INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

University of Alberta

Development of bispecific monoclonal antibodies and their applications in ultrasensitive immunoassays

by

Danzhu Xu 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Fall 1998

University of Alberta

Library Release Form

Name of Author:	Danzhu Xu
Title of Thesis:	Development of bispecific monoclonal antibodies and their applications in ultrasensitive immunoassays
Degree:	Master of Science
Year This Degree Granted:	1998

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

1451 Jeffreys crescent
Edmonton, Alberta
T6L 6T3

Date: June 1, 98

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Development of bispecific monoclonal antibodies and their applications in ultrasensitive immunoassays by Danzhu Xu in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

Dr. M.R. SURESH (Supervisor)

Dr. J. SAMUEL (Chair)

Dr. J. MERCER

Dr. J. SIM

June 1st 98

Abstract

Quadromas producing bispecific antibody (bsMAb) anti-PSA x anti-AP and bsMAb anti-CA125 x anti-AP were developed by a combination of electrofusion and FACS selection. These bsMAbs were purified as immune complexes with alkaline phosphatase (AP) by using a unique method exploiting mimetic affinity chromatography. The immune complexes were efficiently purified under very mild elution conditions minimizing damage to either AP or antibody activity.

BsMAb-AP immune complex based enzyme immunoassays were developed for the detection of PSA and CA125 respectively. In these two tumor marker EIA's the use of AP allowed for the development of protype assays with fast kinetics (conventional assay) and ultrasensitive detection (amplified assay) capabilities. The conventional immunoassays could be utilized in routine clinical use in monitoring prostate and ovarian cancer patients. The ultrasensitive assay could have applications in screening or diagnostics, particularly in detection of very early recurrence of metastasis in patients who have undergone radical prostatectomy or ovaretomy and hence would have virtually no PSA or CA125 in their serum. Ultrasensitive assays would allow the clinician to diagnose disease recurrence early at the micrometastatic stage to take appropriate intervention.

DEDICATED

to

My lovely parents, Xiang Xu and Jin Zhou; my husband Dongxu Qiu and my daughters Tracy and Mary. Without their love and support, this thesis will never be finished.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. M. R. Suresh for his supervision and support; His encouragement during my difficult periods and his constructive suggestions regarding my study have been invaluable. I also like to thank Mr. John E. Yule for his enthusiastic help in revising my thesis. I also like to thank my colleges F.T. Kreutz, Y. Cao and F.Lui for their suggestions in my studies and experiments.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

A280nm	Absorbance at 280 nm
ABTS	2 2'-Azino-di [3-ethyl-benzthiazoline sulfonate]
AC	Alternating current
ACT	α 1-antichymotrypsin
ADH	Alcohol dehydrogenase
AP	Alkaline phosphatase
BDH	Benign prostate hyperplasia
BSA	Bovine serum albumin
BsMAb/bsMAb	Bispecific monoclonal antibody
CA125	Cancer antigen 125
CEA	Carcinoembryonic antigen
C_H	Immunoglobulin heavy chain constant domain
C_L	Immunoglobulin light chain constant domain
CV	Coefficient of variation
Da	Daltons, unit of molecular weight
DC	Direct current
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
IFA	Immunofluometric assay
FBS	Fetal bovine serum
FACS	Fluorescent activated cell sorter
FITC	Fluorescein isothiocyante
HAMA	Human anti-mouse antibody
HAT	Hypoxanthine, aminopterin and thymidine
HRPO	Horse radish peroxidase
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

i.p.	Intra-peritoneal
IRMA	Immunoradiometric assay
LLD	Lowest limit of detection
MAb	Monoclonal antibody
4-MU	4-Methylumbelliferone
4-MUP	4-Methylumbelliferyl phosphate
MW	Molecular weight
NAD	Nicotinamide adenine dinuclotide
NADP	Nicotinamide adenine dinuclotide phosphate
OD	Optical density
OPI	Oxaloacetate, pyruvate, and insulin
OVA	Ovalbumin, hen egg albumin
P92	Hybridoma and monoclonal antibody against alkaline phosphatase
P104	Quadroma and bsMab anti-CA125 x anti-AP
P105	Quadroma and bsMab anti-PSA x anti-AP
PBS	Phosphate-buffered saline pH 7.4
PBST	0.05% Tween 20 in PBS
PEG	Polyethylene glycol
pNPP	p-Nitrophenyl phosphate
PSA	Prostate specific antigen
S-S	Disulfide bond
SD	Standard deviation
SDS-PAGE,	Sodium dodecylsulphate polyacrylamide gel electrophoresis;
TAA	Tumor associated antigen
TRITC	Tetramethylrhodamine isothiocyanate
TSH	Thyroid-stimulating hormone
U	Units
v/v	Volume by volume
V_H	Immunoglobulin heavy chain variable domain
V_L	Immunoglobulin light chain variable domain

CHAPTER 1. INTRODUCTION

1.1. Development of bispecific antibody

In 1906, Paul Ehrlich suggested that molecules with an affinity for certain tissue might be able to serve as carriers of cytotoxic agents to concentrate them on the appropriate target cells *in vivo* (Ehrlich, 1906). Since then immunologists have tried to utilize immunoglobulins as a tool in immunotherapy and immunodiagnosis. Polyclonal antibodies were mostly used at that time and were produced by immunizing the host animals with a desired antigen. However, each batch of polyclonal antibody had to be defined for specificity and affinity due to host variations, and sometimes showed poor specificity and low sensitivity in immunoassays.

In 1975, a landmark discovery was made by G. Kohler and C. Milstein (1975) in generating continuous *in vitro* cultures of fused lymphocytes secreting antibodies of predetermined specificity. The development of monoclonal antibodies (MAb) marked a new era in the diagnosis and treatment of cancer and other immune system related diseases. Today, MAbs conjugated with marker compounds such as radioisotopes, enzymes, and fluorescent molecules are mainly used as immunoprobes in immunoassays (Gosling, 1996; Christopoulos and Diamandis, 1996). In addition, toxins, drugs or isotopes are conjugated to MAbs for targeted immunotherapy (Kosmas et al., 1993; Pietersz and Krauer, 1994). Direct labeling of antibodies to marker or effector agents by chemical conjugation has some major disadvantages. Chemical conjugation could inactivate antibody binding sites as well as cause crucial alterations in the effector agents thus decreasing the efficiency of the immunoconjugates used in immunoassays and immunotherapy (Ishikawa, 1996). Various alternative approaches to direct labeling have been developed, such as the unlabelled peroxidase/anti-peroxidase antibody (Sternberger et al., 1970), bispecific antibodies (Milstein, C. and Cuello, C. 1983), and chimeric antibodies of double specificity (Songsivilai et al., 1989). Bispecific antibodies (bsMAbs) are unique tools which could be used in immunohistochemistry, immunoassays and immunotherapy, because there is no chemical manipulation involved in the interaction

between a bsMAb and the two distinct antigens. The idea of using bsMAbs was first proposed by Nisonoff and Rivers in the early 1960s, but its application was limited since only polyclonal antibodies were available at that time. The development of bsMAbs was based on the concept that a bsMAb capable of binding two different antigens simultaneously might have more efficient and specific cell killing abilities than antibodies chemically linked to effector reagents such as cytotoxic drugs. In 1981, the first bsMAb generated by fusion of two hybridomas was reported by Reading (Reading, 1981). Since then, extensive studies have determined that bsMAbs have more potential applications in immunohistochemistry, immunodiagnosis and immunotherapy ((Milstein and Cuello, 1983; Suresh et al., 1986a; Nolan and O'Kennedy, 1990; Fanger, 1992).

1.1.1. Antibody structure and function

Antibodies are host proteins produced in response to the presence of foreign molecules in the body. Antibodies (or Immunoglobulins) are the major secretary product of B cells and the major component of the system of humoral immunity. Antibodies are a large family of glycoproteins that share key structural and functional features.

The basic structural unit of all classes of immunoglobulins is a symmetric four-chain heterodimer consisting of two identical heavy chains (MW ≈50,000 Da each) and two identical light chains (MW≈25,000 Da each) which form the characteristic Y-shape molecule. Both L and H chains contain constant and variable regions. In the constant regions, amino acid sequences show very little variation among immuglobulins. In contrast, sequences differ in the variable regions among different antibodies determine the specificity and affinity towards the antigen. Hypervariable sites in the variable region show high degree of sequence differences.

Antibodies are divided into five classes, IgG, IgM, IgA, IgE, and IgD, based on the number of Y-like units and the type of heavy chains they have. Monoclonal antibodies (MAbs) are immunoglobulins of a single species derived from cloned B lymphocytes, its fused derivatives or transformants (Suresh et al., 1991). The most common type of monoclonal antibody is the monospecific bivalent form belonging to the IgG class (Fig. 1.1). They are mostly used in immunoassays and immunotherapy. The

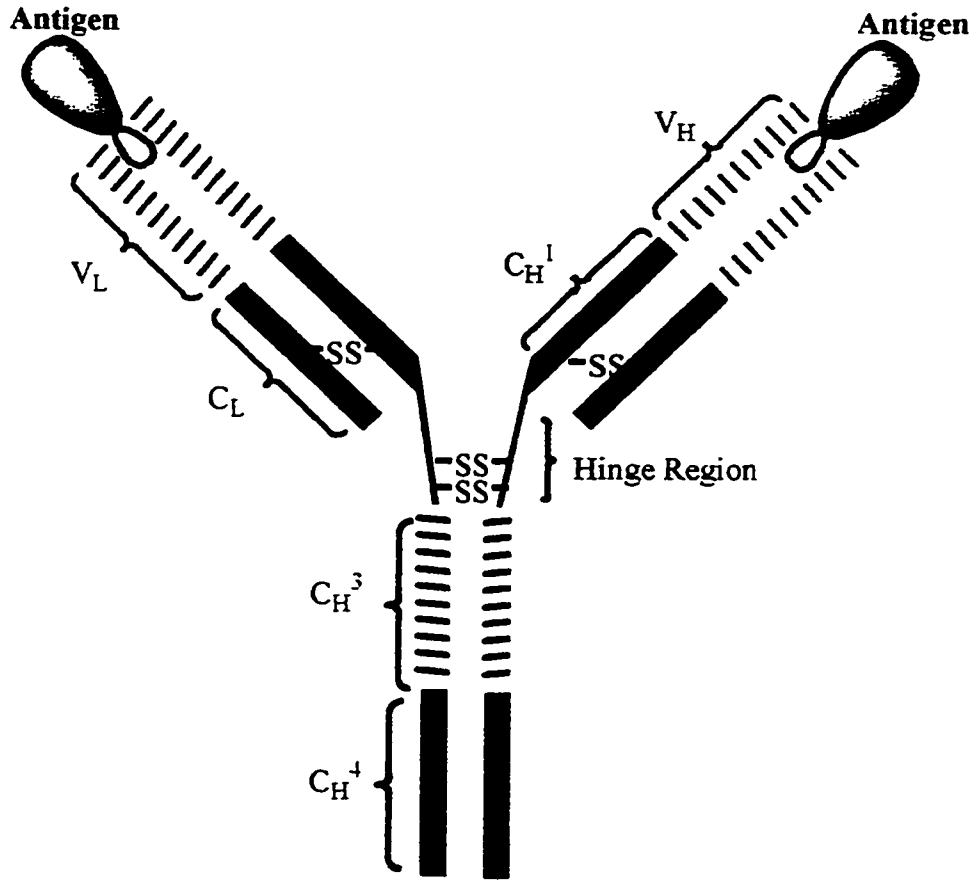


Figure 1.1 Monospecific monoclonal antibody. The regions shown are; C_H=constant region of heavy chain, C_L=constant region of light chain, V_H=variable region of heavy chain, V_L=variable region of light chain.

most important features of antibody structure for *in vitro* assays are binding specificity and affinity.

Antibodies can be prepared having a desired structure and specificity. Polyclonal antibodies, mono/bi-specific monoclonal antibodies, chimeric antibodies with human Fc region and single chain mono/bi-specific antibodies may be obtained using a variety of techniques.

1.1.2. Production of bispecific antibodies

BsMAbs incorporate the binding specificity of two different antibodies into a single molecule (Fig. 1.2). They do not occur naturally but can be created by three different methods:

1) The chemical cross-linking of two MAbs with distinct specificities (Cook and Wood, 1994).

2) Hybrid hybridomas, which are established by somatic fusion of two hybridomas secreting the desired antibodies (Suresh et al., 1986a).

3) Molecular engineering (Mack et al., 1995; Colombia and Morrison, 1997).

Studies have indicated that both the specificity and affinities of the respective paratopes in bsMAbs are similar to those of the parental MAbs (Nolan and O'Kennedy, 1990).

1.1.2.1. Chemical production

Chemical linking can be achieved in two ways:

(1) A direct coupling of the whole antibody molecule of two desired specificities or their Fab fragments (Glennie et al., 1987; Cook and Wood, 1994).

(2) Dissociation and reassociation of heterologous immunoglobulins.

The chemical production of bsMAbs has several advantages. First, this method does not require cell fusion. In addition, the desired bsMAbs are relatively easy to prepare in high yield (Cook and Wood, 1994) and the products are easier to purify (Glennie et al., 1987). Finally they can be used for most *in vitro* applications. However, because of their

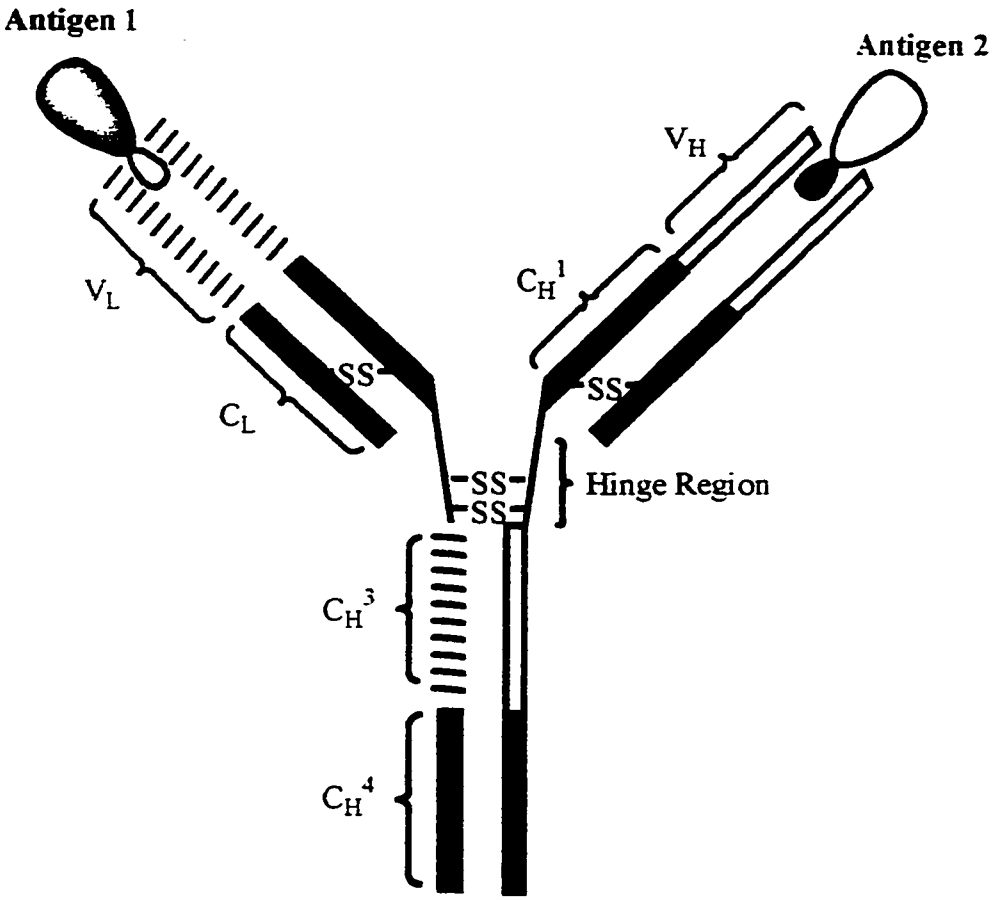


Figure 1.2 Bispecific monoclonal antibody. The regions shown are; C_H=constant region of heavy chain, C_L=constant region of light chain, V_H=variable region of heavy chain, V_L=variable region of light chain.

heterogeneity in size and chemical composition, bsMAbs produced by the chemical method have different physical and biological characteristics from native immunoglobulin molecules and so they are less suitable for animal or human studies.

1.1.2.2. Biological production

BsMAbs produced by hybrid hybridomas are generated by heterologous assembly of two different heavy chain species of both parental antibodies resulting in an antibody with two different antigen binding specificities in one molecule (Suresh et al., 1986a). The biological production of bsMAb involves three major procedures, including fusion of two hybridomas producing different antibodies; selection of quadromas secreting bsMAb from parental hybridomas and purification of bsMAb free from other unwanted antibody species, such as MAbs. The selection of the hybrid hybridomas secreting bsMAb from unfused parental cells is a crucial step. Since both parental hybridomas are resistant to HAT media, which contains hypoxanthine, aminopterin and thymidine, additional selective markers have to be introduced (Staerz and Bevan, 1986; Chervonsky, 1988). There are several methods to mark the parental hybridomas. The traditional method is to backselect one of the hybridomas for HAT sensitivity prior to further fusion. This is carried out by growing the cells in increasing concentrations of 8-azaguanine in RPMI-1640 standard media. The HAT sensitive hybridoma is then further modified for resistance to cytotoxic drugs such as ouabain. Thus, when a HAT-sensitive-ouabain-resistant hybridoma is fused with a HAT-resistant-oubain-sensitive hybridoma (wild-type), only the hybrid hybridoma will survive in the media. However, the selection procedure is time-consuming and sometimes the hybridoma might stop producing MAb.

A simpler method was developed in which a fluorescent marker was used to label hybridoma cells (Stratieva-taneeva et al., 1993). Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are two dyes used for labeling hybridomas. Differentially labeled hybridomas are then fused and the cells containing both fluorochromes are isolated using a fluorescence-activated cell sorter (FACS). A single-cell deposition system equipped with FACS could seed a single cell with double dyes into a 96 well cell culture plate.

The main advantage of developing bsMAbs by fusing two different MAb producing hybridomas over the chemical synthesis and genetic manipulation methods is that the bsMAb are synthesized, associated and secreted by the quadroma as in the case of natural immunoglobulins. Thus the structure and properties of bsMAbs *in vivo* and *in vitro* are similar to that of normal monospecific antibodies. Once the quadroma cell line is established, the secretion of bsMAb is just as the normal hybridoma (Songsivilai and Lacman, 1990). The affinity, isotype and subtype of bsMAbs produced by quadroma can be predicted from the properties of the parental MAbs secreted by hybridomas. However, there are also some disadvantages associated with biological production of bsMAb such as a lower yield of bsMAbs and difficulty with the purification of bsMAbs.

1.1.2.3. Genetic manipulation

The current clinical use of bsMAbs in immunotherapy is primarily as murine antibodies, which will face human anti-mouse antibody response eventually resulting in the formation of immune complexes and rapid clearance of the bsMAb from the circulation. This limits bsMAb application especially when repeated injections are required. These human anti-murine IgGs in a patients' sample interferes with the immunoassays in which murine immunoglobulins are employed. There is also difficulty in producing bsMAb in sufficient quantity and quality required for clinical use. To address these problems, a chimeric bsMAbs developed by a genetic method (Songsivilai et al., 1989). Recently, tetravalent bispecific antibodies with the human IgG Fc region (Coloma and Morrison, 1997) were generated by fusing the DNA encoding a single chain antibody after the C terminus or after the hinge with an antibody of different specificity. The advantages of this approach are:

(1) The HAMA response is reduced.

(2) It yields a homogenous product with all antibodies containing four binding sites which will increase the avidity and specificity.

(3) It is able to produce a large quantity of bsMAbs or even construct multispecific antibodies with desired specificities.

Drawbacks with engineering of bsMAbs could include lower affinity and less efficient *in vivo* targeting to antigens, which needs to be overcome in the future.

1.2. Purification of bispecific monoclonal antibodies

1.2.1. General purification methods

Since the first reports of hybrid-hybridomas producing bifunctional antibodies (Milstein and Cuello, 1983), increased attention has focused on their potential applications in immunodiagnostics (Suresh et al., 1986a; Tada et al., 1989; Auriol et al., 1994) and immunotherapy (Katayose et al., 1996; Guo et al., 1996). These hybrid-hybridomas, with antibody genes inherited from both fusion partners, secrete not only the bsMAb but also antibodies with the monospecific binding capabilities of both fusion parents (Milstein and Cuello, 1984). Random recombination of the heavy and light chains synthesized by a quadroma could lead to ten possible immunoglobulin forms (Milstein and Cuello, 1984). Purification of the desired bsMAb, free from both parental monoclonal antibodies, is desirable for applications in immunoassays and immunotherapeutic studies, since these MAbs will compete with bsMAb for the antigen binding sites. There is no standard method for purification of bsMAb. Methods generally used to purify bsMAbs include ion exchange chromatography (Suresh et al 1986a; Allard et al., 1992) and affinity chromatography on an antigen immobilized column (Kuppen et al., 1993). Ion exchange chromatography lacks specificity and can not remove all MAbs. Double antigen affinity chromatography is based on antibody interaction sequentially with the two antigens which is immobilized on the two different columns. In order to get rid of both parental MAbs, the sample containing the bsMAbs must be passed through both the antigen affinity columns. The antibodies bound on the column are removed with an elution buffer at low pH (2.8-4). Because bsMAbs in the sample must be exposed to low pH twice, they could be easily be denatured. Obviously, there is a need develop simpler and more economical methods to purify bsMAbs for a variety of purposes.

1.2.2. Dye ligand affinity chromatography

Peptide and non-peptide 'biospecific' or 'pseudospecific' mimetic ligands are becoming increasingly popular in the downstream processing of biotech drugs. Dye-

ligand affinity chromatography is used in the isolation and purification of various enzymes (Linder et al, 1989) and non-enzyme proteins (Miribel et al., 1988). These immobilized dyes selectively bind to target proteins from different origins (Miribel et al., 1988). One such target protein, alkaline phosphatase (AP), is bound by MIMETIC blue A6XL matrix in Tricine buffer and subsequently eluted by a phosphate buffer under mild conditions (Fig. 1.3). MIMETIC blue A6XL absorbent was originally used to purify alkaline phosphatase from calf intestinal mucosa extracts (Linder et al, 1989). Since the mimetic affinity absorbents show specific binding to AP, there is potential to employ these absorbents for the purification of a bsMAb/AP immune complex, which could be directly used as an immune probe in a tumor marker immunoassay. There will be no risk of damage to the activity of either AP or bsMAb. In comparing the interactions between AP and bsMAb to AP and absorbents, the antigen antibody interaction is more specific and therefore it is possible to obtain the desired bsMAb/AP immune complex if an excess amount of AP is used.

1.3. Bispecific antibody based two site (sandwich) immunoassay

Immunoassays are based on the observation that in a system containing the analyte and its specific antibody, the distribution of the analyte between the bound and free form is quantitatively related to the total analyte concentration. Most immunoassay configurations can be divided into two large groups:

(1) Competitive immunoassays with limited reagents.

(2) Noncompetitive immunoassays with excess reagents.

In a competitive immunoassay (Fig. 1.4), the analyte and the labeled analyte (tracer) are mixed with a limited amount of anti-analyte antibody. After incubation for a certain period, the bound or the free fraction of tracer is measured and related to the concentration of the analyte in the sample. In noncompetitive immunoassays (Fig. 1.5b), an excess of immunoreactant (antibody or antigen) is added, so that all the analyte is essentially in the form of an immunocomplex. The immunocomplex is then quantified and related to the analyte concentration in the sample (Christopoulost and Diamandis, 1996).

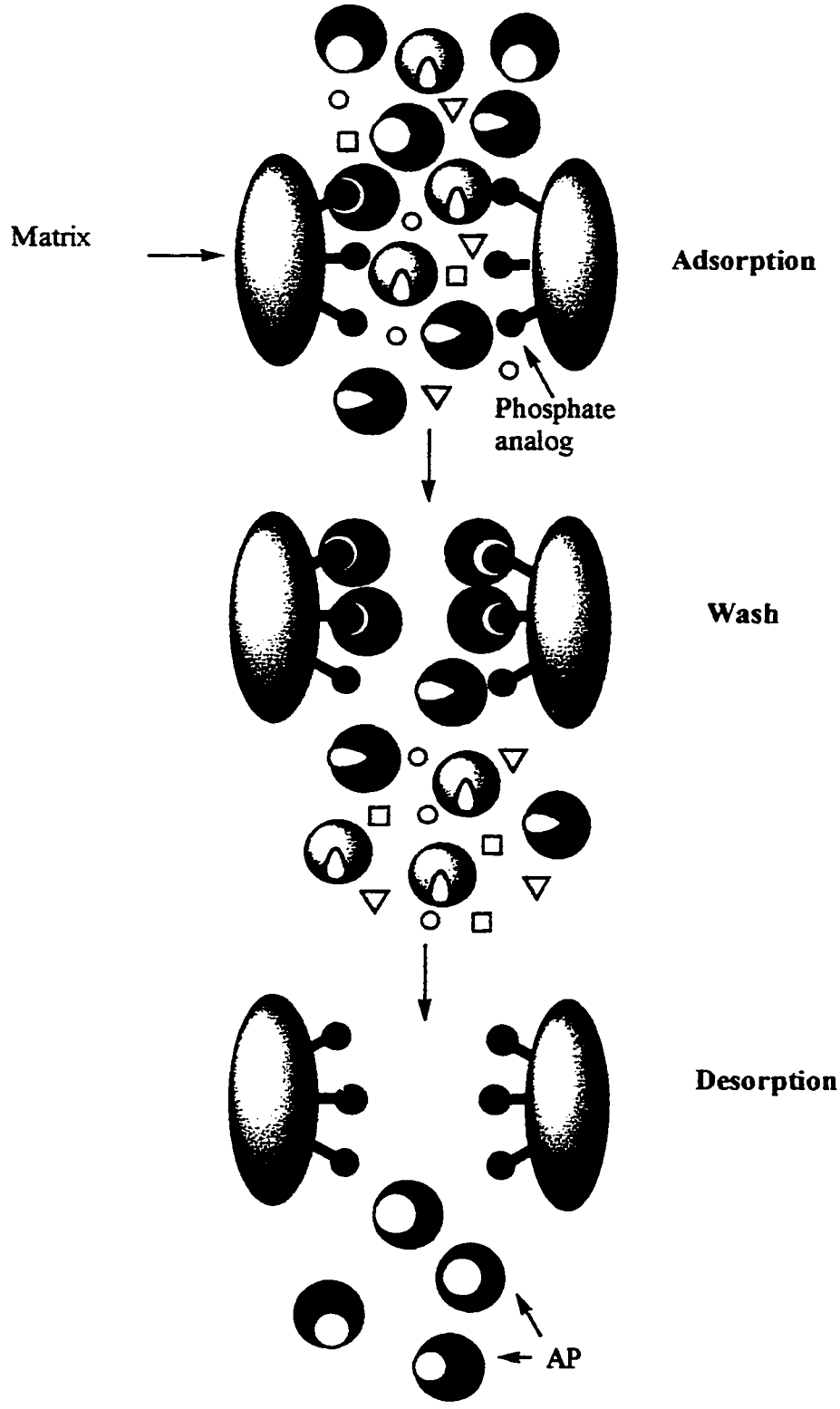


Fig. 1.3 Purification of AP using mimetic ligand A6XL absorbents

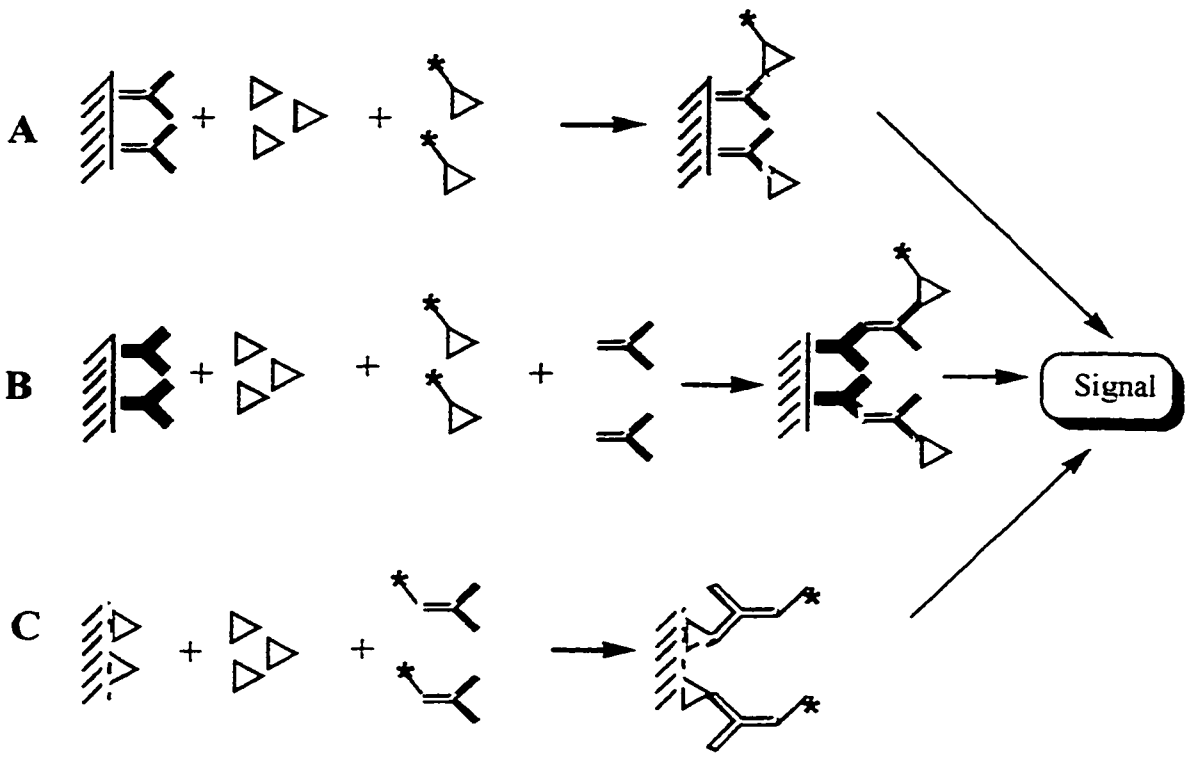


Figure 1.4 Configuration of competitive immunoassays. (A) The immobilized antibody approach. Analyte from the sample competes with labeled analyte for a limited number of antibody binding sites. (B) The use of an anti-immunoglobulin coated solid phase to capture the anti-analyte antibody. (C) The immobilized antigen approach. Here, the analyte from the sample competes with immobilized analyte for binding to labeled antibody molecules. In all cases the signal is inversely related to the analyte concentration (refer to Christopoulos and Diamandis, 1996).

