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DEVELOPMENT OF KITS FOR RADIOIMMUNOMETRIC ASSAYS FOR TUMOUR MARKERS

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FOREWORD

Considerable efforts worldwide continue to be directed towards research for understanding cancer, as well as for management of cancer patients. Apart from national programmes in a number of countries, international organizations like the World Health Organization (WHO) have helped in setting up national cancer control programmes in many developing Member States. These have resulted in an increase in overall survival rates of patients, better quality of life and, in many cases, complete remission of disease. Recent developments in immunology, biotechnology and genetics, as well as availability of sophisticated surgical tools and more effective drugs have enhanced possibilities of treatment and cure for cancer patients. Successful application of better treatment modalities critically depends on the early diagnosis of cancer, well before it spreads to other parts of the body. Among the various methods available for early cancer diagnosis, measurement of 'tumour markers' or 'tumour specific antigens' in serum samples offers several advantages. These include sensitivity, simplicity, reliability, ability to analyse large number of samples at low cost, possibility to use as screening tests and patient tolerance.

Many tumour marker assays have been reported over the years and their role is well recognized and acknowledged in the follow-up of known cancer cases. However, their true potential for use in primary diagnosis or screening of high risk groups is still to be fully realized due to the need to achieve better specificity. Among the various tumour markers, the one for prostate cancer — prostate specific antigen (PSA) — appears to have better specificity, coming close to a tumour specific antigen. Prostate cancer is a commonly encountered cancer in men, and can be effectively treated if detected early. PSA levels in serum appear to provide good correlation with tumour burden. Estimation of free PSA in serum is reported to further improve the diagnosis. In several developed countries routine screening of men above 50 years of age for prostate cancer using serum PSA as marker is recommended.

Radioimmunometric assay techniques offer themselves as attractive candidates for measurement of tumour markers. They are robust, economical and didactic, thus eminently suitable for technology transfer, training and teaching. Preparation of primary reagents is relatively easy. The methodology is flexible. As a result of co-operation projects of the IAEA, many developing Member States have built up indigenous capabilities to perform radioimmunometric assays, which can be extended to development of kits for tumour marker assays.

Considering the need for indigenous development of capabilities to produce reliable kits for radioimmunometric assays for PSA, in 1997 the IAEA initiated a Co-ordinated Research Project (CRP) on Development of Kits for Radioimmunometric Assays for Tumour Markers. Even though the focus of the project was PSA, it was expected that the expertise to be gained by the participants would also help them undertake development of kits for other tumour markers, essentially using the same methodology. Ten laboratories from Europe, Asia, Africa and the Americas participated in the programme. Efforts in the CRP were focussed on developing and validating methodologies for solid phase immunoradiometric assays (IRMA) for both total and free PSA. The procedures and protocols developed in the participating laboratories for preparation of the primary reagents needed for the assays, including purified PSA, matched pair anti PSA MoAbs, ^{125}I labelled MoAb tracer, solid phase bound capture MoAb and PSA standards and the different assay formats standardized, are detailed in the report. This report is expected to serve as a good practical guidebook for any one intending to develop IRMA for free or total PSA.

The IAEA wishes to thank all the participants in the CRP for their valuable contributions, especially M. Venkatesh for help in compiling this report, and M.R. Suresh for the overall support and guidance of the project. The IAEA officer responsible for this publication was D.V.S. Narasimhan of the Division of Physical and Chemical Sciences.

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CONTENTS

1. INTRODUCTION AND SUMMARY OF THE RESEARCH PROJECT

1.1. Preamble

Cancer is one of the major causes of human mortality. For better understanding and management of cancer a great deal of research is going on all over the world in several areas. The concept of "tumour markers" has greatly aided towards this aim. Tumour markers are bio-molecules, which may be hormones or proteins or peptides, present in levels far higher in cancer situations than in normal conditions. *In vitro* assays for tumour markers have made the staging, follow-up and screening for a variety of cancers a simple procedure, enabling better management and better chances of cure [1–3]. These assays have hence become an important limb of cancer management in the recent times. A few valuable tumour markers that are being routinely used are prostate specific antigen (PSA), carcino embryonic antigen (CEA), alfa feto protein (AFP), cancer antigens CA-19.9, 15.3, 125 and others for a range of common cancers such as prostate, colon, liver, pancreas, breast and ovarian cancers respectively. The initial euphoria that tumour markers could unerringly predict the presence and type of cancer lasted only for a short period as many of these markers were present under normal conditions and were not very specific. Many of the markers could be used for follow-up rather than to detect the occurrence. Thus work to identify newer markers with better specificity and sensitivity has been going on.

Among the several known tumour markers, PSA has very high sensitivity (>85%) and specificity (>90%) as a marker for prostate cancer [4–10]. Prostate cancer is one of the most prevalent cancers in men and if diagnosed at an early stage can be treated successfully. Hence, currently PSA is the only marker used for screening the male population over 50 years of age for prostate cancer in many countries [11–15]. The American Cancer Society, USA has recommended that PSA measurement be included as regular analysis in the annual medical examination for senior male population (>50 year age) along with digital rectal examination [16–20]. Often, this kind of mass screening and data collection involves import of a large number of kits and hence prohibitively expensive. Easy and local availability of methods to analyse PSA in human sera would go a long way in mass screening, collection of data from a variety of populations and sustenance of program for a long time to come. Apart from screening for prostate cancer, occurrences of metastasis in organs such as bone have also been shown to be associated with increased PSA levels [21–24]. The recent studies and reports on the significance of high serum PSA levels in other pathological conditions such as liver diseases, lung diseases, breast cancers etc., impart additional importance to the PSA assays [25–34]. Correlation between the serum PSA levels and the extent of malignancy is being researched in various laboratories worldwide [35–38]. Hence, the need for the availability of assay kits for both t-PSA and f-PSA to all the needy clinics and hospitals cannot be over emphasized.

PSA is a serine protease with a molecular weight of ~33 kDa secreted by the prostate cells into the seminal fluid. PSA belongs to the Kallikrein family and has 60–80% homology with human Kallikreins, namely hK1 and hK2. Although PSA is present in seminal fluid at a level of 0.5–2.0 mg/mL, its levels in serum is 0–4 ng/mL Most (70–85%) of PSA in serum is bound to proteins namely, ζ-antichymotrypsin (ζ-ACT) and ζ_2-macroglobulin to a small extent [39–44]. Serum levels of total PSA are increased in prostate cancer patients as well as in benign hyperplasia of prostate. However, the ratio of free to total PSA in benign hyperplasia has been reported to be significantly higher (f/t>0.15) than in prostate cancer (f/t <0.15). This has added a new dimension to the PSA measurement in human sera, namely free and total PSA values and f/t ratio [45–58].

Immunometric assays are the most reliable and easy to perform for measurement of tumour markers in serum samples and a number of immunometric assays such as radioactive, enzyme, chemiluminescent and fluorimetric assays are available for measurement of these tumour markers [59–63]. Immunoradiometric assays (IRMAs) are available for nearly all the known tumour markers. IRMAs are reliable and easy to adopt by most laboratories through the world as these suffer minimum from interference and most laboratories in the world have experience with radioimmunoassays. As

PSA is present in two forms in human serum, accurate measurement of each form would involve careful selection of the antibodies for capture and signal. In the case of total PSA the signal antibody has to be directed to an epitope well removed from the ζ-ACT binding site in order to obtain equimolar response with both free and bound PSA. On the other hand, one of the antisera for IRMA of free PSA should necessarily bind to the epitope in ζ-ACT binding region so that the bound PSA does not give a signal [64–68].

With the view to enable local availability of methods to measure tumour markers, collection of data in local population and critical comparison of the available methods, a CRP on "Development of Kits for Radioimmunometric Assays for Tumour Markers" was started by the IAEA and PSA was taken up as the first priority. In all, ten countries participated in this project with Dr. Mavanur Suresh from University of Alberta, Canada as the research agreement holder and the rest as research co-ordinators.

1.2. Objective

The objective of this CRP was to enable the participant countries to develop IRMAs for both total and free PSA at their own laboratories initially with the key reagents supplied by the IAEA. The participants would achieve expertise in methodology development after investigating various parameters such as reagent stability, storage conditions, performance of the assay system under different assay conditions, validation of the developed system and so on. Expertise gained is expected to be translated to the kit-formulation when the key reagents could either be prepared or obtained from a reliable commercial source.

1.3. Scientific achievements

One of the main highlights of accomplishments is that all the participants could successfully establish sandwich type IRMAs for both total and free PSA using the reagents supplied by the IAEA and validate these assays. All the participants had thoroughly studied the radiolabelling of the monoclonal antibodies (MAbs) and could prepare tracers of required specific activity and quality. Similarly all could prepare solid phase bound capture MAbs of adequate stability and capacity for use in the assays. In a nutshell, the methods for preparation of the various reagents have been standardized. Nearly all the key reagents, namely: standard PSA and matched pairs of MAbs for both total and free PSA, could potentially be indigenously produced by the CRP participants. Many groups have hybridoma facilities to pursue this objective.

In general, the experience of developing these assays has instilled the basic acumen and confidence in all the participants to pursue development of IRMAs for other tumour markers. Some participants have also used their reagents to develop non-isotopic assays and validated them. The following table highlights the achievements of each group.

1.4. Summary

Extensive discussions on all the aspects of assay development and sharing of experience and ideas among the participants resulted in update of information on all aspects. This built-up confidence in the participants to develop their own assays and trouble-shoot when in need. The various aspects of development of assay procedures are summarized here and the individual detailed reports are included in the annexe.

1.4.1. Standard PSA calibrators

A few participants could isolate and purify PSA from seminal plasma using procedures such as affinity purification on a antibody column, liquid chromatography on columns such as affigel blue, DEAE Sepharose, etc. the details of which are included in the individual reports. The calibrator for

Country	Salient feature
Algeria	Use of commercial MAbs and PSA for development of t-PSA IRMA
China	In-house preparation of PSA and all MAbs; supply of kits to local hospitals; complete self-sustenance
Cuba	In-house preparation of PSA and all MAbs; development of ELISAs; complete self-sustenance
Greece	Extensive studies on sample matrix for free PSA assays and coating protocol for rugged solid phase MAbs; use of local tubes as solid phase
India	In-house preparation of PSA and one of the MAbs; self-sustenance in total PSA assays
Islamic Republic of Iran	Use of Avidin-biotin based solid phase coupling of capture MAb; use of polystyrene tubes as well as beads as solid phase
Thailand	Use of locally made polypropylene tubes and magnetisable particles as solid phase; tracer purification by HPLC
Uruguay	Clinical studies with patient samples to revisit the significance of f/t ratio

PSA is a technically involved issue as PSA is present in multiple species (free and bound) and has potential cross reactants in hK1 and hK2, which are very similar to PSA. Several groups from all over the world have addressed this issue, and in a recent international workshop [66,69,70]. Various aspects of PSA calibrators and controversies regarding preparation of PSA calibrators were discussed in detail. The group was adequately aware of the problems and complexity involved. The need to calibrate the in-house calibrators against well established standards was emphasized. Such well established standards could be either the Gold Standard from Stanford University or European Union; or a secondary standard originally standardized against the Stanford standard, such as Hybritech IRMA PSA kit or any other kit that has been standardized. The PSA calibrator supplied by the IAEA has been adequately calibrated for potency as well as absence of cross-reaction with hK1 &2. It was agreed that for self-sufficiency, one could purify PSA from seminal plasma, prepare the calibrators in a buffer solution, calibrate against a known calibrator and finally establish the usability in clinical conditions with limited clinical samples. Depending on the regulatory requirements, the calibration may have to be made in individual countries against Gold standards. In the case of total PSA equimolar response to free and total PSA may have to be proved using PSA-ACT complex [66,71,72].

1.4.2. Monoclonal antibodies

The choice of the matched MAbs was a critical issue. Dr. Suresh has initially screened and identified the three MAbs for the two assays. As many MAbs are known to cross react with human kallikreins hK1 and hK2, Dr. Suresh had carefully screened to ensure that these MAbs did not cross with hK1 and hK2 and resulted in equi-molar assays for total-PSA. These MAbs were initially supplied by Dr. Suresh to all the other participants. This helped the participants to develop assays with authentic reagents to begin with and compare the assays developed using indigenous reagents with these authentic methods.

1.4.3. Tracer

All the participants had used chloramine-T as the oxidant to radioiodinate both the MAbs with ^{125}I. In general, specific activities of ~370–450 kBq/μg (10–12 μCi/μg) were found optimal by the participants and the tracers could be used for at least one month when stored at Ω4ºC. The tracers were purified generally by gel chromatography while HPLC purification was also used and found to yield better quality product with lower non-specific binding and better stability.

It was observed that the quality of radio-iodine was an important parameter that could affect the tracer preparation and that fresh radio-iodine is desirable to get better yield of radio-iodination and better stability of the tracer. Storage of tracer in glycerol as a concentrated solution in order to save the storage space was found suitable by some laboratories. Addition of normal mouse serum (10% net) was found to greatly aid in reduction of non-specific binding in both free and total assays and hence would yield better sensitivities.

1.4.4. Solid phase matrix

All the participants could successfully use tubes as the solid support matrix and some demonstrated successful use of polystyrene beads and magnetic particles also. Although some had difficulties in obtaining good precision and binding with locally produced tubes, the basic causes and methods to solve them could be identified. Some could demonstrate the use of avidin-biotin reaction for preparation of solid phase coupled capture MAb. Based on the collective experience, it was felt that Glutaraldehyde activation for coating MAbs on tubes was not necessary, although may be followed.

Various coating procedures have been optimized and adopted by the participants as detailed in individual reports. In brief, the MAbs supplied could bind to the solid matrix when incubated at a pH 7.5 to 9.5, using 1–2 μg/mL solution. Final washing of the coated tubes with a buffer containing high BSA content (2–8%) or glazing with sucrose solution appears to stabilize the coating and increase the shelf life of the coated tubes.

1.4.5. Assay format

Based on the previous experience, the participants designed different assay formats. Single step assays were followed by some while some others found that two-step assays had better sensitivity and lower non-specific biding. Addition of normal mouse serum was observed to reduce the non-specific binding significantly. This is perhaps due to the prevention of non-specific adherence of tracer MAb molecules (mouse immunoglobulins) by the excess of mouse gamma globulins. Hence normal mouse serum was added either in the tracer as mentioned earlier or in the assay buffer.

There were elaborate discussions on the substitute matrix for standards in both total and free PSA assays. Female serum which normally has insignificant circulating PSA levels was considered the best matrix for total PSA assays. It was felt that female sera could be screened for absence of PSA (less than the detectable limit), pooled and used as the matrix for total PSA assays. However, for free PSA assays it was observed that a lot of caution should be exercized in the choice of matrix for standards. Female serum would be considered an attractive choice, but due to the possible presence of binding proteins such as ζ4ACT, free PSA levels would be depleted depending on the concentration of binding proteins. Hence it was recommended that the free PSA calibrators should be made in the preferred matrix and thoroughly calibrated against known standards or using well established kits. The need to ensure the stability of these standard preparations with time was stressed. Most participants had found assay buffers containing a high BSA concentration (4–8%) to be an usable matrix while a few had used female serum as the matrix for free PSA assays.

1.4.6. Validation

All the participants found that the assay procedures developed had acceptably low variations. In general the intra-assay variations were between 5 to 10% while the inter-assay variations were between 8% and 12%. Recovery of added PSA standards in serum samples and parallelism studies were also carried out by some of the participants. The assays exhibited good parallelism and the recovery was between 90 to 110%. The absence of cross-reaction with hK1 & hK2 and equimolar response in total PSA kits were indirectly established by most participants by correlation of sample values obtained by the developed method and an established kit procedure. This was necessary since hK1 & hK2 are not easily available. Cuba tested the equimolar response by estimating the PSA value

in nine authentic samples supplied by the Stanford University for this purpose. As mentioned earlier, the supplied reagents have already been tested for absence of cross reaction with hK1 & hK2 and equimolar response. As all the participants had gained adequate expertise in preparation of the necessary reagents of good quality and stability and had developed the assay procedures for both total and free PSA, the prospects for preparation and supply of kits in their respective countries and beyond is seen to be bright. No serious problems are expected to be encountered towards local supply of kits by the participant laboratories. China has been supplying the kits since the recent past.

The following tables list the various assay parameters optimized by the participants in the assays developed by them for total and free PSA at a glance.

1.5. Future recommendations

The following recommendations were made.

(a) The participants should co-ordinate and collaborate with local medical laboratories/hospitals to clinically evaluate the developed IRMA methods.

(b) It would be desirable to have an external quality assessment scheme (EQAS) with a panel of samples to ascertain the quality of the reagents and the procedures.

(c) Efforts to obtain PSA-ACT complex and refining the procedures to obtain rugged standard calibrators for free and total PSA should be continued by the participants with an aim to allow assessment in relation to international standards possible.

(d) The participants should establish interactions with health authorities and make benefits of the method available to the cross section of the public.

(e) On similar lines, development of IRMAs for breast cancer marker CA15.3 should be taken up as a continuation of this CRP, as the participants are already familiar with the basic procedures. This would address a key cancer in women.

1.6. Publications resulting from the CRP

INDIA

1. Development of an IRMA for total PSA
   Aruna korde, Ketaki Bapat, Archana Shukla, Meera Venkatesh and M.R.A.Pillai
   Ann. Conf. Society of Nucl. Med. (India), 1999, Indian J. Nucl. Med., 14, p97, 1999.
2. Monoclonal Antibody for Prostate Specific Antigen : Generation and Characterization
   Ketaki Bapat, Aruna Korde, Archana Shukla, Meera Venkatesh and M.R.A.Pillai
   XXVI Annual Conference and Symposium on Cancer Immunology in the New Millennium, Mumbai, India, 2000.
3. Development of an Immunoradiometric Assay for total PSA
   Archana Shukla, Aruna Korde, Ketaki Bapat, Meera Venkatesh and M.R.A.Pillai
   International Conference on Probing in Biological Systems, Mumbai, India 2000.

Assay for total PSA								
Assay parameter	Algeria	China	Cuba	Greece	India	Islamic Republic of Iran	Thailand	Uruguay
MAbs Capture Tracer	Purchased CIS-BIO CIS-BIO	Local P27A10 P27B1	Local CB-4 CB-9	Supplied MAb66 MAb10	Local MAb2S MAbJ2	Supplied MAb66 MAb10	Supplied MAb66 MAb10	Supplied MAb66 MAb10
Tracer* (µCi/µg)	9–10	15–20	15–22	9	10–15	10	30–40	10–15
PSA standard reference for calibration	Purchased NETRIA	Local DPC kit	Local Stanford	Local CIS kit	Local CLIA kit	Supplied CIS kit	Supplied CIS kit	Supplied Netria kit
Solid phase matrix	Tubes - NUNC	Tubes- local	Tubes -	Tubes-	Tubes - NUNC	Beads -USA	Tubes - local	Tubes - NUNC
Coating method	Passive	Passive	Active @	Passive	Passive	Active @	Passive	Passive
Buffer	PB	Bicarb.	PBS	Tris	Bicarb./PB	PB	Bicarb	Bicarb.
PH	7.4	9.6	7.2	7.8	8.5	7.4	8	9.4
MAb (g)	2	2	1	0.5	1	2.5	1	1
Final treatment	BSA 1% +Sucrose 2%	BSA 1%		BSA 1%	BSA + Sucrose 20%	BSA+ Sucrose	BSA 1% +Sucrose 2%	BSA+ Sucrose
Stability	7 weeks, –20°C	6 months		> 6 months, 4°C	3 months, –20°C	6 months	6 months	9 months, –20 °C
Assay parameters Incubation	2 h, RT	1.5 + 2 h 37°C	2 h, RT	18 h, 37°C	3 h, RT	4 h, RT	2 h + 2 h, RT	2 + 18 h, RT
Matrix for standard	Female serum	PB, 5% BSA	Female serum	PBS 4% BSA	Female serum	Female serum	Female serum	10% BSA
Sample vol. (µL)	50	100	100	50	100/50	50	50	25
Tracer (ng/tube)	10	~ 10	~ 7.5	~ 20	8–10	~ 5	4–6	~15
Sensitivity (ng/mL)		0.2	0.025	0.04	0.5		0.05	~1
Validation	QC-CISBIO	QC-DPC	Stanford, CIS-BIO	Hybritech	QC-DPC	QC-DSL	QC-CISBIO	QC-NETRIA
Range (ng/mL)	0–100	0–80	0.025–100	0.04–112	0.5–100	2.5–360	0–200	0–65

* All used Chloramine-T as the oxidizing agent to prepare the tracer by radioiodination with $Na^{125}I$

@ Biotin-Avidin system was used for coating the capture antibody to the tubes.

Assay for free PSA							
Assay parameter	China	Cuba	Greece	Islamic Republic of Iran	India	Thailand	Uruguay
MAbs Capture Tracer	Local P27A10 MAb30	Local CB-9 CB-2	Supplied MAb66 MAb30	Supplied MAb66 MAb30	Supplied MAb66 MAb30	Supplied MAb66 MAb30	Supplied MAb66 MAb30
Tracer* (µCi/µg)	15–22	15–22	9	9	10–20	30–40	10–15
PSA standard reference for calibration	Local DPC kit	Local Stanford	Local CIS kit	Supplied CIS kit	Local CLIA kit	Supplied CIS kit	Supplied Netria kit
Solid phase matrix	Tubes- local	Tubes -	Tubes-	Beads -USA	Tubes - NUNC	Tubes - local	Tubes - NUNC
Coating method	Passive	Passive	Passive	Active @	passive	passive	Passive
Buffer	Bicarb.	PBS	Tris	PB	Bicarb./PB	Bicarb	Bicarb.
PH	9.6	7.4	7.8	7.4	8.5	8	9.4
MAb (g)	2.5	1	0.5	1	1	0.5	1
Final treatment	1% BSA		1% BSA	BYCO-A	BSA + Sucrose 20%	BSA 1% +Sucrose 2%	BSA+ Sucrose
Stability	6 months		> 6 months, 4°C	6 months	3 months,–20°C		9 months,–20°C
Assay parameters Incubation	1.5 + 2 h 37°C		18 h, 37°C	4 h, RT	18 h, 4°C		2 + 18 h
Matrix for standard	PB, 5% BSA		PBS 4% BSA	Female serum	PB, 7.5% BSA	PB, 5% BSA	10% BSA
Sample volume (µL)	100		50	100	100/50	50	25
Tracer (ng/tube)			20		~10	2.5	12
Sensitivity (ng/mL)	0.3		0.03		0.5	0.06	
Validation	QC		HYBRITECH	QC-DSL	QC-CLIA	QC-CISBIO	QC-NETRIA
Range (ng/mL)	0.3–40		0.03–26	2.5–360	0.5–50	0–20	0–65

* All used Chloramine-T as the oxidizng agent to prepare the tracer by radioiodination with $Na^{125}I$
@ Biotin-Avidin system was used for coating the capture antibody to the tubes.

5. Robles, A., Balter, H., Oliver, P., Laiz, J., Berbejillo, J., Gonçalvez, Z., Lopez, A.
Free and Total PSA by IRMA with bulk reagents-XVI Congress of the Latinamerical Association of Societies of Biology and Nuclear Medicine- Iberoamerican Congress on Nuclear Medicine-V Seminar of the Argentinian Association of Nuclear Cardiology-XII Argentinian Congress Argentino of Biology and Nuclear Medicine. 24–28 October, 1999. Buenos Aires-Argentina.
6. Berbejillo, J., Goncalvez,Z., Lopez,A., Robles,A.
Immunoradiometric assay of free and total PSA. VI Congress of the South American Pharmaceutical Federation. 26–28 April, 2000 Montevideo, Uruguay.

REFERENCES

[1] SCHWARTZ, M.K., Current status of tumour markers, Scandinavian Journal of Clinical and Laboratory Investigation **55-**SUPPL. 221 (1995) 5–14.

[2] AZIZ, K., Tumour markers: Current status and future applications, Scandinavian Journal of Clinical and Laboratory Investigation **55-**SUPPL. 221 (1995) 153–155.

[3] GARNICK, M.B., FAIR, W.R., Prostate cancer: Emerging concepts: Part I & II, Annals of Internal Medicine **125** (1996) 118–125, 205–211.

[4] BANGMA, C.H., BLIJENBERG, B.G., SCHRODER, F.H., Prostate-specific antigen: Its clinical use and applications in screening for prostate cancer, Scandinavian Journal of Clinical and Laboratory Investigation **55-**SUPPL. 221 (1995) 35–44.

[5] DIAMANDIS, E.P., New diagnostic applications and physiological functions of prostate specific antigen, Scandinavian Journal of Clinical and Laboratory Investigation **55-**SUPPL. 221 (1995) 105–112.

[6] DUFFY, M.J., PSA as a marker for prostate cancer: a critical review, Ann-Clin-Biochem. **33** (1996) 511–9.

[7] LABRIE, F., et al., Diagnosis of advanced or noncurable prostate cancer can be practically eliminated by prostate-specific antigen, Urology **47** (1996) 212–217.

[8] BECKER, C., LILJA, H., Individual prostate-specific antigen (PSA) forms as prostate tumour markers, Clin-Chim-Acta. **257** (1997) 117–32.

[9] MONTIE, J.E., MEYERS, S.E., Defining the ideal tumour marker for prostate cancer, Urol-Clin-North-Am. **24** (1997) 247–52.

[10] ROSS, K.S., CARTER, H.B., PEARSON, J.D., GUESS, H.A., Comparative efficiency of prostate-specific antigen screening strategies for prostate cancer detection, JAMA **284**(2000) 1399–405.

[11] BANGMA, C., KRANSE, R., BLIJENBERG, B.G., SCHODER, F., The value of screening tests in the detection of prostate cancer. Part I: Results of a retrospective evaluation of 1726 men; Part II: Retrospective analysis of free/total prostate-specific analysis ratio, age-specific reference ranges, and PSA density, Urology **46** (1995) 773–778, 779–784.

[12] FILELLA, X., et al., Co-operative Group For The Diagnosis Of Prostate Cancer, Value of PSA (prostate-specific antigen) in the detection of prostate cancer in patients with urological symptoms. Results of a Multicentre Study, European Journal of Cancer **32A**(1996) 1125–1128.

[13] KOMATSU, K., et al., Physiologic (intra-individual) variation of serum prostate-specific antigen in 814 men from a screening population, Urology **47** (1996) 343–346.

[14] BANGMA, C.H., KRANSE, R., BLIJENBERG, B.G., SCHRODER, F.H., Free and total prostate-specific antigen in a screened population, Br-J-Urol. **79** (1997) 756–62.

[15] MORRIS, J., MCNOE, B., Screening for prostate cancer: what do general practitioners think? N-Z-Med-J. **110** (1997) 178–82.

[16] REISSIGL, A., et al., Comparison of different prostate-specific antigen cutpoints for early detection of prostate cancer: Results of a large screening study. Urology **46**(1995) 662–665.

[17] WOOLF, S.H., Screening for prostate cancer with prostate-specific antigen: An examination of the evidence, New England Journal of Medicine **333**(1995) 1401–1405.

[18] PARKES, C., et al., Prospective observational study to assess value of prostate specific antigen as screening test for prostate cancer, British Medical Journal **311** (1995) 1340–1343.

[19] KANTROWITZ, W., DOYLE, J., SEMERARO, J., KRANE, R.J., Prostate cancer screening in a large corporation population, Journal of Occupational and Environmental Medicine **37** (1995) 1193–1198.

[20] IMAI, K., ICHINOSE, Y., KUBOTA, Y., YAMANAKA, H., SATO, J., Diagnostic significance of prostate specific antigen and the development of a mass screening system for prostate cancer, Journal of Urology **154** (1995) 1085–1089.

[21] LORENTE, J.A., MOROTE, J., RAVENTOS, C., ENCABO, G., VALENZUELA, H., Clinical efficacy of bone alkaline phosphatase and prostate specific antigen in the diagnosis of bone metastasis in prostate cancer, Journal of Urology **155** (1996) 1348–1351.

