6.5 mL of $dH_20$. The reconstituted stock was then diluted to a final volume of 100 µL (minimum required for use with this kit) by addition of 1 µL (1 mM TAP) to 99 µL of PBS to give 10 nanomoles of TAP antigen. The conjugate was prepared as per product insert and an equal volume (120 µL) of 80% (w/v) glycerol was added to the stock solution, which was stored at 4°C until required.

### 2.2.4 Competitive monoclonal *14-8* anti-TAP ELISA

All assay components were removed from storage (-20 or 4°C) and allowed to equilibrate to room temperature (20-25°C) prior to use. Calibrators and samples were prepared and loaded to the assay plate (50 µL/well) before addition of diluted conjugate (50 µL/well), as required. The plate was covered with a plate sealer and incubated at room temperature with uniform shaking (350 ± 10 rpm) for 1 hour. Following incubation, the plate was washed six times with 1X TBST (300 µL/well) before firmly tapping the plate on absorbent paper to ensure complete removal of wash solution. TMB substrate was added (100 µL/well) with no shaking at room temperature in the dark for 15 minutes. Colour development was quenched by addition of Stop Solution (100 µL/well). The plate was read immediately using the Tecan™ Sunrise microplate absorbance reader ($450_{nm}$ with a reference of $620_{nm}$). The reference wavelength absorbance ($620_{nm}$) value was subtracted from the $450_{nm}$ absorbance value for all assays performed in this project.

### 2.2.5 Biotinylation of monoclonal *14-8* anti-TAP antibody

Lightning-Link™ Biotin conjugation kit (Innova Biosciences) was supplied with 100 µg lyophilised biotin per vial. An antibody concentration of 1 – 2.5 mg/mL was recommended for use with this kit. The purified monoclonal anti-TAP antibodies (Lot# B_4168, B_4346 and B_37042) were concentrated, where required, as per section 2.2.6.1, prior to use with this kit. Lightning Link™ rapid modifier solution (10 µL) was added to the anti-TAP antibody before this mixture was added to the lyophilised biotin material. The reaction mixture was incubated for 3 hours before Lightning Link™ rapid quencher solution (10 µL) was added. The biotinylated antibody was stored at 4°C until required for further use.

### 2.2.6 Competitive ELISA with streptavidin-coated plate / biotinylated monoclonal *14-8* anti-TAP antibody

All assay components were removed from storage (-20 or 4°C) and allowed to equilibrate to room temperature prior to use. The streptavidin-coated plate was washed three times (200 µL/well) with 1X TBST, containing 0.1% (w/v) BSA. Biotinylated anti-TAP monoclonal antibody was loaded (100 µL/well) onto the plate, covered with a plate sealer and incubated at room temperature with uniform shaking (350 ± 10 rpm) for 2 hours. The plate was washed three times (200 µL/well) with 1X TBST, 0.1% (w/v) BSA before firmly tapping the plate on absorbent paper to ensure complete removal of wash solution. The remainder of the assay was completed, as described for the monoclonal *14-8* anti-TAP antibody ELISA method, section 2.2.3.

### 2.2.7 Buffer exchange and protein concentration of monoclonal *14-8* anti-TAP antibody

#### 2.2.7.1 Protein concentration by ultrafiltration

Antibodies were concentrated using Vivaspin columns (GE Healthcare) with an appropriate molecular weight cut-off (MWCO: 10 - 30 kDa). The Vivaspin column was pre-rinsed by addition of PBS to half the maximum volume of the Vivaspin unit and centrifuged (4,000 x g) using Eppendorf Centrifuge 5810R rotor A-4-62 at a temperature of 4$^{o}$C for 5 minutes. The PBS was discarded from the upper and lower chambers. Antibody solution was added to the upper chamber and centrifuged (2,500 x g) at a temperature of 4$^{o}$C for 5 minutes. The process was repeated until the sample was concentrated sufficiently. On reaching the required concentration, the concentrated sample was mixed by pipetting and removed. A small volume of PBS was subsequently added to the upper chamber and mixed by pipetting to capture any residual antibody remaining. This solution was pooled with the concentrated sample. Antibody concentration was measured using a Nanodrop spectrophotometer ($A_{280nm}$).

#### 2.2.7.2 Buffer exchange by desalting column

PD-10 columns (GE healthcare) provide a rapid means to buffer exchange small volumes of protein solutions with a maximum sample volume of 2.5 mL per column. The top cap of the PD-10 column was removed and the column storage solution was discarded. The sealed end of the column was cut at the notch using a scissors. The column was equilibrated with 25 mL of PBS. The antibody solution was added to the column and allowed to completely enter the packed bed by gravity flow. If sample volume was <2.5 mL, additional PBS was added to adjust the volume up to 2.5 mL after the sample had entered the packed bed completely. The flow-through solution was discarded before the antibody was eluted with 3.5 mL of 0.05M borate, 0.55% Tween20 (w/v), 0.15M NaCl, pH 8.5 (cross-linking buffer, see section 2.2.8 also) and

collected into a test tube. Antibody concentration was measured using the Nanodrop spectrophotometer ($A_{280nm}$) and stored at 2-8°C or -20°C for short and long-term storage, respectively.

### 2.2.8 Lateral flow test strip – superparamagnetic particle (SPMP) conjugation

The rabbit IgG and chicken IgY antibodies were buffer exchanged into cross-linking buffer, as per section 2.2.6.2. The lyophilised TAP peptide was reconstituted directly into cross-linking buffer due to the limited availability of material. All stock concentrations were confirmed by Nanodrop™ prior to use. A concentration >2 mg/mL was required for this protocol. Magnetic particles were diluted to a 3% (w/v) solid content solution by addition of activation buffer (0.1M 2-(N-morpholino) ethanesulfonic acid (MES), 0.9% NaCl (w/v), pH 4.7, 100 µL total volume) in a 1.5 mL tube. The particle solution was pulled down by a magnetic separator and the supernatant removed. The particle pellet was fully suspended by pipetting with 200 µL of activation buffer before 'pull-down' and removal of supernatant was repeated. The particles were re-suspended in 250 µL of activation buffer and sonicated in a water bath for 2 minutes (1 minute on, 1 minute off, 1 minute on) at a power output of 35W at 44kHz to ensure even dispersion. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide were weighed and dissolved into 50 µL of activation buffer before quickly transferring to the sonicated particles. The reaction mixture was placed in a tube rotator at a 90° angle with slow mixing (20 rpm) at 37°C for 1 hour. The activated particles were pelleted and suspended twice in 300 µL of activation buffer, removing the reaction supernatant on each occasion. The particle pellet was fully suspended in 300 µL of cross-linking buffer before transfer to a clean 1.5 mL tube, followed by particle pull-down and removal of supernatant. The particle pellet was re-suspended in 200 µL of cross-linking buffer and sonicated as above. The required amount of antibody or peptide was added to the sonicated particles and additional cross-linking buffer was added to bring to a final volume of 350 µL, if needed. If this final volume was exceeded following the addition of a large amount of antibody or peptide, no additional cross-linking buffer

was added. The reaction mixture was placed in a tube rotator at a 90° angle with slow mixing (20 rpm) at 37°C for 3 hours. The residual activated COOH groups of the conjugated particles were blocked by addition of 40 µL of cross-linking buffer with 5% (w/v) BSA. This solution was incubated at 37°C for 30 minutes without mixing before washing three times with 400 µL of cross-linking buffer by particle pelleting, supernatant removal and particle resuspension. On the third wash, the particles were transferred to a new 1.5 mL tube and the supernatant removed. The washed particles were fully suspended in 300 µL of 1X Ademtech storage buffer and stored at 4°C until use.

### 2.2.9 Lateral flow test strip – antibody spotting

A base card (MagnaBioSciences) was divided into 5 mm strips using a Lumicolor permanent marker and ruler. The nitrocellulose membrane was attached to the backing card at 1 mm from both sides of the indicated central position (Figure 2.2.8.1.) Goat anti-rabbit IgG or goat anti-chicken IgY capture antibody was diluted to 1 mg/mL in PBS and dispensed by pipette (0.5 µL/strip) onto the nitrocellulose at the required line position (Control Line = 43.8 mm from upper wick end of base card). Streptavidin was diluted to 1 mg/mL in PBS, 0.5% (v/v) green dye and dispensed (0.5 µL/strip) onto the nitrocellulose at the required line position as shown in Figure 2.2.8.1 (Test Line = 54.2 mm, from upper wick end of base card). Both line positions were marked by pencil for guidance, ensuring minimal pressure on the nitrocellulose. Following air-drying for ~5 minutes, the biotinylated antibody was diluted to 0.8 mg/mL in PBS and dispensed (0.5 µL/strip) onto the green streptavidin spot. The spotted strips were stored overnight at room temperature in sealed foil pouches with 2 MiniPax® compact desiccant packets before final assembly, cutting and housing in MICT® cassette casings (see Figure 2.2.11.2).

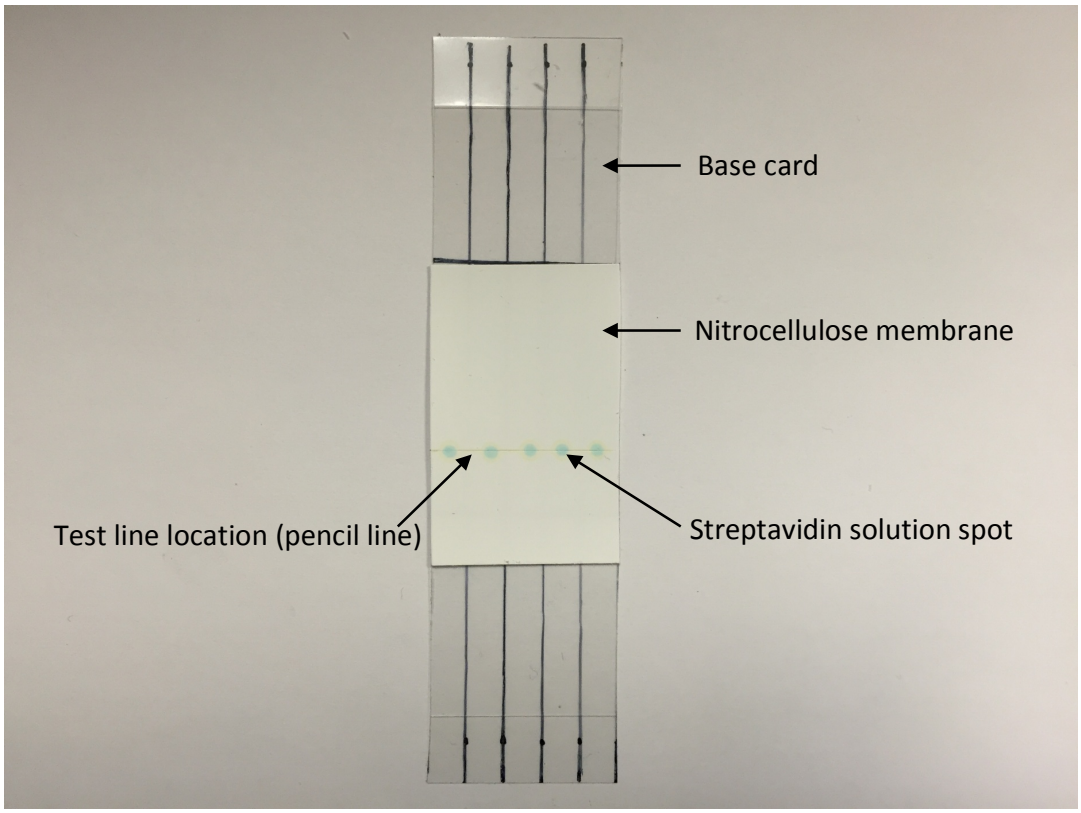



**Figure 2.2.8.1:** Streptavidin solution spotted on nitrocellulose at the test line position.

### 2.2.10 Lateral flow test strip – antibody striping

The base card, nitrocellulose membrane, streptavidin and antibodies were prepared as described for section 2.2.7. A Linomat 5 (CAMAG) instrument was used to stripe the Control and Test Line at the required positions. The Linomat 5 was programmed to dispense 0.1 µL antibody solution per 1 mm at a rate of 500 nL/second. The dispensed volume was equivalent to spotting 0.5 µL per 5 mm strip. The strips were stored overnight at room temperature in sealed foil pouches with 2 desiccants before final assembly, cutting and housing in MICT cassette casings.

### 2.2.11 Lateral flow test strip – device assembly

Sample, conjugate and upper wick pads (Ahlstrom) were supplied in standard sheets of 300 mm width. The desired length of each component (Table 2.2.10.1) was measured and cut using a scissors. Sample pad strips were treated with sample pad buffer by soaking in solution, turning once after 2-3 minutes. The sample pad buffer was formulated to aid in neutralisation of sample pH upon loading onto the assembled test strip. The wetted strips were left to air-dry overnight before storing at room temperature in a sealed plastic bag with 2 desiccants. The test strip assembly was completed as shown in Figures 2.2.11.1 and 2.2.11.2.

**Table 2.2.11.1:** Standard test strip component measurements:

| Component | Length [mm] |
|---|---|
| Sample Pad (SP) | 25.0 |
| Conjugate Pad (CP) | 10.0 |
| Upper Wick (UW) | 25.0 |
| Nitrocellulose (NC) | 39.5 |
| Lamination Tape | 45 .0 |


**Figure 2.2.11.1:** Lateral flow test strip component measurements for assembly.

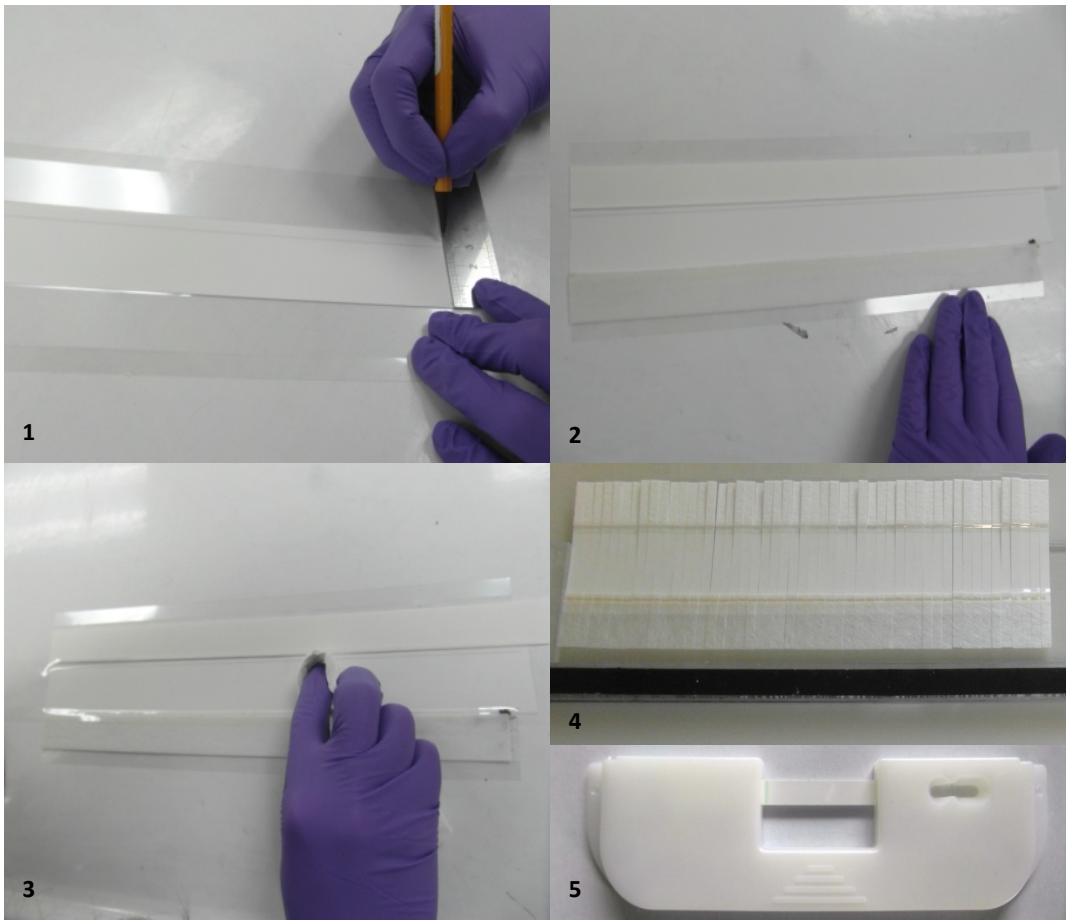



**Figure 2.2.11.2:** Lateral flow test strip assembly. **1**: Overlay position of upper wick and sample pad is measured on NC. **2**: Sample, conjugate and upper wick pads fixed into position. **3**: Lamination of test strips and removal of air bubbles by hand rolling. **4**: Cutting of test card into 5 mm strips. **5**: Closure of test strips into MICT cassettes.

### 2.2.12 Lateral flow test strip – test procedure

All assay components were removed from storage (-20 or 4°C) and allowed to equilibrate to room temperature prior to use. The TAP-SPMP test conjugate and rabbit IgG or chicken IgY-SPMP control conjugates were sonicated and diluted separately in conjugate dilution buffer before pooling and addition of PBS or urine sample to a 1/9 final dilution. For example, to analyse 5 test strips, 480 µL of PBS or urine sample was added to 60 µL pooled SPMP conjugate. PBS was used in place of urine matrix throughout the test optimisation. Pooled conjugate stock consisted of 1

part control conjugate, 1 part test conjugate and 1 part conjugate dilution buffer. The sample/conjugate mixture was loaded by pipette (100 µL/strip) onto the test strip sample pad and incubated for 45 minutes at room temperature before reading in the MICT® instrument. The sample loading was staggered by ~1 minute intervals between test replicates to allow for the time taken to complete MICT® reading.

### 2.2.13 Clinical utility study of TAP – sample collection protocol

Ethical approval was granted by the research ethics committees of the Adelaide and Meath National Children's Hospital (AMNCH), Tallaght, Co. Dublin and by DCU for the collection, storage and analysis of urinary samples at DCU. Patients presenting with acute abdominal pain as a result of suspected acute pancreatitis (AP) were included in this study. Diagnosis of mild, moderate and severe AP was made utilising three different criteria – clinical symptoms observed, Imrie classification score and a C-reactive protein (CRP) level rise of >75 mg/L within two days or a CRP >150 mg/L on Day 2, following presentation to the Emergency Department. CRP was measured in patient plasma. The course of patient treatment was altered according to observations derived from all diagnostic criteria. Urine samples were collected from patients upon admission to the hospital ward and at subsequent time-points of 12, 24, 48 72, 96 and 120 hours, where possible. Individual identifier numbers were assigned to each patient with the patient's sex and age indicated on the label of each collection sample. No other personal information was provided to protect the patient's identity. Patient outcomes were unknown until sample data was provided to AMNCH. Patients with a previous history of chronic pancreatitis and aged <18 years of age were to be excluded from this study. Samples were stored at -20°C as soon as possible after collection and were thawed overnight at +4°C prior to analysis using the competitive ELISA and lateral flow prototypes.

# Chapter 3

# Development of a Monoclonal Anti-TAP ELISA

## 3.1 INTRODUCTION

The advent of the radio-immunoassay (RIA) for detection of antigens or analytes of interest by Yalow and Berson in 1960 was met with instant acclaim from researchers and clinicians alike. However, the safety and waste disposal concerns associated with this detection method encouraged the development and introduction of alternative immunoassay techniques that utilised enzyme-labeled antibodies or antigens for detection of other antibodies or antigens. These enzyme labels interact with substrates to generate coloured, luminescent or fluorescent products that can be measured and quantified. From this work, the enzyme-linked immunosorbent assay (ELISA) was spawned to create a method whose unparalleled and far-reaching impact on scientific research and the diagnostics and medical industries are still being witnessed today (Lequin, 2005). ELISAs are generally highly sensitive and specific for the antigen of interest with wide-ranging detection methodologies. They can be produced at a low-cost and only small amounts of reagents are required. They are flexible and easy to reproduce, thus allowing for adaption from manual to automated high-throughput use (O'Kennedy *et al.*, 1990).

ELISA methods include multiple test formats with two main classification groups – competitive and non-competitive. Competitive immunoassays include formats where enzyme-labeled antigen and unlabeled 'free' antigen in a test sample compete for binding to an antibody immobilised onto a solid phase i.e. the microtitre plate well, or enzyme-labeled antibody and unlabeled antibody compete for binding to an immobilised antigen. The product formed in these assays is inversely proportional to the target antigen/antibody concentration, i.e. the higher the target concentration within the test sample, the lower the amount of enzyme activity detected. In the non-competitive immunoassay format, immobilised antibody binds to antigen in the test sample before a second enzyme-labeled antibody binds the captured antigen at an alternative site. This is also called a sandwich immunoassay and uses both capture and detection antibodies. Furthermore, binding of immobilised antigen by an antibody that is subsequently bound by an enzyme-labeled secondary antibody specific to this antibody is also possible. The resulting enzyme activity of these assays is directly proportional to the target concentration

(O'Kennedy *et al*., 1990; Wild 2006). Competitive assays are often used for the detection of small molecular weight analytes that cannot be detected at two individual sites in a sandwich assay format. The TAP antigen was too small for a non-competitive assay and consequently, a competitive assay format was selected for development of an ELISA prototype (Figure 3.1.1). This format entailed the conjugation of the TAP analyte to a horseradish peroxidase (HRP) enzyme and the coating of the anti-TAP capture antibody to the solid phase by the passive adsorption method. This immobilisation technique is still not fully understood but is thought to involve both charge and hydrophobic interactions. The charge of the coating antibody can be controlled by the pH of the buffer that is used in the coating process. A carbonate buffer (0.05M, pH 9.6) is routinely used for this process together with sufficient temperature control and incubation time to allow for saturation of the solid phase. Any remaining sites on the solid phase that have not been coated are traditionally blocked with inexpensive proteins such as bovine serum albumin to prevent the possibility of non-specific binding of assay components such as the enzyme conjugate to the plastic surface (Deshpande, 1996).

The optimisation of assay conditions including incubation time and the preparation and selection of the optimal buffer formulations and reagents is an essential part of the development process that must also be carefully considered. Buffer solutions provide a controlled aqueous environment (usually by maintaining a constant pH value) for antibody-antigen binding to occur. Phosphate-buffered saline (PBS) (0.15M, pH 7.4) and tris-buffered saline (TBS) (0.05M, pH 7.5) solutions contain buffer salts that help maintain the pH range of reactions. Additional buffer reagents can provide specific functions that warrant their inclusion in the formulation. For example, Tween20 detergent in assay wash buffers can prevent non-specific binding and support homogeneous distribution of the protein in conjugate dilution buffers. Use of 3, 3', 5, 5'-tetramethylbenzidene (TMB) substrate is recommended for assays using horseradish peroxidase (HRP) conjugates due to the rapid development time and improved assay sensitivity versus other substrate alternatives. However, some reagents can bring negative effects, for example, sodium azide used as a preservative to inhibit bacterial growth can also impede HRP activity (Deshpande, 1996).

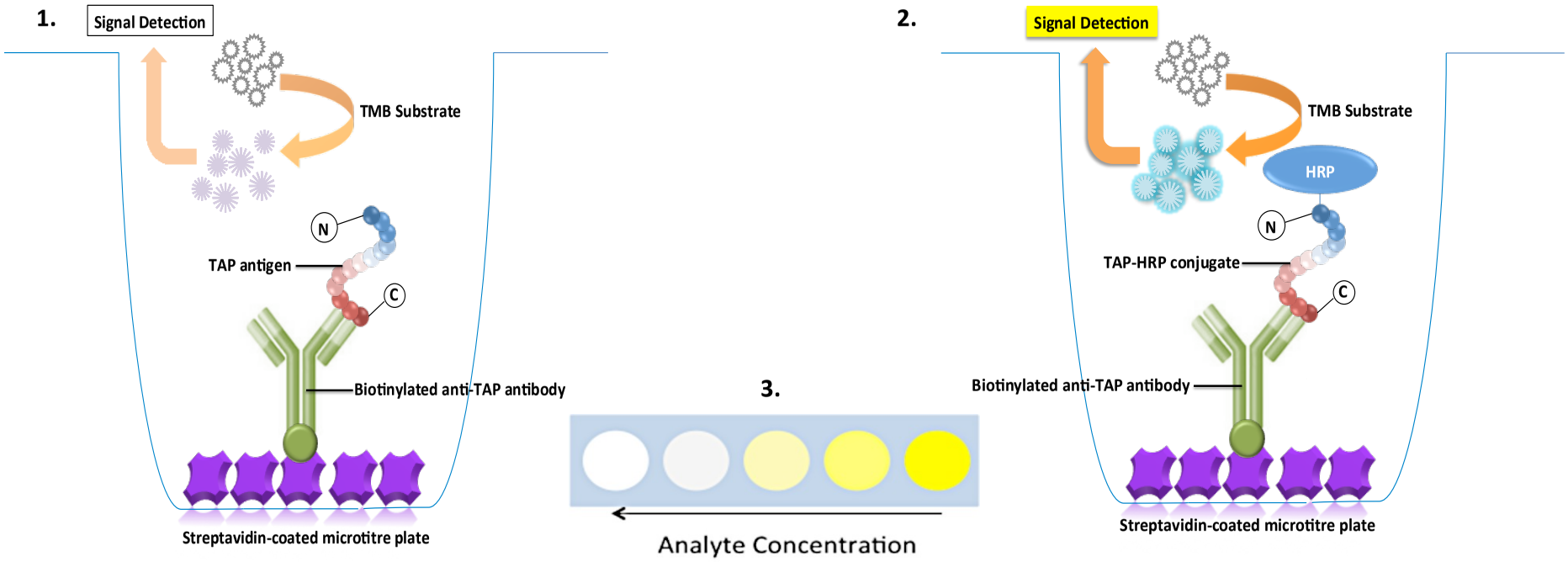



**Figure 3.1.1** Competitive anti-TAP monoclonal ELISA format. Biotinylated anti-TAP capture antibody is incubated with streptavidin-coated microtitre plate wells. Unbound capture antibody is removed by washing. A 1:1 mixture of "neat" (no dilution) urine sample and diluted TAP-HRP conjugate were loaded into the wells. Both "free" and conjugated TAP analyte compete for binding to the capture antibody. Unbound analyte and conjugate are removed by washing and TMB substrate is added to the assay wells. The HRP conjugate oxidises the TMB substrate into a coloured product before the reaction is quenched by addition of sulphuric acid. The absorbance of the coloured product is detectable by spectrophotometric methods. 1: TAP Negative urine sample testing - TAP-HRP conjugate binds to capture antibody when no free antigen is present. 2: TAP Positive urine sample – TAP analyte binds to capture antibody in place of HRP-conjugated analyte. 3: The colour development from the binding reaction is inversely proportional to the analyte concentration present in the wells, i.e. lower colour intensity, more analyte present and, highest intensity for analyte-free sample.

## 3.2 AIMS OF THIS CHAPTER

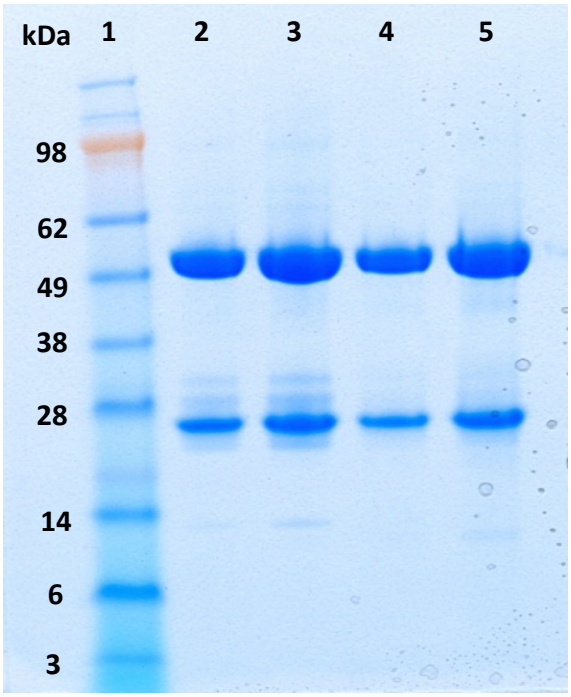
The development of a competitive ELISA prototype for the detection of urinary TAP will be described.

The development of this test will involve the characterisation of the monoclonal anti-TAP antibody, conjugation of the TAP antigen to HRP, optimisation of assay conditions, including plate coating saturation and selection of standard calibration curve and the preparation of internal control samples that can be used to monitor assay performance and establish assay robustness.

## 3.3 RESULTS

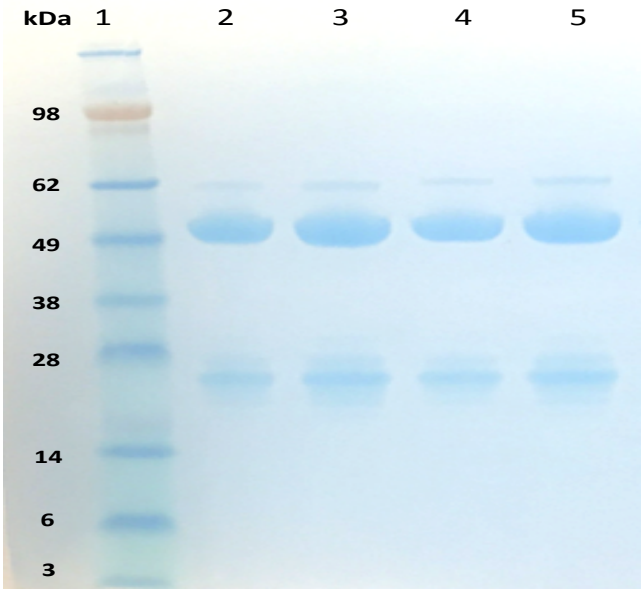
### 3.3.1 Characterisation of rabbit monoclonal *14-8* anti-TAP antibody

The purified rabbit monoclonal anti-TAP IgG antibody was produced by AbCam® from hybridoma supernatant using protein A affinity chromatography. Three individual purified antibody stocks were obtained and assessed by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (see section 2.2.1).




| Lane: | Sample ID | Sample Type |
|---|---|---|
| **1** | MARKER | MARKER |
| **2** | B_4168 (5 µg) | Capture mAb, 1.09 mg/mL |
| **3** | B_4168 (10 µg) | Capture mAb, 1.09 mg/mL |
| **4** | B_4346 (5 µg) | Capture mAb, 0.93 mg/mL |
| **5** | B_4346 (10 µg) | Capture mAb, 0.93 mg/mL |

**Figure 3.3.1.1** SDS-PAGE analysis of rabbit monoclonal anti-TAP IgG antibodies (lots B_4168 and B_4346). The heavy chain domain was observed at ~50kDa and the light chain domain at ~25kDa for both lots of antibody. The heavy and light chains were both consistent with the expected molecular weights of an IgG antibody. Two different concentrations (5 and 10 µg) were run to ensure protein band visibility. As expected, slightly larger bands were observed for the 10 µg samples, nonetheless, 5 µg was sufficient for characterisation. Both lots of antibody were comparable. Additional contaminating bands were observed around the light chain for lot B_4168 (strongest in Lane 2). It was possible that these bands were due to minor contaminants from the supernatant or insufficient denaturation of the antibody. This was feasible as the bands were clearer at the higher concentration of 10 µg. Nonetheless, lot B_4168 was selected for the ELISA prototype development as this had the largest stock availability.




| Lane: | Sample ID | Sample Type |
|---|---|---|
| **1** | MARKER | MARKER |
| **2** | B_37042 (5 µg) | 0.52 mg/mL. EKF 5X Reducing Buffer |
| **3** | B_37042 (8.3 µg) | 0.52 mg/mL. EKF 5X Reducing Buffer |
| **4** | B_37042 (5 µg) | 0.52 mg/mL. 5X Non-Reducing Buffer with fresh 2-mercaptoethanol |
| **5** | B_37042 (8.3 µg) | 0.52 mg/mL. 5X Non-Reducing Buffer with fresh 2-mercaptoethanol |

**Figure 3.3.1.2** SDS-PAGE analysis of rabbit monoclonal anti-TAP IgG antibody (lot B_37042). Heavy and light chain domains were observed at ~50 and ~25kDa, respectively. This was consistent with previous antibody lots (Figure 3.3.1.1). The 8.3 µg sample was run in place of a 10 µg sample due to the low antibody stock concentration (0.52 mg/mL). Concentration by ultracentifugation was not attempted at this time to limit use of antibody stock for future use, if required. Reducing buffer referred to a formulation that included 2-mercaptoethanol and non-reducing buffer contained no 2-mercaptoethanol until addition immediately before use. No difference was observed for protein bands resulting from these buffers, and only very minor contaminating bands were observed when compared with lot B_4168 (Figure 3.3.1.1). The non-reducing buffer formulation appeared to improve the appearance of these additional bands, suggesting that the contaminating bands were due to insufficient denaturation of the antibodies. Extended boiling of samples may have improved this further.

No characterisation of the TAP antigen by, for example, Western blot, was undertaken due to the small size of the peptide (~770 Da) and the limited stock availability for this project. In addition, the exact structure of the synthesized peptide was known and controlled by instructions delivered to Bachem®.

### 3.3.2 Saturation testing for monoclonal *14-8* anti-TAP antibody coated by passive adsorption to the microtitre assay plate and titration of TAP-HRP conjugate

The initial competitive ELISA prototype was prepared by coating the monoclonal anti-TAP antibody onto the microtitre plate surface via passive adsorption. Passive adsorption involves the immobilisation of the capture antibody onto the microtitre plate through van der Waals forces developed during incubation over time (Deshpande, 1996). The TAP antigen was conjugated to HRP using a commercial kit from Innova Biosciences (section 2.2.3) for use in colourimetric signal development in the presence of TMB susbstrate. This kit binds primary amine and lysine groups on the peptide and HRP molecules. The TAP-HRP conjugate competes for binding to the immobilised antibody with TAP antigen calibrator standards prepared at predetermined concentrations or, “free” TAP antigen present in urine samples. Various concentrations (2, 4, 6 and 8 µg/mL) of anti-TAP antibody were coated onto microtitre plates (section 2.2.2) and their saturation points compared i.e. where an increase in immobilised antibody concentration does not lead to enhanced signal development.

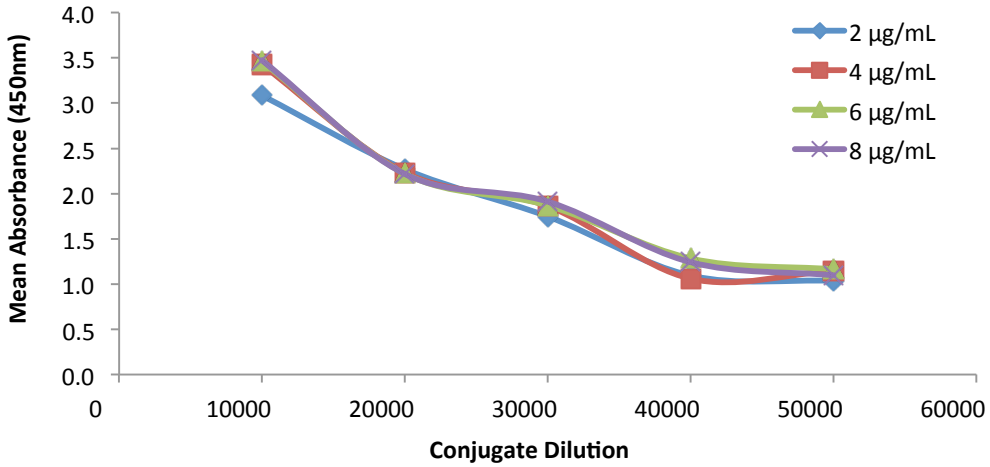



**Figure 3.3.2.1** Saturation testing of monoclonal anti-TAP antibody. No notable difference was observed between coating concentrations, and thus, the 2 µg/mL concentration was selected for further use to minimise antibody use and reduce costs for future manufacture.

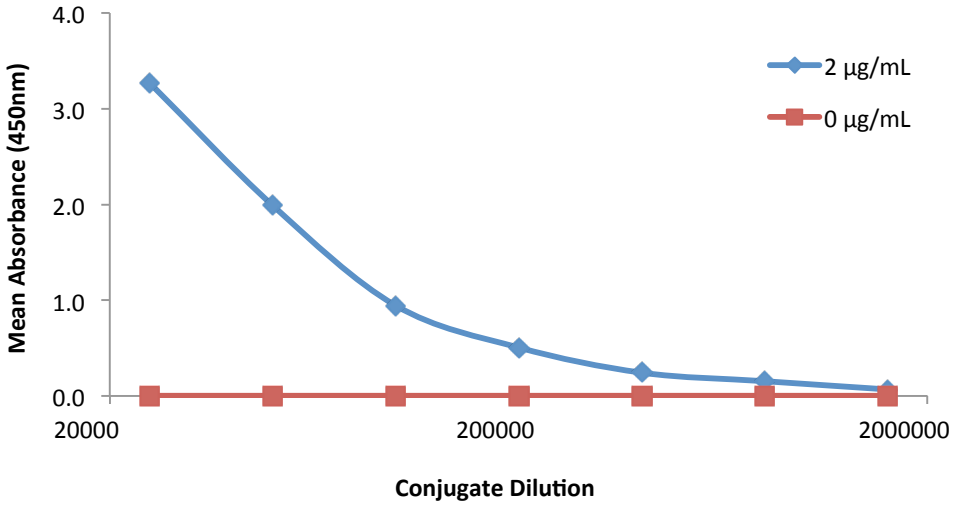



**Figure 3.3.2.2** TAP-HRP conjugate titration using microtitre plates coated with 2 µg/mL anti-TAP mAb. Increasing conjugate dilution resulted in a reduction of the assay signal, with dilutions of 1/25,000 up to 1/1,600,000 tested. Microtitre plate wells coated with buffer only (0 µg/mL) were used as a control to demonstrate that there was no non-specific binding of the TAP-HRP conjugate to the plastic microtitre wells in the absence of immobilised anti-TAP antibody, a highly important characteristic of any robust ELISA.

### 3.3.3 Standard curve generation for monoclonal anti-TAP ELISA prototype (coated by passive adsorption)

To assess the performance of this ELISA prototype, a standard calibration curve was prepared using the TAP antigen diluted into sample diluent (proprietary) as follows:

| Calibrator (nmol/L) | Calibrator Volume (µL) | Diluent Volume (µL) |
|---|---|---|
| 200 (A) | 20 **(i)*** | 980 |
| 100 (B) | 200 (A) | 200 |
| 50 (C) | 200 (B) | 200 |
| 25 (D) | 200 (C) | 200 |
| 12.5 (E) | 200 (D) | 200 |
| 6.3 (F) | 200 (E) | 200 |
| 3.1 (G) | 200 (F) | 200 |
| 0 (H) | 0 | 200 |

***(i)** Intermediate dilution of TAP antigen: $^{1}/_{100}$ = 1 µL (TAP antigen) + 99 µL (diluent). (A) – (H) refers to calibrator names and are used to track dilution preparation. For example, calibrator (B) was prepared at a concentration of 100 nmol/L by the addition of 200 µL of calibrator (A) to 200 µL of sample diluent.

The assay was performed as described in section 2.2.4 using conjugate dilutions ranging from 1/10,000 to 1/50,000.

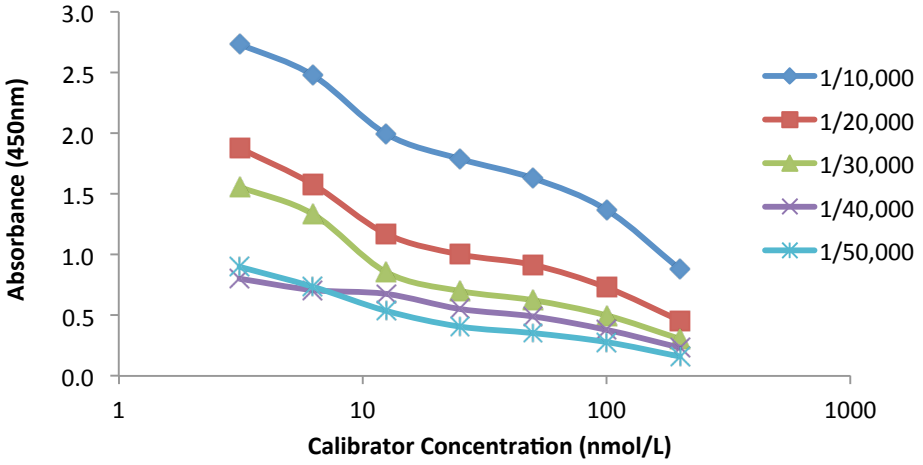



**Figure 3.3.3.1** Standard calibration curve test using various TAP-HRP conjugate dilutions and 2 µg/mL anti-TAP antibody-coated microtitre plates. The calibrator standard range was 0 - 200 nmol/L. This prototype appeared to lack the ability to distinguish between TAP standards while also limiting the amount of conjugate used, i.e. maintaining a higher dilution and thus reducing the stock material requirements. Additionally, producing an acceptable signal that could withstand a certain drop-off over a period of time, ensuring reproducibility of the standard curve was of high importance. This allowance lengthens the shelf life of any product, as a drop-off in activity for biological material is inevitable. Internal observations at EKF Diagnostics have pointed to an OD of approximately 2.5 as a satisfactory target for the highest level (top standard) of a calibration curve. This is reversed for a competitive ELISA where the blank or zero standard should provide the highest signal response. The zero standard OD absorbance varied from 3.1 to 1.0 for conjugate dilutions of 1/10,000 to 1/50,000, respectively.