1.3.1. Two site (sandwich) immunoassay

There are several configurations with two site (sandwich) immunoassays (Fig. 1.5). A known amount of antibody or antigen is immobilized on a solid phase, and then the sample containing analyte (either antigen or antibody) is added and incubated for a certain period. After a washing step to remove unbound antigen or antibody, an antibody labeled with a indicator such as an enzyme, biotin, radioisotope, or fluorescence etc. is added. In the final step, the indicator bound to the immune complex is measured using the appropriate method. The configuration of the immunoassay is determined by the availability of a known antibody or antigen and also the analyte in the sample.

To determine the amount of tumor marker in a patient's sample, a purified MAb specific to the analyte is immobilized on a solid phase such as microtiter plate, polystyrene tube, bead or membrane. After the nonspecific binding sites are blocked by a blocking buffer, the sample containing the tumor marker is incubated with the antibody coated on the solid phase. A MAb conjugated with an indicator or bsMAb together with a labeling reagent is used to quantify the bound tumor marker in the immune complex.

1.3.2. BsMAb- a new reagent used in enzyme immunoassay

Generally, antibody-enzyme conjugates that monoclonal or polyclonal antibodies are covalently coupled to a marker enzyme (e.g., horseradish peroxidase, alkaline phosphatase, urease) are used in most immunoassays. The main advantages are the stability and lone shelf-life of the reagents, simplicity of procedures, and various photometric methods available to measure enzyme activity. In addition, the problems associated with radioisotopes are avoided. However, enzyme immunoassays require the preparation of an enzyme labeled antibody for the specific detection stage. Chemical conjugation techniques have been used to produce most of the enzyme/antibody conjugates to date, despite problems encountered during the conjugation steps including aggregation, non-uniform ratios, inactivation of either molecule, and reduced specific activity (Takahashi and Fuller, 1988; Ishikawa, 1996).

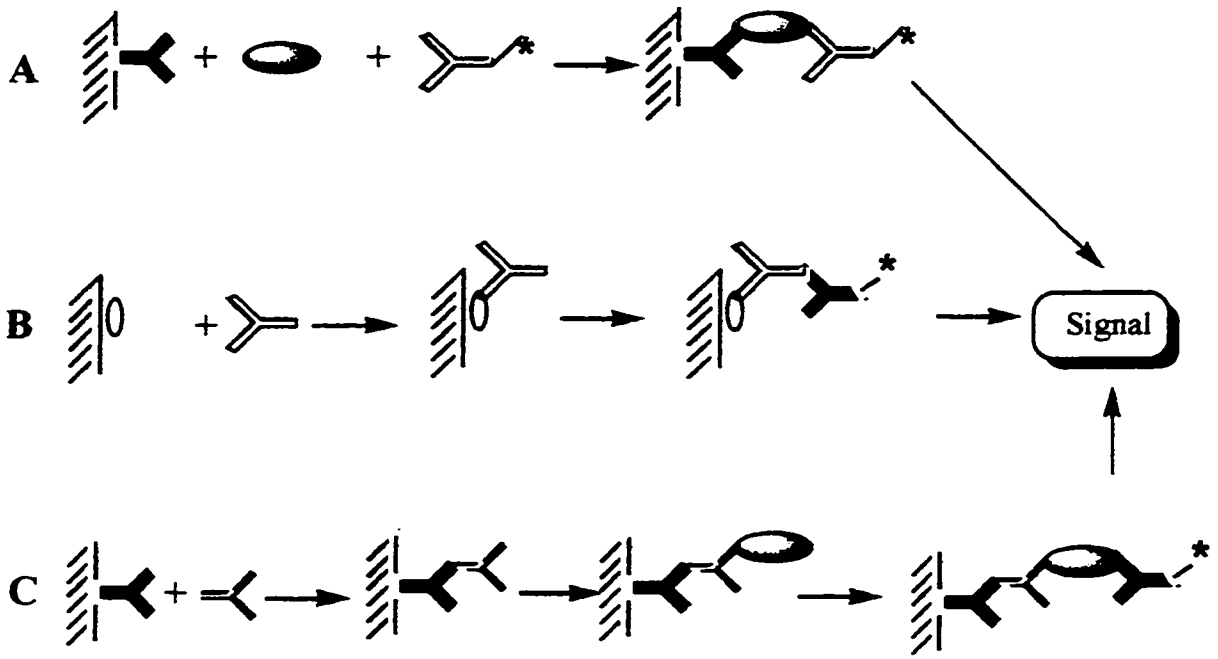


Figure 1.5 (A) A two-sites (sandwich) immunoassay. The analyte is captured by the immunobilized antibody and is then detected by using a labeled antibody. (B) Noncompetive immunoassay for quantification of antibodies. The sample is incubated with an antigen-coated solid phase. The bound antibodies are then quantified by using a labeled anti-immunoglobulin. (C) An immunoglobulin class capture assay. All the immunoglobulins of the class of interest are first captured on a solid phase which is coated with anti-class antibodies. Then the antigen is added and binds only to specific antibodies of the class. The bound antigen is quantified by using a labeled antibody (refer to Christopoulos and Diamandis, 1996).

BsMAbs are uniquely engineered antibodies with two different binding sites (paratopes) in a single antibody molecule. This antibody design has led to the development of a novel detection system employing anti-target antigen x anti-enzyme bsMAbs for immunocytochemistry and immunoassays (Tada et al., 1989; Kenigsberg et al., 1990; Kontsekova et al., 1992; Kreutz and Suresh, 1997; Inouye, 1997). Peroxidase is the most commonly used enzyme in immunoassays. Several investigations on the development of bsMAb anti-target antigen x anti-peroxidase have been reported. A anti-rabbit IgG x anti-peroxidase bsMAb which was developed as a general reagent in immunocytochemistry and enzyme-linked immunoassay has been proved to be superior to the conventional peroxidase-antiperoxidase procedure (Kenigsberg et al., 1990). Anti-tumor marker x anti-peroxidase bsMAbs have been developed for immunodiagnosis and the monitoring of cancer patients. Examples include anti-CEA (carcinoembryonic antigen) x anti-peroxidase bsMAb (Jantscheff, et al., 1993); anti-CA125 (cancer antigen 125) x anti-peroxidase bsMAb (Kreutz and Suresh 1995); and anti-PSA x anti-peroxidase bsMAb (Kreutz and Suresh, 1997). The anti-PSA x anti-peroxidase bsMAb has shown high specificity, excellent detection limit, and fast kinetics in a single-step assay for the detection of a prostate specific antigen.

BsMAbs with one binding arm specific to alkaline phosphatase (AP), a second commonly used enzyme in immunoassay, have been developed (Auriol et al., 1992; Inouye, 1997). The bsMAb developed by Auriol et al., was used in molecular hybridization as a non-radioactive DNA and RNA probe, thus avoiding problems associated with radioisotopes. This bsMAb showed a better signal to background noise ratio in non-radioactive hybridization immunodetection, and high specificity and fast kinetics in southern blot analysis. The bsMAb anti-TSH (thyroid stimulating hormone) x anti-AP demonstrated greatly reduced non-specific binding and an improved detection limit in the TSH assay compared with covalently linked AP-MAb conjugate (Inouye, 1997). Therefore, a bsMAb anti-target antigen x anti-enzyme appears to be a more powerful probe than chemically conjugated MAb-enzyme immunoconjugates in a sensitive immunoassay.

1.4. Tumor markers

1.4.1. Definition

Tumor markers are substances developed in tumor cells and selectively released into body fluids so that they can be quantified by non-invasive analysis. Because of a correlation between marker concentration and tumor mass, tumor markers are useful for clinical monitoring of cancer patients. Today, the term is extended to cell or tissue characteristics, such as cytogenic markers, oncogenes or abnormally expressed proteins with various biological functions (enzymes, receptors, etc.), which help characterize the tumor type and constitute specific biological targets for new anti cancer agents.

1.4.2. Serologic tumor Markers

An ideal serological tumor marker or tumor specific antigen would be a substance which is not present in normal serum. Its detection in serum would be possible with the presence of a minimal tumor burden. Its concentration would accurately indicate tumor progression or regression. At present, very few tumor specific antigens have been recognized and the majority of tumor markers currently defined are tumor associated antigens (TAA). TAA represent either a quantitative over expression of antigens normally expressed by tissues of the same embryonic origin (enzymes such as PSA, peptide hormones, etc.), or the re-expression of cellular antigens selectively expressed during embryogenesis (oncofetal antigens), or more specifically, post-transcriptional variants, for instance by abnormal glycosylation, of cell membrane components (tumor-associated glycolipids and mucins) such as CA125 and CA19.9. CA125 is a glycoprotein mostly associated with ovarian cancer and PSA is a glycoprotein mainly associated with prostate cancer.

1.5. Prostate-specific antigen and prostate cancer

Cancer of the prostate is the most common cancer in men and is second only to lung cancer as the leading cause of male cancer death. The American Cancer Society has projected that 317 000 men will be diagnosed with prostate cancer in the United States in 1996 and that 41400 will die of this disease (Parker et al., 1996). Prostatic cancer is curable through radical prostatectomy if it is diagnosed early and while the disease is still confined to the prostate. Therefore, an assay for tumor markers in the serum or other body fluids for early detection of prostatic cancer is of importance in the management of this malignant disease. An assay for the prostatic tumor marker also is of great value in evaluation of treatment efficiency, in monitoring progression of the disease, and in the early prediction of the recurrence of the disease. Prostatic tumor markers are also a useful tool for differential diagnosis of metastasized tumor of unknown origin, which is crucial in determining the treatment mode.

1.5.1. Prostate-specific antigen (PSA)

PSA is a glycoprotein consisting of a single polypeptide chain with 93% peptide and 8% carbohydrate (Wang et al., 1979). The primary structure of PSA was based on 237 amino acid residues with multiple disulfide bonds in the native form (Schaller et al., 1987). The over-all molecular weight is 34,000 daltons (Wang et al., 1981). PSA is a kallikrein-like, serine protease that is produced exclusively by the epithelial cells lining the acni and ducts of the prostate gland. (Lilja et al., 1985) An abnormally increased serum PSA concentration was found in many BPH (benign prostate hyperplasia) and prostatic cancer patients' sera (Graves et al., 1992b).

Clinically, PSA levels should allow early stage detection of prostate cancer and provide a way to monitor the treatment response, and predict disease recurrence of patients with prostatic cancer (Kuriyama et al., 1981). It was found that PSA levels increased as disease progressed, decreased as disease regressed, and remained relatively constant in patients with stable disease. For a patient who has undergone a radical

prostatectomy, PSA should decrease to undetectable concentrations since all of the source tissue producing PSA has been removed (Price et al., 1991; Lange et al., 1989). If the PSA concentration is higher than the reference of the assay then either prostate was not removed completely or PSA is being produced by residual tumor or metastases of the original tumor.

In addition to prostate epithelial cells, PSA was recently found to be associated with other tissues and fluids/cells including breast milk (Yu and Diamandis, 1995a), breast cyst and amniotic fluids (Yu and Diamandis, 1995b), parotid glands (Van krieken et al., 1993), endometrial tissue, and normal breast tissue (Monne et al., 1994). However the levels of PSA in these tissues or fluids was relatively low and the conventional PSA assay was not able to detect this level. In the other words, ultrasensitive PSA assays are required to measure low levels in these tissues as well as accurately detect early recurrence in patients who undergone radical prostatectomy.

1.5.2. Assays for the measurement of serum PSA

The first PSA assay was a sandwich-type enzyme immunoassay developed by Kuriyaman and associates (Kuriyaman et al., 1980). In this initial report, serum PSA levels from healthy male controls were found to range from less than 0.10 to 2.6 μg/L, with a mean of 0.47. No serum PSA was detectable (<0.10 μg/L) from normal females or female patients with cancer. Male patients with cancer of nonprostatic origin were found to show serum PSA levels similar to those of normal male controls. It was noted that a quantitatively different serum PSA level was presented in patients with prostate cancer than in normal men. It was suggested that the sensitive immunoassay for PSA might be useful to detect and monitor patients with prostate cancer. The early studies on the PSA immunoassay allowed clinical researchers to establish the usefulness of serum PSA in monitoring therapy for prostate cancer (Killian et al., 1985; Hudson et al., 1989).

Since 1980, various improved immunoassays, especially with the use of monoclonal antibodies, have become commercially available. Of these commercial assays, RIMAs and ELISA are the most useful methods (Oesterling et al., 1995). There

are five PSA assays approved by the U.S. Food and Drug Administration and many others are waiting for approval.

The methodology of the PSA assay used by Yang Laboratories as well as Diagnostic Products Corp. is the conventional competitive-inhibition-type polyclonal double-antibody radioimmunoassay (Yang et al., 1989). This assay is based on polyclonal anti-PSA antibodies. Anti-PSA antibodies are immobilized on a solid support. A known amount of PSA labeled with ^{125}I is incubated together with the patient's serum sample. ^{125}I-PSA will compete with the patient's PSA. After the first incubation, ^{125}I-PSA bound to the coated antibodies is separated from unbound free ^{125}I-PSA by a washing step. Finally, the amount of radioactivity from bound ^{125}I-PSA is measured. The concentration of PSA in the patient's serum is determined from a standard curve. An LLD of 0.25 μg/L has been reported. The normal reference ranges for PSA in males is 0-2.7 μg/L.

Hybritech-IRMA (immunoradiomatric assay) and Hybritech-EIA are the dominant PSA assays currently used. The IRMA assay system is basically a two-site immunoradiometric procedure (Myrtle et al., 1983). Two MAbs, each directed against a distinct epitope on the PSA molecule, are used. One of the anti-PSA MAbs is immobilized on plastic beads as a capture to bind only the PSA in the serum sample. The other is labeled with ^{125}I , which serves as the "probe" to quantify the amount of the PSA bound in the immune complex. The manufacturer's state an LLD of 0.1-0.2 μg/L, and a linear range of 2-100 μg/L. The normal reference ranges for PSA in male is 0-4 μg/L. Hybritech-EIA is an improved PSA assay where enzyme labeled anti-PSA MAb is used (Oestering et al., 1995). p-Nitrophenol phosphate is the substrate and the colored product is p-nitrophenol, which has an absorbance at 405 and 450 nm. A standard curve is constructed using six calibrators (0-100 μg/L). The normal reference range is the same as the Hybritech-IRMA assays. The manufacturer claims a LLD of 0.3 μg/L.

Other PSA assay, such as Abbot-IMx™ are based on a microparticle capture ELISA. The patient's PSA binds to the MAb coated on the microparticles. Then a second anti-PSA antibody conjugated with AP binds to the PSA captured on the microparticles. AP will convert its substrate 4-MUP (4-methylumbelliferyl phosphate) into a fluorescent product 4-MU (4-methylumbelliferone) which is measured by front-surface fluorometry. This assay is performed by the IMx™ analyzer. The linear range of this assay is 0-100

μg/L with a LLD of <0.1 μg/L. It correlates well with the Hybritech Tandem-R assay (Vessella et al., 1992).

Recently, studies have found that in serum, two forms of PSA are detected immunologically. The complex of PSA (PSA-ACT) is predominantly bound to alpha-1-antichymotrypsin (MW=100 KD) and is measured in all commercial PSA assays. The free form (MW=30 KD) is present in much lower concentrations than complex PSA and is measured in varying degrees by all commercial PSA assays. An equimolar assay which detects both forms of PSA in equal molar ratios holds the promise of standardization of the PSA assay (Graves et al., 1993).

The value of PSA determination for the diagnosis and monitoring of patients with prostate cancer is now well established. More recently PSA has been proposed as a screening test to complement clinical evaluation of prostate cancer (Graves, 1993; Brawer et al., 1993). However, screening applications are currently controversiale due to unproved patient benefit. Graves et al., (1992) indicated the importance of an ultrasensitive PSA assay that could be used to monitor prostate cancer patients after a radical prostatetomy. Several reports suggested that highly sensitive assays capable of detecting at least 0.1 μg/L PSA would be useful for detecting a relapse months earlier than the conventional assay (Graves et al., 1992a; Yu et al., 1995).

Now more and more new PSA assays have been developed with the aim of increasing sensitivity and specificity of PSA, reducing the duration of the assays as well as facilitating the performance of the assays. Of these assays, RIA has been a traditional method, but has the disadvantages of radio isotope hazard and a long assay time. More EIAs are replacing RIMAs because of its simplicity and long shelf life, but its sensitivity is not as high as that of RIMA, since the enzyme labels on antibodies are limited. FIA has gained recent popularity because of its high sensitivity, however the fluorometer is as yet less popular in a clinical or physician lab.

1.6. CA125 and ovarian cancer

1.6.1. CA125

Ovarian carcinoma is the major cause of death among women with gynecological cancer. The overall cure rate remains relatively low, because diagnosis of this disease usually happens only in the late stages, when cures are difficult to achieve (Hanai et al., 1990). After a woman has received a first-line therapy such as surgery, radiation, or chemotherapy, second-look surgery is often performed to see if the disease has been eradicated, or if it has recurred. CA125 is the most commonly used tumor marker in the recurrence of ovarian cancer prior to second look laprotomy. It has been extremely valuable in patient management (Devine et al., 1992).

CA125 is an antigen of unknown structure associated with a high-molecular weight glycoprotein identified originally by a monoclonal antibody raised to an ovarian carcinoma cell line. CA125 is expressed by greater than 80% of non-mucinnous ovarian carcinomas (Bast et al., 1981). Elevated levels of CA125 have been reported in the serum of a majority of patients with epithelia ovarian carcinoma (Bast et al., 1983) and in some patients with non-gynecological cancer (Haga et al., 1986) as well as with peritoneal mesothelioma (Simsek at al, 1996)

1.6.2. CA125 immunoassays

There is a good correlation between CA125 levels and the histological findings of a second look laparotomy (Rubin et al., 1989). It has been shown that CA125 serum levels correlate well with the response to therapy (Lavin et al., 1987) for ovarian cancer patients. Therefore the measurement of CA125 levels in those patients who received the primary treatment will provide information related to the prognosis or recurrence of the disease before the operation of the second look laparotomy (using an upper limit of 35 U/mL). CA125 may also be helpful in the diagnosis and follow-up of malignant

peritoneal mesothelioma (Simsek et al., 1996). Elevation of CA125 during follow-up of ovarian carcinomas may spare surgical 'second look laparotomy' (Frasci et al., 1996).

Since the first MAb against CA125 was developed in 1981, up to 26 MAbs have been developed and characterized (Nap et al., 1996). These studies have shown that the CA125 antigen has two major antigenic domains, which can be recognized by OC125-like or M11-like MAbs.

There are several types of either polyclonal or monoclonal antibody based immunoassays used for determining the CA125 levels in serum of patients.

Immunoradiomatric assays were developed by several investigators. A homogeneous sandwich IRMA was developed by Bast, Jr. et al in 1983. An anti-CA125 MAb (OC125) was coated on polystyrene beads and ^{125}I-labeled OC125 was used to quantitate the CA125 in the patient's serum. This assay takes 20 hrs with a sensitivity of 1.4 U /mL.

CENTOCOR RIA II is a one step heterogeneous sandwich assay based on MAb OC125 and M11. It takes 20 ± 2 hrs with a sensitivity of 0.3 U/mL.

TRUQUANT OV2 RIA is a two-step sandwich immunoradiometric assay (Krantz et al., 1988). A given specimen containing CA125 is incubated with anti-CA125 MAb (B27.1), which is coated on polystyrene tubes. ^{125}I-labeled MAb B43.13 is added as a probe to detect the CA125 captured by B27.1 and radioactivity is measured by a gamma counter. This assay takes 18-24 hrs with a minimum detectable dose of 9.8 U/mL. B43.13 and B27.2 are OC125-like MAbs, but they bind to different epitopes on CA125 with relatively high affinity.

An antibody-lectin sandwich assay for CA125 was developed by Madiyalakan et al (1996), in which MAb B27.1 was used as capture and ^{125}I-wheat germ lectin was employed as a tracer. This unique format seems able to identify a novel isoform of CA125 with different carbohydrate side chains which might not be recognized by anti-CA125 MAbs.

A new CA125 enzyme immunoassay has also been developed by ABBOTT Inc. The anti-CA125 MAb (OC125) is coated on to the solid phase and OC125 labeled with HRPO is used as the tracer. This assay takes 4 hr with a sensitivity of 5 U/mL. Although the analytical sensitivity of this assay is not as good as that obtained using RIA, the

problems associated with radioisotopes are avoided and the assay is much quicker and simpler.

ABBOTT Inc. has developed another CA125 assay--IMx CA125 which is a 2-step fluorescent immunoassay. This assay consists of sheep anti-CA125 antibody on microparticles and the OC125 monoclonal antibody conjugated to alkaline phosphatase. MUP is the substrate for alkaline phosphatase that converts MUP to a fluorescent product MU. This automated assay has an assay time of 42 min and sensitivity of less than 2 U/mL.

BsMAb anti-CA125 x anti-HRPO based enzyme immunoassay was developed by Kreutz and Suresh in 1995. In this sandwich type assay an anti-CA125 MAb (B27.1) was coated on a 96 well microtiter plate and a bsMAb anti-CA125 x anti-peroxidase was used as a tracer. This 4 hr assay has an analytical sensitivity of 1 U/mL. Comparing to the assays using MAb-enzyme immunoconjugates, bsMAb has increased the analytical sensitivity of CA125 and also simplified the assay procedures.

1.7. Aims, objectives and hypothesis

A. Aims:

Bispecifc MAbs are novel second generation MAbs whose utility in immunoassays has been recognized. Previous work in this area utilized the common enzymes such as peroxidase. In my thesis, the aim was to develop bsMAb against tumor markers incorporating alkaline phophatase, another commonly used marker enzyme which lends itself to conventional and ultrasensitive amplified detection using appropriate substrates and detection methods.

B. Objectives:

(1) To develop a hybridoma secreting monoclonal antibody against alkaline phosphatase (AP) from calf intestine.

(2) To develop two quadromas with AP binding specificity in one arm and anti-tumor specificities in the second arm (anti-CA125 x anti-AP; anti-PSA x anti-AP).

(3) To develop a simple method to purify the enzyme/anti-enzyme bispecific antibody immune complexes using dye ligand affinity chromatography free of the competing monospecific species.

(4) To construct bispecific antibody based conventional immunoassays for CA125 and PSA respectively.

(5) To construct bispecific antibody based ultrasensitive immunoassays for CA125 and PSA respectively using an enzyme amplification system.

C. Hypothesis

(1) Developing bispecific MAb constructs with AP as the enzyme marker specificity would allow us to develop ultrasensitive immunoassays for tumor markers using cyclic enzymatic amplification methods.

(2) Affinity purified bsMAb by mimetic affinity methods would generate immunoprobes approaching the theoretical limit of specific activity with respect to enzyme labeling i.e. one AP molecule bound to every bsMAb to provide the key to ultrasensitive detection methods.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

Alkaline phosphatase (E.C. 3.1.3.1) from calf intestinal mucosa (50 U/mg, solid); diaphorase; alcohol dehydrogenase (ADH); p-iodonitrotetrazolium violet (INT-violet); para-nitrophenyl phosphate (pNPP); tricine and diethanolamine; molecular weight standard for SDS-PAGE (low molecular range); goat anti-mouse IgG (whole molecule); rabbit anti-mouse IgG conjugated with alkaline phosphatase; tetramethylrhodamine isothiocyanate (TRITC); fluorescein isothiocyanate (FITC); Pristane (2,6,10,14-tetramethylpentadecane) and PEG (polyethylene glycol) (MW 1300-1600) were obtained from SIGMA Chemical Co. (St. Louis. MO, USA); Isostrip™ and NADP (NAD free) were obtained from Boehringer Mannheim (Germany). TRUQUANT® OV2™ RIA kit was from Biomira.Inc (Edmonton, AB, Canada). All other reagents were of analytical grade or equivalent purity. Growth factor was from IGEN Inc (Rockville, MD, USA). RPMI-1640 media, L-glutamine, penicillin and streptomycin as well as Fetal Bovine Serum (FBS) were from GIBCO BRL (Gaithersburg, MD, USA). MIMETIC blue AP A6XL adsorbent was obtained from ProMetic BioSciences Inc. (Burtonsville, MD, USA); Dialysis membrane, with a MW cut off of 6000-8000 Da, was from Spectrum Medical Industries, Inc. (Los Angeles, CA, USA). The 96 well ELISA plate was from Nunc (Naperville, IL, USA). PSA (63.5 μg/mL) was purified from LNCap cell supernatant (Kreutz, F.T. 1997). ECM 200 electrofusion system and BTX disposable cuvettes plus were from BTX Inc (San Diego, CA, USA).

2.2. Cell lines and antibodies against tumor marker PSA or CA125

SP2/0 is a myeloma cell line which does not secrete immunoglobulin and is deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT). B87.1 and B27.1 are anti-PSA and anti-CA125 IgG1 monoclonal antibodies respectively which

were kindly provided by Biomira Inc. (Edmonton, Alberta, Canada). P92.3R2.15 is a mouse hybridoma secreting monoclonal antibody IgG1 (P92) against calf intestinal alkaline phosphatase. B80.3 and B43.13 are mouse hybridomas producing anti-PSA and anti-CA125 IgG1 respectively which were also kindly provided by Biomira Inc. P105.2R8.2.1 is a quadroma cell line derived by fusing the P92.3 and B80.3 hybridomas. P104.1R3.2.1 is a quadroma cell line derived by fusing the P92.3 and B43.13 hybridomas. These cell lines were maintained in RPMI-1640 media supplemented with 2 mM L-glutamine, 50 U/mL penicillin and streptomycin as well as 10% v/v of Fetal Bovine serum (FBS).

2.3. Development of hybridoma secreting anti-AP IgG MAb

The entire procedure involving several stages as shown schematically in Fig 2.1 takes approximately 3 months to produce the desired MAb.

2.3.1. Immunization

BALB/c mice were immunized intraperitoneally with crude AP following an immunization protocol described in Table 2.1. Before the intraspleen injection, the mice were bled by tail vein. The titer of anti-AP antibodies in the sera were determined using an enzyme immunoassay in order to select the best mouse responder for antigen boost and fusion.

2.3.2. Enzyme immunoassay for detecting anti-AP antibody

A 96 well ELISA plate was coated with 100 μL of PBS containing goat anti-mouse IgG (whole molecule) diluted as 1:1000 and left overnight at 4°C. The coating solution was discarded and the non-specific binding sites on the plate were then blocked with 5% skimmed milk in PBS. The wells were washed three times with PBS containing

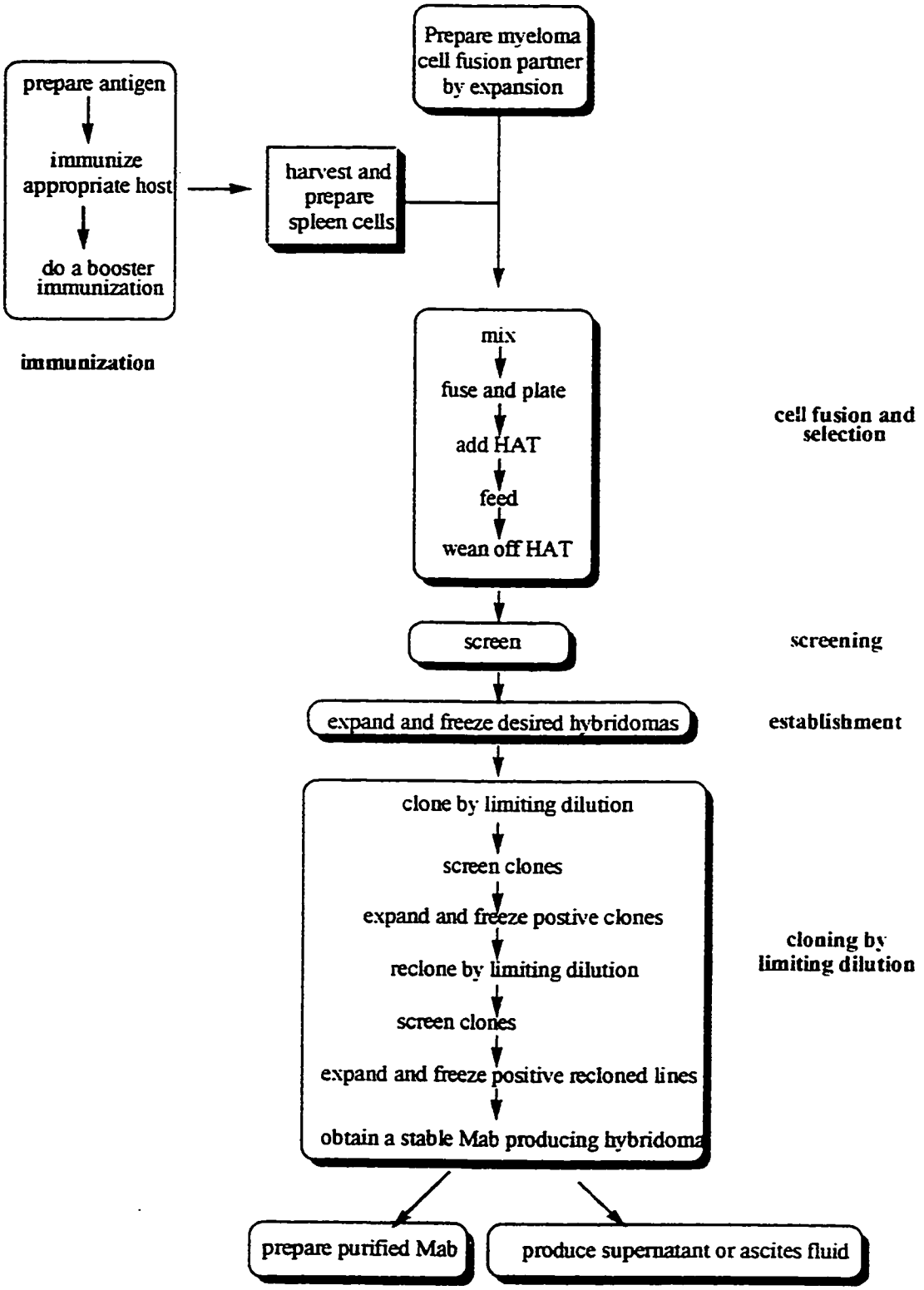


Figure 2.1 Stages of monoclonol antibody production

Table 2.1 Immunization protocol

Time (Days)	Amount of Ag	Ajuvant	injection volumn	Route
1	50 μg	FCA[a]	0.2 mL	IP
8	50 μg	FICA[b]	0.2 mL	IP
10	100 μg	PBS	0.5 mL	IP
12	50 μg	PBS	0.5 mL	IS
15	Fusion			

a=Freund's complete adjuvant

b=Freund's incomplete adjuvant

IP=intraperitoneal injection

IS=Intrasplenic injection

Ag=antigen

0.1% of Tween 20 (PBST). The mouse serum which was diluted 1000 times in PBS was then added to the wells and the plates were incubated for 1 hr at room temperature. After washing, 100 μL of AP (10 μg/mL) was added and the plates were incubated for another hour. Following another washing step, the activity of AP bound on anti-AP antibody was determined by using the substrate p-nitrophenyl phosphate. A 100 μL aliquot of p-nitrophenyl phosphate (1.5 mg/mL) in glycine buffer (pH 10.4) was added to each well. The plate was incubated for 10 to 20 min and the optical density at 405 nm was measured using an ELISA reader (Molecular Device, USA).

