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DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY TEST KIT FOR 1-AMINOHYDANTOIN DETECTION

MISS UMARPHORN CHADSEESUWAN

A Dissertation Submitted in Partial Fulfillment of the Requirements
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คาร์บอกซีฟีนิลอะมิโนไฮแดนโทอิน (CPAHD) ซึ่งเป็น สารอนุพันธ์คาร์บอกซีฟีนิลของสาร AHD ได้ถูกเตรียมขึ้นและเชื่อมต่อกับโปรตีนพาหะ และใช้ในการกระตุ้นภูมิคุ้มกันของหนูไมซ์ หลังจากเทคนิคการหลอมรวมเซลล์ร่างกาย ทำให้ได้โมโนโคลนอลแอนติบอดีรหัส 28/10E/8B/1E ที่มีความจำเพาะสูงต่อสารไนโตรฟีนิลเบนซอล-1-อะมิโนไฮแดนโทอิน (NPAHD) และมีค่าความไวที่ดีโดยมี ค่าความเข้มข้นของสารแข่งขันที่ทำให้ค่าการดูดกลืนแสงลดลง 50 เปอร์เซ็นต์ (IC_{50}) 41.8 ส่วนในพันล้านส่วน (ppb) แอนติบอดีนี้มีความจำเพาะต่อสาร NPAHD CPAHD และสารไนโตรฟูแรนโทอิน และไม่ทำปฏิกิริยาข้ามกับสารที่ไม่เกี่ยวข้อง เมื่อใช้แอนติบอดีที่ผ่านการทำบริสุทธิ์บางส่วน กับรูปแบบ antibody captured competitive indirect ELISA (biotinylated antibody) ได้ค่าความไวที่ดีที่สุด โดยมีค่า IC_{50} และค่าความเข้มข้นต่ำสุดที่ตรวจวัดได้ (LOD) ที่ 4.905 และ 0.113 ppb เมื่อนำวิธี ELISA ไปประยุกต์ตรวจวัด AHD ในตัวอย่างกุ้ง พบว่าอัตราการได้กลับคืนอยู่ในช่วง 85% - 118% สำหรับ Intra-assay และ 98% - 110% สำหรับ Inter-assay จากการวิเคราะห์ปริมาณ AHD ในตัวอย่างกุ้ง ด้วยวิธี ELISA ที่พัฒนาขึ้นกับ LC-MS/MS พบว่าให้ค่าใกล้เคียงกัน ค่าความสามารถในการตรวจวัด (CC_{β}) ของ ELISA ที่พัฒนาขึ้นมีค่าเท่ากับ 0.40 ppb

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UMRPHORN CHADSEESUWAN : DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY TEST KIT FOR 1-AMINOHYDANTOIN DETECTION. ADVISOR : KITTINAN KOMOLPIS, Ph.D., CO-ADVISOR : ASSOC. PROF. TANAPAT PALAGA, Ph.D., ORAPRAPAI GAJANANDANA, Ph.D., 144 pp.

CPAHD, a carboxyphenyl derivative of AHD, was prepared and conjugated to immunogenic carrier protein and used in mice immunization. After conventional somatic cell fusion technique, a monoclonal antibody (MAb), clone number 28/10E/8B/1E, was obtained. The antibody was found to be highly specific for nitrophenylbenzal-1-aminohydantoin (NPAHD) and exhibited high sensitivity with the 50% inhibition concentration (IC_{50}) at 41.8 part per billion (ppb). This antibody showed specificity only against NPAHD, CPAHD and nitrofurantoin, and no cross reactivity to unrelated drugs was found. Using the partially purified antibody, the antibody captured competitive indirect ELISA format (biotinylated antibody) exhibited the best sensitivity with IC_{50} and limit of detection (LOD) values of 4.905 and 0.113 ppb. When applied ELISA to detect AHD in shrimp samples, the recovery rate are in the range of 85% – 118% for intra-assay and 98% – 111% for inter-assay. Analysis of AHD in fortified shrimp samples using the developed ELISA and LC-MS/MS was comparable. Detection capability (CC_{β}) of the developed ELISA was found to be 0.40 ppb.

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LIST OF ABBREVIATIONS

A	Absorbance
Ab	Antibody
Abs	Antibodies
Ag	Antigen
Ags	Antigens
AHD	Aminohydantoin
AMOZ	3-Amino-5-morpholinomethyl-2-oxazolidinone
AOZ	3-Amino-2-oxazolidinone
AP	Alkaline Phosphatase
BCA method	Bicinchonic acid assay
BSA	Bovine serum albumin
C	Constance region
Conc.	Concentration
CPAHD	Carboxyphenyl-aminohydantoin
CR	Cross-reactivity
CV	Coefficient of variation
Da	Dalton (g/mol)
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
FIA	Freund's incomplete adjuvant
FTD	Furaltadone
FZD	Furazolidone
g	Gram

H	Hevy chain
HAT	Hypoxanthine, Aminopterine and Thymidine
HGPRT	Hypoxanthine quanine phosphoribosyl transferase
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	50% of inhibition concentration
Ig	Immunoglobulin
kg	Kilogram
L	Light chain
LC-MS/MS	Liquid chromatography-mass spectrometry/ mass Spectrometry
LOD	Limit of detection
M	Molar
mg	Milligram
ml	Milliliter
MAb	Monoclonal antibody
MAbs	Monoclonal antibodies
MRPL	Maximum required performance limit
ng	Nanogram
NFT	Nitrofurantoin
NFZ	Nitrofurazone
NHS	N-hydroxysuccinimide
NPAHD	Nitrophenyl-aminohydantoin
PAb	Polyclonal antibody
PAbs	Polyclonal antibodies
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with 0.05% Tween 20
PEG	Polyethylene glycol

ppb	Part per billion
Rf	Relative mobility
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
SEM	Semicarbazide
TK	Thymidine kinase
TLC	Thin layer chromatography
TMB	3.3′,5,5′-tetramethylbenzidine
v	Volume
w	Weight
µg	Microgram
%	Percent

CHAPTER I
INTRODUCTION

1.1 Rationale

Nitrofurans are antibiotics widely used for prevention and treatment of animal diseases. When nitrofurans including nitrofurazone, nitrofurantoin, furazolidone and furaltadone are administered to animals, they are metabolized to semicarbazide (SEM), 1-aminohydantoin (AHD), 3-amino-2-oxazolidinone (AOZ) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), respectively. These metabolites are considered to be carcinogenic and mutagenic agents which could post health hazard to consumers. Due to the carcinogenic effects on humans, nitrofurans were banned from use in livestock production in the European Union (Commission Regulation, 1995) and other countries. Therefore, the Food and Drug Administration of Thailand and Europe Union had issued a zero tolerance policy for nitrofurans and their metabolites in food. Consequently, exporters must test their products to ensure that the products are free of the nitrofuran residues. The current standard methods to detect antibiotic residues in food are chemical based methods, such as LC-MS and HPLC. Although, these methods give results with high accuracy, they involve complicated steps and require sophisticated and expensive detecting devices. The immunochemical methods, such as ELISA, have increasingly become popular due to high specificity, low cost and high throughput nature of the methods. Currently, nitrofuran detection test kits for domestic uses in Thailand must be imported, resulting in large amount of budget deficit. Therefore, the overall objective of this research project is to develop an ELISA test kit for detection of AHD to replace the imported test kits as well as to be an optional method for screening a large number of samples before performing confirmation test by chemical methods.

1.2 Objectives of the study

1. To produce and characterize monoclonal antibodies against AHD
2. To develop ELISA test kit for AHD
3. To evaluate the effectiveness of the developed ELISA test kit

1.3 Scope of the study

1. Literature review
2. Preparation of immunogen and coating agent
3. Immunization of mice
4. Production and characterization of monoclonal antibody
5. Development of ELISA test kits
6. Validation of test kit
7. Dissertation writing and manuscript preparation

1.4 Advantages of the study

Currently, large budget is spent on importing nitrofuran detection test kits for domestic use in Thailand. The ability to screen for the presence of AHD using inexpensive ELISA technology will be helpful in the assessment of the health risks posed by AHD.

CHAPTER II

LITERATURE REVIEWS

2.1 Antibiotics

Antibiotics are drug of nature, semisynthetic or synthetic origin. They have been used increasingly for treatment of bacterial diseases in humans and animals. In addition, they are important in animal husbandry because they significantly enhance growth when added to animal feed. However, European Union (EU) legislation has forbidden this practice since 2006. The extensive use of antibiotics has triggered the development of bacterial resistance, which, in recent years, has become an international concern. Much attention has been paid to food-producing animals as potential source of antibiotic resistance bacteria in humans (Chafer-Peric'as *et al.*, 2010).

Several antibiotic families are used in verterinary medicine; β-lactams (pennicillins and cephalosporins), tetracyclines, chloramphenicols, macrolides, spectinomycin, lincosamide, sulphonamide, nitroimidazole, trimethoprim, polymyxins, quinolones, macrocyclics (ansamycins, glycopeptides and aminoglycosides) and nitrofurans.

2.2 Nitrofuran drugs

Nitrofurans belong to a class of synthetic broad-spectrum antibiotic which all contain a characteristic 5-nitrofuran ring (Vass *et al.*, 2008). They are frequently employed in animal production for their excellent antibacterial and pharmacokinetic properties to treat and prevent gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. They are also used as growth promoters in livestock (i.e. poultry, swine and cattle), aquaculture (i.e. fish and shrimp), and bee colonies. It was found by long-term studies with experimental animals that the parent drugs and their metabolites

showed carcinogenic and mutagenic characteristics (Jung *et al.*, 1985), fowl cholera and coccidiosis black heads (Mccalla, 1983).

Parent Compounds	Metabolites	Derivatives
Furazolidone	AOZ	NPAOZ
Furaltadone	AMOZ	NPAMOZ
Nitrofurantoin	AHD	NPAHD
Nitrofurazone	SEM	NPSEM

Figure 2.1 Structure of parent compounds of nitrofurans, their metabolites and their nitrophenyl derivatives.

2.2.1 Metabolism and bioavailability of nitrofurans

Structure of nitrofuran parent drugs, furazolidone (FZD), nitrofurantoin (NFT) nitrofurazone (NFZ) and furaltadone (FTD) and their related compounds are demonstrated in Figure 2.1. The parent compounds are metabolized rapidly after ingestion to form tissue bound metabolites. Due to this instability, effective monitoring of their illegal use has been difficult. The short *in vivo* half-life of the parent drugs (7 to 63 minute) results in rapid depletion of nitrofurans in blood and tissue (Nouws and Laurensen, 1990). However, the formed metabolites (AOZ (3-amino-2-oxazolidinone), AMOZ (3-amino-5-morpholino-methyl-1,3-oxazolidinone), AHD (1-aminohydantoin) and SEM (semicarbazide)) bind to tissue proteins in the body for many weeks after treatment, making them more practice for monitoring public compliance of the EU ban. *In vivo*, these metabolites can be released by natural stomach acids (Hoogenboom *et al.*, 1991).

The stability of the metabolites during the storage and cooking of meat was demonstrated (Cooper and Kennedy, 2007). Eight months storage did not have a significant effect on the residual concentration of nitrofurans in incurred muscle and live pig samples. Several results determined that between 67% and 100% of the residues remained present in the tissue after cooking, frying, grilling, roasting and microwaving (McCracken and Kennedy, 2007).

2.2.2 Mutagenicity and toxicity of nitrofurans

Mutagenicity studies in the 1970's and 1980's revealed the potential effects of nitrofurans in bacterial and mammalian cells. It was suggested that endogenous nitro-reductase was responsible for the *in vitro* reduction of nitrofurans in *E. coli,* leading to the formation of cellular DNA lesion in the stationary phase of bacterial growth (Mccalla *et al.*, 1971). The formation of DNA adduct after bacterial replication causes the induction of error prone DNA repair processes, indicating the mutagenic potency of the drug (Wentzell and Mccalla, 1980).

The toxicity and formation of mutagens in mammalian cells *in vitro* is less understood. Studies suggested that irreversible damage to the DNA of human epithelial cells (HEp-2) as well as hormone disturbances (reflecting endocrine dysfunction) occurred prevalently when cells were exposed to nitrofurans (De Anglis *et al.*, 1999).

2.2.3 Legislation and analytical control

In 1995, the use of nitrofurans for livestock production was completely prohibited in the EU (Commission Regulation, 1995) due to concern about the carcinogenicity of the drug residues and their potential harmful effects on human health. Under EU regulation, countries with products intended for the EU are bound by the same regulations as locally produced food (Commission Decision, 2003), therefore food imported into the EU should be free of nitrofurans. The use of nitrofurans for livestock has also been prohibited in countries such as Australia, USA, Philippines, Thailand and Brazil (Khong *et al.*, 2004). Moreover, it should be noted that nitrofurazone, nitrofurantoin, furaltadone and furazolidone are depicted on the State of California Proposition 65 Carcinogens List (US Environmental Protection Agency, 2008).

The EU has established a minimum required performance limit (MRPL) of 1 µg/kg, for edible tissues of animal origin (Commission Decision, 2003). The illegal use of nitrofurans is controlled by official inspection and analytical services provided by laboratories following the recommendations specified by Council Directive 96/23/EC. According to this document, laboratories performing residual control usually deal with large numbers of samples, with a great variety of residues to be detected in a short period of time. The use of rapid screening methods can improve the effectiveness of residual control in both official and industrial laboratories, although contamination should be confirmed by a suitable instrumental method.

The global nitrofurans crisis during 2002-2003 revealed frequent findings of tissue bound residue in poultry and aquaculture products imported to EU countries from Thailand, China, Taiwan, India, Vietnam, Ecuador and Brazil (Annon, 2008). Moreover, nitrofurans residues were also found in poultry and pork muscle produced in European

countries such as Portugal, Italy, Greece, Romania and Bulgaria (O'Keefe *et al.*, 2004). Later, inspection by EU authorities revealed nitrofurans contamination in products originating from over nine countries in 2007 with the highest incidences being from India (37%), China (37%), Bangladesh (10%) and Thailand (5%) in variety of products including shrimp, honey and canned meat (European Commission, 2008).

Table 2.1 Maximum required performance limits (MRPLs) of various countries.

Countries	Maximum required performance limit (µg/kg or ppb)			
	AMOZ	AOZ	SEM	AHD
European Union	0.3	0.3	1.0	1.0
Germany	0.3	0.3	0.5	1.0
America	x	x	x	x
Australia	x	x	x	x
New Zealand	x	x	x	x
Canada	0.3	0.3	1.0	1.0
Switzerland	0.3	0.3	1.0	1.0
Japan	x	x	x	x
Thailand	0.3	0.3	1.0	1.0

X = Zero tolerance level.
Ref; Vass et al., 2008.

2.3 Analytical methods for monitoring

In general, analytical methods for monitoring antibiotic residues can be classified in two groups (i.e. confirmatory and screening). Confirmatory methods are mostly based on liquid chromatography (LC) coupled to mass spectrometry (MS) to quantify analyte concentrations (Benito-Pena *et al.*, 2009). However, all these method

are time-consuming, expensive, and require complex laboratory equipment and trained personnel. Also, they require tedious sample-preparation procedure based on solid-phase extraction (SPE) and multistep clean-up (Blasco *et al.*, 2009).

Screening methods can detect an analyte or a family at the level of interest, and usually provide semi-quantitative results. The ideal characteristics of a screening method are low rate of false-positive samples, high throughput, ease of use, short analysis time, good selectively and low cost (Gentili *et al.*, 2005).

In general, the reported methods are grouped depending on the type of analytical technique applied, and the corresponding percentages for each one are depicted in Figure 2.2. As can be seen, LC-MS is the most employed analytical method (38%), followed by the LC-UV (18%) and ELISA (18%). However, the development of other screening methods (12%) and biosensors (8%) is increasing considerably (Figure 2.2) (Moreno *et al.*, 2009).

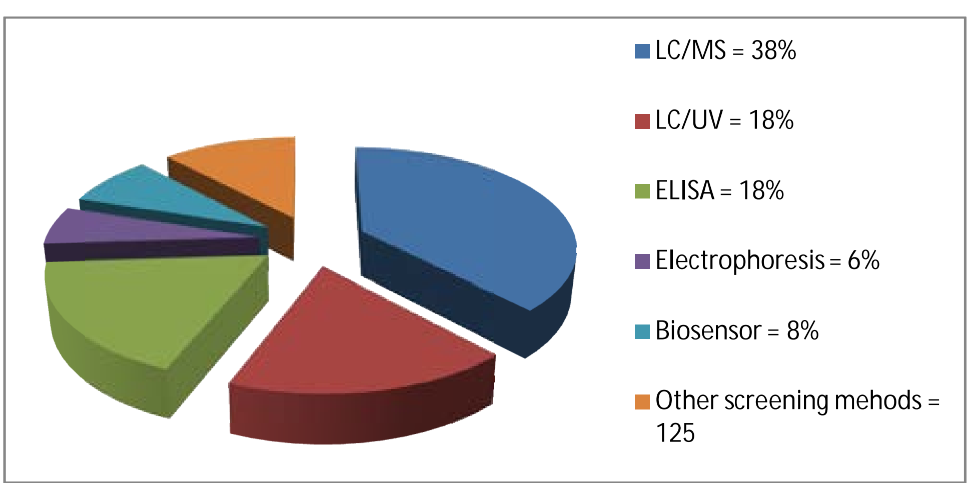

Figure 2.2 Distribution of an analytical methods used for antibiotics determination in food.

2.4 Immunoassays

Immunoassays are semi-quantitative methods characterized by their high specificity, high sensitivity, simplicity and cost effectiveness, which make them particularly useful in routine work. They are based on the specific reaction between antibody and antigen (Cha′fer-Peric′as *et al.*, 2010). Immunoassays applied to antibiotic determination have been classified depending on the detection label (as follows: enzyme-linked immunosorbent assay (ELISA), fluroimmunoassay (FIA) and time-resolved fluoroimmunoassay (TRFIA)).

There are many different immunoassay formats in use to measure antibody and antigen. The type of test chosen will depend on the characteristics of the antigen. It would be an advantage to design a screening test which detects antibody in a format compatible with the antibody's proposed final use.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs are fundamental tools of modern biology and a multimillion dollar biotechnology market in diagnosis and analysis. This powerful technique combines the sensitivity and specificity of antibodies and other binding partners in a format that is easily automated for high-throughput assays (Zheng *et al.*, 2012). ELISA is extraordinary adaptable and take advantage of many combinations of ligand pairs, methods of measuring signal (radioactive, fluorescent, or enzymatics), and experimental strategies. The presence or concentration of an antigen (Ag), antibody (Ab), or other target can be determined in solution with excellent limits of detection, generally in the ng/ml to pg/ml (Howard and Kaser, 2007).

The three classes of ELISA are
(1) Antibody capture assays
(2) Antigen capture assays
(3) Two-antibody sandwich assays

2.5.1 Antibody capture assays

The general protocol is simple; an unlabeled Ag is immobilized on a solid phase, and the Ab is allowed to bind to the immobilized Ag. The Ab can be labeled directly or can be detected by using a labeled secondary reagent that will specifically recognize the Ab. The amount of Ab that is bound determines the strength of the signal (Miura *et al.*, 2008). The three factors that will affect the sensitivity of a labeled Ab assay are (1) the amount of Ag that is bound to the solid phase, (2) the avidity of the Ab for the Ag, and (3) the type and number of labeled moieties used to label the Ab (Figure 2.3). Ab capture assays can be used to detect and quantitate antigens (Ags) or antibodies (Abs) and can be used to compare the epitopes recognized by different Abs (Harlow and Lane, 1988).

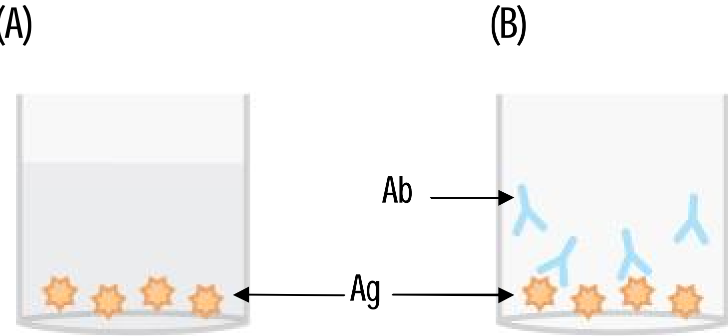

Figure 2.3 Principles of Ab capture assays.

(A) Coat Ag to solid phase

(B) Bind Ab to Ag and quantitate Ab

2.5.1.1 Antibody capture assays – antigen competition

Using an Ab capture assay with the Ag competition variation, the presence and level of an Ag can be determined quickly (Monaci *et al.*, 2011). For this assay, Ag is bound to the solid phase. A sample or test solution, containing an unknown concentration of Ag, is added together with an Ab specific for the Ag. After any unbound Abs are removed by washing, the amount of Ab adhering to the solid phase is determined by using a labeled secondary Ab (Figure 2.4) (Smith *et al.*, 1993). When high concentrations of Ag are found in the test solution, no labeled Ab will bind to the first Ab. When little or no Ag is present in the test solution, high amounts of labeled Ab will bind. To quantitate the levels of Ag, dilutions of the test solution are assayed. Comparing the titration curves for each solution will yield the relative levels of Ag (Harlow and Lane, 1988).

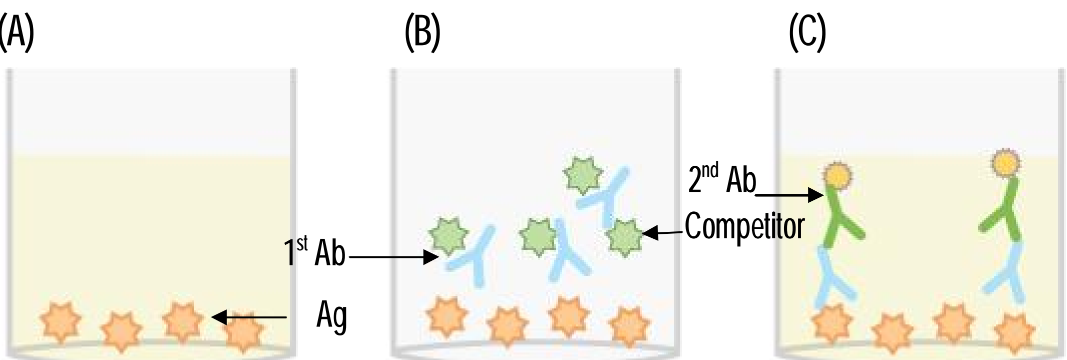

Figure 2.4 Principles of the Ab capture assays – Ag competition.

(A) Coat Ag to solid phase

(B) Add competitor Ag and first Ab

(C) Bind second Ab to first Ab and quantitate second Ab

2.5.2 The antigen capture assays

Ag capture assays are used primary to detect and quantitate Ags. The amount of Ag in the test solution is determined using a competition between labeled and unlabeled Ag (Steinitz and Baraz, 2000). In an Ag capture assay, the Ab is attached to a solid support. A sample of the labeled Ag is mixed with the test solution containing an unknown amount of Ag, and the mixture is added to the bound Ab. The Ag in the test solution will compete with the labeled Ag for binding to the Ab-matrix. The unbound proteins are removed by washing, and the assay is quantitated by measuring the amount of Ag that is bound (Figure 2.5) (Mushens *et al.*, 1993). If the unknown solution contains a high concentration of Ag, it will compete effectively with the labeled Ag, and little or none of the labeled Ag will bind to the Ab. To quantitate the levels of Ag, dilutions of the test solution are performed and assayed. Comparing the titration curves for each solution will yield the relative levels of Ag (Harlow and Lane, 1988).

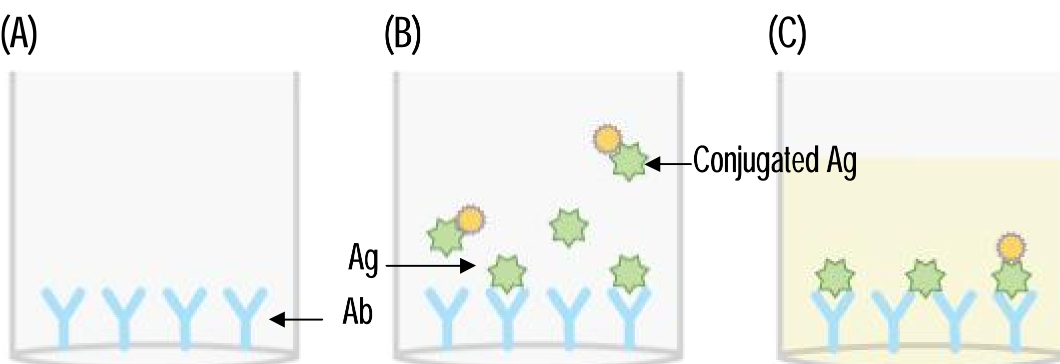

Figure 2.5 Principles of the Ag capture assays.

(A) Coat Ab to solid phase

(B) Add competitor Ag and conjugated Ag

(C) Quantitate conjugated Ag

2.5.3 The two-antibody sandwich assays

These assays are used primarily to determine the Ag concentration in unknown samples. Two-Ab assays are quick and accurate. The assay requires two Abs that bind to non-overlapping epitopes on the Ag. In a two-Ab sandwich assay, one Ab is bound to a solid support, and the Ag is allowed to bind to this first Ab. Unbound Ags are removed by washing, and the labeled second Ab is allowed to bind to the Ag. After washing, the assay is quantitated by measuring the amount of a labeled second Ab that can bind to the Ag (Figure 2.6) (Gut-Winiarska *et al.*, 2000).

To detect and quantitate Ags, the most useful method is the two-Ab sandwich assay. These assays are quick and reliable and can be used to determine the relative levels of most protein Ags. However, they require either two monoclonal antibodies (MAbs) that bind to independent sites on the Ag or affinity-puritied polyclonal antibodies (PAbs) (Castro *et al.*, 1987).

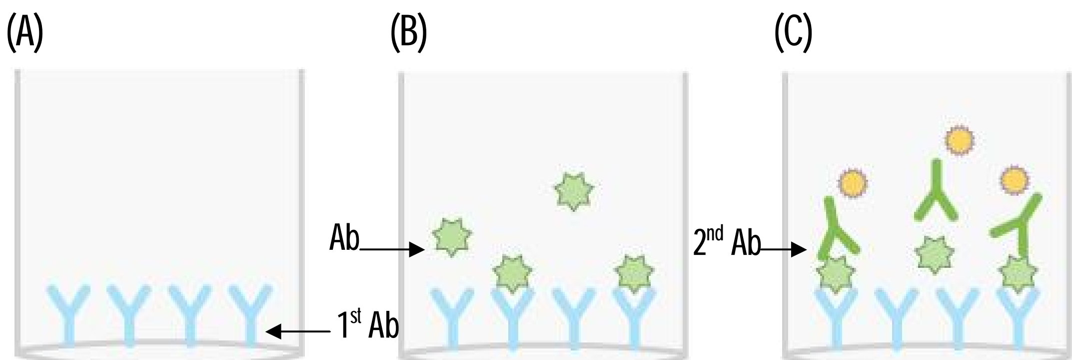

Figure 2.6 Principles of the two-Ab sandwich assays.

(A) Coat first Ab to solid phase

(B) Bind Ag to Ab

(C) Bind second Ab to Ag and quantitate second Ab

Any ELISA can be performed with four variations; the assay can be done in Ab excess, in Ag excess, as an Ab competition, or as an Ag competition. Assays done in Ab excess or as Ag competition are used to detect and quantitate Ags, while Ag excess or Ab competition assays are used to detect and quantitate Abs. The four variations within the three classes yield a total of 16 possible immunoasssays (Figure 2.7 – 2.10) (Chuang *et al.*, 1998).

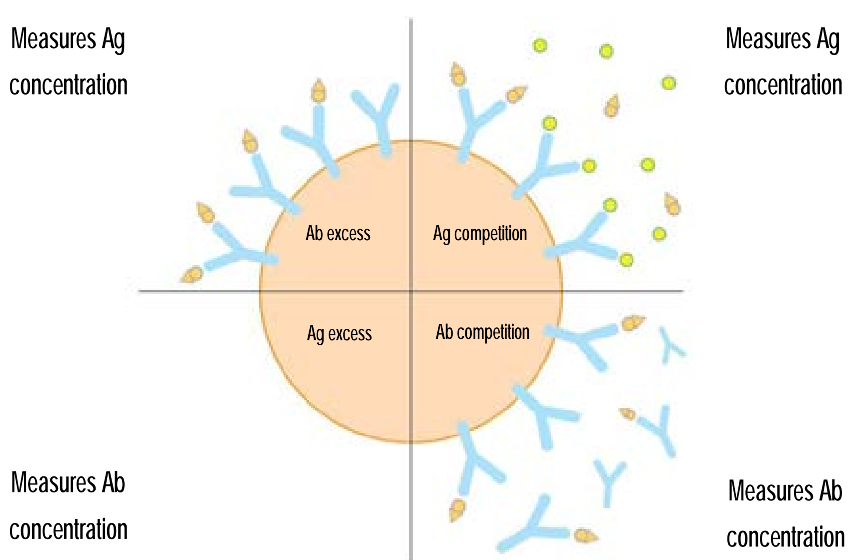

Figure 2.7 A series of summary diagrams illustrates the range of available designs; Ag-capture assays, direct detection.

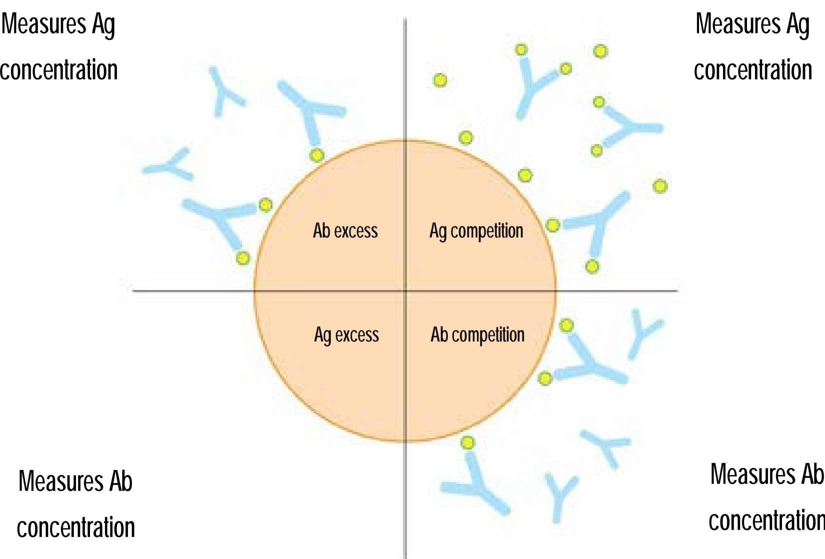

Figure 2.8 A series of summary diagrams illustrates the range of available designs; Ab-capture assays, direct detection.

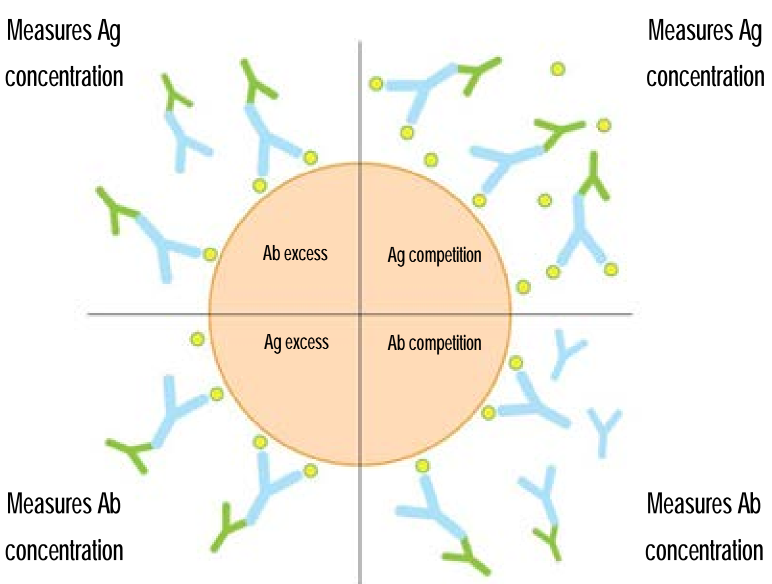

Figure 2.9 A series of summary diagrams illustrates the range of available designs; Ab-capture assays, indirect detection.