[22] KAGEYAMA, Y., KIHARA, K., KAMATA, S., NAGAHAMA, K., YONESE, J., FUKUDA, H., TOSAKA, A., NAGAMATSU, H., ISHIZAKA, K., TSUJII, T., KITAHARA, S., MORITA, T., OSHIMA, H., Relationship between pretreatment serum levels of prostate specific antigen and bone metastasis in prostate cancer, Acta Urologica Japonica **42** (1996) 197–199.
[23] GLEAVE, M.E., COUPLAND, D., DRACHENBERG, D., COHEN, L., KWONG, S., GOLDENBERG, S.L., SULLIVAN, L.D., Ability of serum prostate-specific antigen levels to predict normal bone scans in patients with newly diagnosed prostate cancer, Urology **47** (1996) 708–712.
[24] WOLFF, J.M., BARES, R., JUNG, P.K., BUELL, U., JAKSE, G., Prostate-specific antigen as a marker of bone metastasis in patients with prostate cancer, Urologia Internationalis **56** (1996) 169–173.
[25] MOROTE, J., LORENTE, J.A., Impact of kidney and liver metabolism on serum prostate specific antigen levels, International Journal of Biological Markers **10** (1995) 236–237.
[26] LEVESQUE, M., YU, H., D'COSTA, M., TADROSS, L., DIAMANDIS, E.P., Immunoreactive prostate-specific antigen in lung tumours, Journal of Clinical Laboratory Analysis **9** (1995) 375–379.
[27] GIAI, M., YU, H., ROAGNA, R., PONZONE, R., KATSAROS, D., LEVESQUE, M., A, DIAMANDIS, E.P., Prostate-specific antigen in serum of women with breast cancer, British Journal of Cancer **72** (1995) 728–731.
[28] NADLER, R.B., HUMPHREY, P.A., SMITH, D.S., CATALONA, W.J., RATLIFF, T.L., Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels, Journal of Urology **154** (1995) 407–413.
[29] YU, H., GIAI, M., DIAMANDIS, E.P., KATSAROS, D., SUTHERLAND, D.J.A., LEVESQUE, M.A., ROAGNA, R., PONZONE, R., SISMOMDI, P., Prostate-specific antigen is a new favourable prognostic indicator for women with breast cancer, Cancer Research **55** (1995) 2104–2110.
[30] KADAYIFCI, A., BENEKLI, M., SIMSEK, H., SENCAN, O., Prostatic acid phosphatase and prostate specific antigen in liver disease, International Urology and Nephrology **28** (1996) 67–71.
[31] LAI, L.C., ERBAS, H., LENNARD, T.W.J., PEASTON, R.T., Prostate-specific antigen in breast cyst fluid: Possible role of prostate-specific antigen in hormone-dependent breast cancer, International Journal of Cancer **66** (1996) 743–746.
[32] MELEGOS, D.N., DIAMOANDIS, E.P., Diagnostic value of molecular forms of prostate-specific antigen of female breast cancer, Clinical Biochemistry **29** (1996) 193–200.
[33] ABBATE, I., MUSCI, M.D., PARADISO, A., SCHITTULLI, F., CORREALE, M., Determination of free PSA in breast cancer cytosols by IRMA, International Journal of Biological Markers **11** (1996) 53–54.
[34] MAGKLARA, A., CHEUNG, C.C., ASA, S.L., DIAMANDIS, E.P., Expression of prostate-specific antigen and human glandular kallikrein 2 in the thyroid gland, Clin-Chim-Acta. **300** (2000) 171–80.
[35] LERNER, S.E., et al., Free, complexed, and total serum prostate-specific antigen concentrations and their proportions in predicting stage, grade and deoxyribonucleic acid ploidy in patients with adenocarcinoma of the prostate, Urology **48** (1996) 240–248.
[36] CRITZ, F.A., ET AL., Prostate-specific antigen nadir of 0.5 mg/mL or less defines disease freedom for surgically staged men irradiated for prostate cancer, Urology **49** (1997) 668–72.
[37] MOROTE, J., RAVENTOS, C.X., LORENTE, J.A., LOPEZ-PACIOS, M.A., ENCABO, G., DE-TORRES, I., ANDREU, J., Measurement of free PSA in the diagnosis and staging of prostate cancer, Int. J. Cancer **71** (1997) 756–9.
[38] D'AMICO, A.V., et al., Utilizing predictions of early prostate-specific antigen failure to optimize patient selection for adjuvant systemic therapy trials, J-Clin-Oncol. **18** (2000) 3240–6.
[39] HILZ, H., Molecular forms of prostate specific antigen and their clinical significance, Urologe Ausgabe A **34**(1995) 275–282.

[40] ZHANG, W.M., LEINONEN, J., KALKKINEN, N., DOWELL, B., STENMAN, U.H., Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid, Clinical Chemistry **41** (1995) 1567–1573.
[41] LILJA, H., Prostate-specific antigen: molecular forms and the human kallikrein gene family. Br-J-Urol. **179**-Suppl 1 (1997) 44–8.
[42] SOKOLL, L.J., CHAN, D.W., Prostate-specific antigen. Its discovery and biochemical characteristics. Urol-Clin-North-Am. **24** (1997) 253–9.
[43] ABRAHAMSSON, P.A., LILJA, H., OESTERLING, J.E., Molecular forms of serum prostate-specific antigen. The clinical value of percent free prostate-specific antigen. Urol-Clin-North-Am. **24** (1997) 353–65.
[44] QIAN, Y., SENSIBAR, J.A., ZELNER, D.J., SCHAEFFER, A.J., FINLAY, J.A., RITTENHOUSE, H.G., LEE, C., Two-dimensional gel electrophoresis detects prostate-specific antigen-alpha1-antichymotrypsin complex in serum but not in prostatic fluid, Clin-Chem. **43**(1997) 352–9.
[45] PRESTIGIACOMO, A.F., STAMEY, T.A., Clinical usefulness of free and complexes PSA, Scandinavian Journal of Clinical and Laboratory Investigation 55-SUPPL. **221** (1995) 32–34.
[46] CATALONA, W.J., et al., Evaluation of percentage of free serum prostate-specific antigen to improve specificity of prostate cancer screening, JAMA (Journal of the American Medical Association) **274** (1995) 1214–1220.
[47] DEMURA, T., SHINOHARA, N., TANAKA, M., ENAMI, N., CHIBA, H., TOGASHI, M., OHASHI, N., NONOMURA, K., KOYANAGI, T., The proportion of free to total prostate specific antigen: A method of detecting prostate carcinoma, Cancer **77**(1996) 1137–1143.
[48] ESPANA, F., et al., Prostate-specific antigen and its complexes with alpha 1-antichymotrypsin in the plasma of patients with prostatic disease. Eur-Urol. **30** (1996) 512–8.
[49] PRESTIGIACOMO, A.F., LILJA, H., PETTERSSON, K., WOLFERT, R.L., STAMEY, T.A., A comparison of the free fraction of serum prostate specific antigen in men with benign and cancerous prostates: The best case scenario, Journal of Urology **156** (1996) 350–354.
[50] ELGAMAL, A.A.A., CORNILLIE, F.J., VAN-POPPEL, H.P., WAN-DE-VOORDE, W.M., MCCABE, R., BAERT, L.V., Free-to-total prostate specific antigen ratio as a single test for detection of significant stage T1c prostate cancer, J of Urology **156** (1996) 1042–1049.
[51] WOLFF, J.M., BORCHERS, H., EFFERT, P.J., HABIB, F.K., JAKSE, G., Free-to-total prostate-specific antigen serum concentrations in patients with prostate cancer and benign prostatic hyperplasia, British Journal of Urology **78** (1996) 409–413.
[52] BANGMA, C.H., RIETBERGEN, J.B., KRANSE, R., BLIJENBERG, B.G., PETTERSON, K., SCHRODER, F.H., The free-to-total prostate specific antigen ratio improves the specificity of prostate specific antigen in screening for prostate cancer in the general population [see comments], J Urol. **157** (1997) 2191–6; Comment in 2197–8.
[53] FROSCHERMAIER, S.E., PILARSKY, C.P., WIRTH, M.P., Clinical significance of the determination of non-complexed prostate-specific antigen as a marker for prostate carcinoma, Urology **47** (1996) 525–528.
[54] VOGL, M., MULLER, M.M., HOLTL, W., Clinical usefulness of percentage of free serum prostate specific antigen Clin-Chim-Acta. **258** (1997) 79–90.
[55] REISSIGL, A., KLOCKER, H., POINTNER, J., ENNEMOSER, O., FALK, M., BARTSCH, G., Improvement of prostate cancer screening by determination of the ratio free/total PSA in addition to PSA levels, Prostate **30** (1997) 243–7.
[56] RICCARDO, B., ALBERINO, D., FABRIZIO, T., GINO, C., SIMONE, A., GUIDO, B., CESARE, S., Free to total prostatic specific antigen ratio as a new diagnostic tool in prostatic carcinoma, Anticancer-Res. **17** (1997) 1297–301.
[57] TARLE, M., KRALJIC, I., Free and total serum PSA values in patients with prostatic intraepithelial neoplasia (PIN), prostate cancer and BPH. Is F/T PSA a potential probe for dormant and manifest cancer?, Anticancer-Res. **17** (1997) 1531–4.
[58] MOROTE, J., ENCABO, G., DE-TORRES, I.M., Use of percent free prostate-specific antigen as a predictor of the pathological features of clinically localized prostate cancer, Eur-Urol. **38**(2000) 225–9.

[59] PETTERSSON, K., PIIRONEN, T., SEPPALA, M., LIUKKONEN, L., CHRISTENSSON, A., MATIKAINEN, M.T, SUONPAA, M., LOVGREN, T., LILJA, H., Free and complexed prostate-specific antigen (PSA): *In vitro* stability, epitope map, and development of immunofluorometric assays for specific and sensitive detection of free PSA and PSA-alpha–1-antichymotrypsin complex, Clinical Chemistry **41** (1995) 1480–1488.

[60] MITRUNEN, K., PETTERSSON, K., PIIRONEN, T., BJORK, T., LILJA, H., LOVGREN, T., Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum, Clinical Chemistry **41** (1995) 1115–1120.

[61] MIONE, R., BARICHELLO, M., SARTORELLO, P., LEON, A., BARIOLI, P., GION, M., Third-generation PSA: Ultrasensitive or ultraprecise assay? International Journal of Biological Markers **10** (1995) 229–235.

[62] LEIN, M., JUNG, K., SCHNORR, D., HENKE, W., LEONING, S.A., Strip test for the quick detection of increased concentrations of prostate-specific antigen in blood, European Journal of Clinical Chemistry and Clinical Biochemistry **34** (1996) 511–514.

[63] WANG, T.J., HILL, T.M., SOKOLOFF, R.L., FRANKENNE, F., RITTENHOUSE, H.G., WOLFERT, R.L., Dual monoclonal antibody immunoassay for free prostate-specific antigen, Prostate **28** (1996) 10–16.

[64] LILJA, H., COCKETT, A.T., ABRAHAMSSON, P.A., Prostate specific antigen predominantly forms a complex with alpha 1-antichymotrypsin in blood. Implications for procedures to measure prostate specific antigen in serum, Cancer **70** Suppl. 1 (1992) 230–4.

[65] WENER, M.H., DAUM, P.R., BRAWER, M.K., Variation in measurement of prostate-specific antigen: Importance of method and lot variability, Clinical Chemistry **41** (1995) 1730–1737.

[66] SOKOLOFF, R.L., WOLFERT, R.L., RITTENHOUSE, H.G., Standardization of PSA Immunoassays: Proposals and practical limitations, Journal of Clinical Ligand Assay **18** (1995) 86–92.

[67] JETTE, D.C., KREUTZ, F.T., MALCOLM, B.A., WISHART, D.S., NOUJAIM, A.A., SURESH, M.R., Epitope mapping of prostate-specific antigen with monoclonal antibodies, Clin-Chem. **42** (1996) 1961–9.

[68] STAMEY, T.A., Progress in standardization of immunoassays for prostate-specific antigen, Urol-Clin-North-Am. **24** (1997) 269–73.

[69] TEWARI, P.C, BLUESTEIN, B.I., Multiple forms of prostate specific antigen and the influences of immunoassay design on their measurement in patient serum, Journal of Clinical Ligand Assay **18**(1995)186–196.

[70] SUMI, S., UMEDA, H., KOGA, F., YANO, M., IMAI, T., HOSOYA, Y., HONDA, M., MAEDA, S., SUZUKI, T., YOSHIDA, K.I., NEIGISHI, T., Determination of free type and complex type prostate-specific antigen (PSA): Differences in immunorecognition by Delfia PSA, ACS-PSA and Eiken PA kits, Acta Urologica Japonica **42** (1996) 279–284.

[71] GRAVES, H.C., Issues on standardization of immunoassays for prostate-specific antigen: a review.Clin-Invest-Med. **16** (1993) 415–24.

[72] NAGASAKI, H., WATANABE, M., KOMATSU, N., KANEKO, T., DUBE, J.Y., KAJITA, T., SAITOH, Y., OHTA, Y., Epitope analysis of a prostate specific antigen (PSA) C-terminal-specific monoclonal antibody and new aspects for the discrepancy between equimolar and skewed PSA assays, Clin-Chem. **45** (1999) 486–96.

BIBLIOGRAPHY

General, clinical

AKDAS, A., CEVIK, I., TARCAN, T., TURKERI, L., DALAMAN, G., EMERK, K., The role of free PSA in the diagnosis of prostate cancer, Br-J-Urol. **79** (1997) 920–3.

CHRISTENSSON, A., BJORK, T., NILSSON, O., et al., Serum PSA complexed to alpha-ACT as an indicator of prostate cancer — J. of Urol. **150** (1993) 100–5.

GO, R.S., KLEE, G.G., RICHARDSON, R.L., Use of pleural fluid PSA in the diagnosis of malignant effusion from metastatic prostate cancer, J-Urol. **164** (2000) 459.

HONDA, S.A.A., GOLDSTEIN, A.P., MORITA, T., et al., PSA concentrations in serum in acute illnesses — Clin. Chem. **42** (1996) 1785–8.

HUBER, P.R., MATTARELLI, G., STRITTMATTER, B., VAN-STEENBRUGGE, G.J., SCHMID, H.P., MAURER, A., *In vivo* and *in vitro* complex formation of prostate specific antigen with alpha-1-anti-chymotrypsin, Prostate **27** (1995) 166–175.

HUBER, P.R., SCHMID, H.P., MATTARELLI, G., STRITTMATTER, B., JAN-VAN-STEENBRUGGE, G., MAURER, A., Serum free prostate specific antigen: Isoenzymes in benign hyperplasia and cancer of the prostate, Prostate **27** (1995) 212–219.

HUDSON, M.A., BAHNSON, R.R., CATALONA, W.J., Clinical use of PSA in patients with prostate cancer — J. of Urol. **142** (1989) 1011.

IRANI, J., MILLET, C., LEVILLAIN, P., DORE, B., BEGON, F., AUBERT, J., Serum-to-urinary prostate-specific antigen ratio: A potential means of distinguishing benign prostatic hyperplasia from prostate cancer, European Urology **29** (1996) 407–412.

ISRAELI, R.S., GROB, M., FAIR, W.R., Prostate-specific membrane antigen and other prostatic tumour markers on the horizon. Urol-Clin-North-Am. **24** (1997) 439–50.

JETTE D.C., KREUTZ, F.T., MALCOLM, B.A., et al., Eptiope mapping of PSA with monoclonal antibodies. Clin. Chem. **42** (1996) 1961–9.

KIKUCHI, E., NAKASHIMA, J., ISHIBASHI, M., OHIGASHI, T., ASAKURA, H., et al., Prostate specific antigen adjusted for transition zone volume: the most powerful method for detecting prostate carcinoma, Cancer **89** (2000) 842–9.

LANG, P.H., ERCOLE, C.J., LIGHTNER, D.J., et al., The value of serum PSA determinations before and after radical prostatectomy, J. of Urol. **141** (1989) 873.

MURPHY, G.P., BARREN, R.J., ERICKSON, S.J., BOWES, V.A., WOLFERT, R.,L., et al., Evaluation and comparison of two new prostate carcinoma markers: Free-prostate specific antigen and prostate specific membrane antigen, Cancer **78** (1996) 809–818.

NIXON, R.G., LILLY, J.D., LIEDTKE, R.J., BATJER, J.D., Variation of free and total prostate-specific antigen levels: the effect on the percent free/total prostate-specific antigen, Arch-Pathol-Lab-Med. **121** (1997) 385–91.

OESTERLING, J.E., PSA: a critical assessment of the most useful marker for adenocarcinoma of the prostate — J. of Urol. **145** (1991) 907.

REISSIGL, A., BARTSCH, G., PSA as a screening test — Urol. Clin. of N. Amer. **24** (1997) 315–21.

REISSIGL, A., KLOCKER, H., POINTNER, J., et al., Usefulness of the ratio free/total PSA in addition to the total PSA levels in prostate cancer screening. Urology **48** (1996) 62–6.

PSA variations with population

BLIJENBEG, B.G., KESSLER, A.C., UHL, W., Multicentre evaluation of a new fully automated enzyme immunoassay for total prostate-specific antigen, European Journal of Clinical Chemistry and Clinical Biochemistry **33**(1995) 965–973.

CIATTO, S., BONARDI, R., MAZZOTTA, A., SANTONI, R., Reliability of prostate-specific antigen evaluation according to age-specific range, International Journal of Biological Markers **10**(1995) 182.

CIATTO, S., BONARDI, R., MAZZOTTA, A., SANTONI, R., Reliability of volume or age-adjusted prostate specific antigen to improve diagnostic accuracy, International Journal of Biological Markers **10**(1995) 226–228.

DEANTONI, E.P., Age-specific reference ranges for PSA in the detection of prostate cancer, Oncology-Huntingt. **11** (1997) 475–82, 485; discussion 485–6, 489.

EL GALLEY, R.E.S., PETROS, J.A., SANDERS, W.H., KEANE, T.E., GALLOWAY, N.T.M., COONER, W.H., GRAHAM, S.D., JR., Normal range prostate-specific antigen versus age-specific prostate-specific antigen in screening prostate adenocarcinoma urology **46** (1995) 200–204.

HOFFMAN, B.R., YU, H., DIAMANDIS, E.P., Assay of prostate-specific antigen from whole blood spotted on filter paper and application to prostate cancer screening. Clinical Chemistry **42** (1996) 536–544.

JACOBSEN, S.J., OESTERLING, J.E., Age-specific reference ranges for serum prostate specific antigen levels, Journal of Clinical Ligand Assay **18** (1995) 93–97.

KIROLLOS, M.M., Statistical review and analysis of the relationship between serum prostate specific antigen and age, J-Urol. **158** (1997) 143–5.

NIXON, R.G., WENER, M.H., SMITH, K.M., PARSON, R.E., STROBEL, S.A., BRAWER, M.K., Biological variation of prostate specific antigen levels in serum: an evaluation of day-to-day physiological fluctuations in a well-defined cohort of 24 patients [see comments] J Urol **157** (1997) 2183–90; Comment in: 2197–8.

ORNSTEIN, D.K., SMITH, D.S., RAO, G.S., BASLER, J.W., RATLIFF, T.L., CATALONA, W.J., Biological variation of total, free and percent free serum prostate specific antigen levels in screening volunteers [see comments] J Urol. **157** (1997) 2179–82; Comment in: 2197–8.

RANDELL, E.W., DIAMANDIS, E.P., ELLIS, G., Serum prostate-specific antigen measured in children from birth to age 18 years, Clinical Chemistry **42** (1996) 420–423.

RICHARDSON, T.D., OESTERLING, J.E., Age-specific reference ranges for serum prostate-specific antigen, Urol-Clin-North-Am. **24** (1997) 339–51.

ROEHRBORN, C.G., PICKENS, G.J., CARMODY, T., Variability of repeated serum prostate-specific antigen (PSA) measurements within less than 90 days in a well-defined patient population, Urology **47** (1996) 59–66.

Problems in PSA value interpretation

BLIJENBERG, B.G., KRANSE, R., EMAN, I., SCHRODER, F.H., Some analytical considerations on the measurement of PSA. Eur-J-Clin-Chem-Clin-Biochem. **34** (1996) 817–21.

BRAWER, M.K., DAUM, P., PETTEWAY, J.C., WENER, M.H., Assay variability in serum prostate-specific antigen determination. Prostate **27** (1995) 1–6.

CAREY, R.N., FRYE, R.M., COOK, J.D., KOCH, T.R., HARRIS, E.K., Between-lot/between-instrument variations of the Abbott IMx method for prostate-specific antigen [letter] Clin-Chem. **38** (1992) 2341–3.

HANSELLER, E., KELLER, H., A new mathematical procedure for the assessment of test performance of PSA assays. Scandinavian Journal of Clinical and Laboratory Investigation **55** SUPPL. 221 (1995) 52–56.

JUNG, K., LEIN, M., SCHNORR, D., BRUX, B., HENKE, W., LOENING, S., Comparison between equimolar- and skewed-response assays of PSA: is there an influence on the clinical significance when measuring total serum PSA, Ann-Clin-Biochem. **33** (1996) 209–14.

NARDONE, D.A., Problems with interpreting results of PSA [letter] J-Gen-Intern-Med. **12** (1997): 200; discussion 200-1.

OESTERLING, J, E, JACOBSEN, S, J, KLEE, G, G, PETTERSSON, K, PIIRONEN, T, et al, Free, complexed and total serum PSA: The establishment of appropriate reference ranges for their concentrations and ratios. Journal of Urology **154**(1995) 1090–1095.

ROEHRBORN, C.G., GREGORY, A., MCCONNELL, J.D., SAGALOWSKY, A.I., WIANS, F.H., JR. Comparison of three assays for total serum PSA and percentage of free PSA in predicting prostate histology. Urology **48**-6A Suppl (1996) 23–32.

SEMJONOW, A., BRANDT, B., OBERPENNING, F., HERTLE, L., Discrepancies in assays impair the interpretation of prostate-specific antigen. Urologe Ausgabe A **34** (1995) 303–315.

SEMJONOW, A., OBERPENNING, F., BRANDT, B., ZECHEL, C., BRANDAU, W., HERTLE, L., Impact of free PSA on discordant measurement results of assays for total PSA. Urology **48**-6A Suppl (1996) 10–5.

STAMEY, T.A., Lower limits of detection, biological detection limits, functional sensitivity, or residual cancer detection limit? Sensitivity reports on prostate-specific antigen assays mislead clinicians. Clinical Chemistry **42** (1996) 849–852.

STENMAN, U.H., LEINONEN, J., ZHANG, W., M, Problems in the determination of prostate specific antigen, Eur-J-Clin-Chem-Clin-Biochem. **34** (1996) 735–40.

STENMAN, U.H., LEINONEN, J., ZHANG, W.M., Standardization of PSA determinations. Scandinavian Journal of Clinical and Laboratory Investigation **55**-SUPPL. 221 (1995) 45–51.

TURKES, A., NOTT, J.P., GRIFFITHS, K., Prostate-specific antigen: problems in analysis [see comments] Eur-J-Cancer. **27** (1991) 650–2. Comment in: 1185.

VAN-DUIJNHOVEN, H.L.P., PEQUERIAUX, N.C.V., VAN-ZON, J.P.H.M., BLANKENSTEIN, M., A, Large discrepancy between PSA results from different assays during longitudinal follow-up of a prostate cancer patient. Clinical Chemistry **42** (1996) 637–641.

ZHOU, Z., NG, P.C., VERY, D.L., JR, ALLARD, W.J., YEUNG, K.K., Technicon Immuno 1 PSA assay measures both free and alpha-1-antichymotrypsin-complexed PSA on an equimolar basis. Journal of Clinical Laboratory Analysis **10** (1996) 155–159.

Sample instability

BEI, R., PARANAVITANA, C., MILENIC, D., KASHMIRI, S.V.S., SCHLOM, J., Generation, Purification, and Characterization of a Recombinant Source of Human Prostate-Specific Antigen. Journal of Clinical Laboratory Analysis **9** (1995) 261–268.

BELANGER, A., VAN-HALBEEK, H., GRAVES, H.C.B., GRANDBOIS, K., STAMEY, T., et al., Molecular mass and carbohydrate structure of prostate specific antigen: Studies for establishment of an International PSA Standard. Prostate **27** (1995) 187–197.

CHEN, Z., PRESTIGIACOMA, A., STAMEY, T.A., Purification and characterization of prostate-specific antigen (PSA) complexed to alpha-1-antichymotrypsin: Potential reference material for international standardization of PSA immunoassays. Clinical Chemistry **41** (1995): 1273–1282.

GRAVES, H.C.B., KAMAREI, M., STAMEY, T.A., Identity of PSA and the semen protein p30 purified by a rapid chromatography technique, J. of Urol. **144** (1990) 1510–5.

LEININEN, J., KALKKINEN, N., DOWELL, B., et al., Isolation and characterization of different molecular forms of PSA in human seminal fluid, Clin. Chem. **41** (1995) 1567–73.

MERIO, L., PETTERSSON, K., LOVGREN, T., Monoclonal antibody-based dual-label time-resolved fluorometric assays in a simplified one-step format. Clin. Chem. **42** (1996) 1513–1517.

PIIRONEN, T., PETTERSSON, K., SUONPAA, M., STENMAN, U.H., OESTERLING, J.E., LOVGREN, T., LILJA, H., *In vitro* stability of free PSA and PSA complexed to alpha 1-antichymotrypsin in blood samples. Urology. **48**-6A Suppl (1996) 81–7.

PRESTIGIACOMO, A, F, CHEN, Z, STAMEY, T, A, A universal calibrator for prostate specific antigen (PSA). Scandinavian Journal of Clinical and Laboratory Investigation **55**-SUPPL. 221(1995) 57–59.

PSA standard, purification, preparation

RUSCIANO, D., BERARDI, A., CECCARINI, C., et al., Concomitant purification of prostatic carcinoma tumour makers from human seminal fluid under nondenaturing conditions, Clin. Chem. **34** (1988) 2528–32.

SIMM, B., GLEESON, M., Storage conditions for serum for estimating prostate-specific antigen, Clin-Chem. **37**(1991) 113–4.

THIEL, R.P., OESTERLING, J.E., WOJNO, K.J., PARTIN, A.W., CHAN, D.W., et al., Multicenter comparison of the diagnostic performance of free prostate-specific antigen, Urology **48**-6A Suppl (1996) 45–50.

WANG, M.C., VALENZUELA, L.A., MURPHY, G.P., et al., A simplified purification procedure for human PSA, Oncology **39** (1982) 1–5.

WOODRUM, D., FRENCH, C., SHAMEL, L.B., Stability of free PSA in serum samples under a variety of sample collection and sample storage conditions. Urology. **48**-6A Suppl (1996) 33–9.

Standardization of assays, performances

CHAN, D.W., BRUZUEK, D.J., OESTERLING, J.E., et al., PSA as a marker for prostatic cancer:a monoclonal and polyclonal immunoassay compared, Clin. Chem. **33** (1987) 1916.

CUNY, C., PHAM, L., KRAMP, W., et al., Evaluation of a two-site immunoradiometric assay for measuring non-complexed (free) PSA, Clin. Chem.**42** (1996) 1243–9.

GRAVES, H.C.B., Standardization of immunoassays for PSA: a problem of PSA or a problem of assay design? Cancer **72** (1993) 3141–4.

JUNG, K., STEPHAN, C., LEIN, M., et al., Analytical performance and clinical validity of two free PSA assays compared, Clin. Chem.**42** (1996) 1026–33.

LIEDTKE, R.L., BATJER, J.D., Measurement of PSA by radioimmunoassay. Clin. Chem. **30** (1984) 649.

MURPHY, G.P., The second Stanford conference on international standardization of PSA assays, Cancer **75** (1995)122–9.