2.3.3. Generation of the hybridoma producing anti-AP MAb

The hybridomas were produced by fusing the spleen cells from immunized mice with a SP2/0 myeloma cell line following the fusion procedures outlined below:

(1) Mix SP2/0 myeloma and immunized spleen cells at 1:1-1:20 ratio.

(2) Centrifuge cell mixture at room temperature for 5 min at 500x g. Then remove the supernatant as completely as possible.

(3) Add 1 mL prewarmed 50% PEG slowly, drop by drop to the cell pellet over 1 min, while resuspending the cell by gently shaking for 5 min.

(4) Slowly add 1 mL of RPMI-1640 media for 1 min with gentle shaking.

(5) Slowly add 10 mL of RPMI-1640 media for 3 min with gentle shaking.

(6) Centrifuge 5 min at 500 x g.

(7) Discard the supernatant, add RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 U/mL penicillin and streptomycin, 20% v/v of Fetal Bovine serum (FBS), 10% origen, OPI and HAT.

(8) Resuspend the cell pellet and seed 200 μL of suspension per well in a 96 well tissue culture plate.

(9) Incubate in a humidified, 5% CO_2 incubator at 37°C.

2.3.4. Selection of high affinity anti-AP hybridoma

In order to estimate the affinity of each antibody correctly, the amount of antibody in each supernatant was normalized by limiting the amount of coating antibody to 1:5000 dilution (1μg/mL). The preparation of the ELISA plate for detection of anti-AP MAb is described in section 2.3.2. A plate coated with goat anti-mouse IgG was used, a 100 μL aliquot of cell culture supernatant collected from different clones was added to each well in duplicate and the plate was incubated at room temperature for 1 hr. The plate was then washed 3 times with PBST and 100 μL of AP at varying concentrations was added. The plate was then incubated for another hour at room temperature followed by a washing step. The activity of the AP bound to anti-AP antibody which captured by coating antibody was determined as described in section 2.3.2.

2.3.5. Cloning and recloning of hybridoma

The best positive clone was selected based on the affinity of anti-AP antibody it produced to AP and high stability in the cell culture. The selected clone was recloned twice by limiting dilution to ensure monoclonality. The limiting dilution was done as follows:

After the positive clone was screened, it was transferred to a 24 well plate. The anti-AP antibody activity was tested after 2-3 days. The clone producing anti-AP antibodies with highest OD in ELISA was chosen for limiting dilution as follow.

(1) Resuspend the hybridomas in a standard RPMI-1640 media.

(2) Count the cells.

(3) Make a serial dilution in a six well plate to achieve a concentration of 5-7 cells/mL, then add 100 μL of cell suspension and 100 μL of RPMI-1640 media with 10% of growth factor and 10 % of FBS to each well in a 96 well plate.

(4) Test the supernatant of each clone for the anti-AP antibody activity, and calculate the cloning efficiency as follows:

Cloning efficiency % = Number of positive clones x 100/ Number of clones

The limiting dilution was continued until the cloning efficiency reached 100%. The clone was then expanded and frozen in the liquid N_2 for future use.

This technique also was used for preparing monoclonal quadromas producing desired bsMAbs.

2.3.6. Production of anti-AP MAb

Two methods were used to produce moderate amounts of anti-AP MAb. One was collecting cell supernatant. The other was production of mouse ascites by inoculating hybridoma into BALB/c mice previously primed with 0.5 mL of Pristane. These methods were also used for production of bsMAbs.

2.3.7. Purification of anti-AP MAb

The anti-AP MAb was purified by a combination of ammonium sulfate precipitation with ion exchange chromatography. Firstly, the antibody in the cell supernatant or mouse ascites was precipitated by adding ammonium sulfate to reach a 50% saturation. The precipitate was then pelleted down by centrifugation. The pellet was dissolved in a minimum volume of PBS and then the sample was dialyzed against three changes of binding buffer containing 10 mM NaH_2PO_4 (pH 7.5) which is used in DE-52 anion exchange chromatography (Suresh et al., 1986a). The sample was loaded on to the column at a flow rate of 1 mL/min. And then, the column was washed with binding buffer until no protein is detected in the eluent. Finally the MAb bound on the column was eluted using a 200 mL linear gradient of 10 mM NaH_2PO_4 (pH 7.5), to an equal volume of 100 mM NaH_2PO_4 (pH 7.5). This method was also used for purification of bsMAbs.

2.3.8. Characterize the anti-AP MAbs

2.3.8.1. Isotype

The isotypes of anti-AP antibodies were determined right after the positive clones were screened using an Isostrip™ kit following the manufacture instructions.

2.3.8.2. Cross reactivity of anti-AP MAb

The anti-AP IgG purified by DE-52 column was used to coat a 96 well microtiter plate at 5 μg/mL. AP from different sources were added into the plate at different concentration. The plate was then incubated for 1 hr at room temperature. After three washings, the color was developed using the procedure described in section 2.3.2.

2.4. Preparation of quadromas

2.4.1. Fusion of two hybridomas

2.4.1.1. Fusion of hybridoma B80.3 (anti-PSA) with hybridoma P92.3 (anti-AP)

Two hybridomas B80.3 (anti-PSA) and P92.3 (anti-AP) were labeled with TRITC and FITC respectively (Junker and pederson, 1981) and fused according to the method described by Kreutz et al., 1998. In brief, each hybridoma cell line containing 1-2 x 10^7 cells, grown in logarithmic phase, was prepared and spun down at 1350 rpm for 7 min. The two cell pellets formed were resuspended in 2 mL of TRITC (B80.3) (1.5 μg/mL in serum free RPMI 1640 media with pH 7.4) and FITC (P92.3) working solution (0.5 μg/mL in serum free RPMI 1640 media with pH 6.8) and incubated for 30 min at 37°C. Both cell lines were washed once in RPMI-1640 media followed by two washings with electrofusion solution (sterile 0.3 M glucose with 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$). The pellets were resuspended at $1x10^7$ cells/mL and $2x10^6$ cells labeled with each fluorescence were used for fusion. The cells were fused in a sterilized electrofusion

cuvette (0.2 cm gap) under following conditions: (alignment) 30 sec., 200 V/cm and 60 V of AC; (fusion) 3 pulses of 15 microseconds with a field strength of 1000V/cm DC and amplitude setting 200V. After the fusion procedure, the cells were incubated for two hr at 37°C. The fused cells with both fluorescence signals were selected by FACS and seeded into a 96 well cell culture plate at 1 cell/well. The cells were cultured in the standard RPMI 1640 media consisting of 10% of growth factor and kept in a 37°C incubator supplemented with 5% CO_2. After 14 days, the clones were screened for quadromas secreting bsMAb anti-PSA x anti-AP using the sandwich assay described in section 2.4.2. The best positive clone (P105) with high bsMAb activity in its supernatant was recloned 3 times by limiting dilution technique then expanded and frozen for further study.

2.4.1.2. Fusion of hybridoma B43.13 (anti-CA125) with hybridoma P92.3 (anti-AP)

A P104 quadroma (anti-CA125 x anti-AP) was generated using the same method by fusing hybridomas B43.13 (anti-CA125) and P92.3 (anti-AP).

2.4.2. Screening for the quadromas secreting bsMAb anti-PSA x anti-AP

The microtiter plate was coated with anti-PSA MAb, B87.1 (1 g/well) and incubated overnight at 4°C. The plate was blocked with 5% skim milk in PBS for 1 hr at room temperature. A 25 μL sample of cell culture supernatant was tested together with 50 μL of PSA (260 μg/mL) and 25 μL of AP (40 μg/mL). The plate was incubated for 30 min at room temperature, followed by three times washings with PBST. In the presence of bsMAb anti-PSA x anti-AP, a tetrameric complex will be formed and upon addition of substrate pNPP, bsMAb activity will be detected using the same procedure described in 2.3.2.

2.4.3. Screening for the quadromas secreting bsMAb anti-CA125 x anti-AP

In order to detect the activity of bsMAb anti-CA125 x anti-AP, anti-CA125 MAb B27.1 coated plate (1 μg/100 μL) was used. About 800 U of CA125 was added to the plate and incubated for 3 hr at room temperature. Following a washing step, 50 μL of cell culture supernatant (diluted 2 times with tricine buffer) was added together with 50 μL of AP (20 μg/mL) and incubated for 45 min at room temperature. The color development followed the procedure described in section 2.3.2).

2.5. Purification of anti-AP antibody using mimetic blue A6XL column

The different crude samples (AP, anti-AP MAb, P92; P92-AP immune complex) were dialyzed against 3 changes of 10 mM Tricine-NaOH buffer for 16 hr at 4°C. The samples were applied separately at a flow rate of 3 mL/hr to a column containing 2 mL of mimetic blue AP A6XL absorbent pre-equilibrated with the Tricine buffer. The column was washed with the same buffer until the absorbence of the eluate at 280 nm was negligible. The bound proteins were eluted by 10 mM Tricine-NaOH containing 5 mM potassium phosphate. Approximately 1 mL fractions were collected until the absorbance at 280 nm returned to baseline. The column was finally washed with 0.75 M potassium chloride and 1 mL fractions were again collected.

2.6. Purification of anti-PSA bsMAb and bsMAb-AP immune complex

Diluted mouse ascites with enriched anti-PSA bsMAb activity was fractionated by ammonium sulfate precipitation and then dialyzed against three changes of Tricine buffer (pH 8.5). Five milliliters of crude sample was loaded on a 10 mL mimetic blue column at flow rate of 3 mL/hr overnight at 4°C for maximal binding. After all of the unbound fraction was collected, the column was washed with 100 mL of the 10 mM Tricine buffer. The bound fractions (2 mL/tube) were eluted using a 5 mM phosphate Tricine buffer (pH

8.5), and assayed for bsMAb P105 (anti-PSA x anti-AP) activity. In a parallel purification of bsMAb P105-AP immune complex, 50 mg of AP (20 U/mg) was loaded on the mimetic blue column. Following the washing step, the same amount of bsMAb P105 was loaded, and eluted. The fractions were then assayed.

2.7. Purification of commercial polyclonal IgG conjugated with AP

Four hundred microliliters of commercial AP conjugated IgG (about 6300 U of AP) was diluted to 12 mL with Tricine buffer and dialyzed overnight at 4° C against 3 changes of Tricine buffer. The dialyzed sample was purified on a column containing 10 mL of MIMETIC blue A6XL absorbent which was pre-equilibrated with Tricine buffer as described in section 2.6.

2.8. A Simplified method to purify bulk bsMAb

Fifty milligrams of AP (20 U/mg solid) was dissolved in 5 mL of Tricine buffer pH 8.5 and dialyzed at 4° C against 3 changes of the same buffer. The AP was incubated in 15 mL Tricine buffer with 10 mL of mimetic blue absorbent overnight at 4°C. Then, the absorbents were transferred to a 20 mL column, and unbound AP was collected for reuse. The absorbents were washed with 100 mL of the same buffer and incubated with 15 mL (6 mg of total protein) of bsMAb, which fractionated by ammonium sulfate precipitation in a small flask for 1 hr at room temperature. The absorbents were then transferred back to the column and extensively washed and bsMAb-AP immune complex was eluted with 40 mL of 20 mM phosphate in Tricine buffer (pH 8.5), and the activity of BsMAb-AP immune complex activity was assayed.

AP + MIMETIC blue A6XL absorbents

↓

Incubation overnight at 4°C

↓

Unbound AP was collected (15 min)

↓

Washing absorbents (30 min)

↓

BsMAb + absorbents bound with AP

↓

Incubation for 1 hr at room temperature

↓

Unbound antibodies were collected (15 min)

↓

Washing absorbents (30 min)

↓

Elution of antibody-AP immune complex (30 min)

2.9. Ion exchange purification of anti-CA125 bsMAb

BsMAb P104 was fractionated by ammonium sulfate precipitation from cell culture supernatant and was dialyzed against 10 mM phosphate buffer. A 15 mL of sample was loaded on a DE-52 column (1.8 cm x 18 cm) at flow rate of 0.5 mL/min and purified as described in 2.3.6.

2.10. Assays of enzyme activity

Alkaline phosphatase activities were assayed by measuring the production of the p-nitrophenolate anion at 405 nm (Lindner et al, 1989).

2.11. Protein assay

Antibody concentration was determined by measurement of the absorbance at 280 nm using a mass extinction coefficient of 1.35 absorbency unit per mg/mL of MAb IgG. Relative A280 nm (%) was based on the scale of protein peak on the chart recorded by a A_{280nm} monitor SDS-PAGE was performed according to the published methods (Laemmli, 1970) using a Bio-Rad Mini-protein II dual slab cell along with low molecular weight standards. The gels were developed with silver stain as described earlier (Merril et al., 1990).

2.12. Enzyme immunoassay for analysis of MAb, bsMAb, and their immune complexes with AP

2.12.1. Detecting free MAb and MAb-AP immune complex

The column fractions were assayed at appropriate dilution using the method mentioned in section 2.3.2. The anti-AP MAb activity (free MAb) in fractions was assayed by binding to goat anti-mouse IgG coated plate and subsequently incubating with AP (10μg/mL). The plate was wash three times and p-nitrophenyl phosphate was used as the chromogen for the color reaction. In order to detect pre-formed MAb P92-AP immune complexes, no additional AP was added in the ELISA procedure while testing the column fractions.

2.12.2. Assay for the activity of rabbit anti-mouse IgG-AP immunoconjugates

The activity of rabbit anti-mouse IgG-AP immunoconjugates in the fractions was determined in a similar fashion to the assay described for P105-AP immune complex immunoassay (section 2.4.2). Briefly, a plate coated with 1 μg/100 μL of mouse MAb (B87.1) was used. The samples containing rabbit anti-mouse IgG-AP immunoconjugates were added to the plate and incubated for 1 hr at room temperature. After a washing step,

the activity of MAb-AP immunoconjugates bound to mouse MAb was detected by the colorimetric method described in section 2.3.2.

2.12.3. Detecting free anti-PSA bsMAb, anti-CA125 bsMAb and bsMAb-AP immune complexes

To detect anti-PSA bsMAb (P105), the elution fraction at appropriate dilution was added to a plate coated with 1 μg/100 μL of MAb B87.1. The remaining procedures were previously described in section 2.4.2.

On the other hand, for the detection of the bsMAb P105-AP immune complex, all the procedures are as the same as above for the free bsMAb P105 except that the addition of AP to the assay was omitted. Thus, only pre-formed immune complexes are detected.

The activity of anti-CA125 bsMAb (P104) (anti-CA125 x anti-AP) in elution fractions was assayed using the heterogenous sandwich assay mentioned in section 2.4.3. A 50 μL of elution fraction diluted two times in Tricine buffer was added together with 50 μL of AP (20 μg/mL) and incubated for 45 min at room temperature. The final step is described in section 2.3.2.

In addition, the detection of bsMAb P104-AP immune complex followed the procedures for bsMAb P104 mentioned above excluding addition of AP.

2.12.4. Inhibition assay to estimate contaminating monospecific MAb

In order to estimate contaminating monospecific MAb (anti-CA125 MAb) in the fractions collected both from DE-52 and MIMETIC blue A6XL affinity column, The 96 well ELISA plate prepared for the detection of P104 (anti-CA125 x anti-AP) was used. About 100 μL of solution containing 1000 U/mL of CA125 was added and incubated for 3 hr at room temperature. Following a washing step, 50 μL of aliquot fraction collected from DE-52 column was added to the plate together with 40 μL of bsMAb P104-AP immune complex purified from a MIMETIC blue A6XL column as well as 10 μL of AP (100μg/mL). The plate was incubated for 45 min at room temperature. In a parallel

experiment, 50 μL of aliquot fraction collected from MIMETIC blue A6XL column was assayed using same procedure but omitting the addition of AP. After a wash step, pNPP was added and the color was determined at 405nm after 20 min. The presence of anti-CA125 MAbs in the fraction would exhibit inhibition of bsMAb P104 activity.

2.13. BsMAb-AP based PSA immunoassay

The coating and blocking procedures are as described in section 2.4.2. Fifty microliters of PSA at various concentrations was added into each well together with 50 μL of diluted bsMAb P105-AP immune complex purified using mimetic blue absorbents. The plate was incubated for some time (20 min-2.5 hr) at room temperature and washed a further three times. The color development was carried out using one of two methods as follow.

2.13.1. Conventional assay

A 100μL sample of 5 mmol/L (1.3 mg/mL) pNPP in 0.9 mol /L diethanolamine (DEA) buffer, pH 9.8, containing 0.5 mmol/L $MgSO_4$ was added to each well and incubated for 60 min at room temperature. The optical density at 405 nm was recorded (Johannsson et al., 1986).

2.13.2. Enzyme amplification assay

A 50 μL aliquot of 0.2 M NADP (nicotinamide adenine dinuclotide phosphate) (0.287 mg/mL) in DEA buffer (pH 9.5) was added into each sample well which was ready for color development and incubated for 15 min at room temperature. Amplification was started by adding 110 μL of a cycling solution containing: 0.1 mg/mL ADH, 0.1 mg/mL diaphorase and 0.55 mM INT (mg/mL) in 25 mM sodium phosphate buffer, pH 7.2 including 4% (v/v) ethanol. The enzyme reactions were stopped after a 15

to 45 min incubation by the addition of 50 μL of 0.4 M HCl. Optical density was read at 490 nm using a microtiter plate reader.

2.14. BsMAb-AP immune complex based CA125 ELISA

2.14.1. Conventional ELISA

A 96 well ELISA plate was coated with anti-CA125 MAb B27.1 at 1 μg/100 μL in each well overnight at 4 °C. The plate was then blocked using 5% skim milk in PBS for 2 hr at room temperature followed by three washings with PBST washing buffer. CA125 standard samples with different concentration (Biomira Inc.) were used. The incubation of CA125 was carried out for 3 hr at room temperature. After the wash step, diluted P104-AP immune complex was added and incubated for 45 min. Color development was done by adding pNPP 1.3 mg/mL in DEA buffer, producing a yellow color which was detected at 405 nm.

2.14.2. Amplified ELISA

The steps before detecting the solid-phase bound P104-AP immune complex activity were the same as those described for in the conventional assay. The amplification procedures used were same to in the enzyme amplification assay described for PSA in section 2.13.2.

CHAPTER 3.* RESULTS AND DISCUSSION

This chapter has been divided into three sections representing the three logical stepwise research milestones accomplished.

3.1. Development of the primary hybridoma secreting monospecific anti-AP MAb designated as P92.3.

3.2. Development of bsMAb anti-PSA x anti-AP which secreted by quadroma designated P105 and its application in one step ultrasensitive ELISA for PSA.

3.3. Generation of a second quadroma (designated P104) secreting bsMAb anti-CA125 x anti-AP and bsMAb based CA125 assay.

As alluded to in the first chapter, PSA and CA125 are the most important tumor markers in managing prostate and ovarian cancer patients, respectively. Hence, the development of a rapid and ultrasensitive immunoassay for the detection of ultra low levels of PSA or CA125 would be of great clinical importance, particularly in detecting early recurrence at the micrometastatic stage. In this thesis, my main objective was the development of bsMAb anti-PSA x anti-AP and bsMAb anti-CA125 x anti-AP immunoprobes and the evaluation of their utility in the design of novel bsMAb ELISAs. In order to use this bsMAb as an immunoprobe in ELISA for PSA or CA125, a unique affinity purification method was developed by using mimetic blue absorbents. The purified bsMAb-AP immune complexes were tested in both conventional and enzyme amplified ELISA protocols.

3.1. Development of hybridoma secreting monospecific anti-AP MAb

The distinguishing feature of the bsMAb is its two arms capable of binding two different predetermined antigens simultaneously. In order to develop a bsMAb having one arm against tumor antigen and another arm against a detector molecule such as an enzyme, my first approach was to develop a hybridoma secreting a MAb against one of

*Some of the contents of this chapter has been published and others are being prepared as manuscript for submission:
a) D.Z. Xu, B. Levengle, F. T. Kreutz and M.R. Suresh (1998) J. Chromatog.B, 706 (1998) 217-229

the commonly used enzymes in ELISAs. Alkaline phosphatase from calf intestine is an enzyme commonly used in ELISA, immunohistochemistry, and western blots, because it reacts with various substrates to produce soluble or insoluble color products, as well as luminescent or fluorescent products, which could be detected by different methods.

3.1.1. Immunization and myeloma x splenocyte fusion

Following the immunization protocol described in Table 2.1, a total of 3 mice were immunized with AP (50U/mg) and then the mouse sera were tested for the anti-AP antibody activity by ELISA assay described in section 2.3.2. The mouse with the highest titer serum was selected for hybridoma fusion. The high activity of anti-AP MAb might indicate that more B cells are producing anti-AP antibodies or higher affinity of the anti-AP MAb was present in the immunized mouse. The splenocytes collected from this immunized mouse were then fused with SP2/0 myelomas by polyethylene glycol (PEG) following the protocol described in section 2.3.3. After the fusion, cells were plated in microtiter plates and selected in HAT media, in which only the hybridomas can survive. About 10-15 days after the fusion, the clones that were visible to the eye were screened for the hybridoma producing anti-AP MAb using the method described in section 2.3.2.

The primary screen was performed on goat-anti mouse IgG coated plates. The first incubation was to capture the mouse immunoglobulins from the supernatants of each clone and then the plate was incubated with AP. Following a washing step, bound AP activity was determined using p-nitrophenyl phosphate as a substrate.

The fusion efficiency of the mouse splenocytes with the mouse myeloma SP2/0 was relatively high. Hybridoma cell growth was observed in 95% of the wells. When tested by ELISA, the presence of hybridoma secreting anti-AP antibody was detected in 27% of hybridomas. In all, 100 primary clones were identified with anti-AP antibody activity, of which 10 clones with very high anti-AP MAb activity were expanded into 24 well plates. The anti-AP MAb activity in these supernatants were rescreened after one week to reconfirm the MAb reactivity and the best three clones (P92.1R, P92.2R;

---

b) F.T. Kreutz, D.Z. Xu and M.R. Suresh (1998) A novel electrofusion and FACS sorting method for generating quadromas, Hybridoma, in press.

P92.3R) with high MAb activity were expanded and recloned by the limiting dilution. This was accomplished by seeding the three primary clones at 0.3 cell/well in a microtiter plate and expanding the recloned cultures with the best anti-AP activity.

3.1.2. Characterization of anti-AP antibody

3.1.2.1. Isotype of anti-AP antibody

The cell supernatants were collected from three anti-AP clones and were tested using a commercial isotype strip coated with goat anti-mouse antibodies against different subclasses (IgM, IgG1, IgG2a, IgG2b IgG3, and IgA). The results indicated that all antibodies produced by the three P92 clones were of the IgG1 kappa isotype (Table 3.1).

3.1.2.2. Relative affinity analysis of anti-AP antibody

Before a hybridoma is established for further development of quadromas, it is important to know the affinity of the antibody it secretes, because a bsMAb with a high affinity to the desired antigen and AP is required for developing a rapid and sensitive immunoassay. Instead of measuring absolute antibody affinity which requires either purified AP or purified MAb, the relative affinity of three antibodies were compared using cell supernatants collected from each clone. To compare the relative affinity of each anti-AP antibody, the amount of anti-AP antibody in each supernatant has to be normalized, since the three clones could be secreting different amounts of MAb. Therefore, a limited amount of goat anti-mouse IgG (1 μg/mL) was coated on ELISA plate, and the anti-AP antibodies in supernatants were captured by the goat anti-mouse IgG on the plate. Because the amount of goat anti-mouse IgG coated on the plate was reduced to the minimum to allow detection, it is most likely that each well captured an equal amount of anti-AP MAb from three different clones. The bound anti-AP antibody was incubated with AP at various concentrations, which in turn was detected by the substrate pNPP. The higher optical density at lower AP concentration indicated that the specific anti-AP antibody had relative higher affinity. As demonstrated in Fig. 3.1, among

Table 3.1. Isotype of anti-AP antibodies from P92 clones

	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
P92.1R	–	+	–	–	–	–
P92.2R	–	+	–	–	–	–
P92.3R	–	+	–	–	–	–

"+" positive result on the isotype strip;

"-" negative result on the isotype strip;

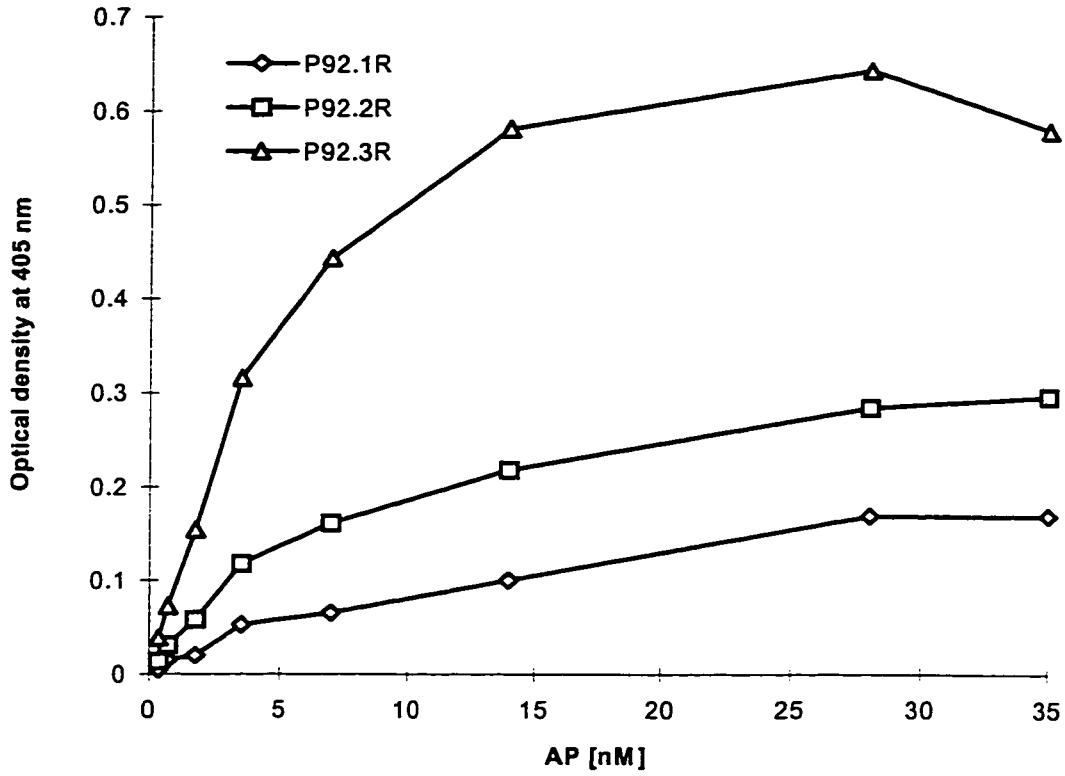


Figure 3.1 Selection of the best anti-AP MAb with high affinity to AP. A limiting amount of goat anti-mouse IgG, immobilized on the plate, was used as the capture reagent. A 100 μL aliquot of cell culture supernatants from each of three different subclones was added into the plate, incubated and washed. Subsequently, 100 μL of AP (roughly 0-35 nM) was added into each well as tracer. The assay was performed as described in section 2.3.4.

the antibodies secreted by three anti-AP hybridomas, the antibody produced by clone P92.3R was the best one with the highest relative affinity to AP. This antibody was able to detect about 0.7 [nM] of crude AP. A final reclone with 100% plating efficiency, designated P92.3R215, was obtained by second limiting dilution and referred to thereafter as P92. This hybridoma P92 and its MAb P92 were used in all the subsequent studies.

3.1.2.3. Preparations of anti-AP antibody

To obtain enough amount of P92 MAb for further studies, hybridoma P92 was then inoculated into BALB/c mice and 10 mL of mouse ascites containing enriched anti-AP MAb was produced. The anti-AP MAb activity could be detected when the mouse ascites was diluted 10,000 times. Anti-AP MAb (P92) was purified from mouse ascites using ammonium sulfate precipitation in combination with ion exchange chromatography (DE-52). The fractions collected from DE-52 column were assayed for anti-AP activity and consequently, the fractions 5 to 10 with high anti-AP MAb activity were analyzed by SDS-PAGE under reducing conditions. The results from reducing SDS-PAGE (data not shown) indicated that fractions 6 and 7 contained less contaminants. In all, about 7 mg of anti-AP IgG was obtained and pooled. The purified P92 could be used at 0.7 μ g/mL in the same ELISA described in 2.3.2.

The DE-52 purification is a general ion-exchange method for the purification of antibody, and the column matrix has a long shelf life and is easy to prepare and maintain. In addition, the elution buffer is mostly near physiological conditions (pH 7-8). However, this method lacks specificity and the purified antibody would have minor contaminants with other proteins such as albumin. Affinity column chromatography on protein A or G is more specific and more efficient in the separation of antibody from other contaminant proteins in mouse serum, but this column has a relatively shorter shelf life and a special preparation is required. Importantly, elution condition is harsh (pH 2-3) and some times the desired antibody might be partially denatured during the elution procedure. Further, this group specific affinity chromatography also co-purified other endogenous mouse antibodies in ascites. In my case, the partially purified anti-AP MAb by the DE-52 method could be used adequately when coated on the plate for further tests.

3.1.2.4. Cross reactivity of anti-AP antibody to AP from human placenta

It was of interest to investigate if anti-AP MAb from mouse could react with the AP from human sources, because this might allow the use of bsMAb anti-tumor marker x human AP in a bsMAb directed enzyme prodrug therapy applications. The anti-AP MAb purified by DE-52 was used for the cross-reactivity test. This was performed in an ELISA plate coated with purified anti-AP MAb as a capture reagent. The AP from both calf intestine and human placenta were added into the plate at different concentrations and the bound AP activity was detected with pNPP. The results, as illustrated in Fig. 3.2, indicated that Anti-AP MAb did not bind to the AP from human placenta. AP from other human organs was not readily available for extensive testing. The MAb bound specifically to AP from calf intestine.