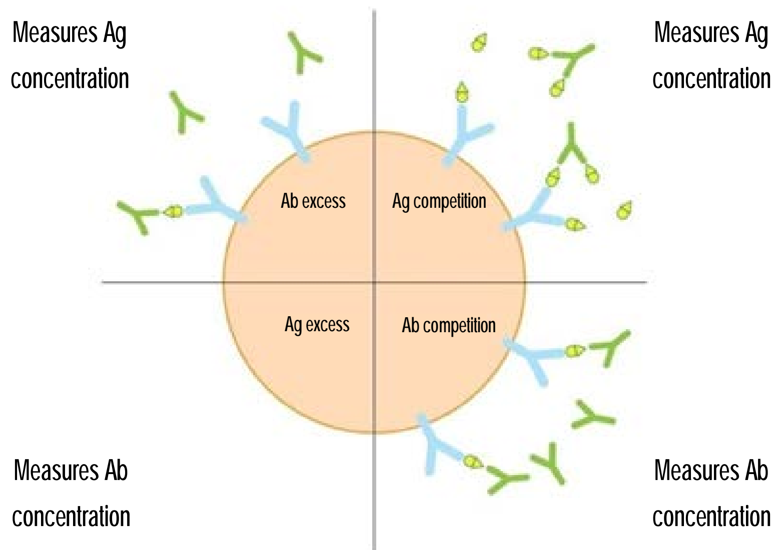

Figure 2.10 A series of summary diagrams illustrates the range of available designs; the two Ab assays.

2.5.4 Detecting the reaction

ELISA reactions can be visualized by eye or quantitated by several methods, including radioactivity, colorimetry, fluorescence, and chemiluminescence. Fortunately, antibodies typically have numerous moieties that can be used for binding labels, including α and ε aminos, carboxyls, sulfhydrals, and carbohydrates (Ellen *et al.*, 1980).

An attractive alternative to radioactivity is to introduce an enzymatic reaction by attaching an enzyme to a secondary antibody or by using avidin-biotin binding or another lectin pair. Biotin-avidin (or streptavidin) binding is often used in the secondary reactions. The very high binding affinity (>10^{15}M) of avidin and biotin makes them a highly desirable ligand pair for many assays. The specificity and strength of the binding reaction allow reaction times to be shorter and allow the use of conditions that reduce nonspecific binding (Castro *et al.*, 1987). Many biotinylating reagents are commercial available, and some often features that may be useful in particular application (Howard and Kaser, 2007). The sensitivity of these reactions is comparable to that of radioactive

detection. In both cases, the color developed after a given time is measured by optical density at a specific wavelength and compared to a standard curve to determine the amount of antibody/antigen present.

Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are the most commonly used enzymes as active catalyst. HRP reacts with hydrogen peroxide to release an oxygen free radical, which in turn reacts with a colometric substrate (Djordjevic *et al.*, 1994). One commonly used substrate is 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), ABTS. This product is solution in aqueous solutions and its reproducibility and high extinction coefficient make it an attractive option. The resulting green color can be measured at 405 nm with conventional plate readers. Another HRP substrate is 3, 3′, 5, 5′-tetra-methylbenzidine. The products of this reaction can be read at 650 nm or, after stopping the enzymatic reaction with acid, read the optical densities at 450 nm (Bos *et al.*, 1981).

AP removes a phosphate from a substrate from a substrate that, in turn, reacts with another component to yield a colored product. ρ-Nitrophenylphosphate yields a soluble bright yellow product that can be quantitate at 405-420 nm (Howard and Kaser, 2007).

Table 2.2 Substrate used for detection in ELISAs

Chromogen	Color	Wave lenght (nm)
Horseradish peroxidase (all with hydrogen peroxide as substrate) 2, 2- azo- bis (3- methylbenzthiazoline- 6- sulfonic acid) (ABTS) o- phenylenediamine (OPD) 3, 3, 5, 5- tetramethylbenzidine base (TMB)	 Green Orange Blue	 405 450 650
Alkaline phosphate ρ- nitrophenylphosphate (ρNPP)	 Yellow	 405-420

2.6 Antibody

The immunoglobulins are an extremely diverse group of plasma proteins which have Ab activity. They have been classified biochemically and antigenically. All share the same basic structure having two heavy chains and light chains usually linked by disulfide bonds (Fig 2.11). The number of intra-heavy chain disulfide bonds varies considerably in different classes and subclasses of immunoglobulins (Scott and Welt, 1997). In most immunoglobulins a single disulfide bond linkes the C terminus of a light chain to a heavy chain. The cysteine residue on the heavy chain which forms this bond is usually between V_H and C_HI domains; in some cases it is near the hinge. All immunoglobulins share the same types of light chains which are termed either lambda or kappa. Any immunoglobulin can have lambda or kappa chains but they are always symmetrical molecules. The ratio of the lambda and kappa chains varies greatly from species to species. Where several Abs have been isolate that bind to the same Ag it is importance to determine if they bind to the same or different epitopes on the target Ag. In addition to the diversity of immunoglobulin type, immunoglobulins show an enormous diversity of structure allowing their recognition of an almost infinite number of Ags. This diversity comes from structural differences at the amino terminus of both heavy and light chains which combine to form the Ag binding site. There are probably at least 10^{11} possible variants (Kerr and Thorpe, 1994).

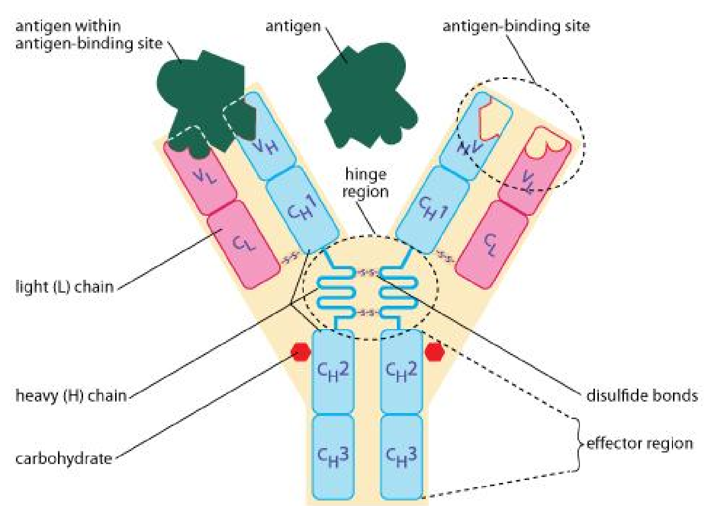

Figure 2.11 Structure of Ab.

Abs can be separated into different classes (IgM, IgG, IgA IgD and IgE) and subclasses (IgG_1, IgG_{2a}, IgG_{2b}) on the basis of the structure of their heavy chains. This reflects the number of genes coding for the heavy chain constant regions. IgG, IgD and IgE are monomeric. IgM and IgA usually exist as polymers of the basic structure. The different classes have different properties and this may be importance in determining the usefulness of the Abs for example in diagnosis and therapeutic application (Shepherd and Dean, 2000).

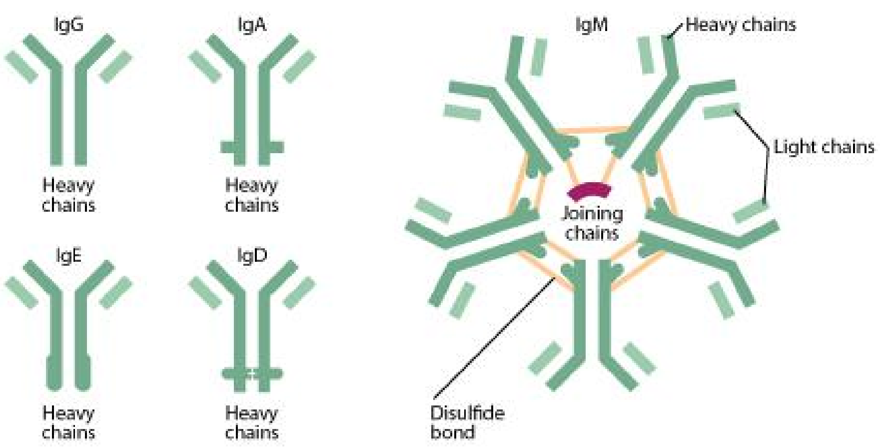

Figure 2.12 Different classes IgM, IgG, IgA IgD and IgE.

Abs can be produced as components of PAb and MAb. The MAb and PAb have different strengths and limitations in ELISAs. Because PAb bind to a wide array of epitopes, they can be particularly valuable as the labeled second Ab in a simple ELISA. MAb bind to a single epitope. Their high specificities and avidities are very useful, but care must still be exercised to ensure that the critical epitopes do not overlap. In addition, the use of MAbs in commercial ELISAs may be subject to patent protection (Howard and Kaser, 2007).

2.7 Choice of antibody production

2.7.1 Animal

The choice of rat or mouse strains for use in MAb production is normally constrained by the sources from which the myeloma cell lines were derived. Thus, the Balb/C mouse strain is preferred since many of the tumors from which myeloma cell lines have been derived were originally induced in such animals and they are therefore compatible for the propagation of hybridoma cells in vivo (Cryer and Liddell, 1991 and Festing *et al.*, 1978).

2.7.2 Antigens and immunogens

An Ag is the molecular entity which has the capacity to react with or bind selectively to an immunoglobulin. For the production of MAbs, and in common with the production of PAbs, the Ag is often also the immunogen (Neuberger *et al.*, 1985). The immunogen is the material which has the capacity, upon administration to the recipient, to elicit Ab production. Some substances can be Ags without being immunogenic and in general only macromolecules have the two properties of antigenicity and immunogenicity. For a substance to be immunogenic it must be a relatively complex structure with a composition which is recognized as foreign by the recipient (Oi *et al.*, 1978). The classes of molecules and structure most commonly used as immunogens

cover an enormous range of natural and synthetic materials including proteins, nucleic acids, carbohydrates, lipids, cellular components, bacteria, viruses and a wide variety of xenobiotics. Most of these are composed of numerous antigenic determinants (epitopes), that is, sequence of continuous or folded molecular structure, each of which may be capable of interacting with a specific Ab. Whether these epitopes are capable of eliciting an Ab response will depend on their recognition by the host as foreign and also on their molecular properties (Cryer and Liddell, 1991).

A number of factors have been investigated, particularly for proteins, with regard to the relationship between molecular properties and immunogenicity. Molecular size plays an important role. Generally, low molecular weight antigens (less than 1000) are not good immunogens. With proteins, both the amino acid composition of the immunogenic determinant and its accessibility will play a part. The overall conformation of the molecular is important, particularly the disposition of active groups at the surface of the macromolecule rather than in the interior of the structure (Bazin, 1982).

When raising polyclonal antisera to large complex molecules, the Ab response to dominant epitopes is likely to mask the response to other epitopes which might be of more interest. Although there are immunization strategies to overcome this problem, in MAb production efforts to isolate Abs against minor epitopes are usually concentrated on refining the specific of the post-fusion screening test, not on pre-fusion immunization.

2.7.3 Adjuvants

Three main classes of adjuvant have been identified. Firstly there is a group known as the "antigen depot" adjuvants. The most commonly used agents of this type include Freund's complete (CFA) and incomplete (IFA) adjuvant and preparations that contain aluminium hydroxide (Fleming and Pen, 1988). Primarily, these adjuvant act by facilitating slow release of the immunogen from the point of administration. The oil-based adjuvants of this class, like Freund's (CFA and IFA) when administrated in the form of stable emulsions containing immunogen, also act to induce the formation of a granuloma which is rich in macrophages immunocompletent cells. Furthermore,

because the droplets containing adsorbed immunogen become transported in the lymphatics, these materials also have a direct effect on the delivery of the immunogen to the antibody-producing cells (Benkirane *et al.*, 1988). Complete Freund's adjuvant is distinguished by the presence of a heat-killed preparation of *Mycobacterium tuberculosis* or *M. butyricum* suspended in the light mineral oil base which also contains emulsifying agents. The presence of the *Mycobacterium* enhances the local inflammatory response and for this reason secondary inoculations of immunogen should be given in the presence of incomplete Freund's adjuvant from which the *Mycobacterium* is excluded (Hildreth *et al.*, 1989).

2.7.4 Routes of administration

The anatomical site at which immunogens are administered also contributes to the overall Ab response. The aim is to create a slow release depot of immunogen, so the site should not be in highly vascular areas that can quickly remove and metabolize the immunogen. In mice and rats, the most appropriate site is subcutaneous, at the scruff of the neck. Subcutaneous injection is also suitable for rabbits, but because of the larger surface area, multiple injection sites are preferred. Intradermal and intramuscular injections are also common routes in the rabbit, but are not normally used in smaller rodents because of their size and skin thickness. Foot pad injections are said to produce good immune responses since they are close to lymphatic systems, but are not advised because of the discomfort caused to the animal. Prefusion boots are usually given via the intravenous rout in order to quickly stimulate β-lymphocyte proliferation (Galfre and Milstein, 1981).

2.7.5 Immunization procedure

The Ab response to primary injection of an immunogen follows a characteristic pattern. Initially, there is a lag phase, followed by a logarithmic increase in Ab levels, then a plateau before levels decline (Hildreth *et al.*, 1989). The whole response is

spread over approximately three weeks. The Abs produced are predominantly IgM. If a secondary challenge with immunogen is presented three to four weeks after the first, then the Ab response is quicker, much greater and lasts for longer. The predominant class of Ab is IgG and the average affinity is increased, particularly if a low immunogen dose is given (Fleming and Pen, 1988).

Once a satisfactory Ab response has been established, animals can be kept as a pool of immune lymphocyte donors for fusion. In practice, it has been shown that the newly challenged and thus rapidly dividing cells preferentially fuse to produce hybridomas. Success is best assured if the final booster injection is given three to four days prior to harvesting the lymphoid cells. If the cells are allowed to reach the peak of Ab production *in vivo*, they are beyond the best state for fusion (Galfre and Milstein, 1981).

The immunogen preparation need not necessarily be pure for the successful production of MAbs. However, to ensure the best chance of yielding specific hybridomas, the presence of the desired Ab should be detectable in the serum of the recipient prior to the final booster dose. Ideally, the specific Ab titre should be at least 1:1000 and preferably more (Boulianne *et al.*, 1984).

Table 2.3 Recommended dosed of immunogen

Immunogen	Preliminary doses	Final booster dose
Soluble or membrane protein	10-100 µg	Up to 500 µg
Nucleic acids	200 µg	200 µg
Eukaryotic cells	2-20 x 10^6	2-20 x 10^6
Bacterial cells	50 µg of protein	50 µg of protein
Viruses	10^7 particles (three doses at weekly intervals)	-
Fungal antigens	20-100 µg	20-100 µg

2.8 Monoclonal antibody

MAb is usually produced as the secretion products of produced in relatively large quantities of consistent quality and characteristics. The overall strategy for the production of MAbs is shown in Figure 2.13. As can be seen, this involves the administration of an Ab-containing preparation to an appropriate recipient animal. The treated animal then becomes the source of sensitized Ab-producing cells. These cells are then mixed with a specially selected immortal cell line and the mixture exposed to an agent which promotes the fusion of cells (Cotton and Milstein, 1973). Under these circumstances fusion between cells takes place a random manner and a fused cell mixture together with unfused cells of each type is the product. The immortal cell partner for the Ab-producing cell is the myeloma cell, itself a cancerous cell derived from the immune system (Coffino *et al.*, 1972). The myeloma cell lines that are used are specially chosen such that, by the use of specific selection media, the only cells to persist in culture from the fusion mixture are those that are hybrids between cells derived from the immunized donor and myeloma cells, i.e. hybridomas. The other possible combinations of fused cells are not able to survive the selection pressure that is applied (Fazehas *et al.*, 1980).

At this stage, the hybrids that survive will still be a very mixed population, not all of which will be able to synthesize Ab. Furthermore, of those that can synthesize Ab, only a few may exist that are able to produce Ab which has the predetermined specificity towards the chosen Ag (Cryer and Liddell, 1991).

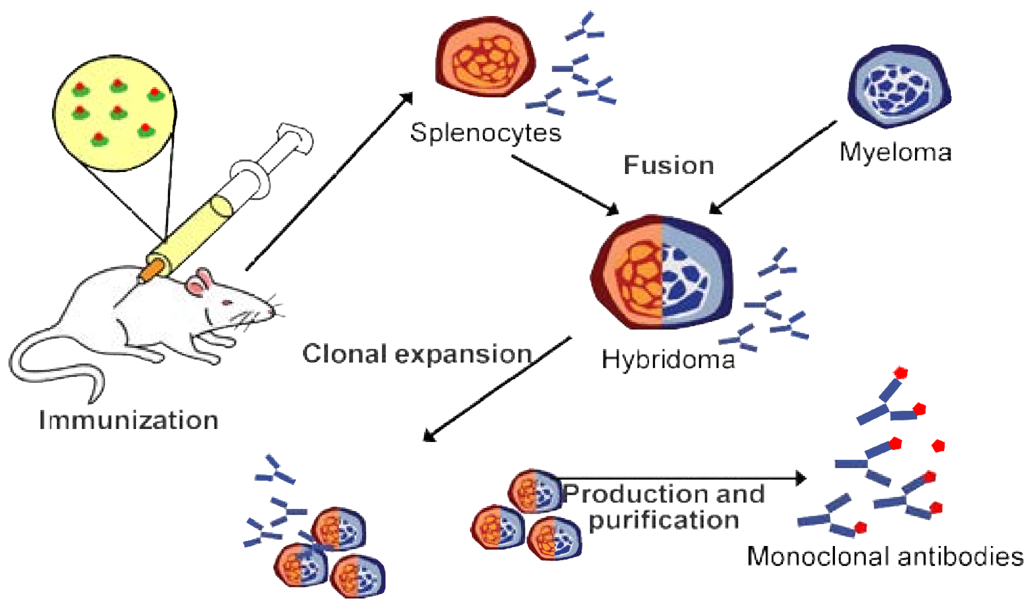

Figure 2.13 The strategy for the production of MAbs.

Cell fusion for the immortalization of spleen cells requires a means of selection for mixed hybrid (myeloma- spleen cell hybrids). As shown in Figure 2.14 and Table 2.4, the favored selection procedure depends on the capacity of cells to use the "salvage" pathway for quanosine production when the main biosynthetic pathway is blocked (usually by the antibiotics aminopterin or amethopterin). This salvage pathway relies on the presence of the enzyme hypoxanthine quanine phosphoribosyl transferase (HGPRT) (Hildreth *et al.*, 1989). Cells lacking the enzyme will die when grown in media containing hypoxanthine, aminopterin and thymidine because both pathways necessary for the formation of the purine precursors of DNA are blocked. However, a cell which is $HGPRT^-$ will grow in the selection medium if it is fused with a cell which is $HGPRT^+$. Myelomas which are $HGPRT^-$ are needed for successful selection of myeloma ($HGPRT^-$) and spleen cell ($HGPRT^+$) hybrids in the hypoxantine- aminopterine- thymidine (HAT) medium (Click *et al.*, 1972).

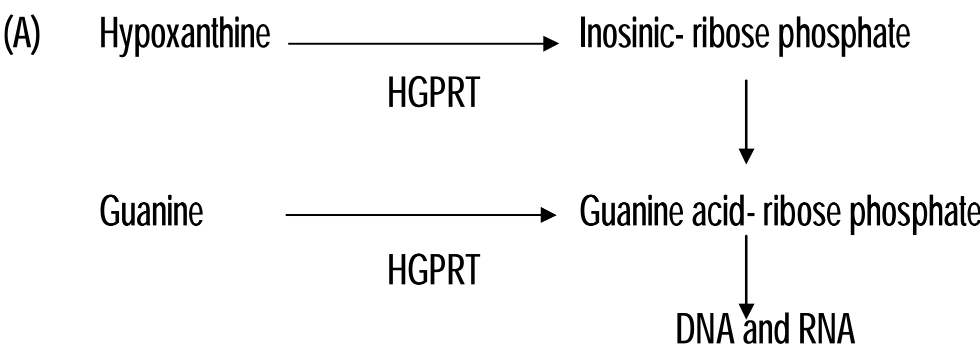

Figure 2.14 (A) Purine biosynthesis salvage pathway. (B) Pyrimidine biosynthesis salvage pathway.

Table 2.4 Post- fusion selection.

Cell type	DNA synthesis		Survival in HAT medium
	Salvage pathway	De novo pathway	
Myeloma	HGPRT$^-$	Aminopterin sensitive	Die (No DNA dynthesis)
Spleen	HGPRT$^+$	Aminopterin sensitive	Die (Finite survive *in vitro*)
Myeloma- Spleen hybrid	HGPRT$^+$	Aminopterin sensitive	Live
Myeloma- myeloma hybrid	HGPRT$^-$	Aminopterin sensitive	Die (No DNA synthesis)
Spleen- spleen hybrid	HGPRT$^+$	Aminopterin sensitive	Die (Finite survival *in vitro*)

2.8.1 Screening of hybridoma cell

In the first screen there are likely to be 180 wells, each containing 150-200 µl of culture supernatant, up to 100 µl of which is available for use in the test. The percentage of cells that can be secreting specific Ab is highly variable, depending upon a number of factors which include the immunogenicity of the immunogen and the specificity of the screening test. On the basis of these first test results a number of wells and selected and the cells they contain transferred to larger (1.5 ml capacity) culture wells. The chosen cells are allowed to expand in number until they approach one third confluency (0.5 x 10^6 cells/ml). This expansion phase should take on longer than seven days but may be completed in a much shorter time if the cells show particularly vigorous growth. The cell population will then be ready for rescreening. It is advisable cells are particularly susceptible to chromosomal loss of Ab production or to the overgrowth by some vigorous non-secreting cells (Fazehas *et al.*, 1980).

Of the wells still shown it contain secreted Ab, a manageable number are selected for cloning, whilst the remainder are frozen for later retrieval if necessary (Alsenz and Loos, 1988). The most usual cloning method, that of limiting dilution, generates 96 new culture wells from each of the previous. Each clone plate will, therefore, yield 96 x 100 µl of culture supernatant for assay, within approximately two weeks. Statistically, only approximately 33% of the 96 wells will contain living cells, but it is rarely practical to select only these for testing. The screening test will need to accommodate all the 96 wells and also possibly several other clone plates at the same time. Of the wells positive for growth, an even smaller proportion, if any, will be secreting specific Ab. However, only those most strongly positive for Ab secretion, also displaying healthy growth, and preferably originating from one clone, should be for further development. It is possible, though unlikely, that a monoclonal cell line secreting specific Ab will be found at this stage, in which case only expansion and propagation of the cell line is required. Normally, several cycles of cloning and screening will be necessary before monoclonal is assured. A good indication of monoclonal is that in the

final clone plate all wells displaying cell growth will also show specific Ab secretion (Birth *et al.*, 1985 and Ellen *et al.*, 1980).

2.8.2 Single cloning

The purpose of cloning is to isolate a single specific Ab-secreting cell from the hundreds of other cells in the culture so that a monoclonal cell line can be established. Three basic cloning techniques may be identified, but the need to freeze and store samples of all the culture prior to and during cloning must not be overlooked (Cordeil *et al.*, 1987). This requirement for continued storage of cell at all stages is essential for the following reason. Firstly, throughout cloning, particularly during the early stages, these is a high probability of chromosome loss and consequent loss of immunoglobulin secretion from the hybrid. Secondly, there is always a risk of overgrowth by non-producing hybrids. Thirdly, the risk of infection and/or mechanical breakdowns should be protected against. The general procedure for the storage of cell is described later (Li *et al.*, 1991).

This is the simplest and most popular method of hybridoma cloning. In practice, there are a number of different procedures but the ultimate objective is to set up at least three very low dilutions of cells resulting in a mean of one cell per well. Within two weeks of cloning, clones should be visible macroscopically and, subjectively at least, some of the wells may be considered to contain monoclonal growth. At this stage each of the cell supernatants should be screened to determine which of them contains the desired Ab. Positive wells which are likely to be of monoclonal origin are expanded. They are then recloned, with a proportion of the cells being frozen and stored (Alsenz and Loos, 1988). The recloning is repeated at least three times, after which the final clone plate should display positive antibody production in virtually all the seeded wells. Cells in the first clone plate producing the desired Ab even though they are unlikely to be of monoclonal origin should not be discarded. The contents of such wells may also be expanded and the cells stored so that they may be cloned at a later stage, should the necessity arise (Galfre and Milstein, 1981).

2.8.3 Characterization

Once a cellular source of MAb has been established in culture, it is usual to obtain a small quantity of ascetic fluid (i.e. from five animals) for further characterization before preparing larger stocks of the Ab. A number of tests need to be carried out in order to relate the outcome to the final application and required specificity of the Ab. This part is concerned with the general characterization of MAb preparations, such as titre, isotyping, affinity and epitope analysis. This is followed by a consideration of Ab purification and labeling methods which will be of use in a variety of use in a variety of applications (Hildreth *et al.*, 1989). In addition, these pilot studies will also include an assessment of the growth characteristics of the hybridoma, its stability on freezing, its suitability for the production of ascites and the suitability of the Ab for direct labeling. Clearly, however suitable the specificity of the Ab may be, it will be of limited use if the hybridoma grows poorly, has a low recovery from frozen stock or will not produce ascetic fluid (Ellen *et al.*, 1980).

2.8.4 Determination of isotypes

The class of Ab may have been selected for by the screening tests used during the establishment of the hybridoma. Adaptation of the existing screening test to include isotype testing, as described above, may seem the simplest approach for the identification of an isotype in a specific case (Alsenz and Loos, 1988). However, isotype-specific reagents are expensive to buy individually and, although potentially thousands of Abs can be tested, many research laboratories have no need for this capacity. It may, be more cost effective, in a research context, to use a commercially available isotype testing kit. Although such a kit will have a smaller capacity, it will contain all the necessary reagents, including standard controls for the isotyping of a number of MAb products. Kits are available, based on several different assay systems, including ELISA, immunodiffusion and red cell agglutination (Hildreth *et al.*, 1989).

2.8.5 Cross-reactivity

An important early characterization test of any panel of Abs is the analysis of whether they react with the same, close or totally different epitopes. The simplest way of testing this characteristic is to label one of the Abs directly and to allow it to compete for Ag with unlabelled Abs of other sources. For this it is necessary to allow serial dilutions of unlabelled Ab (from saturating levels to zero) to bind to Ag, to wash off any excess and to apply the competing Ab label. If the label binding is maximal throughout the whole range of unlabelled Ab concentrations, it may be deduced that the two Abs are directed towards different epitopes. The binding of labelled Ab may be either totally or partially inhibited. This will reflect the existence of competition between the Abs for the same or very close epitopes or the fact that the binding of one Ab causes steric alterations in the molecule to prevent the other binding to its own distinct epitope. However, results should be interpreted carefully, since a high-affinity antibody label may displace a low-affinity Ab at the same epitope (Alsenz and Loos, 1988).

2.8.6 Antibody purification

Supernatant contains Ab and most culture supernatant contains protein from bovine serum that might interfere with some applications of MAbs. However, it is not always necessary to purify the Abs, since they may be used at sufficient dilution and with appropriate controls to make the impurities irrelevant. Purification is necessary if the Abs are to be labelled, since the greater the purity of the labelled Ab the greater the signal-to-nosie ratio in the assay (Burchiel *et al.*, 1984). Polyclonal antisera require affinity purification before labelling but this is not so critical with MAbs. In practice, simple salt fractionation of ascetic fluid may be sufficient, although it may sometimes be necessary to protect the active site of the Ab with an immunoadsorbent. However, elution of the Ab from this absorbent may cause additional damage, eapecially if the Ab is of very high affinity. The effect of the purification procedure on immunoreactivity should always be tested prior to use (Bruck *et al.*, 1982).

2.9 Current methods for nitrofurans detection in foods

Several analytical methods for the determination of nitrofurans residues in various sample matrices have been developed such as honey, muscle tissue, chicken meat and animal feeds. Few methods have been developed for nitrofurans in honey, such as gas chromatography-tandem mass spectrometry (GC-MS/MS) (Sanchez-Brunete *et al.*, 2005), liquid chromatography-mass spectrometry (LC-MS) (Tribalat *et al.*, 2006) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) (Jenkins and Young, 2005; Lopez *et al.*, 2007 and Ortelli *et al.*, 2004). Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESIMS/MS) was developed for determination of nitrofurans metabolites in muscle tissue (Leitner *et al.*, 2001) and chicken meat (Mottier *et al.*, 2005). Xia *et al.* (2008) described a simultaneous identification and quantification of nitrofurans in pork using HPLC couple with ESIMS/MS. Few methods have been also reported for determination of nitrofurans in animal feeds, such as HPLC with ultra violet detection (HPLC-UV), LC-MS (McCraken and Kennedy, 1997) and LC with photodiode-array detection (LC-DAD) and LC;MS/MS (Barbosa *et al.*, 2007). The use of chemiluminescence (CL) combined with flow injection (FI) for the analysis of nitrofurans residues in animal feed samples has been reported by Thongsrisomboon *et al.*, 2010.

As part of the "FoodBRAND" project, an extensive survey of pork was undertaken across 15 European countries. Sample (n = 1500) purchased at retail outlets were analysed for the nitrofurans metabolites AOZ, AMOZ, AHD and SEM using LC-MS/MS determination of nitrobenzaldehyde derivatives. Measurable residues of nitrofurans metabolites were confirmed in 12 samples of which 10 samples were purchased in Portugal (AOZ, 0.3 µg/kg; AMOZ, 0.2-0.6 µg/kg) and one sample each in Italy (AMOZ, 1.0 µg/kg) and Greece (AOZ, 3.0 µg/kg) (O'Keeffe *et al.*, 2004).

In principle, specifically tailored antibodies are capable of detecting nitrofurans metabolites through the use of an enlarged target molecule, which can be formed by a simple derivatisation step. The first antibodies specific for derivatised SEM were produced in two labolatories. The PAbs developed by Cooper *et al.*, (2007) using

3-carboxyphenyl SEM were incorporated into a competitive direct ELISA providing a detection capability for SEM in chicken muscle at 0.25 µg/kg. MAbs raised against the 4-carboxyphenyl SEM hapten by Gao *et al.* (2007) exhibited comparable sensitivity in assay buffer. Vass *et al.* (2008) reported the use of ELISA for screening semicarbazide and its application for screening in food contamination. 3-Carboxybenzaldehyde was used to synthesizing CPSEM. Rabbits were immunized with CPSEM and produced PAbs for apply to SEM detection in porcine.

The formed ρ-nitrophenyl 3-amino-2-oxazolidinone (NPAOZ) is determined by ELISA in the concentration range of 0.05 – 5.0 µg/L after homogenization, protease treatment, acid hydrolysis. Detection capability was 0.4 µg/kg for shrimp, poultry, beef and pork muscle (Diblikova *et al.*, 2005).

Pimpitak *et al.*, (2009) reported the generation of two MAbs with high specificity for AMOZ and the development of MAbs-based ELISA to detect AMOZ in shrimp samples using clones number 2E5.1. When applied in ELISA to detect AMOZ in fortified shrimp samples, the detection capability and limit of detection were 0.3 and 0.16 µg/kg, respectively.

Preparation of PAbs to a derivative of AHD and development of an indirect competitive ELISA for detection of nitrofurantoin residue in water was reported by Liu *et al.*, 2007. Considering that nitrofurans often are used illegally to feed animals through drinking water. The Ab obtained shows excellent specificity and sensitivity toward the parent drug nitrofurantoin due to the structural similarity between AHD and nitrofurantoin. The detection limit was determined to be 0.2 ppb. While Jiang *et al.* (2011) reported a sensitive and specific ELISA for the detection of AHD. The highly specific antibody against AHD was prepared by MAb technology. The Ab exhibited negligible cross reactivity with other nitrofurans. The limit of detection in four kinds of animal tissue (fish, shrimp, pork and chicken) were all below 0.2 µg/kg and recoveries ranged from 75% to 116.7% for fortified samples at levels of 0.2-5 µg/kg.