ROCK, R.C., CHAN, D.W., BRUZEK, D., WALDRON, C., OESTERLING, J.E., WALSH, P.C., Evaluation of a monoclonal immunoradiometric assay for PSA, Clin. Chem. **33** (1987) 2257.

STAMEY, T.A., PRESTIGIACOMA, A.F., CHEN, Z., Standardization of immunoassays for PSA: A different view on experimental observations, Cancer **74** (1994) 1662–6.

ZUCCHELLI, G.C., PILO, A., CHIESA, M.R., et al., — Analytical performance of Free Prostate Specific Antigen immunoassays: Results from an interloboratory survey (technical brief), Clin. Chem. **43** (1997) 2426–8.

2. COUNTRY REPORTS

2.1. ALGERIA

Title of the Project:	Development of Kit for Immunoradiometric Assay of Prostate Specific Antigen
Chief Scientific Investigator:	Benzaid Ahmed
Scientific Co-Investigators:	S. Boudjemai and S. Moussouni

Abstract. Development and optimization of a total PSA IRMA are described here. A matched pair of anti-PSA monoclonal antibodies and pure PSA were procured from a commercial source. PSA standards were prepared and calibrated with the help of a commercial kit. The tracer MAb was labelled with ^{125}I and purified to obtain highly pure tracer (>98%) with low non-specific binding (0.2%), good (~ 80%) maximum binding and long shelf life of at least 8 weeks. The capture MAb was immobilized on polystyrene tubes at 2 μg/tube and an assay was developed for the measurement of total PSA in serum with these reagents. A good correlation was observed between sample values estimated with the developed kit and the commercial kit from CIS-Bio International.

2.1.1. INTRODUCTION

Prostate Specific Antigen (PSA) is a serine protease of the kallikrein group, found almost exclusively in the prostate. It is a glycoprotein with a molecular weight of 34 kDa, which plays a major role in the liquefaction of seminal fluid [1]. In serum, PSA exists simultaneously in two forms, namely, uncomplexed or free PSA (f-PSA) and PSA complexed to anti-proteases ζ1-Antichymotrypsin and ζ2- Macroglobulin [2]. Prostate cancer is one of the major cancers that affects men and PSA has become the most useful biochemical marker for monitoring prostate cancer status, in conjunction with digital rectal exam [3,4]. Total PSA concentration is very low in normal men while it is reported to be elevated in men with prostate cancer [5]. An antibody excess immunoradiometric assay (IRMA) was made commercially available in 1986 [6]. Since then, these assays have been improved upon by several manufacturers and are now available with analytical detection limit below 0.05 ng/mL. The aim of this study was the development of an in-house assay for total PSA measurement in human serum, which would be beneficial to the health program in our country, to manage prostate cancer. The present study concerns the preparation of main reagents used in PSA IRMA assays and development of an IRMA system using them.

2.1.2. MATERIALS AND METHODS

Matched pair of anti-PSA monoclonal antibodies and t-PSA Kits with QC samples were supplied by CIS–Bio-International (France). Polystyrene tubes used for coupling antibodies to solid phase, were obtained from PROMMEDI (Algeria) and NUNC A/S (Denmark). ^{125}I used for labelling was purchased from Nordion, Inc, (Canada). The PSA antigen used for standard preparation was purchased from NETRIA. All other chemical reagents were obtained from NETRIA, SIGMA and FLUKA.

The equipment used in this study includes a single manual gamma counter (Scaler Ratemeter type 6–90 Burnham. UK), a multi-well gamma counter RIA-STAR (Packard–Canberra, including data processing with IAEA immunoassay program) and a rotator (NETRIA).

2.1.2.1. Procedure for t-PSA IRMA recommended by the supplier of reagents

50 µL of standards or samples of PSA and 300 µL of ^{125}I -MAb tracer are added to the tubes coated with capture MAb. The tubes are mixed gently and incubated for 2 h at room temperature. The tubes are then washed twice with 0.33% Tween 20 solution and counted for 60 s. The standard curve is constructed based on the data and sample values read off using this standard curve.

2.1.2.2. Preparation and evaluation of ^{125}I- labelled anti-PSA MAbs

Radioiodination of MAb with ^{125}I was carried out according to the method of Greenwood & Hunter [7] using chloramine-T. The radiochemical purity of ^{125}INa was ensured by Instant Thin Layer Chromatography (ITLC-SG) [8] before use. Radioiodination was carried out in a fume hood in a glass test tube (75×12 mm) as detailed below:

20 µL (50 µg) of anti-PSA MAb
10 µL Phosphate buffer 0.25 M, pH 7.4
9 µL NaI^{125} (° 500 µCi)
10 µL Chloramine-T (0.5 mg/mL in phosphate buffer 0.25 M)
Mix, 60 s
10 µL Sodium metabisulfite (0.5 mg/mL in phosphate buffer 0.25 M)
100 µL KI / BSA (10 mg/mL & 3 mg/mL respectively in phosphate buffer 0.25 M)

The labelled MAb was purified by gel chromatography over a column (150×8 mm) of Sephadex G-25, equilibrated and eluted with 0.05 M phosphate buffer, pH 7.4 containing 0.5% BSA. Fractions of 0.5 mL were collected in test tubes at a flow rate of 20 to 25 mL per hour. The radioiodination yield was estimated from the activity distribution in the elution profile. The fractions corresponding to ^{125}I-MAb, generally occurring between 14th and 15th fraction were pooled, diluted in PBSX-Proteins buffer, pH 7.4 (0.025 M PBSX containing 0.12% BSA, 0.08% BGG and 0.06% EDTA) and stored in aliquots at 4°C and –20°C.

The radiochemical purity (RCP) of the selected fractions was determined using paper electrophoresis. 5µL of tracer to be analysed along with 5µL of potassium iodide as carrier were placed on start line of Whatman No.1 paper bands (300 × 20 mm). Electrophoresis was carried for 1 hour in electrophoresis system (5A, 300V) using 0.05 M barbital buffer, pH 8.6. At the end of the run, the paper was removed, dried and cut into 1 cm segments. The radioactivity in the strips was measured. Radiochromatogram was plotted by counts against fraction number and RCP of tracer was evaluated. The free iodide moves towards the anode and labelled molecule remains at the point of application.

Immunoreactivity of the tracer was evaluated following the protocol for PSA IRMA assays of CIS-Bio-international. The non-specific binding and maximum binding were evaluated. Other parameters such as specific activity and the radioactive concentration were calculated too.The tracer was diluted in PBSX-Proteins buffer, pH 7.4 to get 90,000–100,000 CPM/ 300 µL and stored at 4–8°C.

2.1.2.3. Immobilization of anti-PSA MAbs on local and NUNC polystyrene tubes

The capture MAb AC-PSA:5.2 supplied by CIS-Bio international was immobilized on the local and NUNC polystyrene tubes by passive adsorption in accordance with the optimized CIS-Bio protocol. The steps followed are given below:

a. Dilute anti-PSA-MAb stock solution (5.2 mg/mL CIS) in coating buffer (0.087 M phosphate buffer, pH 7.4 containing 0.05% sodium azide) ~1000 folds to obtain 5 µg/mL.
b. Dispense 0.4 mL of the diluted MAb in each tube and incubate for 24 hours at room temperature.
c. Aspirate the antibody solution and block the surface with 0.5 mL of blocking buffer (Tris- citrate buffer containing 1% BSA, 1% sucrose and 0.1% sodium azide).
d. Aspirate the blocking solution and dry the tubes at 30°C overnight.
e. Store the coated tubes in sealed polyethylene bags at 4 – 8°C.

The coated tubes (local and NUNC) were evaluated according to CIS protocol for PSA IRMA assays. The non-specific binding (NSB) and the maximum binding (Bmax) were measured.

2.1.2.4. Preparation and calibration of PSA standards

PSA standards in a range of 0 to100 ng/mL and quality control samples were prepared in female serum free from PSA, collected from the local hospital, C.H.U.- Bab El Oued. These standards were. calibrated against CIS-Bio t-PSA IRMA in multiplicates of 5 and the mean values were used.

2.1.2.5. Clinical evaluation

The t-PSA concentrations from 36 human sera samples were determined using the in-house t-PSA IRMA Kit and CIS-Bio t-PSA IRMA Kit and the results compared. These tests were performed at the University Hospital of Bab-El-Oued, Algiers.

2.1.3. RESULTS AND DISCUSSION

2.1.3.1. Preparation and assessment of PSA tracer

The radiochemical purity of $Na^{125}I$ determined by ITLC-SG chromatography paper (Fig.1) shows high purity (98%) of the radioiodine and suitability for preparation of the tracer MAb. Although other methods have been used to radioiodinate proteins, the classical method of chloramine -T has been employed and found to yield good quality tracer as depicted in Table I. It is seen that very high labelling yields of ~95% could be achieved which is in compliance with the product specification given by the CIS-Bio (yields of >70%).

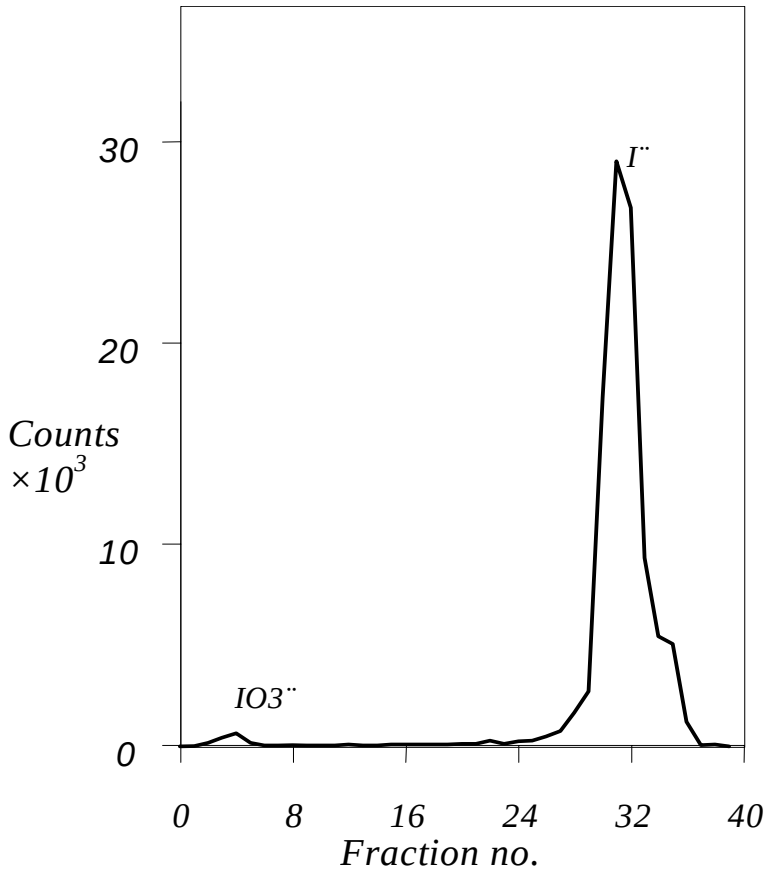

FIG. 1: Purity of NaI-125 obtained by ITLC using Methanol:Water (85:15) as eluant.

TABLE I. QUALITY CONTROL PARAMETERS OF PSA TRACER

^{125}I-MAb Tracer	Yield (%)	Sp. Act.. µCi/µg	Act/mL µCi/mL	R.C.P (%)	Immunoreactivity			
					Bmax (%)		NSB (%)	
					Local	Nunc	Local	Nunc
In-house	95.50	9.55	55.25	98.12	55.36	79.91	0.17	0.21
Cis-Bio	> 70				> 50		0.20	

Fig. 2 shows the elution profile obtained on gel filtration using Sephadex G25. The two peaks, namely of ^{125}I-MAb and free iodide (^{125}I) are separated with good resolution. This procedure allows to obtain tracer solutions with free ^{125}Iodide content lower than 2%. This observation was confirmed by the values of radiochemical purity measured by paper electrophoresis from the selected fractions which are higher than 98% (Fig.3).

The stability of the tracers was studied over a period of ten weeks under storage at 4 and –20°C by estimating the non-specific binding, the maximal binding and radiochemical purity. As shown in Figure 4, the radiochemical purity of tracer decreased slightly from 98% to 88% during the storage period when stored at 4°C. However, the tracer stored at –20°C presented reasonably high radiochemical purity even after 10 weeks.

The immunoreactivity of the tracer was tested using locally coated tubes and CIS standards following CIS protocol. The non-specific binding (NSB) and the maximal binding (Bmax) are listed in Table-I. It can be seen that the Bmax obtained for the tracers prepared in-house are comparable to the CIS kit at >50%. However the maximum binding obtained using Nunc tubes were higher (79%) than the locally available Prommedi tubes (55%). The NSB values are within the expected range (0–0.3%) for Nunc as well as the local and Prommedi tubes. Thus the in-house tracer MAb exhibited good immunoreactivity and low non-specific binding indicating that the radiolabelling conditions are suitable and do not induce any degradation anti-PSA MAb molecule. No significant changes were observed in the non specific binding and maximal binding for at least 7 weeks after preparation when the tracer was stored at 4 and –20°C, indicating the high stability of labelled MAb (Fig.5).

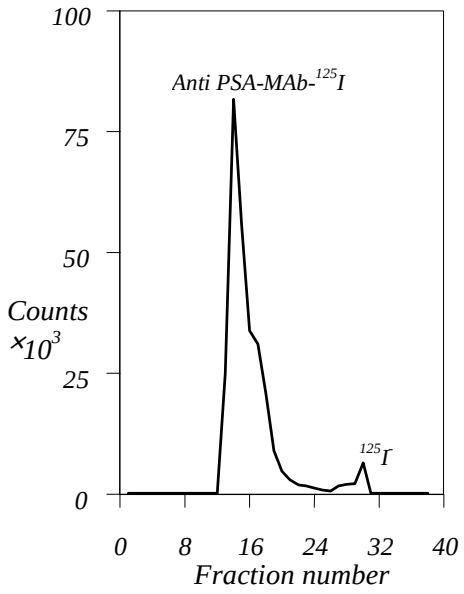

FIG. 2: Elution profile of Anti-PSA Mab on Sephadex G-25.

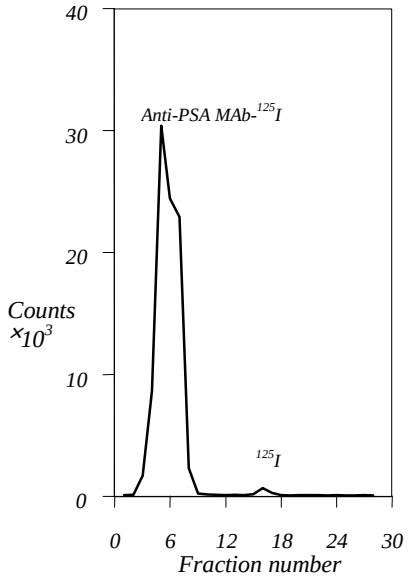

Fig. 3: Radiochromatogram of purity of anti-PSA Mab tracer by paper electrophoresis.

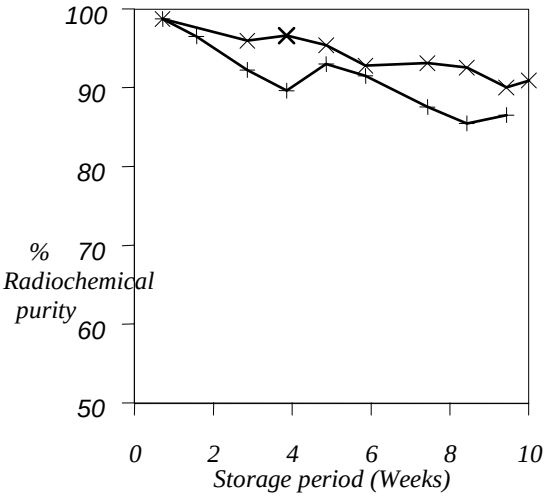

Fig. 4: Radiochemical purity of PSA tracer as a function of time and conditions of storage.
× Stored at –20°C + Stored at 4°C.

The specific activity of the tracer prepared was 9 µCi/µg, which is close to the range reported by others (10 to 25 µCi/µg). The MAb tracers supplied by the commercial company such as Netria, UK also is very close to this specific activity (8.6–9 µCi/µg.)

2.1.3.2. Immobilization of capture MAbs on the local and NUNC polystyrene tubes

The anti-PSA MAb, AC-PSA:5.2 from CIS-Bio, France, was coated on the NUNC and local PROMMEDI polystyrene tubes by passive adsorption at low phosphate buffer concentration. As seen earlier (Table I), the NSB values are comparable in both sets of tubes while the maximum binding was lower in the case of local tubes in comparison to the NUNC tubes. The coated NUNC tubes and CIS-tubes were compared for their performance in the t-PSA IRMAs. The results obtained are summarized in Table II and depicted in Figure 6.

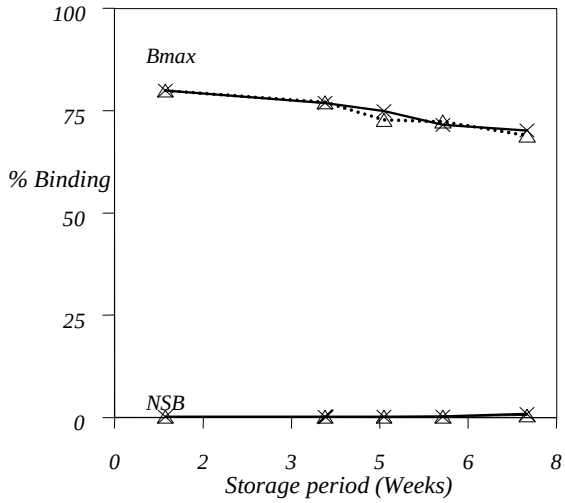

Fig. 5: Immunoreactivity of PSA tracer as function of time and conditions of storage.
÷ Stored at 4°C + Stored at –20°C .

As shown in Table II, the in-house assay for t-PSA based on coated NUNC tubes performed satisfactorily with binding capacity relatively higher than that of CIS coated tubes while the non specific binding for both were within acceptable range (< 0.3%). The laboratory QC sample values as well as CIS QC sample value were within the expected range. These results confirm that the conditions of coating are adequate and the coated NUNC tubes could be used as the solid phase in the in-house t-PSA IRMA kit. In the case of coated PROMMEDI tubes,% C.V. associated with the QC sample analysis were higher than 20% and hence these assays were not further evaluated.

2.1.3.3. Preparation and Calibration of PSA Standards

The standards and controls prepared were calibrated against CIS kit standards and the results are shown in Table III.

2.1.3.4. Clinical evaluation

The results of 36 biological samples evaluated by in-house kit and the commercial CIS-Bio Kit are illustrated in Figure 7. It can be seen that the values correlated well. The correlation coefficient was r = 0.995 and could be expressed by the equation

CIS Kit value = 1.2429 × in-house kit value — 0.2002.

TABLE II. COMPARISON OF IN-HOUSE T-PSA IRMA SYSTEM USING CIS-BIO KIT

		CIS-Bio t-PSA IRMA		In-house t- PSA IRMA (using coated NUNC tubes)	
Q C Parameter	Expected Value ng/mL	Observed Value ng/mL	% C.V.	Observed Value ng/mL	% C.V
Laboratory-QC					
C1		0.64 ± 0.03	5.27	0.58	2.30
C2		2.14 ± 0.10	4.78	2.08	1.72
C3		17.27 ± 0.94	5.44	17.03	0.84
CIS QC C1	2.55–3.55	2.75	3.05	2.69	1.38
% Bmax		61.85		70.92	
% NSB		0.15		0.24	

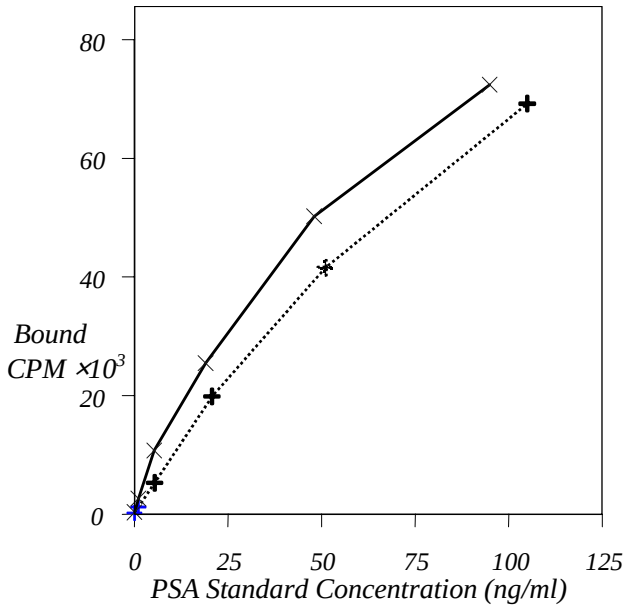

Fig.6: Comparison between CIS standard curve and in-house standard curve for PSA IRMA

.. + ..CIS standard curve × In house standard curve

TABLE III. CALIBRATION OF PSA STANDARDS AND CONTROLS

	Expected values (ng/mL)	Obtained values (ng/mL)	% C.V
Standards			
S_0	0	0	-
S_1	1.00	1.04	5.22
S_2	5.00	5.31	4.20
S_3	20.00	19.00	3.30
S_4	50.00	47.99	3.86
S_5	100.00	94.90	3.39
Lab. Controls			
C1	0.64	0.64	5.27
C2	2.00	2.14	4.78
C3	18.00	17.27	5.44

2.1.4. CONCLUSION

In conclusion, it was seen that

a. anti-PSA MAb from commercial source could be radioiodinated to obtain tracer that was stable for at least 7 weeks that retained the immunoreactivity and the radiochemical purity when stored at –20 °C.
b. anti-PSA MAb from commercial source could be coated on NUNC polystyrene tubes with very good results (Bmax = 70%). The coated tubes were stable when stored in sealed plastic bags at 2–8 °C.
c. IRMA for t-PSA could be developed using the above reagents and standards from commercial source. The standards were found to require careful preparation. The assay performed well with respect to preliminary clinical evaluation.

It is planned to carry out further validation and clinical evaluation of the in-house t-PSA IRMA kit.

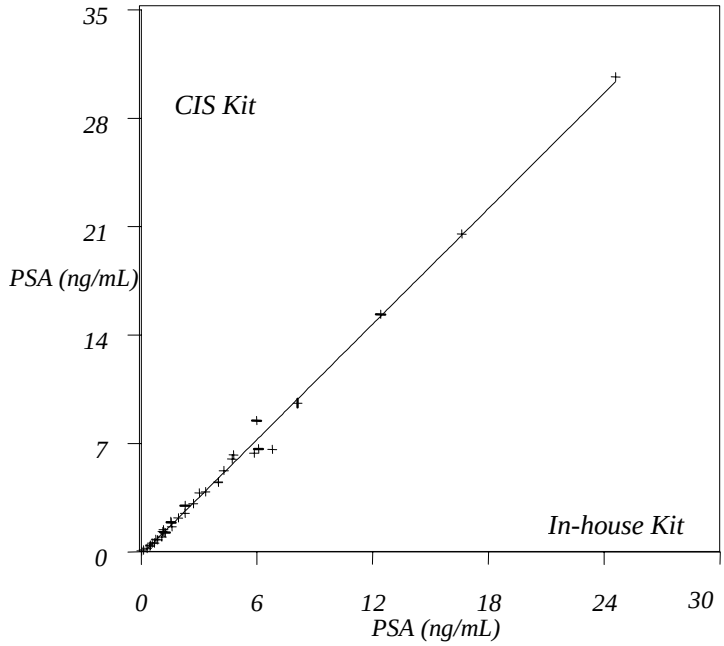

Fig. 7: Relation between the PSA values of biological samples evaluated by CIS kit and in-house kit.

ACKNOWLEDGEMENTS

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BIBLIOGRAPHY

WANG, MC et Al., Biochemical characterization of human prostate specific antigen., Fed. Proc. **40** (1981)1564.

LILJA, H. CHRISTENSSON, A., Clinical. Chem. **37** (1991) 1618.

AMBRUSTER, D. A, Prostate specific antigen: biochemistry, analytical methods and clinical applications., Clin. Chem. **39** (1993) 181.

PARTIN, A. W. OESTERLING, J. E., The clinical usefulness of prostate specific update. J. Urol. **152** (1994) 1358.

KILLIAN, C. S et Al., Cancer research. **45** (1985) 886.

MYRTLE, J. F. IVOR, L. P., Measurement of PSA in serum by IRMA. N.Y Elsevico. Science. Pub (1989) 161.

GREENWOOD, F. C, HUNTER, W. M and GLOVER, J. S., Biochem. J. **89** (1963) 114.

PHANTHETRAN, and WASNWICH, R., Practical. Nuclear. Pharmacy. Publisher, Banyan Enterprises (1979).

2.2. CHINA

Title of the Project:	Development of Kits for Radioimmunometric Assays for Tumour Markers
Chief Scientific Investigator:	Liu Yibing

Abstract. Three hybridoma cell lines secreting monoclonal antibodies against PSA ($P_{27}A_{10}$, $P_{27}B_1$ and $P_{25}B_{11}$) were obtained in our laboratory. IRMAs for total-PSA and free-PSA were developed using these as well as the monoclonal antibodies supplied in the project. $P_{27}A_{10}$ was used for coating tubes. ^{125}I- $P_{27}B_1$ and ^{125}I-PSA MAb 30 from Canada were used as tracers for t-PSA IRMA and f-PSA IRMA, respectively. The sensitivity of t-PSA IRMA was 0.2 ng/mL and the recovery was 95 to 102%. The variations (C.V.%) within and between assays were ~5% and 10–16%, respectively. For the f-PSA IRMA, the sensitivity was 0.19 ng/mL and the recovery was 80%–92.7%.

2.2.1. INTRODUCTION

The importance of serum levels of Prostate-specific antigen (PSA), both total amounts and the free/total ratio, in the early detection of prostate cancer (PCa) is now well established. When conventional cut-off level of 4 ng/mL is used for total-PSA values, the false-positive rate is 65% due to high t-PSA levels in benign prostatic hyperplasia (BPH) or prostatitis. The differentiation between BPH and PCa can be improved by determination of the serum PSA isoforms. But this is tedious and impractical for routine analysis. In the recent past, it has been clearly demonstrated that the f-PSA to t-PSA ratio is significantly lower in PCa patients. Hence, this ratio is considered a vital tool for distinguishing between PCa and BHP.

In order to facilitate screening for prostate cancer and for differential diagnosis of PCa from BPH in prostate, development of assays for t-PSA and f-PSA measurement in serum was undertaken. Towards this aim, various aspects of assay development such as purification of PSA from seminal plasma, raising specific matched monoclonal antibodies, preparation of radiolabelled antibodies for use as tracers and finally optimization of valid assay systems for both free and total PSA were carried out. The initial supply of matched pairs of monoclonal antibodies and partially pure PSA were very important for the development of these assays. An IRMA for total PSA could be fully optimized with indigenous reagents while for free assay the monoclonal antibody used for tracer was supplied by University of Alberta, Canada.

2.2.2. MATERIALS

a. *Monoclonal antibodies for PSA:* MAb66, MAb30 and MAb10 were kindly supplied by Dr. M.R. Suresh, University of Alberta, Canada. All other monoclonal antibodies, such as $P_{27}A_{10}$ were raised in our Lab.
b. *PSA for standards:* PSA for use as standards was purified from seminal fluid.
c. *Solid Matrix for capture:* Polystyrene tubes used for coating MAbs were from a local company.
d. Na^{125}I and Hi Trap ^{TM} Protein G column were from M/s. Amersham, U.K.
e. Normal mouse serum was from local company.
f. DEAE-cellulose and Sephadex G-100 were purchased from M/s. Pharmacia, Sweden.