### 3.3.4 Saturation testing for biotinylated monoclonal *14-8* anti-TAP antibody when used with a streptavidin-coated microtitre assay plate

Due to the perceived lack of sensitivity for the monoclonal anti-TAP antibody and the limited time available to generate an alternative antibody, it was decided that biotinylation of the current anti-TAP antibody using a commercial kit from Innova Biosciences, was the best option (section 2.2.5). The biotin-streptavidin system is a highly specific, non-covalent interaction that has been employed in applications of biotechnology, including immunoassays. As a small molecule, biotin (vitamin H) usually does not alter the biological properties of macromolecule complexes upon its introduction. Biotinylation of antibodies generally involves the irreversible labeling of primary amine groups, such as lysine, using an *N*-hydroxysuccinimide ester attached to a biotin molecule by a long chain which ensures that the biotin is more exposed for binding to the streptavidin protein. Streptavidin is a protein isolated from the bacterium *Streptomyces avidinii* that is used in place of the traditional avidin (from egg-white) due to its lower non-specific binding capacity. Streptavidin contains four biotin-binding sites per molecule, adding to the highly specific interaction that is $10^3$-$10^6$ times greater than the interaction of ligands with their specific antibodies. Through this increased specificity, coating of the anti-TAP antibody onto the microtitre plate and assay sensitivity are improved (Diamandis & Christopoulos, 1991; Ylikotila *et al*., 2009). The biotinylated anti-TAP antibody was incubated with a streptavidin-coated microtitre plate (Fisher Scientific) and the assay performed as described by section 2.2.6.

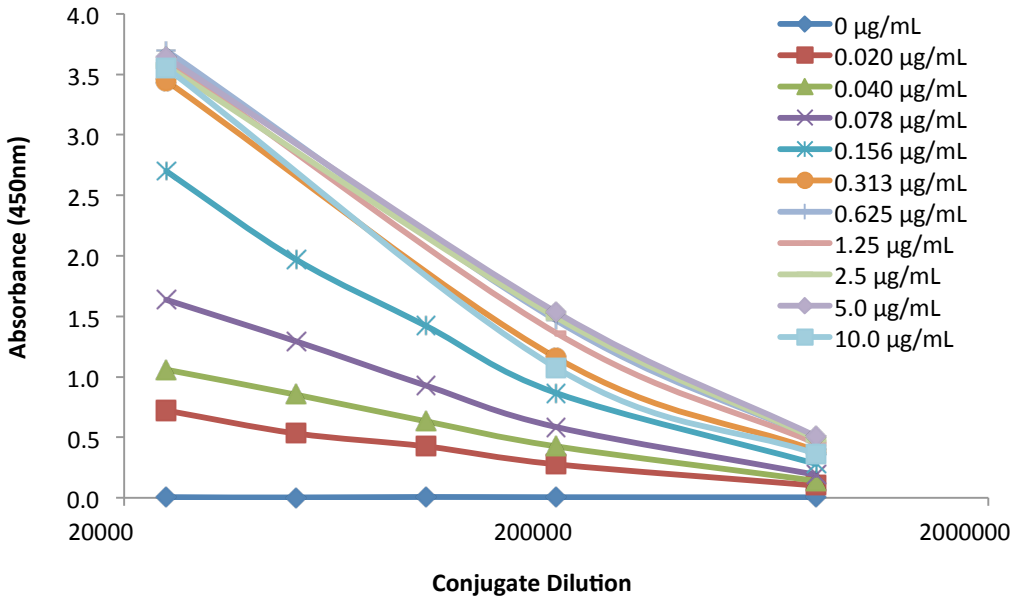



**Figure 3.3.4.1** Checkerboard testing for biotinylated monoclonal anti-TAP antibody incubated with streptavidin-coated microtitre assay plate. Conjugate dilutions of 1/25,000 to 1/800,000 were tested with biotinylated anti-TAP antibody concentrations ranging from 0 – 10.0 µg/mL. The 0.3125 µg/mL concentration was selected for use in this prototype due to similar performance when compared to the higher concentrations of 0.625 – 10.0 µg/mL. The zero µg/mL microtitre plate wells demonstrated that there was no non-specific binding of the TAP-HRP conjugate to the streptavidin-coated microtitre wells in the absence of biotinylated anti-TAP antibody. Increasing conjugate dilution resulted in a reduction of the assay signal towards zero in all other conditions tested, as expected.

### 3.3.5 Comparison of streptavidin / biotinylated anti-TAP ELISA and passive adsorption anti-TAP ELISA

To compare the different ELISA formats; standard calibration curves were prepared for both assays with prepared calibrators of 0, 3.1, 6.3, 12.5, 25, 50, 100, 200, 500 and 1000 nmol/L. Assays were performed as per sections 3.3.3 and 3.3.4, respectively. The calibration curves were prepared as follows:

| Calibrator (nmol/L) | Calibrator Volume (µL) | Diluent Volume (µL) |
|---|---|---|
| 1000 (A) | 50 **(i)*** | 450 |
| 500 (B) | 300 (A) | 300 |
| 200 (C) | 160 (B) | 240 |
| 100 (D) | 300 (C) | 300 |
| 50 (E) | 300 (D) | 300 |
| 25 (F) | 300 (E) | 300 |
| 12.5 (G) | 300 (F) | 300 |
| 6.3 (H) | 300 (G) | 300 |
| 3.1 (I) | 300 (H) | 300 |
| 0 (J) | 0 | 300 |

***(i)** Intermediate dilution of TAP antigen: $^{1}/_{100}$ = 1 µL (TAP antigen) + 99 µL (diluent). (A) – (J) refers to calibrator names and are used to track dilution preparation. For example, calibrator (B) was prepared at a concentration of 500 nmol/L by the addition of 300 µL of calibrator (A) to 300 µL of sample diluent.

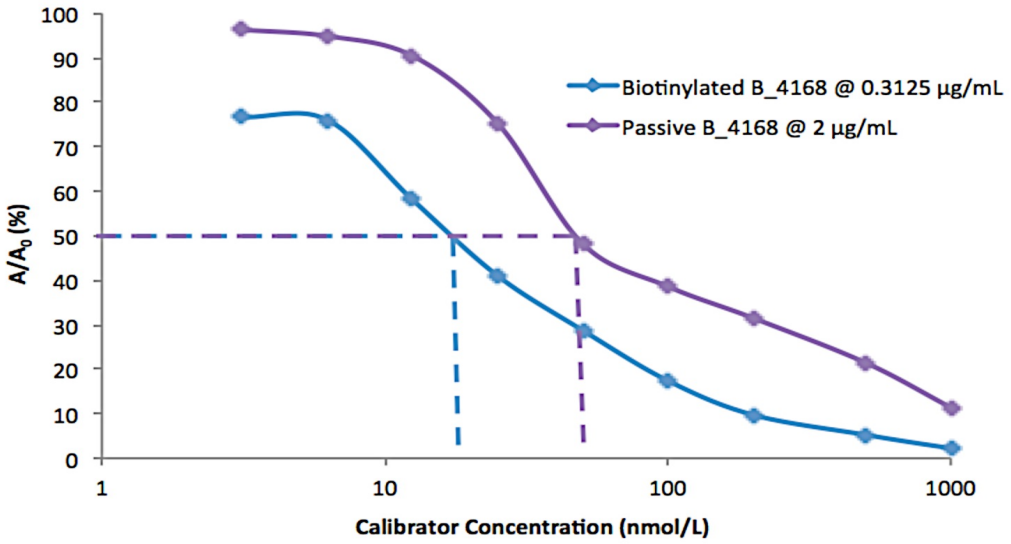



**Figure 3.3.5.1** Comparison of calibration curves for streptavidin/biotinylated anti-TAP ELISA and passive adsorption anti-TAP ELISA formats. The $A/A_0$ values were calculated as calibrator mean OD response divided by zero calibrator mean OD response times 100 to express as a percentage. This calculation normalised the absorbance or OD response for each calibrator (A-I) against the maximum OD response for the zero calibrator (J). B_4168 refers to the lot number of the anti-TAP antibody used. TAP-HRP conjugate was utilised at a 1/50,000 dilution. The half maximal inhibitory concentrations ($IC_{50}$) were 23.3 nmol/L (dashed blue line) and 44.7 nmol/L (dashed purple line) for the streptavidin/biotinylated anti-TAP ELISA and the passive adsorption anti-TAP ELISA curves, respectively (Findlay & Dillard, 2007). The streptavidin/biotinylated anti-TAP ELISA demonstrated an ability to offer greater discrimination between calibrator responses with a more even distribution between calibrator responses overall and importantly, at the lower end of the curve between calibrators 6.3, 12.5 and 25 nmol/L. The passive adsorption anti-TAP ELISA offered greater discrimination at the upper end of the curve, but this was of less value based on TAP concentrations described in the literature for patients of acute pancreatitis (Neoptolemos *et al*., 2001). These results also indicated that the upper limit of the measuring range was close to 1000 nmol/L. Further optimisation could potentially improve the sensitivity of the streptavidin/biotinylated anti-TAP ELISA at this lower limit of the measuring range.

### 3.3.6 Comparison of three monoclonal anti-TAP IgG antibody lots in the streptavidin / biotinylated anti-TAP ELISA

Three individual lots of anti-TAP antibody were received for this project from AbCam International. Each lot of antibody was concentrated to ~1 mg/mL as per section 2.2.7.1, with concentrations confirmed by use of the Nanodrop spectrophotometer ($A_{280nm}$). The ranges of the calibration curves were extended (0 – 1600 nmol/L) in order to determine the entire measurement capacity for the prototype. Calibration curves were prepared for testing with each lot as follows:

| Calibrator (nmol/L) | Calibrator Volume (µL) | Diluent Volume (µL) |
|---|---|---|
| 1600 (A) | 128 **(i)*** | 672 |
| 800 (B) | 300 (A) | 300 |
| 400 (C) | 300 (B) | 300 |
| 200 (D) | 300 (C) | 300 |
| 100 (E) | 300 (D) | 300 |
| 50 (F) | 300 (E) | 300 |
| 25 (G) | 300 (F) | 300 |
| 12.5 (H) | 300 (G) | 300 |
| 6.3 (I) | 300 (H) | 300 |
| 3.1 (J) | 300 (I) | 300 |
| 1.56 (K) | 300 (J) | 300 |
| 0.78 (L) | 300 (K) | 300 |
| 0.39 (M) | 300 (L) | 300 |
| 0 (N) | 0 | 300 |

***(i)** Intermediate dilution of TAP antigen: $^{1}/_{100}$ = 2 µL (TAP antigen) + 198 µL (diluent). (A) – (M) refers to calibrator names and are used to track dilution preparation. For example, calibrator (B) was prepared at a concentration of 800 nmol/L by the addition of 300 µL of calibrator (A) to 300 µL of sample diluent.

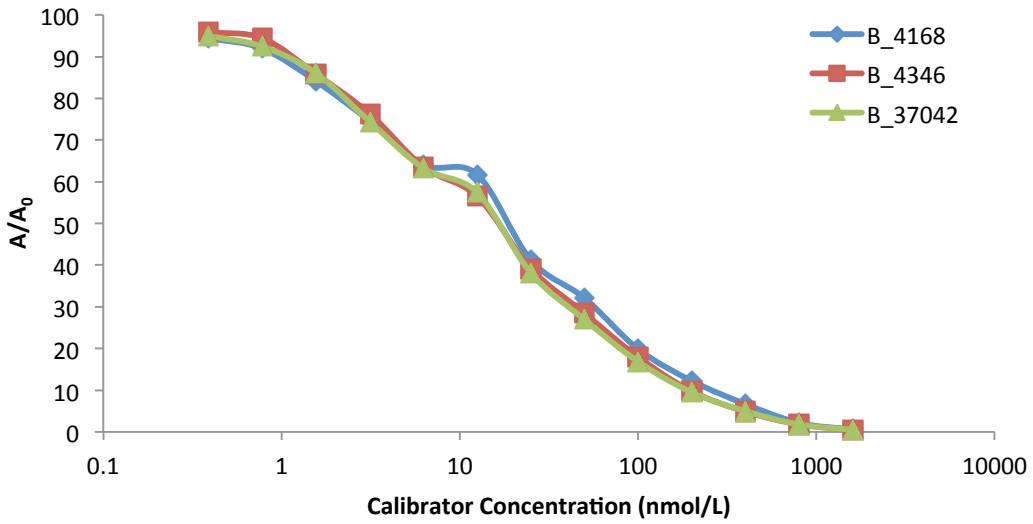



**Figure 3.3.6.1** Comparison of three separate lots of biotinylated anti-TAP antibody. Performance of all three biotinylated anti-TAP antibodies was comparable, demonstrating reproducibility of the method and ensuring that individual batches of prototype could be produced with uniform performance across all batches should any potential commercial use arise. Extension of the calibrator curve at both the lower and upper ends allowed for a clearer definition of the lower and upper limitations to the measuring range. The calibrator dilutions of 0.39, 0.78 and 1.56 nmol/L demonstrated that the curve did not plateau at the 3.1 nmol/L calibrator as the streptavidin/biotinylated anti-TAP ELISA curve for lot B_4168 appeared to indicate in Figure 3.3.5.1. Also, the OD absorbance (<0.01) for the 1600 nmol/L calibrator sample was comparable across all curves and thus, the upper limit of the calibration curve was also determined. With the extended measuring range of the calibration curve defined, optimisation could be pursued.

### 3.3.7 Freeze-thaw stability study of TAP antigen in various matrices

A stability study was designed to determine any effects on the peptide antigen when stored at -20°C and thawed at room temperature for use. This study investigated the effect of the freeze-thaw action on peptide concentration when spiked into three different matrices – $dH_20$, urine and stabilised urine. Stabilised urine had 7.5mM EDTA buffer at a 1/5 dilution (1 part buffer to 4 parts of urine) added. EDTA is a known chelating agent used to bind metal ions required for catalytic activity of metallopeptidases, which could potentially degrade the TAP antigen.

Analyte recovery for initial freeze-thaw cycle in each matrix was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Freeze-Thaw Cycle 1 Concentration}}{\text{Freeze-Thaw Cycle 1 Concentration}} \times 100$$

Analyte recovery in each matrix following initial freeze-thaw cycle (FTx1) was calculated using the following formula:

$$\text{Recovery (\%)} = \frac{\text{Observed Analyte Concentration}}{\text{Freeze-Thaw Cycle 1 Concentration}} \times 100$$

**Table 3.3.7.1** Effect of freeze-thawing on peptide concentration in various matrices:

| Matrix | Sample | FT Cycle | Conc. (nmol/L) | %Rec vs FTx1 |
|---|---|---|---|---|
| $dH_2O$ | 1 | 1 | 289.4 | 100 |
| | | 2 | 294.6 | 102 |
| | | 3 | 315.4 | 109 |
| | 2 | 1 | 161.9 | 100 |
| | | 2 | 150.5 | 93 |
| | | 3 | 178.7 | 110 |
| | 3 | 1 | 86.4 | 100 |
| | | 2 | 82.2 | 95 |
| | | 3 | 95.6 | 111 |
| | 4 | 1 | 23.1 | 100 |
| | | 2 | 23.8 | 103 |
| | | 3 | 25.3 | 110 |
| Urine | 1 | 1 | 249.1 | 100 |
| | | 2 | 227.5 | 91 |
| | | 3 | 240.9 | 97 |
| | 2 | 1 | 145.4 | 100 |
| | | 2 | 138.8 | 95 |
| | | 3 | 143.1 | 98 |
| | 3 | 1 | 74.3 | 100 |
| | | 2 | 75.1 | 101 |
| | | 3 | 71.8 | 97 |
| | 4 | 1 | 19.6 | 100 |
| | | 2 | 18.0 | 92 |
| | | 3 | 20.2 | 103 |
| Urine Stabilised | 1 | 1 | 236.4 | 100 |
| | | 2 | 257.5 | 109 |
| | | 3 | 252.3 | 107 |
| | 2 | 1 | 150.7 | 100 |
| | | 2 | 138.4 | 92 |
| | | 3 | 123.4 | 82 |
| | 3 | 1 | 66.5 | 100 |
| | | 2 | 72.8 | 109 |
| | | 3 | 68.7 | 103 |
| | 4 | 1 | 21.1 | 100 |
| | | 2 | 21.4 | 101 |
| | | 3 | 22.2 | 105 |

Samples 1 – 4 were prepared by spiking TAP antigen into each matrix at approximate concentrations of 200, 100, 50 and 25 nmol/L. Recovery of initial peptide concentration was acceptable (100 ± 10%) across all matrices tested, with the exception of FTx3 for sample 2 in stabilised urine. The higher recovery at FTx3 for the samples stored in $dH_20$ was probably due to assay variation. This demonstrated that storage of the peptide in $dH_20$ was suitable for reconstitution of stocks. Peptide concentration in urine was not affected following up to 3 freeze-thaw cycles and no difference in peptide recovery was observed upon addition of stabilisation buffer to urine. This indicates that urine samples can be collected and stored at -20°C without the need for an additional stabilising step.

### 3.3.8 Optimisation of standard calibration curve for streptavidin / biotinylated anti-TAP ELISA

To finalise the calibration curve for use in performance testing of the ELISA prototype, an extended curve (0, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200, 400, 800, 1600 nmol/L) was tested before selection of the optimal standards to be included in the calibration curve. Each standard was tested in triplicate (n=3) and additionally, Quality Control (QC) urine samples were tested in quadruplicate (n=4). The QC samples were prepared by spiking TAP antigen into pooled bulk urine sourced from an apparently healthy donor (no known clinical illness) group at theoretical concentrations of 200, 110, 50 and 20 nmol/L for QC samples 1-4, respectively. Further discussion of this sample panel is described in section 3.3.9 below. The variation between replicates or the repeatability to produce an OD response close to the average of all replicates of a standard/sample was calculated to demonstrate the robustness of the calibration curve and evaluate operator error. To define the precision between replicates, the coefficient of variation (CV) was determined using the following formula:

$$\text{Precision} = \text{CV}\ (\%) = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

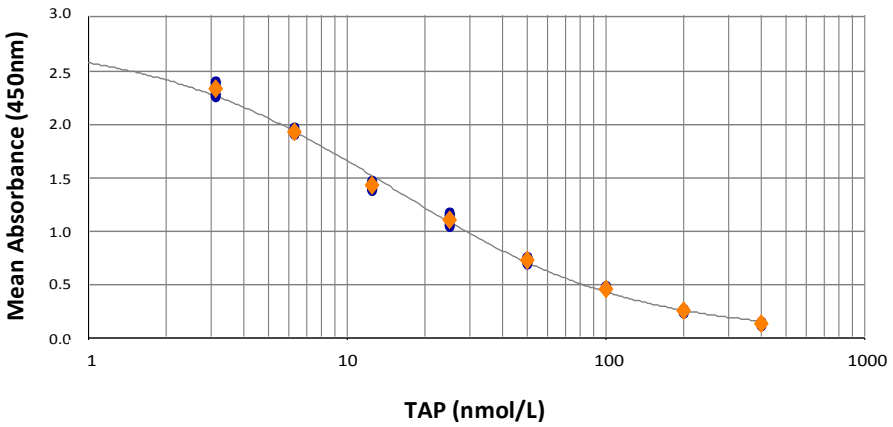
Analyte recovery for calibration curve and QC sample panel was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{\text{Observed Concentration}}{\text{Theoretical Concentration}} \times 100$$

Two separate software programmes were used to plot the standard calibration curve of the streptavidin/biotinylated anti-TAP ELISA. GEN5 MicroPlate Reader software is validated software (US FDA's 21 CFR Part 11 and ISO 9001/ISO 13485 software requirements) provided by Biotek Instruments, Inc. and is used in-house at EKF Diagnostics for ELISA development. This software has security controls that ensure the integrity of all data. MyCurveFit is a free online curve-fitting tool provided by MyAssays Limited that was employed for an independent comparison with the GEN5 software. A four-parameter logistic (4-PL) curve with non-linear regression, originally described by Healy (1972), was employed by both software programmes due to its ability to fit the extremes of a calibration curve better and produce results that are less susceptible to variation compared to alternative methods (Deshpande, 1996). The curve equation utilises four independent parameters which models signal response using the following function:

$$y = d + (a - d) / 1 + (X/c)^{b}$$

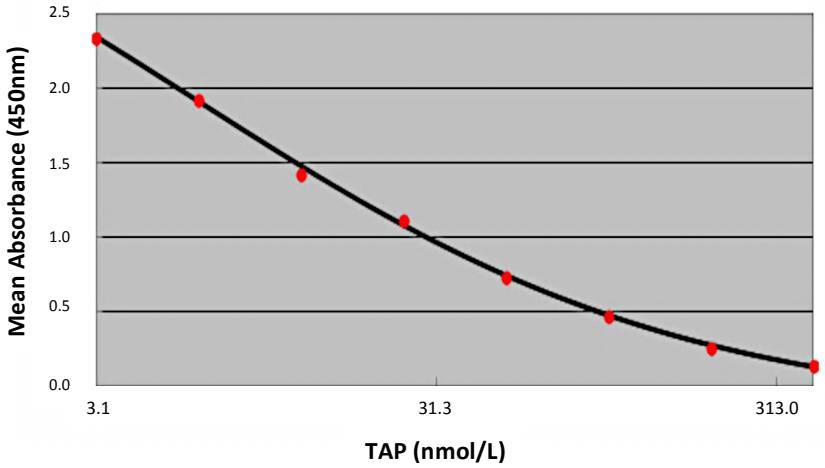
The parameters *a* and *d* refer to responses at zero and infinite calibrator concentration. The parameter *c* refers to the concentration of the calibrator, resulting in a decrease of $A/A_0$ to a value at the midpoint between *a* and *d*, also called $IC_{50}$ value, i.e. where the concentration of an inhibitor (in this case, TAP antigen) causes the OD response to be halved. The parameter *b* determines the steepness of the curve at its centre or any other point. From this, the concentration of any calibrator / sample can be determined using the OD response "X" from the curve equation (Findlay & Dillard, 2007).




| Calibrator (nmol/L) | Mean OD | CV (%) | TAP (nmol/L) | %Recovery |
|---|---|---|---|---|
| 400 | 0.137 | 5 | >420.0 | #VALUE! |
| 200 | 0.256 | 4 | 210.6 | 105 |
| 100 | 0.47 | 2 | 90.5 | 91 |
| 50 | 0.737 | 4 | 47.3 | 95 |
| 25 | 1.115 | 5 | 23.9 | 96 |
| 12.5 | 1.428 | 2 | 14.5 | 116 |
| 6.3 | 1.93 | 1 | 6.3 | 101 |
| 3.1 | 2.336 | 3 | 2.6 | 83 |
| 0 | 2.752 | 3 | <0.2 | #VALUE! |

| Sample | Mean OD | %CV | TAP (nmol/L) | %Recovery |
|---|---|---|---|---|
| QC1 | 0.254 | 4 | 213.3 | 107 |
| QC2 | 0.421 | 2 | 105.6 | 96 |
| QC3 | 0.723 | 2 | 48.6 | 97 |
| QC4 | 1.102 | 3 | 24.4 | 122 |

**Figure 3.3.8.1** GEN5 MicroPlate Reader software generated standard calibration curve for streptavidin/biotinylated anti-TAP ELISA. The 4-PL curve selected to plot the data returned an $R^2$ value = 0.999, showing that the standards fitted closely to the curve regression line. Recovery of TAP concentration for all standards was within 100 ± 20%. Precision testing of the calibration curve over several days would determine the true variability. Recovery of TAP concentration for all QC samples was within 100 ± 10%, with the exception of QC4. Over-recovery of this sample was irrelevant at this time due to the calculation being based on a theoretical concentration. Actual quantified concentration for each QC sample would need to be established over several days of testing.




| TAP Calibrator (nmol/L) | Mean OD | Predicted TAP (nmol/L) | %Recovery |
|---|---|---|---|
| 3.1 | 2.336 | 3.2 | 101 |
| 6.3 | 1.930 | 6.0 | 97 |
| 12.5 | 1.428 | 13.5 | 108 |
| 25 | 1.115 | 23.3 | 93 |
| 50 | 0.737 | 50.3 | 101 |
| 100 | 0.470 | 100.8 | 101 |
| 200 | 0.256 | 213.2 | 107 |
| 400 | 0.137 | 376.7 | 94 |

| Sample | Expected TAP (nmol/L) | Predicted TAP (nmol/L) | %Recovery |
|---|---|---|---|
| **QC1** | 200 | 215.0 | 107 |
| **QC2** | 110 | 117.2 | 107 |
| **QC3** | 50 | 52.0 | 104 |
| **QC4** | 20 | 23.9 | 119 |

**Figure 3.3.8.2** MyCurveFit software generated standard calibration curve for streptavidin/biotinylated anti-TAP ELISA. Mean values from GEN5 data were entered into MyCurveFit.com online account. Recovery of TAP concentration for all calibrators and samples was 100 ± 10%. Slight differences in predicted values were observed compared to GEN5 data, however, these were negligible. The MyCurveFit software, therefore, represented a functional and easy to use tool for plotting calibration curve data in the absence of GEN5 MicroPlate Reader software. As per GEN5, a 4-PL curve fit was selected to plot the data and returned an $R^2$ value = 0.999. This is reflected in the agreement between the expected and predicted concentrations for the calibrators and samples tested.

### 3.3.9 Establishment of urinary TAP quality control sample panel and intra and inter-assay precision for streptavidin / biotinylated anti-TAP ELISA

To establish a set concentration range for each of the QC panel samples and determine the intra and inter-assay precision of the ELISA prototype, five assays were run on five separate days with the calibrator curve tested in duplicate (n=2) and the QC samples tested in triplicate (n=3) on each day. The results were tabulated and analysed using the Analyse-it statistical software. Analyse-it is quality control and method validation tool developed to implement experimental design in line with the Clinical Laboratory Standards Institute (CLSI) guidelines. The CLSI has developed clinical laboratory testing standards based on a consensus among industry, government and healthcare professionals that are routinely implemented in FDA and ISO accredited laboratories around the world as part of their individual quality systems, whether it is sample testing or diagnostic test development laboratories. The CLSI guideline document used for this experiment was *EP5A2: Evaluation of precision performance of quantitative measurement methods; approved guideline*.

A Grubb's (1950) test was performed for each QC sample set to detect any outlier data that deviated from the mean sample value. To do this, the Z value, which is the largest deviation from the sample mean, was calculated as follows:

$$Z = \frac{\text{Mean of dataset} - \text{Individual Measurement}}{\text{Standard Deviation of dataset}}$$

The expected Z value range is defined based on the number of tests performed. For 5 tests, the range is ± 1.71.

| Calibrator (nmol/L) | | Mean | SD | %CV | N | Assay 1 | Assay 2 | Assay 3 | Assay 4 | Assay 5 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Mean OD | | |
| **400** | | 0.120 | 0.015 | 12 | 5 | 0.123 | 0.144 | 0.112 | 0.105 | 0.116 |
| **200** | | 0.227 | 0.018 | 8 | 5 | 0.233 | 0.254 | 0.216 | 0.209 | 0.223 |
| **100** | | 0.410 | 0.038 | 9 | 5 | 0.427 | 0.462 | 0.360 | 0.392 | 0.410 |
| **50** | | 0.703 | 0.040 | 6 | 5 | 0.711 | 0.754 | 0.697 | 0.643 | 0.712 |
| **25** | | 1.092 | 0.054 | 5 | 5 | 1.095 | 1.179 | 1.061 | 1.037 | 1.087 |
| **12.5** | | 1.452 | 0.051 | 4 | 5 | 1.447 | 1.530 | 1.441 | 1.387 | 1.456 |
| **6.3** | | 1.782 | 0.084 | 5 | 5 | 1.787 | 1.856 | 1.861 | 1.750 | 1.657 |
| **3.1** | | 1.909 | 0.101 | 5 | 5 | 2.050 | 1.98 | 1.857 | 1.831 | 1.826 |
| **0** | | 2.599 | 0.065 | 3 | 5 | 2.641 | 2.657 | 2.636 | 2.506 | 2.555 |
| **TAP QC Panel** | **Range (nmol/L)** | | | | | | | **Mean Concentration (nmol/L)** | | |
| **QC1** | 145.2 - 269.6 | 207.4 | 9.9 | 5 | 5 | 210.4 | 207.9 | 190.7 | 210.3 | 217.5 |
| **QC2** | 89.1 - 165.5 | 127.3 | 11.6 | 9 | 5 | 130.7 | 124.7 | 112.5 | 124.3 | 144.5 |
| **QC3** | 43.8 - 81.4 | 62.6 | 6.3 | 10 | 5 | 61.7 | 61.8 | 55.6 | 61.1 | 72.9 |
| **QC4** | 17.9 - 33.2 | 25.5 | 0.8 | 3 | 5 | 24.4 | 25 | 25.7 | 26.6 | 25.7 |

| TAP QC Panel | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean testing (nmol/L)** | **SD** | **Assay 1** | **Assay 2** | **Assay 3** | **Assay 4** | **Assay 5** | **Z Critical (n = 5)** | **Z min** | **Z max** | **Within Limit (Yes / No)** |
| 207.4 | 10 | 210.4 | 207.9 | 190.7 | 210.3 | 217.5 | | | | |
| **QC1** | Z value | -0.3 | -0.1 | 1.7 | -0.3 | -1 | ± 1.71 | -1.02 | 1.67 | Yes |
| 127.3 | 11.6 | 130.7 | 124.7 | 112.5 | 124.3 | 144.5 | | | | |
| **QC2** | Z value | -0.3 | 0.2 | 1.3 | 0.3 | -1.5 | ± 1.71 | -1.47 | 1.27 | Yes |
| 62.6 | 6.3 | 61.7 | 61.8 | 55.6 | 61.1 | 72.9 | | | | |
| **QC3** | Z value | 0.1 | 0.1 | 1.1 | 0.2 | -1.6 | ± 1.71 | -1.63 | 1.11 | Yes |
| 25.5 | 0.8 | 24.4 | 25 | 25.7 | 26.6 | 25.7 | | | | |
| **QC4** | Z value | 1.3 | 0.6 | -0.3 | -1.4 | -0.3 | ± 1.71 | -1.35 | 1.3 | Yes |

**Table 3.3.9.1** Overview of calibration curve and QC sample panel 5 day testing. Precision after 5 days for the calibration curve was <10% for all standards, with the exception of 400 nmol/L. Precision for the QC samples was ≤10% with concentration ranges established based on the mean value of the 5 days ± 30%. These ranges were to be applied internally as quality checks in all future prototype testing. The Grubb's outlier test demonstrated that there was no outlier data, with minimum and maximum values for all assays within the critical Z value range.

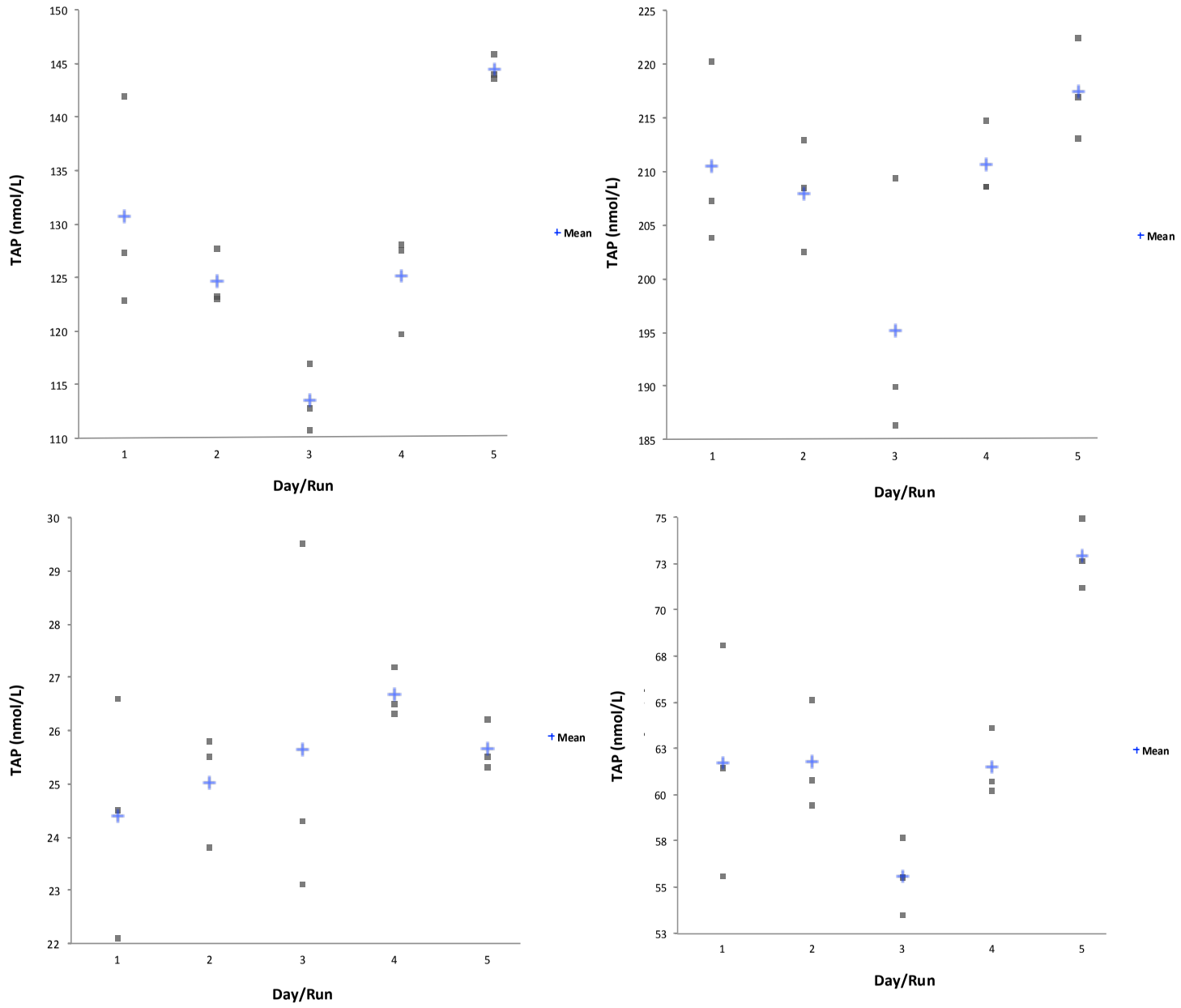



**Figure 3.3.9.1** Intra-assay precision for QC samples 1 - 4, clockwise from top left. The precision for QC1 was 4.1%, 2.5%, 6.3%, 1.7% and 2.2% for days 1 to 5. The precision for QC2 was 7.7%, 2.1%, 2.7%, 3.7% and 0.8% for days 1 to 5. The precision for QC3 was 10.1%, 4.8%, 3.8%, 3.0% and 2.6% for days 1 to 5. The precision for QC4 was 9.2%, 4.3%, 13.3%, 1.8% and 1.8% for days 1 to 5.

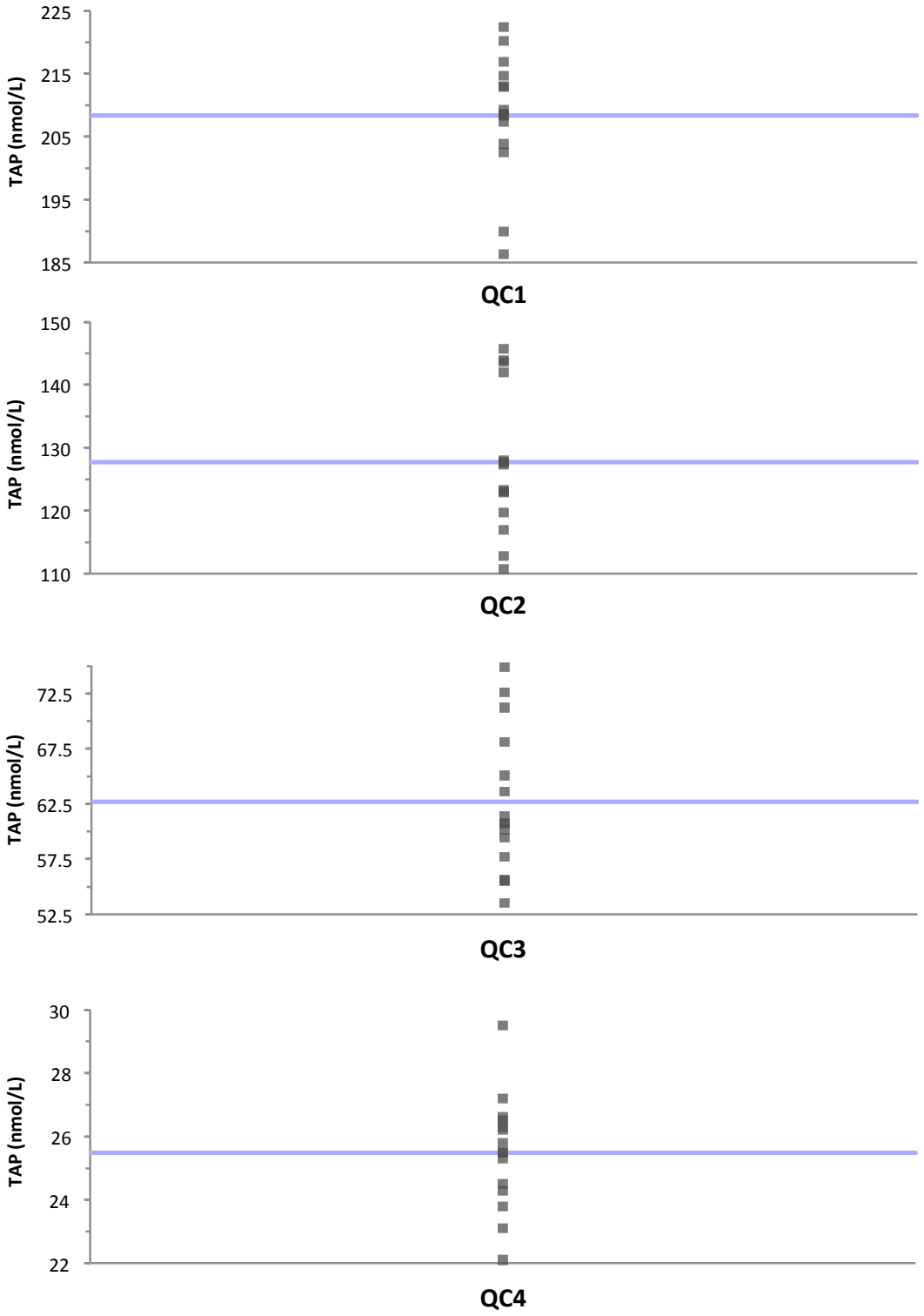



**Figure 3.3.9.2** Inter-assay precision for QC samples 1-4. QC1 = 4.8%, QC2 = 8.9%, QC3 = 10.4% and QC4 = 7.0%. All samples were evenly distributed and were comfortably within an allowable imprecision of %CV = <20%. The blue line represents the mean value of each QC sample as displayed in Table 3.3.9.1.

### 3.3.10 Establishment of TAP positive control sample

A Positive Control (PC) sample was prepared by spiking TAP antigen into a proprietary diluent formulation from EKF Diagnostics to maximise antigen stability. This bulk sample was tested in a single assay (n=22) and quantified from the standard calibration curve (0 – 400 nm/L). Establishment of a positive control sample allows for an additional control that may be run in place of QC samples but also for potential future use in a commercial anti-TAP ELISA. Such a sample can provide a reference check for information on intra- and inter-assay precision and long-term performance of an assay, such as inter-batch variation (Deshpande, 1996).