Moreover, Li *et al.* (2010) reported a broad specificity indirect competitive immunoassay for determination of nitrofurans in animal feeds. This study first reported

the production of a broad specificity PAb for nitrofurans and the development of a heterologous multi-analyte ELISA method. This ELISA method was sensitive, accurate and simple, and showed good agreement with a HPLC method.

CHAPTER III

MATERIALS AND METHODS

3.1 Animals and Cell lines

- Female 8 weeks old BALB/c mice (inbred strain) were obtained from the National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand.
- Myeloma cells P3-X63Ag8 were purchased from the American Type Culture Collection (ATCC; TIB-9).
- Myeloma cells SP2/0-Ag14 were purchased from the American Type Culture Collection (ATCC; CRL8287).

3.2 Chemicals, Antibodies and Kits

- 30% Hydrogen peroxide (H_2O_2)	Merck, Germany
- 40% Acrylamide and Bis-acrylamide solution	Bio-Rad, USA
- Absolute ethanol	Merck, Germany
- Absolute methanol	Merck, Germany
- Acetic acid	Sigma-Aldrich, USA
- Aminopterin	Sigma-Aldrich, USA
- Aminohexanoyl-Biotin-*N*-Hydroxysuccinimide	Zymed, USA
- 1-Aminohydantoin (AHD)	Sigma-Aldrich, USA
- 3-Amino-2-oxazolidone (AOZ)	Sigma-Aldrich, USA
- Ammoniumpersulfate (APS)	Bio-Rad, USA
- BCA^{TM} Protein Assay Kit	Pierce, USA
- Bovine serum albumin (BSA)	Sigma-Aldrich, USA
- Bromophenol blue	Sigma-Aldrich, USA
- Citric acid	Merck, Germany
- Clenbuterol	Sigma-Aldrich, USA

- Coomassie brilliant blue G	Pierce, USA
- D-Glucose	Sigma-Aldrich, USA
- Diethyl ether	Merck, Germany
- Dimethylsulfoxide (DMSO)	Fluka, Switzerland
- di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
- Doxycycline	Sigma-Aldrich, USA
- Ethanol	BHD, England
- 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)	Sigma-Aldrich, USA
- Fetal calf serum (FCS)	Invitromax, USA
- Freund's complete adjuvant (FCA)	Sigma-Aldrich, USA
- Freund's incomplete adjuvant (FIA)	Sigma-Aldrich, USA
- Furazolidone (FZD)	Sigma-Aldrich, USA
- Gentamycin	T.P. drug laboratories (1969) Co., Ltd., Thailand
- Glycerol	Merck, Germany
- Glycine	Sigma-Aldrich, USA
- Horseradish peroxidase-conjugates goat anti mouse IgG (GAM-HRP)	Jackson Immuno, USA
- Hydrochloric acid (HCl)	Merck, Germany
- Hydrogen peroxide (H_2O_2)	Fluka, Switzerland
- Hypoxanthine	Sigma-Aldrich, USA
- L-GGlutamine	Sigma-Aldrich, USA
- Methanol	BDH, England
- Mouse monoclonal antibody isotyping Kit	Sigma-Aldrich, USA
- N-Hydroxysuccinimide (NHS)	Fluka, China
- Oxytetracycline	Sigma-Aldrich, USA
- Penicillin G	Sigma-Aldrich, USA

-	Peroxidase-Rabbit Anti Mouse IgG (Gamma chain specific)	Zymed, USA
-	Polyethylene glycol (PEG)	Sigma-Aldrich, USA
-	Potassium chloride (KCl)	Merck, Germany
-	Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany
-	Pyruvic acid	Sigma-Aldrich, USA
-	Rolitracycline	Sigma-Aldrich, USA
-	RPMI 1640 medium	Biochrom AG, Germany
-	Salbutamol	Sigma-Aldrich, USA
-	Skim milk	Anline, Thailand
-	Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich, USA
-	Sodium carbonate (Na_2CO_3)	Merck, Germany
-	Sodium chloride (NaCl)	Merck, Germany
-	Sodium dihydrogen phosphate (NaH_2PO_4)	Carlo Erba, USA
-	Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, USA
-	Sodium hydroxide (NaOH)	Merck, Germany
-	Sodium pyruvate ($C_3H_3O_3Na$)	Sigma-Aldrich, USA
-	Streptomycin	Sigma-Aldrich, USA
-	Sulfuric acid (H_2SO_4)	Merck, Germany
-	Tetracycline	Sigma-Aldrich, USA
-	N,N,N,N-Tetramethyl-ethylenediamine (TEMMED)	Pierce, USA
-	3,3′,5,5′-Tetramethylbenzidine (TMB)	Sigma-Aldrich, USA
-	Thimerosal	Sigma-Aldrich, USA
-	Thymidine	Sigma-Aldrich, USA
-	Tris (hydroxymethyl) aminomethane (Trisma base)	Merck, Germany
-	Tween 20	Sigma-Aldrich, USA

3.3 Equipments

Equipment	Manufacturer
- -20 °C and -70 °C Freezer	Sanyo, Japan
- 30 °C and 37 °C Incubater	Memmert, Germany
- 4 °C Refrigerator	Mitsubishi Electric, Japan
- 5% CO_2 Incubator (model 311)	Thermo Electron Corporation, USA
- 96-well EIA/RIA plate	Nunc, Denmark
- Autoclave (model ES-315)	Tomy, Japan
- Cell culture dish, 60 and 90 mm	Bibby Sterilin Ltd., UK
- Centrifuge tube 15 and 50 ml	CLP, USA
- Cryotube	Nunc, Denmark
- Filter paper	Whatman, Germany
- Heat block : Thermomixer Compact	Eppendorf, Germany
- High speed refrigerated centrifuge (model J2-21)	Beckman, USA
- Hot air oven (model D06063)	Memmert, Germany
- Hot plate stirrer (model C-MAG HS 10)	Becthai, Thailand
- Hypodermic needle 18G and 21G	Nipro, Japan
- Inverted microscope	Nikon, Japan
- Laminar flow ('clean' model V6)	Lab Survice LTD part, Thailand
- Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic	Harsco Corporation, USA
- Microcentrifuge tube 1.5 ml	Axygen Scientific, USA
- Micropipette P2, P20, P100, P200, P1000 and P5000	Gilson, France
- Microplate reader	Titertek multiskan, Finland
- Microscope (model CH 30RF200)	Olympus, USA
- Nitrocellulose transfer membrane	Whatman, Germany
- Orbital shaker	Bioblock Sciencetific, France

- Petri Dish	Hycon, Germany
- pH meter (model S20-K)	Mettler Toledo, USA
- Pipettes	Eppendorf, Germany
- Precision weighting balance (model AG 204 and PG 4002-5)	Mettler Toledo, Switzerland
- Protein III System for SDS-PAGE	Bio-Rad, USA
- Refrigerated incubater shaker (Innova™ 4330)	New Brunswick Scientific, USA
- Refrigerated Microcentrifuge 6500	Hettich Zentrifugen, Germany
- Semi-dry Electrophoretic Transfer Cell : Trans-Blot® SD	Bio-Rad, USA
- Spectrophotometer (Genesys 20 model 4001/4)	ThermoSpectronic, USA
- Syringe 1 and 5 ml	Nipro, Japan
- Tip 0.01, 0.2 and 1 ml	Axygen Scientific, USA
- Tissue cell culture plate 24-, 48- and 96-well	Corning Incorporation, USA
- Ultra-Pure Water Purification System	Elga, England
- Ultrasonic disruptor (model UD-201)	Tomy, Japan
- Vacuum pump	Iwaki, Japan
- Vortex mixer (model G560E)	Scientific Industries, Inc., USA
- Water bath	Becthai, Thailand

3.4 Experimental procedures

Part I Production of monoclonal antibody

3.4.1 Preparation of antigen and coating agent

3.4.1.1 Synthesis of CPAHD derivative

An excess of 50 mM AHD over 25 mM CBA was stirred overnight in 1 ml pyridine. The desired product in the reaction mixture was detected by thin-layer chromatography or TLC (stationary phase: aluminum silica, solvent system: 5% methanol in chloroform). CBA, AHD, CPAHD were visualized under UV light while AHD and CPAHD were visualized by ninhydrin staining. Mobility of substances were measured and calculated Relative mobility (Rf) following formula. Diagram of synthesis of CPAHD derivative was shown in Figure 3.1. After removal of pyridine by evaporation, pure CPAHD was obtained by recrystallization from dimethylformamide.

$$\text{Relative mobility (Rf)} = \frac{\text{Movement of substance (cm)}}{\text{Movement of solvent (cm)}}$$

3.4.1.2 Preparation of antigen

CPAHD was conjugated to an immunogenic carrier protein BSA via an active ester method. The carboxylic acid on the hapten was activated with EDC and NHS to produce an active ester, which then reacted with amine group of BSA to form an amide bond.

A mixture of the 0.1 M CPAHD, 0.2 M NHS and 0.2 M EDC in 1.5 ml dry 1, 4-dioxane were stirred overnight at room temperature. After centrifugation, this solution was added very slowly while stirring to a mixture solution of 2 ml DMF and 3 ml PBS containing 0.005 M BSA and stirred overnight at 4°C. The mixture was dialyzed against

phosphate buffer (0.1 mol/l, pH 7.4) for 3 days. CPAHD-BSA was freeze-dried and stored at -20°C until use.

The CPAHD-AHD conjugated was checked protein concentration by BCA method. Then, the conjugation was confirmed by two methods, colorimetric reaction of trinitrobenzenesulphonic acid (TNBS) and determination of mass by MALDI-TOF-MS. Mass ratio of CPAHD to BSA was calculated following formula.

$$\text{Mass Ratio of CPAHD: BSA} = \frac{\text{Mass of CPAHD-BSA} - \text{Mass of BSA}}{\text{Mass of CPAHD}}$$

3.4.2 Immunization of mice

Female mice BALB/c, 8 weeks old (National Laboratory Animal Centre, Mahidol University, Thailand), were immunized by intraperitoneal injections with 50 µg CPAHD-BSA in 100 µl sterile PBS emulsified with 100 µl CFA. After three booster doses of CPAHD-BSA (50 µg) in IFA at 2 week intervals, the sera were collected and assayed for anti-CPAHD Abs by an indirect ELISA. Mice were immunized with a final dose of CPAHD-BSA (50 µg) in sterilized normal saline. All procedure involved animals have been approved by IACUC of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

Nitrofurantoin, NFT

1-aminohydantoin, AHD

AHD + 2-NBA → NPAHD

AHD + 3-CBA → CPAHD

Figure 3.1 Chemical structures and chemical reaction to generate of NFT, AHD, NPAHD and CPAHD.

3.4.3 Determination of titre by indirect ELISA

Blood sample was taken from each mouse by tail bleeding, and centrifuged at 2,500 g for 5 min at 4°C to collect the sera. The Ab response was tested against CPAHD-BSA by indirect ELISA as described below. Plates were coated with CPAHD-BSA (3 μg/ml) and incubated at 4◦C overnight. Plates were washed three times with PBS containing 0.05% Tween20 (PBST) and blocked with 5% skimmed milk in PBS. After washing, diluted antisera from mice were added, and the plates were incubated at 37◦C for 2 h. After washing, secondary Ab (goat anti-mouse IgG Abs conjugated with horse radish peroxidase (HRP)) was added and the mixture was incubated at 37◦C for 1 h. Plates were washed and then added substrate solution (tetramethylbenzidine (TMB) and H_2O_2 in 0.15 M citrate buffer pH 5.0 with 0.34% H_2O_2). The reaction was stopped after 10 min with 100 μl of 1 M H_2SO_4, and the absorption was measured at 450 nm using microtiter plate reader. Three day before fusion, mice were given final dose with the same antigen without adjuvant to use as spleen donor for hybridoma production. Serum samples were collected before first immunization and used as negative control.

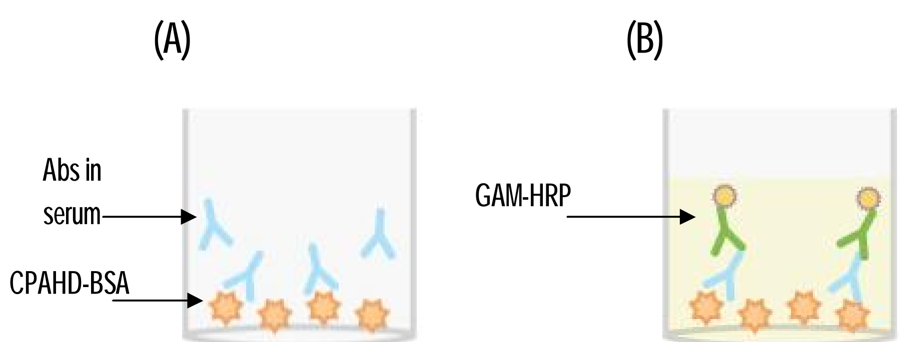

Figure 3.2 Diagram of indirect ELISA for monitoring Ab titre of immunized BALB/c mice.
(A) CPAHD was coated in microtiter plate and Abs were then added.
(B) GAM-HRP was added to system.

3.4.4 Specificity of antibody in sera

Specificity of Ab in the mice sera were tested against the free form of AHD, CBA, CPAHD and NPAHD in the free form. Dilution of mice serum samples was performed to give absorbance (450 nm) value at 1.0 in the indirect ELISA. Concentrations of all competitors were prepared at the level 20 µg/ml. An indirect competitive ELISA was used to check for specific Abs. Plates was coated CPAHD-BSA (3 µg/ml) and blocked as described in indirect competitive ELISA. After washing, competitors and serum were added, and the mixtures were incubated at 37°C for 2 h. Then the plates were washed and assayed as described in indirect ELISA. Finally, the specificity of serum were tested by calculating in term of % competition following this formula.

$$\% \text{ Competition} = \frac{A_{450} \text{ without competitor} - A_{450} \text{ with competitior} \times 100}{A_{450} \text{ without competitor}}$$

3.4.5 Production of monoclonal antibody

3.4.5.1 Myeloma cell lines

Myeloma cell line used for fusion were SP2/0-Ag14 or P3-X63Ag8. They were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified 5% CO_2 incubator at 37°C. The myelomas were maintained in exponential phased growth by subculturing for one week prior to fusion. Approximately 10^7 cells of SP2/0-Ag14 or P3-X63Ag8 myeloma were added into a 50 ml of a polypropylene tube and then centrifuged at 380 g for 5 min. After the supernatant was discarded, RPMI 1640 medium supplemented with 0.2 mg/ml gentamicin was added and then placed in a humidified incubator for using in the fusion step.

3.4.5.2 Isolation of splenocytes

CPAHD-BSA immunized mice were anesthetized with diethyl ether before blood drawn by cardiac puncture, and their sera were pooled and stored at -20°C and used as a positive control in the subsequent immunological assay. The spleen was collected aseptically from euthanized mice and gentle washed in sterile Petri dishes containing RPMI 1640 medium supplemented with 0.2 mg/ml gentamicin to minimize the chance of contamination. Then, the connective tissues were carefully removed as much as possible during extensive soak in the medium. A single cell suspension was prepared by cutting the spleen into small pieces and then crushing by a 10 ml syringe plunger through a steriled grid into the medium. Spleen cells were harvested by centrifugation at 380 g for 5 min and resuspended in 5 ml of the medium for fusion with myeloma cells.

3.4.5.3 Production of hybridoma

Anti-CPAHD MAbs were produced using somatic cell fusion technique. Myeloma cells were added into a 50 ml of polypropylene tube containing the spleen suspension at a 10:1 ratio. After mixing the cells by tapping the tube, 1 ml of pre-warmed (37°C) 50% (w/v) polyethylene glycol (PEG) was added dropwise to the cell suspension using a Pasteur pipette and simultaneously the tube was slightly agitated. The cell suspension was centrifuged and the cell pellet was washed with the same medium in order to remove PEG. The fused cells were suspended in hypoxanthine-aminopterine-thymidine (HAT) selective medium supplemented with 20% FCS. The fused cells (hybridomas) were seeded in 96-well culture plates supplemented with hypoxanthine aminopterin thiamidine (HAT) medium containing 20% fetal calf serum and cultured at 37°C in 5% CO_2 incubator. Half medium of the wells was replaced by fresh HAT medium on day 7 post-fusion. Cell growth in the bottom of the wells was observed using inverted microscope. When cells grow about two-thirds of the wells (10 to 12 days after fusion), the hybridoma culture supernatant in each well was screened for Ab activity against

CPAHD as described below. Cells were grown in HAT medium for 2 weeks and then HAT was substituted by HT medium (HAT medium without aminopterine).

3.4.6 Screening of hybridoma

3.4.6.1 Primary screening by indirect ELISA

Plates were coated with CPAHD-BSA and incubated at 4°C overnight. Plates were washed three times with PBS containing 0.05% Tween20 (PBST) and blocked with 5% skimmed milk in PBS. After washing, culture supernatants or antisera was added, and the plates were incubated at 37°C for 2 hr. After washing, secondary Ab (goat anti-mouse IgG Abs conjugated with HRP) was added and the mixture was incubated at 37°C for 1 h. Plates was washed and then added substrate solution (TMB and H_2O_2 in 0.15 M citrate buffer pH 5.0 in 1 M sodium acetate buffer pH 6 with 0.34% H_2O_2). The reaction was stopped after 10 min with 100 µl of 1 M H_2SO_4, and the absorption was measured at 450 nm using microtiter plate reader. Then the positive samples were further selected by secondary screening.

3.4.6.2 Secondary screening by indirect competitive ELISA

An indirect competitive ELISA was used to screen for Abs specific for free NPAHD and to evaluate the sensitivity and cross reactivity. Plates was coated and blocked as described in indirect ELISA. After washing, competitors (different NPAHD concentrations) and Abs were added, and the mixtures were incubated at 37°C for 2 h. Then the plates were washed and assayed as described in indirect ELISA.

3.4.7 Single cloning of hybridomas by limiting dilution

Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 20% FCS to ensure that the hybridoma was stable and single-cell cloned. From each positive well, cell suspension was diluted with the medium and then mixed with HT medium. The mixture (100 µl) was dispensed into 96-well plate containing 100 µl of HT medium. The plates were placed in a humidified 5% CO_2 incubator at 37°C for 10 to 12 days. Wells containing a single colony were tested for Ab production by ELISA method. The selected hybridomas were cloned at least 3 times to achieve stable Ab producing monoclones. The obtained monoclones were stored in liquid nitrogen for further study.

3.4.8 Cell storage

Stable Ab-producing clones were cultured in RPMI 1640 medium and expanded from wells to plates. Cell pellets were harvested by centrifugation at 380 g for 5 min and mixed with 1 ml of freezing medium, which was kept cold at 4°C. The suspension was transferred to a labeled sterile cryotube using a Pasteur pipette. After closing the cap, the tube was placed into a foam box which was placed into a -70°C freezer for 24 h. After that, the tube was transferred into a container of liquid nitrogen (-196°C) for long-term storage.

3.4.9 Recovery cells from stock

The cryotube from liquid nitrogen was thawed at 37°C in a water bath. The cells were transferred immediately into a tube containing 10 ml of RPMI 1640 medium and centrifuged at 1,500 rpm for 5 min. The cell pellets were resuspended in a new sterile Petri dish with 20 ml of RPMI 1640 medium supplemented with 20% FCS and placed in the humidified 5% CO_2 incubator at 37°C.

3.4.10 Characterization of monoclonal antibody

3.4.10.1 Sensitivity of monoclonal antibody

The indirect competitive ELISA format describe above was used to determine the sensitivity of the Ab. The sensitivity was justified based on 50% maximal inhibition concentration (IC_{50}) which was analyzed using GraphPad Prism 4.03 software. The IC_{50} was calculated using the following formula:

$$IC_{50} = 50\%\ B/B_0$$

where B_0 and B are the average of the absorbance obtained from the indirect competitive ELISA without and with different concentrations of NPAHD, respectively.

3.4.10.2 Cross-reactivity of monoclonal antibody

The indirect competitive ELISA format describe above was also used to determine the cross-reactivity of the obtained MAb. NPAHD, NPAOZ, NPAMOZ and NPSEM were selected as competitors. Concentrations at the range 0.1 – 1000 µg/ml were prepared for this specificity test. The cross-reactivity of a MAb was calculated by the ratio of the IC_{50} of the standard NPAHD to the IC_{50} of the competitors using the following formula:

$$\%\ \text{Cross-reactivity} = \frac{IC_{50}\ \text{of NPAHD}}{IC_{50}\ \text{of competitor}} \times 100$$

3.4.10.3 Isotype determination

Isotypes of MAbs produced by the monoclones were determined using Sigma-Aldrich isotyping kit. The assay was based on a sandwich ELISA principle. The procedure was conducted according to the manufacturer's instruction with slight modification. Plate was coated with 100 µl of isotyping specific antibodies, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA (1:6,000 in PBS) and incubated at 4°C overnight. After washing with PBST, 100 µl of culture supernatant was added, and plates were incubated at 37°C for 2 h. After washing, 100 µl of HRP-Goat anti-mouse IgG (Fab specific) was added and plates were incubated at 37°C for 1 h. The plate was final washed with PBST and then TMB substrate solution was added. The plate was incubated in the dark at RT for 10 min. The reaction was stopped by 100 µl of 1 M H_2SO_4 the absorbance was measured at 492 nm using microplate reader.

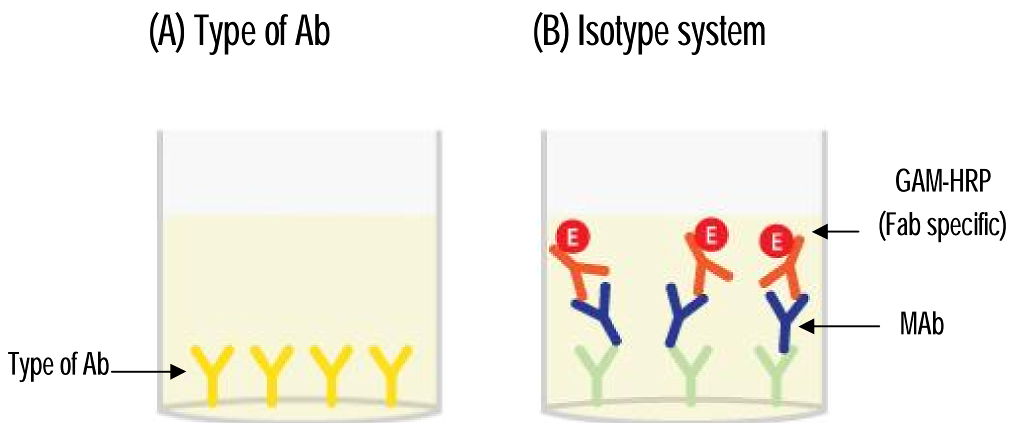

Figure 3.3 Principles method to determine isotype of MAb.

3.4.11 Production of monoclonal antibody

3.4.11.1 Scale up production of the selected hybridoma cell

Selected monoclones were expanded to Petri dish, cell culture bottle and spinner flask containing 500 ml of serum free media and placed in a humidified 5% CO_2 incubator at 37°C for 5-7 days in order to produce large amount of MAb. Cells were removed by centrifugation at 250 g for 5 min and supernatant containing Ab was collected. Ab was purified from the supernatant by protein G affinity chromatography column depending on the isotype of the selected Ab. The amount of protein was quantified using BCA™ Protein Assay Kit (3.4.12.1).

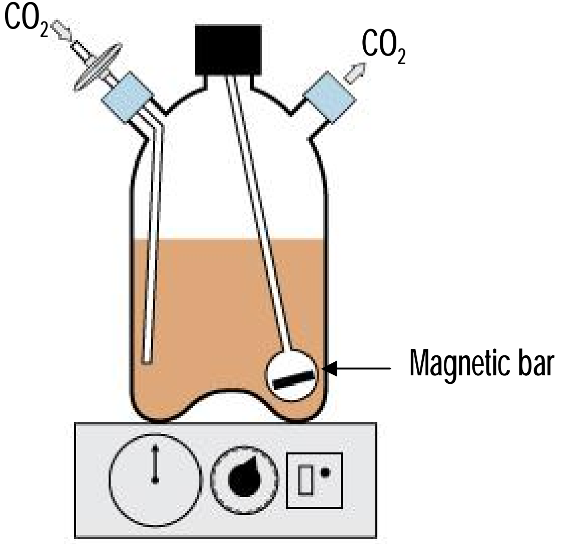

Figure 3.4 Diagram of a spinner flask used to produce the MAb.

3.4.11.2 Purification of monoclonal antibody

Ab was partially purified by sepharose G column chromatography. Sepharose G column was adjusted to a equilibrium state by 20 mM phosphate buffer (PB), pH 7. After setting flow rate at 1 ml/min, supernatant from 3.4.11.1 was added very slowly into column. Unbound protein was washed with 100 ml of PB, pH 7. The desired Ab was eluted from the column with 0.1 M glycine hydrochloric buffer pH 2.7 into test tubes (1ml/tube) that containing 63 µl of 1 M tris-hydrochloric buffer pH 9. The concentration of the Ab was quantified by measuring the absorbance at 280 nm using spectrometer. Fraction containing the antibody were pooled and dialyzed with PBS at 4°C for 3 day.

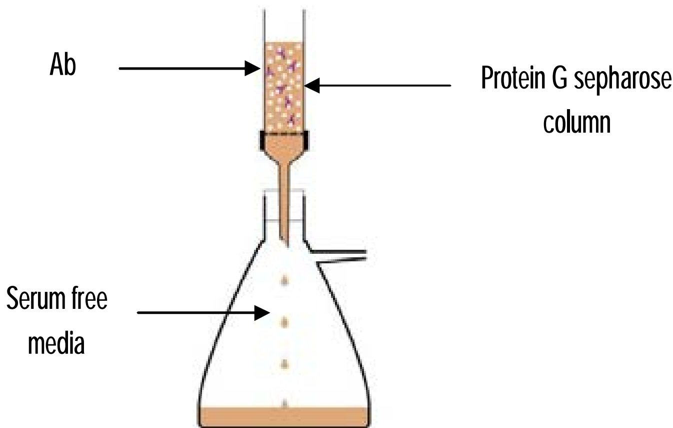

Figure 3.5 Purification of MAb against NPAHD from clone number 28/10E/8B/1E.

3.4.12 Determination of protein

3.4.12.1 Quantification of the amount of protein by Bicinchoninic Acid Assay (BCA assay)

BCA^{TM} Protein Assay Kit was used to quantified the protein concentration. Firstly, Reagent A and reagent B were mixed together at the ratio of 50:1 and used as the working solution. After that, BSA standard solution and the Ab were diluted at the suitable concentration. The 25 µl of samples (BSA standard and Ab) were added into

the well of microtiter plate and 200 µl of the working solution was added in the same well. The plate was incubated in the incubator at 37°C for 30 min. Finally, the absorbance was measured at 540 nm using microplate reader.

3.4.12.2 Determination of antibody concentration

The amount Ab was assayed using the method of Johnstone and Thrope (1987). The dialyzed Ab was diluted and the absorbance was measured at 280 nm. Then, the amount of Ab was determined using the following formula:

Concentration of Ab = OD_{280} / extinction coefficient of IgG

Extinction coefficient of IgG 1 mg/ml at 280 nm is 1.35 (Johnstone and Thrope, 1987).

3.4.12.3 Quantification of the amount of antibody by ELISA

The obtained Ab was tested with Ag using indirect ELISA technique (as described above). Standard graph of relationship between concentration of Ab and the absorbance at 450 nm was created. Pure Ab was prepared as Ab standard and further was compared with the amount of Ab in culture supernatant.

3.4.12.4 Calculating the molecular weight of antibody by SDS-PAGE

SDS-PAGE was performed according to the method described by Laemmli (1970) with slight modification, using a 1.5 mm thick slab gels with 5% stacking gel, 12% separating gel and 15-laned comb for applying samples. All reagents were prepared as described in the Appendix. Total protein concentrations of each of Ab were determined by BCA™ Protein Assay Kit. Two micrograms of proteins from each sample was denatured by mixing with equal volume of SDS staining dye, boiled for 5 min and then

loaded into each well of the gels. Protein markers with a molecular weight ranging from 19 to 118 kDa were used as the standard. Samples were electrophoresed at a constant voltage of 100 volts for 95 min in Western blot running buffer using protein III system. The separated bands of proteins in the gels were either visualized by immersion in staining solution.

Part II Development of ELISA test kit

3.4.13 Development and optimization of the ELISA

3.4.13.1 Preparation of CPAHD-HRP conjugate

The mixture of the 0.044 mol CPAHD, 0.25 mol NHS and 0.1 mol EDC in 0.4 ml dry DMF was slowly stirred. After that, 10 mg HRP in 0.6 ml PBS was added to this solution and stirred for 2 h at room temperature. The mixture was dialyzed against 0.1 M phosphate buffer pH 7.4 for 3 days (two changes of buffer per day). The obtained CPAHD-HRP was then used in the ELISA.

3.4.13.2 Preparation of biotinylated antibody

Biotinylated Ab was prepared by adding biotin to 0.1 M carbonate buffer pH 8.4 containing Ab at a ratio of 100 µg biotin per mg of Ab. The solution mixture was stirred at room temperature for 4 h. Free biotin was then removed by dialysis against three changes of the an excess volume of 0.1 M PBS (pH 7.4). The biotinylated Abs were concentrated and stored at -20°C. The obtained conjugated was then used in the analysis.

3.4.13.3 Optimization of suitable ratio between coating agent and enzyme tracer

The concentrations of coating agent and enzyme tracer were optimized for the suitable ratio for each ELISA format. Concentration of CPAHD-BSA (0.937 – 12.5 µg/ml) and MAb (0.25 – 7.0) were varied for the Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format. Concentration of MAb (0.132 – 7.25 µg/ml) and CPAHD-HRP (0.25 – 10.0) were varied for the Ag captured competitive direct ELISA format. Concentration of CPAHD-BSA (1.0 – 10.0 µg/ml) and biotinyleted Ab (1.0 – 10.0) were varied for the Ab captured competitive indirect ELISA (biotinylated Ab) format.

3.4.13.4 Antibody captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format

A 96-well plate was coated with the suitable concentration of CPAHD-BSA in PBS 100 µl/well by incubation at 4°C overnight. The plate was washed three times with PBST and blocked with 5% skim milk powder in PBS (300 µl/well) by incubation at 37°C for 1 h. The plate was washed and incubated with a mixture of suitable concentration of MAb with various concentrations of analytes, NPAHD at 37°C for 2 h. The plate was washed again and further incubated with goat anti-mouse IgG-HRP (1:5000 in PBS, 100 µl/well) at 37°C for 1 h. After another washing, 100 µl/well of TMB solution was added. The reaction was stopped with 100 µl/well 2 M H_2SO_4 after incubation for 15 min at room temperature. The absorbance at 450 nm was measured by the microplate reader. Sensitivity of competitive indirect ELISA format was analyzed as describe above (Figure 3.6).

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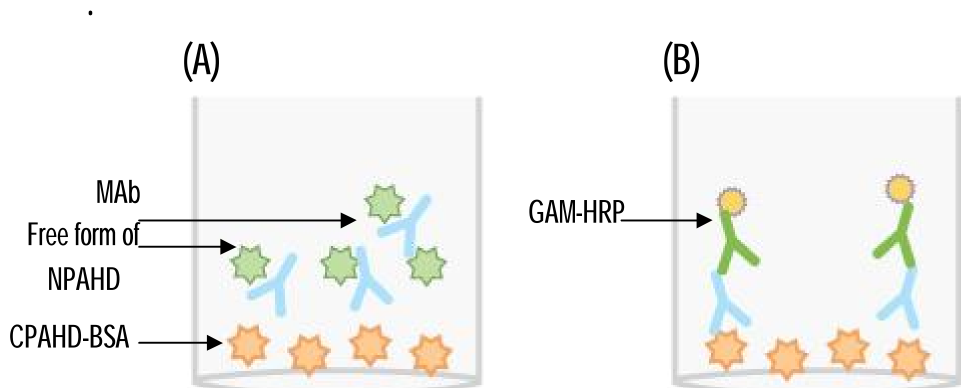


Figure 3.6 Diagram of the Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format.

(A) CPAHD was coated in microtiter plate and then Abs and NPAHD were added.