Hybridoma P92, secreting anti-AP MAb with high affinity, can be fused not only with a hybridoma partner producing anti-PSA or anti-CA125 antibody to provide quadromas (see section 3.2 and 3.3), but also with any splenocytes derived from mice immunized with desired antigen to generate triomas (Tada et al., 1989). The resultant bsMAb with one binding site specific to AP could be used as a new reagent in *vitro* diagnostics. Since AP is the second most common marker enzyme used in immunoassay (Gosling, 1990), there are variety of methods used for the detection of AP such as colorimetry (Ishikawa, 1987), luminometry (Bronstein et al., 1989), and time-resolved fluorimetry (Christopoulos and Diamandis, 1992). In addition, the bsMAb anti-tumor marker x anti-AP might be used in bsMAb directed enzyme prodrug therapy in which the bsMAb will localize AP around the tumor cell and the bound AP will in turn activate the prodrug to the cytotoxic drug to eventually kill the tumor cells (Sahin et al., 1990).

3.2. Development and applications of bsMAb anti-PSA x anti-AP

Immunoprobes used in immunoassays are generally produced by chemically crosslinking antibody with a tracer molecule such as an enzyme or radioisotope. Problems encountered during the chemical conjugation procedure can result in aggregation, non-uniform coupling ratios, inactivation of either molecule, and reduced specific activity. A way to avoid these problems is to develop a bsMAb with one paratope

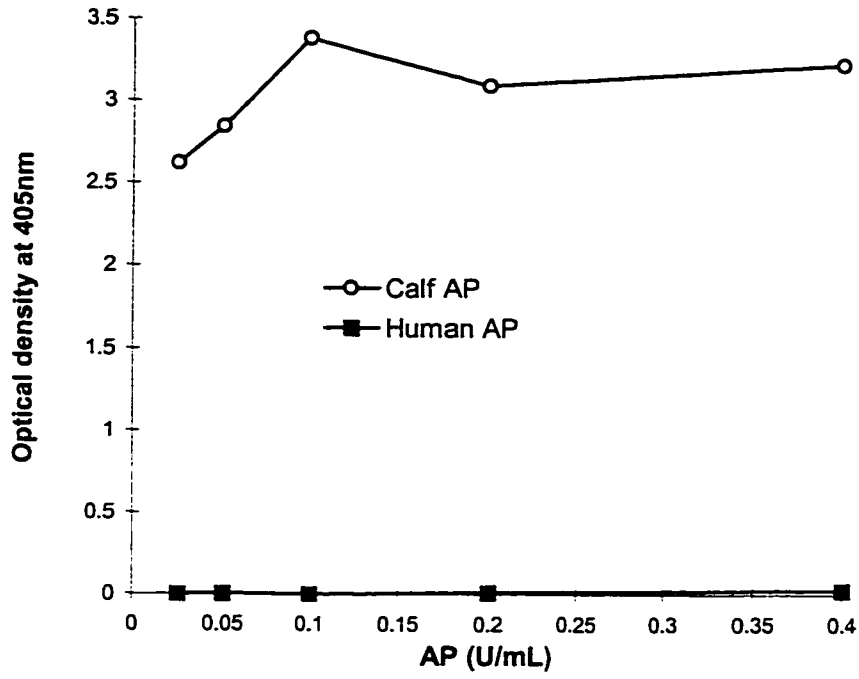


Figure 3.2 The cross reactivity of mouse anti-AP MAb to AP from human placenta. The plate was coated with anti-AP MAb purified from DE52 column and the AP at different concentration from either human placenta or calf intestine were added to the plate. pNPP was used to detect bound AP activity by measuring optical density at 405 nm. Each point represents a mean of triplicate.

specific for AP and second paratope specific for an appropriate tumor antigen such as PSA.

The main advantage of developing bsMAb by the hybridoma technique, over other methods, is that the resultant bsMAb is assembled and secreted by the hybrid hybridoma. Therefore the structure and properties of bsMAbs are generally expected to be similar to that of the normal monospecific antibody secreted by the parental hybridoma. Thus, by using the best anti-AP hybridoma developed above, quadroma secreting bsMAb anti-PSA x anti-AP was developed as described below.

3.2.1. Development of quadroma secreting bsMAb anti-PSA x anti-AP

In order to develop a quadroma secreting bsMAb anti-PSA x anti-AP, a hybridoma producing anti-PSA MAb was required. B80.3 is a well-established hybridoma (Krantz and Suresh, unpublished data) producing anti-PSA IgG1 with high affinity. This clone (provided kindly by Biomira Inc.) was already used in the development of a quadroma producing bsMAb anti-PSA x anti-HRPO (Kreutz and Suresh, 1997). The outline of the fusion of B80.3 with P92 and selection of the potential quadroma cells is illustrated schematically in Fig 3.3b. Briefly, B80.3 and P92 were incubated with two fluorescent dyes, TRITC (red) and FITC (green), respectively, for 30 min at 37 °C in an incubator supplied with 5% of CO_2. Then, the cells were washed to remove the excess amount of dye before the electrofusion. The electrofusion was performed as described in section 2.4.1. After the fusion, the cells were resuspended in standard tissue culture media and incubated for 3 hr. The cells with dual stains were selected by FACS sorter and a single fused cell with two stains was seeded into each well of a microtiter plate. After approximately 10 days, clones were visible to the naked eye. Supernatants collected from each clone were then assayed for the desired bsMAb activity following the procedures in sections 2.4.2. A sandwich assay as shown schematically in Fig. 3.4a, was used for the screening of the positive clone producing bsMAb anti-PSA x anti-AP. A second matched pair of anti-PSA MAb B87.1 (Krantz and Suresh, unpublished data courtesy of Biomira Inc.) was coated on ELISA plate as a capture

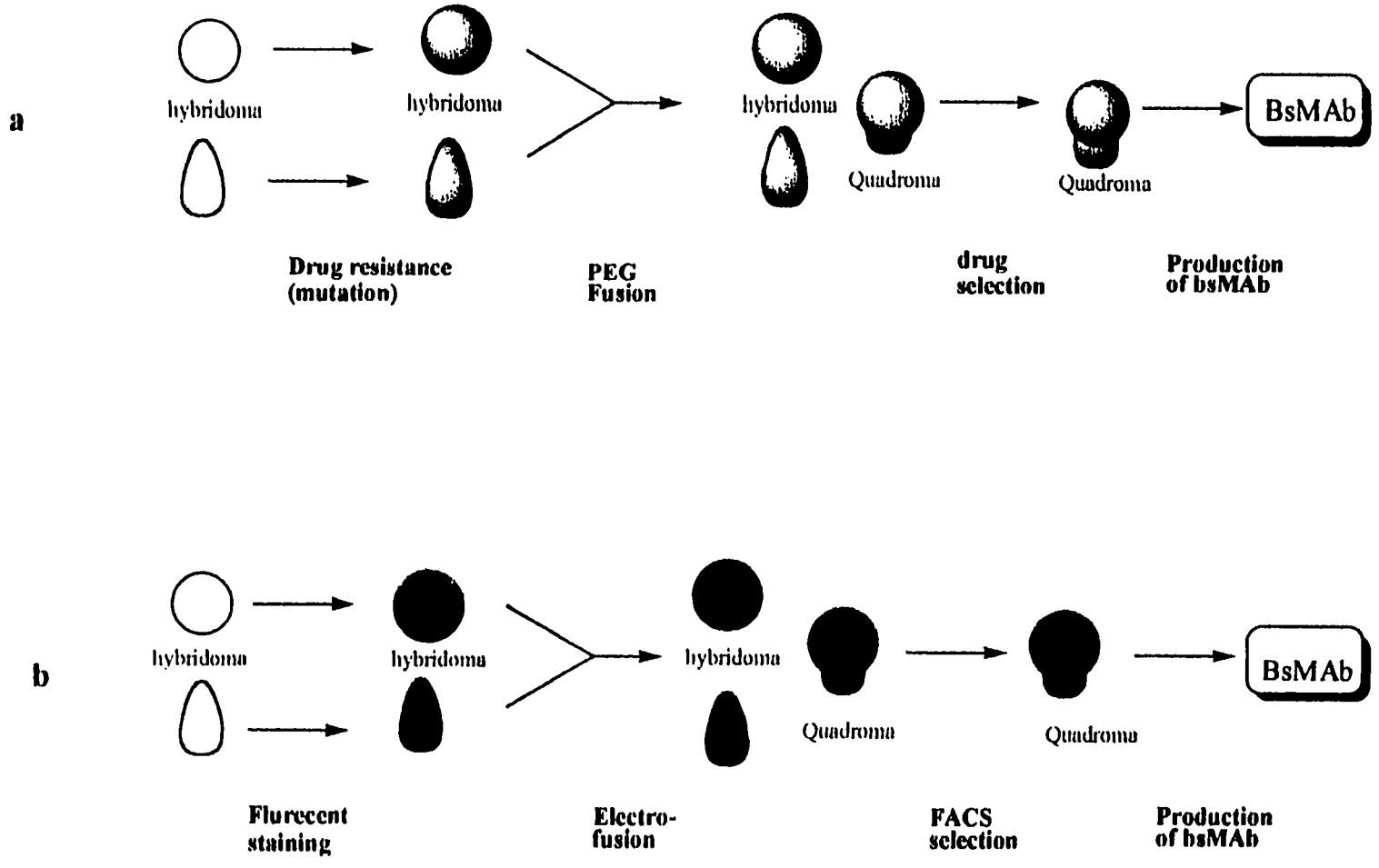


Figure 3.3 Process of quadroma development

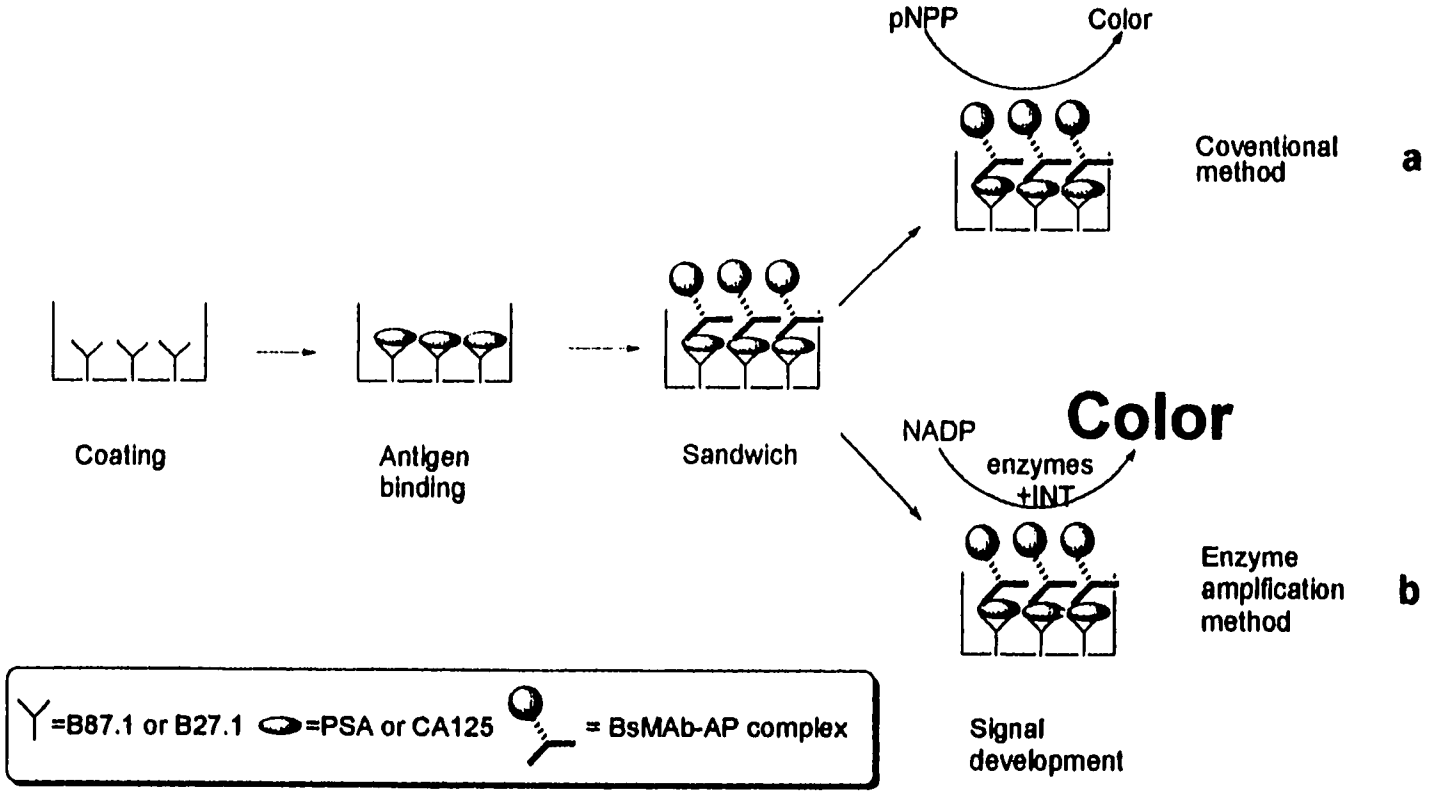


Figure 3.4 BsMAbs-AP based tumor marker EIA

reagent for PSA. PSA was added into B87.1 coated well followed by addition of AP and the supernatant collected for each clone and. After the incubation period, unbound proteins were removed by a washing step and the AP on the PSA sandwich was detected by pNPP at 405 nm. In all, three positive clones with enriched bsMAb anti-PSA x anti-AP were selected out of 33 clones. Of these three clones, P105.2R was selected as the best one based on its consistent high optical density in bsMAb activity assays. Since hybrid hybridomas are highly polyploid (Milstein and Cuello, 1984), they lose chromosomes easily and eventually stop secreting bsMAb. In order to avoid this, we kept recloning until a 100% yield of clones with bsMAb activity was obtained. P105.2R8.2.3 was a monoclonal quadroma obtained by performing recloning by limiting dilution 3 times. The final clone P105.2R 8.2.3, referred as P105, was used for the production of anti-PSA x anti-AP bsMAb by collecting the cell culture supernatant of this quadroma and ascites from the mouse, which was inoculated with this quadroma. About 1 liter of cell supernatant and 20 mL of mouse ascites containing P105 were collected and used in my subsequent studies. In addition, P105 quadroma cells were expanded and stored in liquid N_2 for further use.

Quadromas producing bsMAb can be developed by different methods. The traditional method is a combination of PEG fusion and drug selection (Milstein and Cuello, 1983). PEG fusion is a traditional technique which requires huge population of each fusion partner due to low fusion rate. In addition, a drug selection process is required in order to select the appropriate quadroma from the large population of cells containing both parental hybridomas and their derivatives as shown schematically in Fig 3.3a. This process involves the introduction of additional selective markers to the parental hybridomas, which normally could be achieved in 3 to 6 months. Such markers are necessary because quadromas as well as its parental hybridomas are all resistant to hypoxanthine, aminopterine and thymidine (HAT)-medium. In this quadroma selection process, one hybridoma is maintained with HAT resistance and the other hybridoma is back-selected for HAT sensitivity. The HAT-sensitive second hybridoma is then further modified for the resistance to biological inhibitors or cytotoxic drugs such as ouabain (Staerz, et al., 1986), neomycin (Delau, et al., 1989) etc. When a HAT resistant-ouabain-sensitive hybridoma is fused with a HAT sensitive-oubain-resistant hybridoma, the

quadromas obtained are resistant to both HAT and ouabain and can survive the combined drug selection. Both parental hybridomas will be eliminated in the media containing HAT and ouabain. Unfortunately, these mutation procedures are very time consuming and laborious (Chervonsky, et al., 1988). Moreover, some hybridomas lose the production of antibody during certain mutation steps.

Quadromas were developed using electrofusion in combination with FACS selection as shown schematically in Fig. 3.3b. Since electrofusion is a method which requires a small amount of cells, it can improve the fusion yields and hybridoma recovery by 100-fold (Chang, et al., 1992). FACS selection is based on fluorescence stain on the cell surface and the cells with two fluorescence markers can be selected (Junker and Pedersen, 1981). The staining procedure is short and simple. However, a small cluster (non-specifically aggregated) of unfused hybridomas, which had two different fluorescence signals, might also be selected by the FACS. Since the drug selection procedures were avoided, the fused cells have to be seeded in a very low density to reduce the difficulties in screening of quadromas producing bsMAb. The fusion and FACS selection were done in one day without incorporating a selection marker into hybridomas before the quadroma development. Three quadromas secreting bsMAb anti-PSA x anti AP were obtained respectively in one month, which would have required 3 to 6 months by the traditional method (Fig. 3.3a). Thus, the procedures I used here were simple and efficient. It saved time and reduced cost otherwise spent on intensive tissue culture work using drug selection. It also avoids the use of toxic chemicals (Stratieva-taneeva, et al., 1993) and the risk of losing the antibody producing hybridoma during the drug selection period (Staerz, et al., 1986).

3.2.2. Mimetic ligand based affinity purification of monospecific and bispecific MAbs as immune complexes

The quadroma P105 described above secretes not only bispecific MAb containing the recognition sites for PSA and AP on the same molecule, but also bivalent monospecific antibodies against PSA or AP alone. The former may interfere in the bsMAb based immunoassay, especially at the ultrasensitive level, by competing with the

bsMAb for the binding site on the PSA, thus competing with or diminishing the ability of bsMAb binding. Separation of the bsMAb anti-PSA x anti-AP from monospecific anti-PSA antibody should enhance the bsMAb PSA immunoreaction and improve the limit of the detection.

As discussed previously, the DE-52 method used for the purification of bsMAb lacks specificity and may not adequately resolve the monospecific anti-PSA MAb. Therefore, a novel mimetic, ligand-based affinity purification of the bsMAb, virtually free of the monospecific anti-PSA MAb was investigated and developed. Dye-ligand affinity chromatography has been used to purify many proteins (Linder et al., 1989; Miribel, et al., 1988) due to its "pseudo-specific" binding to different proteins in place of the natural biological ligands, in a selective and reversible manner. The purification of certain proteins can be easily achieved in one of two ways using this matrix and conventional low-pressure chromatography (Miribel, et al., 1988), which can be achieved. One of purification method is by a positive mode wherein the target protein is bound to an absorbent but contaminants are washed off from the absorbent bed as unbound proteins. The bound proteins are subsequently eluted with a suitable eluent containing solutes that compete with the dye-protein interaction. The other is a negative mode method, wherein the contaminants are bound and the target protein passes through the adsorbent bed along with the washing buffer, as unbound protein. In my experiments, the positive mode was chose for the purification of the anti-AP IgG as enzyme bound immune complexes, which was accomplished with a MIMETIC blue A6XL absorbent. The MIMETIC blue A6XL absorbent consists of a blue chromophore linked to a functional phosphoric acid group and demonstrates a high affinity with calf intestinal alkaline phosphatase (Lindner et al., 1989). Since the anti-AP MAb P92 bound the enzyme and yet exhibited enzyme activity, I hypothesized that the mimetic affinity column could co-purify the antibody (monospecific anti-AP as well as MAb with one anti-AP paratope) along with its ability to purify free enzyme. This seemed possible since the antibody likely bound the enzyme away from its phosphatase activity site.

3.2.2.1. Chromatography of free AP or anti-AP MAb alone on MIMETIC blue A6XL column

MIMETIC blue affinity absorbents have been used to purify AP from calf intestine. To test whether these absorbents selectively bind AP, chromatography of commercial crude alkaline phosphatase alone and MAb P92 alone were performed independently as described in detail in section 2.5. Briefly, the sample was extensively dialyzed against Tricine-NaOH buffer to remove all sulfate and phosphate, which inhibit AP binding to the dye matrix. The sample in the Tricine buffer was then applied on the mimetic blue column and the column was washed using Tricine buffer to remove unbound proteins. Elution was monitored by a spectrophotometer at 280 nm. After the absorbance at 280 nm returned to baseline, bound protein was eluted by Tricine buffer containing 5 mM phosphate. The AP activity was detected in various fractions by using substrate pNPP and measuring optical density of the final color product. The MAb P92 activity in the same fractions was detected by an ELISA as described earlier. Comparing elution profiles from both experiments (Fig. 3.5) shows that the MIMETIC blue A6XL absorbent selectively and reversibly binds to AP but not free anti-AP MAb P92. A mild elution condition using Tricine buffer containing only 5 mM of phosphate effectively elutes most of the AP bound to the column.

3.2.2.2. Purification of MAb-AP immune complex

As shown in Fig. 3.5, the MIMETIC blue affinity absorbents selectively bind to AP but failed to bind the free anti-AP MAb. Theoretically, the P92 antibody and the AP antigen forming an enzymatically active immune complex could still bind the affinity column. If AP in the immune complex with anti-AP MAb or bsMAb could bind to MIMETIC blue absorbents, this should allow resolution of the whole complex from other protein contaminants. To test this, a similar experiment as described above was performed to purify MAb P92-AP immune complex formed by mixing 2 mg of crude AP with 1.2 mg of MAb P92. After the sample was applied on the column, the column was washed with Tricine buffer to remove the unbound proteins. Finally the bound proteins were eluted by Tricine buffer containing phosphate and the fractions collected were assayed for the P92-AP immune complex activity using the same assay for P92 MAb

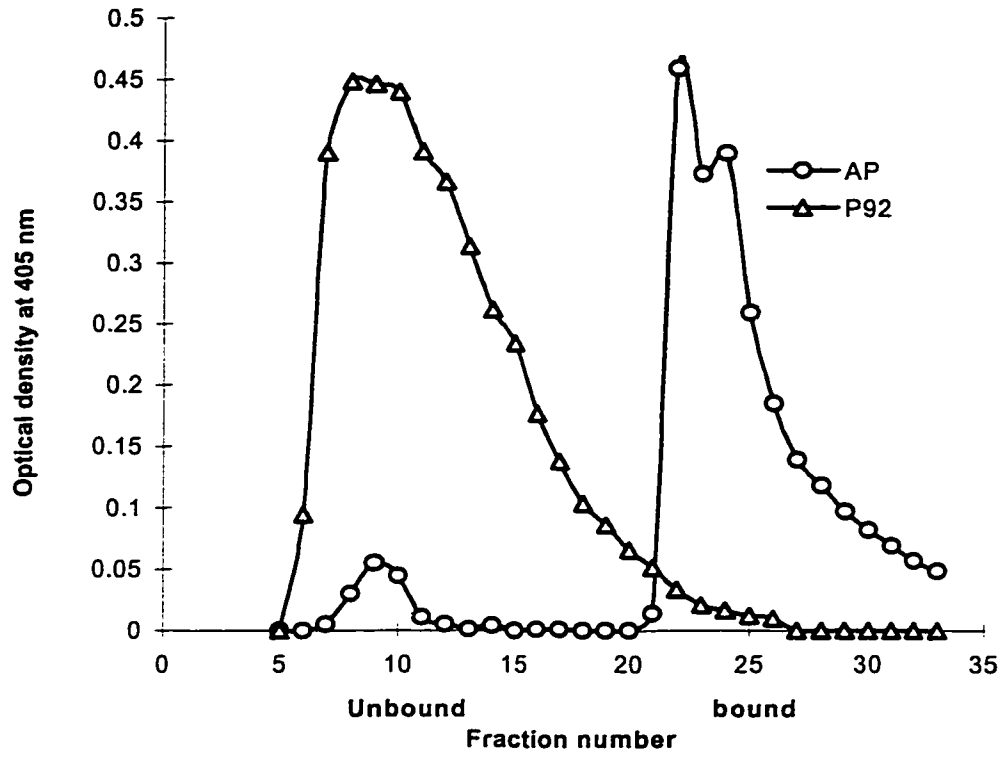


Figure 3.5 Chromatography of a free AP and free anti-AP MAb on mimetic blue A6XL column. A 1 mg sample of crude AP (50U/mg solid) or 1.5 mg of anti-AP MAb (P92) in 10mM Tricine-NaOH buffer pH 8.5 was applied at a flow rate of 3 ml/hr to a column containing 2 ml of mimetic blue A6XL absorbent pre-equilibrated with the same buffer. The column was washed at the points indicated as follows: (unbound) 10 mM Tricine-NaOH buffer pH 8.5, (bound) 5 mM KH_2PO_4 in 10 mM Tricine buffer applied at fraction 18th. Fractions were analyzed for AP activity and anti-AP IgG (P92) activity (optical density at 405nm).

described above, excluding the last step which involves addition of AP. The elution profile (Fig. 3.6) indicated that MAb P92-AP immune complex bound to mimetic blue absorbent and was not washed out by Tricine buffer. However, after the elution using Tricine buffer containing phosphate, both MAb P92 and AP activity were detected in the bound fraction. SDS-PAGE was then performed under reducing condition to locate both MAb P92 and AP (Fig. 3.7). The bands in lane 3 and 4 represents the unbound protein (unbound fraction) and the purified AP (bound fraction) from the chromatography of crude AP (Fig. 3.5) using mimetic blue column, respectively. The bands in lane 5 represents the unpurified crude AP. The multiple bands of purified AP (lane 4) corresponds to MW 50-70 KD. This indicated that the glycoprotein AP has subunits and the MW of the whole molecule is 100-140 KD, which was confirmed by data from several manufacturer. Comparing lanes 3, 4 and 5, it suggested that the mimetic blue column selectively binds AP. The bands in lane 6 and lane 7 represente unbound (proteins) and bound fraction (purified P92-AP immune complex) collected from the chromatography of the mixture of P92 with crude AP, respectively. Lane 8 represents unpurified mixture of P92 and AP. Comparing the bands in lane 6, 7 and 8, the bands in lane 7 indicated that the majority of contaminants were removed and several clear bands were seen, which represented AP and the MAb heavy and light chains at expected MW (Fig. 3.7).

3.2.2.3. Chromatography of free bsMAb and bsMAb-AP immune complex

As demonstrated above, the bivalent anti-AP MAb can be co-purified together with AP as an immune complex using MIMETIC blue column. In this experiment the monovalent bsMAb anti-PSA x anti-AP together with AP was tested to see whether it could bind the column in the same fashion as the bivalent monospecific P92-AP immune complex.

BsMAb anti-PSA x anti-AP secreted by quadroma is contaminated with monospecific antibody anti-PSA, which interferes in bsMAb based immunoassay. The removal of monospecific anti-PSA antibody from the bsMAb anti-PSA x anti-AP will be a crucial step to ensure the specificity and sensitivity of the ultrasensitive assay for PSA using bsMAb P105 as a tracer. The chromatography of bsMAb P105 alone or bsMAb

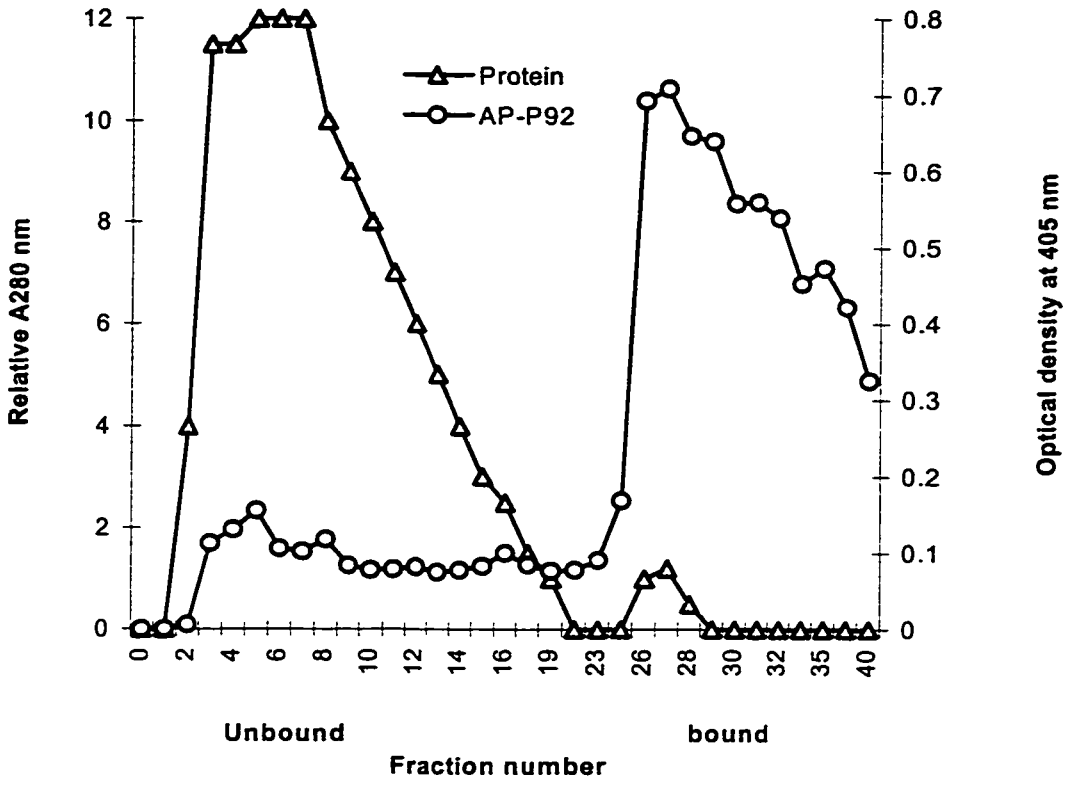


Figure 3.6 Chromatography of MAb-AP immune complexes on mimetic blue column. A 2 mL sample containing a pre-incubated mixture of 2 mg of crude AP (100 U) and 1.2 mg of anti-AP MAb (P92) to form antigen-antibody complexes was purified using the same procedures as described for AP alone. The column was washed at the points indicated as follows: (unbound) 10 mM Tricine-NaOH buffer pH 8.5, (bound) 5 mM KH_2PO_4 in 10 mM Tricine buffer at fraction 19th. Fractions were analyzed for the activity of P92-AP immune complexes (optical density at 405 nm) and protein (Relative A_{280} nm).