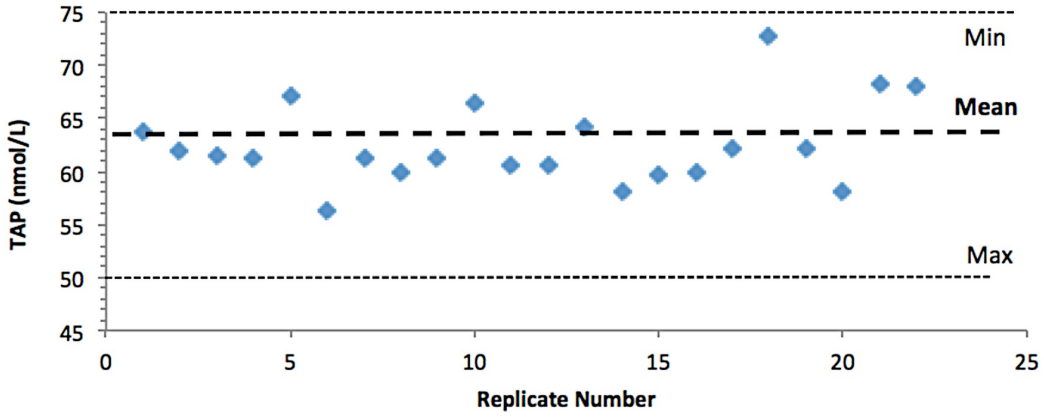



**Figure 3.3.10.1** Measuring interval for Positive Control sample with individual concentration distribution. A quality control range of the mean value (62.5 nmol/L) ± 20% was calculated with the minimum and maximum values for this range being 50 and 75 nmol/L, respectively. This range can be used for future assays run with this PC sample to demonstrate whether quantified results are valid. The variation between replicates was %CV = 5%.

### 3.3.11 Establishment of limit of quantification for streptavidin / biotinylated anti-TAP ELISA

To fully define the measuring range of any assay, the limitations of the assay to accurately measure low concentrations of analyte must be established. There are several parameters that have been defined and used to describe the smallest concentration of an analtye that can be reliably quantified by a measuring procedure. The CLSI defines three individual parameters in relation to assay sensitivity. These are the Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantification (LoQ). The LoB is the highest apparent analyte concentration expected when replicates of a blank sample containing no analyte are tested. The LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. To investigate the differences in results obtained from using the CLSI guideline formula and more traditional methods often employed in laboratories, the LoD was calculated from the same data, as described in Table 3.3.11.1 below. The LoQ is the lowest concentration at which the analyte can not only be reliably detected, but at which some predefined goals for bias and imprecision are met. The LoQ can be equivalent to the LoD or at a much higher concentration and is therefore the most important parameter to define for clinical utility of an assay (Armbruster & Pry, 2008). The predefined allowable imprecision for the LoQ of this prototype was a %CV = ≤10%. While the number of replicates for establishing each of the parameters was less than the figure indicated (60 replicates) in the CLSI document *EP17-A2*: *Evaluation of detection capability for clinical laboratory measurement procedures; approved guideline*, these experiments were sufficient to demonstrate the performance of the ELISA prototype.

The LoB and LoD were calculated using the following formulae:

**Limit of Blank (LoB)** = Mean $_{\text{Blank OD}}$ - 1.645(SD $_{\text{Blank OD}}$)

**Limit of Detection (LoD)** = Mean $_{\text{Blank OD}}$ - 2(SD $_{\text{Blank}}$)

**or**

**Limit of Detection (LoD)** = Mean $_{\text{Blank OD}}$ - 3(SD $_{\text{Blank}}$)

**or**

**Limit of Detection (LoD)** = LoB - 1.645(SD $_{\text{low sample concentration}}$)

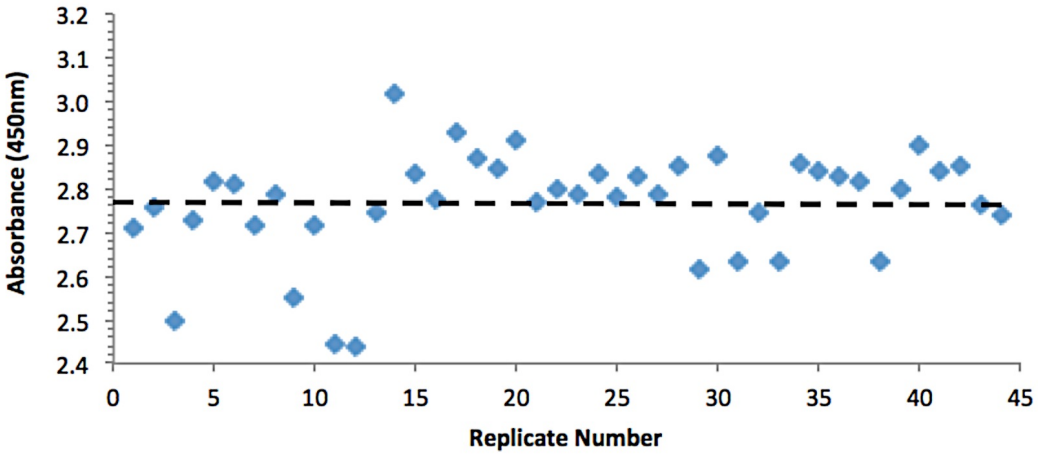



**Figure 3.3.11.1** Determination of LoB for the streptavidin/biotinylated anti-TAP ELISA using 44 replicates of the zero (blank) standard. The variability between replicates was %CV = 4%, with a mean absorbance ($OD_{450nm}$) value of 2.765 (indicated by dashed line). The $OD_{450nm}$ values were inputted into the GEN5 PlateReader software to back-calculate the TAP concentrations for each individual replicate from the standard calibration curve used for assay testing. Using the formula above, the LoB was calculated to be 1.2 nmol/L.

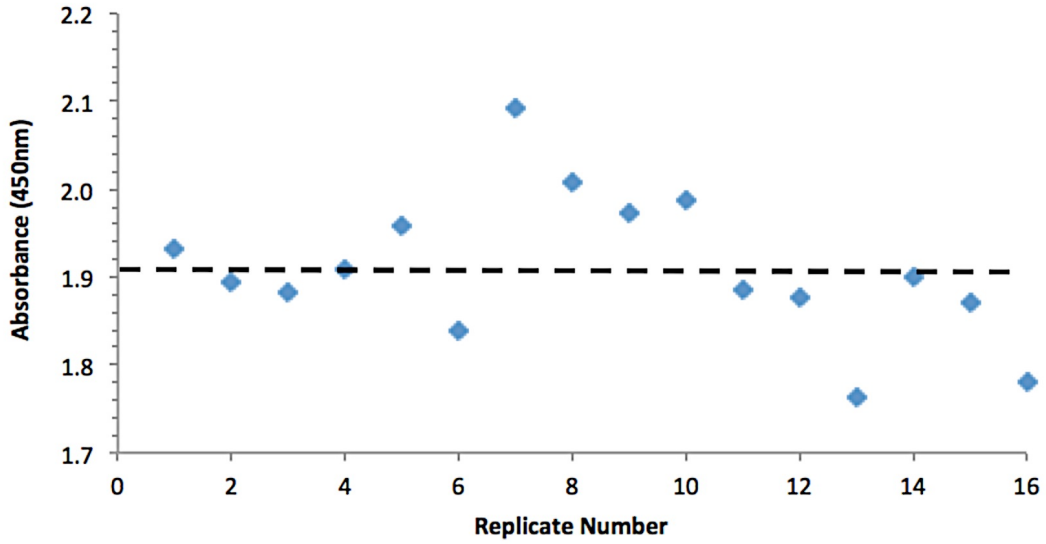



**Figure 3.3.11.2** Determination of LoD for the streptavidin/biotinylated anti-TAP ELISA using 16 replicates of the 3.1 nmol/L TAP standard. The variability between replicates was %CV = 4%, with a mean LoD absorbance ($OD_{450nm}$) value of 1.910. The LoD $OD_{450nm}$ values were inputted into the GEN5 PlateReader software as per figure 3.3.11.1. These concentrations were then utilised in the formulae in table 3.3.11.1. Note, the "+" was replaced by a "-" in these formulae due to the competitive assay format. A "+" would cause the concentrations to be unquantifiable as they would have an $OD_{450nm}$ value greater than the zero standard.

**Table 3.3.11.1** Calculation of limit of detection using various parameters:

| LoD Parameter | LoD (OD) | LoD (nmol/L) |
|---|---|---|
| Mean Blank OD - 2*SD | 2.523 | 1.6 |
| Mean Blank OD - 3*SD | 2.401 | 2.6 |
| Mean Blank OD - 1.645*SD Blank - 1.645*SD Std 3.13 | 2.429 | 2.3 |

With a predefined imprecision requirement of %CV = ≤10% successfully met, the LoQ was equivalent to the LoD and was therefore 2.3 nmol/L.

### 3.3.12 Establishment of linear measuring range for streptavidin / biotinylated anti-TAP ELISA

The relationship between analyte concentration or response, when measured by an analytical method, and a pathophysiological disease process is generally non-linear. However, the measuring procedure must be able to report results with a linear relationship between the response and the recorded analyte concentration. So, for the dilution of a sample of known concentration (quantified from the calibration curve), the measured or expected sample concentration must be directly proportional to the dilution level. It is not possible to interpolate for response values that fall between calibrator standards without a defined linear measuring range. The CLSI document: *EP6-A: Evaluation of the linearity of quantitative measurement procedures: A statistical approach; approved guideline*, defines the ideal matrix for linearity assessment as a patient specimen with an analyte concentration near the expected upper reportable limit that is diluted with another patient's sample having an analyte concentration at the expected or tested lower limit of the assay. It also states that to establish linear ranges, a laboratory should use 7 to 11 concentration levels across the measuring range, with 2 to 4 replicates per level. As a trusted control, the QC1 urine sample was used for the highest concentration (100%) of the linear measuring range to be assessed. This sample was diluted to levels of 90, 80, 70, 50, 40, 30, 20, 10, 5 and 2.5% using donor urine from an apparently normal individual. This donor urine represented the 0% level and was quantified by the ELISA prototype to confirm as "analyte-free" urine. Each of the sample levels was tested in triplicate.

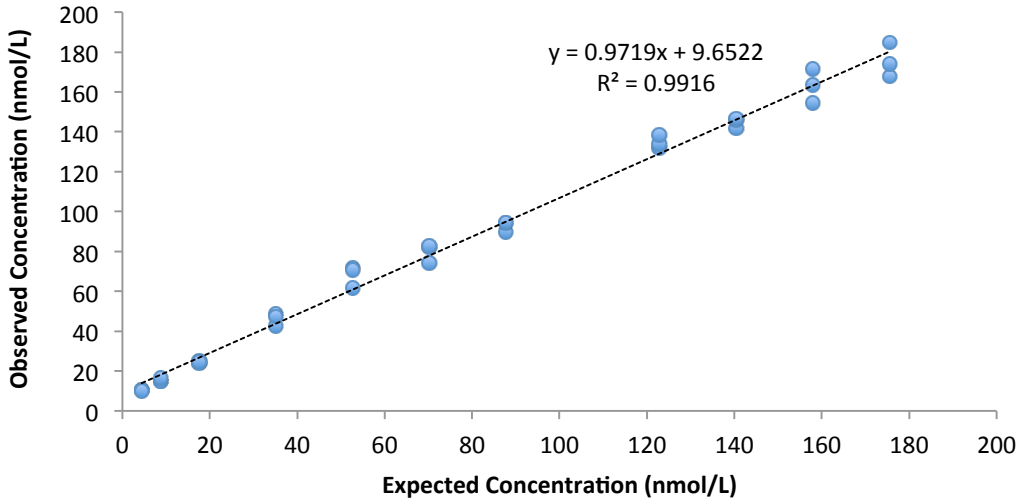



**Figure 3.3.12.1** Linearity of streptavidin/biotinylated anti-TAP ELISA. A strong linear fit ($R^2$ = 0.9916) between the expected and observed TAP concentrations was achieved across the measuring range. The linear measuring range extended from 10.2 – 175.5 nmol/L from the samples tested and the variability between replicates of each sample level was %CV = ≤10%.

### 3.3.13 Streptavidin-coated microtitre plate stability study

To investigate whether the streptavidin-coated microtitre plates purchased from Thermo Fisher Scientific maintained their performance characteristics under specific storage temperature conditions, an accelerated stress stability study was undertaken. Accelerated stability testing allows for a shorter study time where stressed and unstressed product can be compared, thereby reducing the possibility of instability in the measurement. Data taken from storage of a product at a temperature that is higher than the desired storage temperature can be used to predict the degradation rate of the product signal using the Arrhenius equation. This equation states that the relationship between time and stability of a product stored under constant conditions is dependent on the order of reaction and a rate constant that determines the speed of the reaction:

$$k = Ae^{-Ea/(RT)}$$

**or**

$$Ln(k_2/k_1) = -Ea/2.303R*(1/T_2-1/T_1)$$

The parameters $k_1$ and $k_2$ are the rate constants at temperatures $T_1$ (4°C) and $T_2$ (37°C), A is the Arrhenius factor, Ea is the activation energy of the reaction and R is the gas constant. The diagnostic industry simplifies the Arrhenius equation using the "Q Rule" to estimate product shelf-life using certain assumptions. The Q Rule states that product degradation decreases by a constant factor ($Q_{10}$) when the storage temperature is lowered by 10°C. The $Q_{10}$ value can be 2, 3 or 4, ranging from probable to possible results, respectively (Anderson & Scott, 1991). Using this information, the accelerated aging time (AAT) required for predicting the real time stability (RT) was calculated using the following formula:

$$AAT = \frac{\text{Desired Real Time Stability (RT)}}{Q_{10}[^{(T_{AA}-T_{RT})}/10]}$$

where $T_{AA}$ refers to the accelerated aging temperature (°C) and $T_{RT}$ refers to the ambient temperature (°C) (Schueneman & Campos, 2014).

For this study, the microtitre plates were placed in a 37°C incubator for a period of time as indicated in table 3.3.13.1, and then removed and placed at 4°C storage. A microtitre plate was maintained at 4°C throughout as an unstressed control and tested alongside the stressed components. Calibrators, PC and QC samples were run with each of the microtitre plates.

**Table 3.3.13.1** Accelerated stress stability study time-points

| **Incubation at 37°C (Days)** | 1 | 3 | 9 | 18 | 37 |
|---|---|---|---|---|---|
| **Equivalent to storage at 4°C (Months)** | 0.25 | 1 | 3 | 6 | 12 |

| Assigned Expiry (Months @ 4°C) | | | | | | | | | 0.25 | 1 | 3 | 6 | 12 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Timepoint (Days @ 37°C) | | | | | | | | | 1 | 3 | 9 | 18 | 37 | 0 |
| Calibrator | TAP (nmol/L) | | | Mean OD | MIN | MAX | STDEV | %CV | Mean OD | | | | | |
| CAL 1 | 400 | | | 0.112 | 0.102 | 0.118 | 0.0062 | 6 | 0.102 | 0.115 | 0.118 | 0.110 | 0.114 | 0.117 |
| CAL 2 | 200 | | | 0.215 | 0.189 | 0.238 | 0.0188 | 9 | 0.205 | 0.222 | 0.223 | 0.189 | 0.238 | 0.242 |
| CAL 3 | 100 | | | 0.397 | 0.369 | 0.421 | 0.0225 | 6 | 0.369 | 0.421 | 0.402 | 0.379 | 0.415 | 0.360 |
| CAL 4 | 50 | | | 0.733 | 0.702 | 0.766 | 0.0275 | 4 | 0.702 | 0.758 | 0.766 | 0.718 | 0.722 | 0.724 |
| CAL 5 | 25 | | | 1.109 | 1.075 | 1.167 | 0.0390 | 4 | 1.093 | 1.129 | 1.167 | 1.075 | 1.079 | 1.258 |
| CAL 6 | 12.5 | | | 1.463 | 1.411 | 1.570 | 0.0643 | 4 | 1.434 | 1.476 | 1.570 | 1.411 | 1.426 | 1.641 |
| CAL 7 | 6.25 | | | 1.794 | 1.748 | 1.876 | 0.0485 | 3 | 1.789 | 1.772 | 1.876 | 1.787 | 1.748 | 2.059 |
| CAL 8 | 3.13 | | | 2.173 | 2.098 | 2.268 | 0.0618 | 3 | 2.165 | 2.182 | 2.268 | 2.151 | 2.098 | 2.293 |
| BLK | 0 | | | 2.633 | 2.493 | 2.723 | 0.1069 | 4 | 2.493 | 2.692 | 2.713 | 2.543 | 2.723 | 2.771 |
| | | | | Mean %A/$A_0$ | MIN | MAX | STDEV | %CV | Mean %A/$A_0$ | | | | | |
| CAL 1 | 400 | | | 4 | 4 | 4 | 0.1167 | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| CAL 2 | 200 | | | 8 | 7 | 8 | 0.3990 | 5 | 8 | 8 | 8 | 7 | 9 | 9 |
| CAL 3 | 100 | | | 15 | 15 | 16 | 0.4015 | 3 | 15 | 16 | 15 | 15 | 15 | 13 |
| CAL 4 | 50 | | | 28 | 28 | 28 | 0.0440 | 0 | 28 | 28 | 28 | 28 | 27 | 26 |
| CAL 5 | 25 | | | 43 | 42 | 44 | 0.8462 | 2 | 44 | 42 | 43 | 42 | 40 | 45 |
| CAL 6 | 12.5 | | | 56 | 55 | 58 | 1.4963 | 3 | 58 | 55 | 58 | 55 | 52 | 59 |
| CAL 7 | 6.25 | | | 69 | 66 | 72 | 2.5226 | 4 | 72 | 66 | 69 | 70 | 64 | 74 |
| CAL 8 | 3.13 | | | 84 | 81 | 87 | 2.3986 | 3 | 87 | 81 | 84 | 85 | 77 | 83 |
| Sample ID | TAP Range (nmol/L) | | Mean Range | Mean TAP (nmol/L) | MIN | MAX | SD | %CV | Mean TAP (nmol/L) | | | | | |
| PC | 50.0 | 75.0 | 62.5 | 62.4 | 58.2 | 67.5 | 3.5 | 6 | 61.2 | 67.5 | 61.1 | 58.2 | 64.0 | 62.5 |
| QC1 | 145.2 | 269.6 | 207.4 | 169.5 | 148.2 | 187.5 | 14.7 | 9 | 148.2 | 163.3 | 175.8 | 172.5 | 187.5 | 177.3 |
| QC2 | 89.1 | 165.5 | 127.3 | 96.7 | 92.4 | 102.5 | 4.1 | 4 | 93.8 | 102.5 | 92.4 | 95.7 | 99.0 | 114.2 |
| QC3 | 43.8 | 81.4 | 62.6 | 47.5 | 43.8 | 52.2 | 3.2 | 7 | 45.9 | 43.8 | 46.4 | 49.0 | 52.2 | 50.0 |
| QC4 | 17.9 | 33.2 | 25.5 | 19.7 | 18.9 | 22.2 | 1.4 | 7 | 19.3 | 19.1 | 19.2 | 22.2 | 18.9 | 23.0 |

**Table 3.3.13.2** Overview of streptavidin-coated microtitre plate stability study results. The imprecision for the calibrators, PC and QC samples was %CV = ≤10%. All samples tested were within their required specification ranges and thus, the shelf-life of the streptavidin-coated microtitre plates was therefore estimated at least 12 months at 4°C storage.

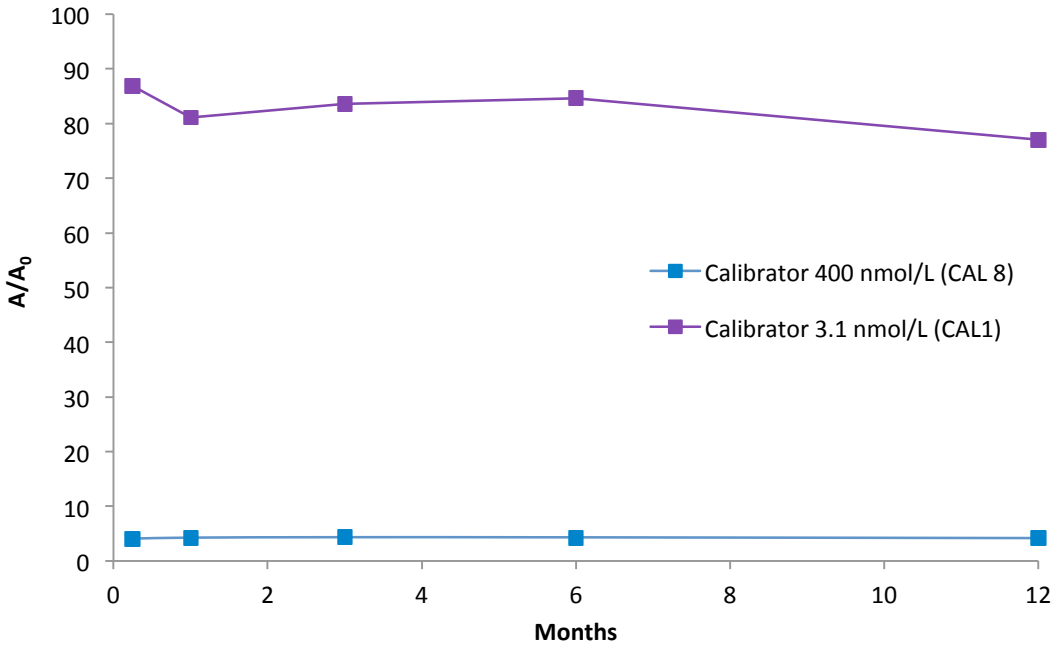



**Figure 3.3.13.1** The $A/A_0$ signal response for TAP calibration standards 3.1 and 400 nmol/L during predicted real time stability period of 12 months. Month "0" refers to control microtitre plate. Response remained constant for both calibrators (CAL1 and CAL8), demonstrating good stability at both low and high TAP concentrations. Response values were taken from Table 3.3.13.2.

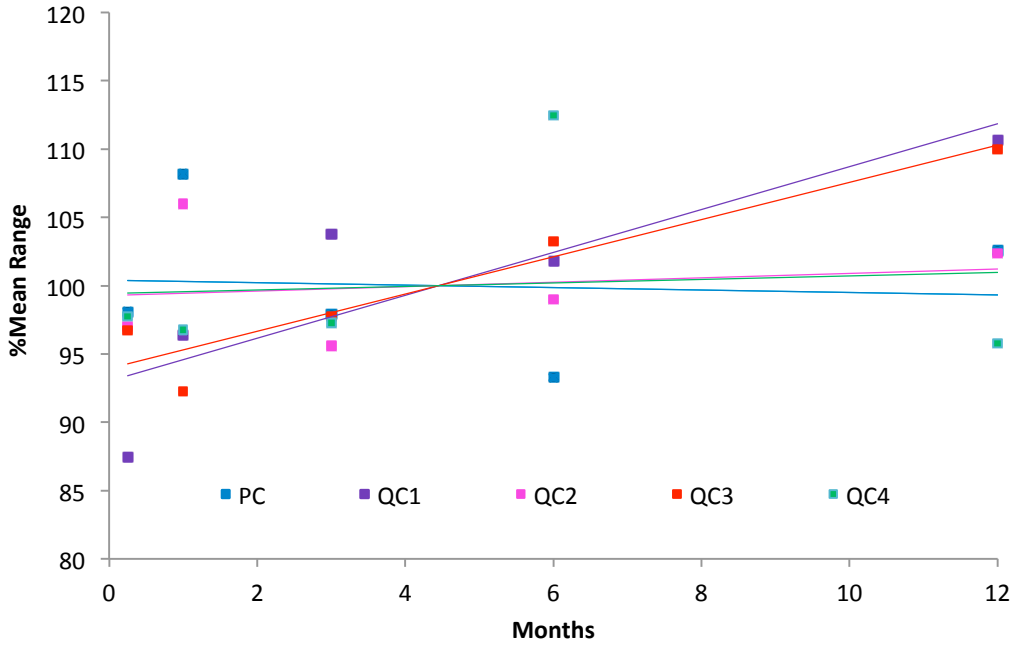



**Figure 3.3.13.2** Trending quantification of PC and QC samples data during predicted real time stability period of 12 months.

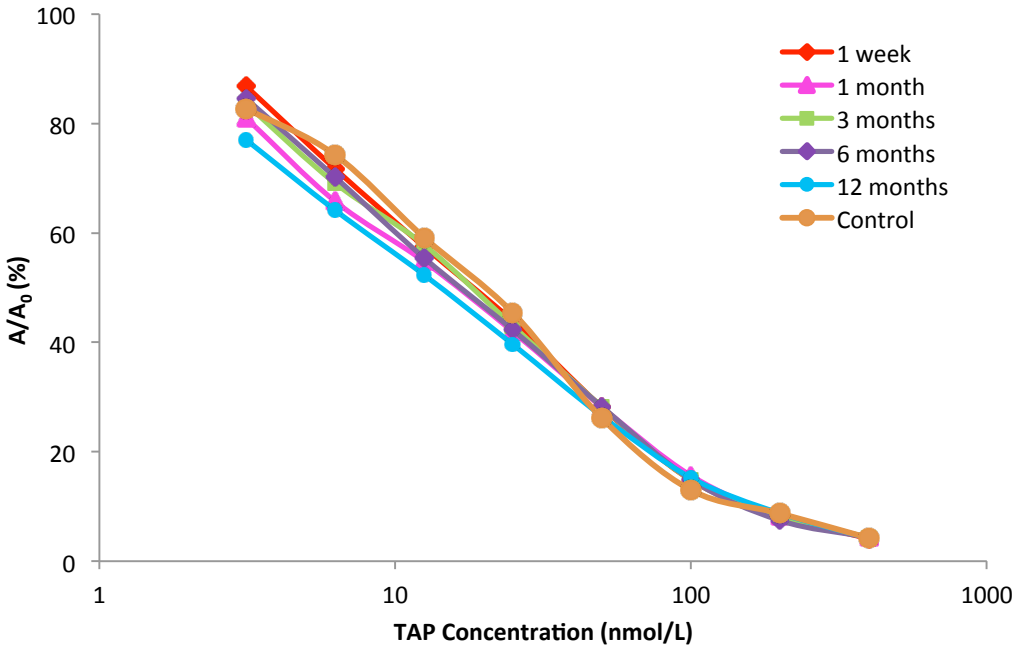
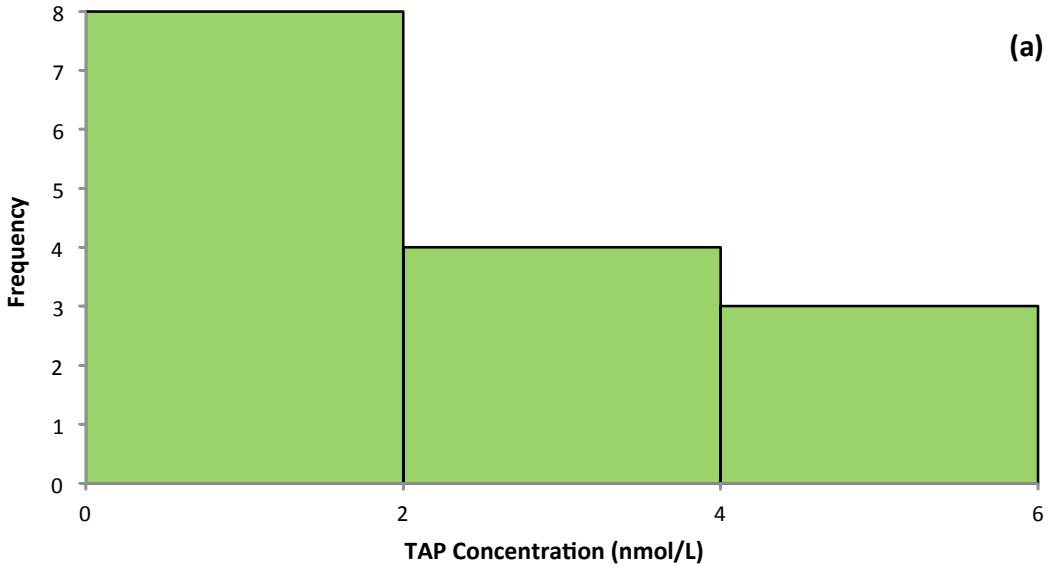



**Figure 3.3.13.3** Overlay of TAP calibration curves during predicted real time stability period of 12 months. As shown for the raw data in the overview table of Figure 3.3.13.1, no major 'drop-off' in assay response signal was observed and thus, the calibration curves for each of the stressed time-points were equivalent to the control in the quantification of the TAP standards.

### 3.3.14 Establishment of a reference range for urinary TAP using "normal" donor group

In order to truly understand a measured analyte test result from a patient sample, it is essential to have a reference range that defines the expected values in a "normal" patient sample, i.e. a person who does not have a medical diagnosis for the disease in question. The most accurate way to achieve this is to collect samples from patients with no clinical symptoms relating to the disease. All test results can subsequently be compared to this range and contribute to therapeutic management decisions in the clinical setting. The CLSI document *C28-A3c: Defining, establishing and verifying reference intervals in the clinical laboratory; approved guideline*, recommends that all laboratories should verify the reference range supplied with any commercial product or establish their own in cases where none is supplied. The reference group for the anti-TAP ELISA consisted of 15 anonymous donor urines that were collected at DCU following research ethics committee approval of the protocol. Due to the time constraints, this donor group was the closest substitute to a group with no known clinical complications relating to acute pancreatitis. All donor urines were tested in duplicate and quantified using the TAP standard calibration curve.




**Frequency Distribution**

| Class | Frequency | Relative frequency | Density | Cumulative frequency | Cumulative relative frequency |
|---|---|---|---|---|---|
| ≥0 to <2 | 8 | 0.533 | 0.2667 | 8 | 0.533 |
| ≥2 to <4 | 4 | 0.267 | 0.1333 | 12 | 0.800 |
| ≥4 to <6 | 3 | 0.200 | 0.1000 | 15 | 1.000 |

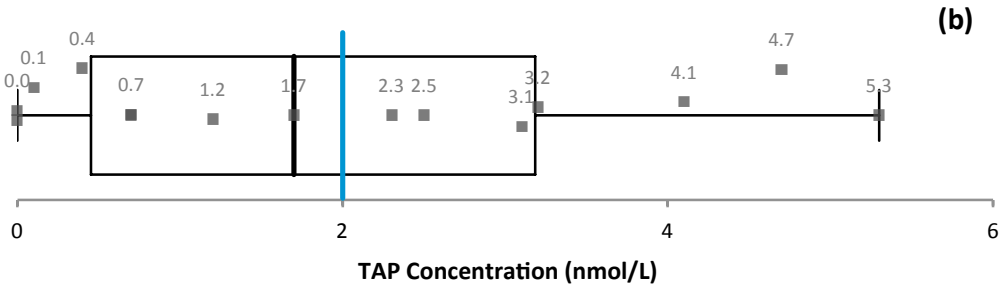



**Figure 3.3.14.1** Frequency distribution plots and data for the TAP reference group - (a) histogram of frequency distribution, illustrating 8 donors between 0 to <2 nmol/L, 4 donors between 2 to <4 nmol/L and 3 donors between 4 to <6 nmol/L and; (b) box-plot frequency distribution, illustrating the mean of the reference range at 2 nmol/L. The TAP reference range was established as 0 - 5.3 nmol/L in this group.

## 3.4 DISCUSSION AND CONCLUSION

The development of an anti-TAP ELISA capable of quantifying the TAP analyte within a defined measuring range and with the pre-desired performance characteristics was described in this chapter. Three separate lots of monoclonal anti-TAP IgG antibody were produced under a license agreement with AbCam® and characterised by SDS-PAGE analysis to demonstrate strong reproducibility. Initial work to establish a competitive anti-TAP ELISA showed a lack of discrimination between calibration curve standards alongside a requirement for a low conjugate dilution that would see the use of large stock volumes. Biotinylation of the anti-TAP IgG antibody and its use with streptavidin-coated microtitre plates demonstrated enhanced discrimination between standards and subsequently, the optimisation and establishment of performance characteristics were outlined for this assay format. The purchase of commercially available streptavidin-coated microtitre plates was made in the interest of the antibody and peptide material limitations for this project. Further cost saving could be made in future through production of streptavidin-coated microtitre plates by alternative methods such as cross-linking with glutaraldehyde (Välimaa *et al*., 2003) or chemical modification such as thiol group introduction (Ylikotila *et al*., 2009).

The streptavidin/biotinylated anti-TAP ELISA standard calibration curve was cross-checked using two separate forms of curve-fitting software to establish the optimal TAP analyte measuring range. The stability of the TAP analyte was then investigated in various matrices by repeated freeze thawing from -20°C to room temperature, an action that would be performed daily in the use of any diagnostic test for patient sample testing. The TAP analyte demonstrated exceptional stability in both $dH_20$ and urine, leaving no apprehension for the next step in prototype assessment with clinical urine samples. To further ensure reproducibility of the assay prototype, several quality control samples were prepared with TAP analyte in urine (QC 1 - 4) and in a specialised buffer formulation for the PC to quantify at various concentrations spread across the assay measuring range. Specification ranges were established for each sample and precision testing indicated very low variability (%CV = ≤ 10%). These controls were essential in determining the prototype's capacity to be utilised in clinical

testing and its potential as a commercial test. The stability of the streptavidin-coated microtitre plate and the established reference range for the TAP analyte were additional experiments that clearly also highlighted the capability of this ELISA prototype to be progressed for use in a clinical sample evaluation and to be considered for potential commercialisation.

The strong performance characteristics and robustness of the streptavidin/biotinylated anti-TAP ELISA outlined in this chapter demonstrate that this prototype has the potential to be commercialised following scale-up production studies that would also include additional performance testing experiments such as testing for endogenous interfering substances found in human urine, including lipids and bilirubin (Dimeski, 2008). Although having a longer test time duration (3h 15mins versus 2h 15 mins total incubation time) compared to the polyclonal anti-TAP ELISA marketed previously, this streptavidin/biotinylated anti-TAP ELISA prototype should provide more reproducible performance due to the use of the anti-TAP mAb. In addition, the overall performance characteristics compared favourably with the polyclonal ELISA and are outlined in Table 3.4.1 below. In fact, the ELISA prototype described in this chapter has undergone much more vigorous performance testing than when compared to the data provided in the polyclonal ELISA product insert (*Appendix 8.1*). In conclusion, the streptavidin/biotinylated anti-TAP ELISA prototype described in this chapter can serve as the reference method for quantification of TAP analyte during the next phase of development – a rapid lateral flow test strip device. The establishment of a "gold standard" reference method, such as an ELISA, is an essential step for the development of any POC device.

**Table 3.4.1** Comparison of performance characteristics for streptavidin/biotinylated anti-TAP ELISA and commercial polyclonal anti-TAP ELISA:

| Parameter | Streptavidin/Biotinylated anti-TAP ELISA | Commercial Polyclonal anti-TAP ELISA |
|---|---|---|
| **Sample dilution** | Not required | 1/4 recommended |
| **Spike recovery (%)** | 100 ± 10% | 100 ± 10% |
| **Intra-assay precision (%CV)** | 0.8 - 13.3% | 10.0 - 16.7% |
| **Inter-assay precision (%CV)** | 4.8 - 10.4% | 11.9 - 18.7% |
| **Measuring range (nmol/L)** | 2.3 - 400 | 0.56 - 800 |

# Chapter 4

# Development of an Anti-TAP Lateral Flow Test Strip Device

## 4.1 INTRODUCTION

The genesis of the lateral flow immunoassay (LFIA) in the 1950s was the result of a confluence of scientific activity, including the latex agglutination test first described by Singer and Plotz in 1956. In the following decades, the LFIA concept was improved, with the human pregnancy test becoming the first major success of this assay format (Gubala *et al*., 2012). Since then, the LFIA has been adapted for large-scale clinical diagnostic use in many fields including infectious and cardiac diseases, cancer and drugs of abuse (Gubala *et al*., 2012; Sharma *et al*., 2015). The LFIA has grown to become the most popular commercial point-of-care (POC) device format of today, with an estimated market size of US$18 billion predicted by 2016 (Gubala *et al*., 2012; St John & Price, 2014). The prevalence of infectious diseases, an aging population and the drive for more home-based and decentralised POC testing, are all significant contributors to this anticipated growth. The LFIA is particularly well suited to this emerging requirement for decentralised testing due to numerous factors such as ease of use, small sample volume requirement, large-scale production within a short timeframe and the ability to be integrated with reader and central information systems (Wong, 2009).

Typically, a LFIA involves the flow of a liquid sample, containing the analyte of interest, through a porous membrane where biological interactions occur from the binding of labeled particles or analyte by immobilised antibodies to produce a signal that can be visualised or detected optically. This lateral flow test strip format would consist of a nitrocellulose (NC) membrane that transports the sample (applied to a sample pad) by capillary action, through a conjugate release pad and onwards to an analyte detection zone before reaching an absorbent pad that terminates flow (Posthuma-Trumpie *et al*., 2009). The format of the lateral flow device for TAP antigen (Figure 4.1.1) was restricted to a competitive assay due to the small size of the peptide. The use of a similar format to the ELISA prototype, described in chapter 3, also allowed for a direct comparison in performance between both assays. The use of a competitive format meant that results would be inversely correlated to analyte concentration i.e. a lower signal response indicates that more analyte is present and the highest response is observed for analyte-free sample.

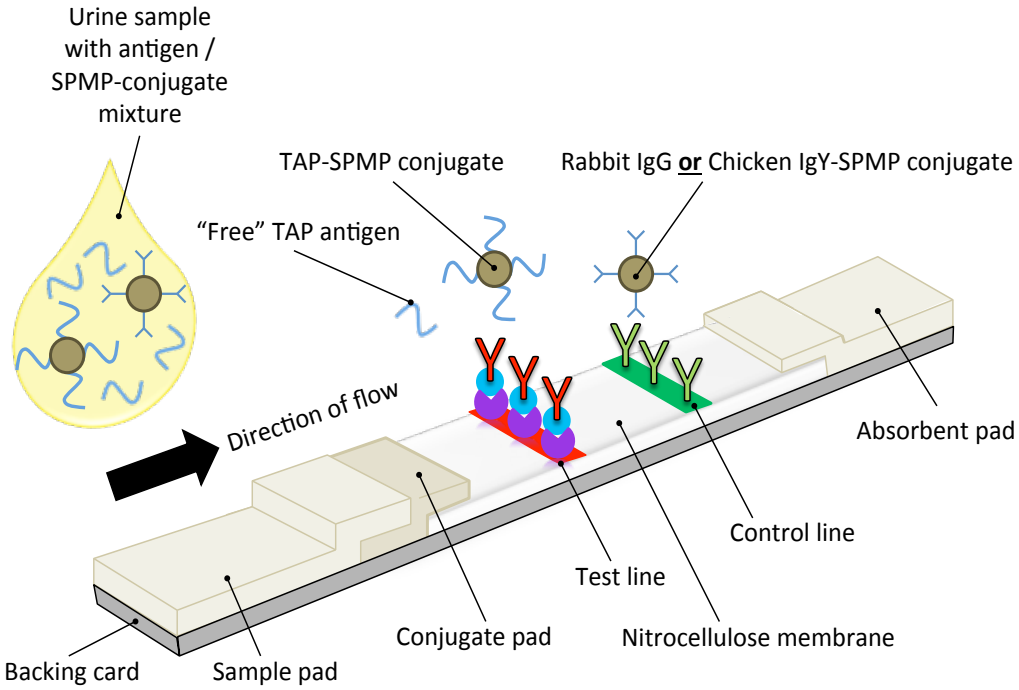



**Figure 4.1.1** Schematic of magnetic LFIA prototype for the detection of TAP antigen. The urine sample is mixed with two independent super-paramagnetic particle (SPMP) conjugates; TAP-SPMP "test" conjugate and polyclonal rabbit IgG or chicken IgY-SPMP "control" conjugate. Sample/conjugate mixture travels through the nitrocellulose membrane with binding occurring at test and control lines before an absorbent pad soaks up any residual particles and "free" antigen. The **Test line incorporates streptavidin, biotinylated anti-TAP capture antibody** which binds to TAP-SPMP conjugate and, **control line incorporates goat anti-rabbit IgG (Prototype 1) or goat anti-chicken IgY (Prototype 2) capture antibodies**. The degree of binding at the test and control regions is read using the magnetic immunochromatographic test (MICT®) instrument from MagnaBioSciences LLC, which quantitatively measures and expresses the signal as a magnetic assay reading (MAR). The MICT® instrument was utilised for all of the magnetic TAP LFIA prototype work described in this chapter.

All lateral flow test strip components were selected based on supplier recommendations for sample flow characteristics, including rate of flow and testing of urine samples. The sample pad for this assay was pre-treated with a proprietary buffer formulated with high concentration salts to neutralise urine sample pH (pH ~6-8) and addition of surfactant to increase wettability of the pad. Human urine can have a pH varying between 5 and 10 and high fluctuations can alter the specificity and sensitivity of capture antibodies by affecting changes in charge densities (Millipore, 2008). Inclusion of a pre-treatment step from the outset assured suitability for future use with clinical samples, if warranted. A glass fibre conjugate pad was also included in all test strips for potential drying of magnetic particle conjugates in future iterations of the assay prototype. Several factors were taken into account for the NC membrane. The pore size in relation to label size and the capillary flow time was considered vitally important for an optimal reaction time. Use of polyethylene material can offer control over membrane pore size, improving material reproducibility and thus, test result reproducibility. A NC membrane is also generally treated with surfactant to make it hydrophilic for test use and it is supported with a plastic layer for handling and cutting purposes (Posthuma-Trumpie *et al*., 2009; Koczula & Gallotta, 2016). An absorbent "upper wick" cotton pad with an absorption capacity greater than the sample volume was also essential. The dispensing buffer used for application of capture antibodies to the NC membrane was PBS.