2.2.3. METHODS

2.2.3.1. Purification of PSA

68 mL of seminal fluid was taken and centrifuged at 1500×g for 30 min and then at 4000×g for 30 min. The supernatant was dialyzed against 0.01M pH 7.8 Tris-HCl buffer for 48 h at 4°C. All

chromatographic work was carried out at 4°C and the eluted fractions containing PSA were identified by PSA RIA.

i. *DEAE-cellulose ion-exchange chromatography*: The dialyzed supernatant mentioned above was applied to a DEAE-cellulose ion-exchange column (2.5 × 24 cm) equilibrated with 0.01 M pH 7.8 Tris-HCl buffer. The column was washed with the same buffer until $O.D._{280nm}$ of the elute was <0.02 and then washed with a continuous linear gradient composed of 250 mL each of the same buffer containing 0 and 0.2M NaCl. The fractions containing PSA were identified by RIA, pooled and concentrated and then dialyzed against 0.01 M pH 7.8 Tris-HCl buffer.

ii. *Gel filtration:* The pooled PSA containing fractions from the above ion-exchange chromatography was applied to a column of Sephadex G-100 (2.1 × 110 cm) equilibrated with 0.01 M pH 7.8 Tris-HCl buffer. The column was eluted with the same buffer at 0.3 mL/min. The fractions containing PSA were identified, pooled and concentrated. It was then dialyzed against 0.01 M pH 7.8 Tris-HCl buffer containing 0.02 M NaCl.

iii. *DEAE-cellulose ion-exchange chromatography:* PSA separated after gel filtration was applied to a column of DEAE-cellulose ion-exchange resin (2.5 × 24 cm) equilibrated with 0.01M pH 7.8 Tris-HCl buffer containing 0.02 M NaCl. The column was washed with the same buffer until $O.D._{280nm}$ of the elute was <0.02. The column was then eluted at 0.4mL/min with a continuous linear gradient composed of 250 mL each of the same buffer containing 0.02M and 0.15M NaCl. The fractions containing PSA were identified, pooled and stored at –20°C.

2.2.3.2. Preparation of monoclonal antibodies against PSA

a. *Immunization*: Female BALB/c mice were immunized with pure PSA. The first injection was 10 µg PSA per mouse in Complete Freund's adjuvant. Boosters were given every four weeks with 5 µg PSA per mouse in Incomplete Freund's adjuvant.

b. *Fusion*:

i. On the third day after booster injection with PSA, the spleen of the mouse was removed in saline and the spleen cells were taken and washed twice with serum-free medium.

ii. $Sp_2/0$ cells were harvested and washed twice with serum-free medium.

iii. The spleen cells and $Sp_2/0$ were mixed in the ratio of 5:1 to which 1 mL of PEG (MW 1500) was added drop by drop and incubated at 37°C for 40 seconds. 20 mL of serum-free medium was added drop by drop and the cells were centrifuged at 800 rpm for 5 minutes.

iv. The cells were suspended in 40 mL of HAT medium and plated on 4 pieces of 96-wells plates containing macrophage.

c. *Screening and cloning:* 7–14 days after fusion the supernatants from the wells were screened for presence of anti-PSA antibodies by PSA RIA. The cells whose supernatants were antibody positive were cloned by limited dilution method. The plates were screened again and single clones secreting antibody were identified and propagated.

d. *Production of ascites:* The cells secreting PSA MAb were injected into the mice which had been treated with pristane a week ago. Ascites were collected in 7–10 days. The affinity constants and titers of the antibodies raised were determined by RIA of PSA.

e. Purification of anti-PSA MAb: 1 mL of ascites was added to 2 mL of pH 4.3 acetate buffer. 75 µL caprylic acid was added to this drop by drop and stirred for 30 min. This was centrifuged at 11000 rpm for 30 min. and dialyzed against 0.05 M pH 7.8 P.B. for 48 h. The purified MAb preparation was stored at 4°C.

f. Purification of PSA MAb ($P_{27}A_{10}$): Anti-PSA MAb $P_{27}A_{10}$ raised in our laboratory was purified by Protein G column also, following the manufacturer's instructions.

2.2.3.3. Development of IRMA method for total PSA

a. *Coating tubes with anti-PSA MAb:* Purified anti-PSA MAb was diluted with 0.1M pH 9.6 bicarbonate buffer to a concentration of 8 µg/mL. 250 µL (2 µg) of this solution was dispensed into polystyrene tubes and incubated over night at room temperature. The tubes were decanted, blocked with 1% BSA and dried at room temperature.

b. *Iodination of anti-PSA MAb:* Chloramine-T was used as the oxidant to radio-iodinate the anti-PSA MAb to a specific activity of 15~20 µCi/µg. The labelled MAb was purified by gel filtration over a Sephadex G-50 column.

c. *Preparation of PSA standards:* PSA which was purified from seminal fluid was diluted to 0 to 40 ng/mL with 0.5 M pH 7.4 P.B. containing 5% BSA.

d. *Assay procedure:* Various parameters such as selection of the MAb for capture and tracer, amount of MAb for coating, incubation time etc. were optimized. Typically, in the assay, 100µL PSA standard or sample is added to the anti-PSA capture antibody coated tube with 100µL of 0.05M pH 7.4 P.B containing 0.2% BSA. The tubes are incubated at 37°C for 1.5 h. and decanted. The tubes are washed twice with 0.025 M pH 7.4 P.B. 200 µL of ^{125}I-MAb (anti-PSA tracer MAb) is then added to each tube and incubated at 37°C for 2 h. The tubes are decanted, washed thrice with 0.025 M pH 7.4 P.B. and counted on ν-counter.

2.2.3.4. Development of IRMA method for free PSA

a. *Iodination of anti-PSA MAb:* Chloramine T was used as the oxidant to radioiodinate MAb30 with ^{125}I to a specific activity of 15~20µCi/µg. The radiolabelled antibody was purified by gel chromatography over a column of Sephadex G-50.

b. *Coating tubes with anti-PSA MAb*: MAb66 as well as the in-house MAb $P_{27}A_{10}$ were used as capture MAbs for developing f-PSA assaya. Purified anti-PSA MAb was diluted with 0.1M pH 9.6 bicarbonate buffer to a concentration of 10µg/mL. 250 µL (2.5 µg) of this solution was dispensed into polystyrene tubes and incubated over night at room temperature. The tubes were decanted, blocked with 1% BSA and dried at room temperature.

c. *Preparation of PSA standards:* Purified PSA was diluted to final concentrations of 0.2, 0.6, 1.4, 4.8, 16 and 24 ng/mL with 0.05M pH 7.4 P.B. containing 5% BSA. These standards were dispensed in small aliquots, lyophilized and stored at 4°C.

d. *Assay Procedure:* The assay procedure was optimized with both capture antibodies mentioned above. These were compared with each other. In general, in the assay, 100µL PSA standards or samples and 100µl 0.05M, pH 7.4 P.B. containing 0.2% BSA were added into the tubes and incubated at 37°C for 1.5 h. The tubes were decanted and washed twice with 0.025M, pH 7.4 P.B. 200µl ^{125}I-MAb30 was then added to each tube and incubated at 37°C for 2 h. The tubes were decanted and washed thrice with 0.025M, pH 7.4 P.B. and counted on ɔ-counter. The developed assay procedure was validated by comparison with the commercial kit from DPC.

2.2.4. RESULTS AND DISCUSSION

2.2.4.1. Purification of PSA

The protein elution patterns (as OD_{280nm}) obtained on using DEAE-Cellulose, Sephadex G-100 and second DEAE-cellulose columns are shown in figures 1, 2 and 3 respectively. The PSA purified by this method showed a single band in PAGE and SDS-PAGE and was used as the immunogen.

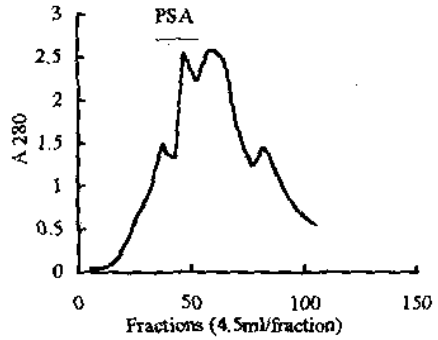

FIG. 1. Ion-exchange chromatography on DEAE-cellulose.
Buffer: continuous linear gradient of 0.01M pH 7.8 Tris-HCl containing 0 and 0.2M NaCl

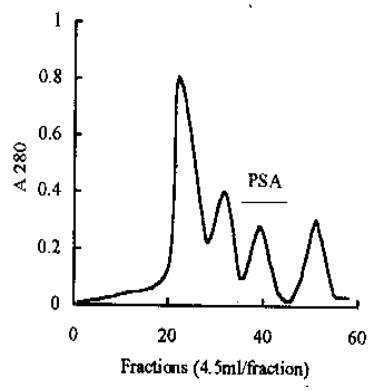

FIG. 2. Gel filtration chromatography on Sephdex G-100
Elution buffer :0.01 M pH 7.8 Tris-HCl buffer

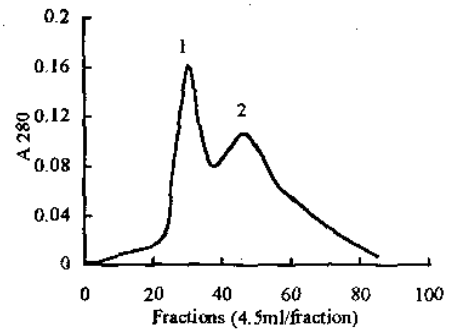

FIG. 3. Ion-exchange chromatography on DEAE-cellulose.
Buffer: continuous linear gradient of 0.01M pH7.8 Tris-HCl containing 0.02 and 0.15 M NaCl

2.2.4.2. Preparation of monoclonal antibodies against PSA

Three cell lines that secreted PSA monoclonal antibody were obtained and named as $P_{27}B_1$, $P_{27}A_{10}$, $P_{25}B_{11}$. The titer and affinity constants of these MAbs are given in the Table I.

The pattern of purification of $P_{27}A_{10}$ which had the highest affinity constant by Protein G column is shown in Figure 4.

Selection of suitable antibody pair for development of t-PSA IRMA: The MAbs were tested for use as both tracer as well as for capture. One polyclonal antibody was also used for coating he tubes (capture). Different combinations of capture and tracer antibodies were tried. The results of these experiments are shown in the Tables II and III.

TABLE I. CHARACTERIZATION OF THE IN-HOUSE ANTI-PSA MAbS

MAb	Titer ($\times 10^4$)	Affinity Constant ($\times 10^{10}$ L/M)
$P_{27}B_1$	1:2.5	1.84
$P_{27}A_{10}$	1:2.5	9.91
$P_{25}B_{11}$	1:8	1.51

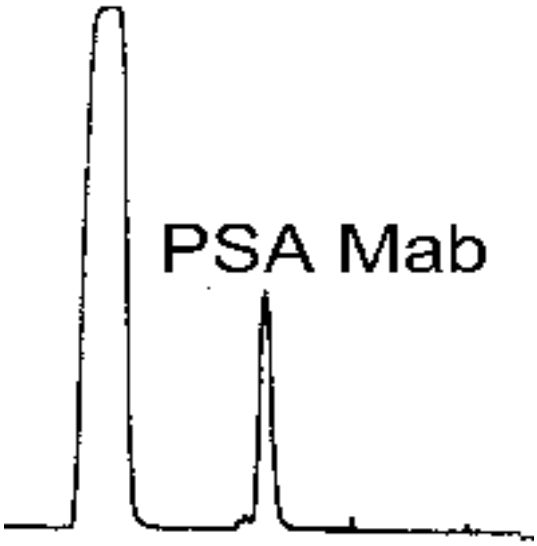

FIG 4. The pattern of purification of $P_{27}A_{10}$ by protein G column

TABLE II. BINDING STUDIES USING VARIOUS IN-HOUSE ANTIBODY COMBINATIONS

Capture MAb	$P_{25}B_{11}$		$P_{27}A_{10}$		PcAb	
Tracer MAb	^{125}I-$P_{27}A_{10}$	^{125}I-$P_{27}B_1$	^{125}I-$P_{25}B_{11}$	^{125}I-$P_{27}B_1$	^{125}I-$P_{27}A_{10}$	^{125}I-$P_{25}B_{11}$
Std. PSA (ng/mL)						
0	119	289	190	375	340	130
0.15	---	808	---	1214	2186	979
0.75	135	3401	185	4550	3434	1957
15	267	32424	267	40095	24378	13769
30	404	31705	404	52490	34049	18926

TABLE III. COMPARISON OF IN-HOUSE MAbS WITH THE MAbS SUPPLIED (CANADA)

Capture MAb	$P_{27}A_{10}$		MAb66	
Tracer MAb	^{125}I-$P_{27}B_1$	^{125}I-MAb10	^{125}I-$P_{27}B_1$	^{125}I-MAb10
Std. PSA (ng/mL)				
0	974	383	856	518
0.3	1566	983	883	822
0.8	2182	1673	810	1333
16	21954	35422	921	32079
40	39104	90790	1091	89314

The data in the table are binding counts (cpm).

It can be seen from the Table II that the combination of $P_{27}B_1$ as the tracer MAb and $P_{27}A_{10}$ as the capture MAb was the best among in-house antibodies. So these were used for developing t-PSA IRMA kit. It is seen from the Table III that the combination of $P_{27}A_{10}$ as capture MAb and ^{125}I-MAb10 donated by Dr. Suresh was also good and the results were similar to those obtained using MAb66 as capture MAb. and ^{125}I-MAb10.

2.2.4.3. Optimization of IRMA method for total PSA

a. *Concentration of the capture MAb for coating tubes:* Table IV depicts the binding as in an assay when different concentrations of capture MAb were used for coating tubes. It is seen from this table that the binding at various coating concentrations were very similar except in the case of 30µg/mL where it was lower. It is possible that two layers of antibodies are coated on to the surface of the tubes when the concentration of coating MAb solution is very high. The second layer when binds to the standard PSA and then to the tracer may be not tightly bound and hence leaching off when the tubes are washed.

b. *Incubation time of the Reaction:* Tables V and VI show the effect of the incubation time in both the steps on the progress of the reaction.

TABLE IV. EFFECT OF COATING ANTIBODY CONCENTRATION

Coating MAb Conc. (µg/mL)	10	15	20	30
Std. PSA (ng/mL)				
0	375	486	437	481
0.3	1187	1118	1102	1054
0.8	2020	2130	1929	1593
16	21241	21236	21188	16811
40	31864	31649	30482	26782

The data in the table are binding counts (cpm).

TABLE V. EFFECT OF INCUBATION TIME IN THE FIRST STEP

Time(h)	0.5	1	2	3
Std. PSA (ng/mL)				
0	837	731	727	528
0.8	1876	1906	1957	2235
16	19266	21212	21314	24890
40	37361	39855	43549	44418
80	49447	55199	44418	58827

TABLE VI. EFFECT OF INCUBATION TIME IN THE SECOND STEP

Time(h)	1	2	3
Std. PSA (ng/mL)			
0	698	895	929
0.8	1591	1971	1937
16	14209	18761	17316
40	28692	41523	47720
80	40480	59062	65020

The data in the tables are binding counts (cpm).

From these tables it can be seen that the reaction proceeds quickly. Nearly 90% reaction is complete within 1 h in the first step and reaches a plateau after 2 h. In the second step, although the binding increases with time at high PSA concentrations, the low end sensitivity does not improve beyond 2 h of incubation. Based on these results, the assay was designed as a two step assay with 1.5 h incubation followed by a 2 h incubation at 37°C.

c. *Sensitivity, recovery and precision:* The sensitivity of t-PSA IRMA, defined as the PSA level at 2SD from the mean zero standard count rate, was 0.2 ng/mL. The recovery of the added standard varied between 94.8% and102%. The intra- and inter-assay variations expressed as% C.V. were ~5% and 10%–16% respectively.

2.2.4.4. Optimization of IRMA method for free PSA

a. *Stability of tracer:* The tracer ^{125}I-MAb30 was tested for its stability at 37°C by evaluating its performance in assay. The results are tabulated in Table VII and it can be seen that even after 15 days at. 37°C, the tracer did not lose its immunoreactivity and could be used in the assay. However, the low end sensitivity was lost in 5 days.

The standard curve with ^{125}I-MAb30 as tracer and $P_{27}A_{10}$ as the capture MAb is shown in Figure 5.

b. *Comparison of different capture antibodies:* Table VIII gives the comparison of the assay binding when MAb66 and MAb$P_{27}A_{10}$ are used as capture antibody with ^{125}I-PSA MAb30 as tracer in the optimized IRMA systems.

Forty-two serum samples were simultaneously assayed with both the IRMA systems (using $P_{27}A_{10}$ and MAb66 as capture antibody). Linear regression analysis of the results suggested good correlation. Y=1.16X–0.19 ; r=0.986. Figure 6 depicts the correlation.

c. *Validation of the CIAE f-PSA IRMA by comparison with DPC kit:* Thirty-one samples including normal subjects and patients were assayed by DPC kit and the developed CIAE kit simultaneously. The results correlated fairly well as seen in the Figure 7.

TABLE VII. STABILITY OF f-PSA TRACER AT 37°C

No. of Days	0	5	7	15
PSA standard (ng/mL)				
0	574	362	312	344
0.3	846	514	400	441
0.8	1287	793	723	737
16.0	16622	12618	13119	14339
40.0	36027	25892	26592	24828

The data in table are binding counts (cpm).

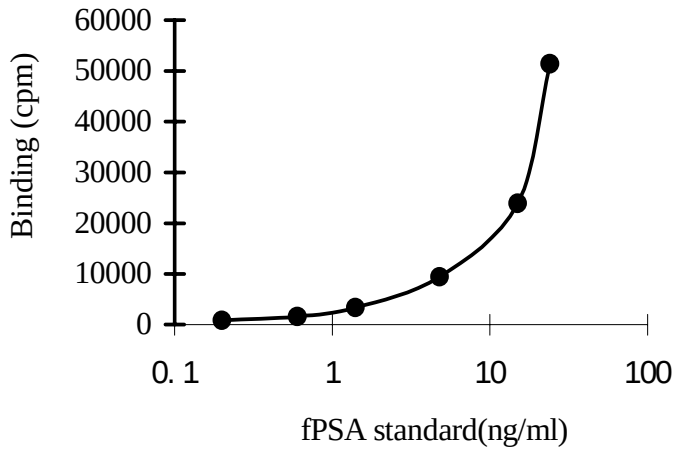

FIG. 5. f-PSA standard crve.

TABLE VIII. COMPARISON OF IN-HOUSE MAb WITH MAb66 FOR CAPTURE IN f-PSA IRMA

Standard PSA (ng/mL)	$P_{27}A_{10}$	PSA MAb 66[#]
0	426	284
0.3	850	943
0.8	1493	1887
16.0	25126	29573
40.0	52481	60396

The data in the table are binding counts (cpm).

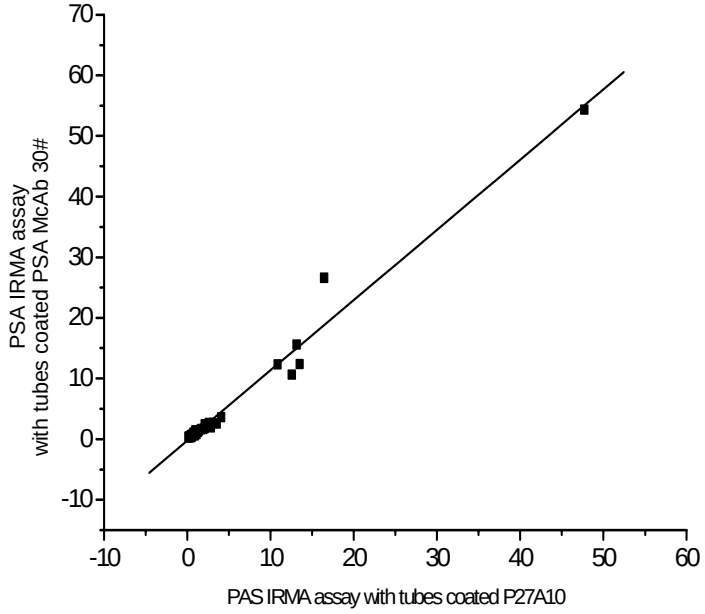

Fig.6. Comparison of f-PSA IRMA assay with MAb66 and $P_{27}A_{10}$ as capture antibody.

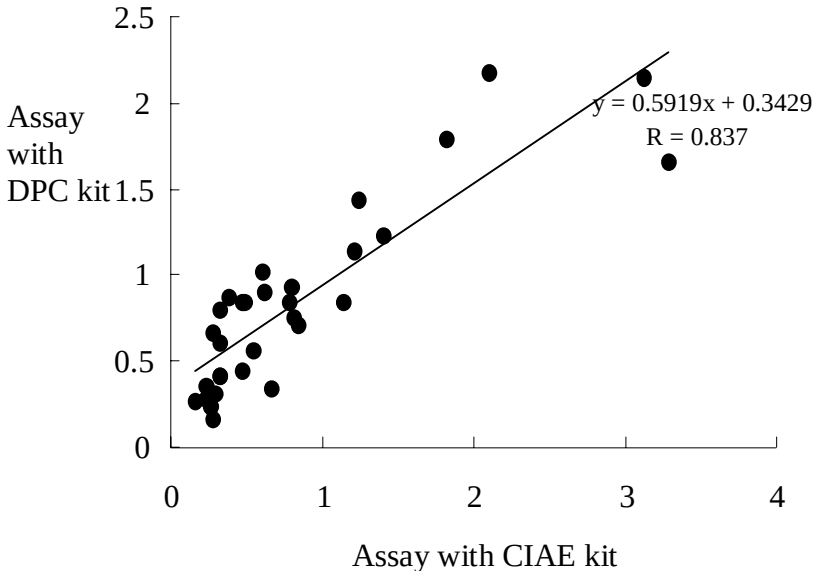

FIG. 7. Comparison of CIAE f-PSA IRMA with DPC kit.

2.2.5. CONCLUSIONS

In conclusion, the following could be achieved at our laboratory as a result of the CRP.

1. Three hybridoma cell lines secreting anti-PSA MAbs were obtained of which two, namely, $P_{27}B_1$ and $P_{27}A_{10}$ were used for developing t-PSA IRMA kit. The sensitivity of this t-PSA IRMA kit was 0.2 ng/mL. The recovery of added standards in the assay was between 94.8% and 102%. The intra- and inter-assay variations were ~5% and 10%–16% respectively. These t-PSA IRMA kits have been used in our hospitals since 1999.

2. f-PSA IRMA methodology was developed with ^{125}I-MAb30 (from Dr. Suresh) as tracer and $P_{27}A_{10}$ as capture antibody. The sensitivity of this assay was 0.19 ng/mL and recovery was between 80% and 92.7%. The result of dilution test was y=72.89x+4.23; r=0.981

3. Comparison of $P_{27}A_{10}$ with MAb66 for use as capture antibody in the f-PSA assay proved that they were very similar. Hence $P_{27}A_{10}$ is being used as the capture antibody at our laboratory.

4. Comparison of the f-PSA values in samples obtained using the commercial DPC kit and the in-house CIAE kit showed that the results were very similar and correlated well.

5. The tracer was fairly stable for 15 days at 37°C. However, the sensitivity of assay deteriorated in 5 days 37°C.

BIBLIOGRAPHY

WAN-MING ZHANG, PATRIK FINNE, JARI LEINONMEN ET AL. Clin. Chem. 1999;45(6) 814–821

SANDRINE MICHEL, GILBERT DELEAGE, JEAN-PHOLIPPE CHARRIER ET AL. Clin. Chem. 1999; 45(5) 638–650

KLAUS JUNG, ULRIKE ELGET, MICHEAL LEIN ET AL. Clin. Chem. 2000; 46(1) 55–62

2.3. CUBA

Title of the Project:	Production of a panel of monoclonal antibodies, specific for PSA, for the diagnosis of prostate cancer using IRMA or RIA"
Chief Scientific Investigator:	Acevedo Castro Boris
Scientific Co-Investigators:	Ruiz Peña Miriam, Perera Negrín Yasser, Hernández Pagarizábal Mireya, Pichardo Díaz Dagmara, Caso Peña Raúl[1], Murugiah Ragavan, Ayala Avila Marta and Gavilondo Cowley Jorge

Abstract. Immunoradiometric assay and Enzyme linked immunosorbent assay were developed for measurement of total-PSA in human serum. Similar assays for free-PSA are also under final optimization and validation. In order to develop in-house assays monoclonal antibodies were raised against PSA at our laboratory, screened and selected. MAbs with good specificity and suitability for use in both total and free PSA IRMAs could be identified. The developed assays exhibited equimolar response towards the different forms of PSA and compared well with commercial t-PSA kits. Using one of our in-house MAbs, immunopurification of PSA from seminal plasma could be achieved and PSA of ~ 91% purity could be obtained.

2.3.1. INTRODUCTION

Monoclonal antibodies against Prostate Specific Antigen were raised at our laboratory and screened for use in total and free PSA assays. These were prepared following conventional hybridoma procedures using P3/x63.Ag8.653 myeloma cells for fusion with splenocytes from a mouse hyper immune against natural PSA. Several stable hybrid cell cultures secreting antibodies specific to natural PSA were obtained. Based on the analytical sensitivity, the best pair of MAbs (CB-PSA.4 and CB-PSA.9) was selected to develop a total PSA assay based on the ELISA and IRMA formats. This ELISA exhibited equimolar response towards the major PSA forms in serum and could detect as low as 0.12 µg/L of natural PSA. Good correlation was obtained with the commercial CIS-bio (France) total PSA assay.

With an aim to obtain pure PSA for use as standard, an immunopurification method was developed using one of the MAbs, namely CB-PSA.2 raised at our laboratory. On eluting PSA under ionic conditions natural PSA of ultra pure (91%) quality could be obtained.

We recommend our anti-PSA MAbs to develop an IRMA assay for total PSA. A Cuban free-PSA assay is under advanced development and evaluation.

2.3.2. EXPERIMENTAL

2.3.2.1. Prostate specific antigen (PSA)

Natural PSA was obtained from Dr. M. Suresh (Alberta University, Canada) with 80% of purity in lyophilized form. This PSA was diluted in distilled water, as recommended by the supplier, to a final concentration of 1 mg/mL, aliquoted, and stored at –80°C in order to avoid degradation.

2.3.2.2. Generation and characterization of anti-PSA MAbs panel

Mice were immunised with human semen eight times every 15 days by the subcutaneous route, at 100 µg of total protein per dose, followed by two doses of 50 µg of PSA. The fusion was performed using the traditional protocol with PEG 42% as fusion agent and the myeloma P3/×63.Ag8.653[1].

Briefly, 100×10^6 splenocytes were fused with 10×10^6 myeloma cells (splenocyte/myeloma ratio 10:1). The fused cell mixture was distributed in conventional 96 well-culture plates at a final concentration of 1×10^5 cells per well. The culture media (RPMI-1460, 10% fetal bovine serum) was supplemented with HAT.

Selection of positive clones was carried out using an ELISA assay. Polystyrene EIA strips (Polysorp, Nunc) were coated with 100 µL/well of the purified natural PSA (1 µg/mL in PBS buffer pH 7.2), at 37°C for three hours The plates were then blocked with 200 µL/well of PBS-containing 1% non fat milk (Oxoid). Supernatants from the wells containing the hybrid cells were diluted 1:2 in PBS and incubated in the PSA coated wells for one hour at 37°C. At the end of incubation, the strips were washed with PBS containing 0.05% Tween 20 (PBS-T), and incubated with commercial HRPO-conjugated anti-mouse IgG polyclonal sheep antibodies (Sigma, A0168), for one hour at 37°C. The strips were emptied and the colour was developed with 0.014% H_2O_2, 0.25% OPD (o-phenylenediamine, Sigma) in citrate-phosphate buffer, pH 5.5, for 10 minutes. The colour development reaction was stopped with 50 µL per well of 2.5 M sulphuric acid and the absorbance of the solution was measured at 492 nm. An unrelated MAb was used as negative control. The positive control consisted of a pool of reactive sera from the mice immunised with the long scheme.