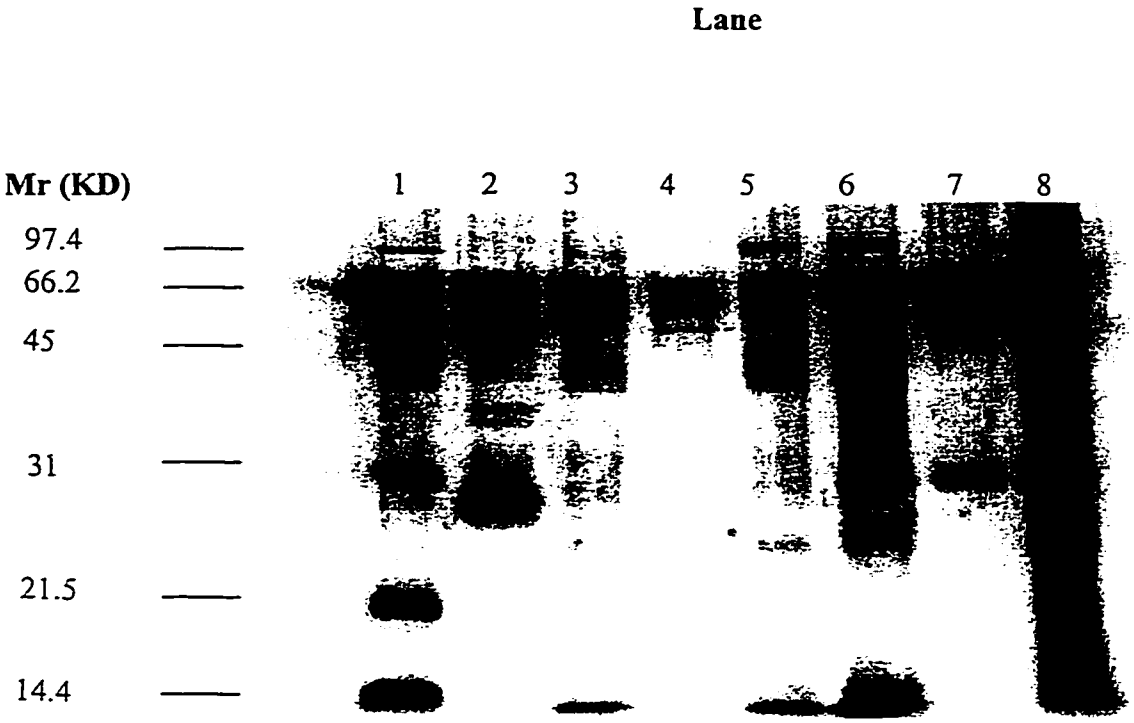


Figure 3.7 SDS-PAGE analysis of the AP and MAb-AP immune complex. Samples were electrophoresed on 12% SDS poly-acrylamide gels and then the proteins were visualized by silver staining. Lane 1: Low molecular weight markers for SDS-PAGE; Lane 2: mouse IgG heavy (50 KD) and light chain (25 KD); Lane 3: unbound fractions from the chromatography of crude AP; Lane 4: bound fractions from chromatography of crude AP; Lane 5: unpurified AP; Lane 6: unbound fractions from purification of P92-AP immune complex; Lane 7: bound fractions from the purification of P92-AP immune complex; Lane 8: unpurified AP-P92 mixture.

P105-AP immune complex was performed on a MIMETIC blue A6XL column as described in section 2.6. The fractions collected were assayed for P105 and P105-AP immune complex activity using the procedures described for screening the quadroma producing bsMAb anti-PSA x anti-AP. The elution profile of bsMAb P105 (Fig. 3.8) showed that most of bsMAb P105 was recovered in the pool of the unbound fraction, which suggested that mimetic blue column did not bind to bsMAb P105 alone. In contrast, majority of bsMAb P105-AP immune complex was recovered in the bound fraction. This result indicated that MIMETIC blue A6XL absorbent bound the bsMAb-AP immune complexes selectively wherein the antibody was tethered to AP and hence the column matrix with its univalent binding arm against AP. Monospecific anti-PSA antibody and other protein contaminants without AP bound on them would thus be separated from bsMAb-AP immune complex.

It is pertinent to note that the P105 quadroma also secretes monospecific anti-AP antibodies, which would be co-eluted with the bsMAb as immune complexes. This monospecific anti-AP antibody enzyme complex is not likely to interfere with the bsMAb based PSA assay. The only requirement is that an excess amount of free AP has to be added to saturate both anti-AP MAb and anti-AP bsMAb. There was no background problem in our ELISA assays due to the presence of the monospecific anti-AP MAbs with the enzyme bound to the paratopes. This simple method allowed us to purify the required bsMAb under mild conditions.

3.2.2.4. Simplified procedure for bulk purification of bsMAb-AP immune complex

In the previous experiment, AP and bsMAb P105 were loaded on the mimetic blue column separately by utilizing a pump at the slow flow rate of 3 mL/hr to ensure the maximum binding between AP and the ligand, as well as the binding between bsMAb P105 and AP. Instead of loading the sample onto the mimetic blue affinity column at very low flow rate, the crude AP was incubated with the absorbents in a flask overnight at 4 °C in batch mode with gentle shaking. Then the absorbents were transferred to an empty column and the excess unbound AP as well as other contaminants were collected and assayed for AP activity. The unbound fraction with high AP activity could be reused directly. The absorbents were then washed extensively with 10 volumes of Tricine buffer,

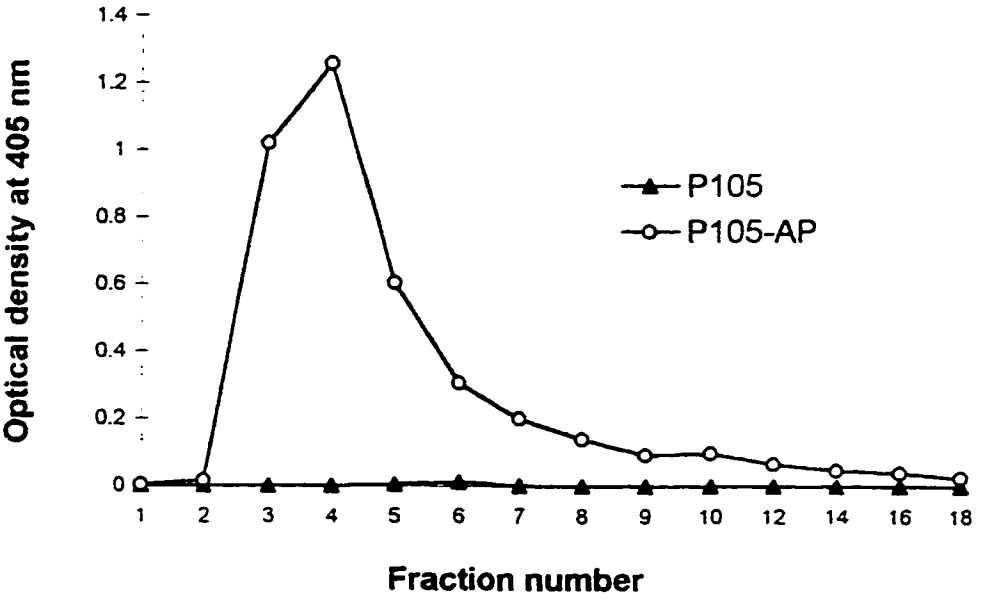


Figure 3.8 Chromatography of free anti-PSA bsMAb and bsMAb-AP immune complexes on mimetic blue column. The samples were loaded separately on to a 10 mL mimetic blue column at flow rate 4 mL/hr overnight at 4°C. After all the unbound fractions were collected, the column was washed with 100 mL of the 10 mM Tricine buffer. The bound fractions of anti-PSA bsMAb (P105) alone or bsMAb P105-AP immune complexes were eluted by using 5 mM phosphate in Tricine buffer (pH 8.5). Fractions of 2 mL were collected and assayed for free anti-PSA bsMAb (P105) and anti-PSA bsMAb/AP immune complex (P105-AP) activities (optical density at 405 nm).

which took about 30 min. Incubation of bsMAb P105 with the absorbents pre-saturated with AP was carried out in a flask at room temperature for 45 min, the absorbents were then transferred back to the column again. The unbound fraction was then collected and assayed for bsMAb P105 activity. If high activity of bsMAb P105 was found in the unbound fraction, it was reused. After another washing step, the bsMAb-AP immune complex was eluted with Tricine buffer containing 20 mM phosphate. Twenty fractions were collected in 30 min. The elution profiles of bsMAb P105-AP immune complex with both short protocol (section 2.8) and long protocol (section 2.6) were similar.

3.2.2.5. Purification of commercial polyclonal IgG conjugated with AP

The MIMETIC blue A6XL affinity chromatography was also investigated to see if it could be either used to purify the widely used chemically linked commercial polyclonal antibody-AP conjugates or to qualify the purity of commercially available conjugates. Purification of commercial polyclonal IgG conjugated with AP was performed on a 10 mL mimetic blue column as mentioned earlier. Fractions (2 mL) were collected and the protein, AP, and conjugate activities in each fraction were assayed. The elution profile of AP conjugates showed that this blue dye absorbent bound to AP conjugates almost quantitatively similar to AP alone (Fig. 3.9). However the results from SDS-PAGE showed that AP-IgG conjugates are heterogeneous in size as indicated by the presence of multiple bands on the top of Lane 5 in Fig 3.10. The fractions of AP-IgG conjugates from MIMETIC blue column were pooled as unbound and bound fractions. The pooled fractions were analyzed for AP-IgG conjugate activity as described in section 2.12.2. The ELISA assay demonstrated that there was very little AP enzyme activity in the unbound fractions. The results of the SDS-PAGE showed that chemically crosslinked AP-IgG conjugates are heterogeneous with a ladder like resolution. Since there were multiple bands on the top of lane 5 in Fig. 3.10, I interpreted them to be due to random crosslinking of AP-IgG heavy chain, and AP-IgG light chain with a number of AP molecules and IgG molecules. The ELISA assay demonstrated that there was very little AP enzyme activity in the unbound fractions, but the results from SDS-PAGE indicated that there were bands corresponding to subunits of IgG and AP molecules in these unbound fractions. This might be due to a fraction of the conjugate, which had lost AP

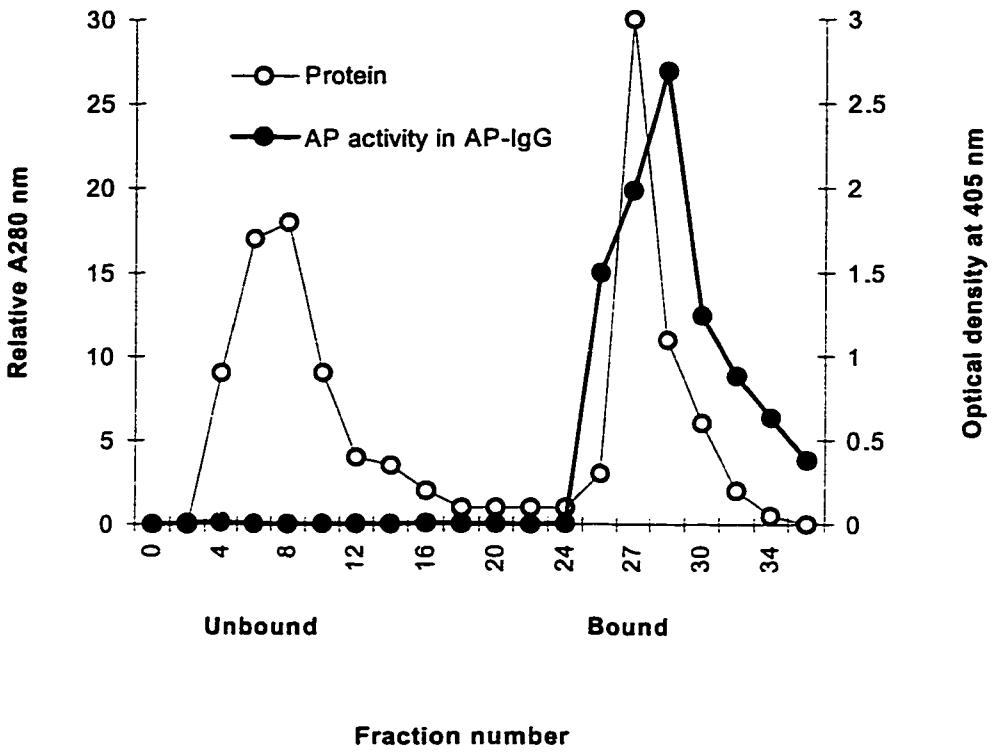


Figure 3.9 Chromatography of commercial polyclonal IgG conjugated with AP on mimetic blue column. 400 μL of polyclonal anti-mouse IgG conjugated with AP (about 1000 U) in 10 mM Tricine-NaOH buffer pH 8.5 (12 mL) was applied at a flow rate of 3 mL/hr to a column containing 10 mL of mimetic blue absorbent pre-equilibrated with the same buffer. The column was washed at the points indicated using: (1) unbound: 10 mM Tricine-NaOH buffer pH 8.5. (2) bound: 5mM KH_2PO_4 in 10mM Tricine buffer applied at fraction 21th. Fractions (2 mL) were analyzed for AP activity in AP-IgG conjugates (optical density at 405 nm). and protein (Relative A_{280} nm).

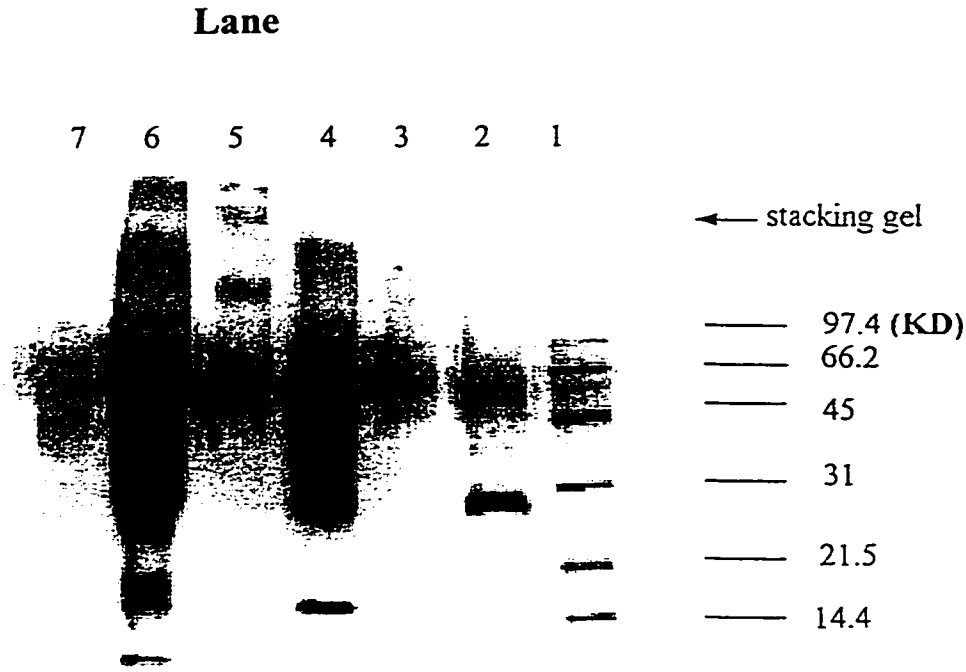


Figure 3.10 SDS-PAGE analysis of fractions collected from the chromatography of AP-IgG conjugates utilizing the mimetic blue column. The electrophoresis was performed on a 12% SDS-PAGE and the proteins were visualized by silver staining. Lane 1: Low molecular weight markers for SDS-PAGE; Lane 2: Mouse IgG heavy and light chains; Lane 3: AP purified from the MIMETIC blue A6XL column; Lane 4: Unbound fractions; Lane 5: Bound fractions; Lane 6: Fractions washed with 0.75 M KCl; Lane 7: unpurified AP-IgG conjugates.

enzyme activity presumably during the chemical conjugation procedures. Thus, the mimetic blue column could be of a great advantage for isolating any functional AP-IgG immunoconjugates made in a laboratory or commercial AP-IgG conjugates with AP activity from the fraction of conjugates without the enzyme activity. This also highlights the unique potential of bsMAbs with an AP binding site giving uniform reproducible enzyme binding as compared with chemical crosslinking procedures which generates random inter- and intra- molecular cross-links and aggregates.

The purification of bsMAb produced by hybrid-hybridomas is a crucial step in the ELISA applications of bsMAb probes. The specific activity and sensitivity of the bsMAb-based immunoassay are affected by the amounts of MAb contaminants. As demonstrated by experiments described above, any contaminants, which do not bind to AP, can be separated from anti-AP bispecific or monospecific antibodies utilizing a MIMETIC blue A6XL absorbent. This unique method allowed us to successfully purify MAb P92-AP and bsMAb P105-AP as enzyme bound immune complexes. In addition, one commercial polyclonal AP-IgG immunoconjugate was purified and qualified by the same chromatography technique.

The following mechanisms might explain why the mimetic blue absorbents can be used to purify the anti-AP MAb-AP and bsMAb anti-PSA x anti-AP-AP immune complexes (Fig. 3.11). 1) There are ligands on the mimetic blue absorbents, which have been found to bind specifically to AP. 2) Both MAb anti-AP and bsMAb anti-AP x anti-PSA can form an immune complex with AP, which are relatively stable in very mild buffer conditions. 3) The binding site of AP with the phosphate analog ligand on mimetic blue absorbents is presumably away from the antibody binding site. As a result, MAb P92-AP and bsMAb P105-AP immune complexes were also bound to the mimetic blue column selectively. Any contaminants not bound to absorbents were washed out by Tricine buffer. MAb P92-AP and bsMAb P105-AP immune complexes were stable in Tricine buffer and still bound to absorbents after this washing step. However, Tricine buffer containing the phosphate ions can bring about desorption of the AP molecules bound to the functional phosphoric acid groups by competing with the binding site. It is worth noting that both MAb P92-AP and bsMAb P105-AP immune complexes are also stable in Tricine buffer containing phosphate and therefore, P105-AP immune complex

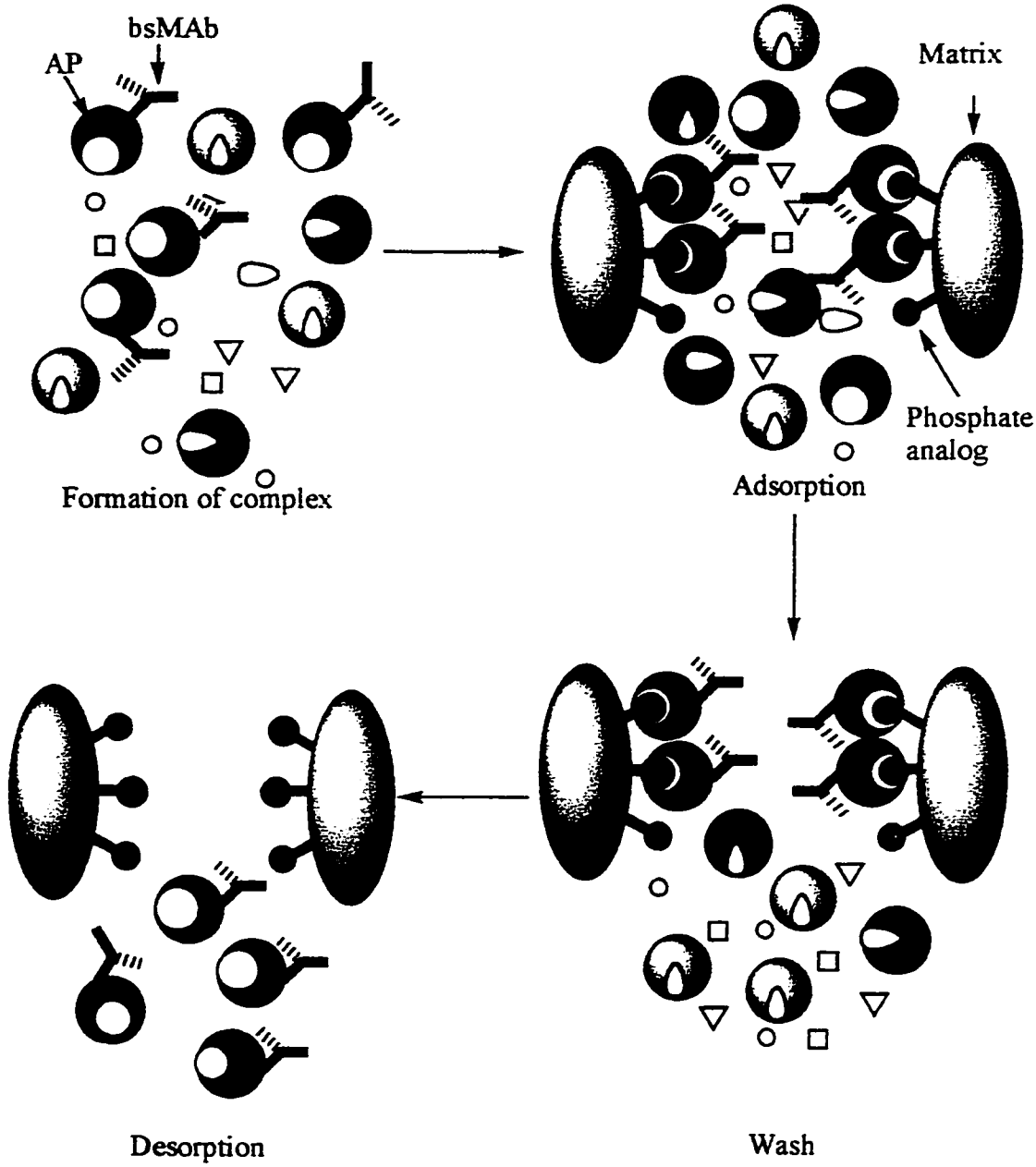


Fig. 3.11 Schemetic representation of purification of bsMAb-AP immune complex using mimetic ligand A6XL absorbents

described in the next section could be used directly in the PSA immunoassay after a simple dialysis step to remove phosphate ions.

3.2.3. BsMAb-AP immune complex based ELISA for the detection of PSA

A lot of effort has been made by clinical investigators during the last few years to develop PSA assays with improved detection limits. The rationale behind developing more sensitive PSA assays is that cancer relapse particularly in patients with total removal of the prostate gland (radical prostatectomy) could be detected earlier if they were monitored with more sensitive immunoassay methods (Yu et al., 1995). In addition, it will be a powerful tool to study the significance of the recent report of the association of PSA in breast cancer. Further, the development of precise immunoprobes would confirm if this molecule in breast cancer is indeed PSA or a related kallikreim. In this section, I described the development of the conventional and ultrasensitive enzyme immunoassays for PSA using bsMAb-AP immune complex as a tracer.

As a first step, a conventional sandwich assay was developed using bsMAb as the tracer with standard or conventional AP substrate pNPP.

3.2.3.1. Development of conventional one step ELISA

The bsMAb anti-PSA x anti-AP was successfully purified as a bsMAb-AP immune complex by using mimetic blue absorbents. To test whether this immune complex was useful as a tracer in a one step ELISA for PSA, several experiments were performed. The principle of the PSA assay is outlined schematically in Fig. 3.4. Briefly, samples containing PSA and P105-AP immune complexes were added into an ELISA plate coated with anti-PSA MAb, B87.1, followed by a certain time of incubation. During the incubation, PSA in the sample was captured by B87.1 MAb immobilized on the ELISA plate and the P105-AP immune complex in the well bound to the captured PSA, resulting in the formation of a sandwich quaternary complex consisting of B87.1, PSA and P105-AP. Following a final wash to remove unbound proteins, the AP substrate was

added. The AP bound in the sandwich converted the conventional substrate, pNPP, to a colored product, which could be detected at 405 nm.

At first, the variation in the incubation time of the sandwich formation and color developing time on the intensity of signal were examined in the presence of excess amounts of PSA (130 μg/L) and a 1: 20 diluted P105-AP immune complex. This one step PSA assay was performed with incubation times from 3 min to 30 min, and the color was developed by incubating pNPP from 5 to 30 min. The results in Fig. 3.12 shows that a optical density 3.0 was achieved in a 3 min incubation plus 20 min color development. In contrast, the optical density was about 1.5 with a 30 min incubation plus 5 min color development. This indicated that the immune reaction among MAb B87, PSA, P105-AP immune complex was very fast but longer color development time was required for the enzyme-substrate reaction. Therefore, the optimal condition for the one step PSA assay was determined from this experiment to be 5 min incubation plus 20 min color development.

To determine the linear range for this bsMAb based PSA assay, a dose-response curve was studied using P105-AP obtained from MIMETIC blue A6XL column as a tracer. Three different dilutions of P105-AP (1:100, 1:50 and 1:25) were used in this assay to determine the optimal dilution of the P105-AP immune complex. Briefly, samples containing different amounts of PSA together with three dilutions of the P105-AP immune complex were added to the MAb B87 coated plate. Then the plate was incubated for 5 min followed by a washing step. The color was then developed by using pNPP for 20 min. This assay was performed using each sample in triplicate. As shown in Fig. 3.13, the 1:25 dilution of bsMAb P105-AP gave the highest signal and a fairly linear response up to 100 μg/L. In addition, when the concentration of PSA in the sample was lower than 50μg/L, the P105-AP immune complex could be used at 1:50 dilution since there was not much difference in the optical density between using the 1:25 and 1:50 dilution of P105-AP. However, when the P105-AP immune complex was diluted to 1:100, the signal intensity of those samples with more than 25 μg/L of PSA were greatly decreased, and the linear range was also reduced to 0-25 μg/L. Linear range is an important parameter in a PSA assay because the PSA concentration in patients vary from 4.0 to 6000μg/L. To determine the PSA precisely in a short range assay, the serum

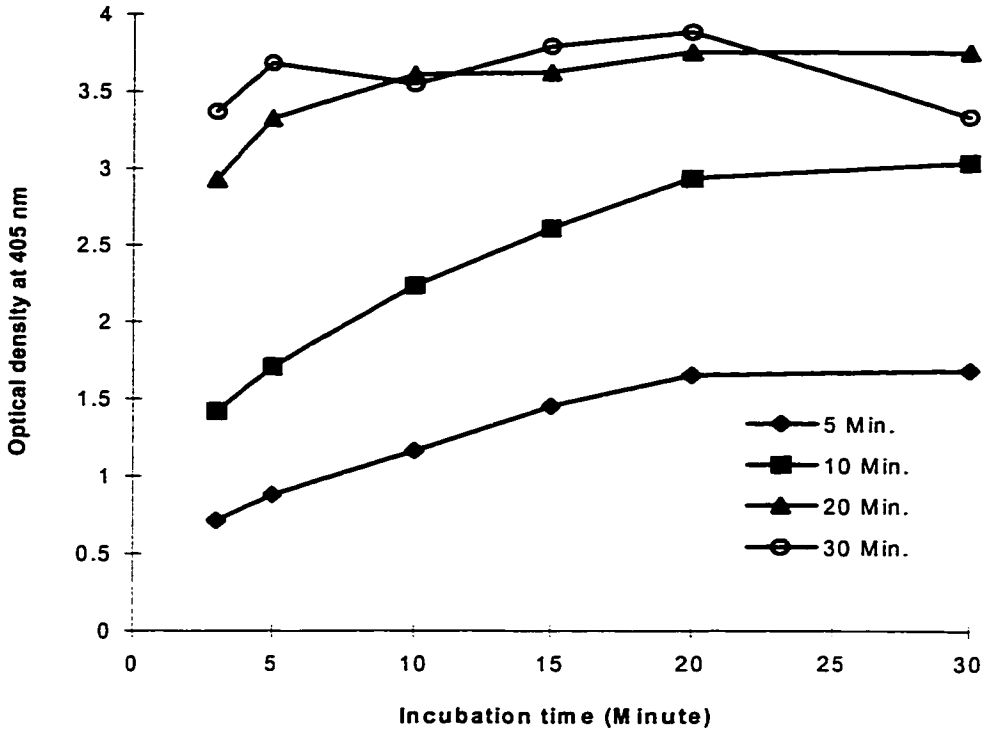


Figure. 3.12 Evaluation of the kinetics of one step conventional assay for PSA. in different color developing time. The sample containing PSA and bsMAb P105-AP immune complex was incubated for different times (5, 10; 20 and 30 min). The activity of bound AP in the ternary complex was determined by pNPP. Finally, the color was developed for 5 min ◆; 10 min ■; 20 min ▲ and 30 min ○. The optical density was determined at 405nm. Each point represents a mean of triplicate measurements. Each point represents the mean of quadruplicate. CV% are from 0.9 to 9.8.

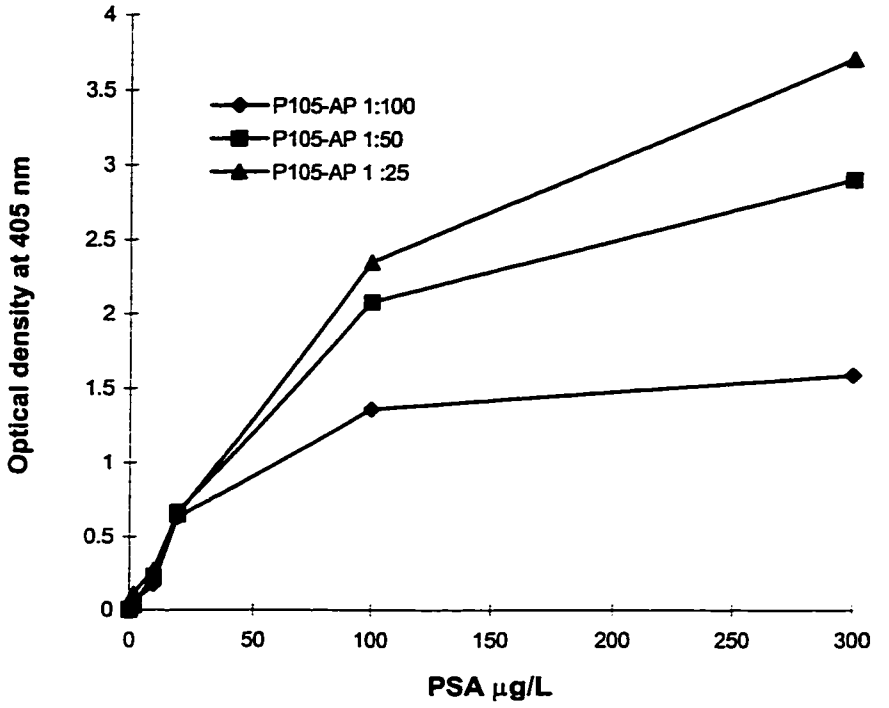


Figure. 3.13 Determination of the optimal dilution of bsMAb-AP immune complex for one step conventional assay for PSA. The ELISA plate was coated with B87.1 at 1 µg/100 µL and blocked with 5% skim milk. Various dilutions of bsMAb P105-AP immune complex were tested with different concentrations of PSA. PSA in the samples were determined by measuring the activity of bound AP at 405 nm as described in section 2.13. Each point represents the mean of triplicate measurements. CV% are from 1.2 to 9.4.

sample would have to be diluted to allow the PSA level to fall into the linear range of the assay.