Magnetic particles have been described for use in medical areas including; as MRI contrast agents, for cell labeling and separation, drug delivery; and more recently as immunoassay labels in LFIAs. Superparamagnetic particles (SPMPs) are magnetic iron oxide or magnetite ($Fe_3O_4$) particles produced by various wet chemistry techniques where external conditions such as pH and temperature are carefully controlled. These techniques control the size and uniformity of the resulting particles with nanoparticles of diameter <100 nm and >200 nm used for *in vivo* and *in vitro* applications, respectively. SPMPs used in LFIAs offer many advantages over the traditional and most widely used LFIA labels of colloidal gold and latex. SPMPs have low background noise due to biological materials lacking magnetism and are inactive until required; becoming magnetic only when placed in a

strong magnetic field and subsequently maintaining a stable signal. The surface of SPMPs can also be extensively modified with various coating chemistries to facilitate bio-conjugation. The resultant magnetic signal at a detection zone can be measured quantitatively through the entire volume of the membrane whereas conventional detection methods with gold and latex particles are interpreted visually and only account for the surface reaction observed (qualitative) (LaBorde & O'Farrell, 2002; Tartaj *et al*., 2003; Nor *et al*., 2012;). SPMP-labeled conjugates were prepared for this assay using EDC-NHS coupling chemistry (section 2.2.7) that utilised the carboxyl-functionalised surface of the SPMPs (Figure 4.1.2) and the amine groups of the TAP antigen or control antibodies.

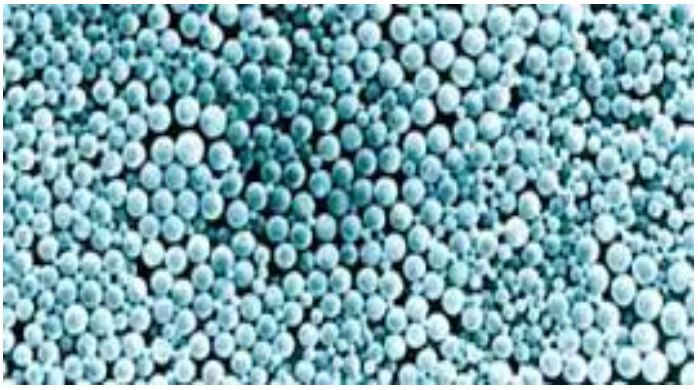


**Figure 4.1.2** TEM image of Estapor® SPMPs, taken from Merck Millipore product information brochure. The SPMPs pictured are uniformly separated or "monodisperse" and demonstrate no sign of particle grouping or aggregation.

The precision or repeatability of the lateral flow prototype was calculated throughout the experimental work by performing multiple measurements (n = 5) within each testing run on the same day. To define the precision between these replicates, the coefficient of variation (CV) was determined using the following formula:

$$\text{Precision} = \text{CV}\ (\%) = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$


**Figure 4.1.3** MICT® platform system is an *in vitro* diagnostic device which can quantitatively detect SPMP-labelled analyte in numerous biological samples. 1: Lateral flow test strips are enclosed in disposable test cassettes which are patented to fit in the MICT® instrument. Test cassettes have barcode information detailing test parameters such as test and control line locations and a standard calibration curve; 2: The MICT® instrument, with a touch-screen interface, is used to measure the magnetic signal of the developed test device; 3: Measurement of particles which accumulate at the test and control regions following biological binding requires excitation of the SPMPs inside a magnetic field. A C-shaped electromagnet is positioned around the test strip and a voltage applied to produce a magnetic field. The resulting magnetic intensity (magnetic assay reading, MAR) is read and converted into analytical units of measurement using mathematical algorithms programmed into the MICT® software. Figure taken from magnabiosciences.com.

## 4.2 AIMS OF THIS CHAPTER

The feasibility of developing a lateral flow test strip prototype for measurement of urinary TAP using the MICT® system will be investigated.

The development of this test will involve profiling optimal concentrations and conditions for use of the same biological materials used in the ELISA prototype, detailed in chapter 3. Conjugation to SPMPs and buffer formulation, which could potentially deliver a quantified TAP concentration within 45 minutes, will also be described. This would offer a significant improvement on the ELISA protocol time and contribute to the important clinical requirement for a device that can report a rapid TAP result.

## 4.3 RESULTS

### 4.3.1 Technical evaluation of MICT® instrument using a commercially available test

Several rapid point-of-care diagnostic tests are currently available to purchase from MagnaBioSciences for detection of biomarkers of acute myocardial infarction and hormones involved in stimulation and maturation of the human reproductive system. A commercial test for cardiac troponin I (cTnI) was selected for internal evaluation of the MICT® system, whereby the manufacturer's performance claims, as stated in the product instructions for use (IFU), would be assessed. In addition to intra and inter-assay precision testing, this evaluation also established a linear measuring range, sample recovery and stability (recombinant cTnI used) and a laboratory reference interval for testing of patient samples. Intra- and inter-assay precision returned higher results (%CV = 14 and 15%, respectively) when compared to the precision characteristics stated in the product IFU. However, a %CV = <20% was still acceptable for further use of this test system as a development tool. An overview of results from the evaluation is displayed below in Table 4.3.1.1.

**Table 4.3.1.1** Cardiac Troponin I technical evaluation - overview of results:

| Parameter | Result | |
|---|---|---|
| ***Intra-assay precision*** | Lot #509B0015:<br>CV Mean 14% (min 10%, max 19%) – 3 samples; triplicate; 5 days; 1 reader; 2 operators | |
| ***Inter-assay precision*** | CV Mean 15% (min 11%, max 19%) – 3 samples; 3 replicates x 5 days | |
| | CV Mean 15% (min 8%, max 23%) – 3 samples; 17 replicates x 1 day, 3 separate lots | |
| ***Sensitivity (Limit of Quantification)*** | Mean LoQ 0.14 ng/mL - Mean Analyte Concentration (n = 20) + 2*SD | |
| ***Linearity*** | Linear fit from 0.2 – 28 ng/mL. Mean Recovery = 121%. | |
| ***Reference Interval*** | 0 – 0.19 ng/mL (n=21) | |
| ***Spike Recovery*** | Heparin Plasma (LOW) | Mean Recovery 113%<br>(Measured Conc. 7.9 ng/mL, Theoretical Conc. 7.0 ng/mL; n = 3) |
| | Heparin Plasma (MED) | Mean Recovery 108%<br>(Measured Conc. 15.2 ng/mL, Theoretical Conc. 14.0 ng/mL; n = 3) |
| | Heparin Plasma (HIGH) | Mean Recovery 101%<br>(Measured Conc. 28.3 ng/mL, Theoretical Conc. 28.0 ng/mL; n = 3) |
| ***Sample Stability*** | Freeze Thaw (LOW) | Recovery 96% after 1 x FT cycle (lithium heparin plasma) |
| | Freeze Thaw (MED) | Recovery 82% after 1 x FT cycle (lithium heparin plasma) |
| | Freeze Thaw (HIGH) | Recovery 88% after 1 x FT cycle (lithium heparin plasma) |

## 4.3.2 Optimisation of LFIA test conditions using rabbit IgG control line configuration

### 4.3.2.1 Covalent coupling of polyclonal rabbit IgG to carboxylated superparamagnetic particles and optimisation of conjugate dilution

Estapor® (Merck) carboxyl-modified superparamagnetic particles (diameter = 200 nm) were used for coupling to primary amines (-$NH_2$) of the rabbit IgG antibody (see Figure 4.3.2.1 (a)). This conjugate would be utilised as part of the control line in Prototype 1 of the lateral flow assay. The particles were conjugated with 310 µg of IgG per 3 mg of particles, which was optimised for use as per the manufacturer's generic protocol, described in section 2.2.8.

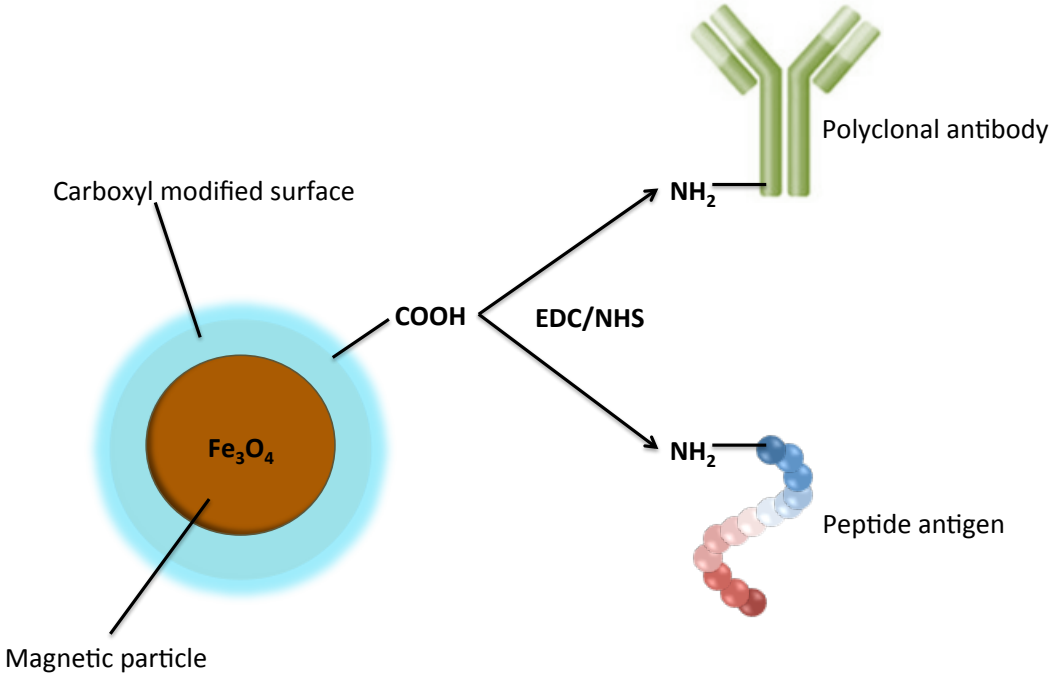



**Figure 4.3.2.1 (a)** Carboxyl-amine group coupling of antibodies or TAP antigen to SPMPs. EDC cross-linker activates carboxyl groups coated on SPMPs in preparation for reaction with primary amine groups of antibodies/target antigen. NHS stabilises this reaction, aiding in the immobilisation of antibodies and/or antigen to the particle surface. A proprietary lyophilisation buffer (LYOPH), which is available commercially to EKF Diagnostics through a partner company, was used for initial assessment of the rabbit IgG-SPMP "control" conjugate. This specialised buffer was developed for freeze-drying of protein conjugates, resulting in improved stability and reproducibility, reduced handling and decreased risk of contamination. It was envisaged that any lateral flow assay prototype would ultimately use this buffer should production scale-up and commercialisation ensue. The conjugate was titrated based on previous knowledge of working dilutions and tested with goat anti-rabbit (GAR) IgG capture antibody spotted at 1 mg/mL on the nitrocellulose membrane. Results are shown in Figure 4.3.2.1 (b) below.

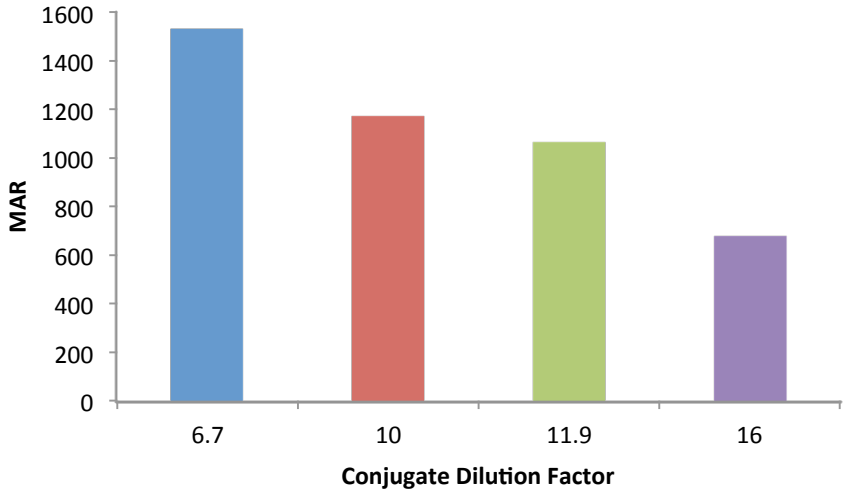



**Figure 4.3.2.1 (b)** Optimisation of rabbit IgG-SPMP conjugate dilution. Dilutions tested were based on previous internal work with similar conjugates at EKF Diagnostics. A 1/16 dilution provided a MAR response adequately within the required response range of 600 - 800 MAR and was selected for use in Prototype 1.

### 4.3.2.2 Selection of optimal conjugate diluent buffer

When formulating the conjugate dilution buffer, it is essential to include components that will maximise uniform distribution of the magnetic particles in the sample solution. Use of detergents such as Tween20 increase the viscosity of the solution, enhancing the homogeneity and maintaining a medium-fast sample flow across the NC membrane. Inclusion of a blocker protein, such as BSA, is also recommended to reduce the possibility of non-specific binding to the NC membrane. Multiple dilution buffers, including PBS (0.01M, pH 7.3); TBS (0.02M, pH 8.2) containing NaCl (0.14M) and Tween20 (1.1%, w/v) (TBST); TBST containing BSA (0.5%, w/v) (TBSTB); Tris buffer containing NaCl (0.15M) and Tween20 (0.5%, w/v) (TNT) and TNT containing BSA (0.5%, w/v) (TNTB) were evaluated for their effects on MAR response at the control line position when tested with the rabbit IgG-SPMP "control" conjugate. The performance of these buffers was compared with the proprietary buffer (LYOPH) described above in Figure 4.3.2.1 (a).

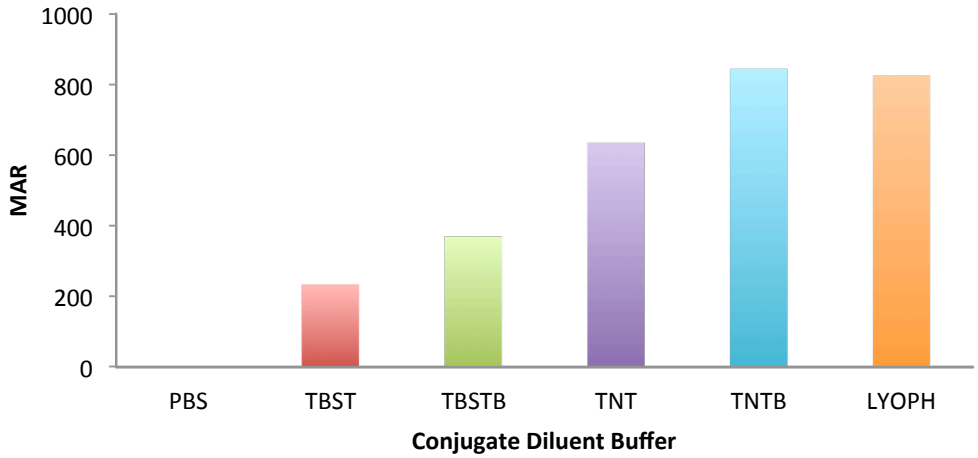



**Figure 4.3.2.2.1** Comparison of conjugate diluent buffer formulations used for preparation of rabbit IgG-SPMP "control" conjugate at a 1/16 dilution. The TNTB buffer returned a MAR response closest to that of the commercial lyophilisation buffer (LYOPH) and thus, was selected for use with Prototype 1.

### 4.3.2.3 Application and optimisation of goat anti-rabbit IgG capture antibody for use on a nitrocellulose membrane at the control line position

Goat anti-rabbit (GAR) IgG was spotted onto the NC membrane at a range of concentrations (1.0, 0.8, 0.5, 0.2, 0.1 mg/mL) via a standard laboratory pipette (see section 2.2.8). MAR response was shown to decrease as GAR concentration was reduced and use of "control" conjugate at 1/16 dilution with 1 mg/mL GAR IgG gave sufficient signal for the control line (approximately 600 - 800 MAR).

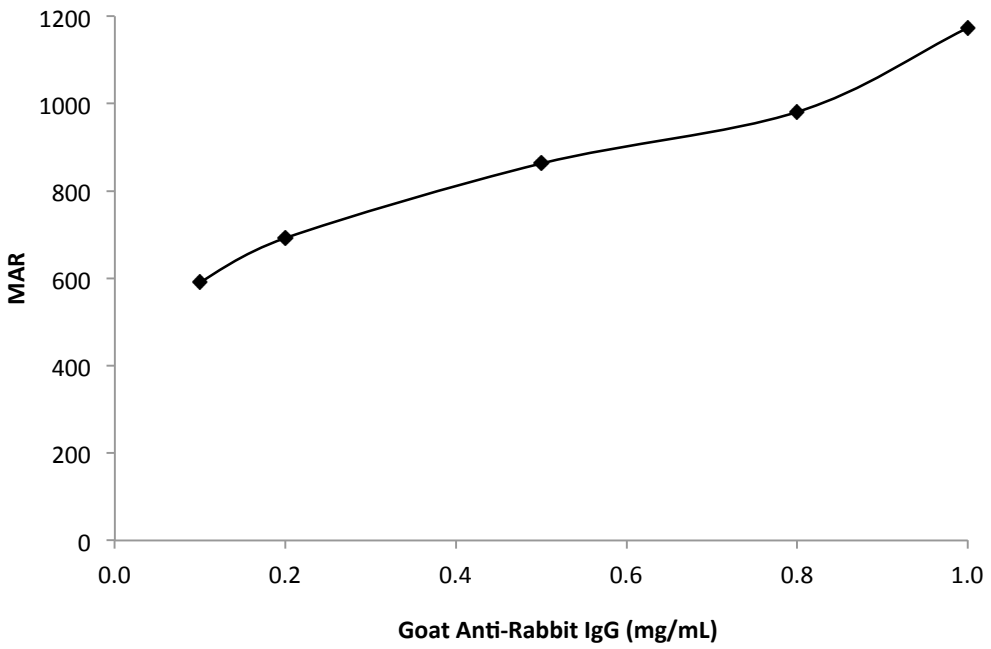



**Figure 4.3.2.3.1** Titration of goat anti-rabbit IgG capture antibody at the control line position. Antibody was prepared in PBS for spotting. A concentration of 1 mg/mL, which gave the highest MAR value, was selected for use in Prototype 1. The rabbit IgG-SPMP "control" conjugate was utilised at a 1/10 dilution for this titration as opposed to the 1/16 dilution employed for subsequent testing events, including those described above in section 4.3.2.1. Unfortunately, there was insufficient GAR material remaining to repeat this experiment at a 1/16 dilution.

The MICT® system is programmed to measure magnetic signal at specific locations on the lateral flow test strip which correspond to the test and control regions. The locations of these positions are communicated to the reader via a barcode printed on each test cassette. To investigate the effect of inaccurate spotting on reported measurements, GAR IgG capture antibody was pipetted up to 2 mm in both directions from the known control line location (43.8 mm). As illustrated in Figure 4.3.2.3.2, spotting at >1 mm in both directions from the control line position resulted in a vast drop in MAR response reported by the MICT® instrument. Therefore, it is highly important to ensure that capture antibody is spotted in the precise location required.

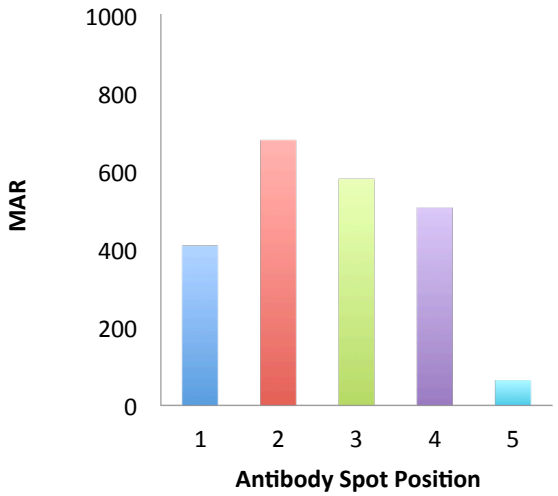



**Figure 4.3.2.3.2** Effect of capture antibody spot position on MAR response. Position 1: 2mm above control line position; Position 2: 1mm above control line position; Position 3: centre of control line position; Position 4: 1mm below control line position; Position 5: 2mm below control line position. Spotting of antibody at the centre or up to 1mm above this position is required to ensure maximum signal intensity.

### 4.3.2.4 Selection of optimal control spot drying conditions

To ensure reproducibility of the lateral flow test strip prototype, it is important to standardise the drying conditions of the antibody spots. The GAR IgG capture antibody was spotted onto the NC membrane as described in section 2.2.9. The wetted membrane was placed into foil pouches and incubated with desiccant to facilitate drying overnight at room temperature (20-25°C) and 37°C. A superior MAR response was observed for strips dried at 20-25°C.

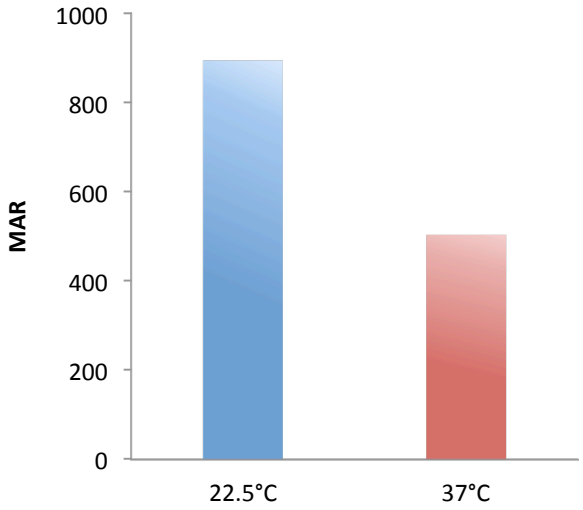



**Figure 4.3.2.4.1** Drying of control line spot overnight at varying incubation temperatures. The GAR IgG capture antibody produced a greater MAR response following drying at 20-25°C. It is possible that the higher temperature of 37°C may have resulted in reduced antibody activity.

#### 4.3.2.5 Immobilisation of "control" conjugate on a glass fibre conjugate pad

To reduce the number of handling steps in the lateral flow assay protocol, drying of the "control" conjugate was attempted by pipetting the diluted conjugate across a clean conjugate pad followed by drying at 37°C for 2 hours prior to assembly in the test strip. On the first assessment, the MAR response was similar between the "wet" and "dry" test methods (638.5 vs. 608.9 MAR), indicating that a dried conjugate format could be pursued. However, on the second attempt responses of 1333.5 MAR and 369.8 MAR were recorded at the test and control line positions, respectively. There was clearly aggregation of SPMPs following release from the pad and, consequently, increased signal at the test line (the first position reached during sample flow). Due to the dragging and deposition of the SPMPs at this location, the control line signal was greatly reduced. To establish the feasibility of a working prototype, it was decided that the liquid format would be sufficient for current development requirements.

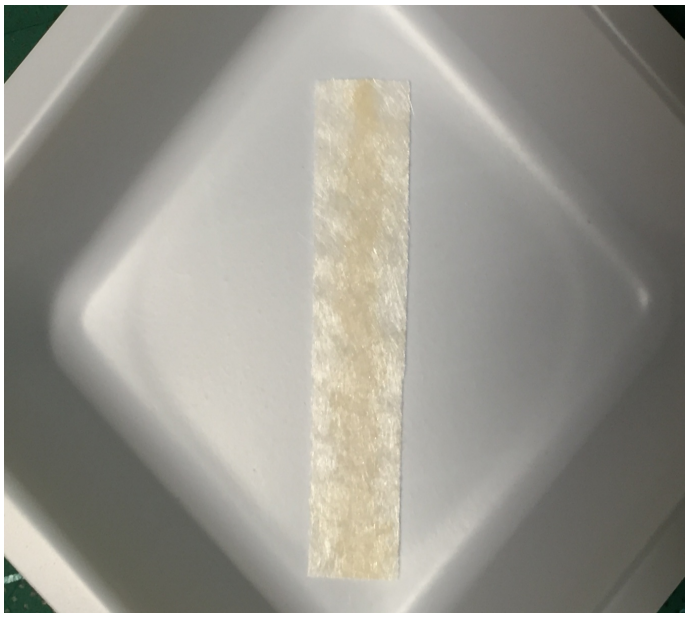


**Figure 4.3.2.5.1** Glass fibre conjugate pad following loading and drying of rabbit IgG-SPMP "control" conjugate. This pad was sufficient for use in 10 separate test strips, each of 5mm width.

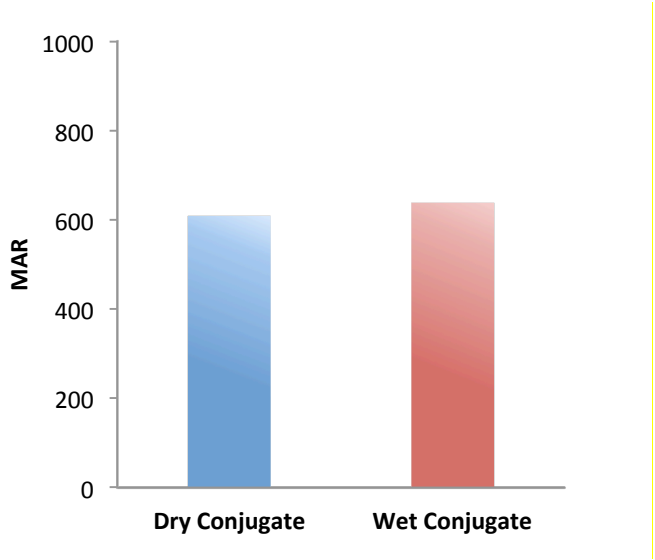



**Figure 4.3.2.5.2** Comparison of lateral flow test strips when using "dry" and "wet" rabbit IgG-SPMP "control" conjugate – first assessment.

### 4.3.3 Optimisation of LFIA test conditions for the test line configuration

#### 4.3.3.1 Covalent coupling of TAP antigen to carboxylated superparamagnetic particles and optimisation of conjugate dilution

Estapor carboxyl-modified SPMPs were used for coupling to primary amines (-$NH_2$) of the TAP antigen, as per sections 2.2.7 and 4.3.2. The particles were conjugated with 0.31, 0.47, 0.94 and 1.5 mg of peptide per 3 mg of particles and the resulting TAP-SPMP conjugates were tested at various dilutions, as shown in Figure 4.3.5.1. The 0.31 and 0.94 mg TAP-SPMP conjugates returned lower MAR response for their respective 1/2 and 1/3 dilutions when compared to the other conjugate preparations and thus, were deemed inadequate for further use. The 1.5 mg TAP-SPMP conjugate maintained a response >900 MAR up to a 1/4 dilution, demonstrating a greater likelihood that the SPMPs had been saturated by the TAP antigen. Further increases in dilution were required to reduce the MAR response for this conjugate when compared to the other TAP-SPMP concentrations. However, the 0.47 mg TAP-SPMP conjugate was able to closely match this response at 1/2 and 1/3 dilutions and thus was selected for further use due to the lower peptide stock requirement.

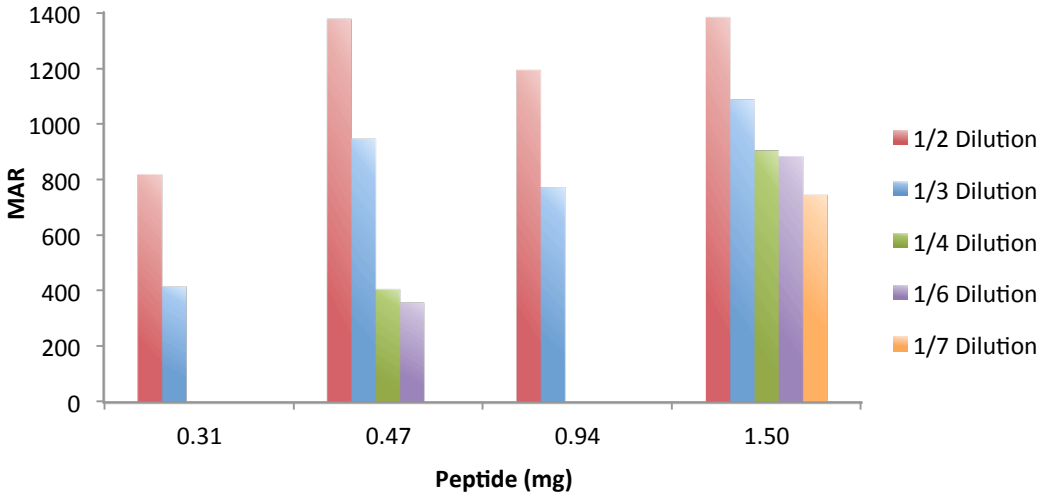



**Figure 4.3.3.1.1** Titration of TAP antigen used in SPMP conjugation protocol and optimisation of TAP-SPMP "test" conjugate dilution. An approximate response of 1000 MAR was targeted for the test line. The 0.47 mg TAP-SPMP conjugate was selected for further use due to similar MAR response seen for 1/2 and 1/3 dilutions when compared to use of 1.50 mg of TAP.

### 4.3.3.2 Titration of streptavidin and biotinylated anti-TAP capture antibody on nitrocellulose membrane at the test line position

Streptavidin protein was prepared at concentrations of 10, 5, 2 and 1 mg/mL in PBS, containing 0.5% (v/v) green dye before spotting onto NC membrane (section 2.2.9). The MAR response decreased as streptavidin concentration increased, potentially demonstrating that saturation of membrane had occurred at 1 mg/mL. The variation in MAR response between replicates may also have contributed to this decreasing trend, e.g. for 10 mg/mL streptavidin, the %CV between replicates was 72%. Nonetheless, use of streptavidin at 1 mg/mL has been demonstrated as an effective working concentration previously (Holstein *et al.*, 2015) and would serve the requirements for development feasibility.

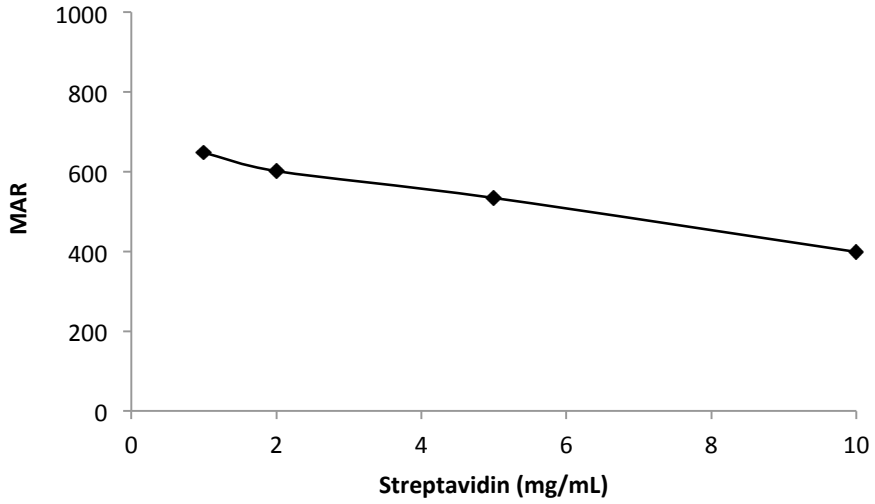



**Figure 4.3.3.2.1** Titration of streptavidin coating concentration on the NC membrane. Biotinylated anti-TAP capture antibody was used at 1 mg/mL. The highest MAR response was observed for 1 mg/mL concentration and was considered sufficient for further use at test line.

Biotinylated anti-TAP capture antibody was prepared at 1.0, 0.8, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL concentrations in PBS prior to spotting onto the streptavidin-coated NC membrane (section 2.2.9). As expected, the MAR response decreased as capture antibody concentration was reduced. Due to the high cost of producing the biotinylated material via a commercial kit and the similar responses recorded for spotting concentrations of 1.0 and 0.8 mg/mL, use of the lower concentration of antibody was selected to reduce project costs. The TAP-SPMP "test" conjugate was utilised at a 1/4 dilution for this titration as opposed to the 1/2 dilution employed for subsequent testing events. This was simply due to initial performance testing of the TAP-SPMP conjugate being completed before the larger scale conjugate titration described above in section 4.3.3.1.

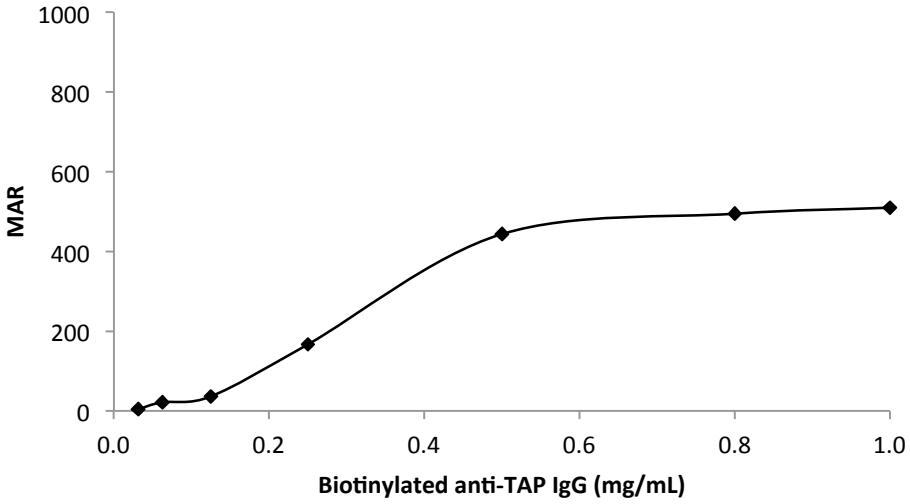



**Figure 4.3.3.2.2 (a)** Titration of biotinylated anti-TAP capture antibody in conjunction with 1 mg/mL streptavidin. A concentration of 0.8 mg/mL was selected for further use as minimal difference in MAR response was observed versus 1.0 mg/mL.

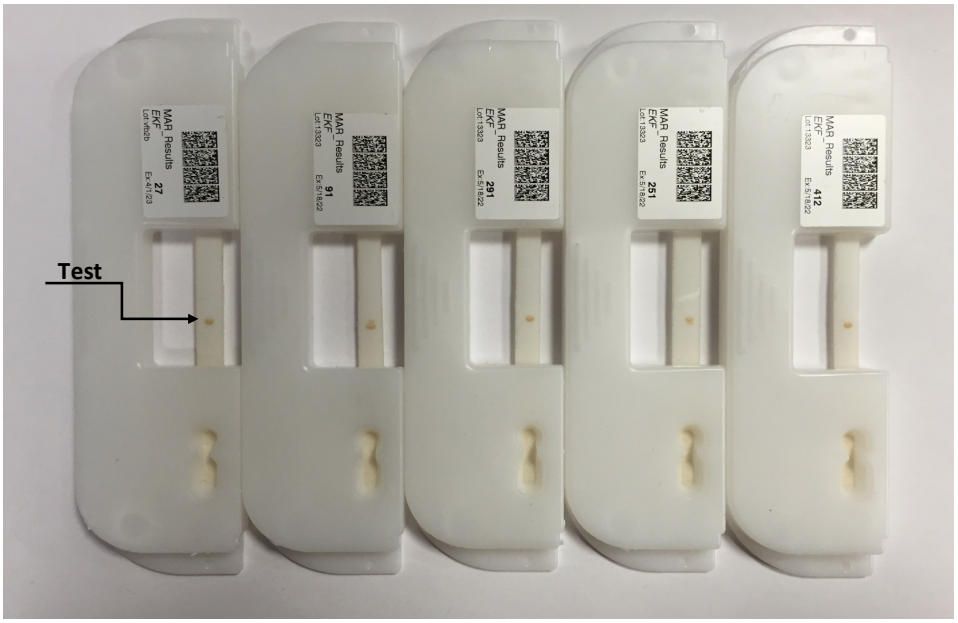



**Figure 4.3.3.2.2 (b)** TAP test line spot devices following incubation for 45 minutes. Visual spot formation was strong following optimisation of streptavidin and biotinylated anti-TAP antibody spot concentrations.

#### 4.3.3.3 Selection of optimal test line spot drying conditions

Numerous drying conditions were assessed for the test line spot in an effort to compare both the incubation conditions used for the ELISA prototype (section 2.2.6) and, other successful lateral flow devices dried at varying temperatures (Pattarawarapan *et al*., 2007) and 37°C (Holstein *et al*., 2015). The incubation/drying conditions investigated were as follows: Condition 1: Streptavidin for 2 hours at 37°C, biotinylated anti-TAP antibody overnight at 37°C; Condition 2: Streptavidin for 2 hours at 20-25°C, biotinylated anti-TAP antibody overnight at 20-25°C; Condition 3: Streptavidin and biotinylated anti-TAP antibody together at 37°C overnight; Condition 4: Streptavidin and biotinylated anti-TAP antibody together at 20-25°C overnight; Condition 5: Streptavidin overnight at 37°C and biotinylated anti-TAP antibody for 2 hours at 37°C; Condition 6: Streptavidin for 24 hours at 20-25°C and biotinylated anti-TAP antibody for 24 hours at 20-25°C (separate days); Condition 7: Streptavidin for 24 hours at 37°C and biotinylated anti-TAP antibody for 24 hours at 37°C (separate days).

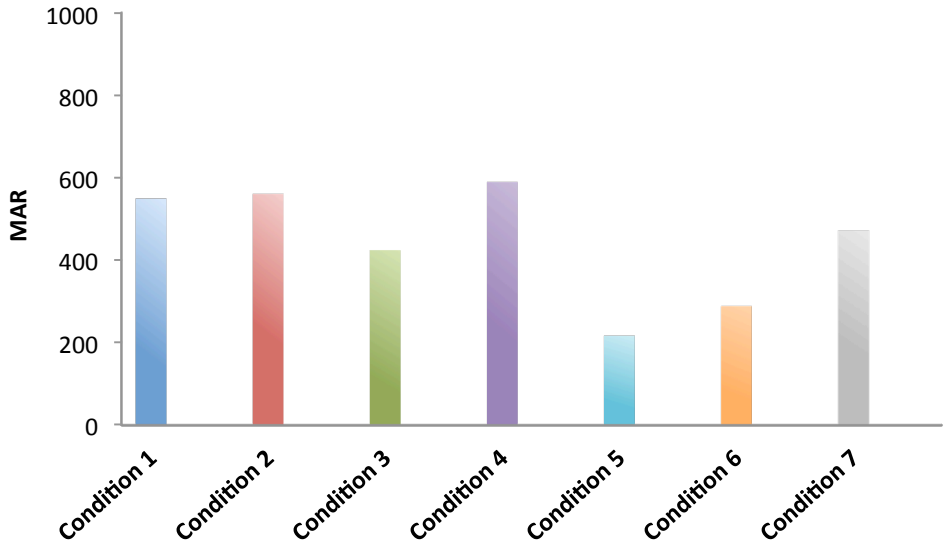



**Figure 4.3.3.3.1** Drying of test line spot using various conditions. Condition 4, whereby the streptavidin and biotinylated anti-TAP capture antibody were incubated together overnight at room temperature, was selected for final test strip preparation. This condition was also selected considering a drying temperature of 37°C appeared to reduce signal for control line spot result (Figure 4.3.2.4.1).

### 4.3.4 Complete LFIA device testing – test and control lines (Prototype 1)

#### 4.3.4.1 Titration of test and control line configurations in combination

Following separate optimisation of test and control line configurations, as per sections 4.3.2 and 4.3.3, finalised lateral flow test strips were assembled containing both test and control spotted reagents. A decrease in the control line MAR response was observed for these devices when compared to testing of the control line alone, as seen in Figure 4.3.2.5.2, for example. Alternative dilutions were tested to increase MAR response for the "control" conjugate in combination with the "test" conjugate (Figure 4.3.4.1.1). However, it was noted that decreasing the "control" conjugate dilution also increased MAR response at the test line, indicating that there was possible cross-reactivity between the test and control lines. A reliable independent control line is an important part of any LFIA POC device, particularly when the device is designed to provide a diagnostic outcome whilst operated by untrained individuals. A specified measuring range can be included in the barcode software of the finalised test to indicate that a device has met the required response at the control line and therefore, the test result can be considered as valid. Hence, the control line ensures that the test strip has been run successfully. Before investigating the potential specificity issue further, it was decided to continue with evaluation of the prototype device to assess performance with TAP antigen-spiked urine samples.