2.3.2.2.1. Epitope screening assay of the MAbs by competition ELISA

In order to identify the PSA epitope profile recognised by our antibodies, a competition ELISA was developed. Polystyrene EIA plates (Polysorp, Nunc) were coated for three hours, with of each of the anti-PSA MAbs (1 µg/mL in PBS buffer pH 7.2) raised in the laboratory. The plates were blocked with PBS-1% BSA for one hour at 37°C. 100 µL of each of the purified MAbs at different concentrations were separately pre-incubated with 100 µL of natural PSA (conjugated to NHS-biotin, Sigma H-1759). The pre-incubations were performed at MAb concentrations of (a) 0 (maximal signal), (b) 0.5, (c) 1 and (d), 2 µg/mL in eppendorf tubes for one hour at 37°C, with shaking. 100 µL of the pre-incubated samples were added to the plates coated with anti-PSA MAbs and incubated for one hour at 37°C. 1:2500 diluted streptavidin-HRPO conjugate (Amersham, UK) was then added to the plates after emptying their contents and incubated for 30 minutes. The development of colour for measuring the enzyme concentration was carried out as detailed above. The assay was performed in duplicate, and the experiment was carried out at least twice.

It is expected that when the MAb was absent in the liquid phase incubation with PSA, the colour signal should maximum and should decrease if the MAbs in liquid phase identify PSA at the same place or in the neighbourhood of region that binds to the coated anti PSA MAb. We assumed that inhibition occurred in those cases where there was more than 50% inhibition. The extent of inhibition were calculated using the following formula:

$$\% \text{ of inhibition} = 100 - (\text{O.D. sample} / \text{O.D. at 0 µg/mL of PSA}) \times 100$$

2.3.2.2.2. PSA epitope recognition by the MAbs using Western Blot

A classical Western Blot technique was developed to characterise the epitope recognition pattern for every MAb. Natural PSA was run in a 12% denaturing (in the presence of 2 mercaptoethanol) or denaturing (absence of mercaptoethanol) sodium-dodecyl-sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane using the semi-dry procedure. The membranes were then blocked with PBS-1% BSA (BDH) by incubation for sixteen hours at 4°C. 750 µL of each MAb in PBS was then incubated for one hour with the membrane. After five washing steps with PBS-T, the membranes were incubated for one hour at 37°C with commercial HRPO-conjugated sheep anti mouse-IgG (Sigma, A0168) following the manufacturer's instructions. The colour was developed with 0.25% 3,3 Diaminobenzidine (Sigma) in PBS for 15 minutes and stopped with water.

2.3.2.3. Capture of biotin-PSA by a direct ELISA method

Polystyrene EIA strips (Polysorp, Nunc) were coated with anti-PSA MAbs 1 µg/mL in PBS buffer pH 7.2, for three hours at 37°C. The plates were then blocked with 200 µL/well of PBS-1% BSA (BDH). Biotin-PSA prepared at our laboratory was diluted to various concentrations (between 100–1.25 ng/mL) in PBS-0.1% BSA and incubated in the wells for one hour at 37°C. At the end of incubation, the wells were washed with PBS-T and incubated with 1:2500 streptavidin-HRPO conjugate (Amersham, RPN 1231) for 30 minutes following the manufacturer's instructions. The colour was developed with 0.014% H_2O_2, 0.25% OPD (O-phenylenediamine, Sigma) in citrate-phosphate buffer, pH 5.5, for 10 minutes. The colour reaction was stopped with 50 µL per well of 2.5 M sulphuric acid and the absorbance was measured at 492 nm. An unrelated MAb that was used as negative control. The positive control consisted of a commercial capture anti-PSA MAb (MAb66 supplied by Dr. M. Suresh, Canada).

2.3.2.4. Optimization and validation of a sandwich ELISA to measure serum total PSA

CB.PSA-4, a MAb raised at our laboratory, was one of the highly suitable MAb for use as capture MAb. Several other MAbs showed promise for use as tracer and form the pair with CB.PSA-4 for assay development. We used these MAbs to develop an ELISA to measure PSA and optimised the system varying the MAbs, reagent concentrations and incubation times. Polystyrene EIA strips (Polysorp, Nunc) were coated with 100 µL/well of CB.PSA-4 MAb (1 µg/mL in PBS buffer pH 7.2) at 37°C for three hours. The wells were blocked with PBS-1% BSA, as mentioned earlier. PSA at different concentrations (100–0.9 µg/L standards from CIS bio-International, France) or natural PSA (100–0.1 µg/L) were used as standards. The assay was set up using these standards and the MAb coated wells. After incubation for 30 minutes at 37°C, the wells were washed with PBS-T and incubated with the tracer biotinylated MAbs prepared at our laboratory. Tracer MAbs were biotinylated following traditional procedures[2]. The amount of biotin-MAb used was varied between 5 and 0.25 µg/mL and the incubation time was varied from 15 minutes to 1 hour. The other steps were similar to the ELISA described in the previous section. The assay was performed in duplicate, and the experiment was carried out at least twice.

The most suitable tracer MAb to pair with CB.PSA-4 was selected and this system was used to estimate within-run and between-assay coefficients of variation (% C.V.) following the traditional procedures described previously [3].

2.3.2.4.1. Evaluation of the in-house t-PSA ELISA for equimolar response

The developed ELISA system was tested for equimolar response. Polystyrene EIA strips (Polysorp, Nunc) were coated with 100 µL/well of CB.PSA-4 MAb (1 µg/mL in PBS buffer pH 7.2) for 3 hours at 37°C and blocked with PBS-1% BSA as before. PSA Standards of concentrations between 100–0.9 µg/L from CIS bio-International, France was used to construct the standard curve. 100 µL of the samples with predetermined PSA values were used as samples in the assay. After incubation for 30 minutes at 37°C, the wells were washed with PBS-T and incubated with 0.5 µg/mL of the tracer CB-PSA.9-biotin MAb for 30 minutes. The assay was carried out as described earlier in the previous section. The assay was performed in duplicate and the experiment was carried out at least twice.

Ten samples were used to test the equimolar response of the assay. These were evaluated by nine different commercial equimolar-PSA assays, namely: Abbot IMx, Enzymun Boehringer Manheim, PSA total CIS bio, Immunotech, Tosoh Inmunolite, Bacarase T etc. The range for each sample was fixed based on the maximum and minimum values obtained on assaying the samples using the above commercial assays.

2.3.2.4.2. Validation of the in-house t-PSA ELISA by comparison with a commercial t-PSA kit

Concordance of the in-house t-PSA kit with an equimolar commercial assay from CIS bio International, France was tested. The t-PSA assay was carried out according to the manufacturer's instructions. The in-house ELISA was performed as described in the previous section, except that the PSA standards used were natural PSA (calibrated using the CIS bio kit) ranging from 0 to 100 µg/L. The assay was performed in duplicate and the experiment was carried out at least twice. Microsoft Software (Microsoft Excel 97) was used to calculate the Pearson coefficient.

2.3.2.5. Optimization and validation of IRMAs for total PSA

Based on epitope characterization experiments and the best pair selection in the free and total PSA ELISAs, we developed IRMA assay for detecting total PSA at the Center for Isotopes of Havana.

2.3.2.5.1. Radioiodination of the MAbs

Ten monoclonal antibodies (PSA-MAB1 to PSA-MAB10) were labelled with ^{125}I using the chloramine-T method[4]. Briefly, 10 µL of iodination buffer (0.1 M Phosphate, pH 7.4), 0.5 mCi carrier-free ^{125}INa and 10µL of chloramine T (1 mg/mL), were added to 40 µg of every anti-PSA MAb at concentrations of at least 2 mg/mL in PBS-thiomersal 0.02%. The solutions were mixed for one-minute and kept at repose for the next minute. The mixing and keeping aside were repeated once more and 10 µL of sodium metabisulphite (2 mg/mL) was added to the reaction mixture to stop the reaction and mixed again. The labelled MAb was purified by gel filtration chromatography over a Sephadex G-25 column (Pharmacia) previously equilibrated with 0.1M Phosphate, pH 7.4-1% BSA. Tracer fractions were eluted using the same buffer and the aliquots were stored as deep-frozen. The specific activity of ^{125}I labelled antibodies was adjusted to approximately 15 Ci/g.

2.3.2.5.2. Assay development

The t-PSA IRMA developed at our laboratory was based on liquid phase reaction between the antigen and the two MAbs. The capture antibody was biotinylated and used with radioiodinated tracer MAb in the assay. At the end of incubation, streptavidin-anti mouse IgG coupled magnetic particles was used to quickly bind to the biotinylated capture MAb and hence as the separation reagent. Several pairs of anti-PSA MAbs were tested for suitability. The assay was optimised varying the incubation time between 0.5 h to 3 h and tracer MAb concentration from 1 to 2×10^5 cpm. The optimsed IRMA system for total PSA consisted in mixing 50 µL of sample or standard with 100 µL each biotin-CB.PSA-4, 10 µg/mL capture MAb and ^{125}I-CB.PSA-9 tracer MAb at 1.25×10^5 cpm/tube. The mixture was incubated two hours at room temperature under constant mixing on a shaker at the end of which 500 µL of avidinated magnetic immunosorbent particles (Institute of Isotopes, Hungary) was added and incubated for 15 minutes at room temperature. In order to separate the bound complexes retained in the magnetic particles, the tubes were placed on a magnetic rack for 5 minutes and the supernatant was discarded. The particles were washed twice with PBS-Tween20, 0.05% discarding the supernatant, each time. The magnetic particles were counted in a gamma counter (Minigamma 1275 LKB, Pharmacia) and the data used for constructing the standard curves and reading sample values.

The optimised total PSA IRMA was evaluated for the biological detection limit, linearity range, analytical detection limit and correlation with a commercial assay (CIS bio-International, France).

2.3.2.6. Immunopurification of natural PSA

With an aim to obtain pure PSA for use as standards, it was decided to purify PSA from seminal plasma using an immunoaffinity column. A suitable MAb was selected by employing a competition ELISA. Polystyrene EIA plates (Polysorp, Nunc) were coated with 1 µg/mL of different anti-PSA MAbs in PBS buffer pH 7.2, for three hours and blocked with PBS-1% BSA for one hour at 37°C. 100 µL of natural PSA, at a concentration that corresponds to O.D. 50, was incubated for one hour at 37°C. The plates were washed with PBS-T and 200 µL of various possible eluting buffers

incubation the plates were washed with PBS-T, the strips were incubated for one hour at 37ºC with 1:2500 streptavidin-HRPO conjugate (Amersham, RPN 1231) for 30 minutes, according to the manufacturer's instructions. The colour was developed with 0.014% H_2O_2, 0.25% OPD (O-phenylenediamine, Sigma) in citrate phosphate buffer, pH 5.5, for 10 minutes and stopped with 50 µL per well of 2.5 M sulphuric acid. Absorbance was measured at 492 nm and the efficiency of elution was calculated using the following formula:

$$\% \text{ elution} = 100 - (1 - \text{ABS sample} / \text{ABS at PBS}) \times 100$$

2.3.2.6.1. Immobilization of CB-PSA.2 to Sepharose CL 4B

Monoclonal antibody (CB-PSA.2) was coupled with a commercial Sepharose-4B matrix activated with cyanogen bromide following the suppliers' (Pharmacia Biotech AG, Sweden) instructions. In brief, 18 mg of the MAb in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was coupled to 3.5 mL of the activated matrix. The solution was mixed and incubated for 3 hours at room temperature. Potential free sites in the matrix were blocked with Glycine, pH 8.

2.3.2.6.2. Purification of natural PSA from seminal plasma

Seminal fluids from 10 healthy males were mixed and collected in the Endocrinology Institute of Havana. Samples were centrifuged at 250×g for 10 minutes and the supernatant material was preserved at –70°C until the purification was carried out. The seminal plasma was diluted (1:5) in 10 mM $NaHPO_4$, pH 7.2, filtered through 0.2 µM membrane (Sartorius, Germany) and loaded on the MAb CB-PSA.2 coupled sepharose-4B column at a constant flow rate of 0.32 mL/min. The column was then washed sequentially with two ionic solutions (2.5 M NaCl pH 8, and 1 M $MgCl_2$ pH 5.5) at 0.5 mL/min. to remove the unwanted proteins. PSA was then eluted with 2 M $MgCl_2$.

PSA concentration in the eluted fractions was estimated using ELISA method described in the previous section. Purity of the fractions was determined by SDS-PAGE technique at 12.5% using an electronic program (Manager Ver 2.0). Western Blot was developed to identify the degraded products of PSA. Briefly, SDS-PAGE at 12.5% was performed and the proteins were transferred into the nitrocellulose membrane (Amersham, RPN203E). After blocking free sites with PBS–2% non fat milk, the nitrocellulose membrane was incubated with CB.PSA-4 at 5 µg/mL for one hour at 37°C. Commercial PSA was used as positive control. Presence of MAbs contaminants in the fractions was estimated by the ELISA method, previously described[5]. Briefly, polystyrene plates were incubated with sheep polyclonal anti-mouse IgG at 10 µg/mL for 16 hours at 4ºC. The plates were blocked with PBS-2% BSA and the fractionsto be tested were added. Pure mouse IgG was used as standard (10 to 0.1 ng/mL). The ELISA was carried out as detailed in section 2.3.2.2.

2.3.2.7. Collection of sera for prostate cancer diagnostic studies.

We collected a panel of human sera from the Institute of Oncology and Radiobiology of Havana. These sera were aliquoted and frozen at –80ºC. PSA contents in these sera were analysed with the Cis bio (France) PSA-total assay, according to the manufacturer's procedures.

2.3.3. RESULTS AND DISCUSSION

2.3.3.1. Generation and characterization of the anti-PSA MAbs panel

Splenocytes from a hyper-immunised Balb/c mouse were fused with parental myeloma cells. Eleven hybridomas exhibited a stable behaviour (positive repeatedly) after being sub-cultured to 24-well plates. The optical densities in ELISA when these MAbs were tested, were at least five times that of negative control. All of them were of subclass IgG1. Evaluation of the pattern of reactivity was by competition ELISA between MAbs pre-incubated with natural PSA and PSA antigen coated on the plastic surface (Table I). Our MAbs were classified in five groups. Some of them recognised a common or neighbourhood epitope to the region bound by commercial MAbs from commercial source.

common or neighbourhood epitope to the region bound by commercial MAbs from commercial source.

A Dot Blot experiment was performed to compare the recognition of native and denatured PSA antigen. MAbs from Group 4 (CB-PSA.6, CB-PSA.7 and CB-PSA.9) and CB-PSA.4 recognised linear epitopes on the PSA molecules because they reacted with similar or higher signal with the PSA antigen under denatured than the non-denatured conditions (data not shown). CB-PSA.4 recognised a sequential epitope and the rest of MAbs from this group reacted with a conformational epitope (Table I). In a molecule, an antigenic region would be recognised and outlined by closed or overlapping epitopes with sequential and/or conformational pattern[6]. Corey E. et al analysed the competition profile of a panel of 10 MAbs against PSA. They demonstrated the presence of at least five antigenic zones on the PSA molecule. One of them presented sequential and conformational epitopes[7]. Group 1 (Table I) had a similar pattern of mixed epitope recognition structure.

TABLE I. ANTI-PSA MAbS GROUPED BASED ON THE EPITOPE CHARACTERIZATION

Group 1	Group 2	Group 3	Group 4	Group 5
MAb 66*	MAb 30**	MAb 10*	CB-PSA.6 (S)	CB-PSA.3
2.1.2. CIS bio 1*	CB-PSA.2	2.1.3. CIS bio 2*	CB-PSA.7 (S)	CB-PSA.10
CB-PSA.1		CB-PSA.8	CB-PSA.9 (S)	CB-PSA.11
CB-PSA.4 (S)				
CB-PSA.5				

*Commercial MAbs against total PSA, **Commercial MAb that only recognized free PSA. CIS bio 1 to capture PSA and CIS bio 2 as tracer were supplied by CIS bio International; S, sequential epitope.

The capacity of each MAb to capture natural PSA under the reaction conditions was different based on their affinity. CB-PSA.4 and the supplied MAb66 (Dr. Suresh) could measure as low as 0.1 µg/L of natural PSA. Affinity of these MAbs were very similar at 3.7×10^{9} LM^{-1} for CB.PSA.4 and 6.9×10^{9} LM^{-1} for MAb66 respectively. The other anti-PSA MAbs assessed could detect natural PSA at levels higher than 1.8 µg/L only.

2.3.3.2. Standardization of a sandwich ELISA for total PSA

Based on competition experiments we evaluated pairs of anti-PSA MAbs that recognised PSA antigen at different zones. PSA concentration range (25 to 0.15 µg/L) covered the relevant clinical interval for medical interpretation. The PSA levels could be classified as

a) Suspected prostate cancer: PSA values higher than 4 µg/L[8],
b) High probability of metastatic prostate cancer: PSA values higher than 10 µg/L[9] and
c) Important to patients who have undergone alternative therapies, for evaluation of the situation: values under 1µg/L[10]

The best sandwich pairs to detect total PSA were CB-PSA.4 as capture antibody, and CB-PSA.9 as tracer. In order to detect a treated patient for possible recurrence of cancer, the system must be capable of minimum detection limit below 0.2 µg/L of PSA. In this t-PSA sandwich ELISA system, the minimum detection limit (MDL) was 0.12 µg/L that corresponded to an OD_{492nm} which was 3 SD away from that of 0 µg/L natural PSA. Linear regression was excellent with regression coefficient $r^2 = 0.997$. Under the experimental conditions, the within-run and between-assay variations (%C.V.) ranged from 2.1% to 3.2% and 2.8% to 6.3% respectively in the range of t-PSA concentrations assayed.

Serum samples from 45 healthy females were analysed with our assay and were found to be below the detection limit of 0.12 µg/L, which is a prerequisite of the assays that recognise PSA in a specific form [11].

Finally a new assay that could be employed in the screening, detection and follow-up of patients with prostate cancer could be standardised. High sensitivity of the assay could attributed to the high affinity of the pair of MAbs used for PSA (3.7×10^{9} LM^{-1} for CB.PSA.4 and 4.7×10^{10} LM^{-1} for CB.PSA-9 respectively) and the effect of amplification due to biotin-streptavidin system.

2.3.3.2.1. Equimolar detection of free PSA and PSA complexed with ACT in the t-PSA assay

One approach to ascertain equimolar response of an assay developed for t-PSA is to compare the developed optimised system with a reference assay such as an established commercial equimolar response assay. It has been observed that different immunoassays for t-PSA detected these 2 forms of PSA in different molar ratios. Graves classified the t-PSA assays available into two types:

(a) "equimolar-response" assay that recognised both free PSA and PSA-ACT complex with equal affinity and

(b) "skewed molar-response" assay which quantified the two forms differently [12].

A set of serum samples containing known values of PSA can serve as secondary serum-based reference materials for calibration of commercial t-PSA immunoassays. Equimolar standardization is an useful procedure for any assay that detects free and bound forms of a ligand in differing molar ratios[13]. For the equimolar evaluation we analysed nine serum samples from the Stanford University. Samples were assessed using nine different commercial equimolar total-PSA assays. The ELISA developed in-house at our laboratory yielded t-PSA values within the range obtained with the equimolar assays for every serum sample (Figure 1). Hence, we concluded that our assay detected free PSA and ACT-PSA complex in an equimolar ratio.

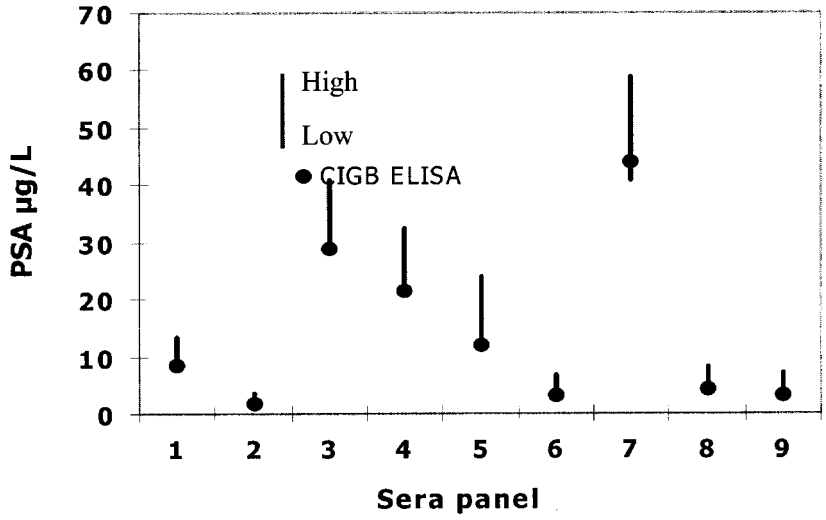

FIG 1. Total PSA shown values in the calibrated Stanford panel of samples in comparison with the values obtained as range using nine commercial total PSA assays with equimolar response.

Assay standardization involves several sequential steps to be optimised. In general, a primary antigen standard should be employed in the standardization of new assay. In accordance with the WHO criteria, Rafferty B. et al recommended the Stanford standard PSA (90:10) to measure total and free PSA[14]. PSA values need to be assigned to PSA-containing serum samples. These secondary serum-based reference materials can be used by manufacturers and regulatory agencies to develop and monitor the performance of PSA assays. In serum, two different forms of PSA are detected immunologically: the free form of PSA with molecular weight of 30 kD and a complexed form of PSA-alpha-1-antichymotrypsin with molecular weight of 100 kD [15].

2.3.3.2.2. Comparison of the ELISA developed with the commercial CIS bio t-PSA assay kit

We evaluated patient samples (n=65) using the in-house ELISA. In parallel, we analysed the same samples with a commercial CIS bio total PSA kit. Samples were classified based on the cut of values commonly employed in clinical prostate evaluation. The results of these assays were found to correlate very well as shown in the Figure 2 in the range of 0 to < 10 µg/L of total PSA(Pearson correlation coefficient r =0.9741).

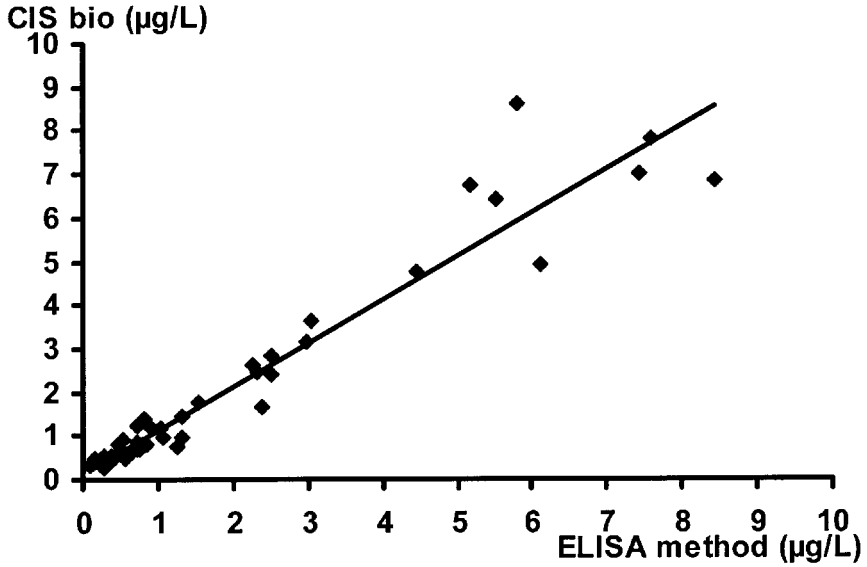

FIG. 2. Correlation of the sample values analysed by CIS bio t-PSA kit and the developed in-house ELISA.

These results confirmed that an ultra-sensitive t-PSA assay has been developed at our laboratory using the pair of high affinity MAbs selected. This assay exhibited equimolar response to free PSA and PSA in PSA-ACT complex in patients' sera. Our assay will be used in the screening and following of patients with prostate cancer.

2.3.3.3. In-house IRMA assay to detect total PSA

The t-PSA IRMA optimised at our laboratory based on liquid phase reaction employed 50 µL of sample, incubation period of 2 hours at ambient temperature. The capture MAb was biotinylated and the tracer MAb was labelled with ^{125}I. Magnetic cellulose particles coupled to streptavidin-anti mouse IgG conjugate was used as the separation reagent which would bind quickly to the biotinylated capture MAb with great affinity. On testing several pairs of anti-PSA MAbs, CB.PSA-4 was found suitable for capture at 1µg/tube/100µL and CB.PSA-9 for use as tracer at 1.25×10^5 cpm/tube (data not shown in detail). This optimised t-PSA IRMA had a minimum detectable limit of 25 pg/mL of PSA.

On comparison of the in-house assay with the commercial CIS Bio t-PSA IRMA as shown in Figure 3, it was observed that the slope of the in-house assay was better than that of the CIS Bio assay. The in-house assay exhibited linearity in the range of 0.025 to 100 ng/mL while the CIS bio assay had linearity only between 1 to 100 ng/mL. In both the cases, hook effect was observed at t-PSA values close to 1 µg/mL. Functional sensitivity was similar in both systems. Analysis of 65 patient samples grouped into different clinically significant groups, by both the in-house IRMA system and the commercial CIS Bio kit gave very good correlation in all the groups. Based on these results we would recommend our anti-PSA monoclonal antibodies for use by other laboratories to develop total PSA IRMA.

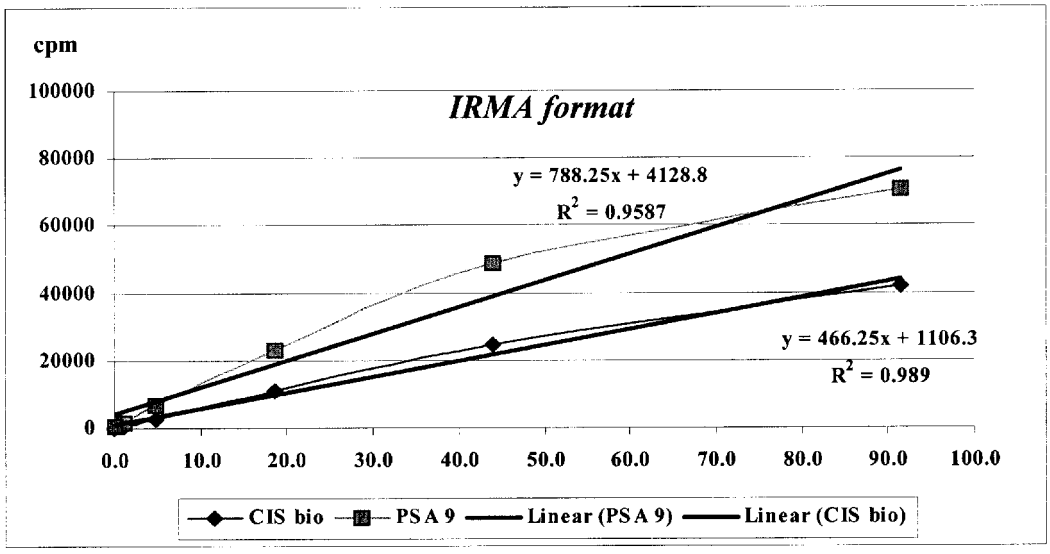

FIG. 3. Typical t-PSA IRMA standard curves: in-house assay and CIS bio assay.

2.3.3.4. Immunopurification of PSA from seminal plasma.

On testing various MAbs for suitability for use in purifying PSA from seminal plasma, CB.PSA-2 when used with 2 M $MgCl_2$, was found most suitable. The results of these studies are shown in Table II.