(B) GAM-HRP was added to system.

3.4.13.5 Antigen captured competitive direct ELISA format

A 96-well plate was coated with MAb in PBS 100 µl/well by incubation at 4°C overnight. The plate was washed three times with PBST and blocked with 5% skim milk powder in PBS (300 µl/well) by incubation at 37°C for 1 h. The plate was washed and incubated with a mixture of CPAHD-HRP and with suitable concentration with various concentrations of analytes at 37°C for 2 h. After another washing, 100 µl/well of TMB solution was added and incubated at room temperature in the dark. After 15 min, the reaction was stopped with 50 µl/well of 2 M H_2SO_4. The absorbance at 450 nm was read using a microplate reader. Sensitivity of competitive direct ELISA format was calculated as describe previously (Figure 3.7).

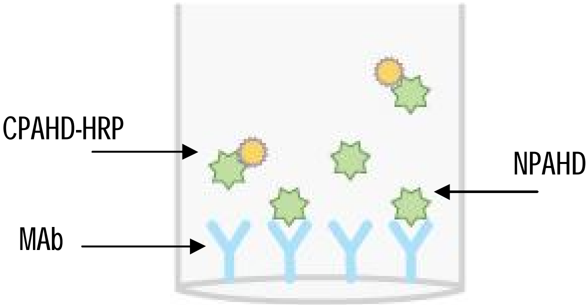


Figure 3.7 Diagram of the Ag captured competitive direct ELISA format.
Ab was coated in microtiter plate and then CPAHD-HRP and NPAHD were added.

3.4.13.6 Antibody captured competitive indirect ELISA (biotinylated antibody) format

A 96-well plate was coated with CPAHD-BSA in PBS 100 µl/well by incubation at 4°C overnight. The plate was washed three times with PBST and blocked with 5% skim milk powder in PBS (300 µl/well) by incubation at 37°C for 1 h. The plate was washed and incubated with a mixture of a suitable concentration of biotinylated Ab with various concentrations of analytes at 37°C for 2 h. The plate was washed again and further incubated with streptavidin-HRP (1:10000 in PBS, 100 µl/well) at 37°C for 15 min. After another washing, 100 µl/well of TMB solution was added and incubated at room temperature in the dark. After 15 min, the reaction was stopped with 50 µl/well of 2 M H_2SO_4. The absorbance at 450 nm was read using a microplate reader. Sensitivity of competitive indirect ELISA format was analyzed as describe previously (Figure 3.8).

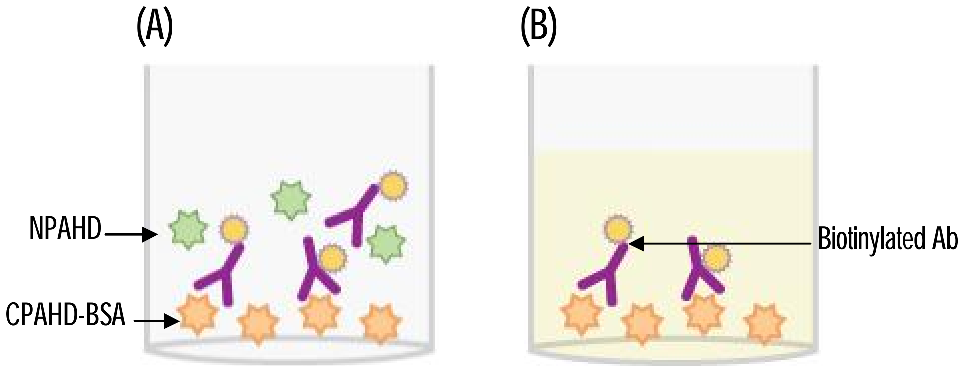


Figure 3.8 Diagram of the Ab captured competitive indirect ELISA (biotinylated Ab) format.

(A) CPAHD was coated in microtiter plate and then Abs and NPAHD were added.

(B) GAM-HRP was added to the system.

3.4.13.7 Effect of incubation temperature and time on sensitivity of the selected ELISA format.

Temperature and time of incubation were studied for the best sensitivity of the prototype test kit and the most practical analysis. These factors were varied for the selected ELISA format only. The conditions were listed in Table 3.1.

Table 3.1 Temperature and time of incubation used in optimization study.

Groups	Temperature (^{o}C)	Time (min)
1	Room temperature	30
2	Room temperature	60
3	Room temperature	90
4	Room temperature	120
5	37°C	30
6	37°C	60
7	37°C	90
8	37°C	120

3.4.14 Evaluation of prototype test kit

3.4.14.1 Sensitivity of prototype test kit

The multiple measurements of B_0 (B_0 are the average of the absorbance obtained from the indirect competitive ELISA without concentrations of NPAHD) of the standard curve were used for calculating the limit of detection (LOD). The LOD was calculated by subtracting the concentration at B_0 with three times of its standard

deviation (SD). SD is the standard deviation obtained from indirect competitive ELISA without competitor of 9 replicates.

3.4.14.2 Specificity of prototype test kit

The cross-reactivity studies were carried out for the selected ELISA by adding various free competitors (nitrofurans: NFT, FZO, NFZ, NPAHD, CPAHD, NPAMOZ, NPAOZ, NPSEM and AHD and other antibiotics: oxytetracycline, tetracycline, rolitetracycline, doxytetracycline, and β-agonist: clenbuterol and sulbutamol) at different concentrations (from 0.1 to 1000 ppb) to compete with the Ab to bind with the coating Ag. The degree of the competition was calculated using the formula described in 3.4.10.2.

3.4.14.3 Precision and accuracy of prototype test kit

The intra and inter variation assay were calculated based on the deviation of the AHD concentration measurement in spiked samples at various concentration. The PBS buffer and shrimp samples were fortified by AHD at the final concentration of 0.2, 0.4. 0.75. 1.5, 3.0 and 6.25 ng/ml. Inter-assay variation was computed from the analysis of 12 replicates of each dilution carried out on 12 different days. Intra-assay variation was measured by the analysis of the 12 replicates of each dilution on a single day. The obtained percentage coefficient of variation (%CV) by replicate analyses ($n=12$) from a single run (intra-variation assay) and from separated runs (inter-variation assay) were calculated using the following formula.

$$\text{Percentage of coefficient of variation (\%CV)} = \frac{SD}{\mu} \times 100$$

where SD is the standard deviation and μ is the mean absorbance of replicate analyses. The %recovery obtained by ELISA was calculated using equation below. Sample recoveries were determined from a standard curve.

$$\text{Recovery (\%)} = \frac{\text{Concentration of measured AHD}}{\text{Concentration of fortified AHD}} \times 100$$

3.4.15 Detection of AHD in fortified shrimp samples

3.4.15.1 Derivatization and extraction procedure

Shrimp sample was thawed and minced. Aliquots were fortified with 100 μl of AHD and carried through the derivatisation and extraction procedure as follows: 4 ml of water, 0.5 ml of 1 M HCl and 100 μl of 10 mM *o*-NBA (in DMSO) were added successively to the homogenate, the mixture was vortexed for 30 s and incubated overnight for 16 h at 37°C. After cooling to room temperature the samples were added, 5 ml of 0.1 M K_2HPO_4, 0.4 ml of 1 M NaOH and 5 ml of ethyl acetate shaken vigorously (30 s) and centrifuged (3000 g, 10 min). The upper ethyl acetate layer was transferred into test tubes and evaporated by dryness at room temperature under nitrogen gas. A mixture of hexane and 0.1 M PBS pH 7.4 (2ml, 1:1 v/v) was added to the residue and mixed thoroughly. The buffer layer containing NPAHD was separated by centrifugation and collected for further analysis.

3.4.15.2 Determination of AHD in shrimp samples

Twenty different samples were purchased from local retail outlets. The samples were used for validation experiments to assess the range of matrix interference. Additionally, the sample were fortified with AHD at different concentrations (0.2 – 3.0 µg/kg). The AHD in the sample were then analysed by competitive indirect ELISA in order to calculate the detection capability (CC_{β}). The recoveries (%) of the added AHD to the blank samples were calculated. The concentration of AHD was also measured by LC-MS/MS method by outside service provide Central Lab. Co., Ltd.

CHAPTER IV

RESULTS AND DISCUSSION

Part I

4.1 Preparation of antigen and coating agent

4.1.1 Synthesis of CPAHD derivative

To detect AHD in the sample, AHD must be first extracted from the tissues by solvent extraction. Then, it must be derivatized in order to prevent the rebinding with the tissue. The most common derivative from used in the detection is NPAHD. In general, a key factor to obtain a specific Ab is the Ag used in the immunization. Since, AHD is a small molecule which is not a good immunogen, it must be conjugated to a carrier protein to improve its immunogenic property. Thus, AHD was derivatized by the attachment of a carboxyphenyl moiety to form CPAHD which offers a carboxylic group to form peptide bond with the carrier protein and offers a structure similar to NPAHD, the target molecule of the detection.

After immunization, reactants and products were observed by TLC (stationary phase: aluminum silica, solvent system: 5% methanol in chloroform). Pyridine, CBA and CPAHD were visualized under UV light while AHD was visualized by ninhydrin staining solution as shown in Figure 4.1. The refractive mobilities of the reactants and products were calculated and shown in Table 4.1. When observed under the UV light (Figure 4.1A), pyridine (lane 1) and CBA (lane 2) were detected at the Rf of 0.83 and 3.5, respectively. While the other reactant, AHD, was observed after spraying the TLC plate with ninhydrin solution (Figure 4.1B) at the Rf of 4.2. If we compared the observed spots in lane 4 (before reaction) and lane 5 (after reaction), we could clearly notice a new spot at the Rf of 0.15 which was expected to be CPAHD. However, the spots of reactants could still be observed, suggesting that the reaction was not completed. After evaporation under N_2, pyridine was removed (lane 6). These results indicated that CBA

reacted with AHD in pyridine and the only possible product, CPAHD, was obtained. The derivatization method used in this research was based on the method previously reported. CPAOZ was generated from the reaction between CBA and AOZ in pyridine (Cooper *et al.*, 2004). In addition, CPSEM was synthesized with the same approach (Vass *et al.*, 2008).

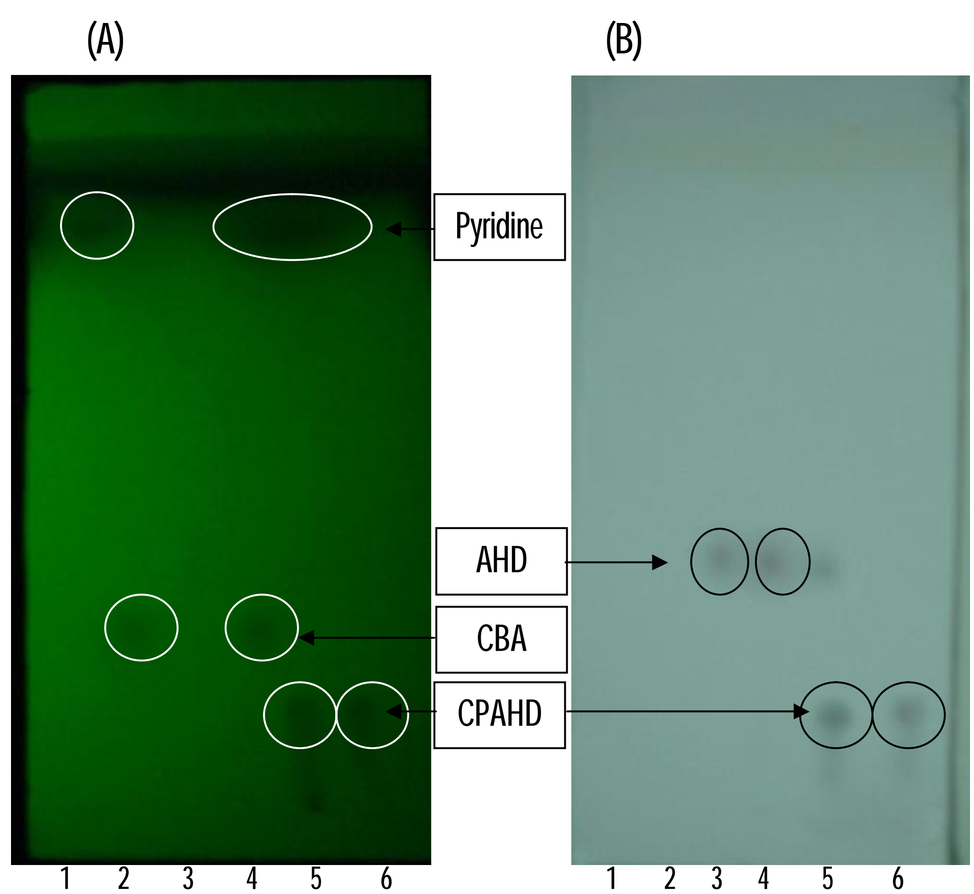


Figure 4.1 Analyses of conjugating mixture by TLC.
(A) Under UV light; (B) After spraying with ninhydrin solution;
Lane 1 = pyridine, Lane 2 = CBA, Lane 3 = AHD,
Lane 4 = mixture before derivatization, Lane 5 = mixture after derivatization
Lane 6 = mixture after evaporation under N_2

Table 4.1 Refractive mobility of chemicals in thin layer chromatography.

Substance	Distance (cm)	Refractive mobility (Rf)
Pyridine	8.3	0.83
CBA	3.5	0.35
AHD	4.2	0.42
CPAHD	1.5	0.15

4.1.2 Preparation of antigens

CPAHD has low molecular weight and is considered to be a hapten which must be coupled to a carrier protein in order to elicit a specific immune response. The conjugation between CPAHD and BSA, the most often used as carrier protein (Liu *et al.*, 2007), was performed. CPAHD was conjugated to BSA using NHS and EDC as the activating and coupling agents. The protein concentration of CPAHD-BSA was checked by BCA method. From the result in Table 4.2, the absorbance at 540 nm of undiluted CPAHD-BSA and 1:4 dilution of CPAHD-BSA were measured and the protein concentration was calculated using BSA standard curves. The concentration of CPAHD-BSA was found to be 2.283 mg/ml. Then, the conjugation was confirmed by two methods, colorimetric reaction of trinitrobenzenesulphonic acid (TNBS) and determination of mass by MALDI-TOF-MS.

In TNBS method, the degree of the reaction between TNBS and uncouplied free amino acid group of the protein can be quantified by measuring the absorbance at 335 nm. Therefore, the reduction in the number of uncouplied amino groups due to the conjugation could result in the decrease of the absorbance (Cooper *et al.*, 2004). The absorbance measurement of the reaction between TNBS and BSA of both before and after conjugation was shown in Table 4.3. The absorbance after conjugation was clearly lower than that before conjugation, thus indicating the attachment of CPAHD to the

amino group of BSA. The percentage of conjugation was calculated based on the absorbance reduction as 40%.

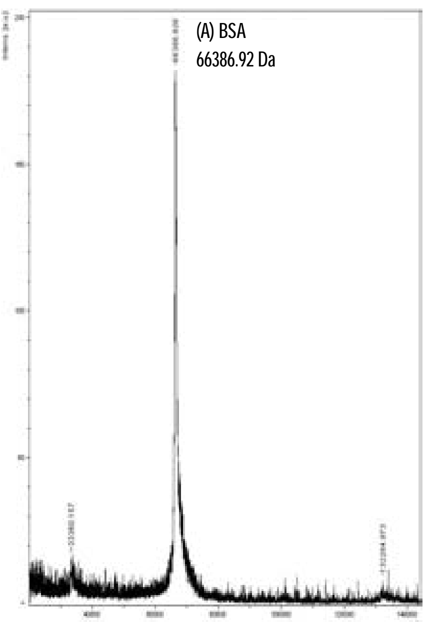
The conjugation of CPAHD-BSA was also confirmed by comparing molecular mass of BSA and that of the conjugated product by MALDI-TOF-MS. The analysis indicated that mass of the conjugate was higher than that of BSA about 3579 dalton (Table 4.4). Using the mass of CPAHD (283.68 dalton), the molecular ratio of CPAHD to BSA (66,386 dalton) was calculated to be 12.4:1. The hapten density is an important factor for the performance of an immunoassay. In this study, the hapten density in immunogen was 12.6 that were appropriate. Based on the above two analysis methods, it can be concluded that CPAHD conjugated on to BSA which was further used as the immunogen.

Table 4.2 Protein concentrations of CPAHD-BSA for immunization determined by BCA method.

Dilution of CPAHD-BSA	Absorbance at 540 nm	CPAHD-BSA concentration (mg/ml)
Undiluted	2.013	2.138
1 : 4	0.519	2.428
Average		2.283

Table 4.3 Percentage of conjugation between CPAHD and BSA carrier protein determined by TNBS method.

BSA concentration (mg/ml)	Absorbance at 335 nm		Percentage of conjugation
	Before conjugation	After conjugation	
50	0.432	0.256	40



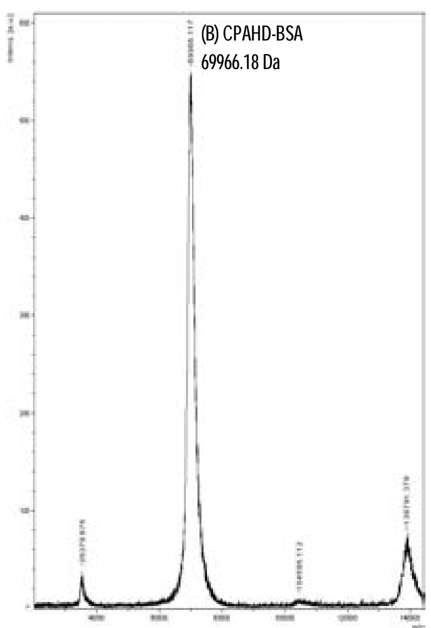


Figure 4.2 Spectra of (A) BSA and (B) CPAHD-BSA from MALDI-TOF-MS analysis.

Table 4.4 The molecular mass of CPAHD, BSA and CPAHD-BSA determined by MALDI-TOF-MS.

Reagents	Molecular mass (Dalton)
CPAHD	283.68
BSA	66,386.92
CPAHD-BSA	69,922.18
Ratio of CPAHD : BSA	12.4 : 1

4.2 Immunization of mice

4.2.1 Determination of sera titer by indirect ELISA

Nine BALB/c female mice, 8 weeks old were immunized by intraperitoneal injections with CPAHD-BSA in CFA. After three booster doses of CPAHD-BSA in IFA at 2 week intervals, the sera were collected and assayed for anti-CPAHD Abs by an indirect ELISA, antiserum titre was monitored by the indirect ELISA.

The titre of Ab was estimated using the absorbance at 450 nm from the indirect ELISA. The Ab titre is usually defined as the lowest Ab dilution to bind significantly to the Ag. The absorbance approximately at 0.100 or 2 time of negative control of each diluted serum were chosen as Ab titre (Lie *et al.*, 2007). The result shown that Ab against CPAHD-BSA was produced at the titre of 4096000, 8192000, 8192000, 8192000, 4096000, 4096000, 8192000, 4096000 and 4096000 for mice number 1-9, respectively (Table 4.5). To ensure the best chance of yielding specific hybridomas, the presence of the desired Ab should be detectable in the serum of the recipient prior to the final boost. Ideally, the specific Ab titre should be at least 1:1000 and preferably more (Liddell and Cryer, 1991).

Table 4.5 Ab titres of serum obtained from 9 BALB/c mice immunized against CPAHD-BSA.

Dilution	Absorbance at 450 nm of each immunized mice								
	#1	#2	#3	#4	#5	#6	#7	#8	#9
1:16000	2.114	2.216	2.059	2.145	2.094	2.011	2.014	2.155	2.135
1:32000	2.086	2.115	1.936	2.176	2.009	1.865	1.962	2.135	2.164
1:64000	2.000	2.077	1.855	2.104	1.965	1.623	1.762	1.936	2.079
1:128000	1.845	1.952	1.618	1.930	1.617	1.421	1.583	1.549	1.807
1:256000	1.512	1.678	1.328	1.328	1.453	1.259	1.328	1.049	1.302
1:512000	0.994	1.279	0.871	0.862	1.098	0.975	1.159	0.630	0.836
1:1024000	0.647	0.857	0.612	0.544	0.820	0.704	0.816	0.376	0.478
1:2048000	0.389	0.550	0.478	0.270	0.501	0.449	0.502	0.202	0.248
1:4096000	0.152	0.208	0.211	0.153	0.188	0.156	0.312	0.096	0.112
1:8192000	0.066	0.081	0.152	0.105	0.067	0.040	0.132	0.088	0.065

Note: # refers to number of mice, highlight refers to titre of each mice.

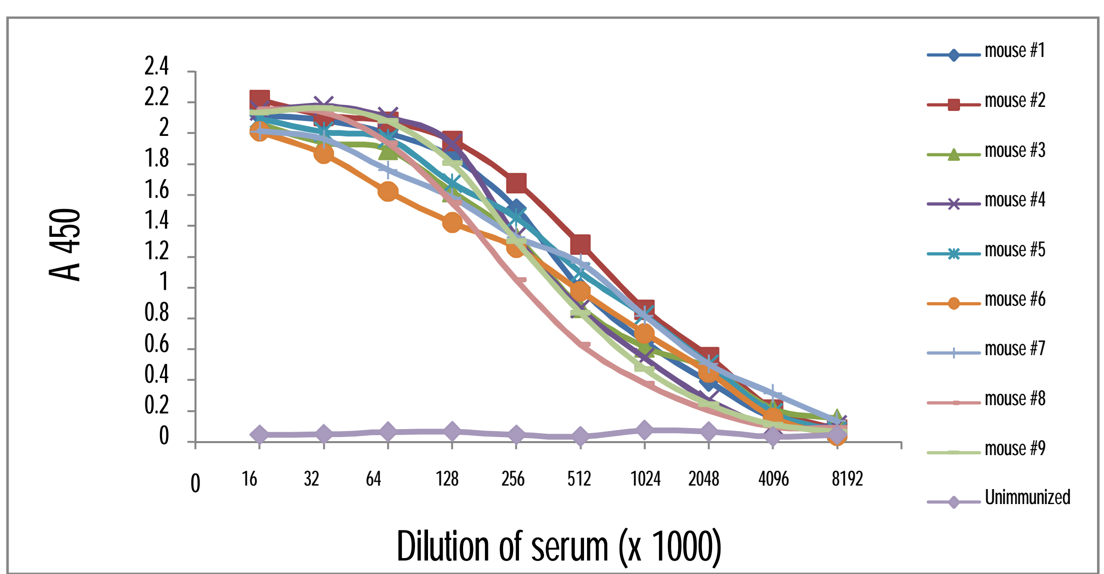


Figure 4.3 Indirect ELISA-dose response curve of Ab in serum from 9 BALB/c mice immunized against CPAHD-BSA.

4.2.2 Determination of the specificity test of serum antibody by indirect competitive ELISA

In the previous experiment, the presence of Ab in the serum was detected based on the ability to bind with the CPAHD-BSA conjugate coated on the well surface. In this section, Ab in serum from nine mice were tested for their binding ability to free molecules of CPAHD, AHD, CBA and NPAHD by indirect competitive ELISA.

These molecules acted as the competitor of the CPAHD-BSA conjugated to bind with the serum Ab. If the Ab can bind with the free competitor molecule, the absorbance obtained from the ELISA should be decreased as compared to the control (no competitor).

The results (Table 4.6) showed that the absorbance values obtained from the ELISA when the competitors were presented, were lower than that of the control. This indicated that serum Abs from all mice can react with all competitors. Among the competitors tested, CPAHD and NPAHD gave relatively highly % competition in the range of 15% - 55%. These results confirmed that the Abs can bind with NPAHD, the target form of the detection (Figure 4.4).

Table 4.6 Specificity of Ab in serum from immunized mice to various competitors.

Mouse number (dilution ratio)	Competitors (20 µg/ml)								
	None (A_{450})	AHD		CBA		CPAHD		NPAHD	
		A_{450}	%C	A_{450}	%C	A_{450}	%C	A_{450}	%C
1 (1:512000)	0.870	0.775	10.91	0.812	7.14	0.416	52.18	0.431	50.46
2 (1:512000)	0.971	0.935	3.71	0.947	2.47	0.493	49.23	0.621	36.05
3 (1:256000)	0.902	0.847	6.10	0.886	1.77	0.597	33.81	0.766	15.08
4 (1:256000)	0.932	0.882	5.36	0.913	2.04	0.418	55.15	0.457	50.97
5 (1:512000)	0.869	0.821	8.37	0.866	3.35	0.539	39.84	0.519	42.08
6 (1:512000)	1.257	1.104	12.17	1.111	11.61	0.683	45.66	0.690	45.11
7 (1:512000)	1.054	1.009	4.27	1.003	4.84	0.511	51.52	0.618	41.37
8 (1:256000)	1.233	1.024	16.95	1.178	4.46	0.626	49.23	0.689	44.12
9 (1:512000)	0.943	0.892	5.41	0.912	3.29	0.450	52.28	0.498	47.19

Note: %C refers to % competition.

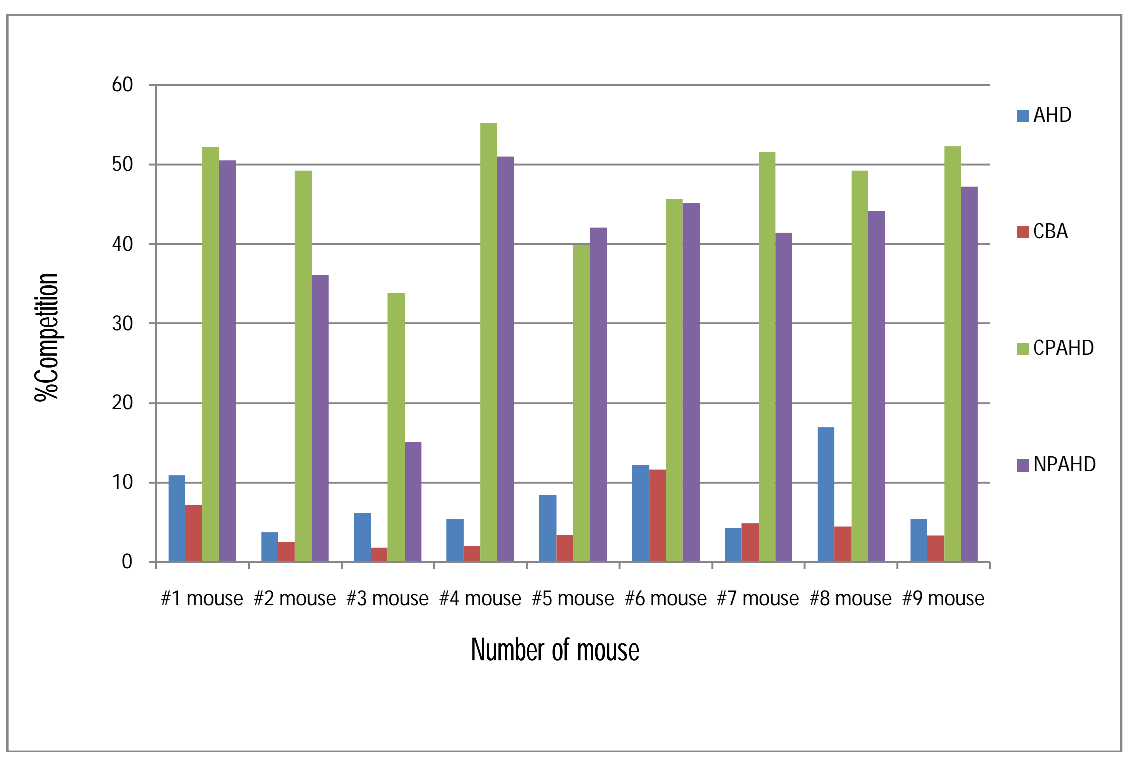


Figure 4.4 Summary of % competition to competitor of each serum of mouse.

4.3 Production of hybridoma

Once stimulated *in vivo*, B-lymphocytes undergo processes of proliferation, differentiation and maturation such that many identical cells capable of producing Ab of a particular nature arise from a single or small number of progenitor cells (Liddell and Cryer, 1991). After nine BALB/c mice were immunized with 50 µg/100µl of CPAHD-BSA as immunogen for 4 times and checked for their response. The spleens were collected and anti-CPAHD MAbs were produced using somatic cell fusion technique.

The cells of the fusion mixture were grown in a selective HAT medium in which, only myeloma-lymphocyte hybrids were survived. Within approximately five days following fusion, small clusters of cells resembling myeloma cells are likely to be hybridoma. Within 10-14 days, these cells were grown sufficiently to secrete enough detectable Abs into the culture supernatant. At this stage cell supernatants were screened to determine which of them contains the desired Ab against NPAHD.

The results of all fusion of spleen and myeloma cell were summarized in Table 4.7. After fusion, cells were seeded into the 96 microtiter plate at different number of wells for each fusion depending on the size of the spleen or the cell density. In fusion lot number 1 to 8, SP2/0-Ag14 myeloma cells were used. Numbers of culture containing Abs which can bind with NPAHD were found in only four fusion trials. Unfortunately, these cultures lose their ability to secrete Ab against NPAHD during the limiting dilution step to produce monoclones. Unlike the previous fusions, P3-X63-Ag8 myeloma cells were used in the 9th fusion trial. After the first screening by the indirect ELISA, 525 clones yielded positive result indicating the ability to produce Abs that can bind with CPAHD-BSA conjugate. The culture supernatants of these clones were then tested by the indirect competitive ELISA, the 2nd screening using 10 µg/ml of NPAHD as the competitor. Only 21 of these clones showed the ability to produce Abs that can bind with free form NPAHD from 3rd screening (Table 4.8).

Positive wells were subcultured at least three times by limiting dilution technique, until monoclones were obtained. The purpose of limiting dilution is to isolate a single specific Ab-secreting cell from the hundreds of other cells in the culture so that a monoclonal cell line can be established. Throughout limiting dilution, particularly during the early stages, there are a high probability of chromosome loss and consequent loss of immunoglobulin secretion from the hybrid. In addition, there is always a risk of overgrowth by non-producing hybrids. Consequently, some hybridoma cells from the experiment lost their capabilities to secrete Ab against NPAHD.

Finally, monoclones number 28/2F/12C, 28/2F/12F, 28/10E/8B/6F, 28/10E/8B/12A, 28/10E/8B/10E and 28/10E/8B/1E were obtained and selected for further experiment (Table 4.9).

Table 4.7 Summary of fusion between splenocytes of immunized BALB/C mice and myeloma cells by somatic cell fusion technique.

Lot of fusion	Number of cultured (well)	Number of survival culture		Number of culture screening Ab		Number of culture secreting NPAHD-bound Ab	
		well	%	well	%	well	%
1	1152	624	54	198	17	23	2
2	864	672	78	571	66	103	12
3	960	768	80	560	58	23	2
4	1152	0	0	0	0	0	0
5	1824	0	0	0	0	0	0
6	3168	108	3	20	0	3	0.1
7	1440	0	0	0	0	0	0
8	1440	0	0	0	0	0	0
9	1548	525	34	90	5.8	21	1.4

Table 4.8 Summary of the positive hybridomas from 9th fusion between 9th spleen of BALB/C mice immunized and myeloma cell type P3-X63Ag8.

Absorbance at 450 nm	1st screen		2nd screen		3rd screen			
					Secreted Ab		Specific with NPAHD	
	well	%	well	%	well	%	well	%
>1.5	387	25	48	3	18	1	11	1
1.0-1.5	58	4	17	1	14	1	6	0.4
0.5-1.0	80	6	25	2	10	1	4	0.3

Table 4.9 Summary of the limiting dilution to obtain monoclone that secrete Ab against NPAHD.