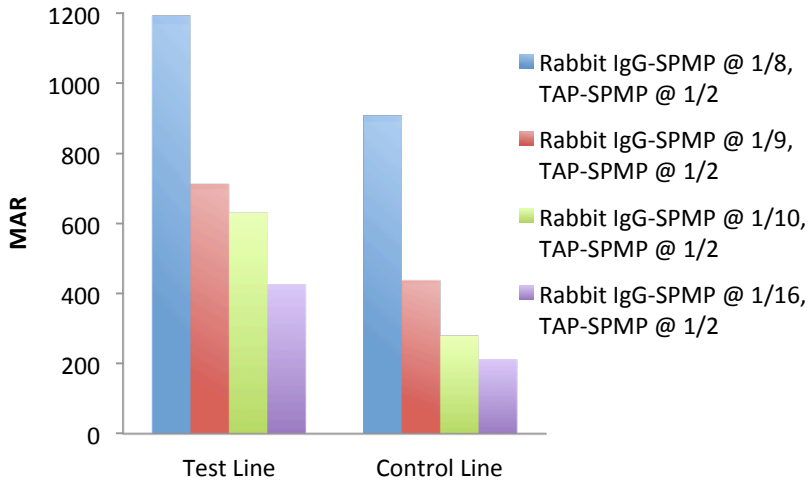



**Figure 4.3.4.1.1** Titration of rabbit IgG-SPMP "control" conjugate with lateral flow test strip devices prepared with test and control line configurations (Prototype 1). TAP-SPMP "test"conjugate remained at 1/2 dilution throughout testing. All diluted conjugates were mixed with PBS prior to loading to test devices (see section 2.2.11). Decreasing "control" conjugate dilution increased MAR response at both the test and control line positions. The combination of "test" and "control" conjugates at 1/2 and 1/8 dilutions, respectively, was selected for use in TAP antigen dose response testing of urine samples.


**Figure 4.3.4.1.2** Lateral flow test strips (Prototype 1) showing test and control line spot. Visual spot formation appeared to be proportional to the intensity of MAR response, particularly at the control line position.

### 4.3.4.2 Dose response curve testing using GAR / rabbit IgG-SPMP control line and biotinylated anti-TAP / TAP-SPMP test line (Prototype 1)

To determine the performance of the lateral flow test prototype, the urinary TAP QC panel established with the ELISA (section 3.3.9) was tested to generate a dose response curve (QC1 = 207.4, QC2 = 127.3, QC3 = 62.6, QC4 = 25.5 nmol/L, mean concentrations as quantified by ELISA). Successful discrimination of varying TAP concentrations would signify the feasibility of an operational prototype that could quantify TAP antigen in patient samples. Urine samples were added to pooled “test” and “control” conjugates (1/2 and 1/8 dilutions, respectively, as per section 2.2.11) before loading to the test devices and reading with the MICT® system.

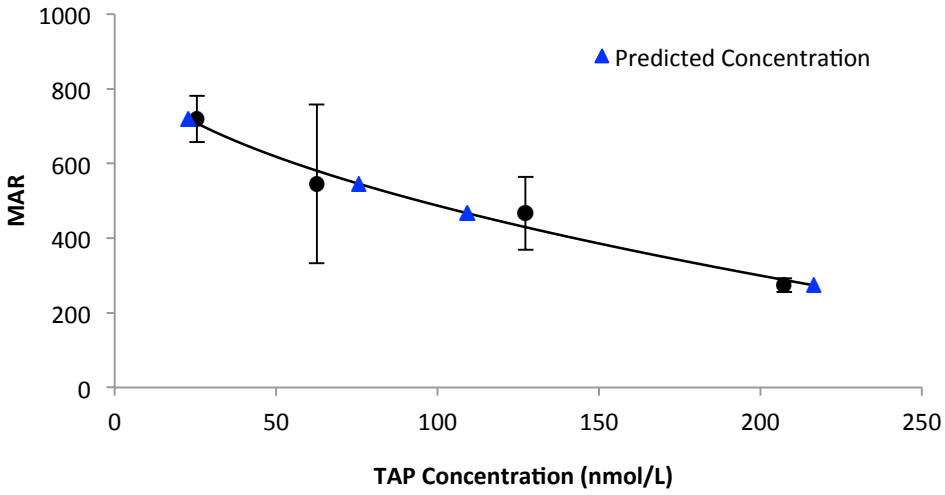



**Figure 4.3.4.2.1** Dose response curve – Test Line. The variability between replicated was %CV = <30%, excluding QC3. A signal response range of 273.8 – 719.3 MAR was demonstrated and some discrimination between QC samples also observed. The back-calculated concentrations for QC1 – 4 were 216.5, 109.3, 75.6, 22.9 nmol/L, respectively, and were similar to the mean concentrations established by the ELISA prototype.

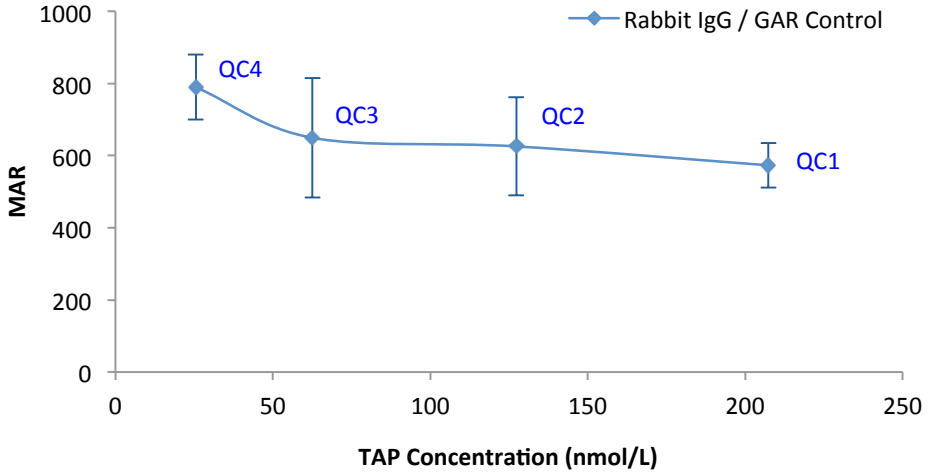



**Figure 4.3.4.2.2** MAR response at control line decreased (from 894.2 – 480.7 MAR) with increasing sample concentration, exhibiting the possibility of cross-reactivity issues due to rabbit IgG-SPMP control conjugate binding to test line capture antibody and therefore reducing the conjugate available to bind and produce signal at the control line position. Potential for aggregation of the "test" and "control" conjugates in the NC membrane also existed. However, this aggregation was not visualised upon inspection of the test strips.

### 4.3.5 Optimisation of LFIA test conditions using chicken IgY control line configuration

#### 4.3.5.1 Covalent coupling of polyclonal chicken IgY to carboxylated superparamagnetic particles and optimisation of dilution for use in lateral flow assay

The unsatisfactory results and potential cross-reactivity issue perceived with prototype 1, lead to the exploration of an alternative control line configuration using a combination of polyclonal chicken IgY-SPMP conjugate and a goat anti-chicken IgY capture antibody. There were several reasons for the selection of chicken IgY for evaluation. In traditional mammalian antibody generation, the animal must be repeatedly bled to obtain large quantities of a target antibody whereas chicken antibodies are transferred from serum into egg yolk, making them easier to obtain in large quantities and so avoiding stress to the animal. Moreover, through the presence of an extra fragment crystallisable (Fc) domain, chicken IgY is not bound by mammalian antibodies such as rheumatoid factor or human anti-mouse antibodies (HAMA), both of which can potentially be found in patient samples (incidence of <1 - 10%) and can be a source of false positives in diagnostic tests (Kricka, 1999; Narat, 2003; Ismail, 2009). Chicken IgY was coupled to carboxyl-modified SPMPs via primary amines as described by sections 4.3.2.1 (a) and 2.2.8.

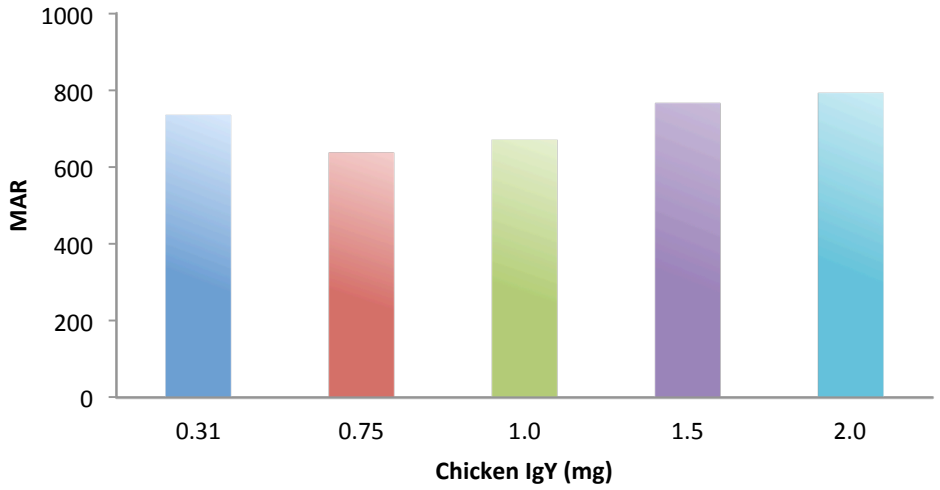



**Figure 4.3.5.1.1** Titration of chicken IgY antibody conjugated to SPMPs. Increasing the amount of chicken IgY antibody used in the conjugation protocol beyond 0.31 mg did not increase the MAR response, suggesting saturation of the particles. Chicken IgY at a concentration of 0.31mg was selected for preparation of the control conjugate in Prototype 2.

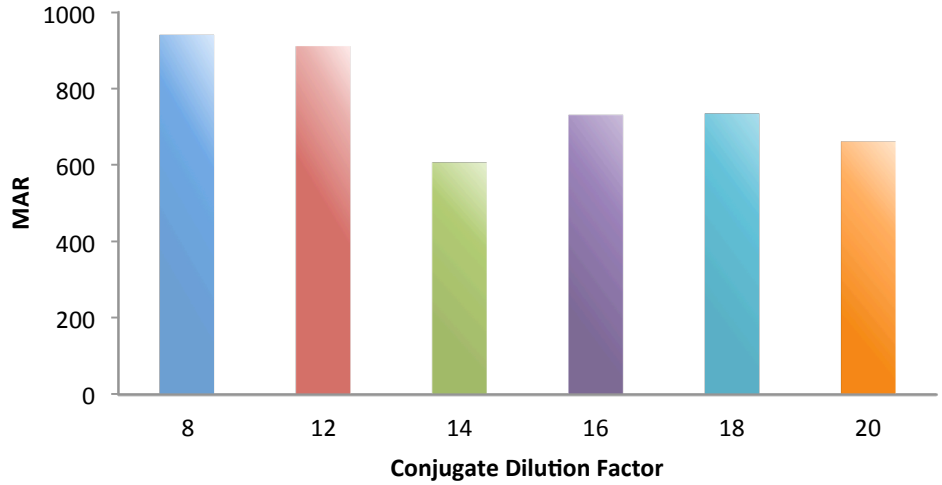



**Figure 4.3.5.1.2** Optimisation of chicken IgY-SPMP conjugate dilution in TNTB buffer. Due to a superior precision between replicates (CV = 14% vs 31%) and a MAR response adequately within the required range (600 - 800 MAR), a 1/18 dilution was selected ahead of a 1/16 dilution for use of the chicken IgY-SPMP conjugate in Prototype 2.

#### 4.3.5.2 Titration of goat anti-chicken IgY capture antibody

Goat anti-chicken (GAC) IgY was spotted onto the NC membrane at a range of concentrations (1.0, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1 mg/mL) via a standard laboratory pipette (see section 2.2.9) and tested with the chicken IgY-SPMP "control" conjugate at a 1/18 dilution.

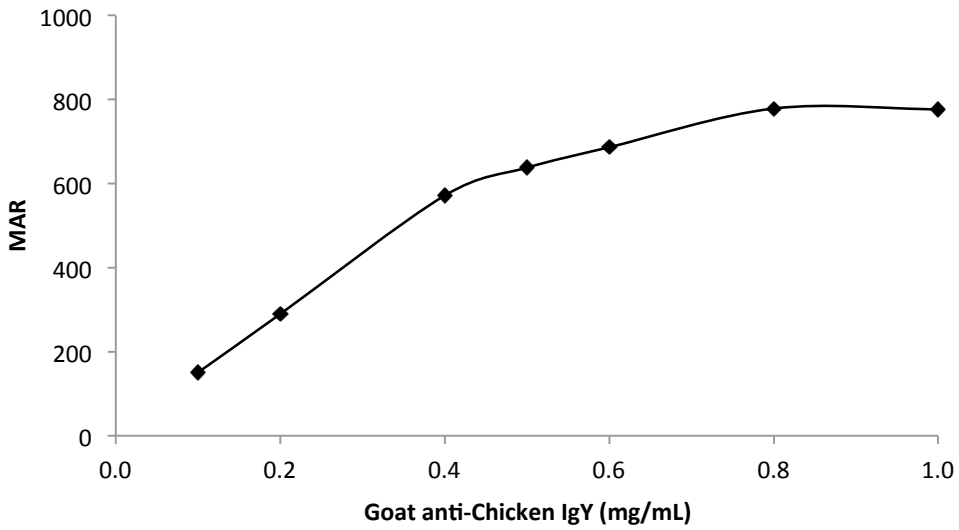



**Figure 4.3.5.2.1** Titration of GAC IgY capture antibody. Antibody was spotted in PBS. As for GAR titration (see 4.3.2.3), concentrations < 1 mg/mL could potentially be used for test strip device. However, internal observations from previous development work have shown that it is generally preferable to maintain antibody concentration at 1 mg/mL for specialised instruments used in production of lateral flow test strips. Consequently, a concentration of 1 mg/mL was selected for use in Prototype 2.

#### 4.3.5.3 Comparison of complete lateral flow test strip device - Prototype 2 vs Prototype 1

Following optimisation of the control line configuration for Prototype 2, finalised lateral flow test strips were assembled containing both test and control spotted regions. The results were compared with Prototype 1.

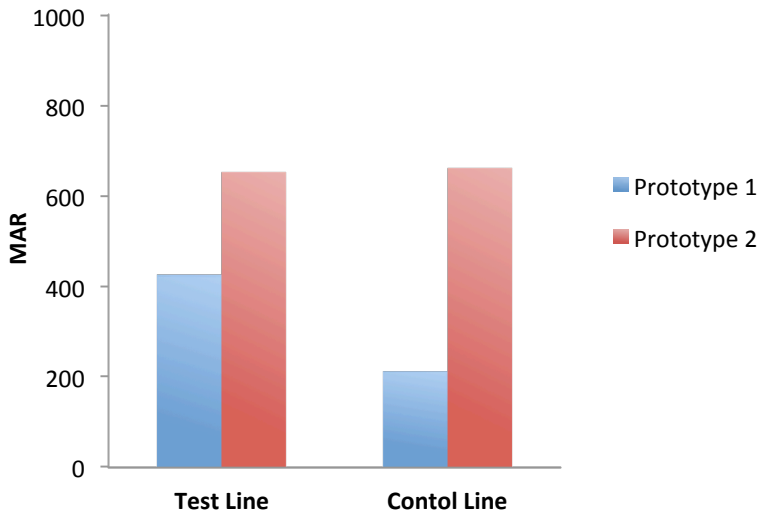



**Figure 4.3.5.3.1** Testing of Prototype 2 lateral flow test strip device compared with previous testing for Prototype 1 (taken from Figure 4.3.4.1). The test and control line configurations of Prototype 2 appeared to operate independently, returning MAR response of 652.1 and 661.1, respectively. The control MAR was similar (661.1 vs. 735.6 MAR) to testing performed with control line only devices (see Figure 4.3.5.2.1). These results were superior to Prototype 1 response of 425.9 and 212.6 MAR for 1/2 and 1/16 dilutions of "test" and "control" conjugates, respectively. These Prototype 1 results were specifically selected for comparison of test performance at the highest "control" conjugate dilution. Higher dilutions were sought to limit the amount of particles present, and reduce the opportunity to impede sample flow across the test strip. Further cross-reactivity testing was required to confirm the Prototype 2 result.

#### 4.3.5.4 Dose response curve testing using GAC / chicken IgY-SPMP control line and biotinylated anti-TAP / TAP-SPMP test line (Prototype 2) – initial assessment

To determine the performance of this lateral flow test prototype, the urinary TAP QC panel samples were added to pooled "test" and "control" conjugates (1/2 and 1/18 dilutions, respectively, as per section 2.2.12) before loading to the test devices and reading with the MICT® system.

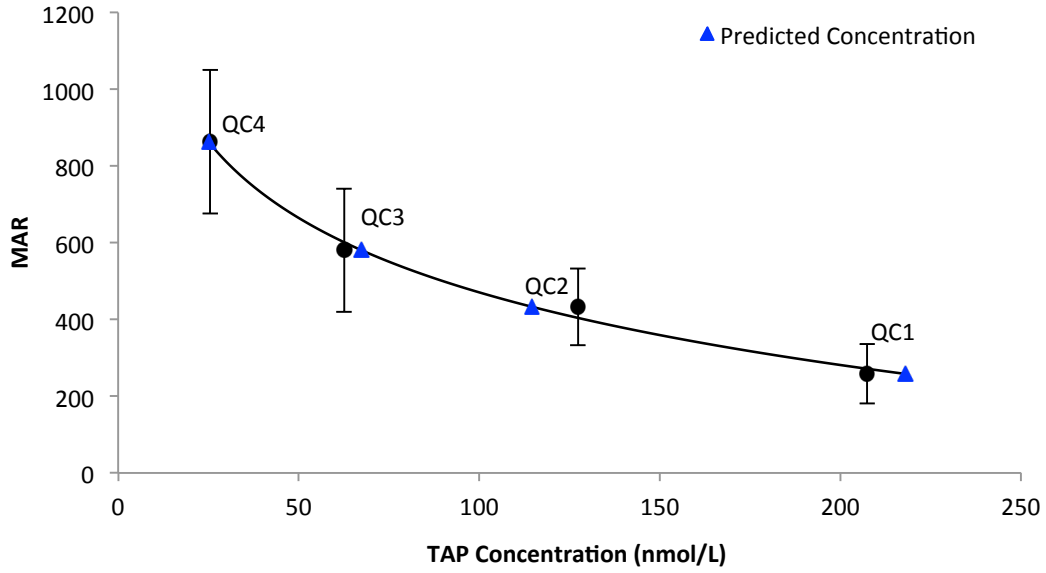



**Figure 4.3.5.4.1** Dose response curve – Test Line. Discrimination between QC samples (MAR range = 258.2 – 862.7) was improved when compared to Prototype 1 (MAR range = 273.8 – 719.3 MAR), particularly at the lower end of the concentration range (QC1, 25.5 nmol/L). The back-calculated concentrations for QC1 – 4 were 217.9, 114.5, 67.4, 25.1 nmol/L, respectively and were very similar to the mean concentrations established by the ELISA prototype (QC1 = 207.4, QC2 = 127.3, QC3 = 62.6, QC4 = 25.5 nmol/L). The improved sensitivity demonstrated would allow users to distinguish between "normal" patients and those with acute pancreatitis.

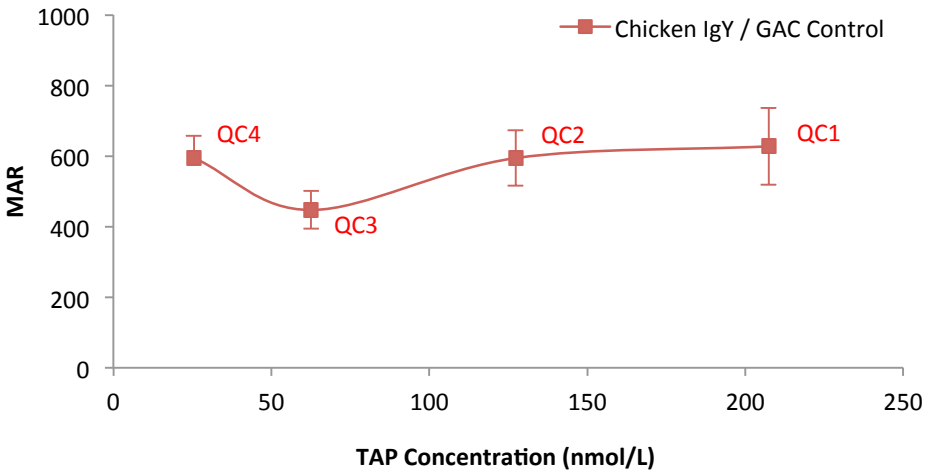



**Figure 4.3.5.4.2** Control line MAR response remained relatively constant (565.9 mean MAR), with the exception of response for QC3 (447.2 MAR). The variation between replicates for each QC sample tested was %CV = <20%, demonstrating good precision.

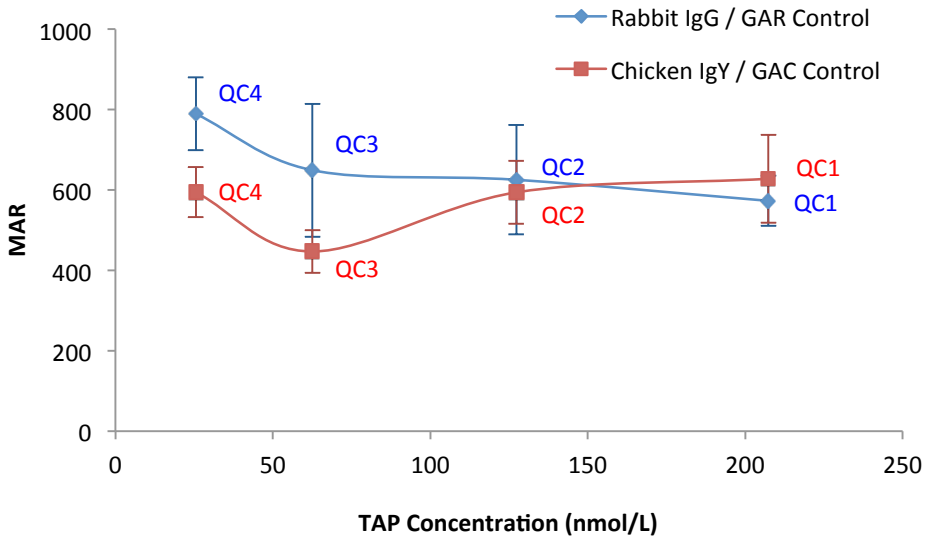



**Figure 4.3.5.4.3** Overlay of control line response for lateral flow prototypes 1 and 2. The use of chicken IgY-SPMP/GAC capture antibody control line configuration for Prototype 2 resulted in a more constant control line response when compared to prototype 1. The control line for prototype 2 therefore appeared to operate independently of the test line.

### 4.3.6 Cross-reactivity testing of biotinylated anti-TAP, goat anti-rabbit IgG and goat anti-chicken IgY capture antibodies for non-specific binding to "test" and "control" conjugates

To ensure that signal increase at the test and control lines was specific, "control" conjugate was incubated independently with spotted anti-TAP capture antibody test strips and, "test" conjugate was incubated independently with spotted GAR IgG and GAC IgY antibody test strips. This process was also repeated for test strips sprayed using the Linomat 5. MAR response was subsequently recorded and compared. For both Prototype 1 and 2, the MAR response for binding of test line to "control" conjugates was low (<20 MAR), demonstrating no non-specific binding. GAR IgG control line binding to 'test" conjugate was comparable (~20 MAR), with higher response recorded for GAC IgY binding of "test" conjugate (~43 MAR). Subsequent comparison of spot and stripe test devices with an increased dilution of TAP-SPMP "test" conjugate indicated that the response at GAC IgY control line could be decreased further (see Figure 4.3.6.3). It is anticipated that the MAR response observed in these experiments would be lowered further through optimisation of the test and control line spraying method using specialised instrumentation for lateral flow test strip preparation.

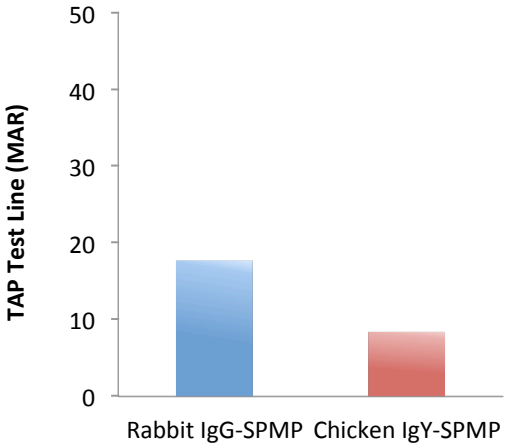



**Figure 4.3.6.1** Specificity testing of biotinylated anti-TAP capture antibody with rabbit IgG and chicken IgY-SPMP "control" conjugates. Minimal cross-reactivity (MAR = <20) was recorded for the anti-TAP capture antibody binding to both "control" conjugates. The MAR response was even lower for binding of chicken IgY-SPMP (<10 MAR) and was considered negligible as it was close to background noise within this lateral flow immunoassay. Rabbit IgG and chicken IgY "control" conjugates were utilised at 1/16 and 1/18 dilutions, respectively.

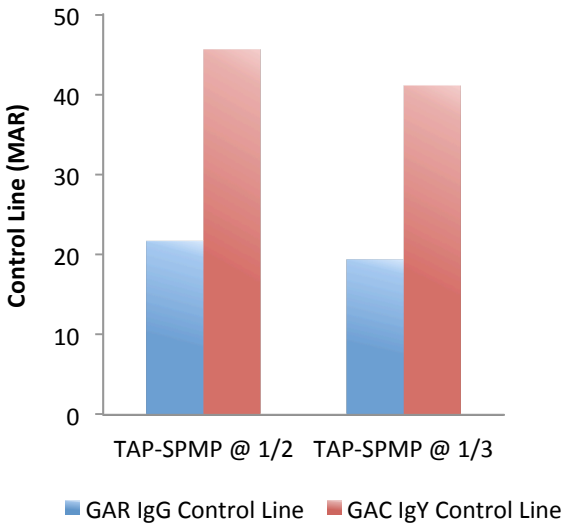



**Figure 4.3.6.2** Specificity testing of GAR IgG and GAC IgY capture antibodies with TAP-SPMP "test" conjugate. Response for GAC IgY control line was double that of GAR IgG control line. Increasing dilution of TAP-SPMP "test" conjugate to a 1/3 dilution marginally lowered MAR response for both prototypes. Further optimisation was investigated as shown in Figure 4.3.6.3.

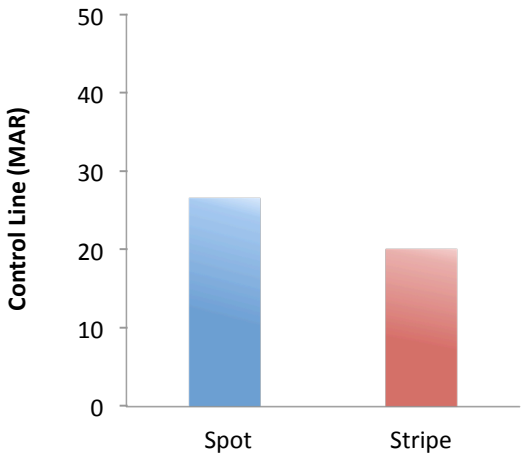



**Figure 4.3.6.3** Specificity testing – spot versus stripe. GAC IgY capture antibody-spotted and striped devices were tested with TAP-SPMP "test" conjugate at a 1/4 dilution as a continuation of testing from Figure 4.3.6.2. The response for each device format was: Spot device = 26.6 MAR, stripe device = 20.1 MAR. Increasing the conjugate dilution from the 1/2 and 1/3 dilutions, shown in Figure 4.3.6.2, reduced the number of particles present in the test strip, and using the Linomat5 for automated striping of the capture antibody improved the non-specific signal further. Further improvement could reasonably be expected following preparation of test strips with more specialised instrumentation specific to lateral flow requirements, such as the Isoflow™ reagent dispenser system.

### 4.3.7 Comparison of test and control line performance - spot vs stripe

In an effort to automate the preparation of the lateral flow test device, the CAMAG Linomat 5 dispenser was utilised for antibody application to the NC membrane. Test line and control lines were striped at 54 and 44mm from the base card end of the absorbent upper wick pad as this was closest the Linomat 5 could conform to the required locations of 54.2 and 43.8 mm, respectively. Striped devices exhibited analogous variation to the spot devices at the control line position and improved precision at the test line position. The MAR response was increased at both positions, offering the opportunity to increase conjugate dilutions and thus decrease the amount of particles present in the test system, which in turn would facilitate a cleaner signal.

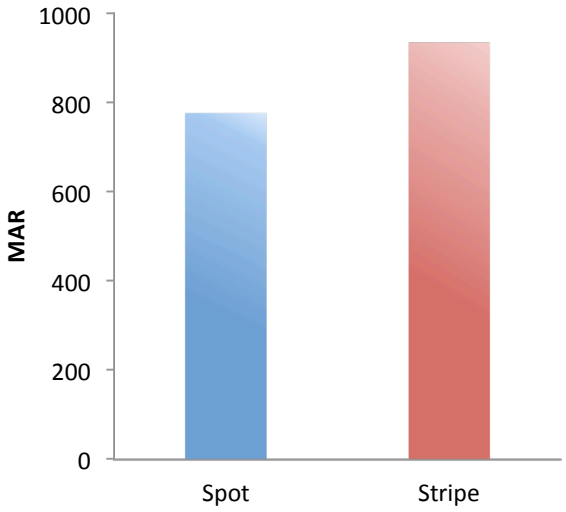



**Figure 4.3.7.1 (a)** Control line response following spotting and striping of the GAC IgY capture antibody on the NC membrane. The variation between replicates for striped devices was consistent with spot devices (%CV = 18 vs. 17%) and an increased mean MAR response was observed (934.7 vs. 776.3 MAR). The control conjugate was utilised at a 1/18 dilution.


**Figure 4.3.7.1 (b)** Lateral flow test devices with GAC IgY control line striped by Linomat.

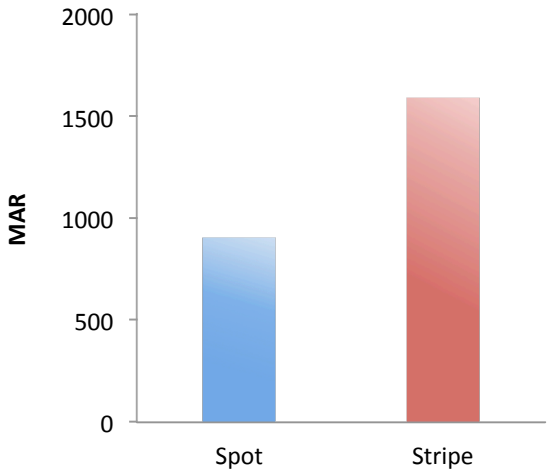



**Figure 4.3.7.2 (a)** Test line response following spotting and striping of streptavidin and biotinylated anti-TAP capture antibody on a NC membrane. The variation between replicates for the striped devices was superior to spot devices (%CV = 13 vs. 21%) and increased mean MAR response was observed (1588.9 vs. 904.6 MAR). Test conjugate was utilised at a 1/4 dilution.


**Figure 4.3.7.2 (b)** Lateral flow test devices with anti-TAP test line striped by Linomat.

### 4.3.8 Dose response curve testing for Prototype 2 – final assessment

#### 4.3.8.1 Dose response curve testing using lateral flow devices with GAC / chicken IgY-SPMP control line and streptavidin / biotinylated anti-TAP / TAP-SPMP test line (Separate devices – antibody spot)

Lateral flow test strips were prepared with a GAC IgY control line only and test line only for assessment with the urinary TAP QC panel and comparison with complete devices containing both the test and control lines. The QC panel samples were added to the "control" conjugate only at a 1/18 dilution (as per section 2.2.12) and "test" conjugate only at a 1/4 dilution (as per section 2.2.12) before loading to the test devices and reading with the MICT® system. TAP-SPMP conjugate (1.5 mg) was utilised for this test event.

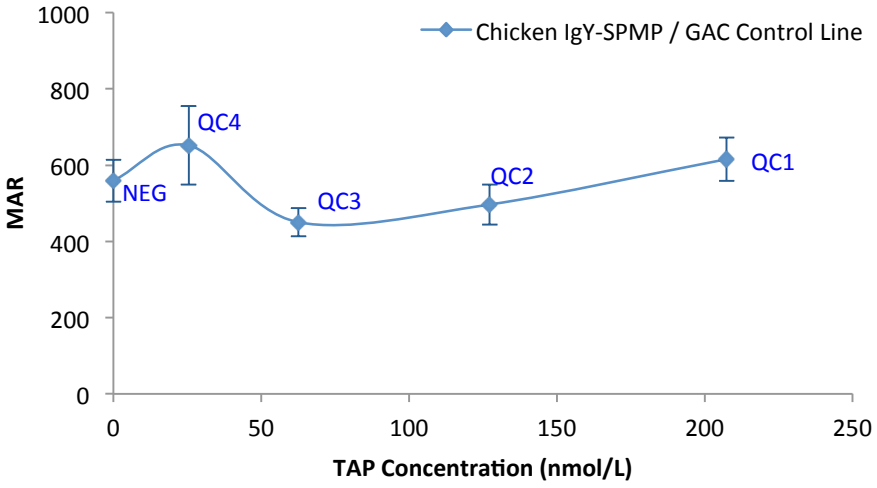



**Figure 4.3.8.1.1** Control line response for spotted device with control line configuration only. The response was lower for QC2 and QC3 samples when compared to QC1, QC4 and negative sample (NEG). Despite this, imprecision and MAR responses for all QC samples were within a satisfactory specification with %CV = <20% and mean control response of ±20%. Although responses bordered the desired specification range (600 – 800 MAR), the signal remained relatively constant across all samples tested and was once again not affected by increasing TAP concentration.

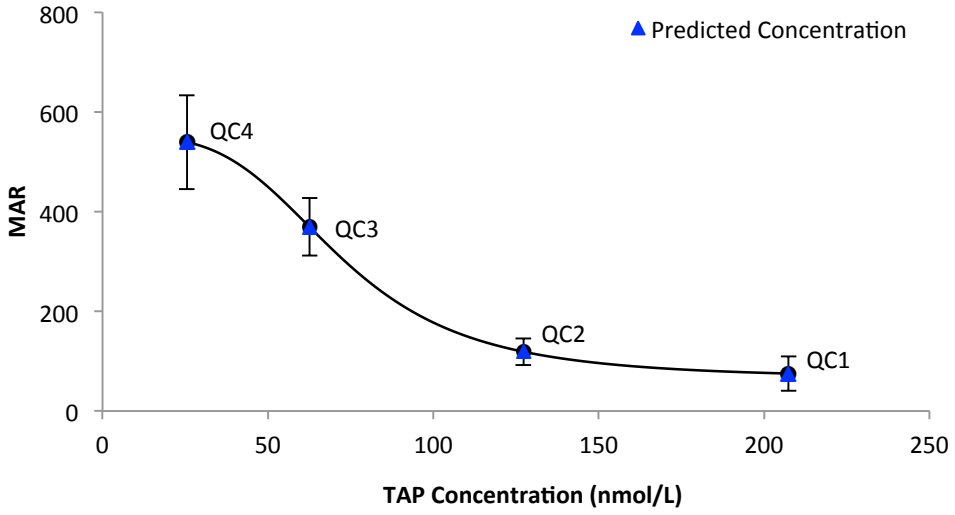



**Figure 4.3.8.1.2** Test line MAR response for spotted device with test line configuration only. Recorded measuring range was 75.2 – 766.0 MAR for QC1 - 4 and negative (NEG) samples, with greater distinction between sample concentrations compared to previous test formats (see Figures 4.3.5.4.1 and 4.3.4.2.1). Imprecision (%CV) between replicates was <20% for the QC3, QC4 and negative samples and, 22% and 46% for QC2 and QC1, respectively. Higher variation observed for QC1 sample was due to variability of MAR signal at the lower end of the measuring range – a common occurrence with most immunoassays where the %CV is higher as values approach zero. Back-calculated QC sample concentrations were uniform with the concentrations established using the ELISA prototype.

### 4.3.8.2 Dose response curve testing using GAC / chicken IgY-SPMP control line and streptavidin / biotinylated anti-TAP / TAP-SPMP test line (Complete device – antibody spot)

Following improved test line performance using 1.5 mg TAP-SPMP "test" conjugate and acceptable precision results for GAC IgY control line on separate devices (section 4.3.8.1), finalised LFIA devices with both test and control lines were assembled for evaluation. In addition, a comparison of spotted versus striped devices was performed. The "test" and "control" conjugates were diluted 1/4 and 1/18, respectively, prior to addition of the urinary TAP QC panel samples, loading onto test devices and, reading with the MICT® system.

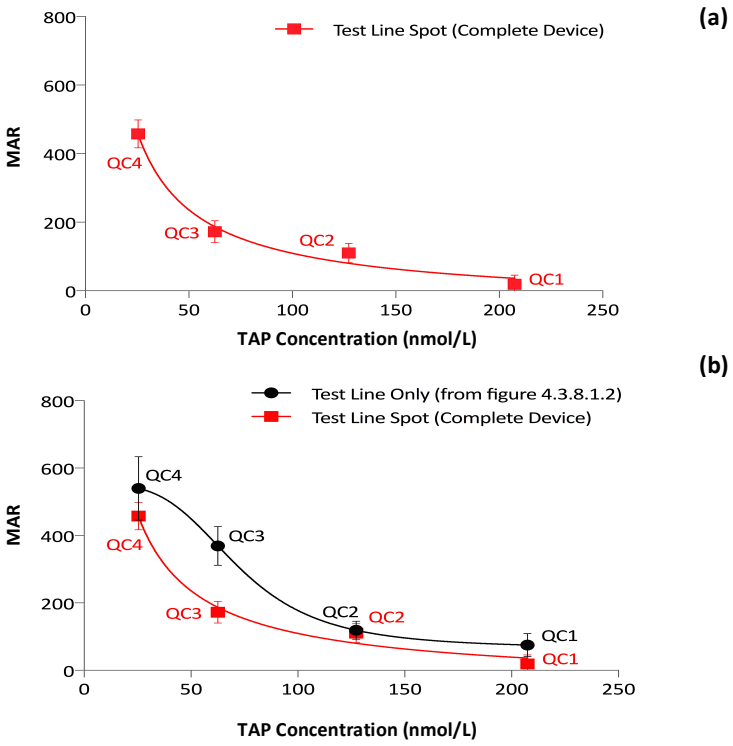



**Figure 4.3.8.2.1** Prototype 2: Test line response for spotted devices. **(a)** Test line response for each QC sample was normalised against the "negative" urine sample response (%MAR/MAR$_0$). Measuring range was found to be 19.0 – 713.5 MAR, with a decreased MAR response observed for QC1 (207.4 nmol/L) and QC3 (62.6 nmol/L) compared to those devices run with the test line only (Figure 4.3.8.1.2). For QC1, this was largely due to the high variation between replicates (%CV = 145%) with some devices returning "0 MAR" values. The increased "test" conjugate dilution may have contributed to the high variation. Precision (%CV) across the other samples tested was more satisfactory – QC2 = 25%, QC3 = 19%, QC4 = 9%, Negative = 13%. Repeat testing of QC3 may potentially show an improvement in overall performance if MAR response were to increase. **(b)** Overlay of test line dose response curves showing devices with test line only (black line curve, see also Figure 4.3.8.1.2) and devices with both test and control lines (complete device: red line) are also displayed. Curves were similar for the complete device, with the exception of the QC3 response. A drop from 369.2 to 172.2 MAR, despite acceptable variability (%CV = 19%), when compared to devices run with test line configuration only, would not have been foreseen. This may be another example of the existing variability in the spotting method.