TABLE II. ELUTION OF IMMUNOSORBENT BOUND PSA WITH DIFFERENT AGENTS : EVALUATION BASED ON ELISA TESTING

	O.D. 492nm	Elution (%)				
	PBS	KSCN	$MgCl_2$	Tris	Urea	Glycine
MAbs	pH 7.2*	3 M	2 M	pH 11.6	8 M	0.2 M, pH 3
CB-PSA.1	1.2	89.7	8.3	91.6	91.6	25
CB-PSA.2	0.8	87.7	76.3	79.6	90.5	60.1
CB-PSA.4	1.4	80.3	45.6	72.9	91.9	15.8
CB-PSA.5	0.5	88.8	–6	80	80	20
CB-PSA.6	0.4	84.4	74	88.8	88.4	57.4
CB-PSA.8	0.6	87.9	11.7	83.3	83.3	16.6
CB-PSA.9	0.5	72.8	0	80	80	20

1 no elution in PBS pH 7.2.

Elution with chaotropic agents (8 M UREA or 3 M KSCN) and basic pH (TRIS pH 11.6) were found to be optimal. However, acid pH agent (Glycine pH 3) presented a low elution performance in comparison with the rest of elution agents. Selection of an ionic strength elution-agent was based on the percent of elution of PSA obtained with CB-PSA.2 (76%) and the lower capability of the ionic agent to disturb the conformational molecular structure of the ligand and the antigen in comparison with a chaotropic agent or extreme pH[16]. 2 M $MgCl_2$ had two additional effects: a) slight chaotropic effect from chloride anion and b) antigen-antibody dissociation effect due to acidic pH after salt hydrolysis. Chaotropic agents (8 M UREA or 3 M KSCN) presented the best elution efficiency (Table II). However, chaotropic agents affected partially or completely the three dimensional structure of the

proteins, in turn affecting the biological activity[16]. Lower stability of MAbs as ligands was seen on employing chaotropic agents[17]. Purification of natural PSA from seminal plasma was carried out in steps as described in Table III. A fraction of natural PSA with > 90% of purity was obtained at the end when 2 M $MgCl_2$ was used for elution, at 23.3% yield. A washing step (2.5 M NaCl with 1 M $MgCl_2$) could eliminate contaminants encountered due to non-specific interaction with the column.

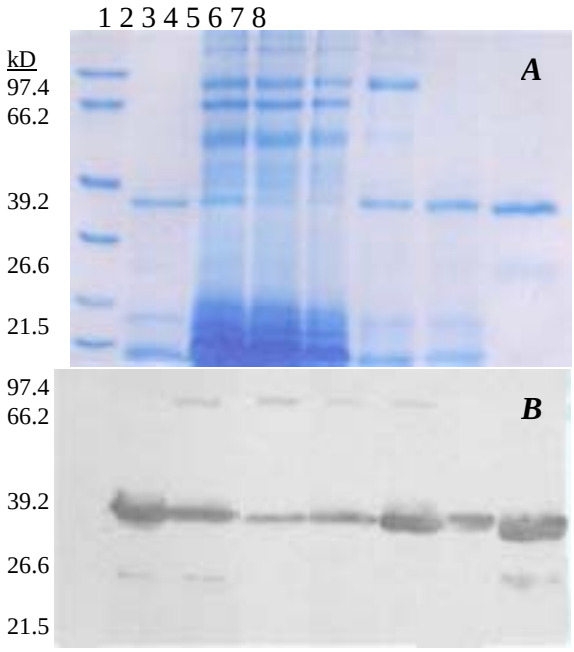

FIG 4. SDS PAGE 12% (A) and Western Blot (B) of natural PSA and purified PSA lane 1: standards from Boehringer Mannheim for MW, lane 2: commercial PSA, lane 3: seminal plasma, lanes 4 and 5: Non bound 1 and 2, lane 6: 2.5 M NaCl eluted, lane 7: 1 M $MgCl_2$ eluted, lane 8: 2 M $MgCl_{2\ eluted}$.

TABLE III. IMMUNOPURIFICATION OF PSA FROM SEMINAL PLASMA

	Fractions				
	Initial*	Non bound	2.5 M NaCl	1 M $MgCl_2$	2 M $MgCl_2$
PSA (mg total)	5.14	1.69	0.49	1.23	1.20
Purity (%)	2.36	1.10	14.75	42.50	91.03
Yield (%)	∝	43.3	9.53	23.92	23.34

* Sample was a mixture of seminal plasma from five patients diluted 1:5 in coupling buffer (50 mL of total volume);% Yield calculated as PSA recovered (mg) with respect to total PSA applied.

Natural PSA was detected in Western blot technique as expected from the description for this molecule (~34 kD, Fig. 4)[18]. Degradation product was detected in the sample (~23 kD) but was absent in the purified material. Degradation of natural PSA has been reported by Frenette G. et. al [19]. Fractions 3–6 presented a band of ~90 kD which is the expected band for the PSA-PCI (PSA and protein-C-inhibitor) complex which constitutes 5% of total PSA in seminal plasma[19]. The degradation products of PSA-PCI bands were also not present in the final material. ELISA did not detect contamination with IgG (Co-elution) in the fractions of the purification.

2.3.4. CONCLUSIONS

1. An ELISA assay was developed in-house using the new panel of anti-PSA MAbs raised at our laboratory
2. High sensitivity (0.12 µg/L) to detect natural PSA was obtained with CB.PSA.4 and CB.PSA-9 as the capture and tracer MAbs respectively. An equimolar response was demonstrated for the developed assay.
3. The standardised total PSA assay presented good correlation with CIS bio total PSA assay and external evaluation was carried out in England with similar results (DPC Immulite total PSA).
4. Natural PSA of 91% of purity could be obtained by inmunopurification employing CB-PSA.2 as the ligand and ionic buffer (2 M $MgCl_2$) as elution agent.

Future plans

1. To standardise an assay to detect free PSA based on the new anti-PSA MAbs.
2. To probe new technologies and products in order to increase the detection of PSA in the new assays.
3. To publish new method to purify PSA from seminal plasma.

REFERENCES

[1] KEARNEY, J.F., RADBRUCH, A., LIESEGANG, B., RAJEWSKY, K., A. new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines, J Immunol, **123(4)** (1979) 1548–50.

[2] LEARY, J., et al., Proc. Nat. Acad. Sci., USA, **80** (1983) 4050.

[3] WILD, D., The Immunoassay Handbook. Mac Millan Press Ltd. (1994) 83–100.

[4] GREENWOLD, F., et al., Biochemical J., **103** (1963): 120–128.

[5] IBARRA, N., CABALLERO, A,, GONZÁLEZ, E., et al., Comparison of different elution conditions for the immunopurification of recombinat hepatitis B surface antigen. J. of Chromatography B, **735** (1999) 271–7.

[6] DAISS, J.L., SCALICE, E.R., Epitope mapping on BIAcore: Theoretical and practical considerations. Methods in Enzymology, **6** (1994) 143–46.

[7] COREY, E., WEGNER, S.K., STRAY, J.E., et. al., Characterization of 10 new monoclonal antibodies against Prostate-Specific Antigen by analysis of affinity, specificity and function in sandwich assays, J Cancer **71** (1997) 1019–28.

[8] CATALONA, W.J., et al., Detection of organ-confined prostate cancer is increased through prostate-specific antigen based-screening, JAMA **270** (1993) 948–54.

[9] VESELLA, R.L., LANGE, P., Issues in the assessment of prostate-specific antigen immunoassays, An update, Urol Clin North, **24** (1997) 261–8.

[10] PARTIN, A.W., OESTERLING, J.E., The clinical usefulness of prostate-specific antigen: Update 1994, J Urol, **152** (1994) 1358–68.

[11] YU, H., DIAMANDIS, E.P., Measurement of serum Prostate Specific Antigen levels in females and in prostatectomized males with an ultrasensitive immunoassay technique, J. Urol., **153** (1995) 1004–8.

[12] GRAVES, H.C., Standardizstion of immunoassays for prostate specific antigen – a problem of prostate-specific antigen or a problem of assay design?, Cancer, **72** (1993) 3141–4.

[13] GRAVES, H.C., Issues on standardization of immunoassays for prostate-specific antigen: a review, Clin Invest Med., **16(6)** (1993) 415–24.

[14] RAFFERTY, B., RIGSBY, P., ROSE, M., STAMEY, T., GAINES, D.R., Reference reagents for prostate specific antigen (PSA): establishment of the first international standards for free PSA (90:10), Clin Chem, **46(9)** (2000) 1310–7.

[15] LILJA, H., CHRISTENSSON, A., DAHLEN, U., et al., Prostate-specific antigen in serum occurs predominantly in complex with with alpha-1 antichymotrypsin, Clin Chem., **37** (1991) 1618–25.

[16] YARMUSH, M.L., PICKARD, K., SUNDARAM, S., et al., Immunoadsorption: strategies for antigen elution and production of reusable absorbents, Biotechnol. Prog., **8** (1992) 168–78.
[17] ALMAGRO, I., AGRAZ, A., FERNÁNDEZ DE COSSIO, M.E., et al., Purificación del AGSHB-R por cromatografía de inmunoafinidad, Biotecnología Aplicada, **11** (1994) 175-9.
[18] QIAN, Y., SENSIBAR, J.A., ZELNER, D.J., et al., Two-dimensional gel electrophoresis detects Prostate-Specific Antigen-ζ-1-Antichymotrypsin complex in serum but not in prostatic fluid, Clin Chem., **43** (1997) 352–9.
[19] FRENETTE, G., GERVAIS, Y., TREMBLAY, R.R., et al., Contamination of purified Prostate-Specific Antigen preparations by Kallikrein hK2, J of Urology **159** (1998) 1375–8.

2.4. GREECE

Title of the Project:	Development of Radioimmunometric Assay for the Determination of Free and Total Prostate-Specific Antigen in Human Serum
Chief Scientific Investigator:	I. Christofidis
Scientific Co-Investigators:	S. Kakabakos, E. Diakoumis, A. Siafaka-Kapadai

Abstract. In the present study, we describe sensitive and reliable immunoradiometric assays (IRMA) for the determination of free (f-PSA) and total (complexed as well as free, t-PSA) prostate-specific antigen in human serum. The assays employ matched pairs of anti-PSA monoclonal antibodies (MAb66 and MAb30 or MAb10). The polystyrene tubes are coated with MAb66 (capture antibody) and the detection antibody MAb30 or MAb10 was labeled with ^{125}I. The minimum detectable dose of f-PSA was less than 0.03 ng/mL, and the assay could determine free PSA concentrations of up to 26 ng/mL; in the case of t-PSA, the minimum detectable dose was less than 0.04 ng/mL while the assay could determine total PSA concentrations of up to 112 ng/mL without dilution of the sample. The intra- and inter-assay variations (%CV) for f-PSA were less than 3% and 15% respectively and for t-PSA less than 2% and 9%, respectively. The results obtained using 44 serum samples correlated well with those obtained by commercially available IRMA kits.

2.4.1. INTRODUCTION

Prostate-specific antigen (PSA) is a (~33 KDa MW) chymotrypsin-like serin protease and a member of the human glandular kallikrein family secreted from the epithelial cells, lining the prostate ducts, into seminal fluid, where its concentration is 0.5–2.0 g/L [1–4]. Due to its sensitivity and organ specificity, PSA is a very valuable protein marker in human blood for the diagnosis of prostate cancer. PSA is also monitored in the follow-up after surgical removal of prostate carcinomas [5–7]. However, patients with benign prostatic hyperplasia (BPH), show total PSA concentrations of up to ~15 ng/mL in serum. Because of this, total PSA concentrations less than 15 ng/mL cannot be used to distinguish between prostate cancer and BPH. Free (uncomplexed) PSA is present in serum accounting for the 5–30% of the total PSA [8–9]. However, PSA is complexed in human serum, with several other proteins and mainly with the serine protease inhibitor ζ_1-antichymotrypsin (ACT), thus, leading to uncertainties in its immunological measurement [10–18]. Currently the ratio of free to total PSA (as well as the ratio of free PSA to the PSA-ACT complex) is being used for better differentiation between prostate cancer and BPH, than the level of total PSA alone. Hence, the accurate measurement of these two markers is of importance for realizing the value of PSA as a tumour marker [19–21]. Thus, the development of a reliable and sensitive immunoassay for the detection of free as well as total PSA is of great importance for the clinical evaluation of the patient status. Current immunoassays focus on two very significant points: better specificity and higher sensitivity [22–26]. It is well known that the use of monoclonal antibodies in sandwich-type assays, which involve pairs of antibodies (one attached onto the solid phase and the other used as the detection system), could increase the specificity and the sensitivity of the assay [27–32]. Good sensitivity may derive from either lower non-specific binding signal or higher detection signal. The former one depends both on the blocking of the solid phase [33–35] and the matrix of the standard solution, whereas the latter one depends on the specific radioactivity of the detection antibody [36–43]. In the case of free PSA, calibrators cannot be made in native human serum due to the presence of ζ_2-macroglobulin which forms a not yet detectable complex with free PSA [44–51]. The objective of this work was to develop a sensitive, reliable and fast IRMA to determine free and total PSA in human serum.

2.4.2. MATERIALS

Sephadex G-75 powder was obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA) and mouse serum were obtained from O.E.M. Concepts Inc.(Toms River, NJ, USA).

L-tyrosine ($C_9H_{11}NO_3$) >99% (TLC) and Trizma®Base were obtained from Sigma Chemical Co. (St Louis, MO, USA). MAb66, MAb30 and MAb10 were provided by the University of Alberta, Canada. PSA was obtained from Genzyme Diagnostics. PSA-ACT complex was obtained from Scripps (San Diego, CA, USA). Carrier-free $Na^{125}I$ (specific radioactivity 17 kCi/g, radiochemical purity 99.9%, iodate <2%) was obtained from MDS Nordion SA. Tandem-R f-PSA and t-PSA kits were products of Hybritech Inc. (San Diego, CA, USA). Polystyrene (75x12 mm) tubes were purchased from VIVE Co. (Athens, Greece). N-chloro-p-toluene sulfonamide and all other reagents were from Merck (Darmstadt, Germany). Doubly distilled water was used throughout this study.

2.4.3. METHODS

2.4.3.1. Radiolabeling of MAb30 and MAb10

A. Solutions for the radioiodination procedure

i. *Phosphate buffer solution:* 0.1 M PBS, pH 7.4.

ii. *Chloramine-T solution*: 0.1 M PBS, pH 7.4, chloramine-T 1mg/mL. The reagent is prepared just before the radioiodination procedure.

iii. *Sodium disulfite solution*: 0.1 M PBS, pH 7.4, sodium disulfite 2mg/mL, L-tyrosine 1mg/mL. The reagent is prepared just before the radioiodination procedure.

iv. *Monoclonal antibody solution*: 0.5 mg of lyophilized MAb30 or MAb10 were diluted in 200 μL of 0.1 M PBS, pH 7.4. Twenty micro liters of this solution were diluted 5 times in 0.1 M PBS, pH 7.4. Twenty aliquots of this were distributed into eppendorf tubes at a working concentration of 10 μg of monoclonal antibody per 20 μL. Both monoclonal concentrated solution and aliquots were stored at –20°C.

v. *Tracer dilution buffer*: 0.2 M PBS, pH 7.4, 0.05% NaN_3, 8% BSA, 10% mouse serum.

B. Radioiodination of MAb30 and MAb10

Into an eppendorf tube containing 20 μL of a 10 μg MAb30 or MAb10 solution and the appropriate volume of $Na^{125}I$ solution, 20 μL of chloramine-T solution were added and the contents were mixed for 30 seconds. The reaction was stopped by adding 50 μL of sodium disulfite L-tyrosine solution. Total counts in the reaction were determined by a NE1612 ν-counter for a period of 6 seconds.

C. Purification of the tracer

The reaction mixture was loaded on a Sephadex G-75 (1×22 cm) column, equilibrated with 0.2 M phosphate buffer saline (PBS), pH 7.4, 0.05% NaN_3, 0.1% BSA. The column was eluted with the same buffer at a flow rate of 20 mL/h. Fractions of high radioactivity were pooled and diluted with the tracer dilution buffer to a concentration of 1.07 μCi/mL providing 200000 cpm per 100 μL (tracer working solution). The tracers were stored at 4°C for 30 days.

2.4.3.2. Preparation of standard calibrator solutions

Nine calibrator solutions in the range of 0–26 ng/mL of f-PSA were prepared using the commercial PSA as the stock solution. In the case of t-PSA, eight calibrator solutions in the range of 0–112 ng t-PSA/mL were prepared by mixing PSA-ACT complex and f-PSA in the ratio of 9:1 [42–43]. For both f-PSA and t-PSA, the calibrators were made by gravimetric dilution in 0.2 M PBS, pH 7.4, 0.05% NaN_3, 4% BSA based on the concentration values for PSA-ACT and f-PSA, provided by the manufacturer. The concentrations of both sets of calibrators were also evaluated by commercial IRMA kits (Tandem-R Hybritech).

2.4.3.3. Preparation of MAb66 coated polystyrene tubes

Polystyrene tubes were coated with MAb66, at a final concentration of 1–2 μg/mL in 50 mM Tris, pH 7.8, 0.05% NaN_3. Five hundred micro liters of this solution, were added to each tube. Tubes

were incubated overnight at room temperature and washed twice with 1 mL of 10 mM Tris, pH 7.8, 0.05% NaN_3, 0.15 M NaCl, 0.05% Tween 20 (washing solution). The tubes were then blocked with 1 mL of 50 mM Tris, pH 7.8, 0.05% NaN_3, 8% BSA (blocking solution) per tube and were incubated overnight at 4^oC. Then, the tubes were washed twice with 1mL of washing buffer, left to dry at room temperature and stored at 4^oC in the presence of a dessicant.

2.4.3.4. Assay procedures

Fifty micro litres of standard solution, or serum sample, and 100 μL of the appropriate tracer working solution were added into polystyrene coated tubes. The contents of the tubes were vortex mixed and were incubated overnight at room temperature. Finally, the tubes were decanted, washed twice with 1mL washing solution per tube and the radioactivity of each tube was measured for 60 seconds in a NE1612 ν-counter. The standard curve of the assay was obtained by plotting the radioactivity (cpm) measured in the tubes corresponding to the calibrators vs. the f-PSA or t-PSA concentration of each calibrator (as the case may be) and drawing the curve between the points.

2.4.4. RESULTS AND DISCUSSION

The objective of the present work was to develop sensitive and reliable IRMA methodologies for the determination of free-PSA and total-PSA in human serum samples. The assays were developed in antibody coated tubes employing two pairs of mouse monoclonal antibodies that present very good specificity against PSA molecule. The monoclonals were provided to our laboratory by the University of Alberta Canada. The first, MAb66, was a common one used for coating of the polystyrene tubes to serve as the capture antibody. The second one of the pair for use as tracer after radioiodination with ^{125}I , was MAb30 for f-PSA assay or MAb10 for t-PSA.

Preparation of antibody coated tubes is an important step since appropriate sensitivity in an IRMA procedure could be derived from careful selection of parameters such as antibody concentration used for coating and the blocking procedure followed. Apart from this, the specific radioactivity of the labeled antibody and the immunoreaction conditions (e.g. matrix of the calibrators, pH and ionic strength of the assay buffer, ingredients of the buffer etc.) could further improve the assay sensitivity. All these parameters were carefully studied in order to optimize the conditions for the preparation of the antibody-coated tubes as well as the assay conditions.

2.4.4.1. Optimization of the antibody coating procedure

The coating of the solid phase (polystyrene tubes) was performed in two types of solutions: (a) Tris buffer, pH 7.8 or and (b) Carbonate buffer, pH 9.6. For blocking the solid phase the same buffers were used with the addition of 1% BSA or 1% casein in each case. Tris buffer gave better results and 1% BSA was found to be better for blocking.

Tubes were coated with MAb66 in Tris buffer at several concentrations ranging from 0.5–5 μg/mL. As it is seen in figures 1 and 2, coating antibody concentration of greater than 1μg/mL was required for both f-PSA and t-PSA assays to obtain adequate sensitivity.

The most effective blocking solution, used after antibody coating to minimise the non-specific binding (NSB), was a 50 mM Tris-HCl buffer, pH 7.8, containing 1% (w/v) BSA. Thus, an antibody concentration of 2 μg/mL in the coating solution and 1% BSA concentration in the blocking solution were adopted in the final protocol.

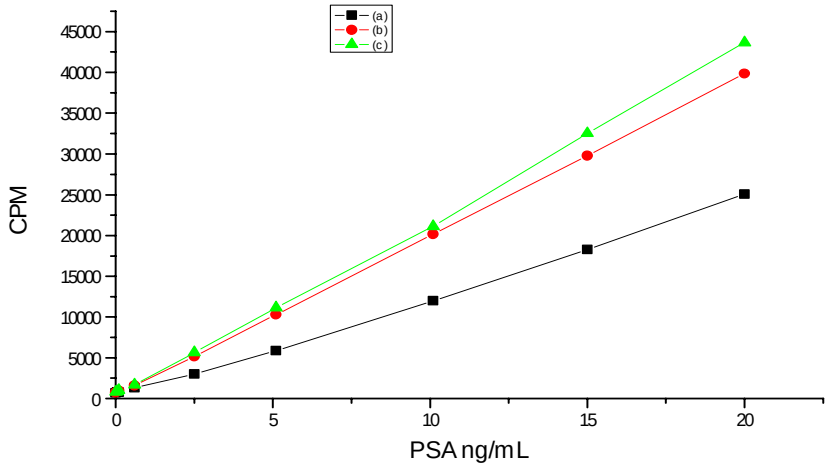

FIG. 1. Calibration curves of free PSA, when test was carried out in: a. 0.5 µg/mL MAb66 coated tubes; b. 1 µg/mL MAb66 coated tubes; c. 5 µg/mL MAb66 coated tubes.

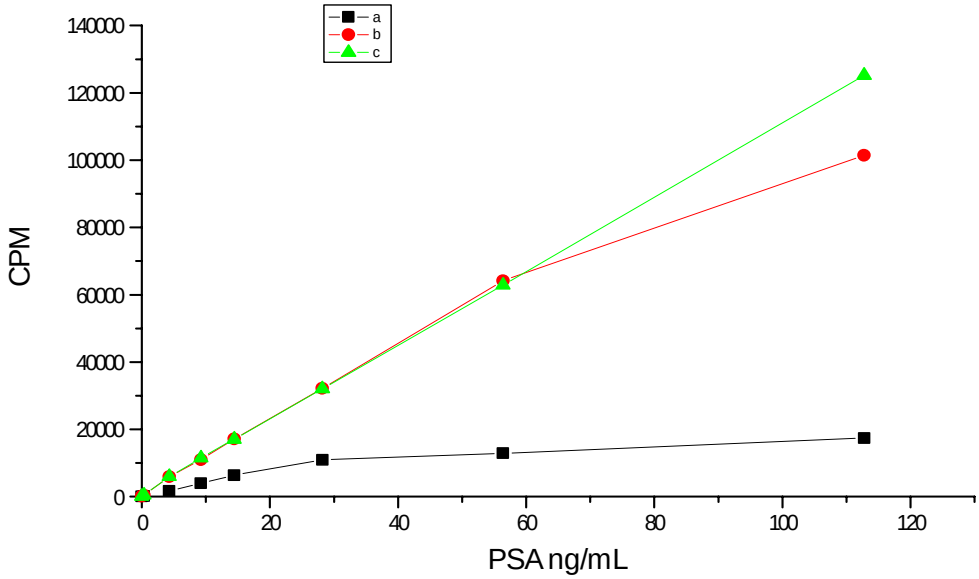

FIG. 2. Calibration curves of total PSA, when test was carried out in: a. 0.5 µg/mL MAb66 coated tubes; b. 1 µg/mL MAb66 coated tubes; c. 2 µg/mL MAb66 coated tubes.

2.4.4.2. Optimization of the free PSA and total PSA assay conditions

Several assay parameters, such as the matrix for the preparation of the calibrators, the assay buffer ingredients and the specific activity of the radiolabeled antibody were optimized in order to achieve appropriate assay sensitivity and accuracy of the free-PSA and total-PSA measurements.

Three types of standard solutions matrix were tested (a) Heat-treated Horse serum, (b) PBS containing casein and (c) PBS containing BSA. We found that PBS containing BSA provided assay with at least 2 times better sensitivity compared to the other two matrices used (FIG. 3.). Thus, PBS containing BSA was used for the preparation of the calibrators.

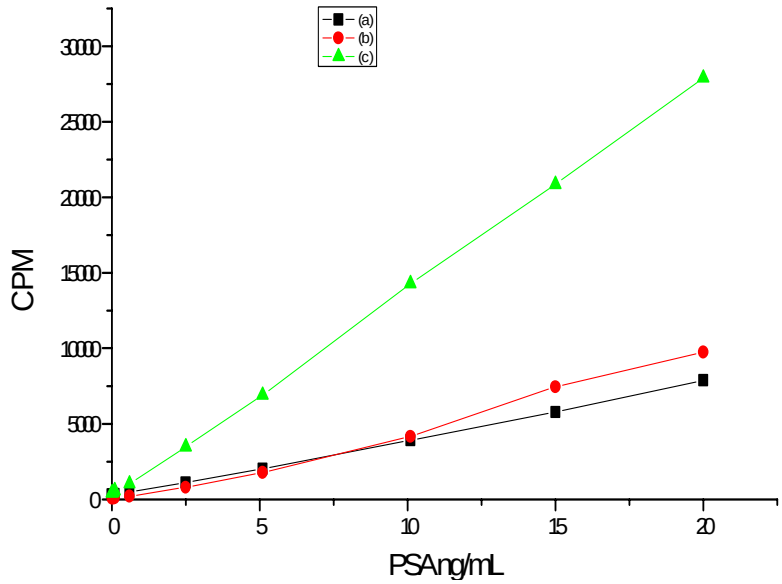

FIG. 3. Calibration curves obtained with different calibration solutions matrix. (a) *Heat-treated horse serum;* (b) *0.2 M PBS, pH 7.4, 4% casein;* (c) *0.2 M PBS, pH 7.4, 4% BSA.*

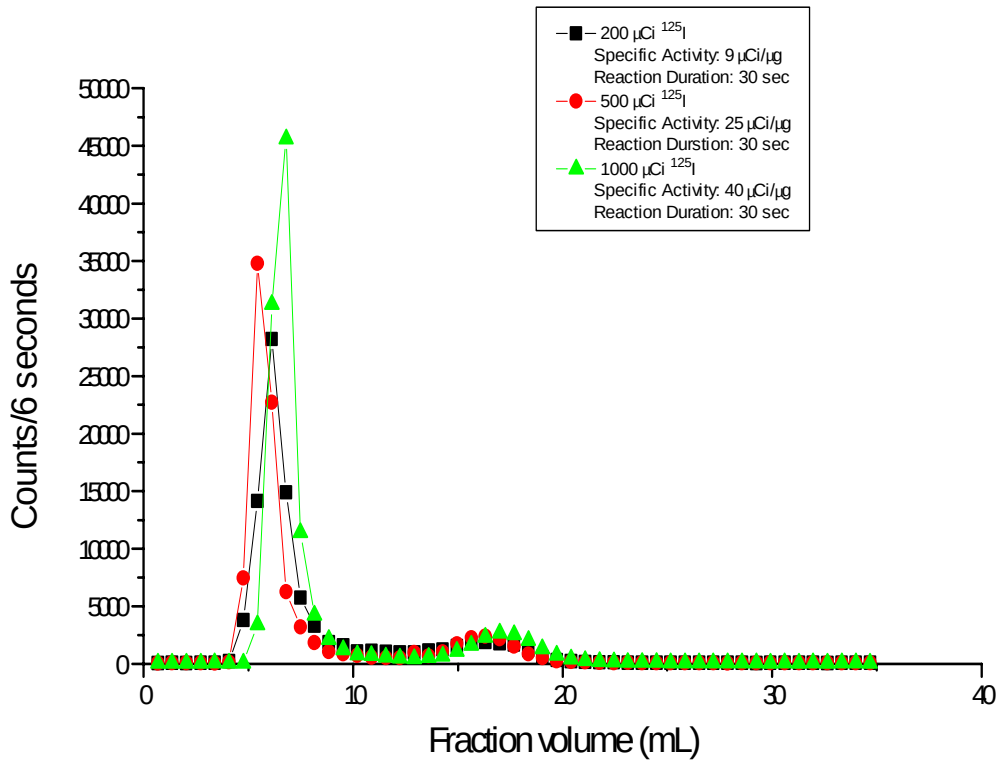

FIG. 4. Elution profile of MAb30 or MAb10 radiolabeled at three different specific activity levels.