Different blocking matrixes were used to test whether they interfered with the assay, particularly when the sample contained low amounts of PSA. To prepare the PSA standards at different concentrations, 1 % ovlbumin (OVA), 1% skim milk, or 1% bovine serum albumin in PBS were used separately as matrixes to dilute the PSA sample. The one step conventional PSA was performed using conditions described above. As demonstrated in Fig. 3.14, there was no significant difference in the optical density reading when BSA, skim milk or OVA was used. The results represent the mean of PSA samples in triplicate and the intra CV was from 0.13 to 6.4 %.

Finally, the lowest limit of detection (LLD) in the one step conventional PSA assay was determined using prostate specific antigen reference material obtained from the College of American Pathologists at concentrations of 0-25 μg/L. The LLD is the least amount of analyte that can be detected with a predetermined confidence usually at 95%. It is defined as the unit value of antigen above the zero value signal plus 2 SD. In this experiment, the LLD was determined using 20 replicates. The standard curve for the detection of PSA is illustrated in Fig. 3.15, which was constructed using 5 calibrators with different concentrations of PSA from 0 to 25 μg/L. The assay was performed as described above. The PSA bound to B87 MAb, which was immobilized on the ELISA plate, was detected using P105-AP immune complex (1:25 dilution). The activity of the AP bound on the sandwich was determined with pNPP. The twenty replicates of PSA free serum gave a mean optical density (OD405) Value (X), of 0.071 and a standard deviation (SD) of 0.002. The cut-off value for this assay was chosen as X +2 SD = 0.075, and accordingly, the lowest limit of detection of 0.05 μg/L was determined, which was well below the mean PSA level (4.0 μg/L) in male blood plasma (Myrtle et al., 1986; McCormack et al., 1995).

BsMAb anti-PSA x anti-HRPO was previously investigated in our lab by fusing hybridoma B80.3 (anti-PSA) with a hybridoma secreting anti-HRPO MAb. The bsMAb-based one step PSA assay reported by Kreutz, F. T. and Suresh, M. R. (1997) showed an excellent correlation with the Hybritech Tandem-E PSA assay which was approved by the FDA for monitoring of patients prostate cancer. As I described above, the bsMAb

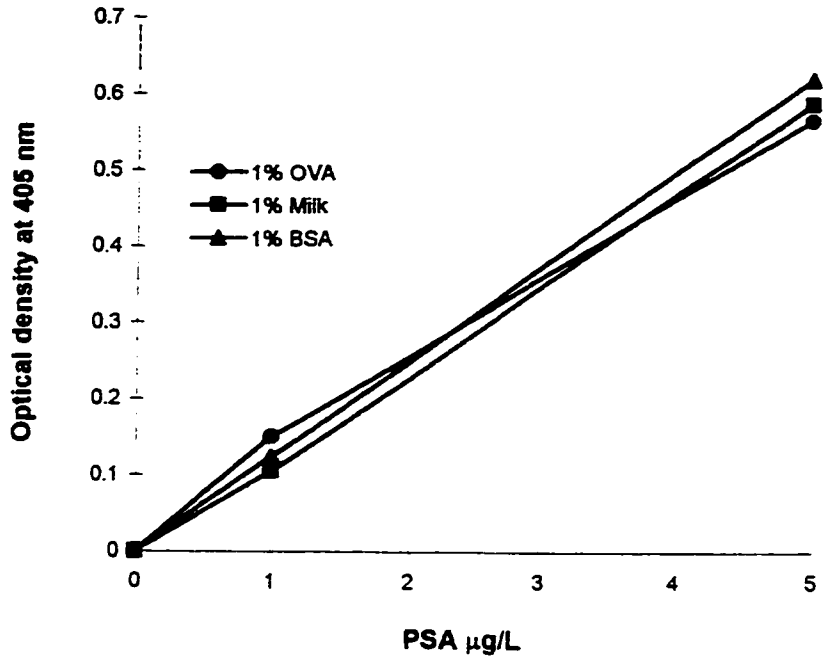


Figure. 3.14. Interference of blocking proteins used in the assay for detection of low amounts of PSA. 1% OVA; 1% skim milk and 1% BSA were tested separately in one step conventional assay for PSA. The PSA in different matrices was determined by measuring bound AP activity as described in section 2.13. Each point represents a mean of triplicate measurements. CV% are from 1.3 to 6.4.

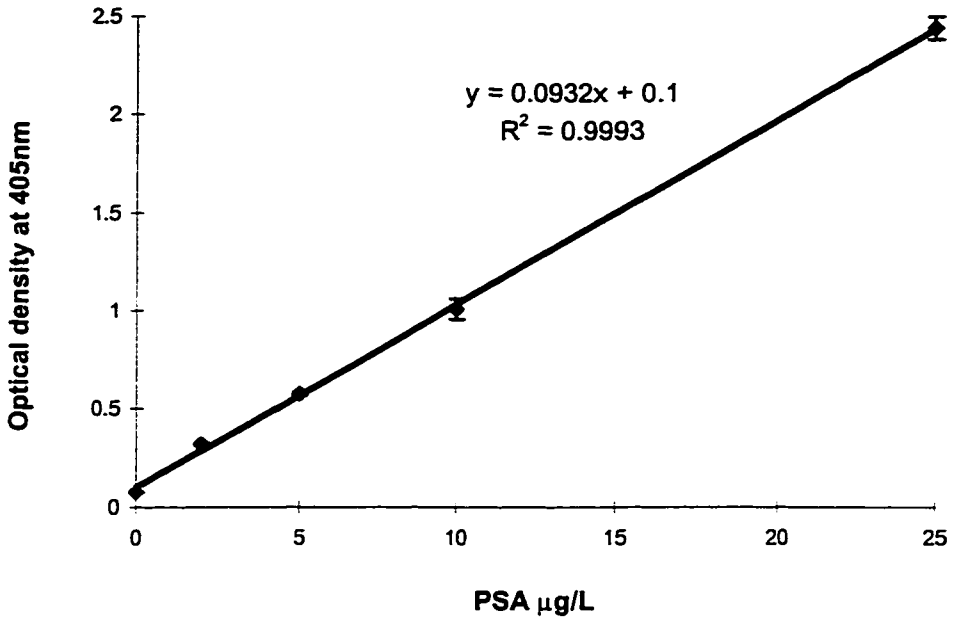


Figure 3.15 Standard curve for PSA in a one step conventional assay. The plate was coated with B87.1 at 1µg/100 µL and blocked with 5% skim milk. Samples containing PSA were added into the plate together with 50 µL of bsMab P105-AP immune complex (1:25 dilution in 1% OVA) and incubated for 20 min at room temperature. PSA was determined by measuring the activity of bound AP at 405 nm by adding substrate pNPP. Optical density at 405 nm was recorded and plotted vs PSA µg/L. Points represent 20 replicates ± SD.

anti-PSA x anti-AP was developed using the same hybridoma B80.3 and a similar PSA assay was developed using the P105-AP immune complex. The bsMAb P105, is assumed to exhibit similar properties to bsMAb anti-PSA x anti-horseradish peroxidase, and needs to be confirmed by further investigation. The detection limit of most commercial PSA assays is between 0.05-0.3 μg/L. It is obvious that the one-step conventional prototype PSA assay using P105-AP immune complex has a sensitivity generally comparable to current commercial assays. In addition, this assay avoids using radioisotopes and the signal is easy to detect using simple spectrometry.

In most conventional PSA assays, both isotopic and non-isotopic labels have been used. RIA, which detects PSA as low as 0.05 μg/L, had been the most useful clinical method in the past. However, because of problems including a short shelf life, the ionizing radiation hazards, a requirement for complex measuring equipment, and long assay times, RIA has been replaced with other methods using non-isotopic labels. ELISA is the most convenient method of immunoassays. Unfortunately, the chemical conjugation of enzyme to MAb often results in a poorly defined mixture of simple and complex, active and inactive species in various proportions. Some MAbs or enzymes might lose their activity during the chemical conjugation procedure (Ishikawa, 1996). In addition, antibody-enzyme conjugates are often much less stable than the antibody or enzyme before the chemical modification. Recently, FIA has become a popular immunoassay because of its high sensitivity. The time resolved fluorometric immunoassay for PSA is able to detect 0.0003 μg/L (Ferguson et al., 1996). However, this assay needs special instruments, which are not available in most clinical laboratories. Therefore, a rapid and more sensitive EIA will be more applicable in screening a large number of samples.

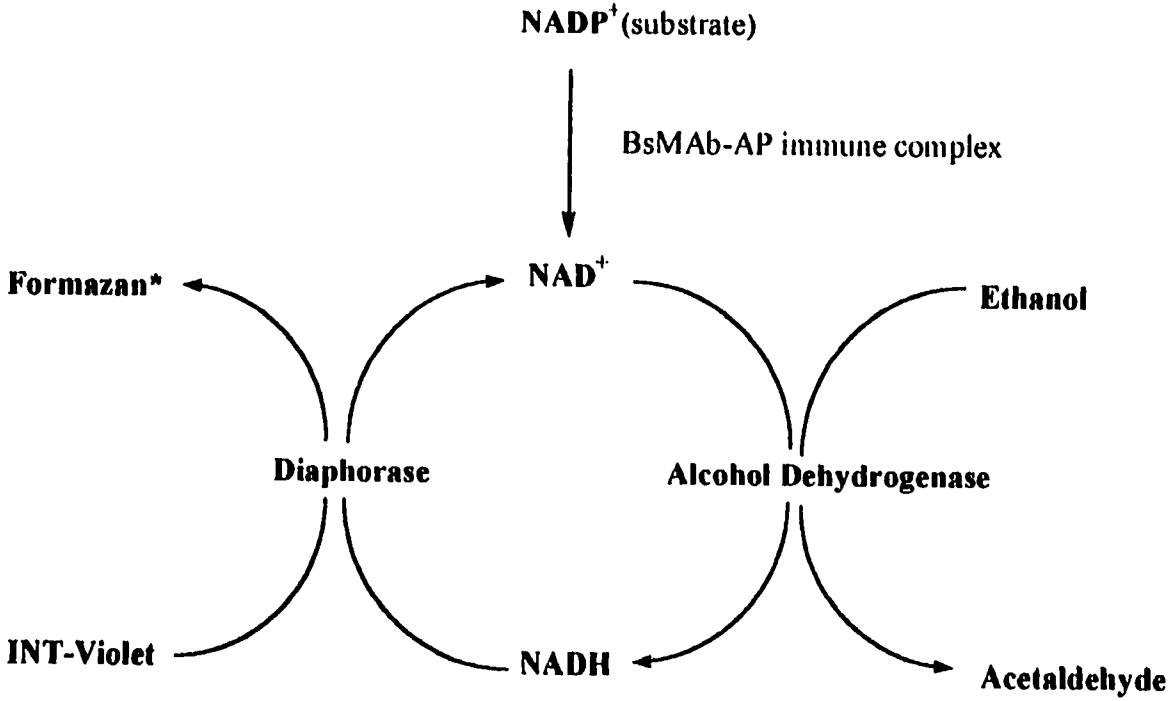
3.2.3.2. Construction of an ultrasensitive ELISA based on bsMAb-AP immune complex for PSA

As mentioned previously, PSA immunoassay has been used to monitor patients who have undergone radical prostatectomy. An ultrasensitive PSA assay could provide information related to recurrence of metastasis at a very early stage. Any small changes of PSA level in these patients might be detected by an ultrasensitive PSA assay. Therefore,

The development of an ultrasensitive ELISA based on the bsMAb-AP immune complex for PSA was further investigated.

The bsMAb-AP immune complex based conventional one step PSA assay was modified by the use of a substrate amplification system to replace pNPP in the final step of conventional assays. Johannsson et al., (1985) reported a highly sensitive colorimetric enzyme immunoassay. The mechanism of the amplification system is shown schematically in Fig. 3.16. The enzyme label (AP) on the immunoconjugates bound on the ELISA plate, converts its substrate NADP into a catalytic activator NAD, which activates a strictly NAD specific redox cycle and results in the formation of a colored formazan as the end product. The amount of end product is proportional to the AP enzyme label bound in the complex formed in the immune reaction. By measuring the absorbance of the colored product, the concentration of the analyte would be determined. It is estimated that each molecule of NAD obtained by cleavage of NADP by AP amplifies the signal 600 times entering the cyclic enzyme coupled reactions (Cook and Self, 1993).

The one step amplified PSA assay was performed using bsMAb-AP immune complex under the same condition as the conventional assay described above. However, to ensure that the antigen-antibody reaction reaches equilibrium, the incubation time was increased to 2.5 hr. The color was developed as illustrated schematically in Fig. 3.16. In brief, samples containing different amounts of PSA were added to a B87 MAb coated plate, and then P105-AP diluted 1:25 was added to the same wells. The plate was incubated and then washed. NADP, the substrate for AP, was then added to the plate and incubated for 15 min. Finally, cycling solution containing diaphorase, an alcohol dehydrogenase and INT-violet was added and incubated for 20 min. The optical density was recorded at 490 nm by an ELISA reader. As shown in Fig. 3.17, application of the amplification system to the bsMAb-AP based ELISA for PSA resulted in a significant increase in signal intensity and detection sensitivity over that achievable with the conventional substrate pNPP. In general, a more sensitive assay is associated with a high background problem. The affect of color development time was evaluated. As show in Fig 3.18, the background of the optical density increased when color development time



*Colored end product

Figure 3.16 ELISA amplification system (refer to Bates, 1987)

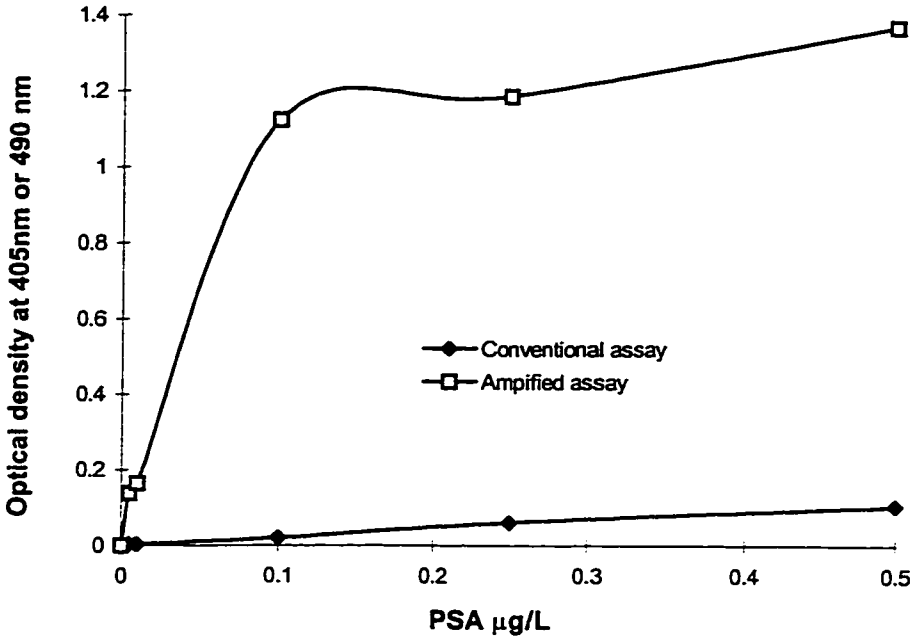


Figure 3.17 Comparison of one step conventional and amplified ELISA for PSA. The plate was coated with B87 at 1 µg/100 µL and 100 µL of PSA together with bsMAb P105-AP immune complex was added and incubated for 2.5 hr at room temperature. PSA was determined by measuring the activity of bound AP using conventional and amplifed methods as described in 2.13. Optical density at 405 nm or 490 nm was plotted vs PSA concentration. Each point represents the mean of three determinations. CV% are from 0.4 to 6.4.

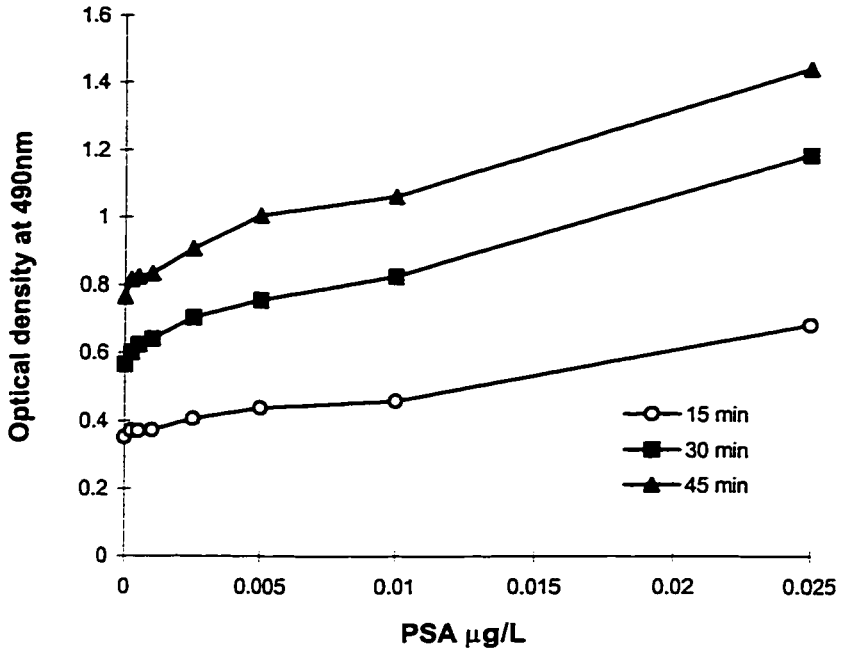


Fig.3.18 Evaluation of the color development time in amplified PSA assay. Different concentrations of PSA samples were used in the amplified ELISA for PSA as described previously. The optical density of color was determined in different time (15 min, 30 min and 45 min) after the cycling solution was added. Each point represents the mean of triple determination in the same assay. CV% are from 0.7 to 9.5.

was prolonged. However, the signal to noise ratio was also increased. Additional studies should be done to decrease the background of the signal to acceptable levels.

The LLD in this amplified PSA assay was further determined using a standard curve for low concentration of PSA. Twenty PSA free samples (blank) gave a mean optical density (OD405) value (X), of 0.7712 and a standard deviation (SD) of ± 0.0079. The cut-off value for this assay was chosen as X+ 2SD = 0.786, and, accordingly, the lowest limit of detection was determined to be 0.00025 μg/L or 250 fg/L. The amplified PSA assay was performed in triplicate with intra-assay CV% of 0.8-14%. The interassay CV% needs to be further evaluated. As described above, when the pNPP was used as the substrate, the lowest limit of detection was 0.05 μg/L. However, using the substrate amplification procedure, this limit dropped to 0.00025 μg/L, indicating a 200 fold increase in sensitivity over that achievable with pNPP. Cook and Self (1993) used fluorimetric determination of resorufin, formed from nonfluorescent resazurin with the same enzyme amplification system used for detection of proinsulin in a MAb-AP conjugates based immunoassay. Since the fluorescent system greatly extended the range of measurement, they were enable to measure 17 amol/L of proinsulin. Generally, bsMAb/AP immune complex has several advantages over MAb-AP covalently conjugated by chemical manipulation. The noncovalent crosslinking between bsMAb and AP molecules fully retains bsMAb antigen-binding capacity. In addition, the noncovalent crosslinking has no batch to batch variation and the preparation is simple which does not require enzyme with high purity. Therefore, using the bsMAb/AP immune complex, the sensitivity of PSA might be further increased by using the fluorimetric determination of resorufin.

The bsMAb-AP immune complex based PSA assay described above has two important features. First, enzyme labeling of the detecting antibody was not achieved by chemically conjugation but by the formation of the bispecific antibody-antigen (enzyme) immune complex. This ensured uniform labeling with one AP per bispecific MAb to result in the development of a rapid conventional assay which might be used in rapid screening, diagnostics and monitoring of prostate cancer. Second, a substrate amplification system for the enzyme AP was used which resulted in a significant increase in sensitivity of the immunoassay. The ultrasensitive PSA assay has the potential to

detect ultra-low levels of PSA in monitoring of patients who have undergone radical prostatectomy. Thus, minute increases in serum PSA during the remission phase could indicate recurrence due to micrometastasis and allow early intervention. Furthermore, this assay could also be applied to non-invasive methods such as capillary blood (Hoffman et al., 1996), sweat or other body fluids (Yu and Diamandis, 1995a, 1995b) which contains very low amount of PSA and for some difficult forensic applications.

3.3. Development and applications of bsMAb anti-CA125 x anti-AP

The successful development of a bsMAb-AP based ultrasensitive ELISA for PSA encouraged us to look for another bsMAb anti-CA125 x anti-AP. As reviewed in Chapter 1, CA125 is a tumor marker (tumor associated antigen) used for monitoring ovarian cancer patients. It has been found that CA125 levels in the serum provide information that relates to the prognosis or metastasis of ovarian cancer (Bast et al., 1983; Frascie et al., 1996) and malignant peritoneal mesothelioma (Simsek et al., 1996).

3.3.1. Generation of quadroma producing bsMAb anti-CA125 x anti-AP

Quadroma producing bsMAb anti-CA125 x anti-AP was developed by fusing the hybridoma P92 (anti-AP) with a well characterized hybridoma B43.13 which produces anti-CA125 MAb with high affinity and specificity (Krantz, et al., 1988). MAb B43.13 has been used in several immunoassay applications (Krantz, et al., 1988; Kreutz and Suresh, 1995). In the fusion of B43.13 with P92, a hybridoma described previously, 200 cells with double fluorescence were selected by FACS and seeded into 96 well cell culture plates at 1 cell per well. Sixteen clones were obtained after 10 days. Positive clones with anti-CA125 x anti-AP activity were screened out by sandwich ELISA as shown schematically in Fig. 3.4a. Briefly, the ELISA plate was coated with anti-CA125 MAb, B27.1. A sample containing CA125 was added and incubated for 3 hr, followed by the washing procedure. The cell supernatant containing bsMAb anti-CA125 x anti-AP together with AP was further incubated in the plate. Finally, the bound AP on the

tetrameric complex was enzymaticlly measured using substrate pNPP. The OD 405 nm of each sample was recorded and compared with the positive and negative control. The best P104.1R clone was recloned three times until 100% efficiency was achieved and expanded. The final quadroma, coded P104.1R.3.2.1, referred to thereafter as P104, was inoculated into BALB/c mice for ascites production. In addition, 1 L of cell culture supernatant of the cell line was collected for further studies.

Fluorescent label on a hybridoma has been used in the generation of hybrid hybridoma (Junker and Pedersen, 1981; Jantschff et al., 1993). However, the main drawback with this method is the selection of a considerable percentage of non-fused heterofluorescent cell cluster. To overcome this, a single cell cloning is required to isolate the quadromas from parental hybridomas. Jetscheff et al (1993) used FACS selection in combination with HAT medium selection to eliminate the non-fused heterofluorescent cell cluster selected by FACS. However, they had to spent a lot of time on the generation of a HAT sensitive hybridoma before the hybridoma fusion. The reason I used fluorescent labeling hybridoma was to save time, labor, and cost of development of quadromas producing bsMAb. In my experiments, single cell cloning right after FACS selection followed by another two 0.3 cell cloning ensures the separation of quadroma from non-quadromas. In addition, high fusion yield (Chang et al., 1992) provided by electrofusion ensures the selection of fused quadroma with higher possibility.

3.3.2. Purification of bsMAb and its immune complex

A MIMETIC blue A6XL affinity absorbent was successfully used for the purification of anti-PSA bsMAb-AP immune complex (section 3.2.2). To demonstrate the superiority of the mimetic blue affinity absorbents for purifying the bsMAb with one arm bound to AP, I compared this new method with DE52 chromatography, a traditional method for the purification of bsMAb (Suresh et al., 1986a). Equal amounts of bsMAb P104 were purified by both methods. All of the fractions from both columns were assayed for bsMAb P104 and monospecific anti-CA125 MAb activity as described in detail in sections 2.12.4 and 2.12.5 respectively. The bsMAb P104 was assayed by a sandwich CA125 assay, in which P104 was used as a tracer together with AP. The AP

bound in the sandwich complex was detected using pNPP. The higher OD over blank at 405 nm indicated the higher activity of bsMAb P104. The contaminating monospecific anti-CA125 MAb in the various fractions was estimated by an inhibition assay. A known amount of P104-AP immune complex purified by MIMETIC blue A6XL column was added into each aliquot of fractions collected from either the DE-52 column or MIMETIC blue A6XL column, and then the bsMAb activity in each sample was assayed as described above. Tricine buffer was used as blank. The optical density of blank and sample were measured at 405 nm and used to calculate the inhibition of P104-AP activity (%) as follow:

$$\text{Inhibition (\%)} = (OD_{blank}\text{-}OD_{sample}) \times 100 / Od_{blank}$$

The results shown in Fig. 3.19 and Fig. 3.20 indicated that bsMAb P104 was contaminated by anti-CA125 MAb in most of the fractions from DE-52 column. In contrast, the majority of the monospecific anti-CA125 MAb was recovered in the unbound fraction using MIMETIC blue A6XL affinity purification. There was very little inhibition of bsMAb P104 activity found in the fractions eluted from the mimetic blue affinity column (Fig. 3.20), which confirmed that anti-CA125 MAb was separated from the main peak of bsMAb P104.

The elution profiles of bsMAb P104 and anti-CA125 MAbs from the MIMETIC blue A6XL column gave further proof of the general applicability of this method, in addition to successfully purifying the bsMAb P105 binding to PSA, described in section 3.2. Contaminants such as anti-CA125 MAbs were removed from the bsMAb P104-AP immune complex (Fig. 3.20). Higher sensitivity of the bsMAb-AP based ELISA for detecting CA125 would likely be achieved with high specific activity immunoprobes.

Ion exchange chromatography has been used to purify bsMAbs (Suresh, et al., 1986a). However, it has some drawbacks including (1) lack of specificity in resolving the described species, (2) the requirement of a large volume of eluent with a gradient concentration of salt (300 mL) and (3) labor intensive collection and assay of large number of fractions. More importantly, some bsMAbs could not be separated from other contaminants such as monospecific antibody (Fig. 3.19). These monospecific antibodies

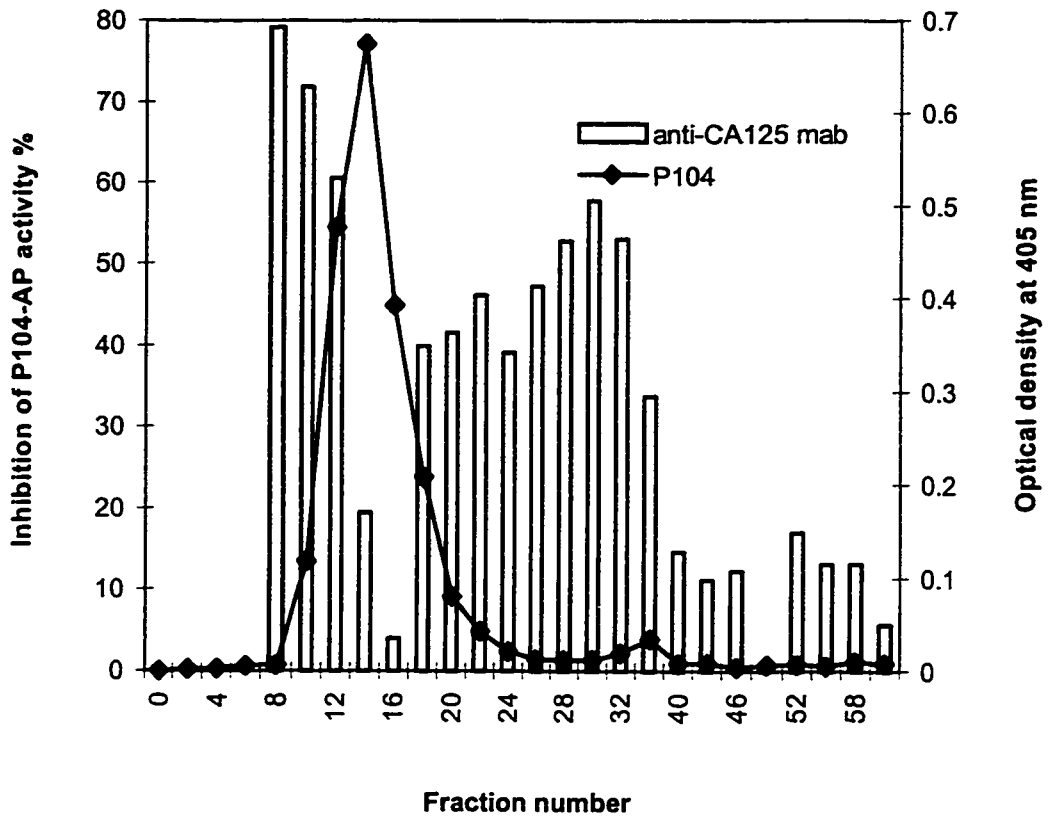


Figure 3.19 Elution profile of anti-CA125 MAb and bsMAb from DE52 column. A 15 mL sample containing anti-CA125 bsMAb (P104) was loaded on to the DE52 column at flow rate of 0.5 mL/min. After washing off the unbound protein, bsMAb P104 was eluted with 150 mL of each 10 mM to 100 mM phosphate buffer, pH 7.5. The inhibition % was determined as described in section 3.3.2 which is proportional to the amount of anti-CA125 MAb in each fraction. The activity of P104-AP immune complex was determined as described in section 2.14.1 (optical density at 405 nm).