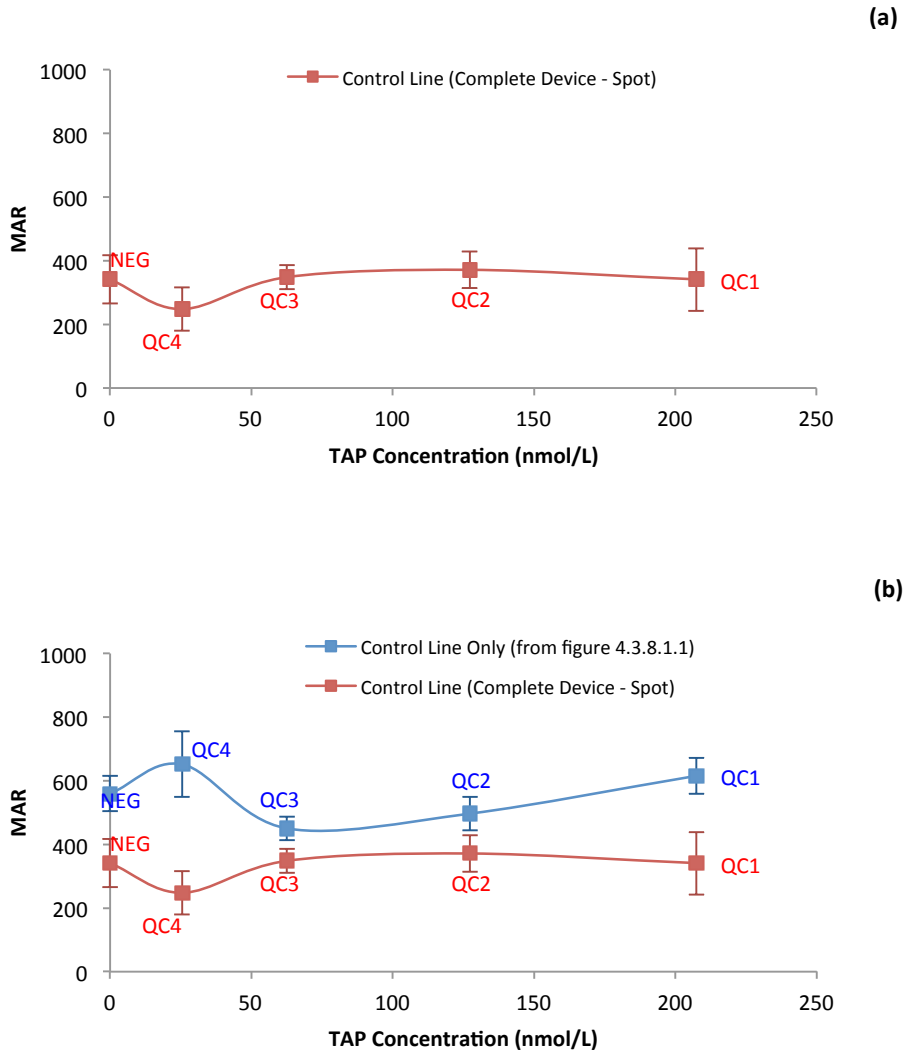



**Figure 4.3.8.2.2** Prototype 2: Control line response for spotted devices. **(a)** Control line response was lower than previous testing (see Figures 4.3.8.1.1 and 4.3.5.4.2). The reason for this was unknown. However, with the exception of QC4 (25.5 nmol/L), control response was within ±20% of mean control response across all samples tested. Response also remained constant across QC1 - 3 and negative urine sample, with variation between replicates (%CV) being <30% – QC1 = 29%, QC2 = 16%, QC3 = 11%, QC4 = 28%, Negative = 22%. **(b)** Overlays of control line dose response testing using devices with control line only (blue line) and, devices with both test and control lines (complete device: red line)) are also shown.

### 4.3.8.3 Dose response curve testing using GAC / chicken IgY-SPMP control line and streptavidin / biotinylated anti-TAP / TAP-SPMP test line (Complete device – antibody stripe)

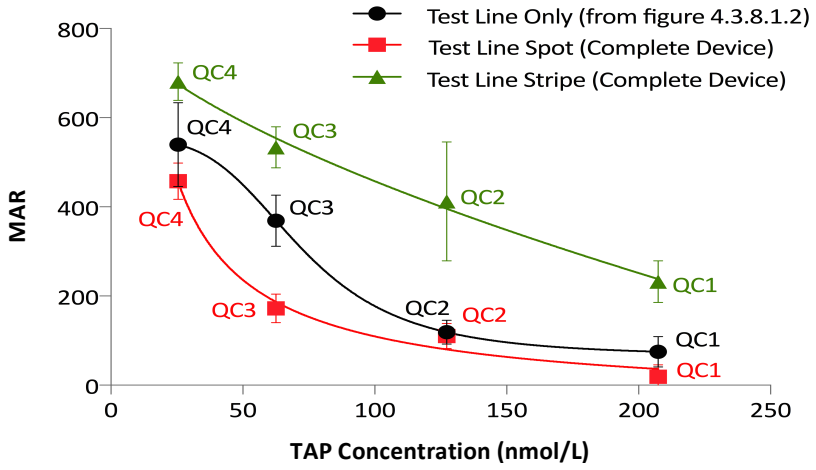



**Figure 4.3.8.3.1** Prototype 2: Striped devices - Test line MAR response for striped lateral flow test devices. The "test" and "control" conjugates were used at the same dilution as the spot devices (1/4 and 1/18 respectively). The results for all QC samples were normalised against the "negative" urine sample response (%MAR/$MAR_0$). Recorded measuring range was 232.3 – 712.2 MAR, showing 12-fold higher response for QC1 (232.2 vs. 19.0 MAR). This demonstrated the potential for the striped device to extend the TAP concentration measuring range beyond ~200 nmol/L, if required. MAR response was comparable to spot device for negative urine sample (712.2 vs. 713.5 MAR), indicating the maximum signal response of the current prototype. There was less distinction between negative, QC4 and QC3 samples in comparison to the spot device prototype (%MAR/$MAR_0$ of spot device: Negative = 100%; QC4 = 64%; QC3 = 24%; QC2 = 15%; QC1 = 3%), (%MAR/$MAR_0$ of stripe device: Negative = 100%; QC4 = 96%; QC3 = 75%; QC2 = 58%; QC1 = 33%), indicating that the stripe device may have a reduced sensitivity. This was not ideal as the reliability to distinguish between response values is paramount to a prototype that must be capable of segregating patient prognosis based on differences in TAP levels. Imprecision (%CV) for all samples tested was <20%, with the exception of QC2 (32%). Overlay of test line dose response curves showing devices with test line only and devices with both test and control lines (complete device, spot) are also displayed.

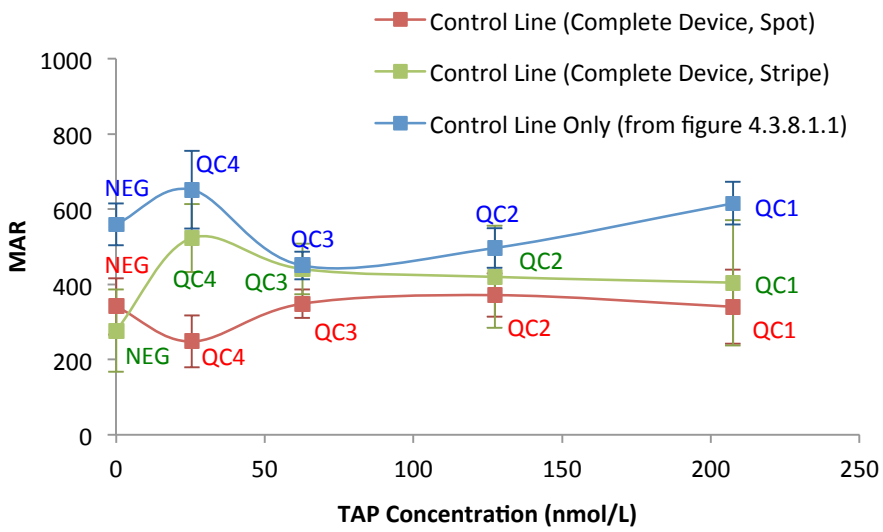



**Figure 4.3.8.3.2** Prototype 2: Striped devices - Control line response was generally higher compared to spot device testing. Mean response of all samples was 413.0 MAR for striped devices and 330.4 MAR for spot devices. QC1 - 3 control line responses were within ±20% of the mean control response. QC4 (25.5 nmol/L) and negative urine (0 nmol/L) produced a higher and lower response (523.2 and 276.8 MAR, respectively) than the 413.0 ± 20% MAR range, and were therefore outside of this mean control range. This was due to the variability observed between replicates, with precision results across the devices being predominantly inadequate (%CV = >20%) – QC1 = 41%, QC2 = 32%, QC3 = 15%, QC4 = 17%, Negative = 39%. Although these results demonstrated an increased overall MAR response at the control line position and hence; indicated the feasibility of a striped lateral flow device prototype, variability arising from the application method still existed. Overlay of control line dose response testing is also shown comparing devices with control line only and, devices with both test and control lines (complete device, spot).

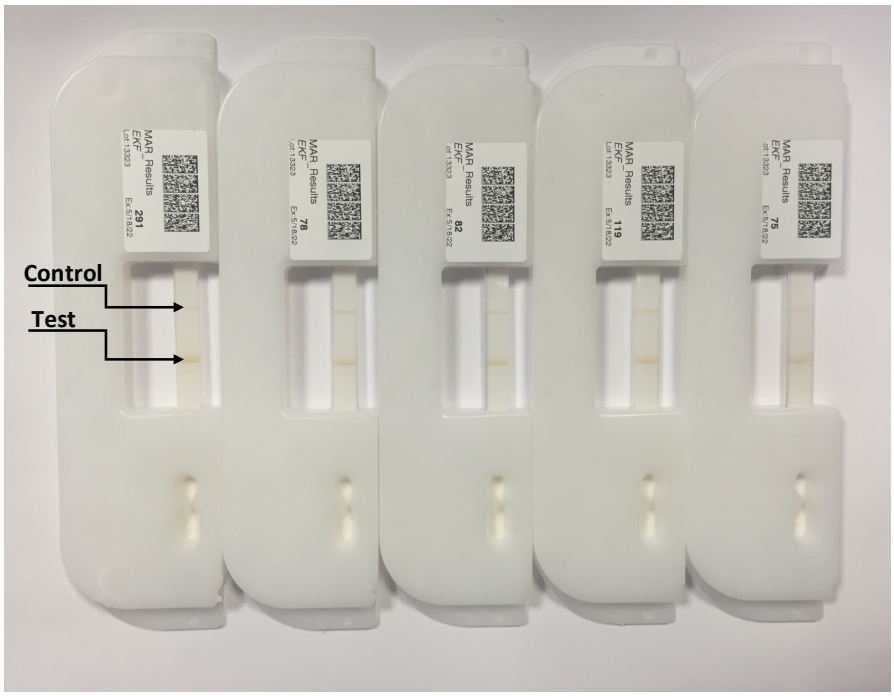



**Figure 4.3.8.3.3** Prototype 2 devices with test and control lines striped by Linomat.

## 4.4 DISCUSSION AND CONCLUSION

The feasibility of developing a lateral flow test strip device capable of distinguishing between varying concentrations of urinary TAP was described in this chapter. Initially, a lateral flow device (Prototype 1) utilising a rabbit IgG-SPMP conjugate and goat anti-rabbit IgG capture antibody for the control line was constructed and evaluated. Issues were observed for this prototype when both the test and control lines were combined. However, specificity testing demonstrated that there was negligible cross-reactivity between the capture reagents and the opposing test or control SPMP conjugates. Therefore, stability of the rabbit IgG-SPMP conjugate may have contributed to the difficulties encountered for this prototype. An alternative independent control line configuration was investigated using a chicken IgY-SPMP conjugate and goat anti-chicken IgY capture antibody (Prototype 2). This control line configuration was shown to be reproducible and functioned independently of fluctuating TAP antigen concentrations. While variation between replicates was higher than desirable for certain samples tested, this did not reduce the ability of Prototype 2 to distinguish between TAP antigen concentrations when spiked into urine. Repeat testing with the urinary TAP QC panel samples and Prototype 2 could not be performed due to funding availability at this time. However, future work would investigate the points raised surrounding improvement of the dose response curve shape.

Once feasibility of a functional test strip that met the predefined requirements was demonstrated, initial assessment of alternative buffer formulations for antibody spotting was undertaken. This work included addition of surfactants, such as Synperonic F108 and Triton X-100, at very low concentrations (0.006%, w/v) in PBS (Mujawar, 2001). Unfortunately, high variation was observed between replicates as well as the migration of antibody spot location across the NC membrane on inclusion of Synperonic F108. MAR responses for antibody spots using Triton X-100 were shown to be lower than use of PBS alone. Use of carbonate buffer (100 mM, pH 9.6) was also assessed due to its potential to aid in the formation of a more uniform spot (Mujawar, 2001). The MAR response using the carbonate buffer was slightly lower than with use of PBS and no improvement to spot formation was observed. Thus, the use of PBS for antibody spotting was deemed suitable for prototype development.

Blocking of the NC membrane with proteins such as BSA to reduce non-specific binding and background noise in the assay is sometimes applied in lateral flow test strip preparation (Mansfield, 2014). The MICT® system used for this project had the ability to monitor background signal throughout all testing events and consequently, indirectly aided test strip preparation by eliminating the need for an additional blocking step prior to use. The barcode supplied with the test cassette contains an algorithm to instruct the MICT® system to read three individual positions across all test strips which correspond to two test regions (54.2 mm and 49mm from upper wick end of the base card, respectively) and a control (43.8 mm) region. The raw data from this project continuously and conclusively demonstrated that it was unnecessary to include a nitrocellulose-blocking step during test strip preparation. In addition to providing a simple method to monitor non-specific binding or deposition of particles in the NC membrane, this barcode also allows for the inclusion of another test line. This feature would facilitate the development of a multiplex test strip capable of measuring two individual markers with an independent control line, should such a need arise.

The CAMAG Linomat 5 dispenser instrument was utilised for capture antibody striping on the NC membrane to reduce the hands-on requirement of the spotting method and thus potentially improve variability observed. The striping method was not applied in the first instance due to the concern that the limited materials available would be exhausted before prototype feasibility was established. Ultimately, the striping method was refined to ensure that the same volume of capture antibody solution was used for application to the NC membrane (0.1 µL per 5 mm, totaling 0.5 µL per test strip). Of course, an additional volume was still required to prime the dispenser. As discussed by Wong *et al.* (2009) and the Millipore Corporation (2008), it is imperative to optimise the final width of the dried capture line as signal intensity increases with decreasing capture line width. This is due to the same amount of particles being bound and concentrated in small area within the required line location versus a larger area. When the antibody solution was striped onto the NC membrane for Prototype 2, the width of the dried capture line was narrower than the striped line width following initial application on the NC membrane. However, during testing, some binding was observed at the horizon beyond the actual reading location for the test line. Numerous factors can affect the width of the dried capture line, including the

thickness of the NC membrane, the distance of the striping head from the membrane and the rate of striping (Millipore, 2008). It was believed that the force of spraying antibody solution onto the NC membrane caused the capture antibody to travel outside the required line location. Attempts to reduce the rate of application did not curtail the extent to which the solution travelled across the NC membrane. The capillary flow rate of the membrane can also cause the capture line width to spread. Still, this issue was only observed with the test line and may therefore simply require optimisation of test line reagents rather than the excessive and laborious assessment of NC materials. The final recorded result was not affected but demonstrated that the Linomat 5 instrument was truly inadequate for the specialised preparation of LFIA test strips.

While the Prototype 2 lateral flow test device will require further optimisation to improve variability observed during this work, testing of urinary QC samples indicated that discrimination between varying TAP concentrations was possible. Therefore, evaluation with TAP positive and negative clinical urine samples from patients with confirmed cases of acute pancreatitis should be undertaken as the next step to confirm that the prototype is capable of distinguishing between highly contrasted concentrations of native TAP in the urine matrix.

# CHAPTER 5

# Clinical Evaluation of Anti-TAP ELISA and LFIA Prototypes

## 5.1 INTRODUCTION

Clinical evaluation is an essential step in fully defining the performance of any medical device. This assessment provides both feedback on product performance in the field and relevant clinical data for the biomarker of interest. It also verifies clinical safety, thus identifying potential risks. Consequently, it is best practice to begin a clinical evaluation of any test device prototype at the earliest convenience, once technical performance and reproducibility have been established. This allows the manufacturer to stagger the development process, gaining sufficient information prior to marketing or submitting a device for conformity assessment by the regulatory authorities. Moreover, the clinical evaluation process is ongoing throughout the life-cycle of any test device and, therefore, it is highly beneficial to begin this process as soon as possible (European Commission, 2009).

The EU has sought to provide guidelines on clinical evaluation through continued consultation with relevant parties in the medical device industry, including National Competency Authorities and Notified Bodies. These guidelines seek to work towards a uniform application in member states and are summarised in the document "*Clinical Evaluation: A Guide for Manufacturers and Notified Bodies (MEDDEV 2.7.1 Rev.3)*". They are not legally binding but, are relevant to the Medical Devices Directive (Directive 2007/47/EC) and the international standards ISO 14155-1: 2011, and 14971: 2007 relating to clinical investigations and medical device application. The scope of the clinical study must include a defined patient cohort that the device is intended to analyse and the identification of the current treatment standards in this area, including any harmonised guidelines or scoring systems, diagnostic biomarkers or imaging detection methods and treatment or surgical interventions employed by the clinician. These considerations must be described in complete detail in a clinical sample collection protocol and submitted to the hospital ethics board for approval. Additionally, a patient consent form must be drafted to supply potential study participants with sufficient information that is clear and easily understood by a non-scientific audience. Sample collection can only commence once ethical approval is granted (Friedman *et al*., 2010). As mentioned before, previous clinical studies have been undertaken with a polyclonal anti-TAP ELISA that

performed well in the prediction of patients with AP who would subsequently develop severe disease. A literature search was completed to assess the sample collection protocols of these various studies and guide the drafting of the clinical protocol for the study discussed herein. This study would seek to establish an exploratory phase assessment of device capability to measure TAP antigen in 10 - 50 clinical urine samples, with blinded interpretation of data required to avoid bias and uphold diagnostic accuracy (Weinstein *et al*., 2005).

There are several approaches for measuring the diagnostic performance of a test to detect a patient with disease versus a patient without disease. These include negative predictive value (NPV), positive predictive value (PPV) and, sensitivity and specificity analyses. The NPV and PPV refer to the probability that a test measurement will lead to the correct diagnosis of a patient. The NPV is the proportion of patients who are not likely to have disease given a negative result and the PPV is the proportion of patients who are likely to have disease given a positive result. The PPV and NPV are particularly pertinent to patient management in the clinical setting, providing likelihood of whether a patient does or does not have the disease based on the test result (Akobeng, 2007). Thus, a patient with a negative result can require only minor observation or be discharged from hospital, reducing the demand placed on resources. This would be of particular relevance to the treatment of AP, where segregation of patients into mild, moderate and severe categories is paramount to patient management and outcome. Sensitivity and specificity are properties of the diagnostic test, indicating accuracy of performance. Altman and Bland (1994) defined sensitivity as “the proportion of true positives that are correctly identified by the test” and specificity as “the proportion of true negatives that are correctly identified by the test”. In this way, the sensitivity and specificity are post-treatment parameters that are defined from known patient outcomes and cannot be used to define the probability that a test result is an indicator of disease or no disease. A test with high sensitivity will detect a greater amount of patients with the disease such that, a negative result would indicate that a patient is highly unlikely to have the disease. However, this is also reliant on a high specificity, where a negative result is truly negative and therefore, a patient with a positive result is likely to have the disease (Akobeng, 2007). Consequently, neither

parameter should be reported without the other (Weinstein *et al*., 2005). As PPV and NPV are parameters that can be defined post-test, their value is greater in the clinical setting where patient management is the highest priority (Akobeng, 2007).

The validity of diagnostic performance is calculated using sensitivity, specificity and PPV and NPV as follows (Indrayan & Sarmukaddam, 2001):

$$\textbf{Sensitivity} = \frac{\text{True Positive (n)}}{\text{True Positive (n) + False Negative (n)}} \times 100$$

$$\textbf{Specificity} = \frac{\text{True Negative (n)}}{\text{True Negative (n) + False Positive (n)}} \times 100$$

$$\textbf{Positive Predicitve Value (PPV)} = \frac{\text{True Positive (n)}}{\text{True Positive (n) + False Positive (n)}} \times 100$$

$$\textbf{Negative Predicitve Value (PPV)} = \frac{\text{True Negative (n)}}{\text{True Negative (n) + False Negative (n)}} \times 100$$

## 5.2 AIMS OF THIS CHAPTER

The analysis and evaluation of a patient group with confirmed clinical cases of acute pancreatitis using the competitive anti-TAP ELISA prototype, developed as per chapter 3, will be described.

The clinical utility of the TAP biomarker to segregate patients based on potential to develop mild, moderate and severe conditions of acute pancreatitis will be explored. The analysis of the clinical urine samples will also include dipstick urinalysis testing to assess the urine matrix collected from each of the study participants.

In addition, the lateral flow test strip device prototype, described in chapter 4, will be evaluated for its ability to distinguish between TAP positive and negative clinical urines. This will further serve the feasibility investigation of an anti-TAP lateral flow test strip.

## 5.3 RESULTS

### 5.3.1 Stratification of patient cohort according to clinical criteria of the AMNCH, Tallaght

#### 5.3.1.1 Patients treated as having mild acute pancreatitis

A total of 15 patients were included in this clinical evaluation of TAP as a predictive biomarker for stratification of mild, moderate and severe AP, with sample collection taking place from October 2015 to April 2016. Of these 15 patients, 8 were male and 7 were female with a median age of 48.5 and 36 years, respectively (see Table 5.3.1.1.1 below). The Adelaide and Meath National Children's Hospital (AMNCH), Tallaght treatment regime for AP patients incorporates three separate criteria to segregate patients into the mild, moderate and severe categories. These criteria include clinical symptom assessment such as for peripancreatic complications; Imrie score allocation and C-reactive protein (CRP) measurement in heparinised plasma across the first two days following presentation to the emergency department (ED). A CRP level rise of >75 mg/L within these two days or a measurement of >150 mg/L on day two are considered to be indicators of a potential case of severe AP. Although not included in clinical decision-making at AMNCH, Tallaght, amylase measurements were also recorded from serum samples for all patients and are plotted in this chapter for information purposes. Amylase is commonly measured internationally as part of pancreatitis diagnosis (Vissers *et al.*, 1999) Both CRP and amylase test measurements were made at the AMNCH, Tallaght central laboratory using the Cobas® clinical chemistry analyser from Roche/Hitachi.

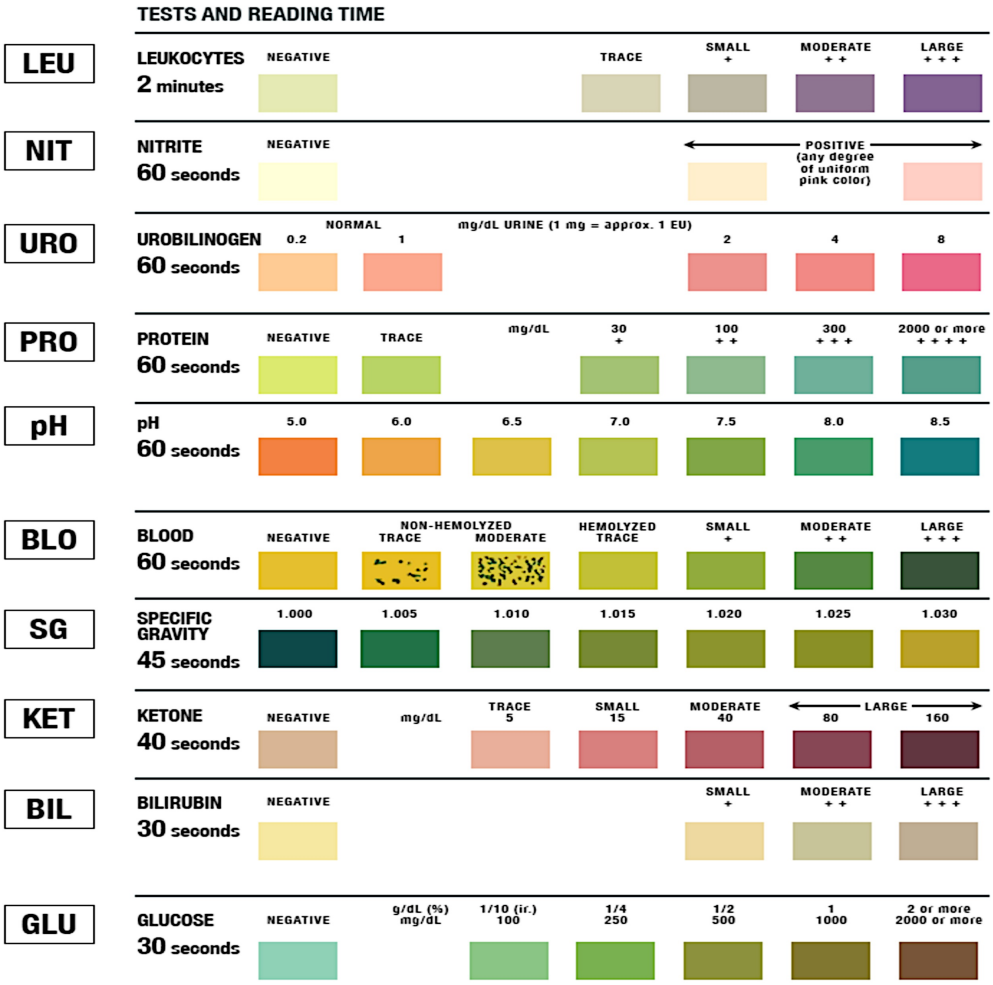
All sample time-points collected (as described by section 2.2.13) for each patient admitted to the AMNCH, Tallaght were analysed in duplicate using the competitive anti-TAP ELISA prototype. The analysis was performed as per section 2.2.6 with the TAP standard calibration curve (0 – 400 nmol/L) and Positive Control (PC) sample run in duplicate. Furthermore, each urine time-point was also analysed by dipstick urinalysis using Multistix 10SG reagent strip tests (Siemens). These test strips are generally utilised for the detection of urinary tract infections (UTI) through

leukocyte and nitrate analysis but also provide extensive information on blood and protein content as well as urine pH. Thus, a more comprehensive patient profile could be assembled from a single dipstick test. A full list of the tests available with this strip is outlined in Table 5.3.1.1.2.

**Table 5.3.1.1.1** Patient cohort breakdown based on Sex and Age. IQR = interquartile range:

| Sex | # of Patients | Median Age (IQR) (Years) | <30 # | 30 - 40 # | 40 - 50 # | 60 - 70 # | 70 – 80 # |
|---|---|---|---|---|---|---|---|
| **Male** | 8 | 48.5 (23-77) | 1 | 2 | 2 | 1 | 2 |
| **Female** | 7 | 36 (27-69) | 1 | 3 | 1 | 2 | 0 |

**Table 5.3.1.1.2** Siemens Multistix 10SG reagent strip test colour chart for interpretation of dipstick results:



Test strips are immersed into patient urine and then removed and compared with the chart above for information on 10 different test criteria that are graded accordingly. The colour development times for each test parameter were taken into account prior to recording of the final results. The presence of nitrites from gram-negative bacteria or leukocyte esterase from neutrophils can indicate a UTI. Urine specific gravity (USG) is an indicator of a patient's hydration status, measuring the density of the urine compared to water. USG can indicate patients that may have impaired renal function. Glucose is normally filtered and completely reabsorbed by the kidney. However, if this reabsorption ability is exceeded, glycosuria can occur and be indicative of diabetes and liver and pancreatic diseases. Urinalysis is recommended within two hours of sample collection (Simerville *et al.*, 2005). However, for this study, analysis was not performed until freeze thawing of urine specimens and thus, results expressed for each parameter within this chapter are for information purposes only.

**Table 5.3.1.1.3** Clinical study parameters categorised in relation to causes and treatment of AP from patient information supplied by AMNCH, Tallaght:

| Parameter | Etiology | | | |
|---|---|---|---|---|
| **Treated as Mild AP** | Alcohol | Gallstones | Idiopathic | Other |
| n | 2 | 2 | 2 | N/A |
| Men/Women | 2 (100%)/0 | 1 (50%)/1(50%) | 0/2 (100%) | N/A |
| Age (Years) | 36 (23-48) | 71 (69-72) | 48 (30-65) | N/A |
| Admission Delay/Symptom Onset (Days) | 1 (1) | 3 (2-3) | 1 (1) | N/A |
| Hospital Stay (Days) | 5 (5) | 8 (7-9) | 4 (1-7) | N/A |
| **Treated as Severe AP** | | | | |
| n | 5 | 3 | N/A | 1 |
| Men/Women | 4 (80%)/1 (20%) | 0/3 (100%) | N/A | 1 (100%)/0 |
| Age (Years) | 36 (32-60) | 34 (27-46) | N/A | 77 (77) |
| Admission Delay/Symptom Onset (Days) | 3 (1-6) | 2 (1-7) | N/A | 1 (1) |
| Hospital Stay (Days) | 16 (8-19) | 10 (6-30) | N/A | 21 (21) |

Note: value ranges are median (interquartile range)

The “Other” etiology referred to steroid-induced AP and was most likely associated with the patient’s underlying respiratory disease. Of the 15 patients in total, six were treated as cases of mild AP and nine were treated as cases of severe AP using the AMNCH clinical treatment protocol. The median admission delay for patients treated as mild was 1 day and for patients treated as severe was 2 days. The median hospital stay was 4 and 10 days for the mild and severe patient groups, respectively.

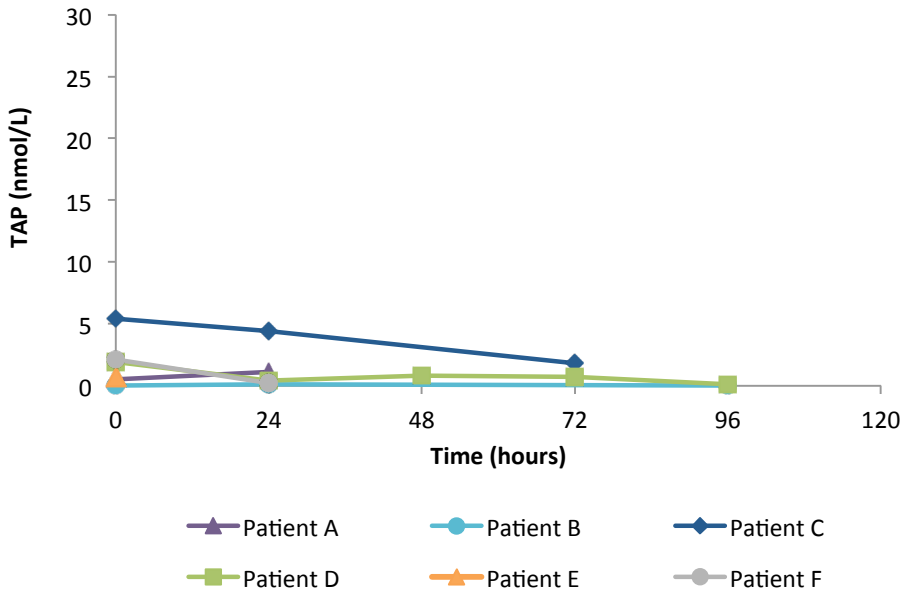



**Figure 5.3.1.1.1** Mean TAP concentrations for patients treated as having mild AP defined by the AMNCH, Tallaght clinical criteria. TAP values were low for all samples analysed with a maximum concentration of 5.4 nmol/L recorded from the zero hour time-point of Patient C. This matched closely with the maximum concentration recorded for reference range testing (5.3 nmol/L, section 3.3.14). Despite limited or non-uniform time-point collections for some patients, TAP concentrations were predominantly lower than the reference range (0 - 5.3 nmol/L) established from the apparently healthy donor group at DCU (see section 3.3.14). The six patients of this treated as mild AP group were an even split of male and female patients. The causes of AP in the three male patients were gallstones (Patient B) and alcohol (Patients C and D). The causes of AP in the three female patients were idiopathic (Patient A and F) and gallstones (Patient E). Patient A was listed as idiopathic, however, a subsequent X-ray computed tomography (CT) scan showed no sign of pancreatitis, and Patient F had reported pancreatitis previously at 15 years and may potentially have signaled the presence of reoccurring or chronic pancreatitis.

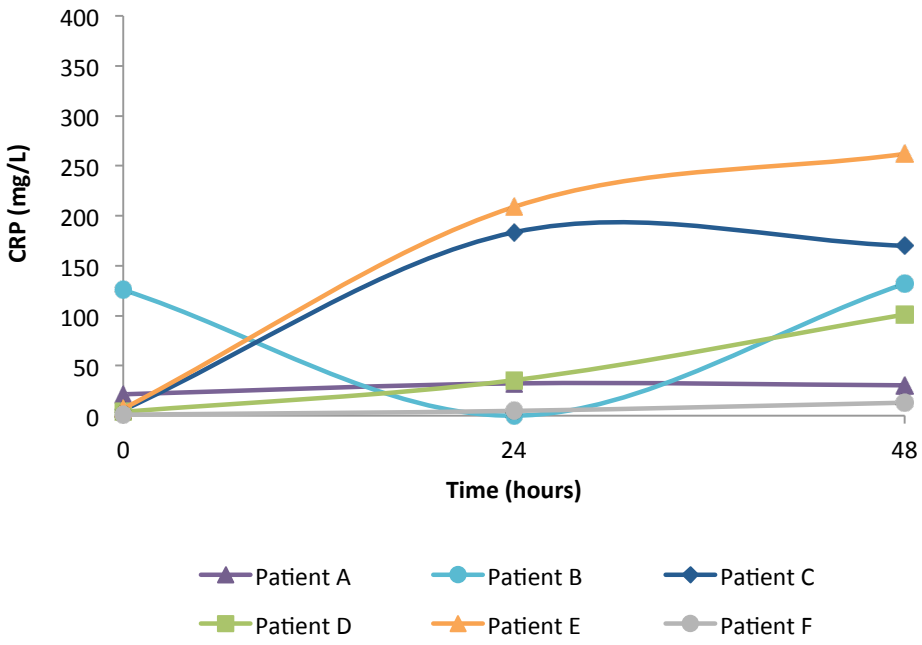



**Figure 5.3.1.1.2** CRP concentrations for mild AP patients as defined by the AMNCH, Tallaght clinical criteria. The CRP measurements were taken on presentation to the ED and in the morning of Days 1 and 2 (equivalent to 24 and 48 hours). Although a large decrease in CRP level (126 to 0 mg/L) was recorded for Patient B between admission and Day 1, the CRP measurement returned to 132 mg/L on Day 2. As the overall CRP level was less than the specified level of <150 mg/L on Day 2, the patient was treated as mild (see section, 5.3.1.1). The overall CRP level increases observed for Patient C and E were considered closely, with Patient C showing a drop in CRP level (184 to 170 mg/L) from Day 1 to 2 and thus, being categorised as improving and therefore had mild AP. The rise in CRP level for Patient E was due to ascending cholangitis from the apparent gallstones and was therefore not localised to the pancreas. However, the raised CRP level for this patient meant that additional clinical acumen was required to establish a diagnosis of mild AP. For Patient D, the rise in CRP level between Day 1 and 2 was <75 mg/L and thus, was defined as mild. All of these patients were in contrast to the low CRP levels recorded for Patient A and F across the two days from admission. Nonetheless, the CRP biomarker specifications validated at the AMNCH, Tallaght designated the majority of these patients as having mild AP based on CRP measurement. The established reference range for AMNCH, Tallaght was 0 - 5 mg/L.

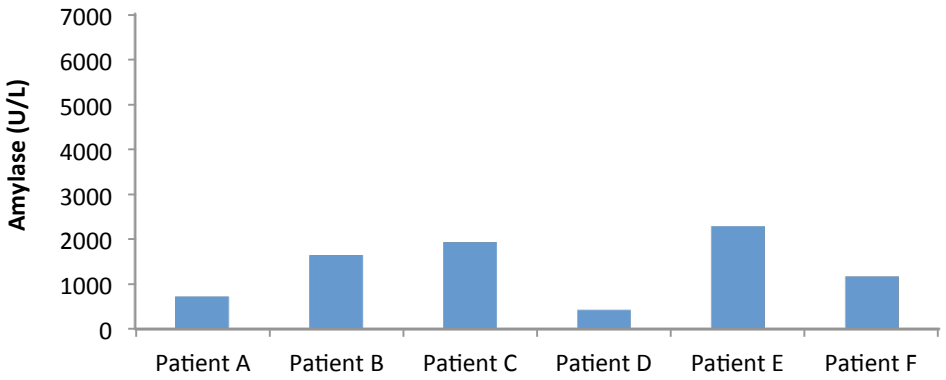



**Figure 5.3.1.1.3** Amylase concentrations for patients treated as having mild AP as defined by the AMNCH, Tallaght clinical criteria. The highest amylase measurement recorded for patients treated as mild was 2300 U/L for Patient E. The established reference range for AMNCH, Tallaght was <100 U/L.

The Glasgow (Imrie) score (as described in section 1.3.1) took into account various biochemical risk factors including glucose and blood urea nitrogen (BUN) levels and ranged from 0 – 1 for this patient group, where a score of ≤3 was defined as mild AP by AMNCH, Tallaght guidelines (Steinberg & Tenner, 1994). Dipstick urinalysis showed that patient sample pH ranged from 5.0 - 7.5 with some samples containing trace amounts of protein. Normal urinary pH can range from 4.5 - 8.0 (Simerville *et al*., 2005). A high level of protein in urine is referred to as proteinuria, however, only repeated testing can confirm that this is a recurring issue that may be caused by kidney disease due to diabetes or high blood pressure. Small levels of protein can often be detected in the urine of healthy individuals during stressful periods or following exercise (Carroll & Temte, 2000). Patient C had sample time-points with a small amount of bilirubin "+" and Patient E had sample time-points with a trace amount of non-hemolysed blood as listed in the colour chart in Table 5.3.1.1.2. Only very high levels of bilirubin are indicative of liver disease, however, it should not be present in the urine of a healthy individual (Simerville *et al.,* 2005). Non-hemolysed blood in urine can occur from kidney or UTI but can also occur during the female menstrual cycle (Kelly *et al.,* 2009). The other test parameters for dipstick urinalysis generally returned negative or inconsequential results. None of these test parameters were at levels that were expected to cause interference with the biomarker measurements.

### 5.3.1.2 Patients treated as having severe acute pancreatitis

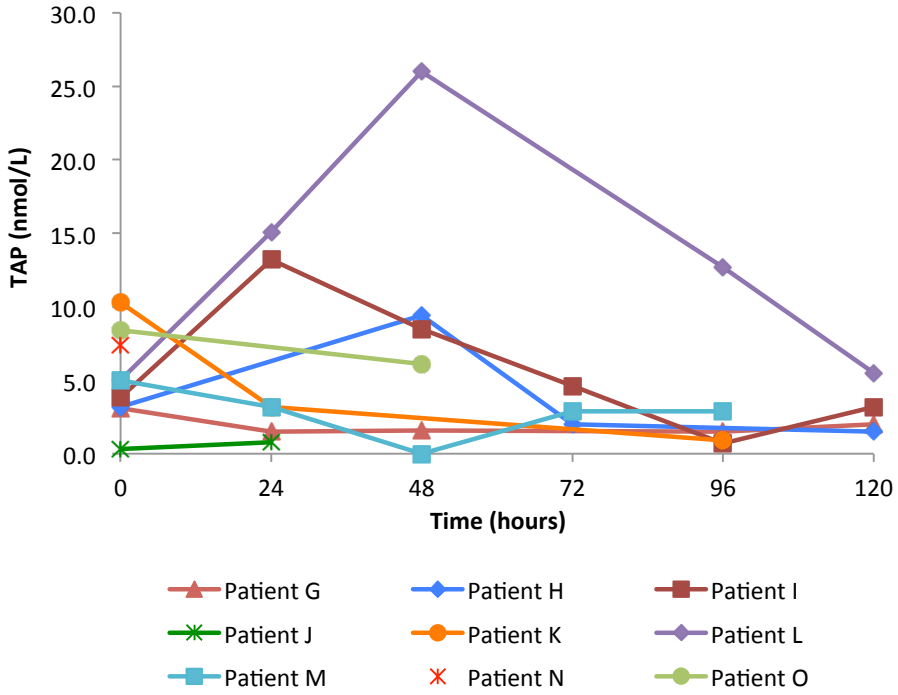



**Figure 5.3.1.2.1** Mean TAP concentrations for patients treated as having severe AP defined by AMNCH, Tallaght clinical criteria. This patient group consisted of 5 males and 4 females with the prevailing cause of AP being alcohol and gallstones for the males and females, respectively. Patient N was the only female with a cause of AP recorded from alcohol. Gallstones were the cause of AP in the male Patient O, with Patient I having underlying respiratory disease that resulted in steroid-induced AP. This patient demonstrated the second highest peak in TAP concentration at 24 hours (13.2 nmol/L). The revised Atlanta classification indicates that systemic complications can be related to exacerbations of underlying co-morbidities and are more likely to occur in the early phase of AP (Banks *et al*., 2013). The highest TAP concentration recorded was 26.0 nmol/L at 48 hours for Patient L, a female patient with gallstones. The admission of Patient K and Patient O at 2 and 4 days following onset of symptoms, respectively, may explain the higher TAP concentrations recorded (10.3 and 8.4 nmol/L) for these patients at zero hours and the subsequent concentration drop-off recorded. Perhaps this may demonstrate that TAP antigen release into urine reaches a critical mass within a short window of time from symptom onset and would very much be an early prognostic marker. Clear profiles of TAP concentration increase to decrease were observed over time for several patients,

however, Patient G and Patient J did not share this profile. The TAP levels for Patient G did not significantly change from the zero hour baseline result across all of the time-points analysed. It should also be noted that Patient J was admitted to the hospital a reported nine days after symptoms and only supplied two sample time-points for analysis from a total 10 day length of stay. Subsequent analysis of this patient cohort would demonstrate that this group in fact contained both mild and moderate cases of AP when using the revised Atlanta classification, with Patient G and Patient J designated as cases of mild AP under this classification.