In addition, we found that when normal mouse serum was added in the assay buffer (labelled antibody working solution) the NSB was further reduced to barely 0.05% of the total counts added per tube.

The specific radioactivity of the tracer antibody, affects the sensitivity of the assay significantly. Purification patterns of differently labelled MAb30 and MAb10 are presented in Figure 4. Labelled antibody with specific radioactivity from 9–40 µCi/µg was used. The high specific activity MAb30 or MAb10 (25–40 µCi/µg) resulted in assays with higher sensitivity (less than

0.02 ng PSA/mL) compared to the 9 µCi/µg one, which resulted in a detection limit of less than 0.03 ng PSA/mL for the f-PSA assay and less than 0.04 ng PSA/mL for the t-PSA assay. However, as the specific activity increased, the working range of the assays decreased as shown in the figures 5 and 6. Hence, the low specific radioactivity MAb30 or MAb10 (~9 µCi/µg) was selected for our final protocol.

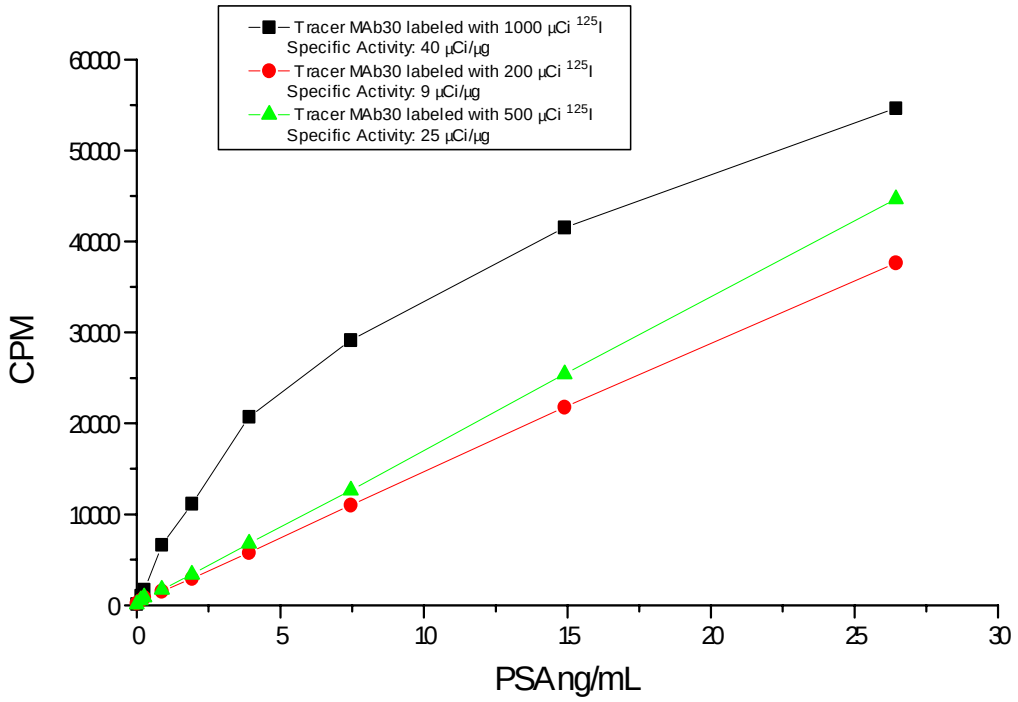

FIG. 5. Standard curves of fPSA obtained by three differently radiolabeled MAb30 antibodies.

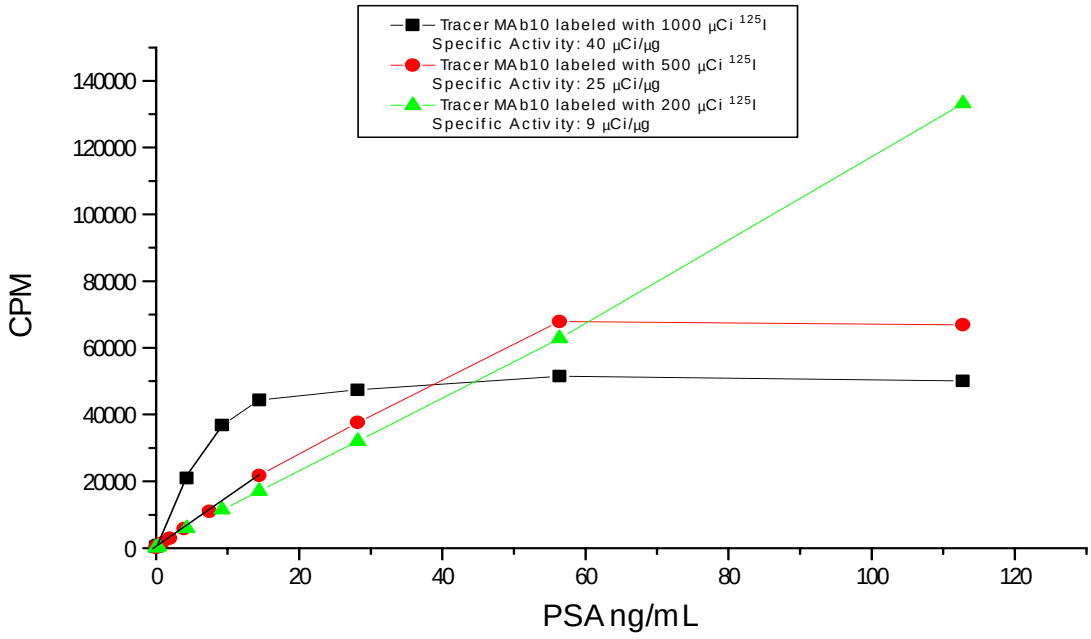

FIG. 6. Standard curves of tPSA obtained by three differently radiolabeled MAb10 antibodies.

2.4.4.3. Analytical characteristics of the f-PSA assay

A typical calibration curve of the free PSA assay is presented in Figure 7. The detection limit of the assay, defined as the concentration corresponding to a signal 2 SD above the mean of 30 replicates of the zero calibrator, was 0.03 ng/mL. The working range of the assay was up to 26 ng/mL.

The accuracy of the assay was estimated in terms of recovery and parallelism experiments. The analytical recovery was studied by spiking increasing amounts of f-PSA to a low f-PSA standard solution. The results are shown in Table I. The recovery of added analyte in 5 different trials, carried out in duplicate, ranged from 94–101% with an average of 99%.

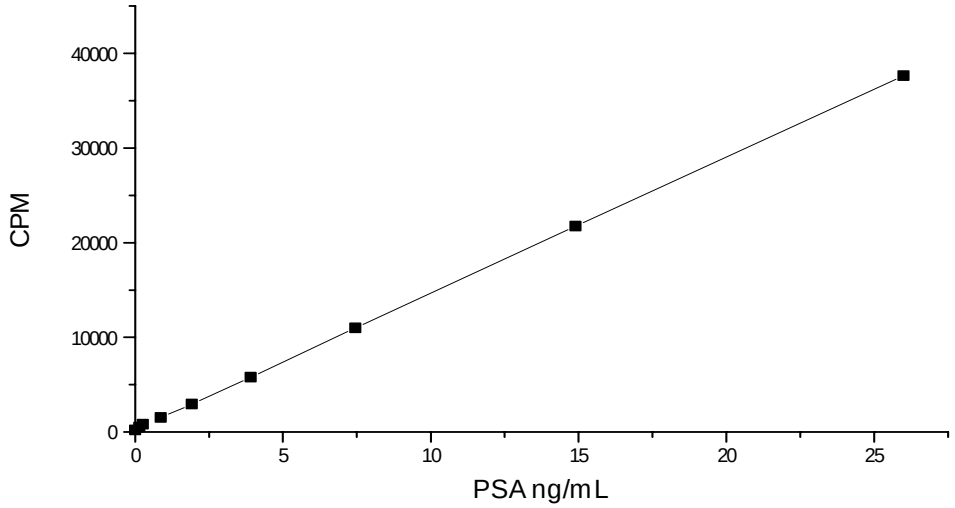

FIG. 7. Typical calibration curve of the free PSA assay.

TABLE I. ANALYTICAL RECOVERY OF f-PSA IN LOW f-PSA STANDARD SOLUTION

Original Concentration of f-PSA in sample ng/mL	Concentration of f-PSA added ng/mL	Concentration of f-PSA observed ng/mL	Recovery%
0.8	0	0.8	100
0.8	0.32	1.05	94
0.8	0.61	1.4	99
0.8	1.24	2.02	99
0.8	2.21	3.03	101

The dilution effect was studied by diluting four human serum samples with elevated f-PSA concentrations with f-PSA zero standard solution. The results were compared to the calculated ones. As it is shown in Figure 8, the values determined for the diluted samples, correlated well with the expected values.

The within- and between-assay variations determined for the f-PSA assay developed are presented in Table II. The within-assay variability was determined by assaying 3 serum samples according to the routine protocol 10 times in the same run. The CVs varied between 3–6%. The between-assay variability was determined by assaying 3 serum samples, according to the routine protocol, 10 times, in 10 different runs in duplicate. The CVs varied between 8 and 15%.

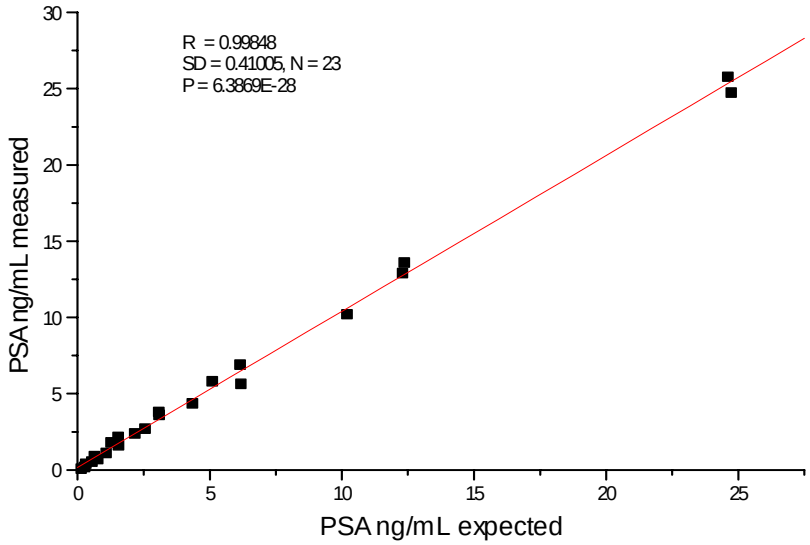

FIG. 8. Parallelism testing: the expected f-PSA values are compared with the estimated f-PSA values, for four human serum samples with high PSA concentrations diluted with f-PSA zero standard solution.

TABLE II. STATISTICAL VARIATIONS IN f-PSA ASSAY

	Mean f-PSA ng/mL	CV%
Within-Run	1.12	6
	2.39	3
	5.73	4
Between-Run	0.91	15
	2.05	8
	4.83	9

When 30 human serum samples obtained from healthy individuals were tested, the concentration of f-PSA ranged from less than 0.03 ng/mL up to 2.85 ng/mL (median, 0.8 ng/mL), as shown in Figure 9.

The assay developed was compared with a commercially available IRMA kit, namely Hybritech Tandem-R f-PSA IRMA kit. As it is shown in Figure 10, the values obtained by the assay developed for 20 healthy individuals were in good agreement with those determined by the commercial kit.

2.4.4.4. Analytical characteristics of the t-PSA assay

A typical calibration curve of the total-PSA assay is presented in Figure 11. The detection limit of the assay, defined as the concentration corresponding to a signal 2 SD above the mean of 30 replicates of the zero calibrator, was 0.04 ng/mL. The working range of the assay was up to 112 ng/mL.

The accuracy of the assay was estimated in terms of recovery and parallelism experiments. The analytical recovery was studied by spiking a certain amount of purified PSA-ACT to a low PSA concentration male or female serum and performing the assay to determine the t-PSA in the samples both before and after the addition of PSA-ACT. The results are shown in Table III. The recovery of added analyte in 12 different trials, carried out in duplicate, ranged from 95–102% and the average recovery was 99%.

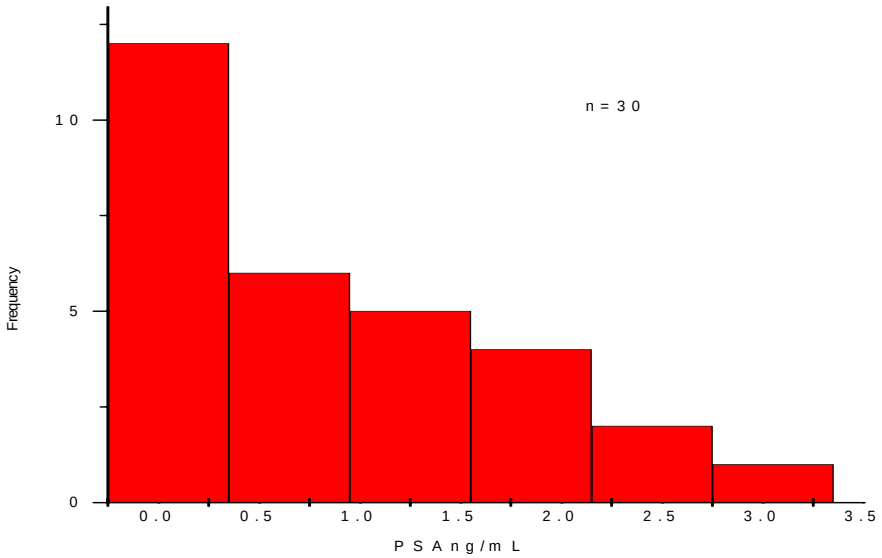

FIG. 9. Frequency distribution of free PSA concentrations obtained using serum samples from 30 healthy individuals.

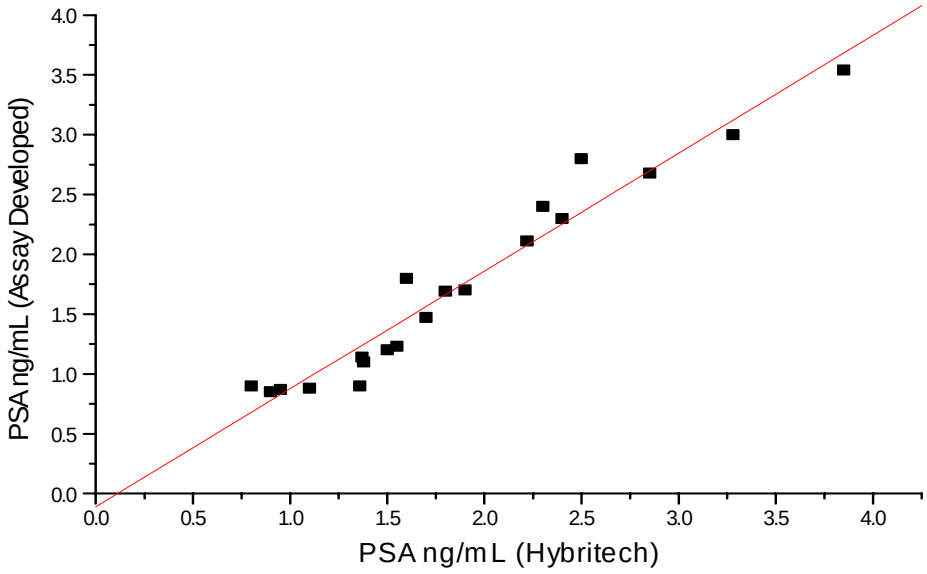

FIG. 10. Correlation of the f-PSA values obtained using the assay developed (Y axis) with those estimated using the Hybritech Tandem-R IRMA f-PSA assay (X axis). A: -0.111, B: 0.986, R: 0.973 (n=20, SD=0.196, P<10^{-5}).

The dilution effect was studied by diluting eight high-PSA value human serum samples with zero PSA standard solution. The results were compared with the calculated values. As it is shown in Figure 12, the values determined for the diluted samples, correlated well with the expected values.

The within- and between-assay variations determined for the assay developed are presented in Table IV. The within-assay variation was determined by assaying 3 serum samples according to the routine protocol 10 times in the same run. The CVs varied between 2–5%. The between-assay variability was determined by assaying 3 serum samples, according to the routine protocol, 10 times, in 10 different runs in duplicate. The CVs ranged between 3 and 9%.

When 30 human serum samples obtained from healthy individuals were tested, the concentration of t-PSA ranged from 0.04 ng/mL up to 4.00 ng/mL (median, 0.61 ng/mL), as shown in Figure 13.

The assay developed was compared with a commercially available IRMA kit, namely Hybritech Tandem-R t-PSA IRMA kit. As it is shown in Figure 14, the values obtained by the assay developed, for 24 healthy individuals were in good agreement with those determined by the commercial kit.

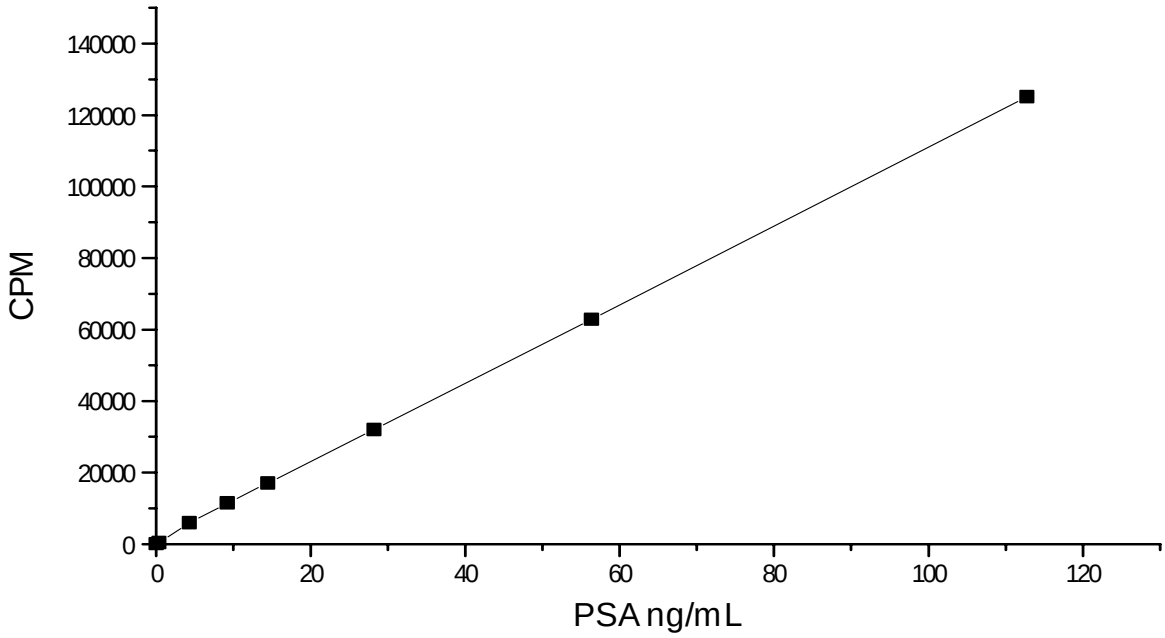

FIG. 11. Typical calibration curve of the total PSA assay.

TABLE III. ANALYTICAL RECOVERY OF t-PSA IN LOW t-PSA STANDARD SOLUTION

Low PSA concentration male (M) or female (F) serum (ng/mL)	Concentraton of PSA-ACT added (ng/mL)	Concentration of t-PSA (observed ng/mL)	Recovery%
M 0.3	1.5	1.71	95
M 0.4	1.5	1.88	99
M 0.5	1.5	1.94	97
M 0.6	1.5	2.02	96
M 0.7	1.5	2.22	101
M 0.9	1.5	2.41	100
M 2.8	1.5	4.27	99
F 0	1.5	1.5	100
F 0	1.5	1.51	101
F 0	1.5	1.53	102
F 0	1.5	1.47	98
F 0	1.5	1.49	99

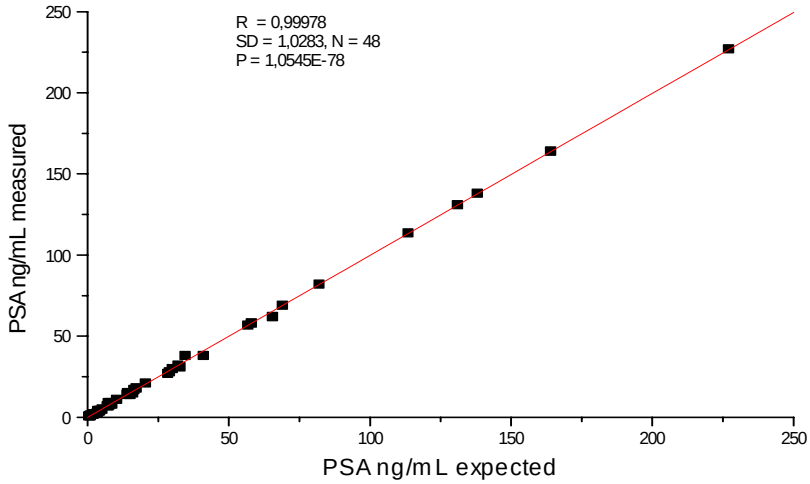

FIG. 12. Parallelism testing:. Comparison of the calculated t-PSA values with the estimated t-PSA values, for eight human serum samples with high PSA concentrations diluted with PSA zero standard.

TABLE IV. STATISTICAL VARIATIONS IN t-PSA ASSAY

	Mean t-PSA ng/mL	CV%
Within-Run	0.68	3
	14.1	2
	54.0	5
Between-Run	2.8	3
	7.1	6
	122	9

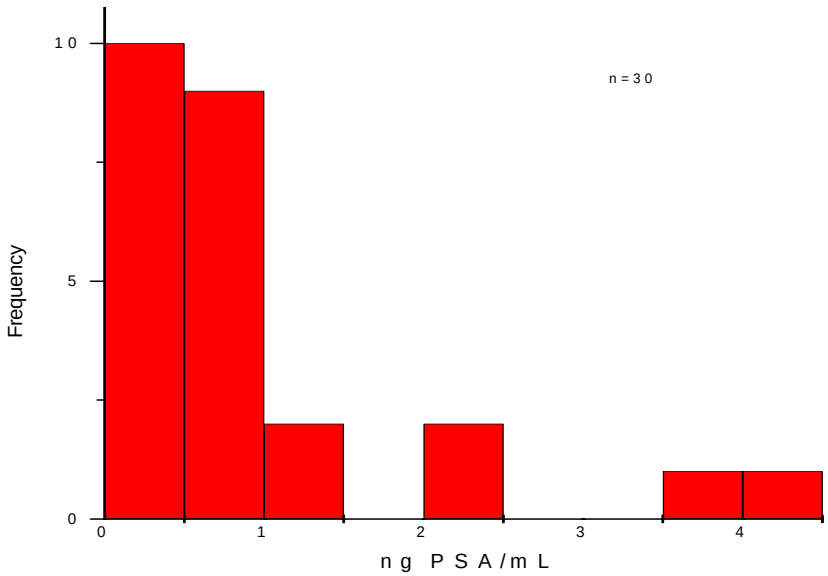

FIG. 13. Frequency distribution of total PSA concentrations obtained using serum samples from 30 healthy individuals.

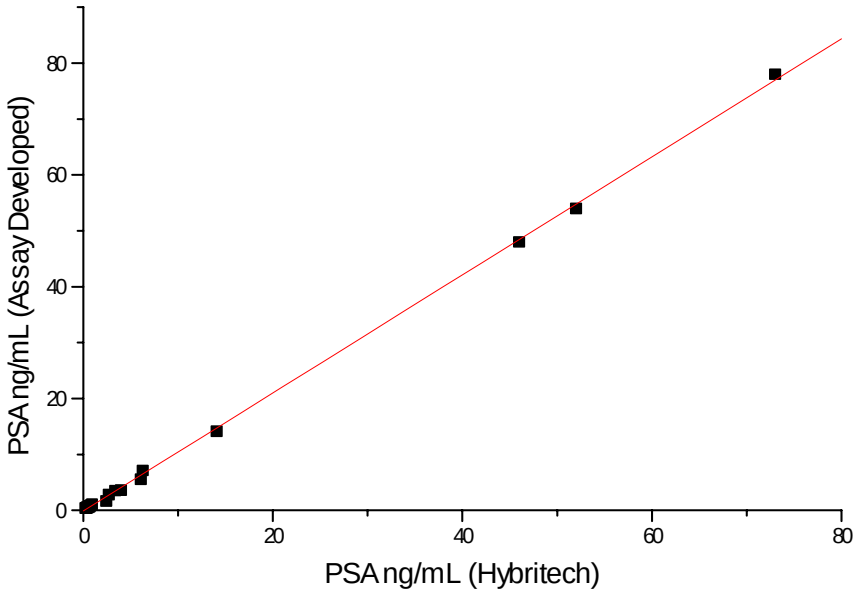

FIG. 14. Correlation of the t-PSA values obtained using the assay developed (Y axis) with those provided by the Hybritech Tandem-R IRMA t-PSA assay (X axis). A: -0,118, B: 1.056, R: 0.999 (n=24, SD=0.456, P<10^{-5}).

2.4.5. CONCLUSION

In conclusion, we could demonstrate the development of sensitive and reliable immunoradiometric assay procedures for the determination of free and total PSA in human serum using matched pairs of highly specific anti-PSA monoclonal antibodies. The working ranges of the assay were between 0.03 ng/mL (detection limit) and 26 ng/mL for f-PSA and between 0.04 ng/mL (detection limit) and 112 ng/mL for t-PSA. The assays were precise (Intra- and inter-assay CVs less than 3% and 15% for f-PSA, and less than 2% and 9% for t-PSA, respectively) and accurate as indicated by the recovery and parallelism experiments. The assays are capable of determining f-PSA and t-PSA in human serum samples and the results obtained with this assay correlate well with those obtained using commercial IRMA kits.

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REFERENCES

[1] WATT, K.W., LEE, P.J.M., TIMKULU, T., CHAN, W.P., LOOR, R., Human prostate-specific antigen: structural and functional similarity with serine proteases, Proc. Natl. Acad. Sci., USA, **83** (1986) 3166–3170.

[2] SCHALLER, J., AKIYAMA, K., TSUDA, R., MARTI, T., RICKLI, E.E., Isolation, characterization and amino-acid sequence of seminoprotein, a glycoprotein from human seminal plasma, Eur. J. Biochem. **170** (1987) 111–130.

[3] HENTTU, P., VIHKO, P., cDNA coding for the entire human prostate specific antigen shows high homologies to the human tissue kallilrein genes, Biochem. Biophys. Res. Commun. **160** (1989) 903–910.

[4] LUNDWALL, A., LILJA, H., Molecular cloning of the human prostate specific antigen cDNA, FEBBS Lett. **214** (1987) 317–322.