Clone number	Limiting dilution		
	1st	2nd	3rd
10	10/6A, 10/2D, 10/1B	10/6A/12D, 10/2D/7G	-
12	12/9C, 12/10A, 12/10C	12/10C/1A	-
26	26/12G	-	-
28	28/12C, 28/10E, 28/2A, 28/2F	28/2F/12C, 28/2F/12F, 28/12C/1G, 28/12C/5A, 28/12C/5C, 28/12C/5E, 28/10E/8B, 28/10E/8C, 28/10E/11E	28/10E/8B/6F, 28/10E/8B/10E, 28/10E/8B/12, 28/10E/8B/1E
58	58/7F, 58/8C	58/7F/8C, 58/7F/10A, 58/8C/8C	-
60	60/4C	60/4C/4F	-
80	80/3B, 80/4D	80/3B/3E	-

4.4 Characterization of monoclonal antibodies

4.4.1 Sensitivity test

Since several clones were obtained, they were screened for the most sensitive Ab based on both the IC_{50} and the LOD values by the indirect competitive ELISA using NPAHD as competitor. Table 4.10 showed that clone 28/2F/12C, 28/2F/12F, 28/10E/8B/6F, 28/10E/8B/12A, 28/10E/8B/1E and 28/10E/8B/10E have IC_{50} values at 74.8, 112.7, 103.7, 293.3, 47.0 and 41.8 ppb, and LOD values at 31.5, 14.5, 27.2, 72.3 14.5 and 15.9, respectively (Figure 4.5 – Figure 4.10).

However, the LOD values were still higher than the MRPL currently enforced at 1 ppb. These could be due to the reason that conditions such as concentration of antibody and competitor used in the ELISA were not optimized for each Ab. There was a study reported that the immunoassay with less coating Ag (1:100 and 1:200) was more sensitive than that with higher coating Ag (1:10 and 1:50) (Franek *et al.*, 2006). This indicated that the obtained MAb from clone 28/10E/8B/1E could be sensitive enough to detect NPAHD at the current MRPL.

Table 4.10 The IC_{50} and LOD of MAb against NPAHD determined by indirect competitive ELISA.

Monoclone number	IC_{50} (ppb)	LOD (ppb)
28/2F/12C	74.8	31.5
28/2F/12F	112.7	14.5
28/10E/8B/6F	103.7	27.2
28/10E/8B/12A	293.3	72.3
28/10E/8B/10E	47.0	14.5
28/10E/8B/1E	41.8	15.9

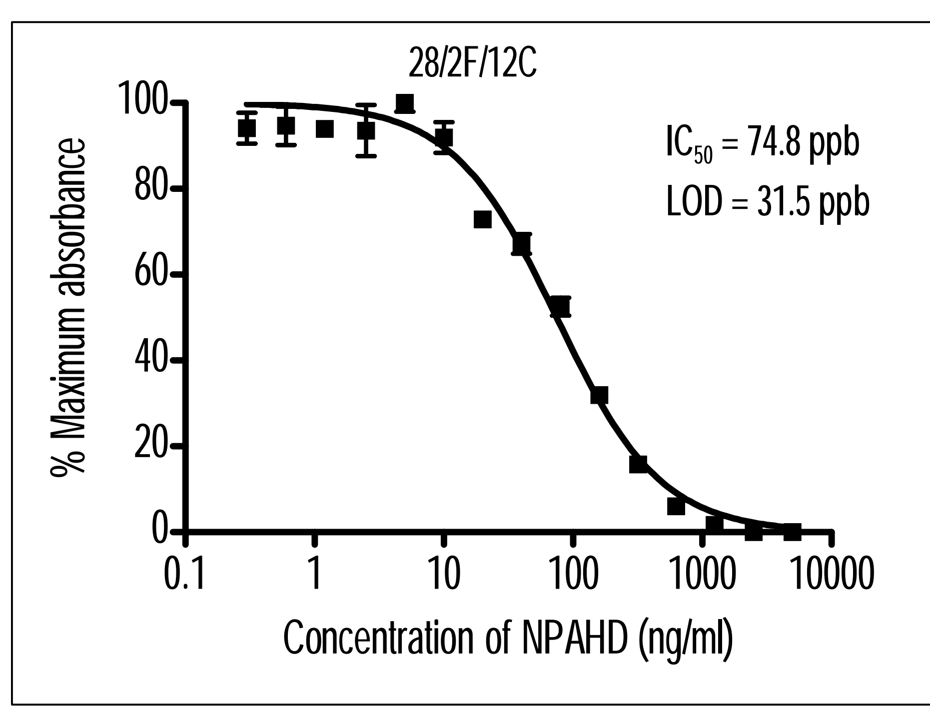


Figure 4.5 Indirect competitive response curve of MAb clone 28/2F/12C against NPAHD.

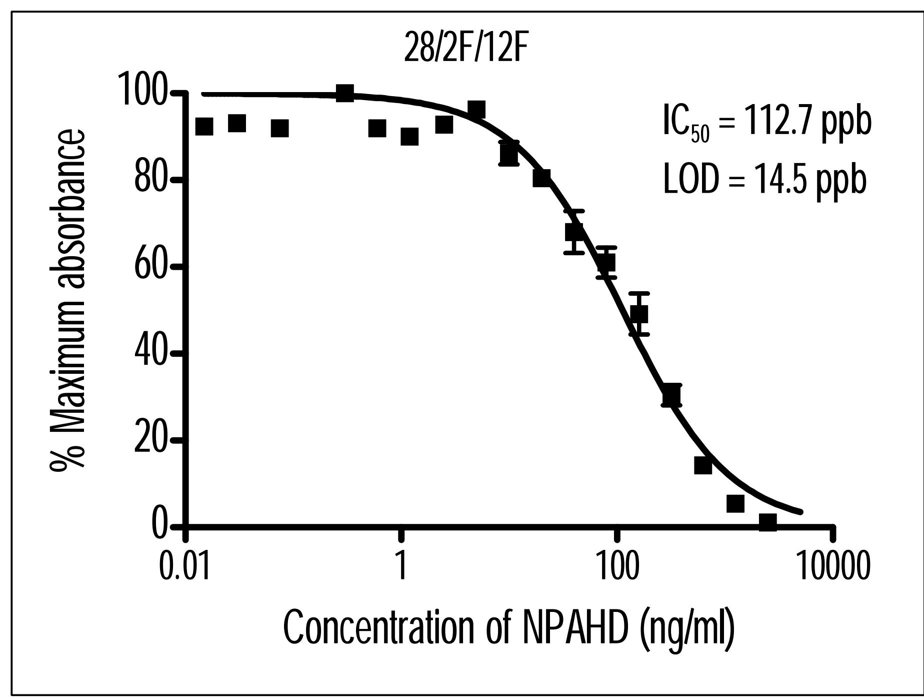


Figure 4.6 Indirect competitive response curve of MAb clone 28/2F/12F against NPAHD.

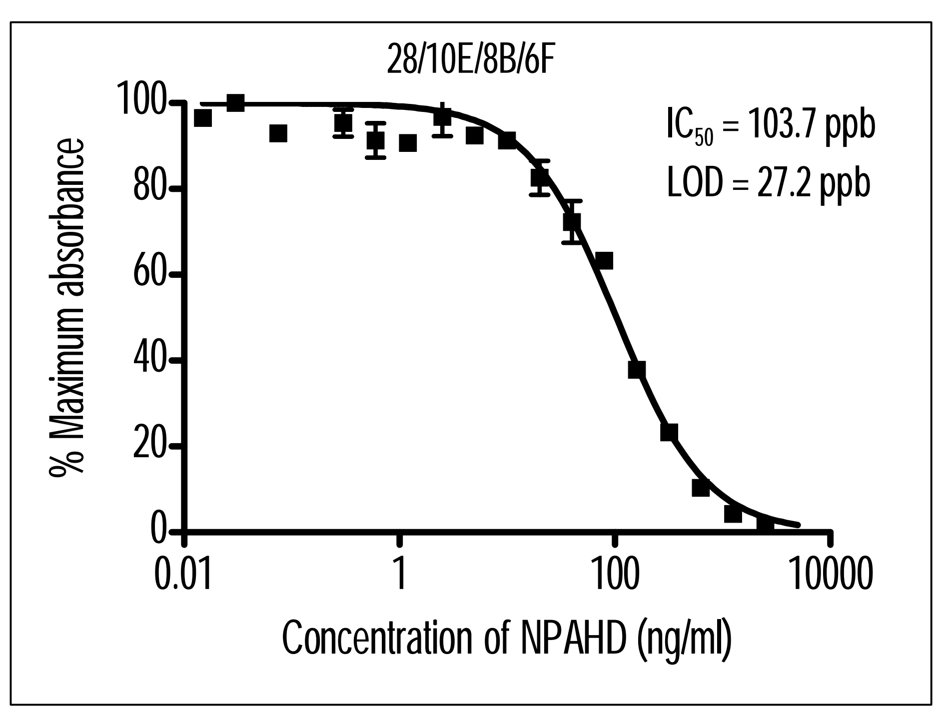


Figure 4.7 Indirect competitive response curve of MAb clone 28/10E/8B/6F against NPAHD.

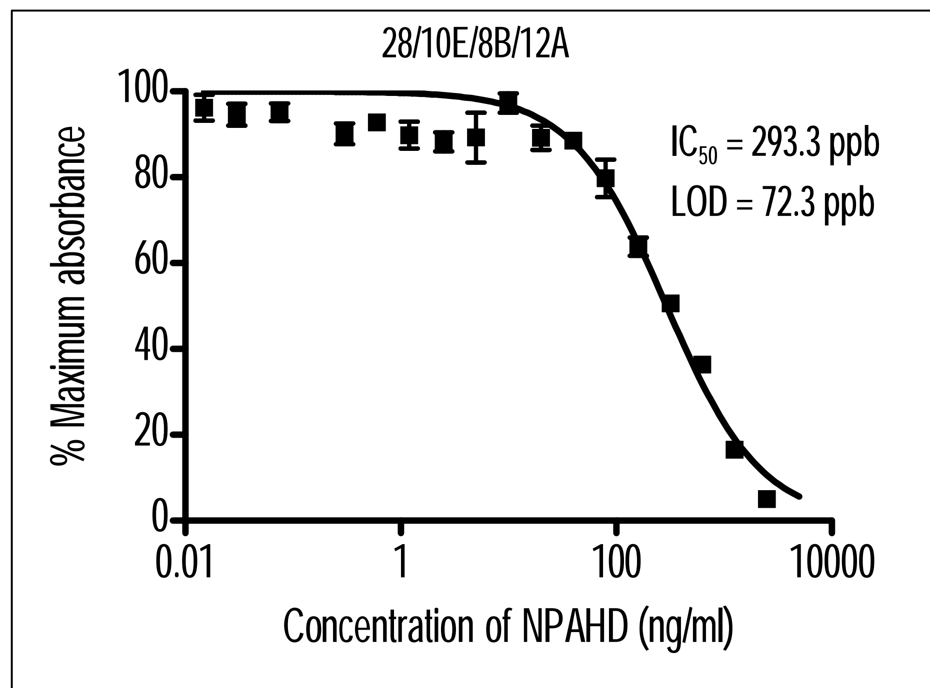


Figure 4.8 Indirect competitive response curve of MAb clone 28/10E/8B/12A against NPAHD.

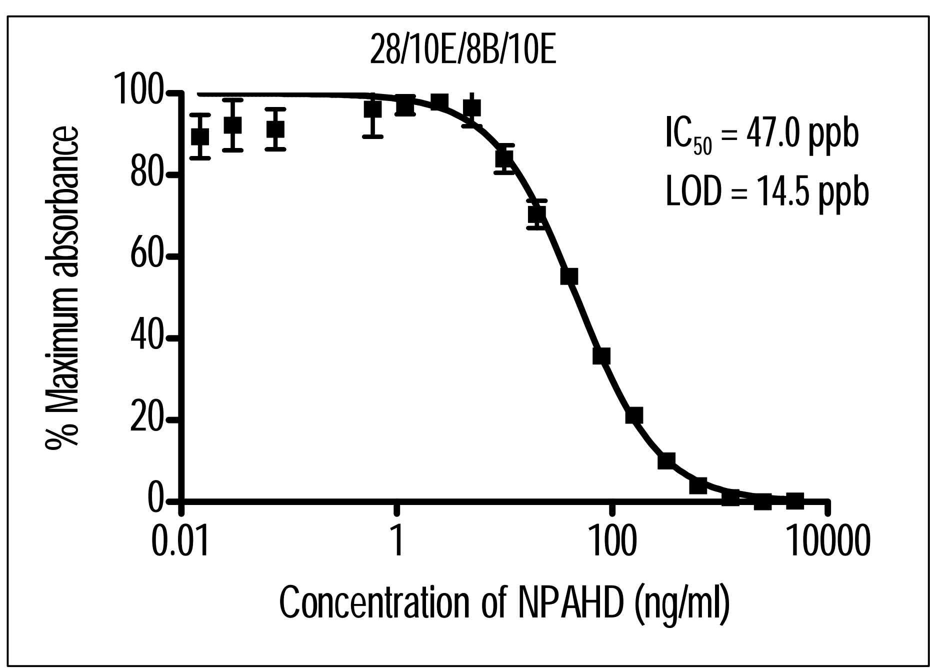


Figure 4.9 Indirect competitive response curve of MAb clone 28/10E/8B/10E against NPAHD.

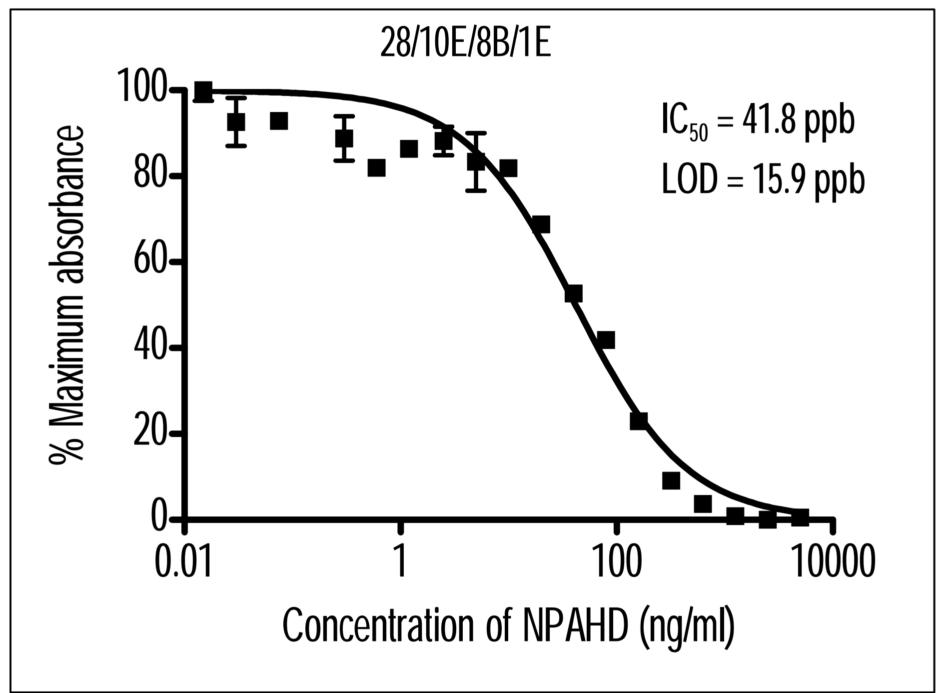


Figure 4.10 Indirect competitive response curve of MAb clone 28/10E/8B/1E against NPAHD.

4.4.2 Determination of antibody isotype

The isotype of MAbs was determined using Mouse MAb Isotyping Kit (Sigma). The absorbance values of all MAbs were measured at 450 nm by indirect ELISA method (shown in Table 4.11). The results showed that MAb from clone number 28/2F/12C, 28/10E/8B/12A and 28/10E/8B/1E yielded high absorbance when Ab specific to IgG_1 was used as the coating Ab while that of MAb from clone number 28/2F/12F and 28/10E/8B/6F reacted to coating Ab specific for IgG_{2a} and IgG_{2b}, respectively.

Therefore, it can be concluded that MAb from clone number 28/2F/12C, 28/10E/8B/12A and 28/10E/8B/1E were IgG_1, 28/2F/12F was IgG_{2a} and 28/10E/8B/6F was IgG_{2b} isotype. As expected, all MAb produced were IgG subclass. This could be due to the reason explained by Liddell and Cryer (1991). The Abs produced are predominantly IgM. If a secondary challenge with immunogen is presented three to four weeks after first, then the Ab response is quickly, much greater and lasts for longer. The predominant class of Ab is IgG and the average affinity is increased, particularly if a low immunogen dose is given.

Table 4.11 Determination of Ab isotype by ELISA.

MAb from clone number	Absorbance at 450 nm					
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgM	IgA
28/2F/12C	1.221	0.234	0.311	0.411	0.273	0.376
28/2F/12F	0.186	0.958	0.178	0.238	0.163	0.222
28/10E/8B/6F	0.351	0.112	0.892	0.182	0.170	0.194
28/10E/8B/12A	0.993	0.273	0.210	0.185	0.174	0.212
28/10E/8B/10E	0.935	0.167	0.182	0.231	0.194	0.214
28/10E/8B/1E	0.891	0.104	0.178	0.187	0.349	0.105

Highlight refers to isotype of each monoclone.

4.4.3 Selectivity test

MAbs from 6 clones were further characterized by determining its cross-reactivity with other nitrofurans derivatives in aqueous buffer as described by Cooper *et al.*, 2007. The indirect competitive ELISA using nitrofurans derivatives of interest as the competitors was performed. The cross reactivity data in Table 4.12, demonstrated the highly specific nature of this Ab. All MAbs showed high specificity against NPAHD. With the exception that, MAb from clone number 28/2F/12C and 28/10E/8B/10E were cross-reacted with NPSEM at 21.2% and 30.2%, respectively. These might be due to structure similar of NPAHD and NPSEM. So, MAb from clone number 28/10E/8B/1E was selected for the ELISA development.

Table 4.12 % Cross-reactivity of MAbs with various nitrofuran derivatives using indirect competitive ELISA.

Clone number	% Cross reactivity			
	NPAHD	NPSEM	NPAMOZ	NPAOZ
28/2F/12C	100	21.2	<0.03	<0.03
28/2F/12F	100	<0.03	<0.03	<0.03
28/10E/8B/6F	100	<0.03	<0.03	<0.03
28/10E/8B/12A	100	<0.03	<0.03	<0.03
28/10E/8B/10E	100	30.2	<0.03	<0.03
28/10E/8B/1E	100	<0.03	<0.03	<0.03

4.5 Production and partial purification of monoclonal antibody

4.5.1 Culture expansion and antibody purification

In order to produce a large amount of the desired MAb for further study, the culture of clone 28/10E/8B/1E was expanded from petridish to 50 ml flask and 500 ml spinner flask. Stirring of such cultures is best done slowly with a magnet encased in a glass pendulum. The stirring speed should be between 30 and 100 rpm, sufficient to prevent cell sedimentation, but not so fast as to create shear forces that would damage the cells (Freshney, 2005). Cells were separated from the culture supernatant by centrifugation. Then, the Ab was purified by chromatography using protein G sepharose column. Ab content eluted from the column was monitored by measuring the absorbance at 280 nm. In addition, the present of the Ab was also confirmed by the indirect ELISA. The absorbance of the eluted fractions from both measurements were plotted and shown in Figure 4.11. The result showed that fraction 7-14 yield high absorbance in both measurements.

Furthermore, the protein and Ab contents were also quantified by the BCA method and the extinction coefficient method, respectively. The results were shown in Figure 4.12 and were found to be in a good agreement with the previous results. The Ab solutions from fraction 7-14 were then pooled and the total protein contents were determined.

The protein and Ab concentrations before and after purification step were summarized in Table 4.13. The total protein content was reduced from 1,925 mg to 13.9 mg while the Ab content was decreased from 55.0 to 10.7 mg. This indicated that most of the unwanted proteins were removed. The % recovery was also calculated to be 19.9%. In general, protein G has a high affinity to bind to the Fc region of IgG and become a universal immunochemical agent used for purification of IgG. Ab can be captured from culture supernatant. Yield and purity are high and the methods are applicable to the majority of IgG Abs in common use (Kerr and Thrope, 1994). In this research, reuse and maintain of the column might reduce the column efficiency,

resulting in low %recovery. In addition, the Ab might be damage during the elution step. Most Abs can be eluted with buffer at pH 3.2-3.5. However, elution from protein G generally requires a lower pH. The low pH necessary for desorption may lead to denaturation and/or aggregation of the Ab (Shepherd and Dean, 2000).

Table 4.13 Protein and Ab contents from clone number 28/10E/8B/1E.

Purification	Volume (ml)	Amount of protein		Amount of Ab		% Recovery
		Conc. (mg/ml)	Total (mg)	Conc. (mg/ml)	Total (mg)	
Before	500	3.85	1,925	0.11	55.0	-
After	8	1.74	13.9	1.34	10.7	19.5

Note: Conc. refers to concentration.

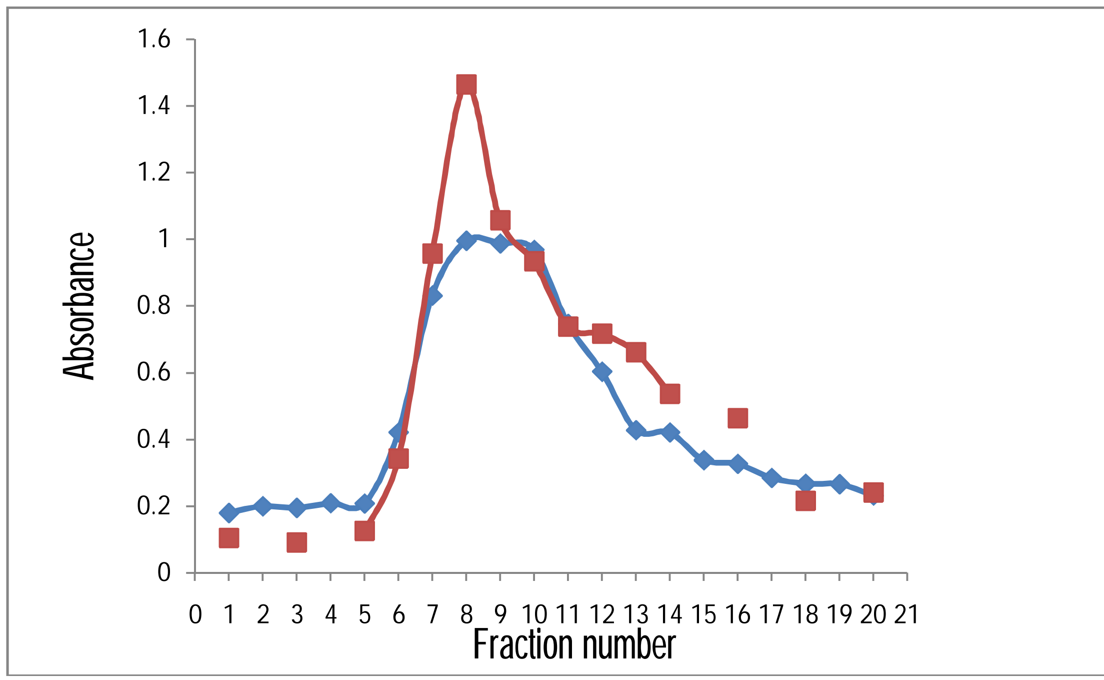


Figure 4.11 Chromatogram of purified Ab of clone 28/10E/8B/1E from protein G column.

absorbance at 280 nm

absorbance at 450 nm from indirect ELISA method

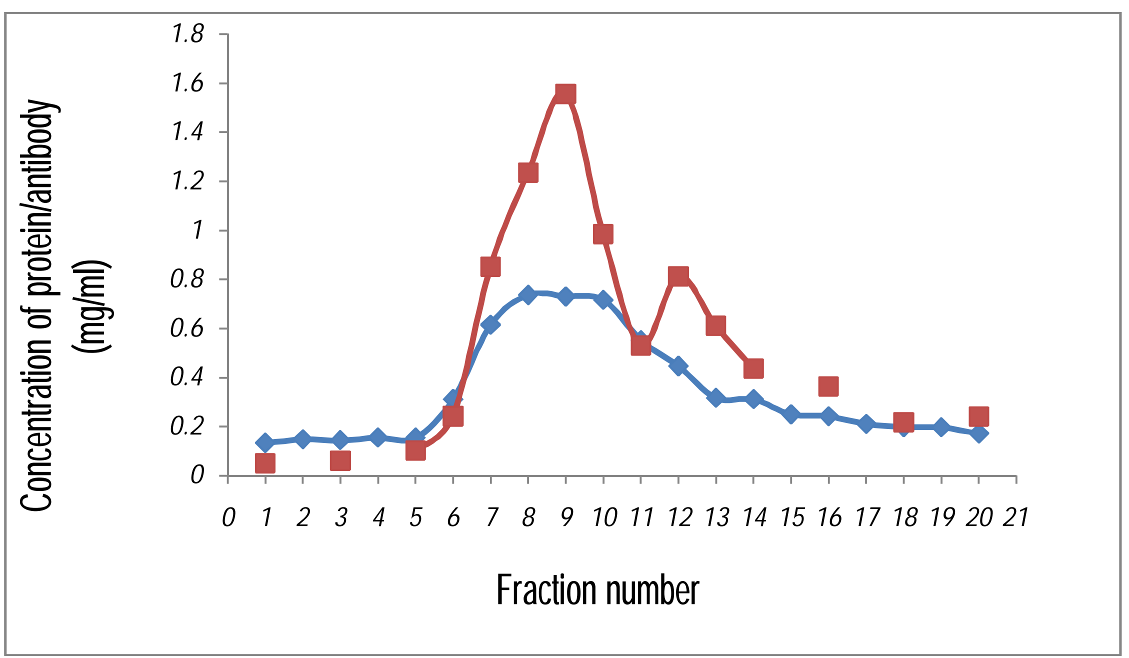


Figure 4.12 Chromatogram of concentration of Ab of clone 28/10E/8B/1E by measuring the absorbance.

Ab concentration

Protein concentration by BCA method

4.5.2 Quantification of the molecular weight of antibody by SDS-PAGE

Molecular weight of the purified MAb from clone number 28/10E/8B/1E was determined using SDS-PAGE (Figure 4.13). Molecular weight of polypeptide of MAb was determined by using a correlation between retention mobility and logarithm of molecular weight. The results shown that these finding were similar to these reported in many researches (Khamjing, 2008) the molecular weights of the heavy chain and the light chain of the MAb were at 44.9 kDa 28.7 kDa, respectively.

Table 4.14 SDS-PAGE analysis of MAb from clone number 28/10E/8B/1E against NPAHD.

Marker	Molecular weight (kDa)	Distance (cm)	Retention mobility or Rf
β-galactosidase (*E.coli*)	118	1.27	0.09
BSA (Bovine plasma)	90	2.96	0.20
OVA (Chicken egg white)	50	6.14	0.42
Carbonic anhydrase (Bovine erythrocytes)	34	9.95	0.68
β-lactoglobulin (Bovine milk)	26	12.70	0.87
Lysozyme (Chicken egg white)	19	14.61	1.00

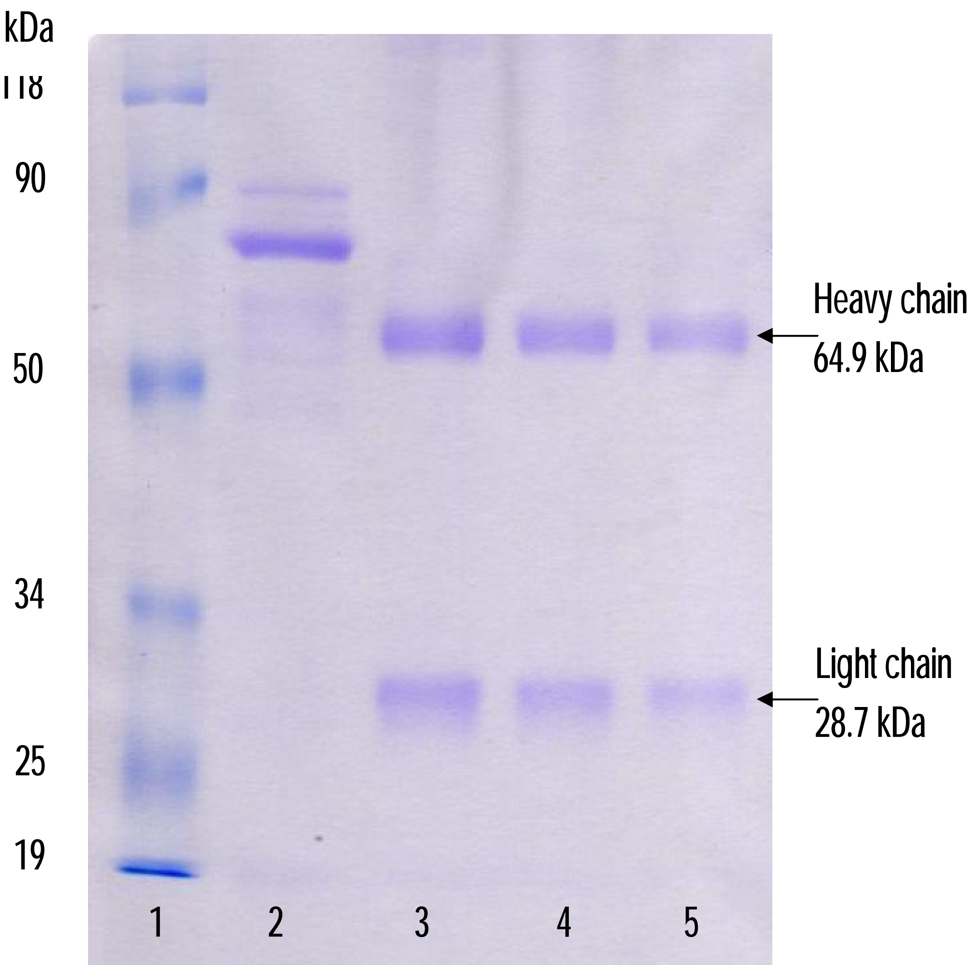


Figure 4.13 Purity of purified Ab from clone 28/10E/8B/1E by SDS-PAGE.
Lane 1 = Marker Lane 2 = Media Lane 3 = 5 µg/ml purified Ab
Lane 4 = 3 µg/ml purified Ab and Lane 5 = 2 µg/ml purified Ab

Part II

To detect drug residues in food and food products, it is a common practice to combine low-cost, high-volume screening methods with high-cost but low-volume confirmatory methods to ensure efficient and cost-effective detection of the contaminants.

ELISA studied in this research, is a well-accepted convenient and widely used as screening method to detect drug residues (Liu *et al*, 2007). ELISA can provide an inexpensive, high sensitive and fast screening alternative for the detection of samples containing trace amount to low-molecular weight analytes such as nitrofurans (Vass *et al.*, 2008).

4.6 Development and optimization of ELISA for AHD detection

In order to obtain the most suitable ELISA with the lowest IC_{50} and LOD, three ELISA formats were investigated. Concentration of Ab and Ag, incubation time and temperature were optimized for each format studied. The concentration of both Ag and Ab are the important factor for the quantitative analysis. Therefore, the concentrations of both Ag and Ab must be at the suitable ratio to allow the best competition of the AHD in the sample.

The optimal dilutions of coating Ag and Ab were determined by the checkerboard titration method. The suitable ratio of the Ag and the Ab were justified by the resulting absorbance. The typical recommended absorbance value were 1.0 – 1.5 (Gao *et al.*, 2007; Vass *et al.*, 2008; and Li *et al.*, 2010)

In case of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format, CPAHD-BSA at the concentration of 7.5, 5.0, 2.5 and 0.937 µg/ml and MAb against NPAHD at the concentration of 0.50, 0.625, 1.25 and 4.0 µg/ml were selected for, respectively (Figure 4.15).

MAb against NPAHD at the concentration of 5.0, 2.5, 1.25 and 0.625 μg/ml and CPAHD-HRP at the concentration of 0.625, 1.25, 2.50 and 5.0 μg/ml were selected for Ag captured competitive direct ELISA format (Figure 4.16).

In the last format, Ab captured competitive indirect ELISA (biotinylated Ab) CPAHD-BSA at the concentration of 7.5, 5.0, 2.5 and 1.25 μg/ml and biotinylated Ab as 1st Ab at the concentration of 1.25, 2.5, 2.0 and 5.0 μg/ml were selected, respectively (Figure 4.17).

These suitable concentrations were further used to investigate the sensitivity of the three ELISA format in the next section.

Table 4.15 The optimal dilution of coating Ag and Ab using Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format.