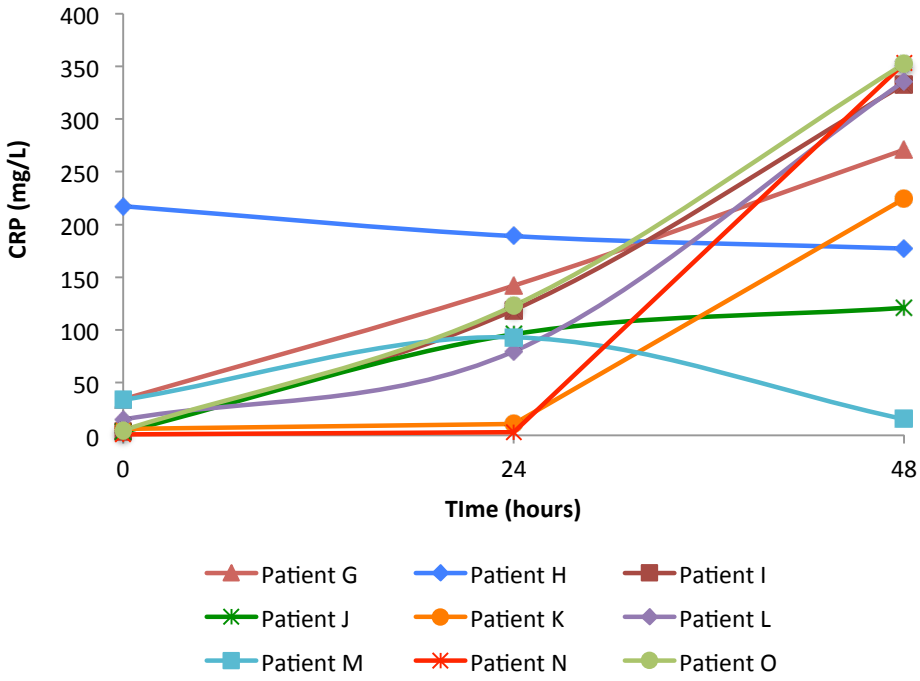



**Figure 5.3.1.2.2** CRP concentrations for patients treated as having severe AP defined by AMNCH, Tallaght clinical criteria. Plasma samples from Patients G, J, M, I and O were subject to a CRP level increase >75 mg/L, generally by Day 1, and were consequently treated as cases of severe AP. As the CRP level for Patient J between Day 1 and 2 increased only slightly (96 to 121 mg/L), an additional measurement was made on Day 3 (148 mg/L) to confirm that the CRP level was not beginning to decrease, and would have potentially indicated that this patient should be considered for treatment as mild. This patient was subsequently designated as mild under the revised Atlanta classification and the TAP levels remained below the initial baseline measurement (Figure 5.3.1.2.1). In spite of the decrease in CRP level on Day 2 for Patient M (from 93 to 15 mg/L) and, an indifferent TAP concentration profile (see Figure 5.3.1.2.1), these results were not flagged as indicators of improving condition and thus, this patient was treated as having severe AP. This decision was complimented when the patient was also designated as an Atlanta moderate. Patient H maintained a CRP level >150 mg/L across the two days of assessment, remaining at the higher end of the established CRP specification for AMNCH, Tallaght. Patients K, N and L showed a rise in CRP level to >150 mg/L on Day 2, therefore adequately meeting the change in CRP level specification requirement. When analysed for the TAP biomarker, Patient K showed an overall “drop-off” in TAP levels from a high initial

baseline value of 10.2 nmol/L (Figure 5.3.1.2.1). However, this patient was designated as mild under the revised Atlanta classification and Patient L (designated as moderate under Atlanta) did demonstrate the largest increase in TAP levels observed for this study. Unfortunately, only a baseline TAP measurement was recorded for Patient N despite a hospital stay lasting 17 days and thus, a more accurate TAP profile of the only female patient with alcohol-induced AP and moderate classification under Atlanta, was not afforded. While there was clearly some time-varied sample collections, albeit more a lack of sample time-point availability for TAP measurement when compared to CRP levels, the opportunity to explore the distribution of the biomarkers values at important time-points still existed and is discussed at a later stage in this chapter.

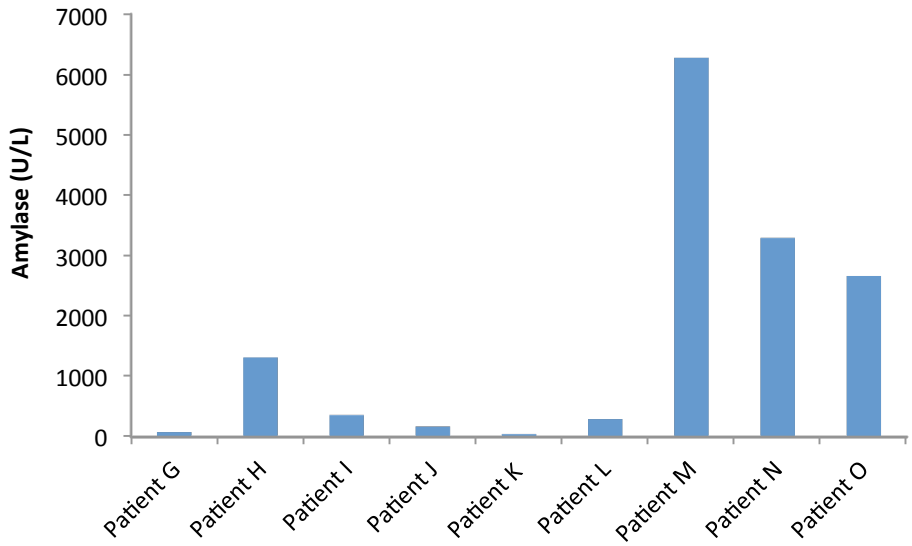



**Figure 5.3.1.2.3** Amylase concentrations for patients treated as having severe AP defined by AMNCH, Tallaght clinical criteria. The highest amylase measurement recorded for patients treated as severe was 6276 U/L for Patient I. The closest amylase value to this was 3295 U/L for Patient L. Interestingly, both of these patients also demonstrated large increases in CRP and TAP levels, with Patient O also showing a significant increase in CRP level by Day 1 (Figures 5.3.1.2.1 and 5.3.1.2.2). However, as can be seen from this data, no overall pattern could be established which could significantly contribute to clinical decision making for segregation of patients into cases of severe AP. Amylase values fluctuated from as low as 46 to 6276 U/L and thus, were of no beneficial significance.

The Glasgow (Imrie) score for this patient group ranged from 0 – 2, where a score of ≥3 was defined as severe AP by AMNCH, Tallaght guidelines. These results exhibited the fact that Imrie score did not particularly contribute to the clinical decisions taken for this study. Dipstick urinalysis showed that patient sample pH ranged from 5.0 – 8.5. Upon comparision to the colour chart in Table 5.3.1.1.2, ketone levels (15 – 80 mg/dL) were raised (ketonuria) in several sample time-points for Patients G, J, H, I and L. Ketonuria can result from uncontrolled diabetes, however, it can also occur during fasting periods (Simerville *et al*., 2005). Patient nutrition would have been closely controlled within this group. Also from this analysis, sample time-points from Patient I displayed large hemolysed blood content (hematuria) and a moderate level of bilirubin; traces of protein and large bilirubin content for Patient L and, high hemolysed blood content for Patient O. The high blood content may have been indicative of potential kidney or UTI issues in addition to the AP diagnosis (Kelly *et al*., 2009). A raised bilirubin level may have pointed to biliary obstruction, which was likely as several patients were reported to have evolving (Patient O) or definitive (Patient L) peripancreatic collections, i.e. bile blockages from issues such as gallstones (Simerville *et al*., 2005). These patients were also designated as moderate under the revised Atlanta classification. Once again, the dipstick testing served only to offer additional screening information of these patients.

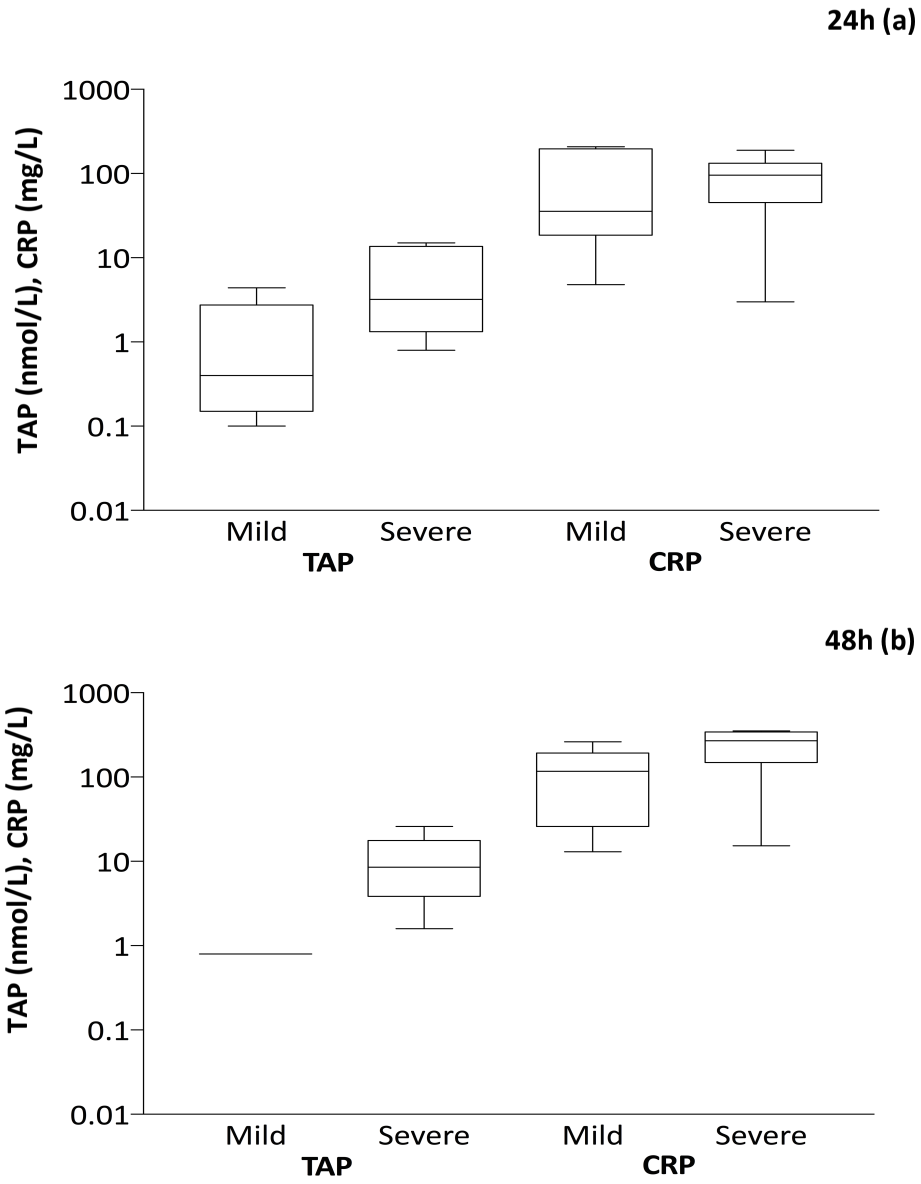



**Figure 5.3.1.2.4** Distribution of TAP and CRP levels after 24 **(a)** and 48 **(b)** hours for patients treated as mild or severe according to the AMNCH, Tallaght clinical criteria. Boxes = 25$^{th}$ and 75$^{th}$ percentiles; line in box = median; outer bars = 95$^{th}$ percentiles. At both the 24 and 48 hours time-points, the TAP biomarker demonstrated better discrimination between the patient groups treated as mild and severe when compared to the distributions for CRP levels. The slight improvement for CRP distribution in the severe group at 48 hours possibly hinted at the fact that this inflammatory marker often does not reach a peak until 48 - 72 hours (Meher *et al.,* 2015). The non-specific nature of the CRP marker was further underscored by the reporting of one of the highest CRP levels observed for this study for Patient E, who suffered from the gallstone-induced inflammatory condition of cholangitis. The lack of available time-points for TAP analysis caused the single line distribution noted for the mild group in **(b)**.

### 5.3.2 Stratification of patient cohort according to clinical criteria of the revised Atlanta classification

#### 5.3.2.1 Atlanta mild

As previously discussed in section 1.3.2, the revised Atlanta classification is designed to provide a standardised and consistent international criterion to aid in the assessment of clinical severity and evaluation of potential treatments of AP. It is not intended to be a management guideline and recognizes that the severity of AP is an evolving condition that has the propensity to change over the course of the disease. In the early phase of AP, the illicit host response to pancreatic injury gives rise to an initial systemic inflammatory wave that lasts for approximately one week. If this cascade continues, the risk of developing organ (local and multiple) failure is increased. Local complications such as peripancreatic collections present during the early phase of AP are not sufficiently reliable to define the severity of AP alone. However, the presence and duration of organ failure can distinguish between moderate and in particular, severe AP. The absence of organ failure and local or systemic complications is associated with mild AP whereas both moderate and severe AP are attributed to organ failure and local or systemic complications. The duration of organ failure distinguishes between moderate AP (transient, resolving within 48 hours) and severe AP (persistent, non-resolving). The evaluation of persistent systemic inflammation and local complications during the late phase of AP can further distinguish between moderate and severe AP, aiding in surgical intervention or specialist care. However, the identification of patients as potentially severe is essentially based on the early phase characteristics of AP. Due to the evolving nature of AP, the aggressive treatment of patients as potentially severe using the AMNCH, Tallaght clinical criteria may have limited the presence of local complications and persistent organ failure in this study and, could therefore be the reason why there was no cases of severe AP observed under the revised Atlanta classification. Consequently, the difference between the TAP and CRP biomarker levels for the patients classified as mild and moderate are discussed herein.

**Table 5.3.2.1.1** Clinical study parameters categorised in relation to the revised Atlanta classification of acute pancreatitis:

| Parameter | Etiology | | | |
|---|---|---|---|---|
| **Mild AP** | Alcohol | Gallstones | Idiopathic | Other |
| n | 4 | 3 | 2 | N/A |
| Men/Women | 4 (100%)/0 | 1 (33%)/2(67%) | 0/2 (100%) | N/A |
| Age (Years) | 40 (23-49) | 69 (34-72) | 48 (30-65) | N/A |
| Admission Delay/Symptom Onset (Days) | 2 (0.5-3) | 3 (2-7) | 1 (1) | N/A |
| Hospital Stay (Days) | 7 (5-10) | 9 (7-10) | 4 (1-7) | N/A |
| **Moderate AP** | | | | |
| n | 2 | 3 | N/A | 1 |
| Men/Women | 1 (50%)/1 (50%) | 1 (33%)/2 (67%) | N/A | 1 (100%)/0 |
| Age (Years) | 48 (36-60) | 32 (27-46) | N/A | 77 (77) |
| Admission Delay/Symptom Onset (Days) | 4 (1-6) | 2 (1-4) | N/A | 1 (1) |
| Hospital Stay (Days) | 18 (17-19) | 16 (6-30) | N/A | 21 (21) |

Note: value ranges are median (interquartile range)

The “Other” etiology referred to steroid-induced AP and was most likely associated with the patient’s underlying respiratory disease. Of the 15 patients in total, nine patients were classified as having mild AP and six as moderate AP. No patients were classified as having severe AP for this study. The median admission delay for both mild and moderate patients was 2 days and the median hospital stay was 7 and 18 days for the mild and moderate patients, respectively.

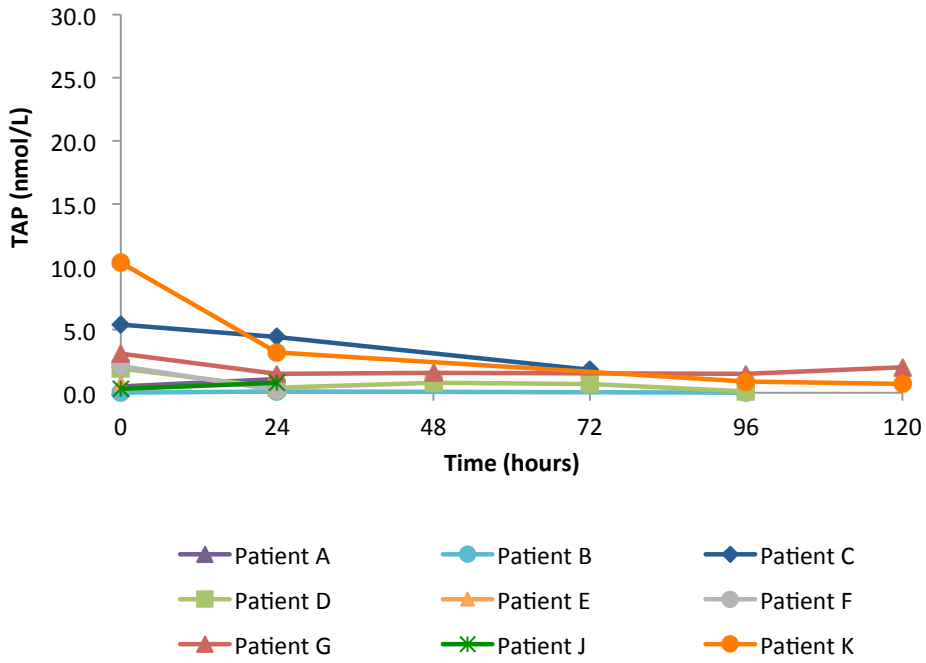



**Figure 5.3.2.1.1** Mean TAP concentrations for patients classified as having mild AP using the revised Atlanta classification. In comparison to the 6 patients treated as mild by the AMNCH, Tallaght criteria (Figure 5.3.1.1.1), nine patients (five male, four female) were considered as having mild AP using this classification. The three additional patients included were Patients G, J and K. TAP levels remained the same between both figures with the exception of Patient K who showed a high initial baseline value of 10.2 nmol/L before a rapid drop-off to levels similar to other mild patients. Therefore, the TAP values of these mild patients were again largely within the established reference range (0 - 5.3 nmol/L). Alcohol was the predominant cause of mild AP with all 4 cases of alcohol-related AP associated with male patients (Patients C, D, G, K). The causes of AP in the four female patients were an even split between idiopathic (Patients A and F) and gallstones (Patients E and J). Patient A was listed as idiopathic however; subsequent X-ray computed tomography (CT) scan showed no sign of pancreatitis. A single male patient was diagnosed with gallstone-related AP (Patient B).

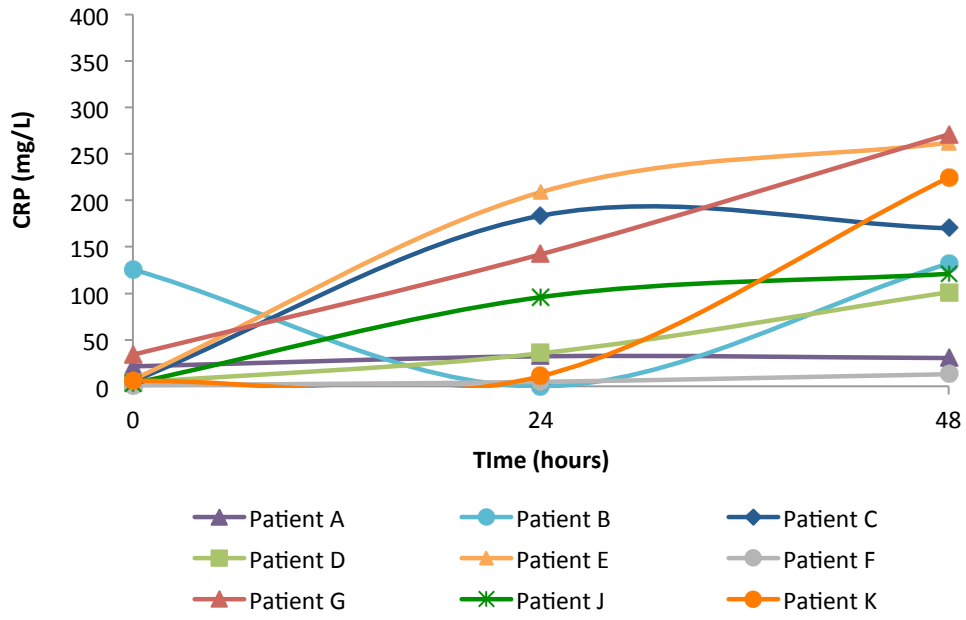



**Figure 5.3.2.1.2** CRP concentrations for patients classified as having mild AP using the revised Atlanta classification. The three additional patients included (Patients G, J and K) in comparison to the patients treated as mild by the AMNCH, Tallaght criteria (Figure 5.3.1.1.2) were treated as severe according to the clinical criteria employed by the AMNCH, Tallaght. Under this treatment guideline, both Patient G and Patient J demonstrated an increase in CRP level >75 mg/L within Day 1 (24 hours) and Patient K had a CRP >150 mg/L by Day 2 (48 hours).

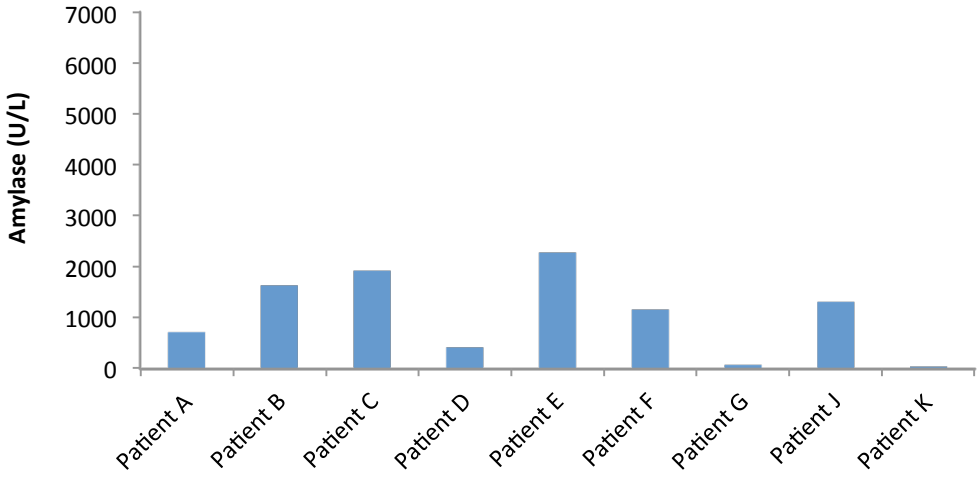



**Figure 5.3.2.1.3** Amylase concentrations for patients classified as having mild AP using the revised Atlanta classification. The highest amylase level recorded for this group was 2283 U/L for Patient E. Patients B, C, F and J also recorded raised amylase levels of 1165 - 1926 U/L. The established reference range for AMNCH, Tallaght was <100 U/L.

### 5.3.2.2 Atlanta moderate

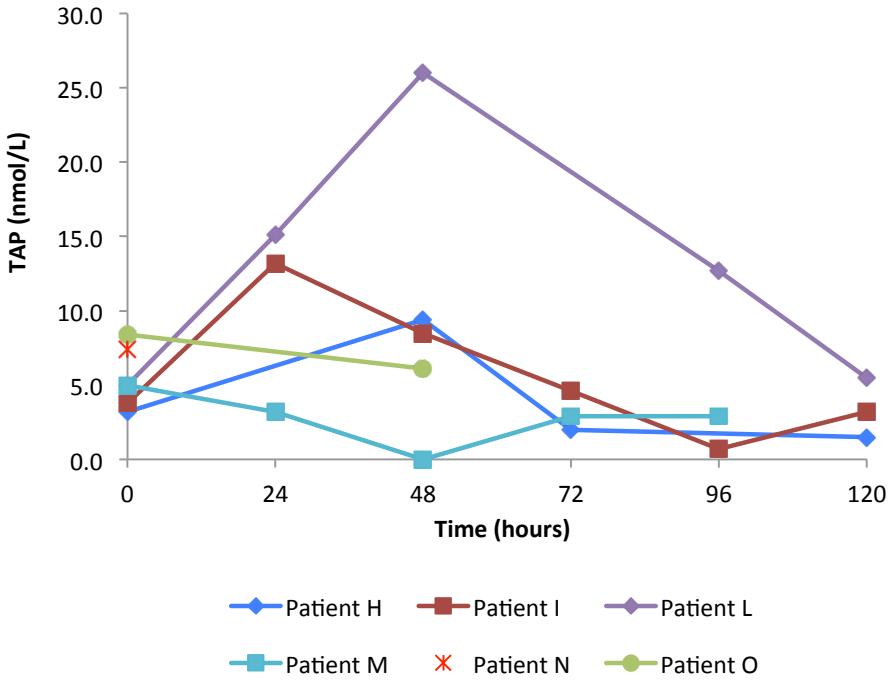



**Figure 5.3.2.2.1** Mean TAP concentrations for patients classified as having moderate AP using the revised Atlanta classification. The removal of data for Patients G, J and K who were treated as severe under the AMNCH, Tallaght clinical criteria (Figure 5.3.1.2.1), refined the results to exclude those patients with a TAP level <5.3 nmol/L for the first 48 hours following admission, with the exception of Patient M who's TAP level never increased outside of the established reference range (0 – 5.3 nmol/L). In addition, the CRP level for this patient demonstrated a spiked trend whereby an increase of ~60 mg/L occurred by Day 1 but quickly fell to 15 mg/L by Day 2 (Figure 5.3.2.2.2). This patient was subsequently diagnosed with a pancreatic pseudocyst, i.e. a fluid-filled lesion of the pancreas that can develop following AP, chronic pancreatitis or pancreatic trauma (Rabie *et al*., 2014). This cystic body carried no malignant potential (Sahani *et al*., 2012). Therefore, this was a local complication, occurring in the peripancreatic region and could have been diagnosed in the later phase of the AP episode. The use of multiple clinical criteria, combining biomarker scores and clinical acumen of symptoms and complications at AMNCH, Tallaght ensured that this patient received early aggressive treatment to avoid any potential severity. This illustrates that neither biomarker (CRP/TAP) appeared to be capable of detecting AP in this patient and was conducive to the fact that there is rarely a single answer for prognosis of this complicated disease.

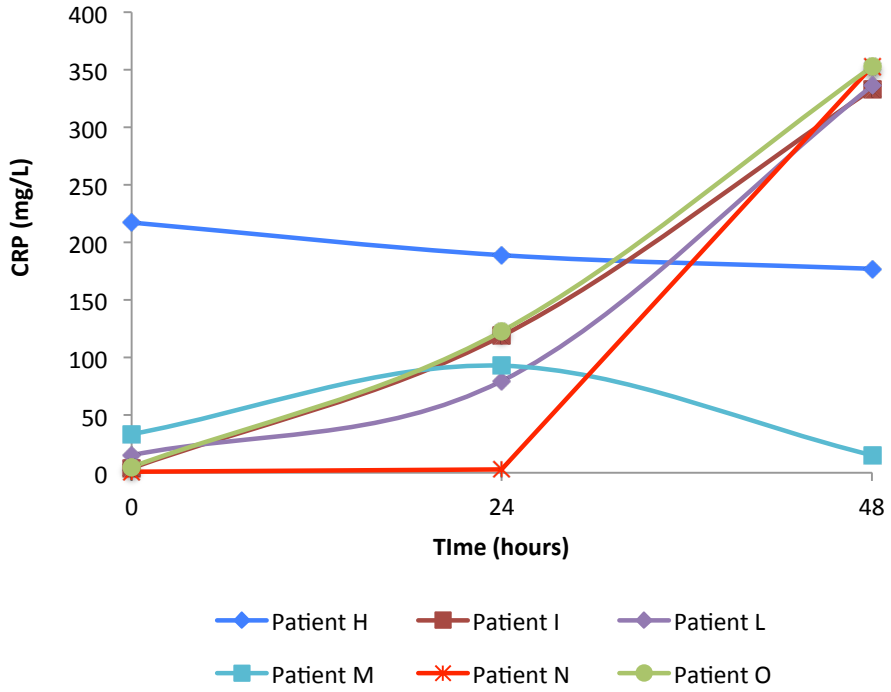



**Figure 5.3.2.2.2** CRP concentrations for patients classified as having moderate AP using the revised Atlanta classification. As discussed in Figure 5.3.2.2.1 above, the alternative trend of CRP level for Patient M can be seen in comparison to the other patients included in the moderate AP group. The CRP biomarker, like TAP, did not demonstrate an overall increasing trend for this patient that would have corresponded with the increasing marker responses observed for patients such as Patient I and Patient L. The CRP results for this patient demonstrate that the various clinical criteria employed at the AMNCH, Tallaght are paramount to confirm the condition of a patient and that the use of a single biomarker for stratification of AP patients is not currently possible.

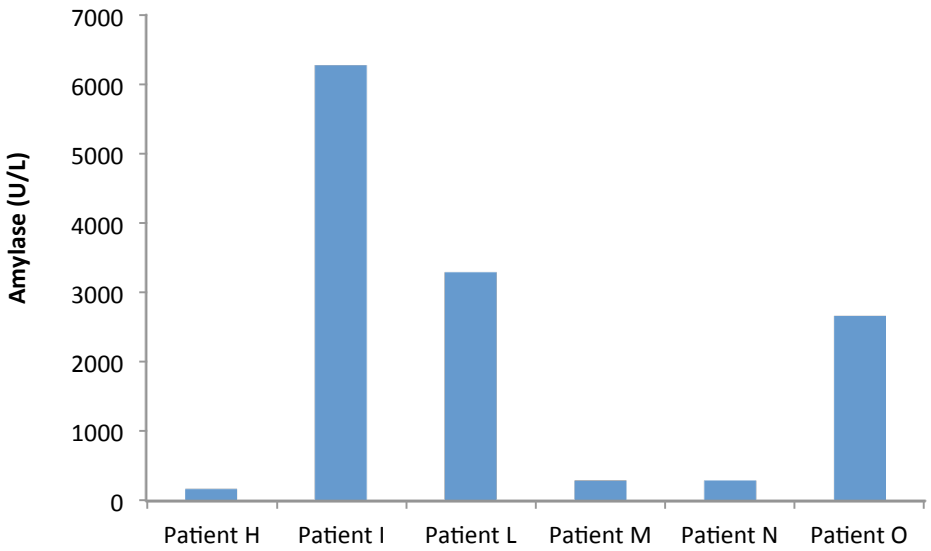



**Figure 5.3.2.2.3** Amylase concentrations for patients classified as having moderate AP using the revised Atlanta classification. The highest amylase level recorded for the Atlanta moderate patient group was 6276 U/L for Patient I. High levels were also observed for Patient L (3295 U/L) and Patient O (2664 U/L). Both Patient I and Patient L also demonstrated large increases in CRP and, in particular, TAP levels (Figure 5.3.2.2.1). Patient O also showed a significant increase in CRP level by Day 1 (Figure 5.3.2.2.2). The other patients (Patients H, M and N) had serum amylase levels of 293, 291 and 172 U/L, respectively. The removal of data for Patients G, J and K who were treated as severe under the AMNCH, Tallaght clinical criteria (Figure 5.3.1.2.3), refined the results to include only those patients who presented with an amylase level greater than the established reference range of 0 - 100 U/L.

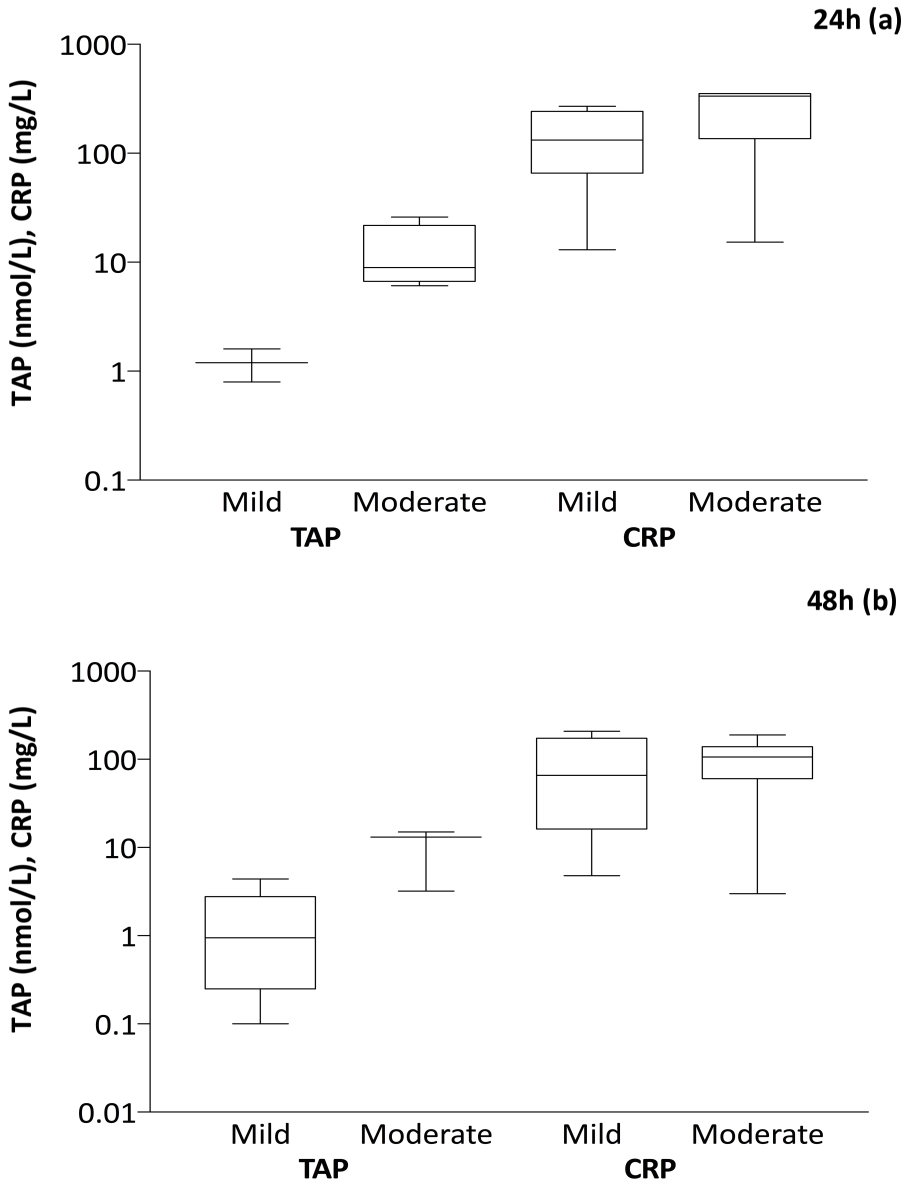



**Figure 5.3.2.2.4** Distribution of TAP and CRP levels at 24 **(a)** and 48 **(b)** hours for patients classified as mild and moderate using the revised Atlanta classification. Boxes = 25$^{th}$ and 75$^{th}$ percentiles; line in box = median; outer bars = 95$^{th}$ percentiles. Greater discrimination was observed between the mild and moderate patient groups using the TAP biomarker at 48 hours when compared to the time-point at 24 hours. The area under the receiver operator characteristic (ROC) curve was 0.81, 0.94 and 0.80 at 0h, 24h and 48h, respectively (Weinstein *et al.*, 2005). No discrimination could be made between the mild and moderate patient groups using CRP level at 24 hours. Minor discrimination was observed at 48 hours using CRP level and may have indicated an improving trend in the following hours. As also indicated in Figure 5.3.1.2.4, these results suggest the less specific nature of the CRP biomarker, which is a generalised inflammatory marker. This idea was further compounded by the reporting of one of the highest CRP levels observed for this study for presence of the inflammatory condition of cholangitis in Patient E. The area under the ROC curve was 0.52, 0.52 and 0.79 at 0h, 24h and 48h, respectively.

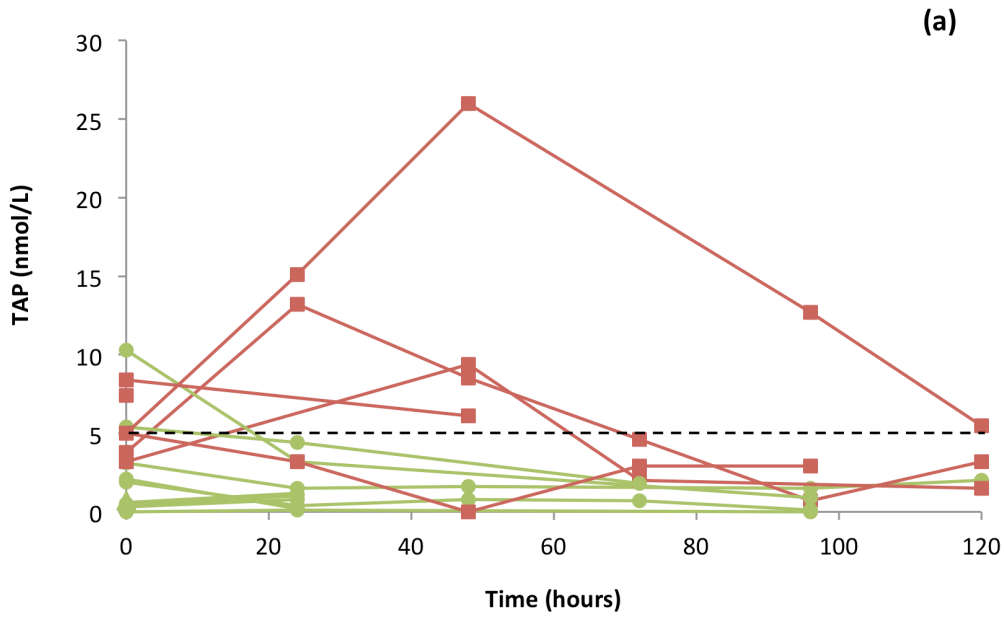



**Figure 5.3.2.2.4 (a)** Overview of TAP concentrations for patients segregated as mild (green) or moderate (red) using the revised Atlanta classification. A cut-off value of 5.3 nmol/L (dashed line) based on the established reference range was used to distinguish between these groups. From a total of 15 patients, six patients were determined to be true positives (moderate AP) and nine patients were determined to be true negatives (mild AP). One patient was determined to be a false negative (Patient M) for TAP and no patients were determined to be falsely positive as the TAP level for mild AP Patient K rapidly decreased into the reference range within 24 hours. Consequently, using the Atlanta classification of severity for this exploratory clinical cohort, the sensitivity and specificity of TAP were 85.7% and 100.0%, respectively. The negative predictive value (NPV) and positive predictive value (PPV) were 90.0% and 100%, respectively. The areas under the receiver operator characteristic (ROC) curve were 0.81, 0.94 and 0.80 at 0h, 24h and 48h, respectively (*Appendix 8.2*).