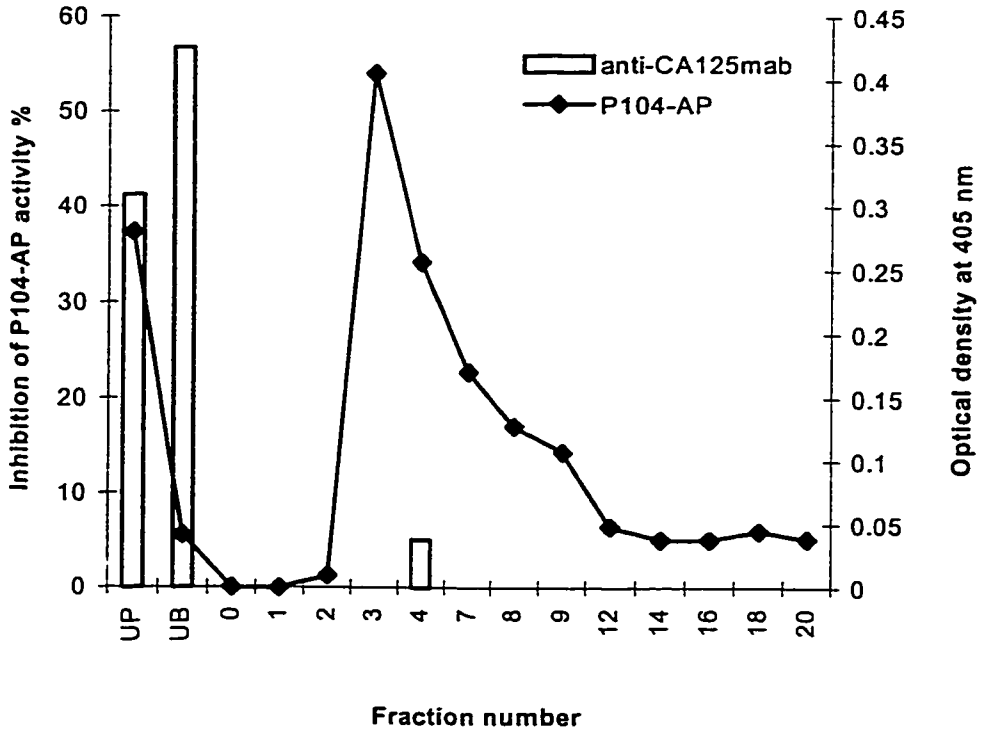


Figure 3.20 Elution profiles of anti-CA125 MAb and bsMAb-AP (P104-AP) immune complex from MIMETIC blue A6XL column using short purification protocol described in section 2.8. UP is an unpurified sample; UB was unbound fraction collected. The inhibition % was determined as described in section 3.3.2 which is proportional to the amount of anti-CA125 MAb in each fraction. The activity of P104-AP immune complex was determined as described in section 2.14.1 (optical density at 405 nm).

could compete with bsMAb molecules, reducing the specific activity and sensitivity of the bsMAb based immunoassay (Milstein and Cuello, 1983; Suresh, 1986b). In contrast, as demonstrated, the mimetic affinity method is simple and effective to remove monospecific MAb (anti-CA125 MAb). The purification is achieved in a short period of time with simple and mild procedures. The elution profile showed that most of the bsMAb-AP immune complex was recovered in 30 mL of elution buffer. In addition, both the enzyme and bsMAb collected in the unbound fraction can be reused without being dialyzed.

3.3.3. BsMAb-AP immune complex--a sensitive probe for detection of CA125

3.3.3.1. Construction of conventional ELISA

As discussed previously, the quadroma P104 was developed using hybridoma B43.13. The MAb B43.13 has been used in a TRUQUANT OV2 RIA (Biomira) which is a two step sandwich immunoradiometric assay. In this assay, anti-CA125 MAb B27.1 is used as a capture reagent, which is coated on polystyrene tubes. A given specimen containing CA125 is added into the tube and ^{125}I-labeled MAb B43.13 is used as a probe to detect the CA125 captured by B27.1. The bound radioactivity is measured using a gamma counter. This assay takes 18-24 hrs with a minimum detectable level of 9.8 U/mL (Manufacture instruction).

In my thesis, an alternative CA125 assay using bsMAb anti CA125 x anti-AP as a non-radioactive tracer was developed. Because B43.13 and B27.1 are both OC125-like MAbs but they bind to different overlapping epitopes with relatively high affinity, B27.1 was chose as a capture and a bsMAb anti-CA125 x anti-AP as a tracer. To generate bsMAb anti-CA125 x anti-AP, a hybridoma producing anti-CA125 MAb B43.13 was fused with the hybridoma P92 (anti-AP). The bsMAb P104-AP immune complex was purified by MIMETIC blue A6XL affinity chromatography and used directly in the immunoassay as a tracer. A heterogeneous sandwich assay based on bsMAb anti-CA125 x anti-HRPO was reported previously from our lab and the LLD of this assay was 1 U/mL (Kreutz and Suresh, 1995). In this assay, the incubation time was 3 hr for CA125

binding to B27.1 and 45 min for bsMAb-HRPO binding to CA125. In my approach, I tried to shorten the assay time and increase or at least maintain the sensitivity of the CA125 assay achieved in the previous format. Thus, the same assay format as bsMAb anti-CA125 x anti-HRPO based CA125 assay was used. BsMAb anti-CA125 x anti-AP, purified from MIMETIC blue A6XL affinity column as bsMAb-AP immune complex, was used in a CA125 immunoassay as a tracer. Of the two paratopes of bsMAb P104, one binds with AP and the other binds to CA125. Theoretically, one B27.1 molecule on the plate could bind one molecule of CA125 detected later by at least one bsMAb P104-AP immune complex. Furthermore, the activity of the AP can be detected by its enzymatic activity, which gives a final product with color or fluorescence. For example, pNPP, is converted by AP to a yellow product, which is detected at 405nm. MUP is a fluorogenic substrate for AP, it is converted by AP to MU, which is detected by fluorimetry (Porstmann and Kiessigo, 1992).

First of all, the blocking matrixes, 6% BSA or 1-3% OVA was tested for blocking the non-specific sites in the CA125 immunoassays, in which P104-AP immune complex (1:20 dilution) was used as a tracer. The CA125 assay results demonstrated that both matrices did not increase the signal of blank (data not shown). Since BSA is relative expensive, OVA was used in subsequent experiments. As discussed previously, P104-AP immune complex was co-purified with excess amount of AP when a mimetic blue absorbent was used. The optimal dilution of P104-AP immune complex could avoid higher background due to excess amount of AP. The CA125 assay was performed using TRUQUANT (Biomira Inc.) calibrators, containing 0-35 U/mL of CA125 in order to test the background problem at low levels of the tumor antigen. P104-AP immune complex at different dilutions was used as a tracer in the second incubation. The results from Fig. 3.21 showed that OD of blanks were > 0.1 when the P104-AP immune complex in either 1:5 or 1:10 dilution was used. In the 1:40 dilution of P104-AP immune complex, OD of calibrator with 35 U/mL was too low to be detected (ODsample-ODblank < 0.1), which tends to decrease the accuracy of the assay. The 1:20 dilution was found to be the preferred dilution, which showed a relatively higher signal to noise ratio in the conventional assay for CA125.

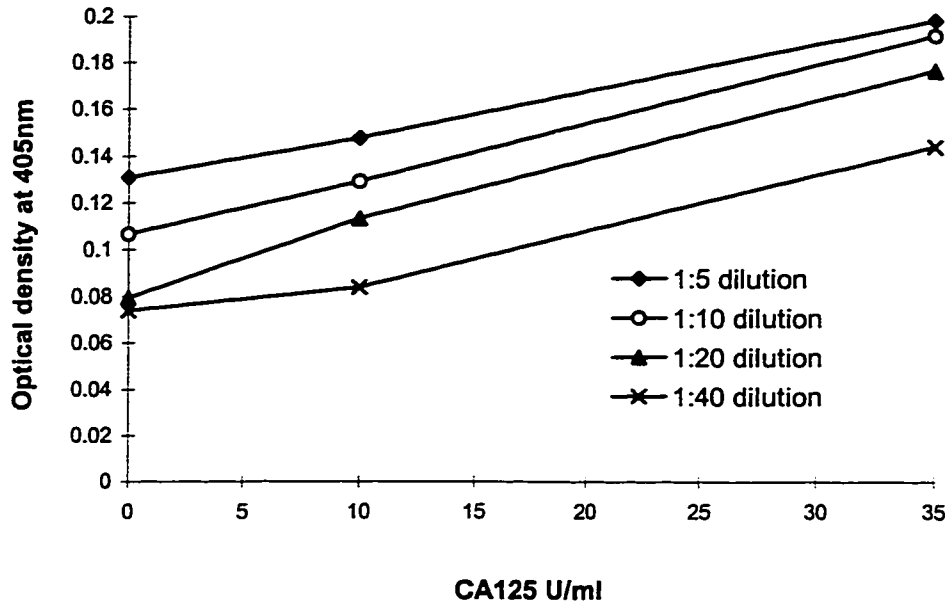


Figure 3.21 Determination of the optimal dilution of bsMAb-AP immune complex in a conventional ELISA for CA125. The microplate was coated with B27.1 at 1 μg/well and CA125 was detected using different dilutions of anti-CA125 bsMAb /AP immune complex (P104-AP) as described in section 2.14.1. Each point represents the mean of three determinations. CV% are from 0.4 to 7.6.

The CA125 incubation time in the CA125 assay was also evaluated as the next parameter. Six calibrators containing 0-1000 U/mL of CA125 were added in triplicate into an ELISA plate coated with anti-CA125 MAb, B27.1, and incubated for 30 min, 90 min and 180 min . The incubation time for P104-AP immune complex was 45 min. Fig. 3.22 illustrated that the 90 min and 180 min incubation resulted in fairly linear curves with a range of 0-1000 U/mL. When the 30 min incubation was performed, the optical densities were decreased as expected and the linear range of the curve was between 200-1000 U/mL. The 10 replicates of calibrator with 0 U/mL CA125 (blank) were used for the determination of LLD. A mean optical density value of blank plus 2 SD gave sensitivity for the assay. The LLD of the assay was then determined from the standard curve. Conventional two step ELISA was further constructed under the preferred conditions. The standard curve (Fig. 3.23) showed a very good correlation between the CA125 concentration (0-1000 U/mL) and optical density of pNPP. This indicated that P104-AP immune complex could be used as a new tracer in the sandwich ELISA for CA125.

A number of CA125 immunoassays have been described (Bast et al., 1983; Capstick et al., 1991; Kreutz and Suresh 1995; Madiyalakan et al., 1996). Among these assays, there are RIA, MAb based ELISA, and bsMAb based ELISA. Except for the bsMAb based assay (Kreutz and Suresh 1995), other assays require overnight incubation with the sample containing CA125. This could be due to a combination of low specific activity of the tracer and the loss of antigen binding during chemical conjugation procedures. BsMAb provide us with powerful immunoreagents which recognize two antigens simultaneously in a one to one relationship. This property allows the immunoprobe to reach the absolute theoretical limits of specific activity, therefore improving the sensitivity of the immunoassay. The bsMAb anti-CA125 x anti-HRPO based CA125 assay demonstrated a fast kinetics and high sensitivity, thus showing high promise for application in manual and automated CA125 in the clinic (Kreutz and Suresh 1995). As I expected, using bsMAb anti-CA125 x anti-AP as a tracer, the LLD and linear range of the assay was closer to that achieved using bsMAb anti-CA125 x anti-HRPO based CA125 under the same conditions. However, when the first incubation time for CA125 was reduced from 3 hr to 1.5 , the LLD of the assay was decreased from 2 U/mL to 10 U/mL (Table 3.2). That might be due to steric hinderance between the AP (100-140

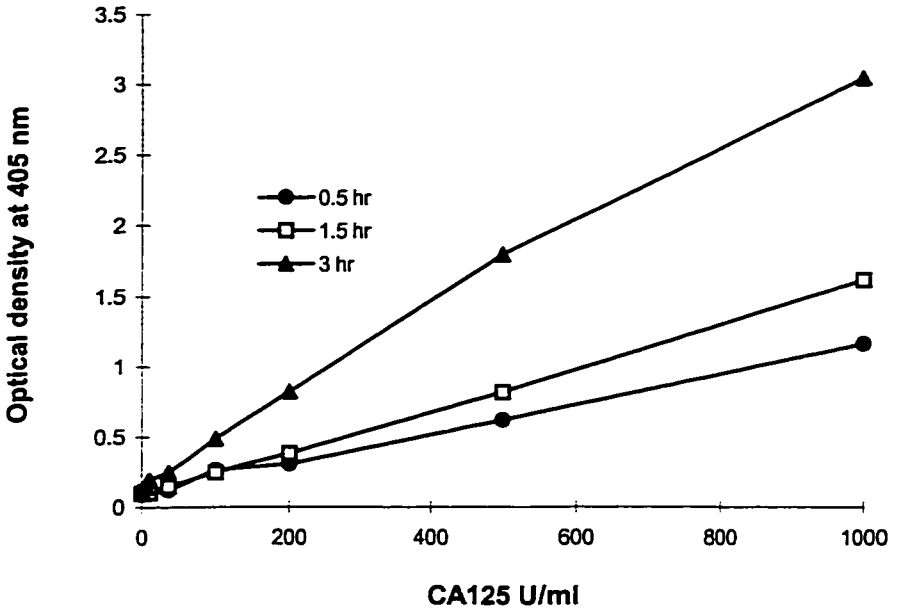


Figure 3.22 Conventional sandwich ELISA with different incubation times for CA125. The ELISA plate was coated with 1μg/well of B27.1 MAb. Samples containing different amounts of CA125 were added into the plate and incubated for 0.5 hr, 1.5 hr and 3 hr. The CA125 in the samples was determined by measuring bound anti-CA125 bsMAb/AP immune complex (P104-AP) activity after a 45 min incubation as described in section 2.14. Each point represents the mean of three determinations. CV% are from 0.3 to 10.5.

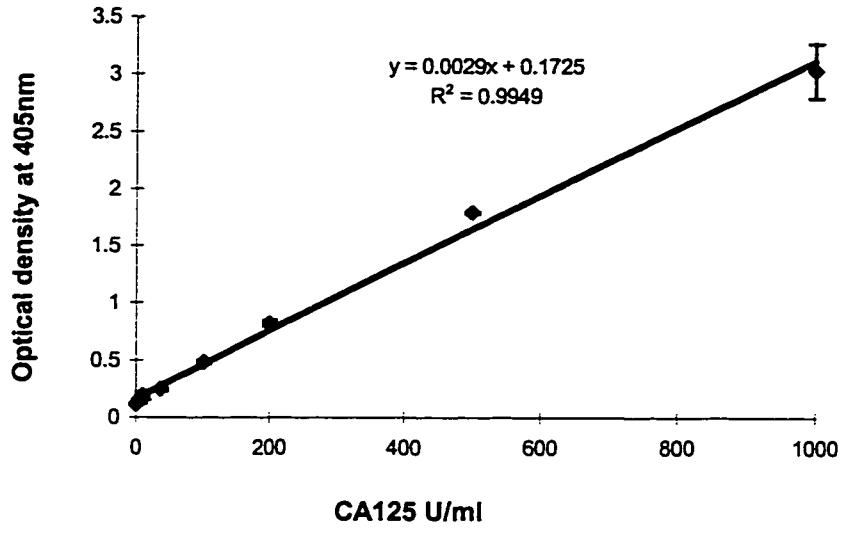


Fig. 3.23. Standard curve of CA125 in two step conventional ELISA. The procedures before the color development were the same as described in 2.14.1. The plate was coated with B27.1. The calibrators containing different amounts of CA125 were added in the plate and incubated for 3 hr at room temperature. Following a washing step, P104-AP immune complex (1:20 dilution) was added and incubated for 45 min followed by another washing step. Finally, the AP substrate, pNPP was added and the optical density of the color was measured after 20 min at 405nm.

KD) bound on bsMAb and CA125, to slow down the speed of bsMAb binding to CA125, which is a subunit of the whole antigen with a minimum size of 210 KD. Alternative approaches might be used in future studies such as using a substrate which could produce a more intensive signal (enzyme amplification system) or a more sensitive detection method (fluorimetry).

3.3.3.2. Amplified ELISA for CA125

The previous study showed that P104-AP immune complex could be used as a tracer in the conventional CA125 assay. In this section, the development of an ultrasensitive CA125 assay using the enzyme amplification system was further investigated (Self, 1985). I expected that the ultrasensitive CA125 assay might detect very low amount of CA125. The serum CA125 levels in the patients who have undergone radical ovarectomy is expected to be very low. Therefore, an ultrasensitive CA125 assay might be able to detect minute changes of CA125 levels which might indicate a metastasis or early recurrence of the tumor. The conventional CA125 assay format was utilized as described above, but another AP substrate, NADP, was used. Samples contain CA125 were first added into an ELISA plate immobilized with B27 MAb in the first 3 hr incubation. Then P104-AP immune complex was added in the second incubation. Final color was developed by adding an AP substrate, NADP, followed by a cycling solution consisting of diaphorase, ADH and INT-violet (Fig. 3.16). Finally, amplified signal was determined by measuring the optical density at 490 nm.

Several factors will affect this CA 125 assay and the additional investigations were carried out with lower amounts of CA125. First of all, optimizing the dilution of purified P104-AP immune complex was a crucial step in this assay in order to keep the background to the minimum. Four samples containing 0-2.5 U/mL of CA 125 were used in the first incubation. Then P104-AP immune complex at different dilutions (20-100 times) was used in the second incubation. The dilution curve was plotted as optical density of each sample vs. dilution factor of P104-AP immune complex. The results in Fig. 3.24 indicated that the amplified system using P104-AP immune complex as tracer increased the signal from both specific and non-specific binding of the AP. For example,

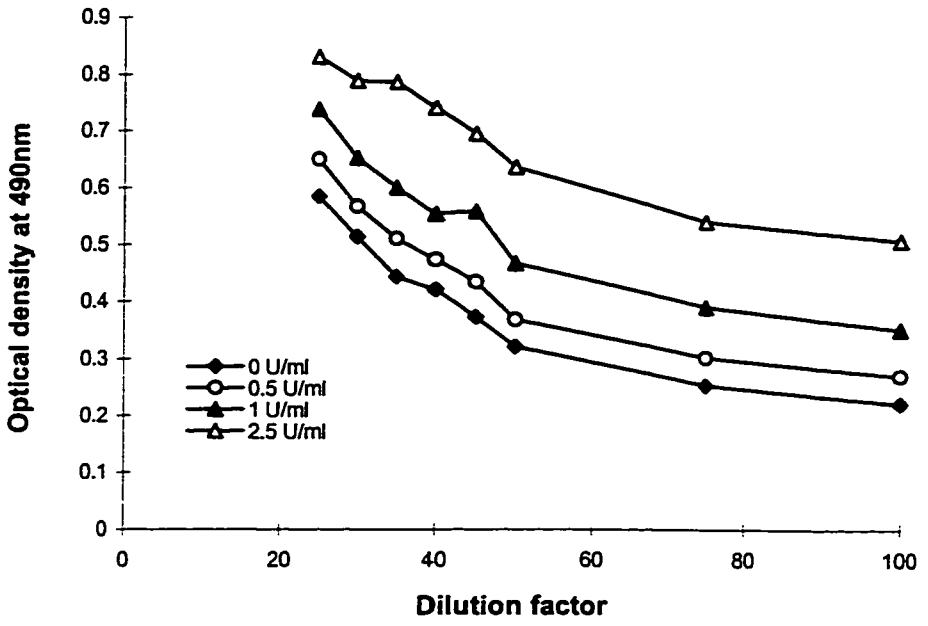


Figure 3.24 Optimization of the dilution of bsMAb-AP immune complex used in amplified CA125 sandwich ELISA. The microplate was coated with B27.1 MAb at 1 μg/well. The CA125 in different samples was detected using anti-CA125 bsMAb/AP (P104-AP) immune complex in different dilution as described in section 2.14. Optical density at 405 nm was recorded and plotted vs dilution factor of P104-AP immune complex. Each point represents the mean of three determinations. CV% are from 0.5 to 7.8.

when 1:25 dilution of P104-AP immune complex was used, the optical density of blank was 0.6 which was unacceptably high. However, when dilution was 1:100 times, the optical density of blank was reduced to 0.2. Therefore 1:100 times dilution of P104-AP was used as preferred dilution for the amplified ELISA.

To demonstrate the signal amplification over conventional ELISA, the two CA125 assays were performed parallel using either the conventional substrate or enzyme amplification system as described above. Fig. 3.25 shows an increased sensitivity for CA125 in the amplified assay compared with the conventional method. The OD of the sample with 10 U/mL of CA125 was 68 times higher than that obtained by conventional method. The linear range of the amplified assay appears from 1-10 U/mL. Furthermore, The CA125 incubation time was evaluated for the amplified assay. The CA125 assay was performed in three different incubation times (30 min, 90 min and 180 min) during the CA125 incubation. The rest of the procedures were as described above. The LLD of the three amplified CA125 assay times were determined. As shown in table 3.2. The LLD of the three CA125 assays were 0.53, 0.164 and 0.06 respectively. The results indicated that the sensitivity of the CA125 assay was affected by the incubation time for the B27.1 MAb capturing CA125.

As described above, the conventional assay using pNPP as substrate is able to detect CA125 at a LLD of 2.08 U/mL with linearity range up to 1000 U/mL in 4 hr and 45 min of signal generation. The amplified CA125 assay is able to detect 0.53 U/mL of CA125 in less than 2 hr. Although the amplification assay can decrease the detection limit 4 fold, the linear range was smaller. As mentioned previously, the CA125 assays currently used in the clinic utilize 35 U/mL of CA125 as the cut off value (Lavin et al., 1987). Therefore, a rapid and non-radioisotope CA125 assay with a acceptable LLD would be more valuable in the clinic. As I discussed in section 3.2.3.2, using resazurin to replace INT in the enzyme amplification system, a fluorescent final product, resorufin, could be detected by fluorimetry (Cook and Self, 1993). The fluorescent system holds promise to greatly extending the measurable range and increase the sensitivity of the immunoassay for CA125 in a shorter period. The development of the ultrasensitive assay opens the door to detecting minute alterations in CA125 levels that could be triggered by emergence of micrometastasis following primary treatment.

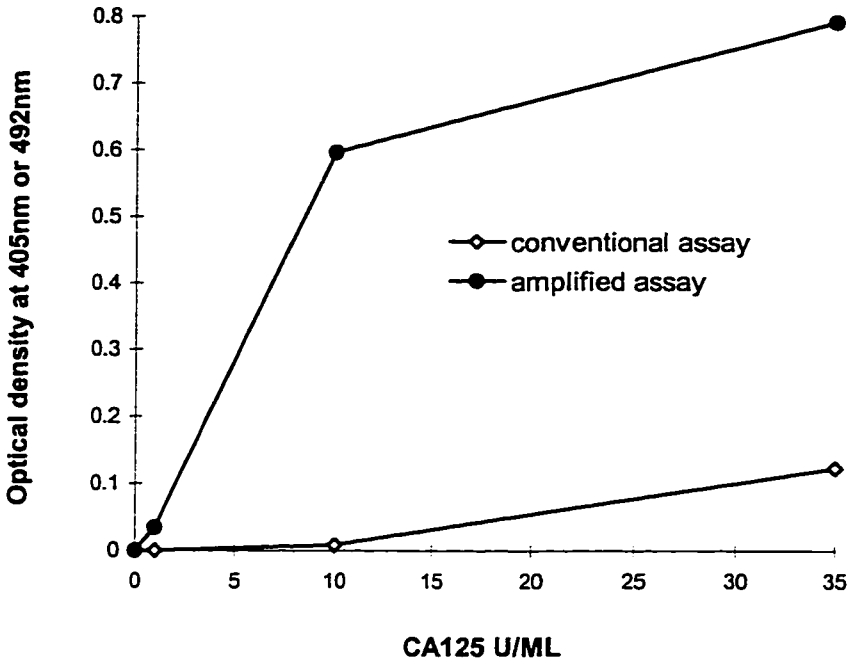


Figure 3.25 Amplified assay vs. conventional assay for the detection of CA125. BsMAb P104-AP immune complex was used to detect CA125 captured by B27.1 coated on the ELISA plate. The AP activity was determined by either amplified or conventional method as described in section 2.14. The optical density was plotted vs CA125 (U/mL). Each point represents the mean of triple determination in the same assay. CV% are from 0.5 to 11.1.

Table 3.2 Summary the LLD of CA125 assays under different conditions

Incubation time for the 1st step	Conventional assay	Amplified assay
3hr	2.08	0.06
1.5 hr	10	0.164
0.5 hr	13.2	0.53

LLD--lowest limit detection (U/mL)

CHAPTER 4. SUMMARY, CONCLUSIONS AND FUTURE WORK

In this project, bsMAbs were developed using classical hybridoma technology. Initially, a hybridoma producing anti-AP antibody was generated by fusion of myeloma SP 2/0 with the splenocytes collected from mouse immunized with alkaline phosphatase from calf intestine. The best hybridoma P92 secreting anti-MAb with highest affinity was established and the anti-AP MAb was purified and partially characterized. The results indicated that anti-AP MAb (P92) belongs to the IgG1 subclass and does not bind to the AP from human placenta (section 3.1.1).

The quadromas producing bsMAbs anti-PSA x anti-AP (P105) and anti-CA125 x anti-AP (P104) were developed by fusion of hybridomas B80.3 (anti-PSA) or B43.13 (anti-CA125) with P92 (anti-AP) respectively. The hybridoma fusion partners were labeled with two different fluorescence markers for 30 min before the electrofusion and selection of double fluorescent quadromas by FACS. Three quadromas producing bsMAb anti-PSA x anti-AP were screened from 33 clones. Among the three positive clones, P105.2R was the best one with a consistent high level secretion of the bsMAb ELISA activity (section 3.2). This modified FACS fusion procedure avoids the incorporation of drug selection markers on hybridomas which normally takes 3-6 months. The second quadroma secreting bsMAb anti-CA125 x anti-AP was developed using the same FACS fusion procedure (section 3.3.1). Both quadromas were recloned, expanded and stored in liquid N_2. Mouse ascites with enriched P105 or P104 activity were collected from mice injected with the quadromas and stored at -20°C for further purification. In addition, bulk tissue culture supernatants of the quadromas were pooled and collected as an additional source of the bsMAb.

The purification of bispecific antibody from other antibody species secreted by the same quadroma is a crucial step for the further development of rapid and sensitive immunoassays for PSA or CA125 respectively. A MIMETIC blue A6XL affinity absorbent which was originally designed for the purification of AP from calf intestine was utilized. I hypothesized and rationalized that the mimetic ligand might selectively bind to AP even if presented as an AP/anti-AP immune complex, effectively eliminating contaminants such as monospecific anti-PSA antibody and other impurities as unbound

material and in a subsequent washing step. The elution involves a competition wherein the phosphate ion competes with AP bound to the ligand (phosphate analog). The elution procedure is mild (5 mM phosphate in 10 mM tricine buffer) which is unlikely to damage either AP or bispecific antibody. In order to successfully perform the mimetic affinity procedure, phosphate in the crude AP or antibody sample should be removed by dialysis. A set of experiments demonstrated that this MIMETIC blue affinity absorbent selectively binds to AP and AP/anti-AP immune complexes as well as commercial AP-antibody conjugate. Most contaminants such as monospecific anti-PSA antibody, monospecific anti-CA125 antibody and non-specific mouse IgG in the commercial polyclonal antibody or mouse ascites were removed from AP/bsMAb immune complex bound to the column (section 3.2.2, 3.3.2). More importantly, the AP/anti-AP immune complexes are obtained using the same elution conditions used for AP alone. This affinity purification method provides high specific activity bsMAbs with an AP already bound to its site. The uniform reproducible enzyme binding to the bsMAb results in superior immunoconjugate in comparison with chemical crosslinking procedures which resulting random inter- and intra- molecular cross-links and multiprotein aggregates. This simple and unique purification method allowed us to further investigate the bsMAb based immunoassay for PSA or CA125. It is important to note that this affinity purification also co-purifies the monospecific anti-AP antibody along with the bsMAb. However, the later species does not interfere with immunoassays and can be washed away prior to substrate addition.

A series of experiments were carried out towards developing conventional enzyme immunoassays for PSA and CA125, respectively, using the purified AP/anti-AP bsMAb immune complexes. A sandwich format was used in PSA assay, in which a matched pair of anti-PSA MAb (B87) was coated on the ELISA plate. The AP/anti-AP bsMAb was used as a tracer as described in section 3.2.3. The one step PSA assay was evaluated at various incubation times and signal development times. Results suggested that the conventional PSA assay could detect PSA as low as 0.05 μg/L in 30 min. A preferred dilution of the AP/anti-AP bsMAb immune complex pooled from the mimetic affinity purification was determined as 1:25. The blocking matrix, 1% ovalbumin, 1% BSA and 1% skim milk were tested, and none of them increased the background of the signal.

In a similar fashion, the AP/anti-CA125 bsMAb immune complex was tested as an immunoprobe in the conventional sandwich ELISA for CA125. In this assay, the anti-CA125 MAb B27.1, was used as the capture antibody and the AP/anti-CA125 bsMAb immune complex was used as the tracer. Firstly, the preferred dilution of AP/anti-CA125 bsMAb immune complex was determined as 1:20 based on the signal to noise ratio when using different concentration of CA125. Then the first incubation with CA125 was evaluated at different time (0.5 hr, 1.5 hr, 3 hr). The results suggested that a 3 hr incubation with CA125 could detect CA125 as low as 2 U/mL which is far below the general clinical cut off value (35 U/mL) for most CA125 assay.

The unique advantage of AP as the enzyme marker in bsMAb is its ability to lend itself to cyclic enzymatic amplification for enhanced sensitivity. Further experiments were conducted to test the AP/anti-AP bsMAb immune complex in a amplified PSA assay as well as amplified CA125 assay. The co-enzyme NADP was used as a substrate for AP and an enzyme amplification system was used to amplify the signal as described in section 3.2.3.2 and 3.3.3.2. The amplified PSA assay resulted in a phenomenal increase in the signal intensity and detection sensitivity over that achievable with the conventional assay. The ultrasensitive assay could detect PSA as low as 0.00025 μg/L making this the most sensitive PSA assay developed to date. However, the problems such as high background and narrow linear range of the assay (< 0.1 μg/L) remains to be solved. Comparing with conventional assay, the amplified CA125 assay also showed a 68-fold increase in signal intensity when a sample containing 10 U/mL of CA125 was used. When the first incubation step with CA125 was also varied from 0.5 hr, 1.5 hr and 3 hr, and the LLD of CA125 was found to be 0.53, 0.164, and 0.06 U/mL respectively.

In conclusion, using a unique electrofusion in combination with FACS selection, two quadromas producing bsMAb anti-PSA x anti-AP and anti-CA125 x anti-AP were developed. Exploiting MIMETIC blue A6XL affinity chromatography, two bsMAb-AP immune complexes were purified in a single step to have high specific activity. These two immune complexes were used directly in the enzyme-based immunoassays for both tumor makers, namely PSA and CA125. The lowest detection limits of the conventional assay for PSA and CA125 were 0.05 μg/L and 2.08 U/mL, respectively. In the amplified assay format, these lowest detection limits were decreased to 0.25 pg/mL and 0.53 U/mL

for PSA and CA125, respectively. Currently, these are two of the most sensitive immunoassays reported for these important tumor markers.