Concentration of MAb (μg/ml)	Absorbance at 450 nm					
	Concentration of coating agent, CPAHD-BSA (μg/ml)					
	12.5	10.0	7.5	5.0	2.5	0.937
7.0	2.637	2.532	2.444	2.322	1.919	1.726
6.0	2.471	2.436	2.341	2.269	1.781	1.553
5.0	2.433	2.407	2.288	2.171	1.740	1.239
4.0	2.212	2.166	2.071	1.945	1.562	1.009
1.25	1.851	1.772	1.624	1.528	1.045	0.857
0.625	1.478	1.411	1.307	1.104	0.812	0.735
0.500	1.233	1.153	1.057	0.902	0.655	0.573
0.250	1.146	0.843	0.812	0.793	0.651	0.558

Highlight refers to selected combination of MAb and coating agent.

Table 4.16 The optimal dilution of coating Ag and Ab using Ag captured competitive direct ELISA format.

Concentration of CPAHD-HRP (µg/ml)	Absorbance at 450 nm					
	Concentration of coating agent, MAb (µg/ml)					
	7.25	5.00	2.50	1.25	0.625	0.312
10.0	2.270	2.231	2.117	1.892	1.584	1.486
7.50	1.882	1.582	1.491	1.444	1.287	1.235
5.00	1.690	1.592	1.350	1.246	1.110	0.896
2.50	1.519	1.438	1.244	1.096	0.887	0.757
1.25	1.302	1.252	1.153	0.954	0.825	0.733
0.625	1.298	0.996	0.890	0.763	0.696	0.563
0.500	0.819	0.740	0.705	0.666	0.543	0.476
0.250	0.670	0.538	0.519	0.479	0.390	0.322

Highlight refers to selected combination of CPAHD-BSA and coating agent.

Table 4.17 The optimal dilution of coating Ag and biotinylated Ab using Ab captured competitive indirect ELISA (biotinylated Ab) format.

Concentration of biotinylated Ab (µg/ml)	Absorbance at 450 nm					
	Concentration of coating agent, CPAHD-BSA (µg/ml)					
	10.0	7.50	5.00	2.50	1.25	1.00
10.0	2.467	2.471	2.436	2.354	1.796	1.410
7.50	2.277	2.193	2.071	1.930	1.590	1.267
5.00	1.950	1.802	1.802	1.204	1.063	0.882
2.50	1.554	1.233	1.068	1.110	0.793	0.751
2.00	1.338	1.308	0.888	0.924	0.743	0.715
1.25	1.151	0.919	0.861	0.773	0.724	0.675
1.00	0.908	0.877	0.809	0.747	0.711	0.642

Highlight refers to selected combination of biotinylated Ab and coating agent.

4.6.1 Antibody captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format

Competitive ELISA is the method of choice when the target Ag is very small. In the Ab-captured format, NPAHD in the sample competes with immobilized CPAHD to bind with the Ab. The amount of NPAHD was then quantitated, and the amount of labeled GAM-HRP that bound is inversely proportional to the amount of NPAHD in the system. HRP activity is measured indirectly by the rate of transformation of H-donor. HRP reacts with hydrogen peroxide to relase an oxygen free radical, which in turn reacts with a colorimetric substrate (Howard and Kaser, 2007). Four different combinations of Ag and Ab concentration were tested for the lowest value of IC_{50} and LOD. The result in Table 4.18 shown that using 7.5 µg/ml of CPAHD-BSA as coating agent and 0.5 µg/ml of 1st MAb gave the best IC_{50} and LOD values of 12.15 and 0.258 ppb, respectively for this format (Figure 4.14 – Figure 4.17).

Table 4.18 IC_{50} and LOD of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format.

Concentration of coating Ag (µg/ml)	Concentration of MAb against AHD (µg/ml)	IC_{50} (ppb)	LOD (ppb)
7.5	0.5	12.15	0.258
5.0	0.625	16.4	1.600
2.5	1.25	13.93	0.756
0.937	4.0	15.02	0.432

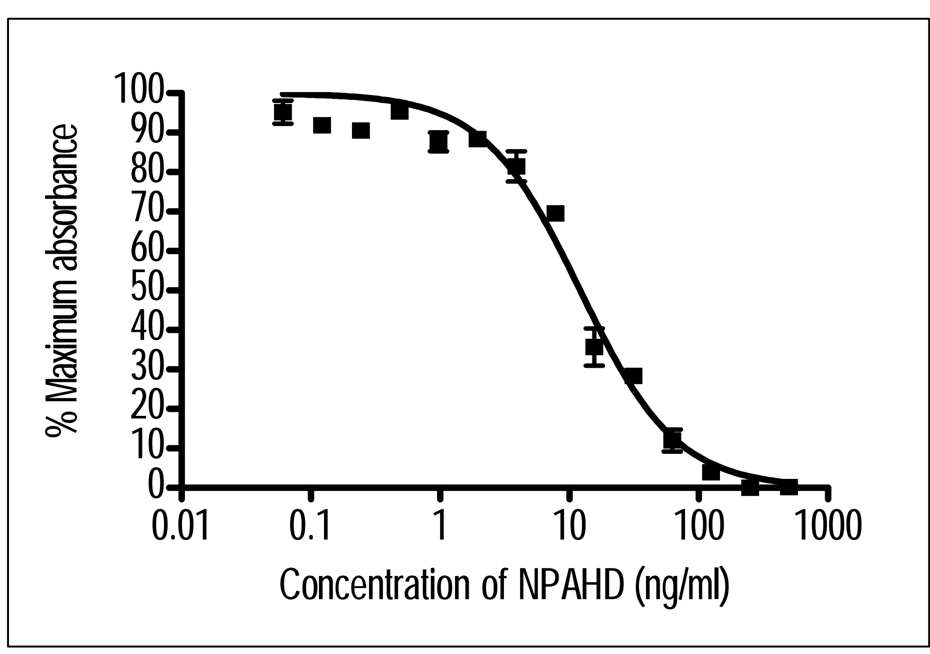


Figure 4.14 Response curve of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format using coating Ag at 7.5 µg/ml and MAb at 0.5 µg/ml.

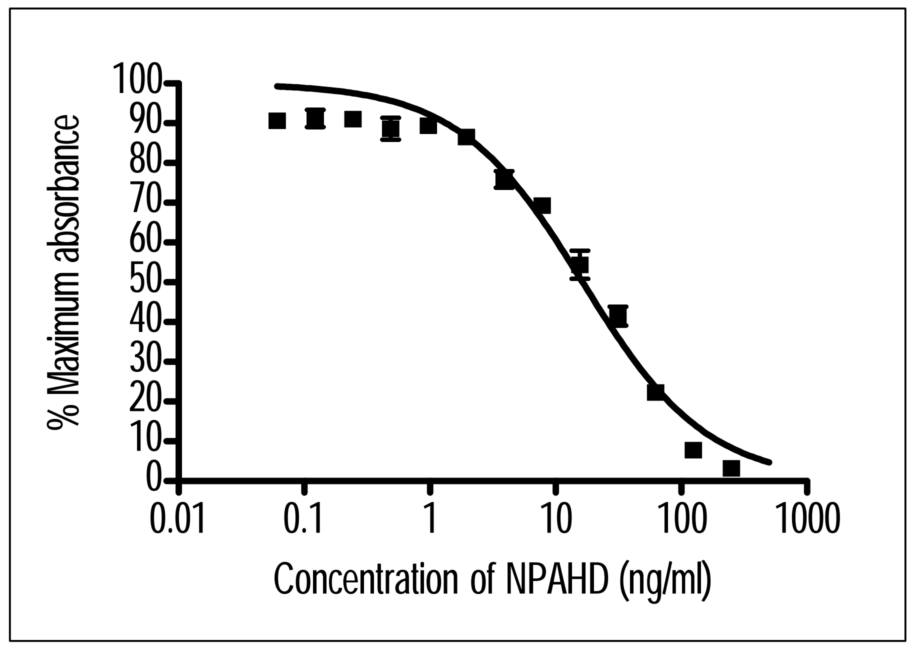


Figure 4.15 Response curve of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format using coating Ag at 5.0 µg/ml and MAb at 0.625 µg/ml.

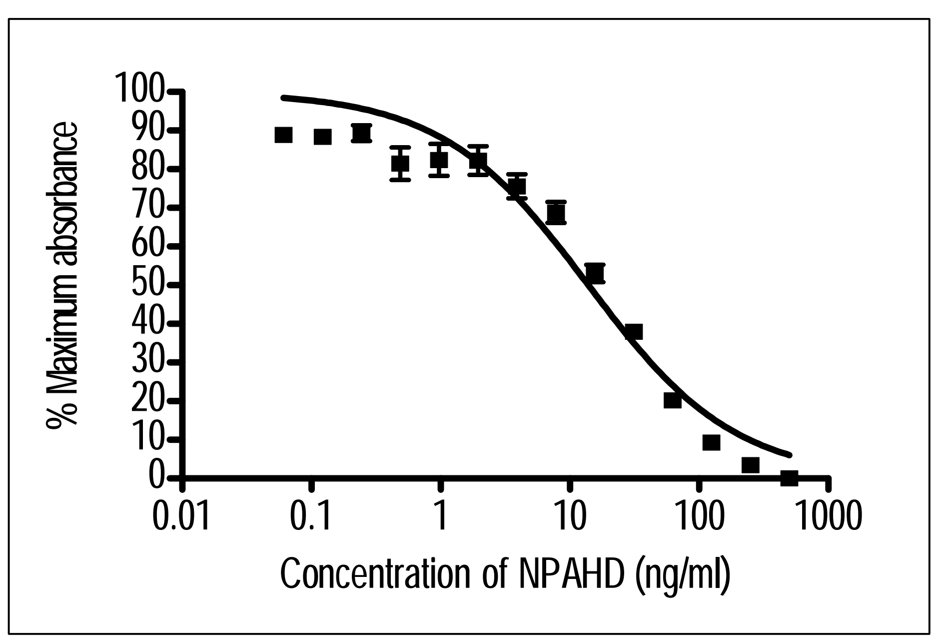


Figure 4.16 Response curve of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format using coating Ag at 2.5 µg/ml and MAb at 1.25 µg/ml.

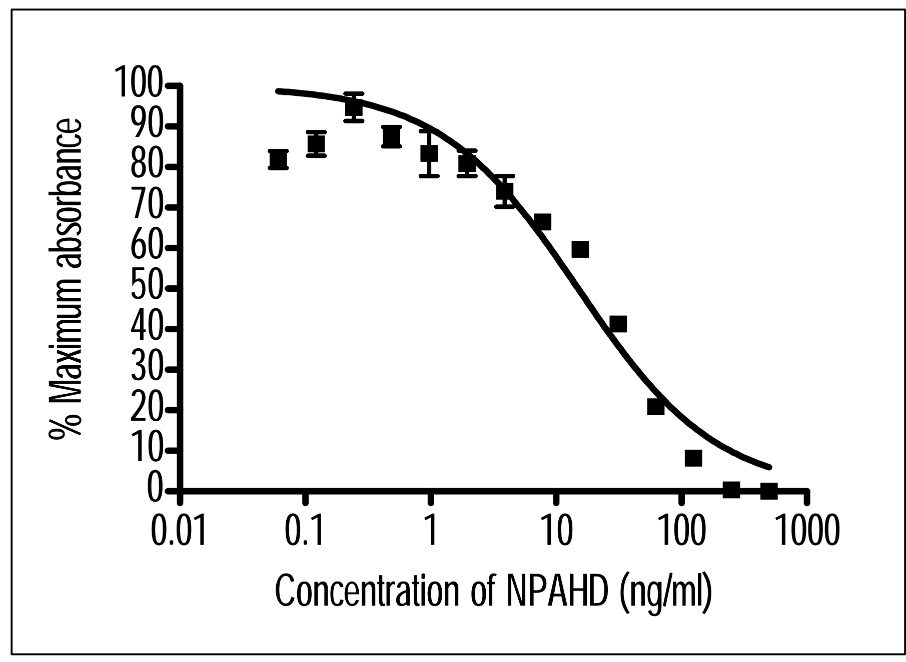


Figure 4.17 Response curve of Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format using coating Ag at 0.937 µg/ml and MAb at 4.0 µg/ml.

4.6.2 Antigen captured competitive direct ELISA format

In this assay, CPAHD was conjugated to the detecting molecule, HRP. NPAHD in the sample competes with the HRP-labeled CPAHD to bind with the immunized Ab. The label was then quantitated, and the amount of labeled CPAHD-HRP that bound is inversely proportional to the amount of NPAHD as bound unlabeled Ag. The IC_{50} and LOD values of the selected conditions were shown in Table 4.19. The result shown that using 2.5 µg/ml of MAb as coating agent and 1.25 µg/ml of CPAHD-HRP gave the best IC_{50} and LOD values of 72.99 and 7.5 ppb, respectively. As compared to the previous format, this format was less sensitive (Figure 4.18 – Figure 4.21).

Table 4.19 IC_{50} and LOD of Ag captured competitive direct ELISA format.

Concentration of MAb (µg/ml)	Concentration of CPAHD-HRP (µg/ml)	IC_{50} (ppb)	LOD (ppb)
5.0	0.625	86.29	14.22
2.5	1.25	72.99	7.50
1.25	2.5	79.46	28.78
0.625	5.0	88.94	25.31

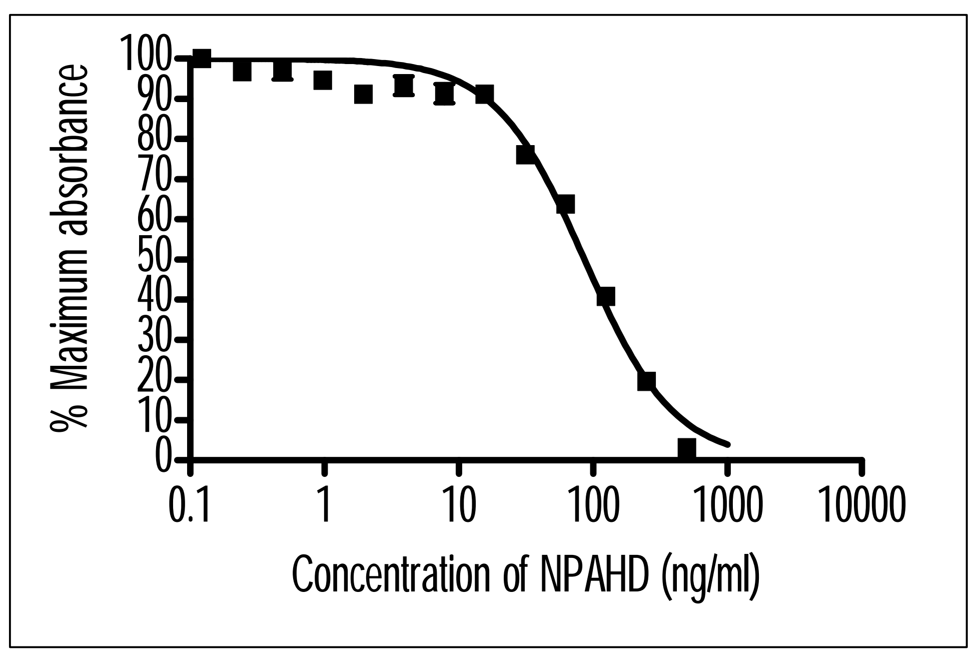


Figure 4.18 Response curve of Ag captured competitive direct ELISA format using MAb at 5.0 µg/ml and CPAHD-HRP at 0.625 µg/ml.

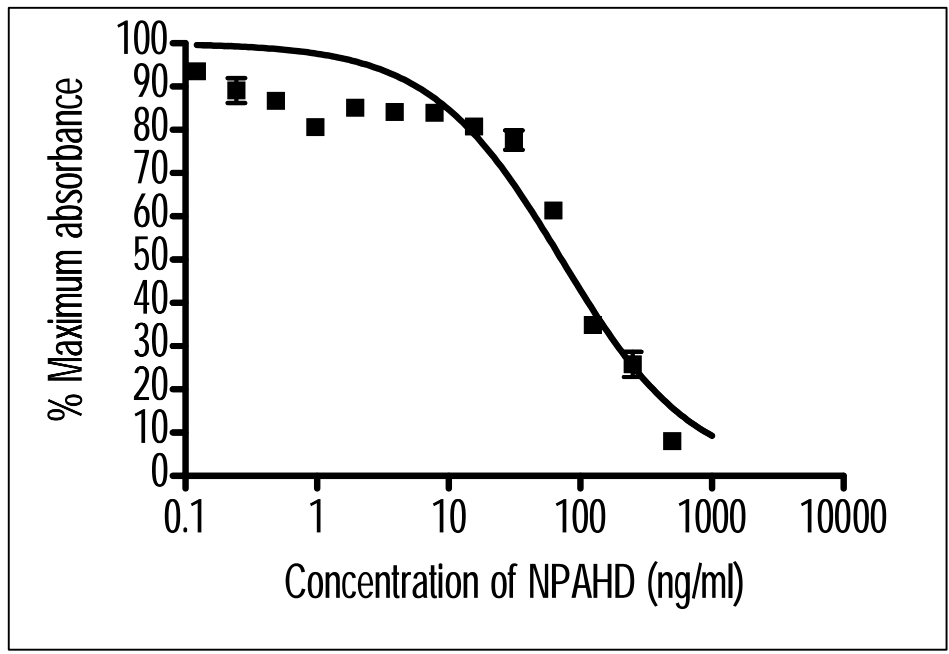


Figure 4.19 Response curve of Ag captured competitive direct ELISA format using MAb at 2.5 µg/ml and CPAHD-HRP at 1.25 µg/ml.

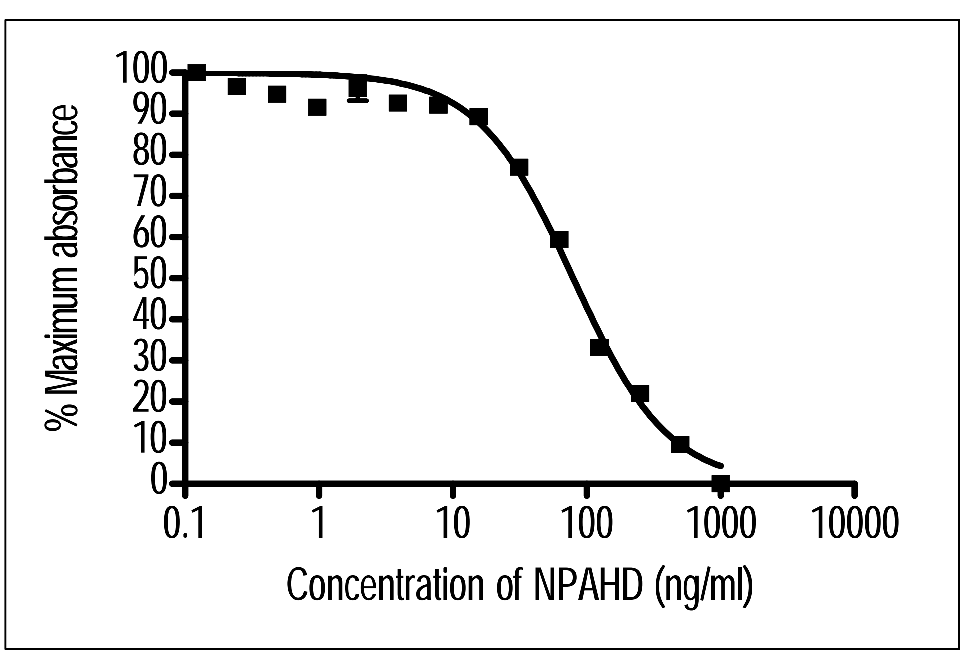


Figure 4.20 Response curve of Ag captured competitive direct ELISA format using MAb at 1.25 µg/ml and CPAHD-HRP at 2.5 µg/ml.

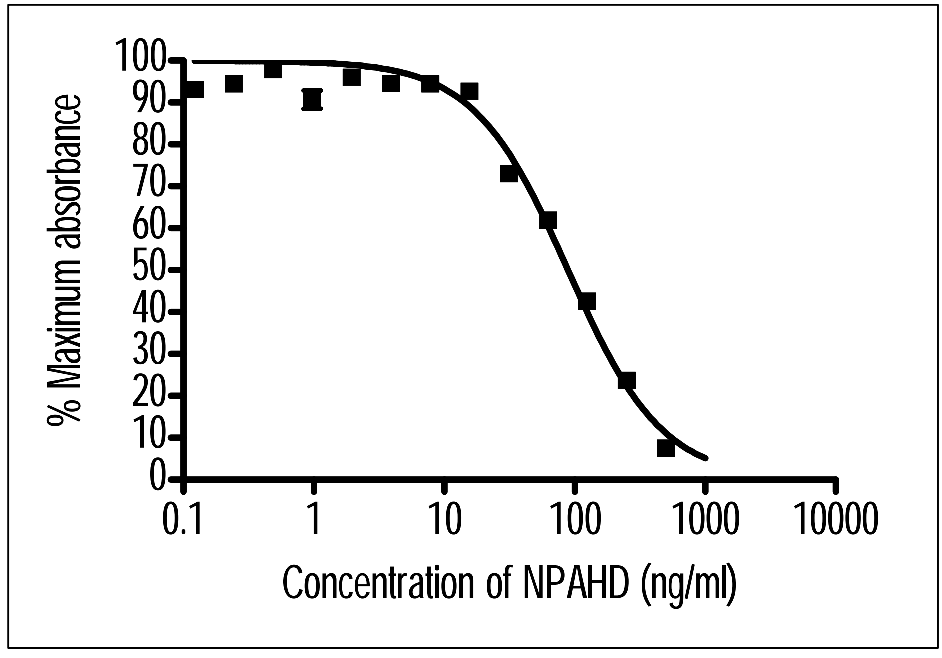


Figure 4.21 Response curve of Ag captured competitive direct ELISA format using MAb at 0.625 µg/ml and CPAHD-HRP at 5.0 µg/ml.

4.6.3 Antibody captured competitive indirect ELISA (biotinylated antibody) format

Many assays were able to take advantage of the signal-enhancing biotin-streptavidin system (Wilchek and Bayer, 1984; Guesdon et al., 1979; Kendall *et al.*, 1983). Biotin conjugated to Ab can be detected by streptavidin conjugated to a variety of different induces, many of which are available commercially.

Four selected concentration method of CPAHD-BSA conjugate and biotinylated Ab were used to obtain the lowest IC_{50} and LOD value. The result (in Table 4.20) shown that using 2.5 μg/ml of CPAHD-BSA as coating agent and 2.0 μg/ml of biotinylated Ab gave the best IC_{50} and LOD values of 5.307 and 0.091 ppb in this format, respectively (Figure 4.22 – Figure 4.25). Among the three formats tested, the Ab-captured formats were better than the Ag-captured format. In addition, the tracking system of the Ag-Ab binding is also important. The results showed that the system with the biotin-conjugated Ab and streptavidin-HRP was better than the HRP-labeled goat anti-mouse IgG system as expected due to the increase of signal from biotin and streptavidin binding. Kerr in 1994 reported on the enhanced enzyme immunodetection systems. Enzyme immunoassay is extremely sensitive and can be used for the detection and quantification of most Ags, there are some instances where increased sensitivity is necessary because of the low level of Ag or because of the need for rapid detection. The biotin-avidin or streptavidin system allows increase in sensitivity. However, this system was the most complicated one among the three tested format.

Table 4.20 IC_{50} and LOD of Ab captured competitive indirect ELISA (biotinylated Ab) format.

Concentration of coating Ag (µg/ml)	Concentration of biotinylated Ab (µg/ml)	IC_{50} (ppb)	LOD (ppb)
7.5	1.25	6.52	0.214
5.0	2.5	8.57	0.397
2.5	2.0	5.31	0.091
1.25	5.0	6.35	0.273

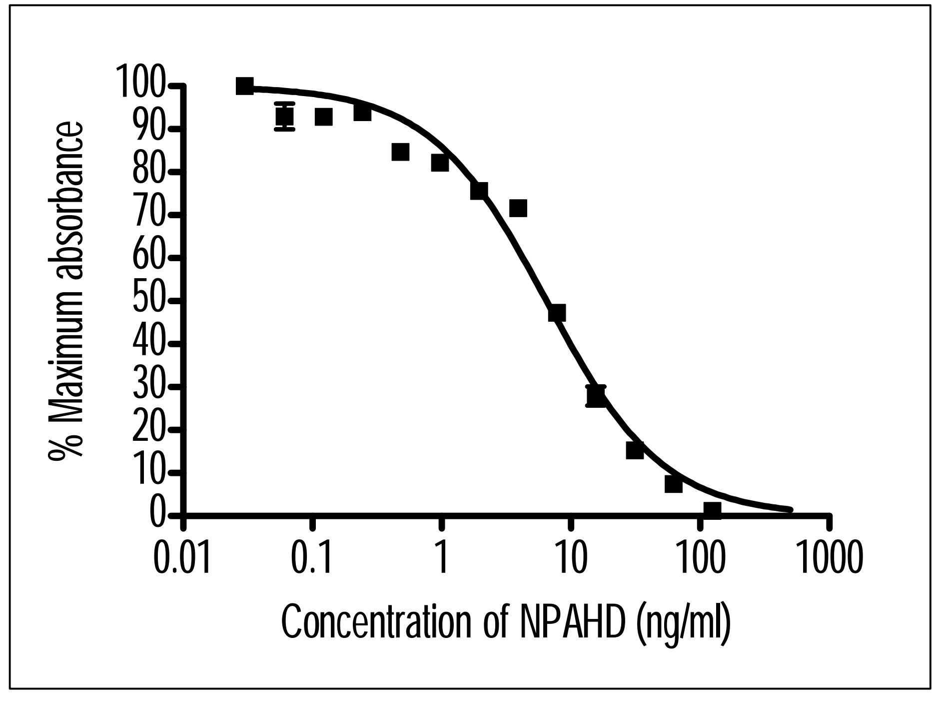


Figure 4.22 Response curve of Ab captured competitive indirect ELISA (biotinylated Ab) format using CPAHD-BSA at 7.50 µg/ml and biotinylated Ab at 1.25 µg/ml.

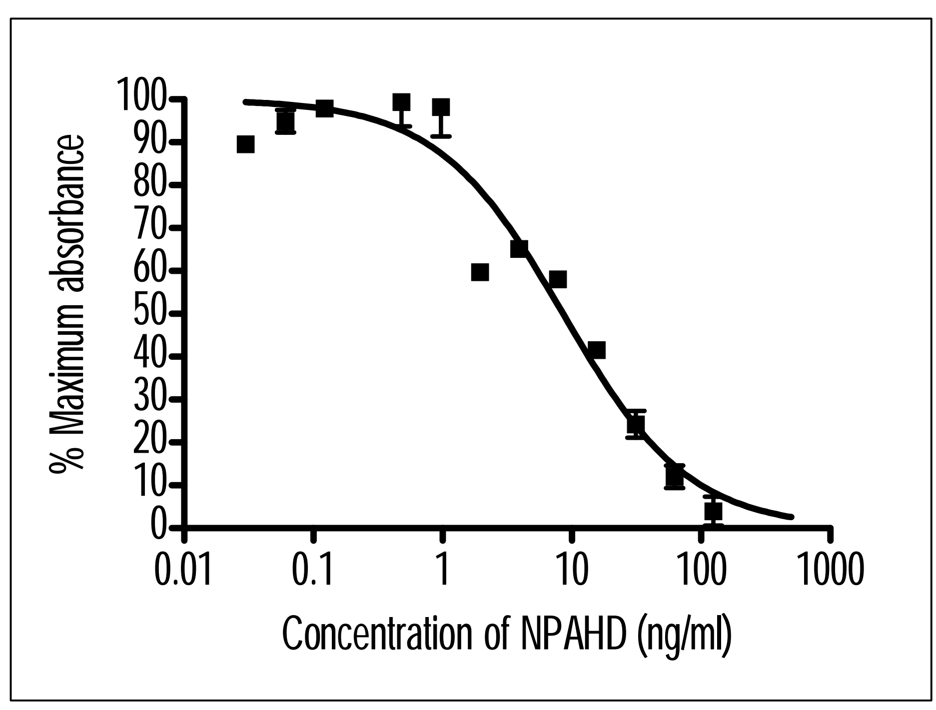


Figure 4.23 Response curve of Ab captured competitive indirect ELISA (biotinylated Ab) format using CPAHD-BSA at 5.0 µg/ml and biotinylated Ab at 2.5 µg/ml.

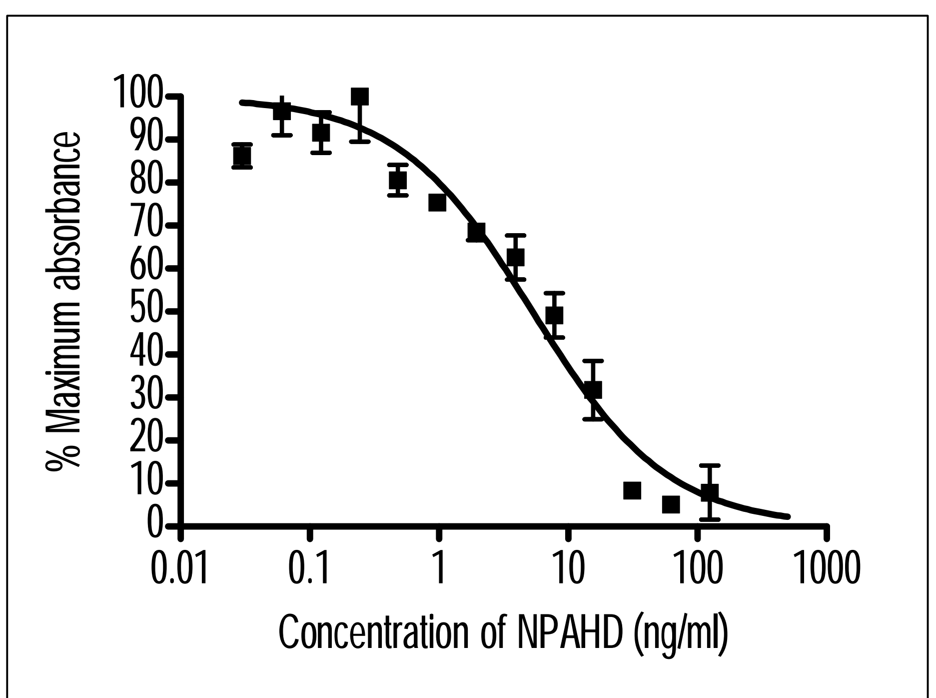


Figure 4.24 Response curve of Ab captured competitive indirect ELISA (biotinylated Ab) format using CPAHD-BSA at 2.5 µg/ml and biotinylated Ab at 2.0 µg/ml.

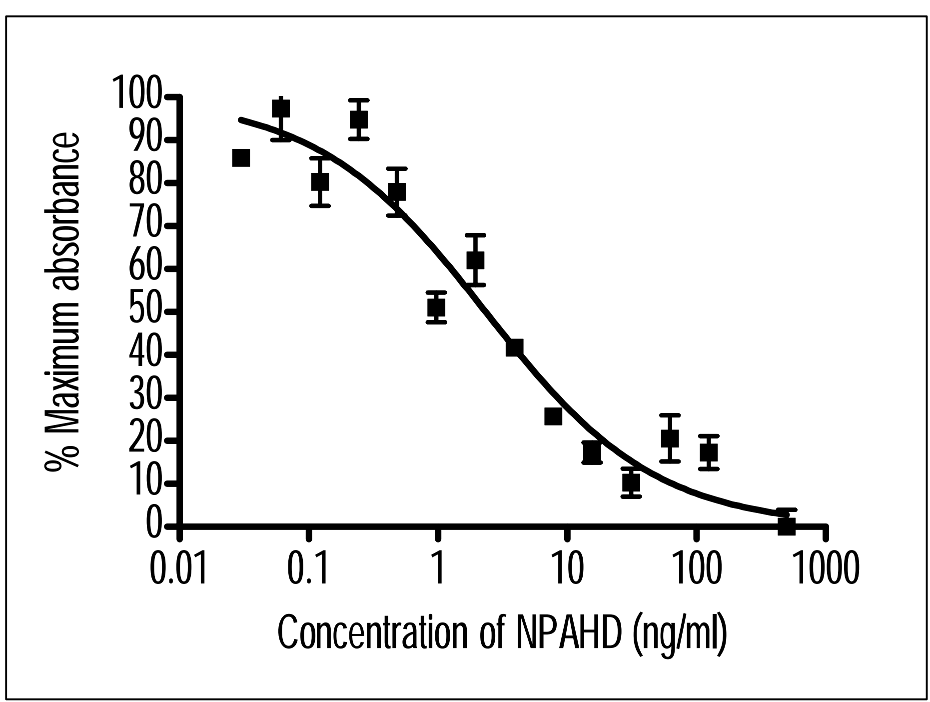


Figure 4.25 Response curve of Ab captured competitive indirect ELISA (biotinylated Ab) format using CPAHD-BSA at 1.25 µg/ml and biotinylated Ab at 5.0 µg/ml.