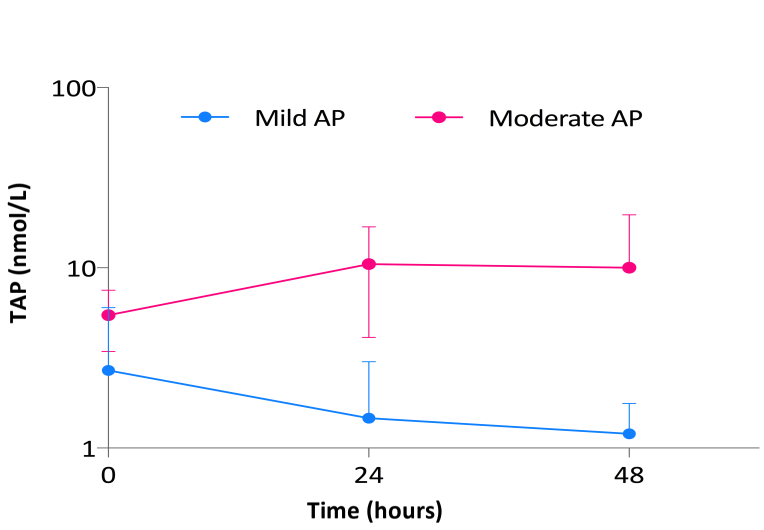
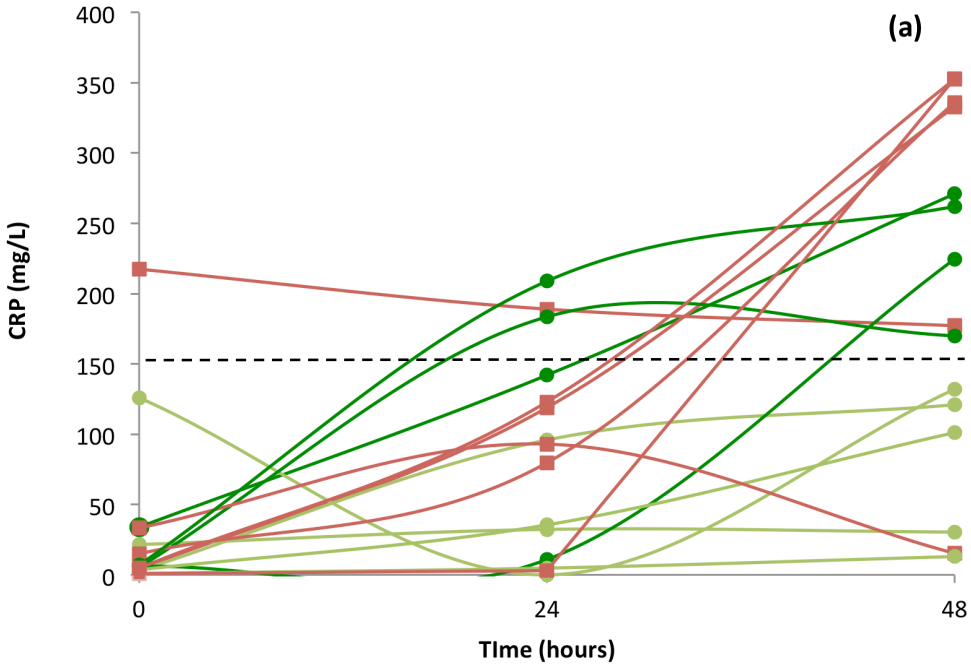



**Figure 5.3.2.2.4 (b)** Mean TAP concentrations in patients with mild and moderate AP during the first 48h in hospital. A clear separation was observed between these groups from the outset of sample collection. Mean TAP values for mild AP were 0h = 2.7; 24h = 1.5; 48h = 1.2 nmol/L and, for moderate AP were 0h = 5.5; 24h = 10.5; 48h = 12.5 nmol/L.



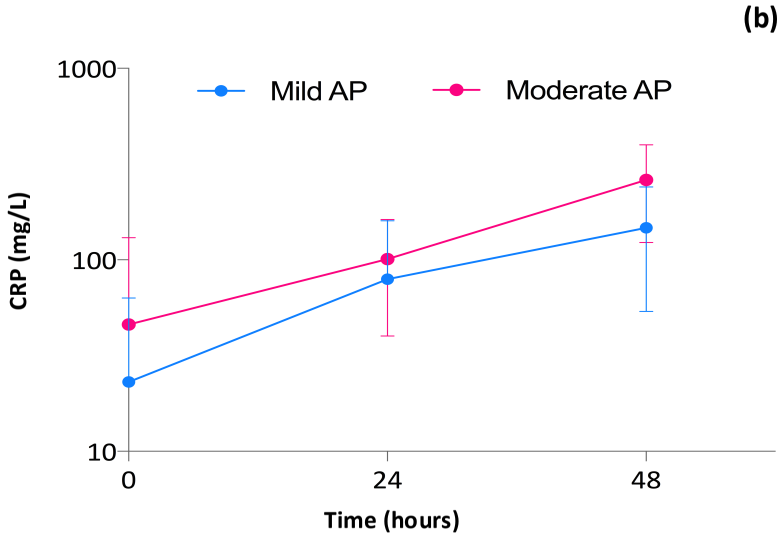




**Figure 5.3.2.2.5 (a)** Overview of CRP concentrations for patients segregated as mild (green) or moderate (red) using the revised Atlanta classification. A cut-off value of 150 mg/L (dashed line) based on the AMNCH CRP specification at 48h was used to distinguish between these groups. From a total of 15 patients, six patients were determined to be true positives (moderate AP) and nine patients were determined to be true negatives (mild AP). One patient was determined to be a false negative (Patient M) and four patients were determined to be false positives (Patients E, G, J, and K indicated by dark green). For this exploratory clinical cohort, the sensitivity and specificity of CRP were 85.7% and 69.2%, respectively. The negative predictive value (NPV) and positive predictive value (PPV) were 90.0% and 60.0%, respectively. The areas under the ROC curve were 0.52, 0.52 and 0.79 at 0h, 24h and 48h, respectively (*Appendix 8.2*).

**Figure 5.3.2.2.5 (b)** Mean CRP concentrations in patients with mild and moderate AP during the first 48h following admission to the ED. The separation was less clear with an increasing trend observed for both groups. Mean CRP values for mild AP were 0h = 23.1; 24h = 79.3; 48h = 147.2 mg/L and, for moderate AP were 0h = 45.9; 24h = 101.1; 48h = 261.2 mg/L.

**Table 5.3.2.2.1** Overview of performance to predict moderate acute pancreatitis at 48 hours after hospital admission, as defined by the revised Atlanta classification:

| 48h after admission | Sensitivity (%) | Specificity (%) | NPV (%) | PPV (%) |
|---|---|---|---|---|
| **TAP** | 85.7 | 100.0 | 90.0 | 100.0 |
| **CRP** | 85.7 | 69.2 | 90.0 | 60.0 |
| **AMNCH, Tallaght Classification** | 100.0 | 75.0 | 100.0 | 66.7 |

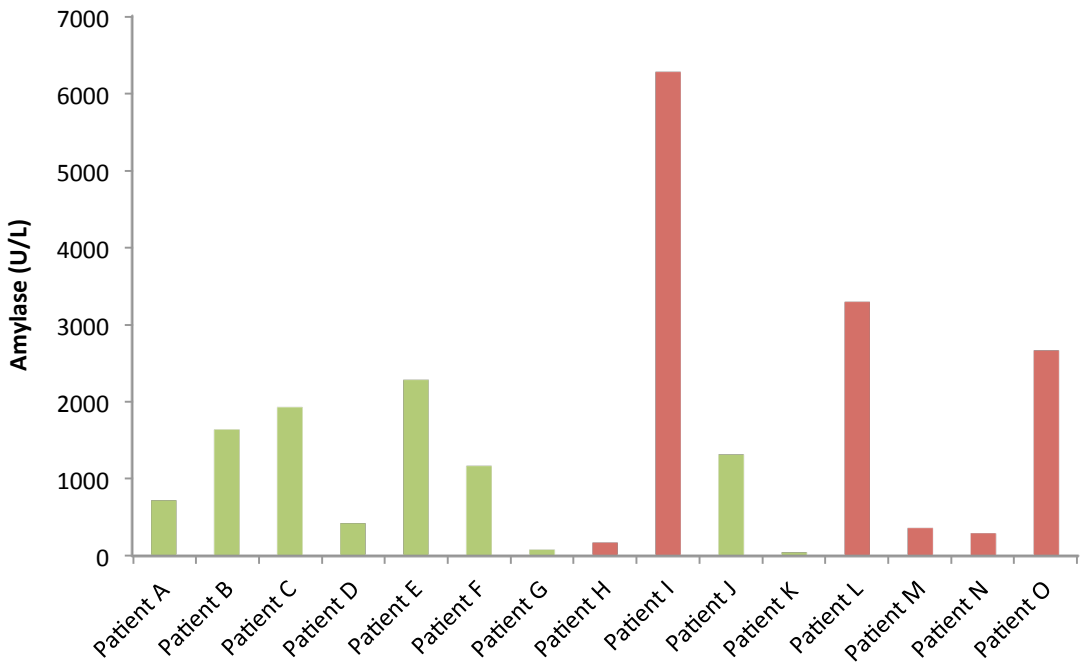



**Figure 5.3.2.2.6** Overview of amylase concentrations for patients segregated as mild (green) or moderate (red) using the revised Atlanta classification. A large proportion of the mild patients demonstrated serum amylase levels that were significantly raised, with both Patient C and Patient E having results that were not completely distant from that of Patient O, a confirmed case of moderate AP. In addition, the lowest amylase levels recorded for the moderate AP patients were comparable to some of the mild AP patients. This once again highlights that no overall pattern could be established which could significantly contribute to clinical decision-making for segregation of patients.

### 5.3.3 Lateral flow prototype – clinical feasibility assessment

In order to determine the feasibility of the anti-TAP LFIA prototype to distinguish between TAP positive and negative clinical samples, urine from Patient L (TAP = 26.0 nmol/L, 48 hours) and from Patient D (TAP = 0.4 nmol/L, 24 hours) was selected for the positive and negative samples, respectively. The selection of this negative sample was due to this time-point returning the lowest possible concentration of TAP that was recorded from the clinical analysis as distinguishable from a zero concentration. These samples were tested using both antibody spot and stripe LFIA prototypes, with test conjugate only (Figure 5.3.3.1) and test and control conjugates concurrently to represent a complete device (Figure 5.3.3.2). All devices used the Prototype 2 control line configuration of a goat anti-chicken (GAC) IgY capture antibody immobilised onto the nitrocellulose (NC) membrane and a chicken IgY antibody conjugate chemically linked to superparamagnetic particles (SPMPs). This testing was performed as per sections 2.2.12 and 4.3.8.

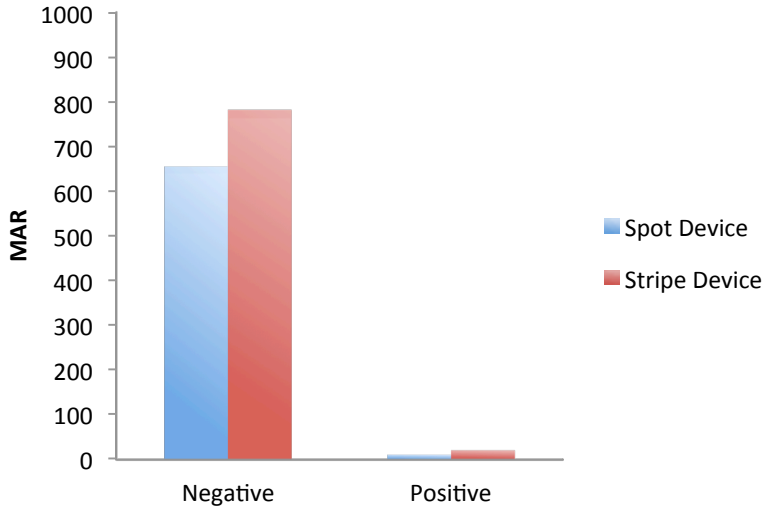



**Figure 5.3.3.1** TAP positive and negative clinical urine samples analysed using the anti-TAP LFIA prototype (test and control lines, complete device) and TAP-SPMP "test" conjugate only. As demonstrated by dose response curve testing in section 4.3.8, the negative sample returned a high MAR response (Spot device = 655.4; Stripe device = 782.1 MAR) in comparison to the positive sample (Spot device = 11.2; Stripe device = 20.6 MAR). The MAR response was slightly raised for the striped device in comparison to the spot device and was in agreement with

previous results as shown in Figure 4.3.8.3.1. The contrasting MAR response between the positive and negative samples indicated the capability of the LFIA prototype to distinguish between varying TAP concentrations in clinical urines and was complimentary to the previous dose response curve analysis. The optimisation of a finalised LFIA prototype that could quantify urinary TAP within a defined measuring range was therefore possible.

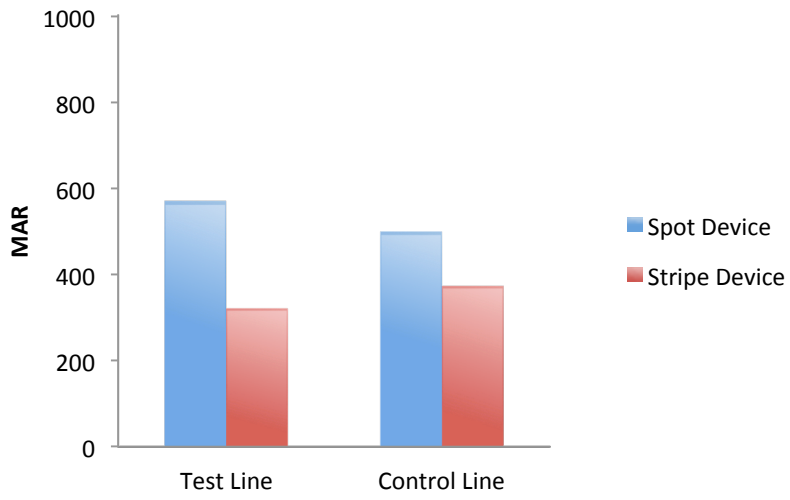



**Figure 5.3.3.2** TAP negative clinical urine samples analysed using the anti-TAP LFIA prototype (complete device) and **both** TAP-SPMP "test" and chicken IgY-SPMP "control" conjugates. The negative sample returned a lower test line response when compared to Figure 5.3.3.1. The reported values for the spot device were 570.0 and 320.7 MAR at the test and control lines, respectively. This control line response was similar to previous spot device testing for Prototype 2 (Mean = 330 MAR, Figure 4.3.8.3.2). The control line response for the stripe device (373.0 MAR) was also not very dissimilar to this previous testing (Mean = 413.0 MAR). However, the test line for both the spot and stripe devices had previously returned a higher response for negative sample testing (Spot = 713.5 MAR; Stripe = 712.2 MAR). This difference in signal response may have arisen from analysis of alternative urine matrices (apparently healthy donor versus clinical, diseased). However, the test line response (320.7 MAR) for the striped device was clearly reduced and raised the question of variability in the antibody application method. A sample flow issue was observed for testing of the TAP positive urine sample and resulted in response values <60 MAR. A lack of visual development at the test and control lines further supported that the sample/conjugate mixture had not migrated across the entire device. Unfortunately, there were insufficient project materials to repeat this analysis.

## 5.4 DISCUSSION AND CONCLUSION

The analysis of an exploratory cohort of patients with acute pancreatitis (AP) using the anti-TAP ELISA prototype (chapter 3) and the feasibility of the anti-TAP LFIA prototype (chapter 4) to discriminate between TAP positive and negative clinical urine samples was described in this chapter. The revised Atlanta classification is the only clinically based system for the description and management of AP that is widely accepted by clinicians and radiologists alike (Banks *et al*., 2013). This classification system was used to compare the performance of the clinical criteria utilised at the Adelaide and Meath National Children's Hospital (AMNCH), Tallaght, and the TAP and CRP biomarkers in their ability to stratify the severity of AP into mild, moderate and severe categories. This exploratory study confirmed that the AMNCH, Tallaght clinical assessment methodologies were excellent at ensuring that no potentially severe patients were excluded from a treatment regime designed to reduce the likelihood of developing symptoms of severe AP. In this 15 patient cohort, 6 patients were treated as having mild AP and 9 patients were treated as potentially severe AP. The revised Atlanta classification subsequently determined that there were 9 patients with mild AP and 6 patients with moderate AP. No Atlanta severe cases and no deaths were observed in this study. Therefore, 3 patients were treated as potentially severe prior to their establishment as actual cases of mild AP but most importantly, all Atlanta moderates were treated as severe by AMNCH, Tallaght. Consequently, the sensitivity, specificity, NPV and PPV of the AMNCH, Tallaght classification method to predict moderate AP at 48h after admission was 100.0%, 75.0%, 100.0% and 66.7%, respectively. Certain patients had extended hospital stay periods lasting for as long as one month and there was every possibility that treating patients as severe from the beginning could prevent complications which would score them as severe using the revised Atlanta classification. The length of hospital stay very clearly rose for the moderate AP category (see Table 5.3.2.1.1) and demonstrated the additional burden on hospital resources that arises from this group. These figures offer a projection of the burden that would be faced for treatment of patients with severe AP. The excellent NPV (100%) of the AMNCH, Tallaght classification was a particularly valuable outcome of this analysis as NPV decreases the demand placed on hospital resources, allowing greater focus to be applied to more severe patients requiring intensive management (Neoptolemos *et al*., 2001).

Measurement of plasma CRP levels following patient admission to the Emergency Department (ED) and up to 48 hours is performed as part of the AMNCH, Tallaght clinical stratification criteria. CRP has been shown to correlate well with severity of AP (Wilson *et al.,* 1989) but this inflammatory marker often does not reach a peak until 48 - 72 hours (Meher *et al*., 2015) and is part of the wave of acute-phase proteins produced by the body in response to inflammation or infection (Jain *et al*., 2011). CRP therefore lacks specificity for AP but is a good diagnostic tool for measuring the level of inflammation, which could potentially correlate to disease severity. This lack of specificity was highlighted with Patient E returning one of the highest CRP levels in this study while suffering from cholangitis. For this study, the sensitivity, specificity, NPV and PPV of CRP to predict moderate AP at 48h, relative to clinical assessment by revised Atlanta; was 85.7%, 69.2%, 90.0% and 60.0%, respectively. In comparison, the analysis of urine samples collected from patients at set time-points following hospital admission using the anti-TAP ELISA prototype demonstrated a strong capacity for the TAP biomarker to predict the severity of AP in the early phase of the disease. The sensitivity, specificity, NPV and PPV of TAP to predict moderate AP at 48h, relative to clinical assessment by revised Atlanta; was 85.7% and 100.0%, 90.0% and 100% respectively. From these results, TAP was clearly highly specific to AP with a considerable NPV and PPV. The activation of trypsinogen to trypsin and the ensuing release of TAP into urine is a recognised mechanism that precedes all clinical events in AP (Gorelick, 2003). The proportional proliferation of TAP to trypsinogen activation makes this peptide a biomarker candidate for use in the early predication of AP severity. As the first study of TAP under the revised Atlanta classification, it was demonstrated that TAP could potentially provide additional support in clinical decision making for stratification of AP patients at the early stages of disease and appeared to be superior in this role when compared to CRP. The increase in TAP levels at an early stage of disease is also an important characteristic for any useful POC biomarker. While a further and larger cohort analysis is required to support these results, TAP would compliment the assessment of AP patients by CRP, if not further improve this process. Unfortunately, there was insufficient data available from this study to truly determine the prognostic performance of TAP at the 24h time-point. The TAP levels observed for this study were complimentary of an original clinical study performed with a radioimmunoassay format by Gudgeon *et al*., (1990). In fact, if the TAP cut-off value of ≥2 nmol/L proposed by this group for the prediction of severe AP upon admission were applied to the data from this study, TAP would have a 100% record for sensitivity, specificity, NPV and PPV. The TAP levels for this study were below those of several other clinical studies of note

(Neoptolemos *et al*., 2001; Khan *et al*., 2002; Liu *et al*., 2002; Johnson *et al*., 2004) where a TAP cut-off value of 35 nmol/L was described for the prediction of severe AP. It is important to note that these studies largely defined severity of AP based on the initial Atlanta classification, which did not include a moderately severe AP category (Bradley, 1993). In addition, the majority of these studies enrolled patients who were admitted within <48h after the onset of symptoms of AP. Unfortunately, the same could not be said for this study (see Table 5.3.2.1.1). Consequently, there was potential for lower TAP levels to feature more prominently in the data. Still, it was decided to include all of these patients to capture a broader analysis and reflect the exploratory nature of this clinical study. The higher TAP levels recorded for the previous studies may potentially be explained by the use of a polyclonal ELISA format for the analysis. The potential for binding multiple target antigen epitopes when using a polyclonal assay could significantly contribute to the raised TAP levels observed. Perhaps the binding of a single epitope or isoform of the TAP biomarker by the monoclonal antibody used for the ELISA prototype described in this work could be the reason for the lower TAP levels observed. As analysis of both these assay formats could not be performed in parallel, and no gold standard measurement of TAP currently exists, a direct comparison of the TAP levels could not be obtained from this patient cohort. This does not take away from the functionality of this assay, or indeed the performance as seen in this study, but simply should be noted as a topic of further investigation. Nonetheless, this assay format was entirely capable of stratifying the patients with mild and moderate AP based on TAP levels, under the revised Atlanta classification. In addition, the concurrent rapid increase and decrease observed in TAP levels for mild and moderate patients by 24h after admission, respectively (Figure 5.3.2.2.4 (b)), again suggests another advantage of TAP as a potential biomarker for early prediction of severity and therefore, early treatment intervention.

The capability of the anti-TAP LFIA prototype to distinguish between TAP positive and negative clinical urines was investigated to determine the feasibility of an anti-TAP POC device. The use of the TAP-SPMP test conjugate alone in this system clearly demonstrated that this discrimination was possible. Unfortunately, the addition of the control conjugate resulted in a reduced signal response at both the test and control lines and this result could not be reviewed further due to the costing and financial constraints of this project. Future work with the LFIA prototype would demand the purchase of materials to repeat positive clinical sample testing and the optimisation of conjugate dilutions used in complete device testing to prove that test and control line responses could operate within specific and reproducible ranges.

# CHAPTER 6

# Overall Conclusions and Future Work

## 6.1 OVERALL CONCLUSIONS

The aim of the work presented in this thesis was to develop assays for the detection and quantification of urinary trypsinogen activation peptide (TAP). It is now accepted that the inappropriate intracellular activation of the "zymogen" trypsinogen to form trypsin in acinar cells is a major event in the pathogenesis of acute pancreatitis (AP). The improper activation and proliferation of zymogens causes auto-digestion of the pancreatic tissue and can potentially trigger necrosis and the effluence of these activated enzymes into the bloodstream, provoking the release of inflammatory cytokines and a subsequent systemic inflammatory response. Continued organ failure and septic infection can result, causing increased incidence of mortality. TAP is a small, fragmented peptide that is proportionally produced as a by-product to any trypsinogen activation before its expeditious excretion into the urine. As this mechanism occurs at the earliest phases of AP and the structure of TAP is highly conserved due to its small size, TAP is a potential biomarker for the early detection of AP and would be most proficient in a point-of-care (POC) test device capable of rapid reporting of results.

The early detection of AP is not sufficient alone in the management of this evolving disease and thus, a biomarker of AP must also exhibit the capability of predicting those patients who will develop severe AP. Previously, several multicenter clinical studies exploring the viability of the TAP biomarker as a predictor of AP severity have been completed using a polyclonal anti-TAP capture antibody in a competitive ELISA format. However, continued generation of the polyclonal antibody proved difficult and poor reproducibility of the test method followed. The strategy employed for this project involved the production of a monoclonal anti-TAP capture antibody under a contract agreement with an industrial partner. This novel antibody was characterised by SDS-PAGE analysis to confirm purity prior to its application in an ELISA prototype for the detection and quantification of the TAP biomarker in the urine matrix. The demonstration of an ELISA prototype capable of serving as a "gold standard" reference method was paramount to the subsequent development of a lateral flow test strip device. Finally, a small-scale clinical study was undertaken using the ELISA prototype to analyse the clinical utility of the TAP biomarker. The feasibility of the LFIA prototype to distinguish between TAP positive and TAP negative urine samples from patients with confirmed AP was also demonstrated with a reduced test duration. The various segments of this work are categorised below.

- Three individual lots of *14-8* monoclonal anti-TAP IgG capture antibody, provided by AbCam International, were characterised by SDS-PAGE analysis with heavy and light chain domains observed at ~50 and 25kDa, respectively, and no notable contaminating bands present.
- The immobilisation of the *14-8* monoclonal anti-TAP IgG capture antibody in a competitive ELISA test format utilising a TAP-HRP conjugate competing with "free" TAP calibrator standard demonstrated insufficient sensitivity due to the limited discrimination between calibrator standards.
- An alternative ELISA format using biotinylated monoclonal anti-TAP IgG capture antibody and a streptavidin-coated microtitre plate demonstrated clear discrimination between calibrator standards and increased TAP-HRP conjugate dilution. Three individual lots of biotinylated monoclonal anti-TAP IgG capture antibody produced comparable standard curves.
- Freeze-thaw stability of the TAP antigen was demonstrated in both urine and $dH_2O$, indicating that clinical urine samples could be collected and stored at -20°C without degradation of the peptide structure. Stability in $dH_2O$ would allow for reproducible stock dilutions of calibrator standards from -20°C storage.
- The standard curve dynamic measuring range was selected as 0 – 400 nmol/L with a back-calculated TAP antigen recovery of 100 ± 10%.
- The intra- and inter-assay precision (%CV) of the ELISA prototype was established using quality control (QC) urine samples spiked with TAP antigen and ranged from 1.7 – 10.1% and 4.8 – 10.4%, respectively.
- A positive control (PC) sample, consisting of TAP antigen spiked into a proprietary diluent to maximize stability, was established with a mean concentration of 62.5 nmol/L. A controlled concentration range of 50 – 75 nmol/L was established for future use of this sample in ELISA prototype testing.
- The Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantification (LoQ) of the ELISA were established as 1.2, 2.3 and 2.3 nmol/L, respectively.
- The linear measuring range was determined to be between 10.2 – 175.5 nmol/L from dilution of the highest urinary QC sample (QC4) concentration. The linear fit

was $R^2$ = 0.9916 with variability (%CV) between replicates ≤10% for each sample level.

- The streptavidin-coated microtitre plate was estimated to be stable for at least one year at 4°C from an accelerated stress study at 37°C.
- A "normal" reference range for urinary TAP was established from an apparently healthy donor group as 0 – 5.3 nmol/L.

**Chapter 4. Development of an anti-TAP lateral flow test strip device**

- Technical evaluation of the MICT® instrument using a commercially available test for the detection of cardiac troponin I (cTnI) demonstrated acceptable performance (intra-/inter-assay precision, LoQ, linearity, reference range, spike-recovery and sample freeze thaw stability) for use of the MICT® system as a development platform.
- An optimised conjugation protocol for the coupling of polyclonal rabbit IgG and chicken IgY to superparamagnetic particles (SPMPs) as "control" conjugates and TAP antigen to SPMPs as a "test" conjugate was established. An optimised dilution buffer formulation was also selected for use with the SPMP conjugates in the LFIA prototype.
- Spotting and striping of capture antibodies onto the nitrocellulose (NC) membrane at separate test and control line positions was investigated and optimised with regard to stock concentrations, application methods and volumes, and drying conditions. The test line comprised immobilised streptavidin and biotinylated monoclonal anti-TAP IgG capture antibody. The control line comprised either goat anti-rabbit IgG (Prototype 1) or goat anti-chicken IgY (Prototype 2).
- Dose response curves using urinary QC samples with known TAP concentrations, which were previously established with the ELISA prototype, were analysed via striped and spotted LFIA devices. Results indicated that discrimination between varying urinary TAP concentrations was possible using the LFIA device and paved the way for preliminary feasibility testing with TAP positive and TAP negative clinical samples.

**Chapter 5. Clinical Evaluation of anti-TAP ELISA and LFIA prototypes**

- An exploratory clinical study of urine samples from 15 patients with AP was designed in collaboration with the Adelaide and Meath National Children's Hospital (AMNCH), Tallaght to assess the clinical utility of the TAP biomarker for the detection and prediction of AP severity.
- The analysis of this patient cohort was completed using the ELISA prototype and the results for the TAP biomarker were scrutinized against the clinical criteria employed by AMNCH, Tallaght and the international Atlanta classification guideline for the stratification of patients according to AP severity.
- Using the TAP biomarker with the Atlanta classification definition of severity, one patient was determined to be a false negative (Patient M) and no patients were determined to be falsely positive. The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of TAP to predict the development of moderate AP at 48h after admission was 85.7%, 100.0%, 90.0% and 100%, respectively. The areas under the receiver operator characteristic (ROC) curve were 0.81, 0.94 and 0.80 at 0h, 24h and 48h, respectively. Mean TAP values for mild AP were 0h = 2.7; 24h = 1.5; 48h = 1.2 nmol/L, and for moderate AP were 0h = 5.5; 24h = 10.5; 48h = 12.5 nmol/L.
- Using the C-reactive protein (CRP) biomarker with the Atlanta classification definition of severity, one patient was determined to be a false negative (Patient M) and four patients were determined to be false positives (Patients E, G, J, and K). The sensitivity, specificity, NPV and PPV of CRP to predict the development of moderate AP at 48h after admission was 85.7%, 69.2%, 90.0% and 60.0%, respectively. The areas under the ROC curve were 0.52, 0.52 and 0.79 at 0h, 24h and 48h, respectively. Mean CRP values for mild AP were 0h = 23.1; 24h = 79.3; 48h = 147.2 mg/L, and for moderate AP were 0h = 45.9; 24h = 101.1; 48h = 261.2 mg/L.
- TAP was superior to CRP for the stratification of patients with mild and moderate AP and also for ability to predict the development of moderate AP.
- The striped and spotted LFIA prototype devices (Prototype 2) adequately distinguished between TAP positive and TAP negative clinical urine samples when using the "test" conjugate only.

- The TAP negative clinical urine sample analysed using both TAP-SPMP “test” and chicken IgY-SPMP “control” conjugates returned a reduced test line response for both the striped and spotted devices when compared to previous testing during development. This difference in signal response may have risen from analysis of alternative urine matrices (apparently healthy donor versus clinical, diseased) or potential variability in the antibody application method. However, the control line response for the striped and spotted devices was similar to previous device testing during development for Prototype 2.
- A sample flow issue was observed for testing of the TAP positive urine sample and resulted in a signal response considerably lower than expected. A lack of visual development at the test and control lines further implied that the sample/conjugate mixture had not migrated across the entire device. Unfortunately, there were insufficient project materials available to repeat this analysis.

## 6.2 FUTURE WORK

The keys aspects of any future work undertaken in relation to this project would include the following important areas detailed below.

### 6.2.1 Further performance testing of ELISA prototype in preparation for commercialisation as a Research Use Only (RUO) product

- Interference testing for possible endogenous interfering substances found in human urine, including lipids and bilirubin.
- Accelerated stress stability study of biotinylated anti-TAP IgG capture antibody.
- Further characterisation of biotinylated anti-TAP IgG capture antibody using GE Healthcare BiaCore system to analyse the affinity and interaction with the TAP antigen.
- Real-time stability study of urinary QC panel samples stored at -20°C.

### 6.2.2 Optimisation of lateral flow test strip device

- Finalise conjugate dilution when using test and control conjugates concurrently with test device.
- Real-time stability study of SPMP conjugate stocks.
- Use of specialised instrumentation commonly used for preparation of lateral flow test strips, e.g. IsoFlow™ reagent dispenser and precision testing to demonstrate robustness of manufactured devices.
- Preparation and selection of standard curve measuring range for use in the conversion of magnetic signal into a quantifiable TAP concentration.
- Method comparison of finalised LFIA prototype with ELISA reference method for the quantification of the TAP biomarker in urine.

### 6.2.3 Clinical evaluation of large-scale patient cohort containing a minimum of 50 patients

- Confirmation of initial exploratory study findings through continued patient recruitment, sample collection and biomarker analysis using ELISA “gold standard”.
- Confirmation of LFIA prototype capability to distinguish between TAP positive and TAP negative clinical urine samples.
- Evaluation of LFIA prototype performance in ongoing exploratory clinical study.

# Chapter 7

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# Chapter 8

Appendices

## PERFORMANCE CHARACTERISTICS

### MEASURING RANGE

The calibration curve range covers 0.14 - 200nM, corresponding to 0.56 - 800nM in samples diluted 1/4 in Assay Diluent. This range may be extended by increasing sample dilution.

### SENSITIVITY

The minimum detectable concentration of TAP was determined by calculating the concentration corresponding to the optical density of the mean minus two standard deviations for 24 replicates of the zero calibrator. The detection limit of Biotrin TAP EIA is 0.2nM which is equivalent to 0.8nM in samples diluted 1/4.

### SPECIFICITY

Biotrin TAP EIA is highly specific for Trypsinogen Activation Peptide. No significant cross-reactivity is observed with either the parent pro-enzyme Trypsinogen or the active enzyme Trypsin.

### REPRODUCIBILITY

**Intra-Assay Precision**

**URINE:**

| | H | | | M | | | L | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | |
| Batch 1 | 129.9 | 11.8 | 9.1 | 27.7 | 2.7 | 9.8 | 7.9 | 0.8 | 10.1 | 9.7% |
| Batch 2 | 133.6 | 10.8 | 8.0 | 26.7 | 3.98 | 14.8 | 5.9 | 1.3 | 21.9 | 14.9% |
| Batch 3 | 92.5 | 11.9 | 12.9 | 17.3 | 2.5 | 14.3 | 5.5* | 0.99 | 18.1 | 15.1% |
| Mean CV% | | | 10.0 | | | 13.0 | | | 16.7 | |

*n = 19

**PLASMA:**

| | H | | | M | | | L | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | |
| Batch 1 | 145.2 | 9.28 | 6.4 | 34 | 5.2 | 15.3 | 9.1 | 2.02 | 22.1 | 14.6% |
| Batch 2 | 113.7 | 9.2 | 8.1 | 26.9 | 4.0 | 15.4 | 5.2 | 1.13 | 21.5 | 15% |
| Batch 3 | 87.9 | 6.92 | 7.9 | 21.8 | 3.1 | 14.3 | 6.2 | 1.4 | 22.5 | 14.9% |
| Mean CV% | | | 7.5 | | | 15 | | | 22 | |

Table 1: Intra-assay Precision
Three plasma and three urine samples of known concentration were assayed in replicates of 20 at 1/4 dilution, in each of three validation batches.



**Inter-Assay Precision**

**URINE:**

| | H | | | M | | | L | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | |
| Batch 1 | 126 | 14.1 | 11.2 | 26.8 | 2.95 | 11 | 8.6 | 1.51 | 17.6 | 13.3% |
| Batch 2 | 148.6 | 15.4 | 10.3 | 25.9 | 3.55 | 13.7 | 10 | 1.87 | 19.2 | 14.4% |
| Batch 3 | 148.2 | 21.2 | 14.3 | 33.5 | 4.48 | 13.4 | 8.0 | 1.55 | 19.2 | 15.6% |
| Mean CV% | | | 11.9 | | | 12.7 | | | 18.7 | |

**PLASMA:**

| | H | | | M | | | L | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | Mean (nM) | S.D. | CV% | |
| Batch 1 | 141.6 | 14.9 | 10.5 | 28.3 | 2.91 | 10.3 | 8.8 | 1.75 | 19.8 | 13.5% |
| Batch 2 | 168.6 | 24.8 | 14.7 | 28.2 | 4.02 | 14.3 | 6.7 | 0.93 | 13.7 | 14.2% |
| Batch 3 | 126.2 | 10.4 | 8.2 | 25 | 4.48 | 17.9 | 5.9 | 0.65 | 11.1 | 12.4% |
| Mean CV% | | | 11.1 | | | 14.2 | | | 14.9 | |

Table 2: Inter-assay Precision
Three plasma and three urine samples of known concentration were assayed in duplicate in ten individual assay runs for each of three validation batches.

### RECOVERY

| | Plasma | Urine |
|---|---|---|
| Batch 1 | 105.5% | 104.5% |
| Batch 2 | 102.5% | 95.7% |
| Batch 3 | 118% | 97.7% |
| **Mean % Recovery** | **108.6%** | **99.3%** |

Table 3: Three plasma and three urine samples containing low, medium and high base levels of TAP were spiked at three different levels with synthetic TAP to yield concentrations spanning the clinical range of the test system. All samples were assayed over three kit batches and mean % recovery calculated.

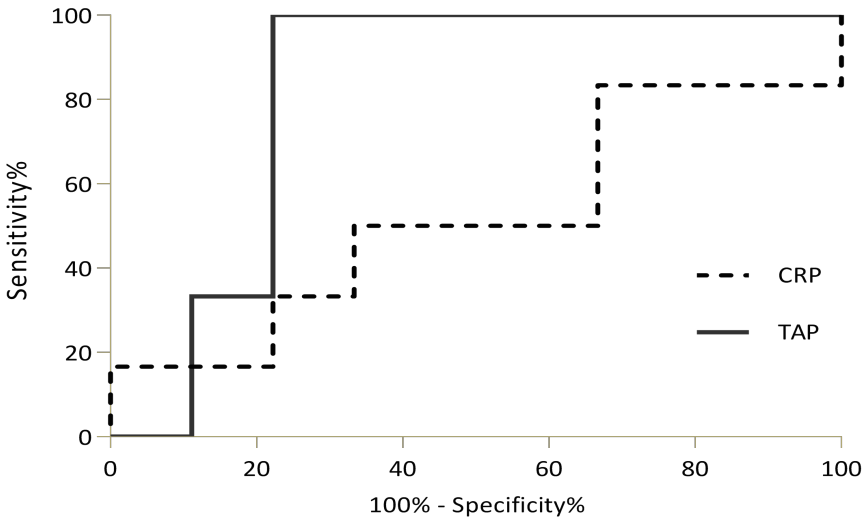
### WARRANTY

The performance data presented here were obtained using the procedure described. Any change or modification of the procedure, not recommended by Biotrin International, may affect the results, in which case Biotrin International disclaims all warranties, expressed, implied or statutory, including implied merchantability and fitness for use. In the case of such an event, Biotrin International shall not be liable for damages, direct or consequential.

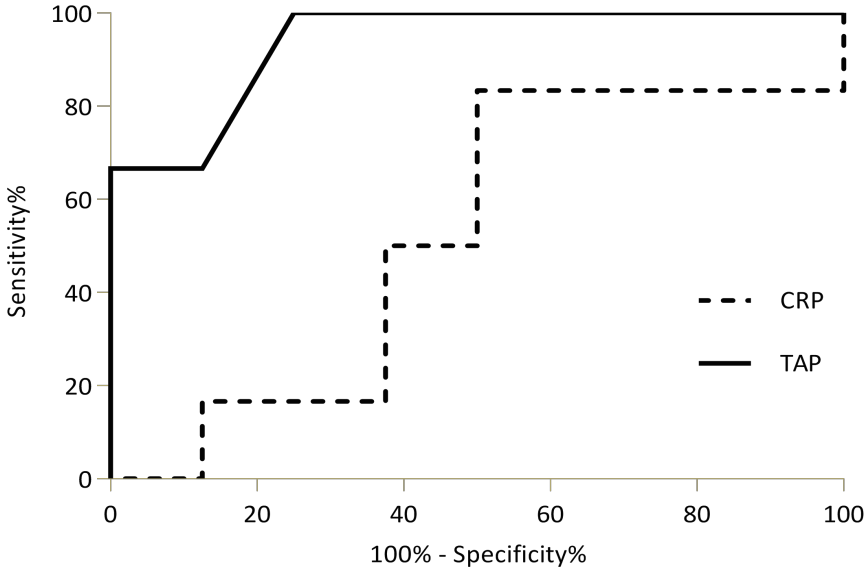


**Appendix 8.1** Polyclonal anti-TAP ELISA performance characteristics taken directly from the Biotrin International "Trypsinogen Activation Peptide ELISA" product insert.

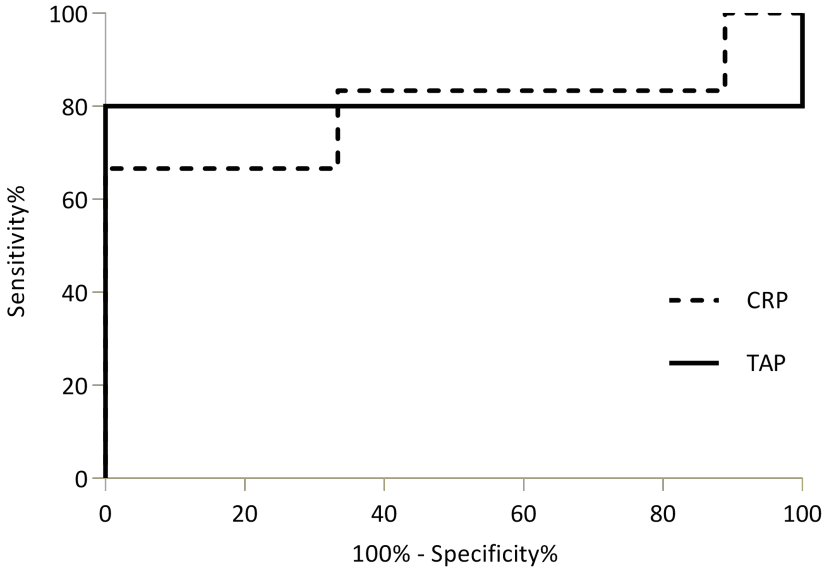
**(a)**


100
80
60
40
20
0
Sensitivity%
0
20
40
60
80
100
100% - Specificity%
CRP
TAP

**(b)**


100
80
60
40
20
0
Sensitivity%
0
20
40
60
80
100
100% - Specificity%
CRP
TAP

**(c)**




**Appendix 8.2** Receiver operator characteristic (ROC) curves for prediction of moderate acute pancreatitis (AP) by trypsinogen activation peptide (TAP) and C-reactive protein (CRP) biomarkers. Both TAP and CRP are graphed with (a) = 0h; (b) = 24h; (c) = 48h after hospital admission. The area under the ROC curve for TAP was 0.81, 0.94 and 0.80 at 0h, 24h and 48h, respectively, and for CRP was 0.52, 0.52 and 0.79 at 0h, 24h and 48h, respectively.