The generation of the two key quadromas in this thesis allowed the development of novel prototype ultrasensitive immunoassays as predicted for CA125 and PSA. Future work in this area would initially be in the area of assay optimization and validation of key components of the assays. This would set the stage for clinical evaluation in ovarian and prostate cancer patients who have undergone primary radical surgical removal of the involved organ. For example, in the case of radical prostatectomy, post-operative PSA levels should be virtually undetectable. This group of patients could be monitored serially every 2-3 months by the ultrasensitive bsMAb immunoassay to detect ultralow level of PSA in serum, putatively indicating micrometastatic recurrence of prostate cancer well ahead of other physical and biochemical detection methods. Potentially, as a result of this early diagnosis effective second line therapeutic treatment could have a positive benefit before the spread and establishment of cancer metastases.

Other potential future applications of the two bsMAbs developed in this thesis include immunohistochemistry immunofluorescent assays and targeted enzyme based prodrug therapeutics. Alkaline phosphatase is a versatile enzyme with numerous substrates generating a variety of endpoints. Bromo-chloroindolyl phosphate is a substrate which upon cleavage of the phosphate group forms a colored precipitate and this can be generated at the site of the immunohistochemical reaction to identify CA125 and PSA producing tissue sections. Similarly, methyl umbelliferryl phosphate could be used as a cryptofluorecent substrate to generate the fluorescent methyl umbelliferrone for applications in fluoroimmunoassays. Lastly, by employing mitomycin phosphate a non-toxic prodrug, these bispecific antibodies and AP targeted to tumor cells *in vitro* and *in vivo* could selectively cleave the prodrug to the cytotoxic mitomycin at the site of tumor. Thus, the basic design and development of AP based bsMAbs allows further exploration of these novel applications in cancer diagnostics and therapeutics.

References

Allard, W.J., Moran, C.A., Nagel, E., Collins, G., Largen, M.T. (1992) Antigen binding properties of highly purified bispecific antibodies. *Mol. Immunol.* **29**:1219-1227.

Amoroso, A.R., Clark, J.I., Litwin, S., Hsieh-Ma, S., Shi, T., Alpaugh, R.K., Adams, G.P., Wolf, E.J., Ring, D.B., Weiner, L.M. (1996) Binding characteristics and antitumor properties of 1A10 bispecific antibody recognizing gp40 and human transferrin receptor. *Cancer Res.* **56**:113-120.

Auriol, J., Guesdon, J.L., Mazié, J.C., Nato, F. (1994) Development of a bispecifc monoclonal antibody for use in molecular hybridization. *J. Immunol. Methods* **169**:123-133.

Bast, Jr, R.C., Freeney, M., Lazarus, H., Nadler, L.M., Colvin, R.B., Knapp, R.C (1981). Reactivity of a monoclonal antibody with human ovarian carcinoma. *J. Clin. Invest.* **68**:1331-1337.

Bast, Jr, R.C., Klug, T.L., St. John, E. Jenison, E., Niloff, J.M., Lazarus, H., Berkowitz, R.S., Leavitt, T., Griffiths, C.M., Parker, L., Zurawski, V.R., Knapp, R.C. (1983) A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *N. Eng. J. Med.* **309**:883-887.

Bates, D.L. (1987) Enzyme amplification in diagnostics. *TIBTECH.* **5**:204-209.

Berek, J.S., Knapp, R.C., Malkasian, G.D., Lavin, P.T., Whitney, C., Niloff, J.M., Bast,R.C. (1986) CA 125 levels correlated with second-look operations among ovarian cancer patients. *Obstet. Gynecol.* **67**:685-689.

Brawer, M.K. and Lange, P.H. (1989) Prostate specific antigen in management of prostatic carcinoma. *Urol.* **33**:11-16.

Brawer, M.K., Beatie, J., Wener, M.H., Vessella, R.L., Preston, S.D., Lange, P.H (1993) Screening for prostatic carcinoma with prostate specific antigen: Results of the second year. *J. Urol.* **150**: 106-109.

Brennan, M., Davison, P.F., Paulus, H. (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science* **229**: 81-83.

Bronstein, I., Voyta, J.C., Thorpe, G.H.G., Kricka, L.J., Armstrong, G. (1989) Chemiluminescent assay of alkaline phosphatase applied in an ultrasensitive enzyme immunoassay of thyrotropin. *Clin. Chem.* **35**:1441-1446.

Chang, D C., Saunders, J.A., Chassy, B.M., Sowers, A.E. (1992) Overview of electroporation and electrofusion. In: D.C. Chang, J.A. Saunders, B.M., Chassy,

A.E. Sowers (Eds), Guide to electroporation and electrofusion. Academic Press, San Diego, CA.

Capstick, V., Maclean, G.D., Suresh, M.R., Bodnar, D., Lloyd, S., Shepert, L., Longenecker, B.M., Krantz, M. (1991) Clinical evaluation of a new two-site assay for CA125 antigen. *Int. J. Biol. Markers.* **6**:129-135.

Chen, I.W., Sperling, M.J., Heminger, L.A., Kaplan, L., Maxon, H.R. (1985) Prostate specific antigen (PSA) in detection of prostatic cancer. *Clin. Chem.* **31**:984.

Chervonsky, A.V., Faerman, A.I., Evdonina, L.V., Jazova, A.K., Kazarov, A.R., Gussev, A.I. (1988) A simple metabolic system for selection of hybrid hybridomas (tetradomas) producing bispecific monoclonal antibodies. *Mol. Immunol.* **25**:913-915.

Christopoulos, T.K. and Diamandis, E.P. (1996) Immune function and antibody structure. In Diamanids, E.P., Christopoulos, T. K. (eds.), Immunoassay. p 13, Academic Press Inc, San Diago, CA.

Christopoulos, T.K. and Diamandis, E.P. (1992) Enzymatically amplified time-resolved fluorescence immunoassay with Terbium chelates. *Anal Chem* **64**:342-346.

Christopoulos, T.K. and Diamandis, E.P. (1996) Immunoassay configurations. In. Diamanids, E.P., Christopoulos, T. K. (Eds.), *Immunoassay.* p 227. Academic Press, San Diago, CA

Coloma, M.J., Morrison, S.L. (1996) Design and production of novel tetravalent bispecific antibodies. *Nature Biotechnol.* **15**:159-163.

Cook, A.G., Wood, P.J. (1994) Chemical synthesis of bispecific monoclonal antibodies: potential advantages in immunoassay systems. *J. Immunol. Methods.* **171**:227-237.

Cook, D.B., Self, C.H. (1993) Determination of one thousandth of an attomole (1 zeptomole) of alkaline phosphatase: application in an immunoassay of proinsuline. *Clin Chem.* **39**:965-971.

De lau, W.B.M., Van loon, A.E., Heije, K., Valerio, D., Bast, B.J.E.G. (1989) Production of hybrid hybridomas based on HAT^s-$neomycin^r$ double mutants. *J. Immunol. Methods* **117**:1-8.

Devine, P.L., McGuckin, M.A., Ward, B.G (1992) Circulating mucins as tumor markers in ovarian cancer. *Anticancer Res.* **12**:709-718.

Diamandis, E.P.and Yu, H. (1995) New biological functions of prostate-specific antigen? *J. Clin. Endocrinol. Metab.* **80**:1515-1517.

Diamandis, E.P., Yu, H., Sutherland, D.J.A. (1994) Detection of prostate-specific antigen immunoreactivity in breast tumors. *Breast Cancer Res. Trat.* **32**:291-300.

Ehrilich, P. (1906) *Collected studies on immunity* Vol. 2. p442. John Wiley, New York.

Ferguson, R.A., Yu, H., Kalyvas, M., Zammit, S., Diamandis, E.P.(1996) Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the immulite® immunochemiliminescent third-generation assay: potential application in prostate and breast cancers. *Clin. Chem.* **42**:675-684.

Fanger, M.W. (1992) Bispecific antibodies. *Crit. Rev. immunol.* **12**:101-124.

Frasci, G., Conforti, S., Zullo, F., Mastrantonio, P., Comella, G., Comella. P., Persico, G., Laffaioli, R.V.(1996) A risk model for ovarian carcinoma patients using CA125. *Cancer* **77**:1122-1130.

Glennie, M.J., McBride, H.M., Worth, A.T., Stevenson, G.T. (1987) Preparation and performance of bispecific F(ab'γ)$_2$ antibody containing thioether-linked Fab'γ fragments. *J. Immunol.* **139**:2367-2375.

Gosling, J.P. (1990) A decade of development in immunoassay methodology. *Clin. Chem.* **36**:1408-1427.

Graves, H.C.B., Wehner, N., Stamey, T.A. (1992a) Ultrasensitive radioimmunoassay of prostate-specific antigen. *Clin. Chem.* **38**:735-742.

Graves, H.C.B. (1992b) Prostate-specific antigen comes of age in diagnosis and management of prostate cancer. *Clin. Chem.* **38**:1930-1932.

Graves, H.C.B. (1993) Issues on standardization of immunoassays for prostate-specific antigen: a review. *Clin. Invest. Med.* **16**:415-424.

Guesdon, J.L., Avrameas, S. (1980) Lectin immuno test: Quantitation and titration of antigens and antibodies using lectin-antibody conjugates. *J. Immunol. Methods* **39**:1-13.

Guo, Y.J., Che, X.Y., Shen, F., Xie, T.P., Ma, J., Wang, X.N., Wu, S.G., Anthony, D.D., Wu, M.C. (1997) Effective tumor vaccines generated by in vitro modification of tumor cells with cytokines and bispecific monoclonal antibodies. *Nature Med.* **3**:451-455.

Haga, Y., Sakamoto, K., Egami, H., Yoshimura, R., Mori, K., Akagi, M. (1986) Clinical significance of serum CA125 values in patients with cancers of the digestive system. *Am. J. Med. Sci.* **292**: 30-34.

Hanai, A. (1990) Trends and differentials in ovarian cancer: incidence, mortality and survival experience. *Acta Pathol. Microbiol. Immunol. Scand.* **98.** supp **12**:1-20.

Harlow, E.D.and Lane, D.(1988) Storing and purifying antibodies. In Harlow, E.D., Lane, D (Eds.) Antibodies: A laboratory manual, p298. Cold Spring Habor Laboratory, New York.

Hoffman, B.R.,Yu, H., Diamandis E. (1996) Assay of prostate-specific antigen from whole blood spotted on filter paper and application to prostate cancer screening. *Clin Chem.* **42**:536-544.

Hudson, M.A., Bahnson, R R., Catalona, W.J. (1989) Clincal use of prostate specific antigen in patients with prostate cancer. *J. Urol.* **142**:1011-1017.

Huang, Z., Olson, N.A., You, W. Haugland, R.P. (1992) A sensitive competive ELISA for 2,4-dinitrophenol using 3,6-fluorescein diphosphate as a fluorogenic substrate. *J. Immunol. Methods* **149**:261-266.

Inouye, K. (1996) Bispecific-Ab-based immunoassay of thyroid-stimulating hormone. *CancerImmunol. Immunother.* **45**:159-161.

Ishikawa, E. (1987) Development and clinical application of sensitive enzyme immunoassay for macromolecular antigen--a review. *Clin. Biochem.* **20**:375-385.

Ishikawa, E. (1996) Labeling of antibodies and antigens. In Diamanids, E.P., Christopoulos, T. K. (eds.) Immunoassay. p191-204, Academic Press Inc, San Diego, CA.

Jacobs, I., Bast, Jr, R.C. (1989) The CA125 tumor-associated antigen. *Hum. Reprod.* **4**:1-12.

Jacobs, N., Mazzoni, A., Mezzanzanica, D., Negri, D.R.M., Valota, O., Colnaghi, M.I., Moutschen, M.P., Boniver, J., Canevari, S. (1997) Efficiency of T cell triggering by anti-CD3 monoclonal antibodies (mAb) with potential usefulness in bispecific mAb generation. *Cancer Immunol. Immunother.* **44**:257-264.

Jantscheff, P., Winkler, L., Karawajew, L., Kaiser, G., Bottger, V., Micheel, B. (1993) Hybrid hybridomas producing bispecific antibodies to CEA and peroxidase isolated by a combination of HAT medium selection and fluorescence activated cell sorting. *J. Immunol. Methods* **163**:91-97.

Johannsson, A., Ellis, D.H., Bates, D. L., Plumb, A.M., Stanley, C.J. (1986) Enzyme amplification for immunoassays detection limit of one hundredth of an attomole. *J. Immunol. Methods* **87**:7-11.

Junker, S., Pedersen, S. (1981) A universally applicable method of isolating somatic cell hybrids by two-color flow sorting. *Biochem. Biophys. Res. Commun.* **102**:977-984.

Karawajew, L., Behrsing, O., Kaiser, G., Micheel, B. (1988) Production and ELISA application of bispecific monoclonal antibodies against fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). *J. Immunol. Methods* **111**:95-99.

Katayose, Y., Kudo, T., Suzuki, M., Shinoda, M., Saijyo, S., Sakurai, N., Saeki, H., Fukuhara, K., Imai, K., Matsuno, S. (1996) MUC1-specific targeting immunotherapy with bispecific antibodies: inhibition of xenografted human bile duct carcinoma growth. *Cancer Res.* **56**:4205-4212.

Kenigsberg, R.L. and Cuello, A.C. (1990) Production of a bi-specific monoclonal antibody recognizing mouse kappa light chains and horseradish peroxidase--applications in immunoassays. *Histochem.* **95**:155-163.

Kenigsberg, R.L., Elliott, P., Cuello, A.C. (1991) Two distinct monoclonal antibodies raised against mouse nerve growth factor--generation of bi-specific anti-nerve growth factor anti-horseradish peroxidase antibodies for use in a homogeneous enzyme immunoassay. *J. Immunol. Methods* **136**:247-257.

Killian, C.S., Yang, N., Emrich, L.J., Vargas, F.P., Kuriyama, M., Wang, M C., Slack, N, H, Papsidero, L.D., Murphy, G.P., Chu, T.M. (1985) Prognostic importance of prostatic-specific antigen for monitoring patients with stage B2 to D1 prostate cancer. *Cancer Res.* **45**:886-891.

Klug,T.L., Bast, Jr, R.C., Niloff, J.M., Knapp, R.C., Zurawski, Jr,V.R. (1984) Monoclonal antibody immunoradiometric assay for an antigenic determinant (CA125) associated with human epithelial ovarian carcinomas. *Cancer Res.* **44**:1048-1053.

Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**:495-497.

Kosmas, C., Linardou, H., Epenetos, A.A. (1993) Advances of monoclonal antibody tumor targeting. *J. Drug Targeting* **1**:81-91.

Krantz, M.J., Maclean, G., Longenecker, B.M., Suresh, M.R. (1988) Abstract, *J. Cell Biochem. Suppl.* **12E**:139.

Kreutz, F.T. and Suresh, M.R. (1995) Bispecific Monoclonal anti-CA125 x anti-peroxidase antibodies in the measurement of the ovarian carcinoma antigen. *J. Tumor Marker Oncol.* **10**:45-53.

Kreutz, F.T. and Suresh, M.R. (1997) Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. *Clin. Chem.* **43**:649-656.

Kuppen, P.J.K., Eggermont, A.M.M., Smits, K.M., Van Edendenburg, J.D.H., Lazeroms, S.P.G., Van de Velde, C.J.H., Fleuren, G.J. (1993) The development and purification of a bispecific antibody for lymphokine-activated killer cell targeting against the rat colon carcinoma CC531. *Cancer Immunol. Immunother.* **36**: 403-408.

Kuriyama, M., Wang, M.C., Lee, C.L., Papsidero, L.D., Killian, C.S., Inaji, H., Slack, N.H., Nishiura, T., Murphy, G.P., Chu, T.M. (1981) Use of human prostate-specific antigen in monitoring prostate cancer. *Cancer Res.* **41**:3874-3876.

Kuriyama, M., Wang, M.C., Papsidero, L.D., Killian, C.S., Shimano, T., Valenzuela, L., Nishiura, T., Murphy, G.P., Chu, T.M. Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. *Cancer Res.* **40**:4658-4662.

Lange, P.H., Ercole, C.J., Lightner, D.J., Fraley, E.E., Vessella, R. (1989) The value of serum prostate-specific antigen determinations before and after radical prostatectomy. *J. Urol.* **141**:873-879.

Lavin, P.T., Knapp, R.C., Malkasian, G., Whitney, C.W., Berek, J.C., Bast, Jr, R.C. (1987) CA125 for the monitoring of ovarian carcinoma during primary therapy. *Obstet. Gynecol.* **69**:223-227.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lilja, H. (1985) A killikrein-like serine protease in prostatic fluid leaves the predominant seminal vesicle protein. *J. Clin. Invest.* **76**:1899.

Linder, N.M., Jeffcoat, R., Lowe, C.R. (1989) Design and applications of biomimetic anthraquinone dyes, Purification of calf intestinal alkaline phosphatase with immobilised terminal ring analogues of C.I. reactive Blue 2. *J. chromatogr.* **473**:227-240.

Loebel, J.E. (1991) Tosoh AIA-1200/AIA-GOO automated immunoassay analyzers. *J. Clin. Immunoassay* **14**:94-102.

Mack, M., Riethmuller, G., Kufer, P. (1995) A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. USA.* **92**:7021-7025.

Madiyalakan, R., Kuzma, M., Noujaim, A.A., Suresh, M.R. (1996) An antibody-lectin sandwich assay for the determination of CA125 antigen in ovarian cancer patients. *Glycoconjugate J.* **13**:513-517.

McCormack, R.T., Rittenhouse, H.G., Finlay, J.A., Sokoloff, R.L., Wang, T.J., Wolefert, R.L., Lilja, H., Oesterling, J.E. (1995) Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. *Urol*, **45**:729-744.

Milstein, C. and Cuello, A.C. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature* **305**:537-540.

Milstein, C. and Cuello, A.C. (1984) Hybrid hybridomas and the production of bispecific monoclonal antibodies. *Immunol. Today* **5**:299-304.

Miribel, L., Gianazza, E., Arnaud, P. (1988) The use of dye-ligand affinity chromatography for the purification of non-enzymatic human plasma proteins. *J. Biochem. Biophys. Method* **16**:1-15.

Monne, M., Croce, C.M., Yu, H., Diamandis, E.P. (1994) Molecular characterization of prostate-specific antigen mRNA expressed in breast tumor. *Cancer Res.* **54**:6344-6347.

Myrtle, J.F., Schackelford, W., Bartholmew, R M., Wampler, J. (1983) Prostate-specific antigen: quantitation in serum by immunoradiometric assay. *Clin. Chem.* **29**:1216.

Nap, M., Vitali, A., Nustad, K., Bast, Jr., R.C., O'Brien, T.J., Nilsson, O., Seguin, P., Suresh, M.R., B rmer, O.P., Saga, T., de Bruijn, H.W.A., Nozawa, S., Kreutz, F.T., Jette, D., Sakahara, H., Gadnell, M., Endo, K., Barlow, E.H., Warren, D., Paus, E., Hammarström, S., Kenemans, P., Hilgers, J. (1996) Imminohistochemical characterization of 22 monoclonal antibodies against the CA125 antigen: 2nd report from the ISOBM TD-1 workshop. *Tumor Biol* **17**:325-331

Niloff, J.M., Bast, Jr, R.C., Schaetzl, E.M., Knapp, R.C. (1985) Predictive value of CA125 antigen level in second-look procedures for ovarian cancer. *Am J Obstet Gynecol.* **151**:981-986.

Nisonoff, A. and Rivers, M.M. (1961) Recombination of a mixture of univalent antibody fragments of different specificity. *Arch. Biochem. Biophys.* **93**:460-462.

Nolan, O. and O'Kennedy, R. (1990) Bifunctional antibodies: concept, production and applications. *Biochim. Biophys. Acta* **1040**:1-11

Nolan O. and O'Kennedy R. (1992) Bifunctional antibodies and their potential clinical applications. *Int. J. Clin. Lab. Res.* **22**:21-27

Oesterling, J.E., Chan, D.W., Epstein, J.I. (1988) Prostate specific antigen in the preoperative and postoperative evaluation of localized prostatic cancer treated with radical prostatectomy. *J. Urol.* **139**:766-772.

Oesterling, J.E., Moyad, M.A., Wright, G.L., Beck, G.R. (1995) An analytical comparison of the three most commonly used prostate specific antigen assays: Tandem-R, Tandem-E, and Imx. *Urol.* **46**:524-532.

Parker, S.L., Tong, T., Bolden, S., Wingo, P.A.(1996) Cancer statistics, 1996 [comment citation in Medline]. *CA Cancer J. Clin.* **46**:5-27.

Pietersz, G. A. and Krauer, K. (1994) Antibody-targeted drugs for the therapy of cancer. *J. Drug Targeting.* **2**:183-215.

Porstmann, T and Kiessig, S.T. (1992) Enzyme immunoassay techniques. *J. Immunol. Methods* **150**: 5-21.

Price, A., Attwood, S.E.A., Grant, J.B.F., Gray, T.A., Moore, K.T.H. (1991) Measurement of prostate-specific antigen and prostatic acid phosphatase concentrations in serum before and 1-42 days after transurethral resection of the prostate and orchidectomy. *Clin. Chem.* **37**:859-863.

Reading, C. (1981) in Tom, B.H. and Allison, J.P., (eds.) *Hybridomas and Cellular Immortality*, Plenum Press New York. pp. 235-250.

Rubin, S.C., Hoskins, W.J., Hakes, T.B., Markman, M., Reichman, B.S., Chapman, D. and Lewis, Jr, J.L. (1989) Serum CA 125 levels and surgical findings in patients undergoing secondary operations for epithelial ovarian cancer. *Am J Obstet. Gynecol.* **160**:667-671.

Sahin, U., Hartmann, F., Senter, P., Pohl, C., Engert, A., Diehl, V., Pfreundschuh, M. (1990) Specific activation of the prodrug mitomycin phosphate by a bispecific anti-CD30/anti-alkaline phosphatase monoclonal antibody. *Cancer Res.* **50**:6944-6948.

Schaller, J., Akiyama, K., Tsuda, R., Hara, M., Marti, M., Rickli, E.E. (1987) Isolation, characterization and amino-acid sequence of gama-seminoprotein, a glycoprotein from human seminal plasma. *Eur. J. Biochem.* **170**:111-120.

Self, C H. (1985) Enzyme amplification-A general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J. Immunol. Methods* **76**:389-393.

Simsek, H., Kadayifci, A., Okan, E. (1996) Importance of serum CA125 levels in Malignant Peritoneal Mesothelioma. *Tumor Biol.* **17**:1-4.

Smith, W., Jarrett, A.L., Beattie, R.E. and Corvalan, J.R.F. (1992) Immunoglobulins by a hybrid-hybridoma: analysis of chain assemblies. *Hybridoma.* **11**:87-98

Songsivilai, S., Clissold, P.M., Lachmann, P.J. (1989) A novel strategy for producing chimeric bispecific antibodies by gene transfection. *Biochem. Biophy. Res. Commun.* **164:** 271-276.

Songsivilai, S. and Lacman, P. J. (1990) Bispecific antibody: A tool for diagnosis and treatment of disease. *Clin. Exp. Immunol.* **79**:315-321

Staerz, U.D. and Bevan, M.J. (1986) Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity. *Proc. Natl. Acad. Sci. USA* **83**, 1453-1457

Stamey, T.A., Graves, H.C.B., Wehner, N., Ferrari, M., Freiha, F.S. (1993) Early detection of residual prostate cancer after radical prostatectomy by an ultrasensitive assay for prostate specific antigen. *J. Urol.* **149**:787-792

Sternberger, L.A., Hardy, P.H., Cuculis, J.J., Meyer, H.G. (1970) The unlabeled antibody enzyme method of immunohistochemistry; preparation and properties of soluble antigen-antibody complex (horseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* **18**: 315-333.

Stratieva-Taneeva, P.A., Khaidukov, S.V., Kovalenko, V.A., Nazimov, I.V., Samokhvalova, L.V., Nesmeyanov, V.A. (1993) Bispecific monoclonal antibodies to human interleukin 2 and horseradish peroxidase. *Hybridoma* **12**:271-285.

Suresh, M.R., Cuello, A.C., Milstein, C. (1986a) Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol.* **121**:210-228.

Suresh, M.R., Cuello, A.C., Milstein, C. (1986b) Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. *Proc. Natl. Acad. Sci. USA* **83**:7989-7993

Suresh, M.R., Noujaim, A.A., Longenecker, B.M. (1991) Recent developments in monoclonal antibodies. in: Cheremisinoff, P.N. and Ferrante, L.M. (eds), *Biotechnology Current Progress*. Vol. 1, pp.83-101. U.S.A. Technomic Publ.

Tada, H., Toyoda, Y., Iwasa, S. (1989) Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassay for human lymphotoxin. *Hybridoma* **8**:73-83.

Takahashi, M. and Fuller, S.A. (1988) Production of murine hybrid-hybridomas secreting bispecific monoclonal antibodies for use in urease-based immunoassays. *Clin.Chem.* **34**:1693-1696.

Van Krieken, J.H.J.M. (1993) Prostate marker immunoreactivity in salivary gland neoplasms. *Am. J. Surg. Pathol.* **17**:410-414.

Vessella, R.L., Noteboom, J., Lange, P.H. (1992) Evaluation of the abbott IMx automated immunoassay of prostate-specific antigen.*Clin.Chem.* **38**:2044-2054.

Walsh, P.C. (1992) Why make an early diagnosis of prostate cancer. *J. Urol.* **147**:853-854.

Wang, M.C., Valenzuela, L.A., Murphy, G.P., Chu, T.M. (1979) Purification of a human prostate specific antigens. *Invest. Urol.* **17**:159-163.

Wang, M.C., Papsidero, L.D., Kuriyama, M., Valenzuela, L.A., Murphy, G.P. (1981) Prostate antigen: a new potential marker for prostatic cancer. *Prostate* **2**:89-96.

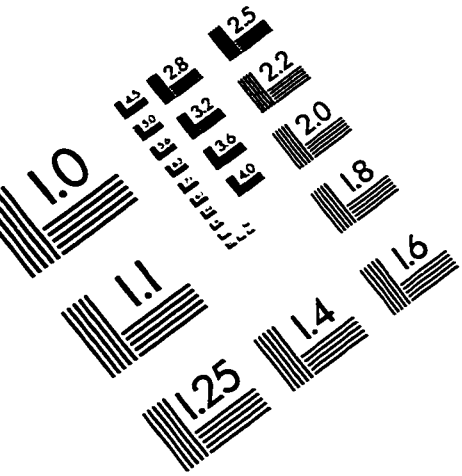
Yang, N. (1989) Pros-check PSA: a double antibody radioimmunoassay for prostate-specific antigen. In Catalona, W. J., Coffey, D. S. and Karr, J. P.(Eds): *Clinical Aspects of Prostate Cancer*. pp. 172-178. Elsevier Science, New York:

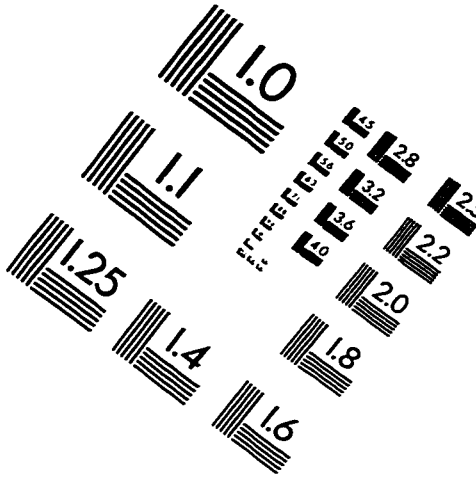
Yu, H., Diamandis, E.P., Prestigiacomo, A.F., Stamey, T.A. (1995) Ultrasensitive assay of prostate specific antigen used for early detection of prostate cancer relapse and estimation of tumor doubling time after radical prostatectomy. *Clin. Chem.* **41**:430-434

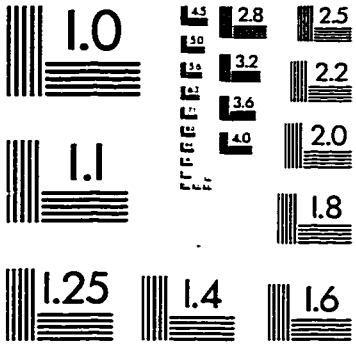
Yu, H. and Diamandis, E.P., (1995a) Prostate-specific antigen in the milk of lactating women*Clin. Chem.* **41**:54-58.

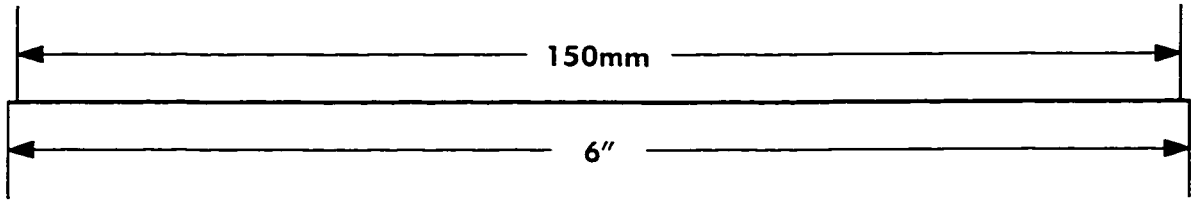
Yu, H. and Diamandis, E.P. (1995b) Prostate-specific antigen in the amniotic fluid. *Clin. Chem.* **41**:204-210.

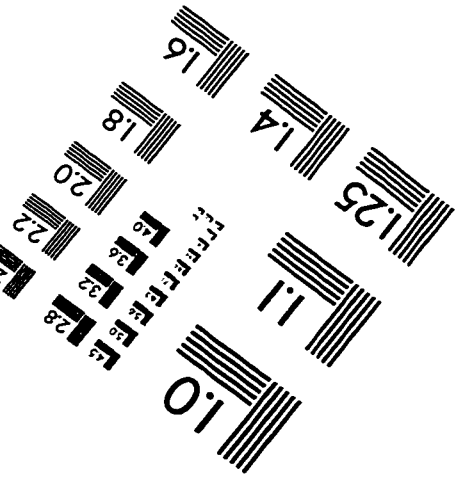
IMAGE EVALUATION
TEST TARGET (QA-3)

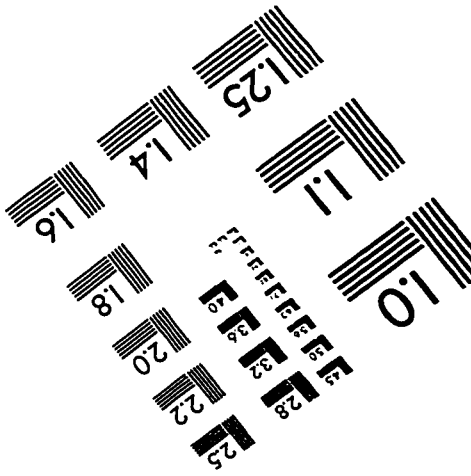












APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989