4.6.4 Effects of incubation temperature and time on sensitivity of the selected ELISA format.

Effects of incubation time and temperature were investigated using the optimized condition obtained previously. The results showed that the sensitivity increased as the incubation time increased. This might be due to relative low association rate and dissociation rate constant of the Ab (Chen *et al.*, 2010). The differences in incubation at room temperature and at 37°C showed no obvious effect on the IC_{50} and LOD. Thus, the condition of incubation at room temperature for 60 minutes was selected for further investigation since it offerd enough sensitivity for the detection under the convenient working condition (Table 4.21).

Table 4.21 Effect of incubation temperature and time on sensitivity of the Ab-captured competitive indirect ELISA (biotinyted Ab).

Temperature (°C)	Time (min)	IC_{50} (ppb)	LOD (ppb)
Room temperature	30	13.43	3.363
	60	4.905	0.113
	90	5.566	0.155
	120	5.210	0.120
37	30	12.28	3.560
	60	5.772	0.211
	90	6.190	0.310
	120	5.307	0.091

4.7 Specificity of prototype test kit

Similar to the sensitivity, cross reactivity of the Ab is one of the major criteria to rationalize the application of the obtained Ab whether it is suitable for test kit development. The specificity of the developed assay was evaluated by determination of the cross-reactivity against selected compounds. The percentages of cross reactivity of the MAb 28/10E/8B/1E with various competitors were quantified and showed in Table 4.22.

The MAb showed specificity for NFT (parent form, 876%), NPAHD (detecting derivative, 100%) and CPAHD (metabolite derivative form, 287%) but showed no significant cross reactivity against other tested compounds including other nitrofurans (< 0.03%). It is understandable that the Ab shows a high cross-reactivity toward CPAHD because it was the modified hapten used to link to carrier protein BSA to prepare the immunogen (Liu *et al.*, 2007). It is well-known that Abs elicited to haptenic conjugates show a preferential recognition to the part of the molecule that is farthest from the attachment site of the hapten to the carrier protein. Both CPAHD and NPAHD have

moieties composed of the condensation of AHD and benzaldehyde, but their cross-reactivities were different. Considering that their only structure differences are the substituents in the benzene ring, it is possible that the phenyl moiety plays a certain role for Ab recognition despite this part being close to the linking point of hapten and carrier protein. According to their molecular structures, nitrofurans have the same central group (O_2N-furan ring-CH=N) and the majority of their structure (O_2N-furan ring-CH=N-N-H) is as same as the conjugated CPAHD hapten (Li *et al.*, 2010). Therefore, this MAb showed high cross-reactivity against NFT.

Cooper *et al* (2004), reported about Ab either specificity for FZD, the parent compound of AOZ. However, nitrofurantoin has been known to be metabolized rapidly *in vivo*, and as a consequence such cross-reactivity is unlikely to occur in food detection. These results suggested that the MAb 28/10E/8B/1E is suitable for the development of a test kit specific only for NFT and NPAHD.

Table 4.22 % Cross-reactivity of MAb 28/10E/8B/1E with various substances.

Competitors	%Cross-reactivity
Nitrofurans	
NFT	876
NPAHD	100
CPAHD	287
AHD	<0.03
FZD	<0.03
FTD	<0.03
NFZ	<0.03
NPAMOZ	<0.03
NPAOZ	<0.03
NPSEM	<0.03
Antibiotics	
Oxytetracycline	<0.03
Tetracycline	<0.03
Rolitracycline	<0.03
Doxycycline	<0.03
β-Agonists	
Clenbuterol	<0.03
Salbutamol	<0.03

4.8 Detection of AHD in spiked shrimp samples

A very important step in the evaluation of any analytical procedure is assessment of the matrix effect. To evaluate the use of the obtained Ab and the interference of sample matrix, various concentrations of AHD in spiked shrimp sample and buffer were quantified by the Ab-captures indirect competitive ELISA (biotinylated Ab). The sample preparation used in this study was modified from a previous paper (Pimpitak *et al.*, 2009).

In order to assessment of the detection capability of the ELISA, shrimp samples were fortified with several concentration of analyte or AHD. The samples were extracted and determined for the analytes using the selected ELISA format at room temperature for 60 minutes for incubation. The sample derivatization and extraction procedure was performed according to the established method for AHD analysis by LC-MS/MS, which includes tissue homogenization, acid hydrolysis of AHD from tissue-bound moieties, derivatization of AHD with *o*-NBA, sample neutralization and extraction of NPAHD using ethyl acetate. The derivatization of nitrofurans metabolites was done by using *o*-NBA to enlarge analyte mass prior to detection (Cooper *et al.*, 2007). The derivatization step is required to isolate residues from the matrix to produce a derivative with a chromophore or higher molecular mass suitable for UV and MS detection (Diblikova *et al.*, 2005).

As shown in Table 4.23, the % recoveries of AHD in both shrimp samples and buffer were not dramatically different, thus indicating that the matrix effect was insignificant based on the extraction method used in this study. This result supported the finding of Pimpitak *et al.*, (2009) in the detection of AMOZ in shrimp samples. The accuracy of the detection was quantified in term of %recovery. Recovery values were calculated using AHD calibration curve in assay buffer (Figure 4.25). The recovery rates are in the ranges of 91-113 % and 85-119% of PBS and shrimp samples for intra-assay, respectively and 96-112% and 98-112% of PBS and shrimp samples for inter-assay, respectively. In general, the common acceptable %recovery value for ELISA is between 80% and 120% (Kramer *et al.*, 2010). The results showed that the % recoveries of all

samples were between 85% and 119%, indicating that the accuracy of this ELISA is in an acceptable range.

The precision of the analysis was investigated in term of coefficient of variation (CV) of both intra and inter-variation assay. Most of the obtained % CV were less than 10% with the exception of the inter-variation assay at 6.25 ppb of shrimp sample (17.8%).

Table 4.23 Intra-assay and inter-assay coefficient of variation (CV) in the determination of NPAHD levels in PBS and shrimp samples by Ab-captures indirect competitive ELISA (biotinylated Ab).

NPAHD conc. (ppb)	Intra-assay [a]				Inter-assay [b]			
	B/B_0	SD	%CV	%Recovery	B/B_0	SD	%CV	%Recovery
PBS								
0.20	88.6	0.04	1.10	99.5	88.3	2.12	2.40	101
0.40	72.5	0.02	0.72	108	74.9	2.12	2.83	96.4
0.75	61.6	0.01	1.78	97.5	60.5	2.54	4.20	103
1.50	44.3	0.02	2.10	112	46.4	2.49	5.36	102
3.00	33.2	0.01	1.18	96.3	32.4	1.99	6.13	100
6.25	19.1	0.01	1.75	91.2	17.0	1.60	9.35	101
Shrimp samples								
0.20	86.2	0.04	2.02	112	86.5	1.10	1.27	110
0.40	70.8	0.02	1.07	117	71.9	1.52	2.12	112
0.75	60.7	0.03	2.42	102	59.8	2.35	3.94	107
1.50	43.2	0.02	2.51	119	46.1	2.99	6.49	103
3.00	33.9	0.01	1.86	93.2	32.1	2.99	9.31	102
6.25	20.5	0.02	4.97	85.3	17.5	3.11	17.8	99

[a] Intra assay variation was determined by 12 replicates in a single day

[b] Intra-assay variation was determined by 6 replicates in 12 different days.

4.9 Comparison detection of AHD in shrimp samples by ELISA and LC-MS/MS

As the use nitrofurans is banned, all the ELISA positive finding must be re-analyzed by a confirmation method to identify specific nitrofurans (Li *et al.*, 2010). The accuracy of the analysis was also determined by the comparative detection of AHD in fortified shrimp samples at different concentrations using ELISA developed in this study and LC-MS/MS the most acceptable method for AHD quantification (Niessen, 1998, and Hu *et al*, 2007).

Blank shrimp samples fortified with AHD at the level of 0.2 – 3 ppb were used as the sample. The %recovery and correlation of the two methods were shown in Table 4.24 and Figure 4.26, respectively. The developed ELISA gave the %recovery in the range of 80.6 – 113.9% while LC-MS/MS gave in the range of 80.0 – 116%. The %recovery in both methods showed no systematic derivation and were in the same range of accuracy. The correlation coefficient value between the two methods was 1.163 with the R^2 of 0.987. There results were comparable with reported by Diblikova *et al.*, 2005. The regression analysis of ELISA results and comparative LC-MS/MS method showed extremely good correlation between the two methods within the concentration range of 0-10.5 µg/kg as determined by $y = -0.078 + 0.612x$ ($r = 0.99$, $n = 8$) for AOZ detection in shrimp sample.

Table 4.24 Comparison of quantitative analysis of AHD by ELISA and LC-MS/MS in fortified shrimp samples.

Fortified conc. (ppb)	ELISA		LC-MS/MS	
	Measured conc. (ppb)	% Recovery	Measured conc. (ppb)	% Recovery
3.00	2.47	82.4	2.99	99.7
1.00	0.94	93.9	0.93	93.0
0.50	0.56	113.9	0.58	116
0.40	0.42	104.6	0.32	80.0
0.30	0.27	89.4	0.25	83.3
0.20	0.16	80.6	0.19	95.0

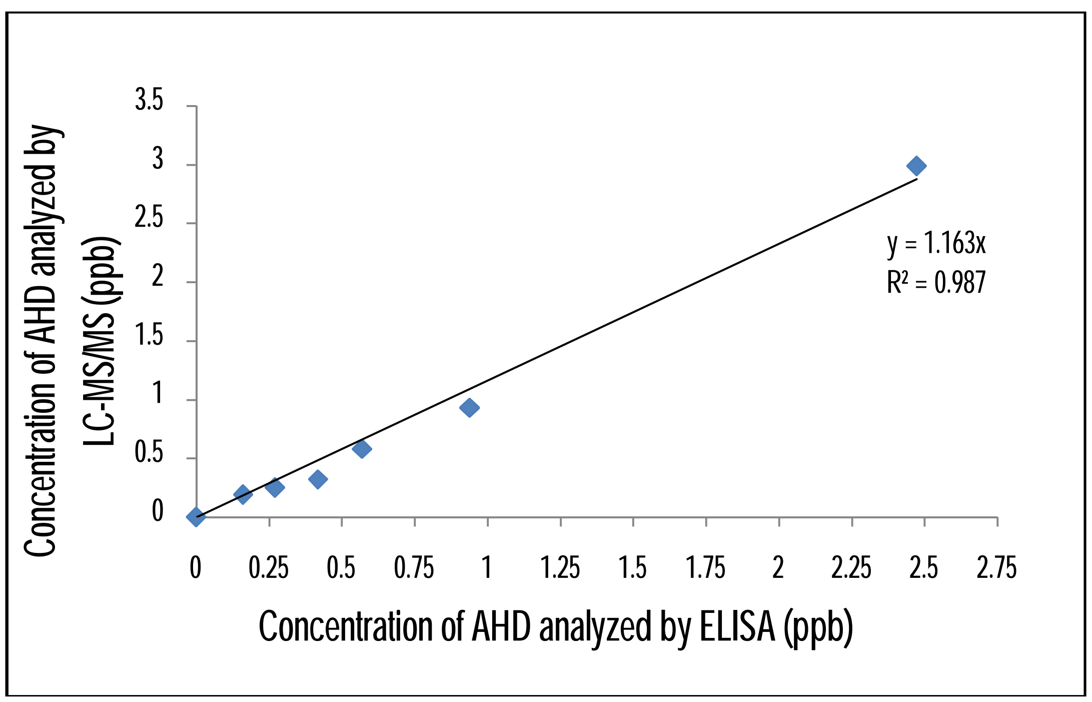


Figure 4.26 Comparative determination of AHD by ELISA and LC-MS/MS for AHD.

4.10 Detection capability and assay recovery for shrimp samples

Detection capability (CC_{β}) of the assay was analyzed using twenty different shrimp blank samples fortified AHD. The investigations were performed by the Ab captured competitive indirect ELISA (biotinylated Ab) format at room temperature for 60 minutes for incubation. Analysis of 20 blank samples by ELISA yielded values ranging from 0.09 to 0.24 ppb (mean 0.16 ppb). When these same 20 samples were fortified with AHD at 0.4 ppb, values ranged from 0.33 to 0.64 ppb (mean 0.49 ppb). There was no overlap between the two groups of blank and fortified samples. Base on this data, the detection capability of the assay was declared for shrimp to be 0.4 ppb when a threshold value of 0.33 ppb (the lowest observed 0.4 ppb fortified sample) is applied to analysis (Figure 4.27). These results were comparable with those reported by Vass *et al.*, 2008, where the CC_{β} determined for porcine tissue was 0.3 µg/kg when a threshold of 0.11 µg/kg was applied. In addition, CC_{β} of SEM in chicken muscle was reported to be with the 0.25 µg/kg and threshold of 0.21 µg/kg (Cooper *et al.*, 2007). Since the MRPL of AHD has been set at 1 ppb, the developed ELISA with the CC_{β} of 0.40 ppb could be confidently applied for AHD detection.

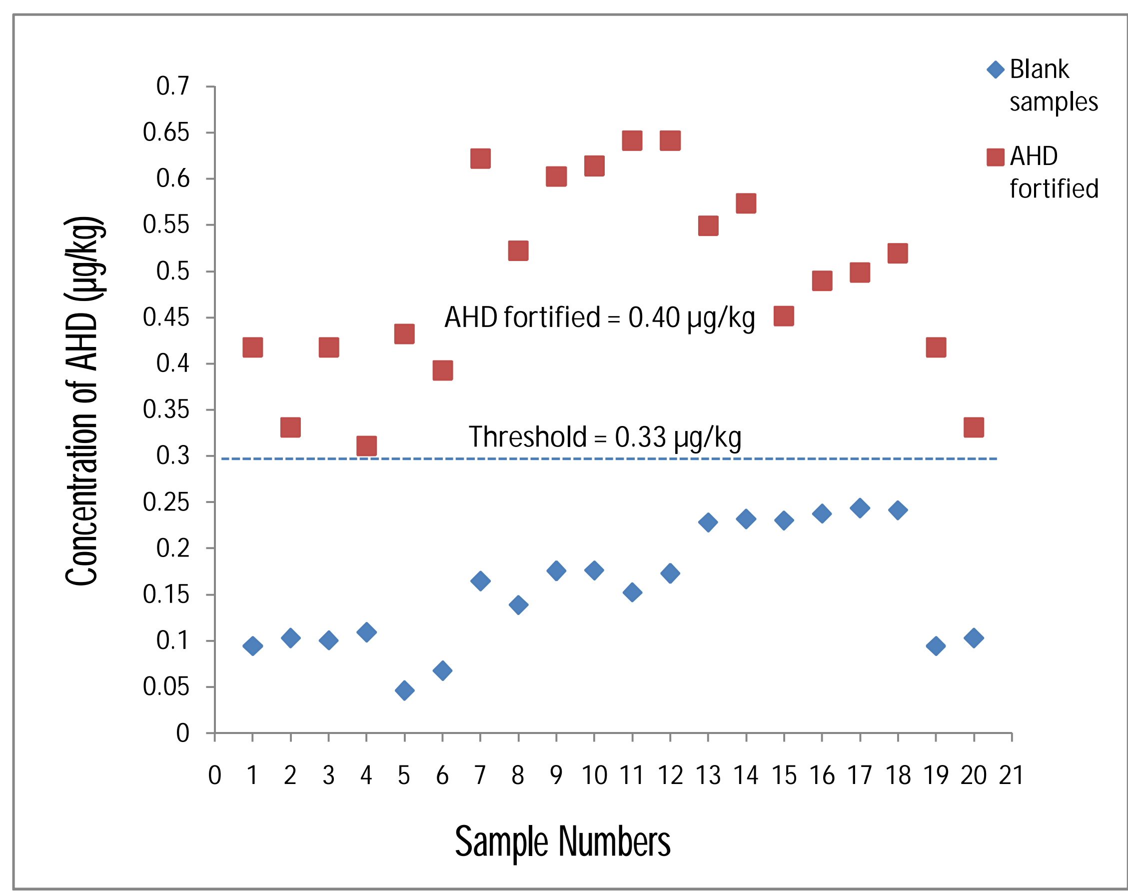


Figure 4.27 Detection capability of the Ab captured competitive indirect ELISA (biotinylated Ab) format for detecting AHD in shrimp samples.

CHAPTER V

CONCLUSION AND SUGGESTION

5.1 Conclusion

AHD was derivatized with CBA in pyridine to form CPAHD which was observed by TLC. CPAHD was then attached to BSA by carbodiimide reaction. The percentage of conjugation was determined by TNBS method and found to be 40%. In addition, the conjugation of CPAHD-BSA was also confirmed by comparing molecular mass of BSA and that of the conjugated product by MALDI-TOF-MS. The molecular ratio of CPAHD to BSA was calculated to be 12.4:1. The CPAHD-BSA conjugate was then used as immunogen for immunization of BALB/c mice and coating agent of ELISA method.

Nine BALB/c female mice, 8 weeks old were immunized by intraperitoneal injections with CPAHD-BSA. The sera were collected and assayed for anti-CPAHD Abs by an indirect ELISA. The result shown that Ab against CPAHD was produced at the titre of 4096000, 8192000, 8192000, 8192000, 4096000, 4096000, 8192000, 4096000 and 4096000 for mice number 1-9, respectively. The competitors such as AHD, CBA, CPAHD and NPAHD at the concentration 20 μg/ml were used in the indirect competitive ELISA. Cross-reactivity was calculated in term of % competition. The results suggested that Ab reacted with CPAHD and NPAHD in the range of 15% - 55% and reacted with AHD and CBA lower than 17%.

The spleens of immunized mice were collected and anti-CPAHD MAbs were produced using somatic cell fusion technique. Myeloma cell type SP2/0-Ag14 were used in cell fusion for 8 trial, resulting in unstable monoclones. Then, myeloma cell type P3-X63-Ag8 was used in the 9th fusion, yielding 21 clones which secreted Abs that can bind with NPAHD. After limiting dilution step, six monoclone namely 28/2F/12C, 28/2F/12F, 28/10E/8B/6F, 28/10E/8B/12A, 28/10E/8B/10E and 28/10E/8B/1E were obtained.

All MAbs were tested for their sensitivities in term of IC_{50} and LOD which were found to be in the range of 41.8 - 293 ppb and 14.5 – 72.3 ppb, respectively. The isotype of MAbs was determined using Sigma's Mouse Monoclonal Antibody Isotyping Kit. The absorbance values of all MAbs were measured at 450 nm by indirect ELISA method. It was concluded that clone number 28/2F/12C, 28/10E/8B/12A and 28/10E/8B/1E were IgG_1, 28/2F/12F was IgG_{2a} and 28/10E/8B/6F was IgG_{2b} isotype.

Then, MAbs were investigated for their specificity. Among these clones, MAbs from clone 28/2F/12C and 28/10E/8B/10E cross-reacted with NPSEM at 21.2% and 30.2%, respectively, while the other four MAbs did not cross-reacted with other nitrofurans as compared with NPAHD. MAb from clone 28/10E/8B/1E was selected for further studies due to its highest sensitivity.

Monoclone 28/10E/8B/1E was cultured in spinner flask to produce a large amount of the Ab which later purified by protein G sepharose column. The %recovery was 19.5%. The total protein content was 13.9 mg and the amount of Ab was 10.7 mg. The molecular weights of the heavy chain and the light chain of the Ab were found at 64.9 kDa and 28.7 kDa, respectively, by SDS-PAGE.

To obtain the most sensitive ELISA, three formats of ELISA were investigated under the suitable concentrations of Ag and Ab employed in each format. The Ab captured competitive indirect ELISA (goat anti-mouse IgG-HRP conjugate) format gave the best IC_{50} and LOD values of 12.15 and 0.258 ppb, respectively, while the Ag captured competitive direct ELISA format gave the best IC_{50} and LOD values of 72.99 and 7.5 ppb, respectively. But the Ab captured competitive indirect ELISA (biotinylated Ab) format was the most sensitive format with the IC_{50} and LOD values of 5.307 and 0.091 ppb, respectively.

The effects of incubation time and temperature were studied using the optimized condition previously obtained. The difference in incubation temperature at room temperature and 37°C showed no obvious effect on the IC_{50} and LOD values. Thus, incubation at room temperature for 60 minutes was selected for further investigation

since if offers enough sensitivity for the detection under the convenient working condition.

The percentages of cross reactivity of the MAb 28/10E/8B/1E with various competitors were quantified. The MAb showed specificity toward NFT (parent form, 876%), NPAHD (100%) and CPAHD (287%) (metabolited derivative form) but showed no significant cross reactivity to other tested chemicals including other nitrofurans (< 0.03%).

To evaluate the use of the obtained Ab and interference of sample matrix, various concentrations of AHD in spiked shrimp sample and buffer were quantified by the Ab-captures indirect competitive ELISA (biotinylated Ab). The % recoveries of AHD in both shrimp samples and buffer were not dramatically different, thus indicating that the matrix effect was insignificant based on the extraction method used in this study. Most of the obtained % CV were less than 10% with the exception of the inter-variation assay at 6.25 ppb of shrimp sample (17.84%). In case of intra-variation assay, the % recovery were in the ranges of 91-113 % and 85-119% for AHD in PBS and shrimp samples, respectively. For inter-variation assays, the % recovery of the AHD in PBS buffer and in shrimp samples was found to be 96-112% and 98-112%, respectively. The results showed that the % recoveries of all samples were between 85% and 119%, indicating that the accuracy of this ELISA was in an acceptable range.

The accuracy of the ELISA analysis was also determined by the comparative detection of AHD in fortified shrimp samples at different concentrations using ELISA and LC-MS/MS. It was found that there was a good agreement in the detected concentration in the range of 0.2 – 3.0 ppb obtained by these two techniques. The correlation coefficient value between the two methods was 1.163 with the R^2 of 0.987.

Detection capability (CC_β) of the assay was analyzed using twenty different shrimp blank samples. The matrix-specific threshold values, define as the lowest observed blank sample value, at 0.33 was applied for the selection of positive samples. When blank samples were assayed at the fortified concentration of 0.40 µg/kg, no

overlap between the matrix-specific threshold value and fortified samples was observed. Base on this data, detection capability of the assay was 0.40 ppb for shrimp samples.

5.2 Suggestion

This prototype test kit using MAb from clone number 28/10E/8B/1E with Ab captured competitive indirect ELISA (biotinylated Ab) format were developed. The developed ELISA showed excellent properties in term of sensitivity and accuracy as a screening method for AHD detection at the current enforced level of MRPL.

The further direction should focus on following step, expiry date of test kit and suitable condition for sample collection.

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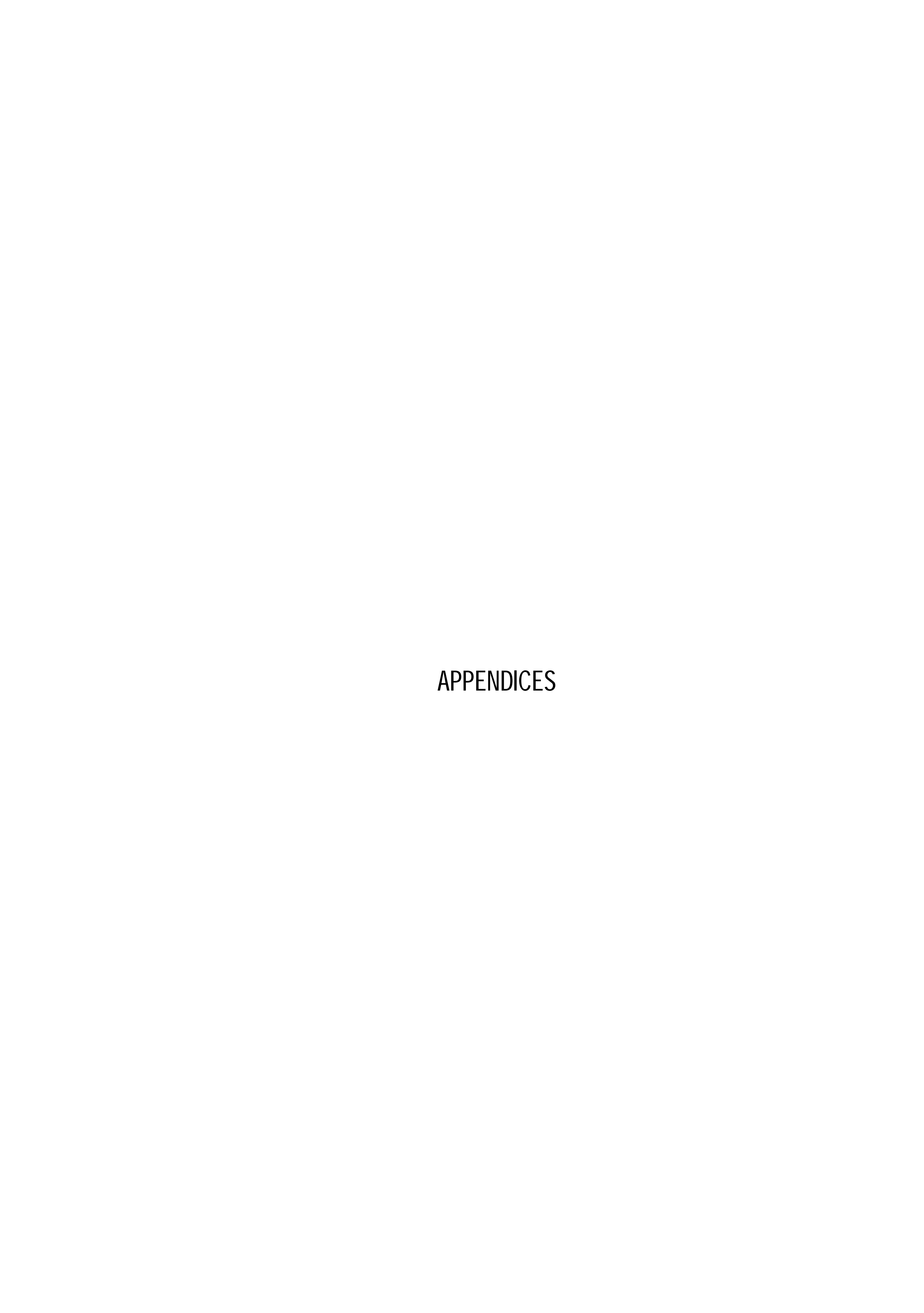


APPENDICES

APPENDIX A

Reagents

1. 2x treatment buffer (10 ml)

1 M Tris-Cl pH 6.8	1.25	ml
10% SDS	4	ml
Glycerol (87%)	2.29	ml
2-mercaptoethanol	1	ml
Distilled water adjusted volume to	10	ml

2. 1 N HCl

HCl (37%)	8.28	ml
Distilled water adjusted volume to	100	ml

3. 1 N NaOH

NaOH	4	g
Distilled water adjusted volume to	100	ml

4. 0.85% NaCl

NaCl	8.5	g
Distilled water adjusted volume to	1000	ml

APPENDIX B

Media and reagents for hybridoma production

1. Stock 100x HT

Hypoxanthine	0.1360 g in 20 ml distilled water
Thymidine	0.0388 g in 20 ml distilled water

Dissolve the solutions above and total volume was adjusted to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 ^{o}C before use.

2. Stock 100x HAT

Hypoxanthine	0.1360 g in 20 ml distilled water
Aminopterin	0.0018 g in 20 ml distilled water
Thymidine	0.0388 g in 20 ml distilled water

Dissolve the solutions above and total volume was adjusted to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 ^{o}C before use.

3. RPMI 1640 medium

RPMI 1640 (Roswell Park Memorial Institute)	10.43	g
$NaHCO_3$	2	g
L-glutamine	0.1	g
Glucose	2	g
Sodium pyruvate	0.11	g
Distrilled water	1000	ml

The solution of penicillin G and streptomycin were added to a final concentration of 100,000 units and 100 mg per litre, respectively. The medium was sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 ^{o}C.

4. HT medium

RPMI 1640 medium	1000	ml
100x HT	10	ml

The medium was mixed well and then sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

5. HAT medium

RPMI 1640 medium	1000	ml
100x HAT	10	ml

The medium was mixed well and then sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

6. 50% (w/v) polyethylene glycol (PEG)

PEG was thawed in a 60 °C water bath. The solution was prepared by dissolving 2 g of PEG in 2 ml of RPMI 1640 medium. The solution was divided into aliquots with the volume of 1 ml and stored at 4 °C. The aliquot of PEG solution was placed in a humidified 5% CO_2 incubator at 37 °C before use.

7. Freezing medium (10% DMSO)

Dimethyl sulfoxide (DMSO)	10	ml
RPMI 1640 medium	90	ml

The medium was stored at 4 °C before use.

APPENDIX C

Buffers and reagents for SDS-PAGE

1. 10% SDS

Sodium dodecyl sulfate (SDS)	10	g
Deionized water adjusted volume to	100	ml

2. 10% APS

Ammonium persulfate (APS)	1	g
Deionized water adjusted volume to	10	ml

3. 1 M Tris-HCl pH 6.8

Trisma base	12.11	g

The small volume of deionized water was added and pH was adjusted with 1 N HCl to 6.8, and then deionized water was added to reach 100 ml final volume.

4. 1.5 M Tris-HCl pH 8.8

Trisma base	18.17	g

The small volume of deionized water was added and pH was adjusted with 1 N HCl to 8.8, and then deionized water was added to reach 100 ml final volume.

5. 2x Laemmli buffer (SDS-dye) (10 ml)

1 M Tris-HCl pH 6.8	1	ml	
10% SDS	4	ml	(4% v/v)
Glycerol (87%)	2.29	ml	(20% v/v)
Bromphenol blue	0.001	g	
HPLC water adjusted volume to	10	ml	

The solution was stored at -20 ^{o}C.

6. SDS staining dye

2x Laemmli buffer (SDS-dye)	900	µl
2-mercaptoethanol	100	µl

The solution was stored at -20 °C.

7. SDS-polyacrylamide gel preparation

7.1 12% separating gel (8 ml)

Sterile water	3.436	ml
40% Acrylamide and Bis-acrylamide solution	2.4	ml
1.5 M Tris-HCl pH 8.8	2	ml
10% SDS	0.08	ml
10% APS	0.08	ml
TEMED	0.004	ml

7.2 5% stacking gel (2 ml)

Sterile water	1.204	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
1 M Tris-HCl pH 6.8	0.504	ml
10% SDS	0.02	ml
10% APS	0.02	ml
TEMED	0.002	ml

8. 5x running buffer for SDS-PAGE

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
Deionized water adjust volume to	1000	ml

For working solution, 1x running buffer was prepared by diluting 100 ml of 5x running buffer to total volume 500 ml using deionized water.

9. Staining and destaining solution

9.1 Staining solution

Coomassie brilliant blue R-250	2.5	g
Methanol	500	ml
Acetic acid	100	ml
Distilled water adjust volume to	1000	ml

The solution was stored in a dark bottle.

10. Destaining solution

10.1Destain I (50% methanol, 10% acetic acid)

Methanol	500	ml
Acetic acid	100	ml
Distilled water adjusted volume to	1000	ml

10.2 Destain II (5% methanol, 7% acetic acid)

Methanol	50	ml
Acetic acid	70	ml
Distilled water adjusted volume to	1000	ml

Procedure of gel staining

After electrophoresis, the separating bands of proteins in the gel were visualized by immersion in staining solution (10.1) for 1-2 h. The gel was then washed in destain I (10.2.1) for 1 h with 1-2 changes and followed by destain II (10.2.2) until the gel was cleared.

APPENDIX D

Buffers and reagents for immunoassay

1. 0.15 M Phosphate buffer saline (PBS), pH 7.4

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Distilled water adjusted volume to	1000	ml

Adjusted pH to 7.4

2. 0.05% Tween 20 in PBS (PBST)

Tween 20	500	μl
PBS	1000	ml

3. 5% skim milk

Skim milk	5	g
PBS	100	ml

4. 5% skim milk in PBST

Skim milk	5	g
PBST	100	ml

5. 0.15 M Phosphate Citrate buffer, pH 5.0

Na_2HPO_4	9.5	g
Citric acid	7.3	g
Distilled water adjusted volume to	1000	ml

Adjusted pH to 5.0 and the buffer was stored at 4 ^{o}C in a dark bottle before use.

APPENDIX E

1. Antibody purification using protein G column

Antibody was partially purified by protein G column chromatography. The amount of protein and antibody were quantified by measuring the absorbance at 280 nm and 450 nm, respectively (Table E1.).

Table E1. Absorbance at 280 and 450 nm of protein purification using protein G column.

Fractions	A_{280}	A_{450}
1	0.180	0.105
2	0.200	-
3	0.195	0.091
4	0.209	-
5	0.208	0.125
6	0.321	-
7	0.831	0.957
8	0.995	1.465
9	0.987	1.057
10	0.968	0.934
11	0.746	0.739
12	0.603	0.717
13	0.428	0.661
14	0.421	0.837
15	0.338	-
16	0.327	0.363
17	0.285	-
18	0.267	0.216
19	0.266	-
20	0.232	0.240
21	0.234	-

2. Determination of protein concentration by BCA™ Protein Assay Kit

The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitative determination of total protein using bovine serum albumin (BSA) as a protein standard.

2.1 Preparation of a standard curve of BSA

BSA stock solution (2 mg/ml) was diluted in PBS to get the final BSA concentration of 0, 0.125, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00 mg/ml (Table E2). Each of concentrations was used as standard for determination of the protein concentration of samples. The protein standard curve was prepared by plotting the 540 nm absorbance value for each BSA standard versus its concentration in mg/ml (Figure E1).

Table E2. Concentration of BSA and the 540 nm absorbance values for protein standard curve.

BSA concentration (mg/ml)	A_{540}
0.000	0.000
0.125	0.157
0.250	0.244
0.500	0.502
0.750	0.755
1.000	0.972
1.500	1.250
2.000	1.732

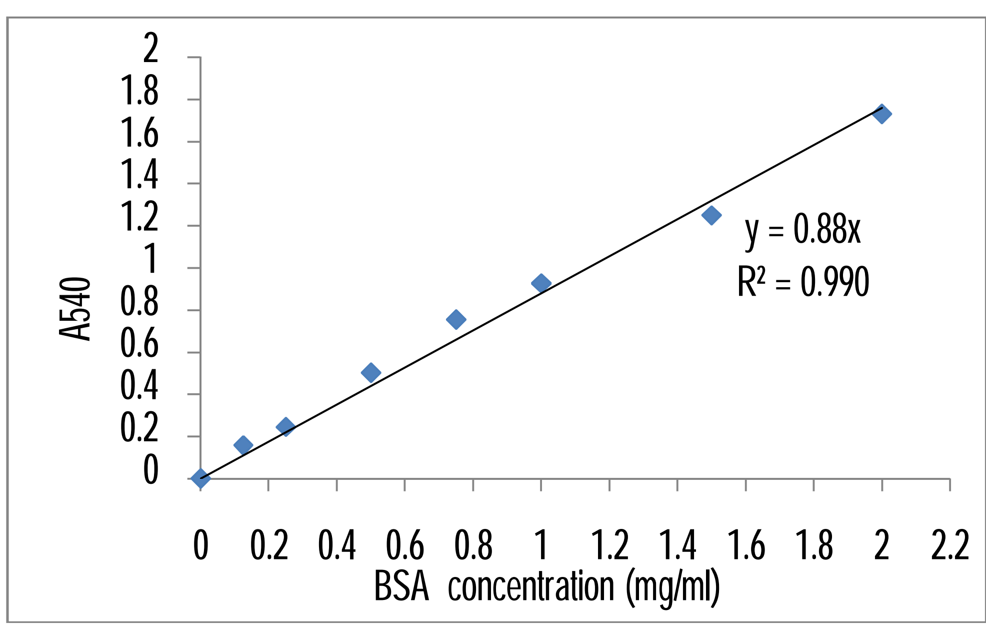


Figure E1. BSA standard curve by BCA™ Protein Assay Kit.

3. Molecular weight determination

The molecular weight of an unknown protein could be determined directly from a semilog graph of the molecular weight of protein marker versus their mobility (Table E3 and Figure E2). The relative mobility (Rf) of the protein was calculated according to the following formula:

$$\text{Relative mobility (Rf)} = \frac{\text{Distance of the protein migrate}}{\text{Distance of the tracking dye migrate}}$$

Table E3. Molecular weight of protein markers and their relative mobility.

Marker	Molecular weight (kDa)	Distance (cm)	Retention mobility or Rf
β-galactosidase (*E. coli*)	118	1.27	0.09
BSA (Bovine plasma)	90	2.96	0.20
OVA (Chicken egg white)	50	6.14	0.42
Carbonic anhydrase (Bovine erythrocytes)	34	9.95	0.68
β-lactoglobulin (Bovine milk)	26	12.70	0.87
Lysozyme (Chicken egg white)	19	14.61	1.00

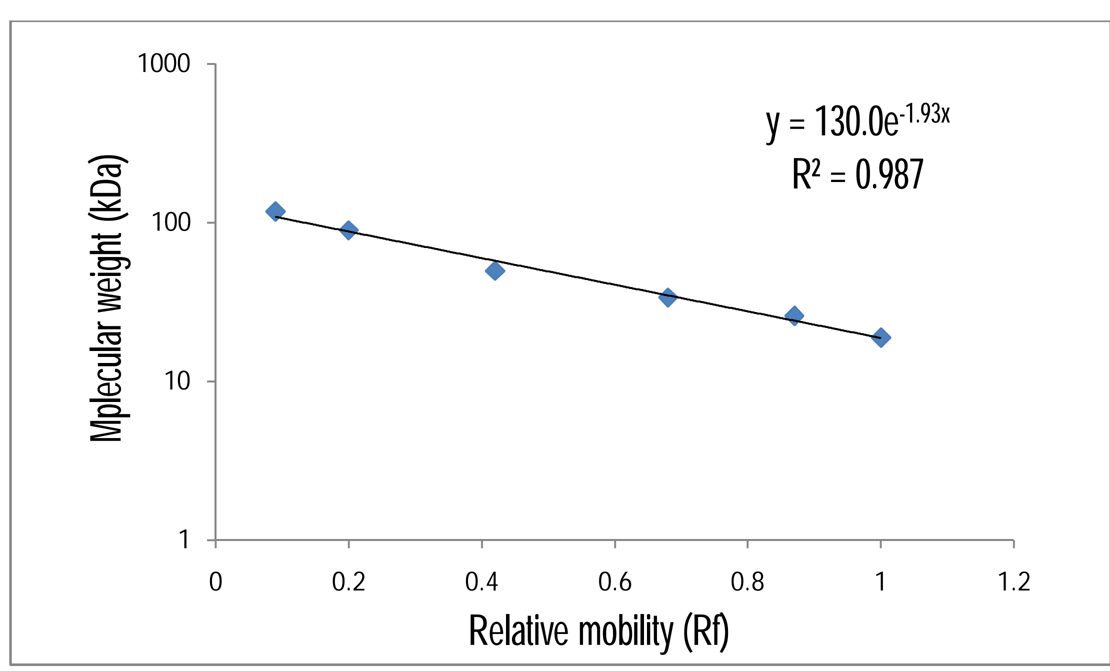


Figure E2. Standard curve of protein marker separated by SDS-PAGE.

APPENDIX F

AHD detection in shrimp samples

1. Standard curve for fortified shrimp sample

AHD stock solution (2 mg/ml) was diluted in PBS to get the final AHD concentration of 0, 0.125, 0.50, 0.750, 2.50, 4.00 and 6.50 mg/ml (Table F1). Each of concentrations was used as standard for determination of the AHD concentration of samples using antibody captured competitive indirect ELISA (biotinylated antibody) format. The AHD standard curve was prepared by plotting % B/B_0 from 450 nm absorbance value for each AHD standard versus its concentration in µg/ml (Figure F1).

Table F1. Concentration of AHD in assay buffer and % B/B_0 for standard calibration curve.

AHD concentration (µg/ml)	%B/B_0
0.000	100
0.125	86.98
0.500	74.74
0.750	62.69
2.500	47.78
4.000	31.23
6.500	16.74

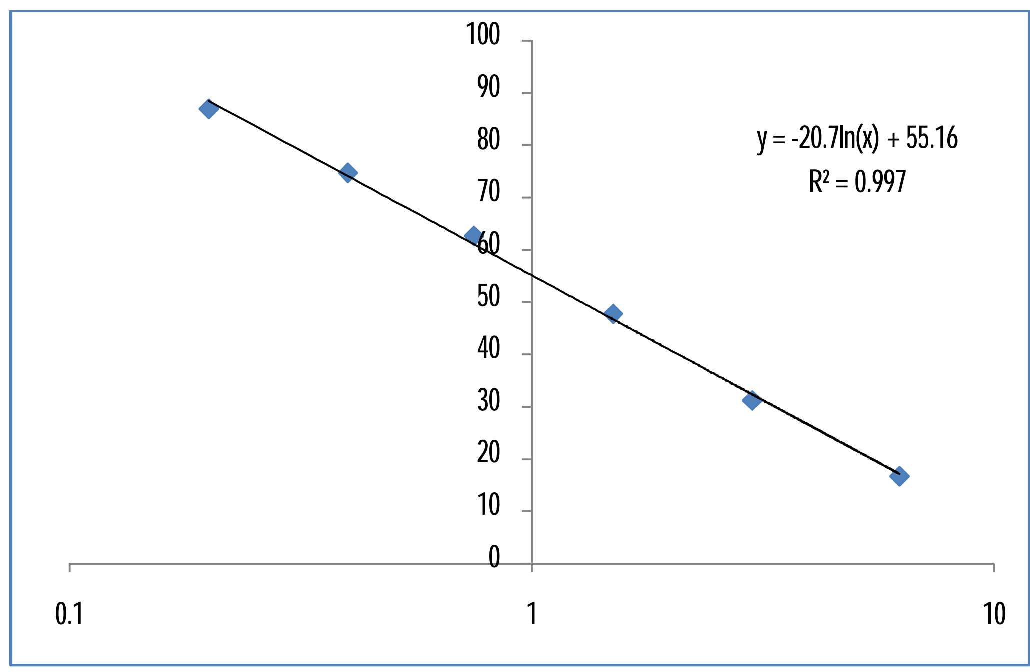
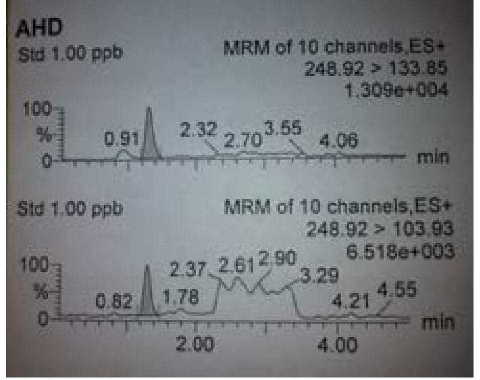


Figure F1. AHD standard calibration curve in assay buffer.

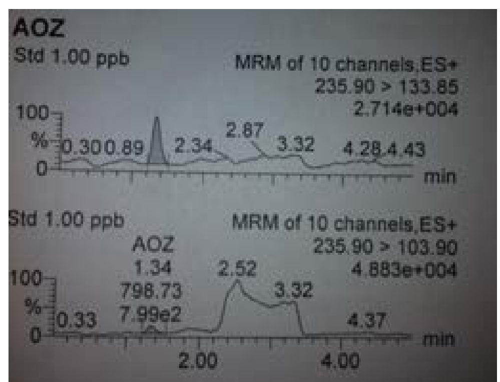
2. AHD detection by LC-MS/MS method

2.1 Standard of nitrofurans metabolites (ppb) by LC-MS/MS method

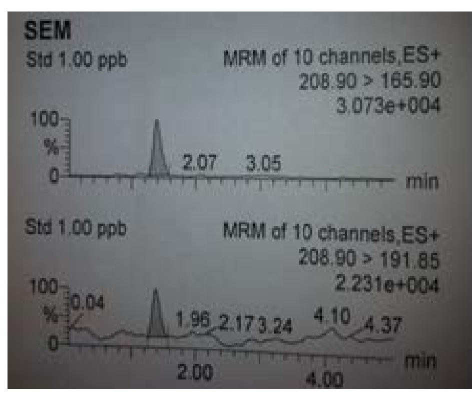
(A) Standard AHD



(B) Standard AOZ



(C) Standard SEM



(D) Standard AMOZ

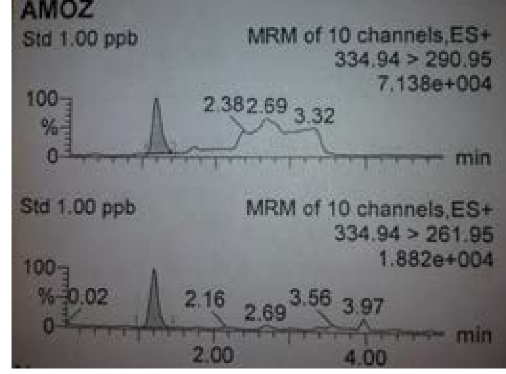
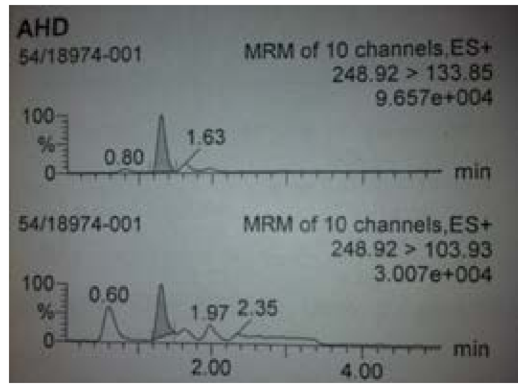


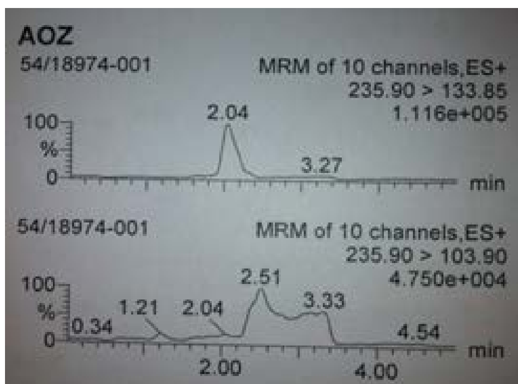
Figure F2. Chromatogram of AHD standard by LC-MS/MS method.

2.2 Shrimp sample was fortified with 3 ppb of AHD.

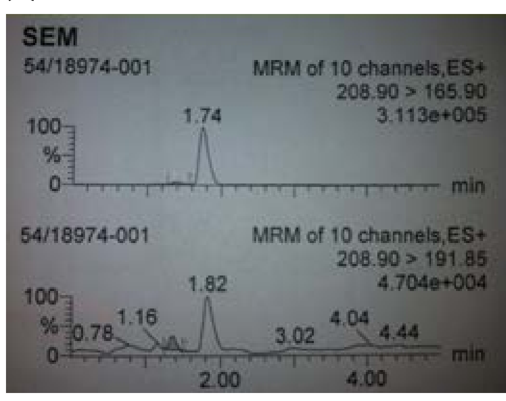
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

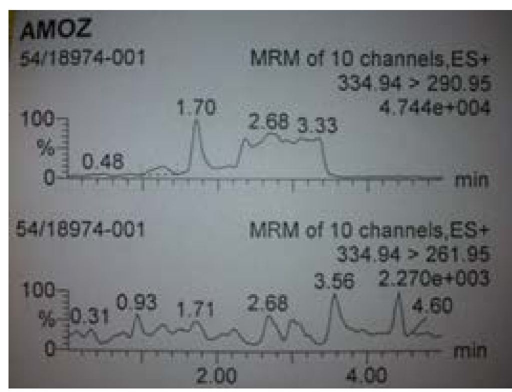
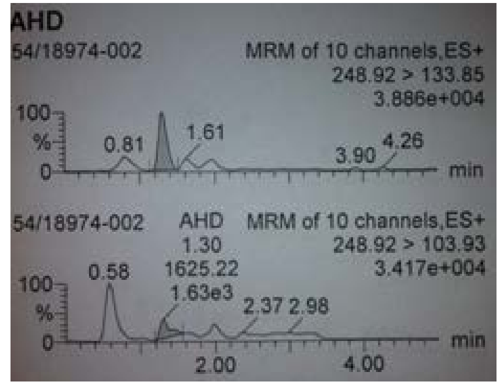


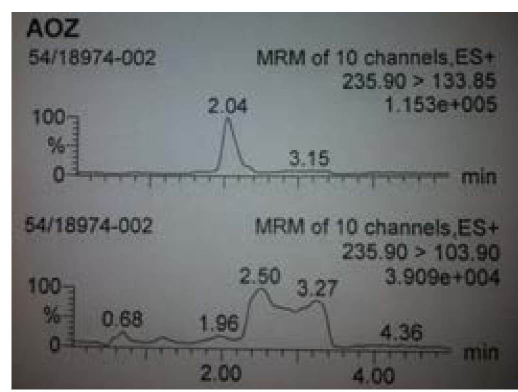
Figure F3. Chromatogram of 3 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

2.3 Shrimp sample was fortified with 1 ppb of AHD.

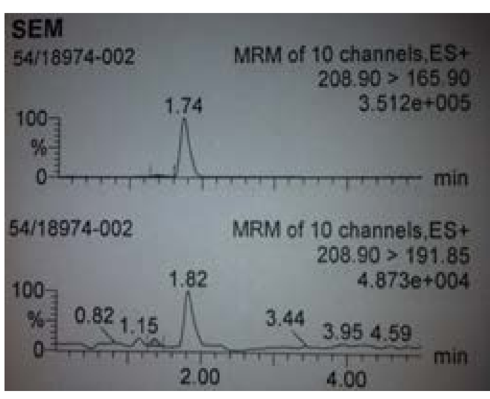
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

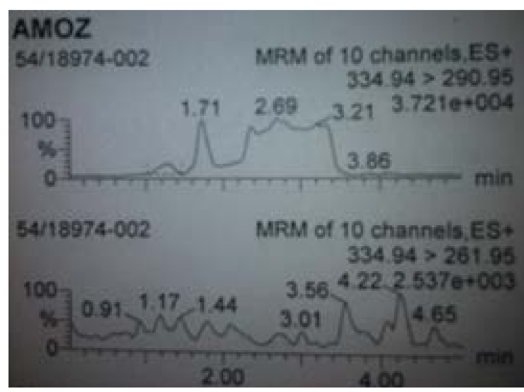
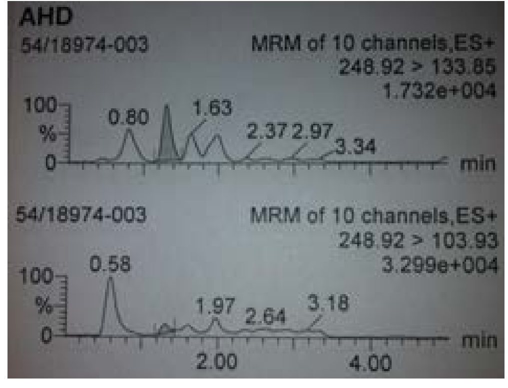


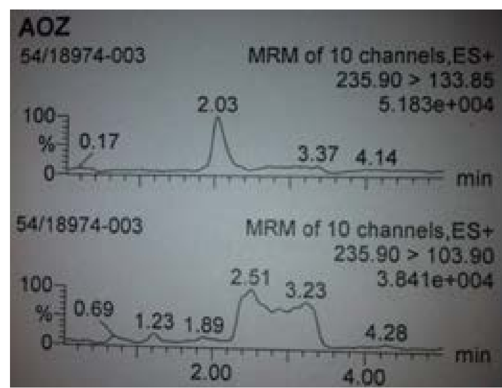
Figure F4. Chromatogram of 1 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

2.4 Shrimp sample was fortified with 0.5 ppb of AHD.

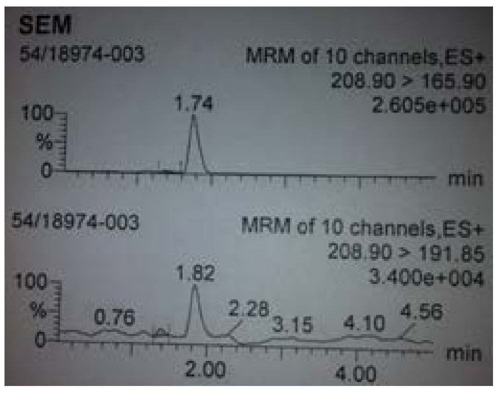
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

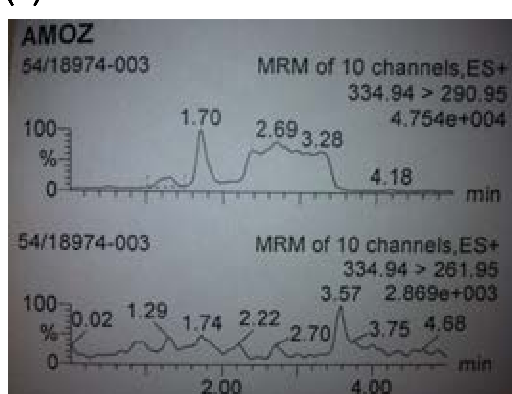
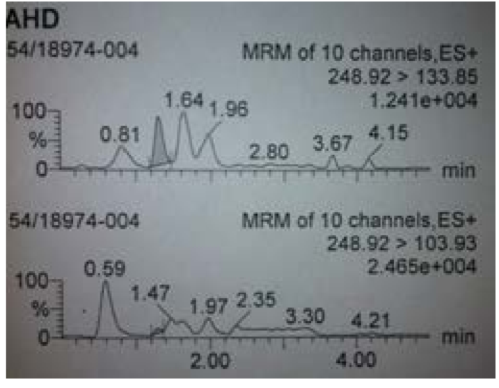


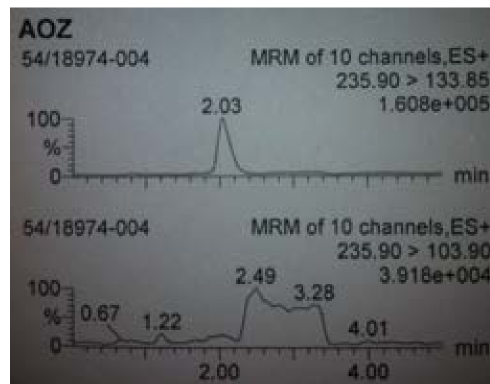
Figure F5. Chromatogram of 0.5 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

2.5 Shrimp sample was fortified with 0.4 ppb of AHD.

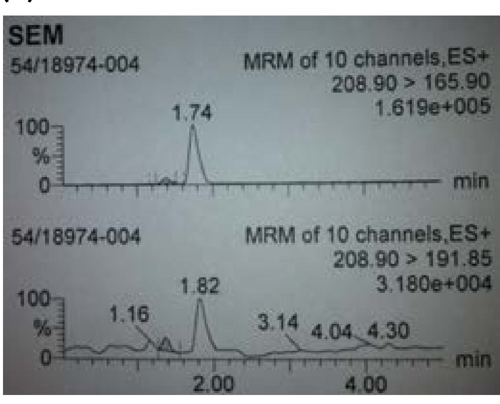
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

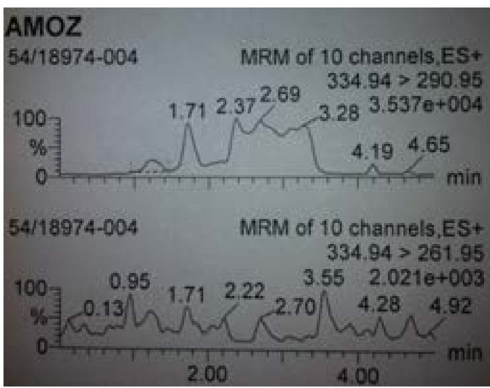
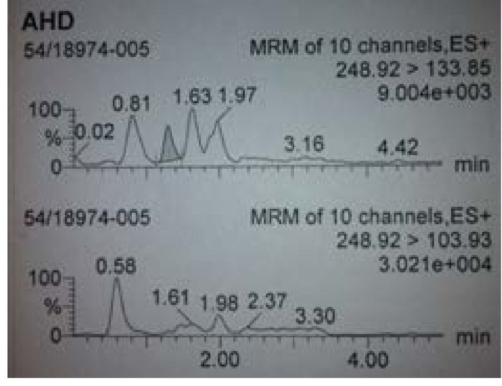


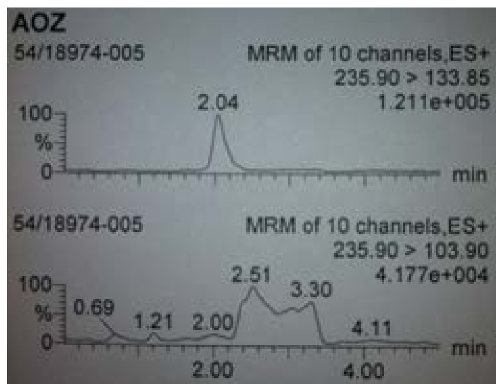
Figure F6. Chromatogram of 0.4 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

2.6 Shrimp sample was fortified with 0.3 ppb of AHD.

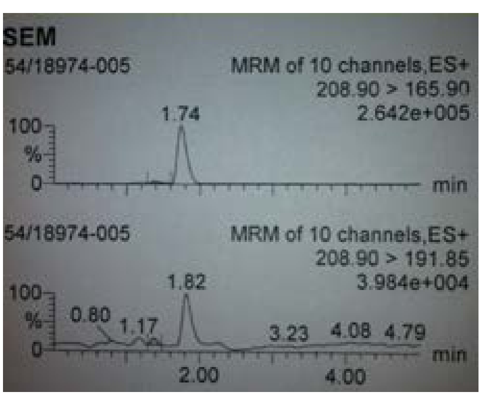
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

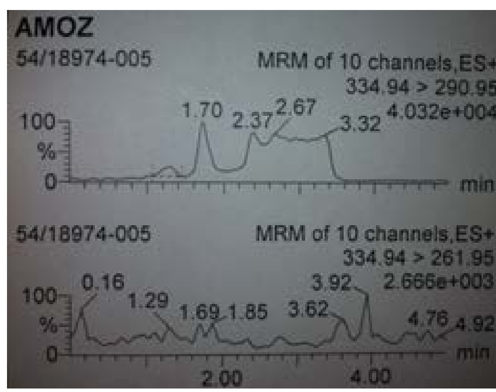
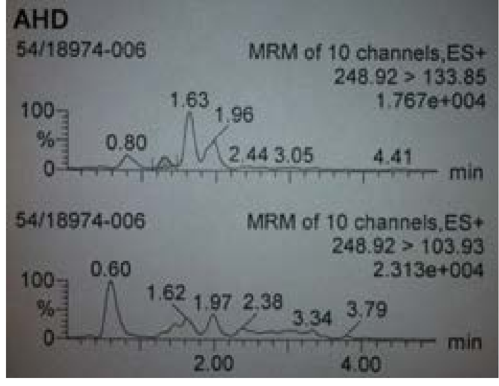


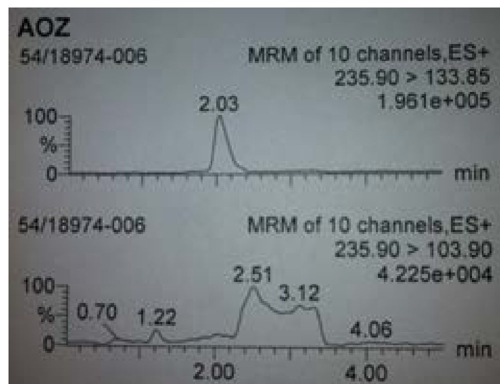
Figure F7. Chromatogram of 0.3 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

2.7 Shrimp sample was fortified with 0.2 ppb of AHD.

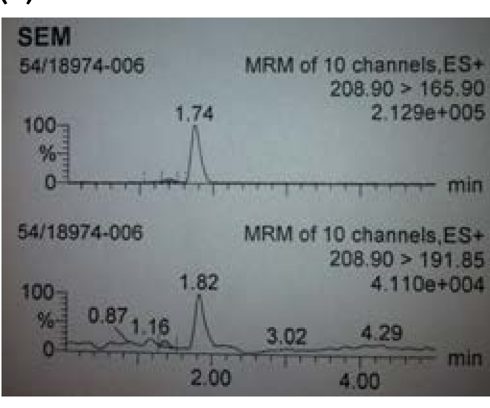
(A) AHD



(B) AOZ



(C) SEM



(D) AMOZ

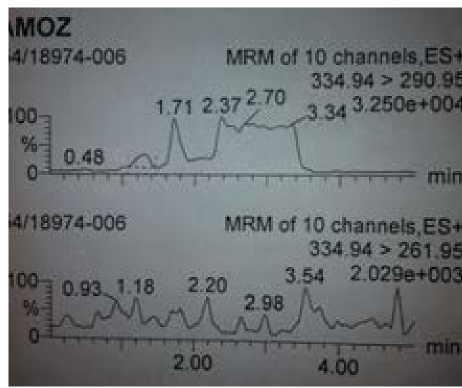


Figure F8. Chromatogram of 0.2 ppb of AHD fortified in shrimp samples by LC-MS/MS method.

3. Detection capability (CC_{β}) of antibody captured competitive indirect ELISA (biotinylated antibody) format.

Table F2. %B/B_0 and AHD concentration using Ab captured competitive indirect ELISA (biotinylated Ab) format in 20 shrimp samples.

Shrimp samples	Without fortified AHD		With fortified 0.4 μg/kg of AHD	
	B/B_0	AHD Conc* (μg/kg)	B/B_0	AHD Conc* (μg/kg)
1	103.99	0.095	73.24	0.470
2	102.19	0.103	78.07	0.331
3	102.70	0.101	73.25	0.417
4	100.96	0.109	79.36	0.311
5	118.71	0.046	72.54	0.432
6	110.87	0.068	74.53	0.392
7	92.47	0.165	65.02	0.621
8	96.01	0.139	68.62	0.522
9	91.13	0.176	65.66	0.602
10	91.06	0.176	65.27	0.614
11	94.08	0.152	64.37	0.641
12	91.45	0.173	64.37	0.641
13	85.72	0.228	67.58	0.548
14	85.40	0.232	66.69	0.573
15	85.53	0.231	71.64	0.451
16	84.89	0.238	69.96	0.489
17	84.37	0.244	69.58	0.498
18	84.56	0.241	68.75	0.519
19	103.99	0.094	73.25	0.417
20	102.19	0.103	78.07	0.331

*Conc. = Concentration

BIOGRAPHY

Miss Umarphorn Chadseesuwan was born in Petchaboon, Thailand on November 6, 1980. She received bachelor's from the Department of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, in 2003. She completed her master in the same field and university in the Master in 2007. Present, she subsequently enrolled in the Doctoral's degree of Program in Biotechnology, Faculty of Science at Chulalongkorn University in 2007.

Academic presentation:

Chadseesuwan, U., Puthong, S., Gajanandana, O., Palaga, T., and Komolpis, K. (2011). PRODUCTION OF MONOCLONOL ANTIBODIES AGAINST 1-AMINOHYDANTOIN. Proceedings of 2011 International Conference on Asia Agriculture and Animal (ICAAA 2011), Volume 13 p.16-20. Hong Kong, China. July 2-3, 2011. (Oral presentation) (Full text in CD-ROM)

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