[5] STAMEY, T.A., YANG, N., HAY, A.R., MCNEAL, J.E., FREIHA, F.S., REDWINE, E.A.., Prostate-specific antigen as a serum marker for the adenocarcinoma of the prostate, N. Engl. J. Med. **317** (1987) 909–1016.
[6] CHU, T.M., Prostate-specific antigen and early detection of prostate cancer, Tumour Biol. **18** (1997) 123–134.
[7] SOKOL, L.J., CHAN, D.W., Total, free and complexed PSA: analysis and clinical utility, J. Clin. Ligand Assay **21** (1998) 171–179.
[8] WANG, M.C., VALENZUELA, L.A., MURPHY, G.P., CHU, T.M., Purification of a human prostate-specific antigen, Invest Urol, **17** (1979) 159–163.
[9] WANG, M.C., VALENZUELA, L.A., MURPHY, G.P., CHU, T.M., A simplified purification procedure for human prostate antigen, Oncology, **39** (1982) 1–5.
[10] STENMAN, U.H., LEINONEN, J., ALFTHAN, H., RANNIKKO, S., TUHKANEN, K., ALFTHAN, O., A complex between prostate-specific antigen and Ŭ$_1$-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer, Cancer Res. **51** (1991) 222–226.
[11] LILJA, H., CHRISTENSSON, A., DAHLEN, U., MATIKAINEN, M.T., NILSSON, O., PETTERSSON, K., LOVGREN, T., Prostate-specific antigen in serum occurs predominantly in complex with Ŭ$_1$-antichymotrypsin, Clin. Chem. **37** (1991) 1618–1625.
[12] LEINONEN, J., LOVGREN, T., VORNANEN, T., STENMAN, U.H., Double-label time-resolved immunofluorometric assay of prostate-specific antigen and the complex between prostate-specific antigen with ζ_1-antichymotrypsin, Clin. Chem. **39** (1993) 2098–2103.
[13] CHRISTENSSON, A., BJORK, T., NILSSON, O., DAHLEN, U., MATIKAINEN, M.T., COCKETT, A.T., et al., Serum prostate specific antigen complexed to Ŭ$_1$-antichymotrypsin as an indicator of prostate cancer, J. Urol. **150** (1993) 100–105.
[14] CHRISTENSSON, A., LILJA, H., Complex formation between protein C inhibitor and prostate-specific antigen *in vitro* and in human semen, Eur. J. Biochem. **220** (1994) 45–53.
[15] CHRISTENSSON, A., LAURELL, C.B., LILJA, H., Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors, Eur. J. Biochem. **194** (1990) 755–763.
[16] LEINONEN, J., ZHANG, W.M., STENMAN, U.H., Complex formation between PSA isoenzymes and protease inhibitors, J. Urol. **155** (1996) 1099–1103.
[17] ZHANG, W.M., LEINONEN, J., KALKKINEN, N., STENMAN, U.H., Prostate specific antigen forms a complex and cleaves Ŭ$_1$-protease inhibitor *in vitro*, Prostate **33** (1997) 87–96.
[18] BECKER, C., LILJA, H., Individual prostate-specific antigen (PSA) forms as prostate tumour markers, Clin. Chem. Acta., **257** (1997) 117–132.
[19] MOORE, R.A., Free PSA as a percentage of the total: where do we go from here? [Editorial], Clin. Chem. **43** (1997) 1561–1562.
[20] MEYER, A., JUNG, K., LEIN, M., SCHNORR, D., LOENING, S.A., Factors influencing the ratio of free to total prostate specific antigen in serum, Int. J. Cancer **74** (1997) 630–636.
[21] WOODRUM, D.L., BRAWER, M.K., PARTIN, A.W., CATALONA, W.J., SOUTHWICK, P.C., Interpretation of free prostate specific antigen clinical research studies for the detection of prostate cancer, J. Urol. **159** (1998) 5–12.
[22] STAMEY, T.A., Lower limits of detection, biological detection limits, functional sensitivity, etc., Clin. Chem. **62:6** (1996) 849–852.
[23] DIAMANDIS EP, YU H, MELEGOS D. Ultrasensitive prostate specific antigen assays and their clinical application. Clin Chem 1996;42:6, 853–857.
[24] INTERNATIONAL ATOMIC ENERGY AGENCY, Development of kits for radioimmunometric assays for tumour markers (Report of a Research Co-ordination Meeting, Edmonton, Canada) Vienna (1999).
[25] HAGE, D.S., Immunoassays, Anal. Chem. **65** (1993) 420R.
[26] CHARD, T., An introduction to radioimmunoassay and related techniques, 2nd ed. (TS Work and E Work Eds.), Elsevier, Amsterdam, 1982.
[27] CATT, K., TREGEAR, G.W., Solid-phase radioimmunoassay in antibody-coated tubes, Science **158** (1967) 1570–1572.

[28] BUTTLER, J.E., Perspectives, configurations and principles. In: Immunochemistry of solid-phase immunoassay. (J.E. Buttler, Ed.) CRC Press, Boca Raton (1991) 3.
[29] TIJSSEN, P., Practice and theory of enzyme immunoassays, (RH Burdon and PH van Knippenberg Eds.) Elsevier, Amsterdam (1985).
[30] PETERMAN, J.H., TARCHA, P.J., CHU, V.P., BUTTLER, J.E., The immunochemistry of sandwich ELISAs IV. A comparison of the antigen capture capacity of antibodies adsorbed or covalently attached to polystyrene, J. Immunol. Method **11** (1988) 271.
[31] CANTARERO, L., BUTTLER, J.E., OSBORNE, J.W., The adsorptive characteristics of proteins for polysterene and their significance in solid-phase immunoassays, Anal. Biochem. **105 (**1980) 375–383.
[32] PETROU, P.S., KAKABAKOS, S.E., KOUPPARIS, M.A., CHRISTOFIDIS, I., Antibody coating approach involving gamma globulins from non-immunized animal and second antibody antiserum, J Immunoassay **19** (1998) 271.
[33] SHIRAHAMA, H., SUZAWA, T., Adsorption of bovine serum albumin onto styrene/acrylic acid copolymer latex, Colloid Polymer Sci. **263** (1985) 141–146.
[34] BULL, H.B., Adsorption of bovine serum albumin on glass, Biochem. Biophys. Acta. **19** (1956) 464–471.
[35] QUALTIERE, L.F., ANDERSON, A.C., MEYER, P., Effects of ionic and non-ionic detergents on antigen-antibody reactions, J. Immunol. **119** (1977) 1645–1651.
[36] FREEDLENDER, A., CATHOU, R.E., Iodination (In: Kirkham, K.E., Hunter, W.M., Eds.), Radioimmunoassay Methods: European Workshop, Edinburgh, Churchill Livingstone (1971) 94–96.
[37] HEBER, D., ODELL, W.D., SCHEDEWIE, H., et al., Improved iodination of peptides for radioimmunoassay and membrane radioreceptor assay, Clin. Chem. **24** (1978) 769–799.
[38] GREENWOOD, F.C., HUNTER, W.M., The preparation of ^{131}I labelled human growth hormone of high specific radioactivity, Biochem. J. **89** (1963) 114–123.
[39] THORELL, J.I., JOHANSSON, B.G., Enzymatic iodination of polypeptides with ^{125}I to high specific activity, Biochem. Biophys. Acta. **251** (1971) 363–369.
[40] KROHN KA, KNIGHT LC, HARWIG JF, ET AL. Differences in the sites of iodination of proteins following four methods of radioiodination. Biochim Biophys Acta 1977; 490:497–503.
[41] BROWN, N.S., ABBOTT, S.R., CORRIE, J.E.T., Some observations on the preparation, purification and storage of radioiodinated protein hormones (In: Hunter, W.M., Corrie, J.E.T., Eds.) Immunoassays for Clinical Chemistry, 2nd ed. Edinburgh, Churchill Livingstone (1983) 267–276.
[42] BARTOLINI, P., RIBELA, M.T.C.P., Influence of chloramine T iodination on the biological and immunological activity of the molecular radius of the human growth hormone molecule, J. Immunoassay **7** (1986) 129–138.
[43] HUNTER, W.M., The preparation and assessment of iodinated antigens (In: Kirkham, K.E., Hunrwe, Q.M., Eds.) Radioimmunoassay Methods: European Workshop. Edinburgh: Churchill Livingstone (1971) 3–23.
[44] GRAVES, H.C.B., Standardization of immunoassays for prostate-specific antigen: a problem of prostate-specific antigen complexation or a problem of assay design? Cancer **72** (1993) 3141.
[45] GRAVES, H.C.B., Issues on standardization of immunoassays for prostate-specific antigen: a review, Clin. Invest. Med. **16** (1993) 415.
[46] STAMEY, T.A., Standardization of immunoassays for prostate-specific antigen: a different view based on experimental observations, Cancer **74** (1994) 1662.
[47] BANGHAM, D.R., COTES, P.M., Standardization and standards. Br. Med. Bull **30** (1974) 12.
[48] WORLD HEALTH ORGANIZATION, Technical Committee on Biological Standardization., Annex 4, Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances, WHO Technical Report Series 800, Fortieth Report (1990) 181.
[49] MARCOVINA, S.M., ALBERTS, J.J., DATI, F., et al., International federation of clinical chemistry standardization project for measurements of apolipoproteins A-I and B, Journal of the International Federation of Clinical Chemistry **3** (1991) 186.

[50] MURPHY, G.P., The second Stanford conference on international standardization of prostate-specific antigen assays, Cancer **75** (1995) 122.
[51] STAMEY, T.A., Second Stanford conference on international standardization of prostate-specific antigen immunoassays, 1–2 September 1994, Urology **45** (1995) 173.

2.5. INDIA

Title of the Project:	Development of Radioimmunometric Assays for the Measurement of Total and Free Prostate-Specific Antigen in Human Serum
Chief Scientific Investigator:	Meera Venkatesh
Scientific Co-Investigators:	Ketaki Bapat, Archana Mukherjee, Aruna Korde, Suresh Subramanian and M.R.A. Pillai

Abstract. Radioimmunometric assays were developed for the measurement of total and free PSA in human serum samples. Initially the reagents supplied were used to develop the assays. These were then used to compare and optimise the in-house reagents and assay systems. Standard PSA was prepared by purification from seminal plasma. Monoclonal antibodies were raised at our laboratory against PSA. For total PSA, IRMAs were optimized using the in-house reagents and compared with commercial kits as well as the IRMA system developed using the supplied authentic reagents. In the case of free PSA, the assay was optimized using the supplied reagents alone. The in-house t-PSA IRMA employed an in-house MAb (MAb2S) for capture, a matching commercial MAb (MAbJ2) for tracer, polystyrene tubes as the solid matrix and a single step assay at ambient temperature for 3 hours. The assay range was from 1 to 100 ng/mL and the sensitivity was 0.5 ng/mL. The in-house assay correlated well with the IRMA based on the authentic reagents supplied (MAb66 and MAb10, purified PSA) as well as commercial kits. Screened female serum was used as the sample matrix supplement in standards. In the case of f-PSA, MAb66 and MAb30 were used as the capture and tracer antibodies respectively. The assay the assay range was 0.5–20 ng/mL, had a sensitivity of 0.5 ng/mL and was carried out at 4°C for 18–24 h. 7.5% BSA in the assay buffer was used as the sample matrix supplement in the standards.

2.5.1. INTRODUCTION

Prostate specific antigen (PSA) is a well-established tumour marker with high specificity and sensitivity for prostate cancer [1–11]. The presence of PSA in serum in complexed form (predominantly PSA-ACT) and free form and the significant difference in their ratios in malignant and benign situations has led to the high diagnostic potential of free and total PSA values [12–23]. However, development of immunometric assays for total-PSA and free-PSA will require careful selection of the antibody pairs in order to avoid skewed response to the two forms of PSA [24–27]. Hence selection of appropriate pairs of monoclonal antibodies (MAbs) and validation of the assay are major steps in the development of these assays [28–32]. PSA, a serine protease, originally secreted into the seminal plasma in the free form, undergoes complexation only in serum. The concentrations of the complexing proteins such as ζ_1-antichymotrypsin and ζ_2-macroglobulin play a role in the proportions of free and bound form. The stability of the various forms in stored serum samples is hence poor as also the ratio of f/t. All these factors make thorough validation necessary before the assay procedures for PSA, both total and free, are used for clinical interpretations [33–36]. Availability of authentic key reagents will hence aid in a great way in the endeavour of development of these assays.

Development of IRMAs for total and free PSA was taken up at our laboratories and the supply of matched pairs of MAbs and pure PSA by Dr. M. Suresh, University of Alberta, Edmonton, Canada, as a part of the IAEA-CRP helped to realise this aim. In order to be self-sufficient in the long run, we attempted to purify PSA from seminal plasma and to raise suitable monoclonal antibodies against PSA, at our laboratory. The reagents supplied by the IAEA as well as our in-house reagents were used to develop IRMAs for t-PSA.

2.5.2. MATERIALS AND METHODS

Matched monoclonal antibodies, MAb66, MAb10 and MAb30, 70% pure PSA and affigel blue were supplied by Dr. M. Suresh, University of Alberta, Canada through the IAEA.

Partially pure PSA was a gift from the Institute of Research in Reproduction, Mumbai. The semen samples and a few analysed patient samples were obtained from Dr. Phadke's Pathology Laboratory, Mumbai. The analysed samples for PSA were obtained from "Tata Memorial Hospital", Mumbai.

All the chemicals/biochemicals needed for the hybridoma technique were purchased from the Sigma Chemical Company. Pure PSA for radioiodination was obtained from Scripps, USA. The inorganic salts and solvents were from SD Fine Chemicals, India or or Sarabhai Chemicals, India.

Detachable polystyrene wells – Immulon and "Star" polystyrene tubes were purchased from Nunc. Magnetic cellulose particles were from Scipac, UK.

Isotyping kit for antibody isotyping was purchased from Boehringer Mannheim.

The SP2/0 and Fox-NY myeloma cell lines were obtained from the National Centre for Cell Sciences, Pune, India. The animals were raised at our animal house and all animal experiments were carried out in compliance with the rules laid by the Animal Ethics Committee of our Insitute.

2.5.2.1. Standard PSA

PSA purified from seminal plasma and the PSA supplied by Dr. M. Suresh, were both tried as possible standard PSA materials. Partially purified PSA preparations were found to be of high potency, although not equivalent to the protein content. When tested on SDS-PAGE, these showed several bands of proteins. On purification by HPLC, single band in SDS-PAGE at molecular weight ~30,000 similar to a commercial preparation (Scripps) was obtained. But, the potency was far less (at least 100 times lesser) than that expected from the protein content. Based on reported methods [37,38], a modified method, briefly described in the Annex, was then adapted to purify PSA from seminal plasma.

Partially pure PSA preparations were used for immunizing mice (for monoclonal antibodies) while to obtain PSA for use as standards in the assay, native–PAGE of the partially purified fractions was carried out at preparative levels. The protein bands isolated, concentrated, lyophilised and stored at a minimum of 1 mg/mL protein level in tris buffer (0.1 M, pH 8). The PAGE purified preparations (bands with best potency) and the PSA supplied by the IAEA were explored for use as standards after estimating their potency using a commercial IRMA kit. PSA standard supplied by Dr. Suresh was reconstituted in 0.05 M phosphate buffer to a concentration of 1 mg/mL and stored in aliquots of 200 μg/vial in air-tight plastic vials. The stock standards were then used to prepare working standards at desired levels in an appropriate matrix (either female serum or 0.05 M phosphate buffer, pH 7.5, 7.5% BSA). Standards of concentrations 1, 2, 5, 10, 20, 50 and 100 ng/mL were used in the t-PSA assay while 0.5, 1, 2, 5, 10 and 20 ng/mL were used in f-PSA assays.

2.5.2.2. Anti-PSA monoclonal antibodies

Monoclonal antibodies were raised against PSA at our laboratory using the partially pure PSA preparation to immunise the BALB/c mice. The procedures followed were in the lines generally followed in hybridoma laboratories [39,40]. In brief, it was as follows:

a. Immunization — BALB/c mice were immunised with partially pure PSA at 50–70 μg primary injection followed by 50 μg booster doses at monthly intervals for 3–4 months through intraperitoneal route; the immunogen was emulsified 1:1 in Freund's complete adjuvant for the primary dose and Freund's incomplete adjuvant subsequently.
b. Fusion-fusion was carried out three days after the last booster. ~10^7 spelnocytes from the spleen of the immunised animal and 10^6 myeloma cells were fused using 30% PEG. Two fusions were carried out with Sp2/0 myeloma cells while one was tried with FOX-NY myeloma cells.
c. Selection — the fused hybridoma cells alone were preferentially grown in the nutrient media (DMEM with 10% FCS) by "HAT Selection"

d. Screening — for identification of antibody secreting wells; binding of the supernatant from the wells to ^{125}I-PSA as well as binding to anti-mouse antibody–ALP enzyme assay were both used to identify the cells secreting specific antibody
e. Clone selection and isolation — limiting dilution was followed to isolate clones derived from single cells. The wells that were positive for antibody production were propagated further at very low cell concentrations (average 0.3 cell/well) to isolate clones. Sturdy clones that were antibody secreting over a long period of time were selected.
f. Antibody production and purification — the isolated specific antibody producing clones were grown to confluence, the cells separated and injected intra-peritoneally into BALB/c mice to grow ascites. The ascitic fluid rich in MAb was treated to purify the antibodies (by caprylic acid precipitation, ammonium sulphate precipitation and dialysis), concentrated and stored for further studies. These MAbs were characterised for their isotype using the isotyping kit.

The usability of the in-house MAbs as capture antibody or as tracer antibody was tested by comparison with the supplied antibodies, namely MAb66 and MAb10. All the antibodies to be tested including MAb66 were coated on tube surfaces and checked for binding using MAb10 tracer. In order to have a kit formulation at a later stage, commercial antibodies were also tested. Similarly, tracers were made using the in-house MAbs and tested for binding on MAb66 coated surfaces.

2.5.2.3. ^{125}I-labelled radiotracers

MAb10 and MAb30 supplied by Dr. Suresh and the in-house monoclonal antibodies were labelled with ^{125}I using Chloramine-T as the oxidant. Tracers of specific activities ranging from 222 to 444 kBq/μg (6–12 μCi/μg) were prepared. In brief, to 10 μg (5 μL) of the MAb in 30 μL 0.5 M phosphate buffer (pH 7.5), ~11–22 MBq (300–600 μCi) ^{125}INa and 10 μg Chloramine-T (10 μL) were added and mixed for 60 s. The reaction was stopped by addition of 112.5 μg (15 μL) sodiummetabisulfite. The labeled product was purified by gel filtration using Sephadex G-75 (1×30 cm). The elution was carried out with 0.05 M phosphate buffer (pH 7.5) containing 0.1% BSA. The tracer fraction was collected and stored under refrigeration after appropriate dilution with the same buffer. Percentage iodination yield and purity of the fraction were estimated by paper electrophoresis. Pure PSA from Scripps, USA was radioiodinated in a similar way and used for screening the antibody secreting clones.

The tracers prepared were tested for use in IRMAs with either MAb66 or in-house MAbs. For this, the potential tracer MAbs were individually added to the capture MAb coated tubes along with standard PSA at two levels of concentration (low and high), incubated as in an assay and tested for binding. The tracers were studied for their stability with time by following their assay performance.

2.5.2.4. Solid matrix for coating capture MAb

Polystyrene tubes, wells and magnetic cellulose were tried as the possible solid matrices for coupling the capture MAb, MAb66.

Magnetic cellulose that was existing in our laboratories was used for these studies. 200 mg of particles in 5 mL suspension was activated with carbonyl diimidazole, coupled to MAb66 (in bicarbonate buffer, 18 h, R.T.) and then blocked with Glycine. The detailed procedure given in the earlier IAEA-TECDOC (No. 914, Nov. 1996) on this subject was followed. The coated magnetic cellulose particles were stored as suspension at 2.5 mg/mL in 0.05 M phosphate buffer. The effect of amount of antibody coated cellulose on the binding at different PSA levels and the variation due to the presence of serum were studied to test its usability in regular assays.

Immulon wells, plain polystyrene tubes and ‘star tubes’ (with larger area) were tested for coating efficiency and stability. Initially both types of tubes were coated by:

a. passive coating, wherein ~ 1 μg MAb/tube was added in 0.1 M bicarbonate buffer, pH 8.5 and left over night; and
b. chemical coupling after activating the polymer surface with gluteraldehyde. In this case, the tubes were treated with 2.5% gluteraldehyde in bicarbonate buffer for 18 h at 4°C followed by 1 μg MAb solution in bicarbonate buffer for 18 h at 4°C.

In both the cases, the tubes were blocked with 1% BSA (in 0.05 M phosphate buffer, pH 8.5) for 3–18 h at 4°C. After each step the tubes were washed with buffer containing 0.1% Triton X-100 in 0.05 M phosphate buffer, pH 7.5 and stored cold in airtight bags. Passive coating was followed for optimization of the assay. Coating with bicarbonate buffer as well as 0.1 M phosphate buffer, pH 8.5 were tried and compared. The immulon wells were also coated in exactly the same way as the tubes by passive adsorption. The coated tubes/wells were tested periodically for binding and sensitivity of the assay. The effect of glazing with sucrose, storage under vacuum etc. on the stability of the coated surface were studied.

2.5.2.5. Optimization and validation of the total-PSA IRMA

Six in-house MAbs were tested as capture antibody. The MAbs which matched MAb10 tracer, were further used to develop assay systems. The supplied reagents MAb66 and MAb10 and the in-house reagents were used and optimized for obtaining feasible t-PSA IRMA procedures. Initially a t-PSA IRMA was optimised using MAb66 and MAb10 tracer. This system was compared with a commercial IRMA kit (IZOTOP, Hungary) and was then in turn used for comparing different systems developed using in-house reagents.

The effect of amount of capture MAb, tracer MAb, reaction temperature, time, assay buffer composition etc. on the assay performance were studied for each system. The various standards obtained were also compared for their performance in the assays. In particular, an IRMA system employing the in-house MAb2S (as capture MAb) and a commercial matched MAbJ2 (as tracer MAb) were thoroughly optimised with respect to amount of coating antibody, amount of tracer added, solid matrix used, reaction time, temperature and pH etc. with an aim to supply kits. Typically, the t-PSA IRMAs were carried out at ambient temperature (~25°C) for 3 h in a single step employing 100 μL sample/standard. Screened female serum with PSA values below detectable limits was used as the matrix for standards.

Several samples were obtained from the local cancer hospital and a pathology laboratory which had analysed them for t-PSA levels by ELISA. The PSA levels in these samples were estimated using the optimised systems were as well as a commercial IRMA kit. The sample values obtained by the various IRMAs were then compared with each other.

2.5.2.6. Optimization of the free-PSA IRMA

The supplied MAbs, MAb66 and MAb30 could alone be used for development of an IRMA for free-PSA assay, since we could not succeed in obtaining a matching MAb for use as tracer to our in-house MAbs. As in the case of t-PSA IRMA, the various assay parameters were optimised for the f-PSA using the supplied matched MAbs. Star tubes were coated with MAb66 at 0.5, 1, 2 and 5 μg/400 μL 0.1 M phosphate buffer/tube as detailed in an earlier section, to study the effect of amount of MAb used for coating. Tracers prepared at two different specific activities (222 and 444 kBq/μg or 6 and 12 μCi/μg) were used in different amounts in the f-PSA IRMA to study the effect on sensitivity. The reaction time, temperature, pH, effect of two-step assay were also studied.

Various matrices were tested as standard matrix equivalent to the sample matrix, which is human serum. These included, screened female serum, bovine serum, control processed serum replacement (CPSR — a serum like synthetic supplement supplied by Sigma Chemicals Company) and 0.05 M phosphate buffer, pH 7.5 containing 7.5% BSA or 4.5% BSA+1.5% BGG. The effect on binding and the minimum detectable dose were used for comparing these matrices. Apart from this,

female serum and 7.5% BSA in buffer were used for preparing a couple of standards and given for analysis at a pathology laboratory, using Chemiluminescent Immunoassay (CLIA) method. Samples from patients that have been analysed for f-PSA levels by ELISA or CLIA were obtained from the local pathology laboratories. These samples were analysed by the developed IRMA system for checking the correlation.

2.5.3. RESULTS AND DISCUSSION

2.5.3.1. Standard PSA

The PSA obtained on purification from the seminal plasma could be calibrated against the supplied PSA and used as standards in assays. Standard curves constructed for t-PSA IRMAs using these standards is depicted in Figure 1. It is seen that the supplied 70% pure PSA calibrated standard and the in-house purified PSA preparation have yielded similar results, thus proving the usability of the in-house preparation in further assays for t-PSA.

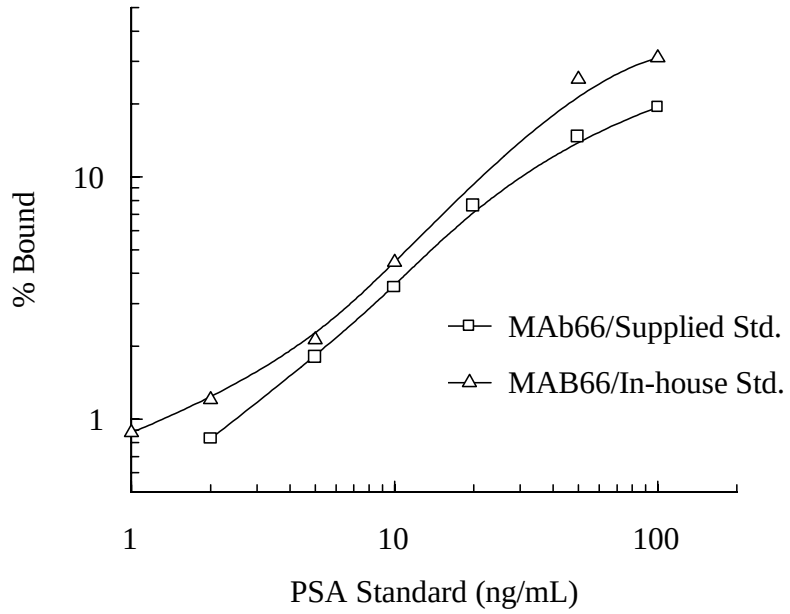

FIG. 1. Comparison of in-house Standard with the Supplied Authentic Standard, in t-PSA IRMA.

However, at each step of purification, the yield dropped considerably. Particularly after the last step of purification through affigel blue, the drop has been very steep as shown in Table I. This perhaps indicates heavy losses during washing with lower salt buffers and hence would benefit from modification that could prevent the loss but still purify PSA. Hence, affinity purification by anti-PSA MAb bound solid phase columns is planned in collaboration with a Chemical Technology Teaching Institute which has the know-how for a novel material for large through put of samples.

2.5.3.2. Anti-PSA Monoclonal Antibodies

Of the three attempts to raise monoclonal antibodies against PSA, the first one did not yield any useful clone though there were several growth positive clones. The second time, a large number of growth positive and immunoreactive (binding ^{125}I-PSA and anti-mouse antibody–ALP) wells were present. The best among them, 4A6, exhibiting high binding with ^{125}I-PSA (55%) was used for limiting dilution to pick single clones. There were 79 positive single clones which were grown to confluence and frozen for further studies. Of these, six sturdy ones with good immune binding were grown as ascites in mice. The third attempt of using FOX-NY myeloma did not yield any positive clones.

TABLE I. RECOVERY OF PSA ON PURIFICATION FROM SEMINAL PLASMA

Sample	Recovery Yield	PSA Concentration
1. Seminal plasma	-	4.7×10^2 mg /mL
2. Sample after CM-Sephadex chromatography	56%	1.7×10 mg/mL
3. Sample after ammonium sulfate fractionation	11%	1.8×10 mg/mL
4. Sample after Affigel Chromatography	<1%	6 μg/mL

The usability of in-house MAbs was tested by comparison with the supplied antibodies, namely MAb66 and MAb10. The following Table II shows the binding of MAb 10 tracer to the tubes coated with the six selected MAbs at two different levels of PSA as standards. It is observed that except MAb1B2 the rest five are responding to the changes in PSA concentration. But, the extents of response are different. Among these, MAb19 and MAb2S are comparable to MAb66 and hence were pursued further for coating as capture antibody. All these MAbs on isotyping were found to be of class IgG_1 with kappa chains (IgG_1, ρ).

Although several positive clones were isolated, we could not succeed in getting a matched pairs for free or total PSA measurement. On testing various commercially available MAbs, one designated MAbJ2 could be identified for use as tracer with MAb2S (as the capture antibody). This pair yielded an assay very similar to the assay developed with the IAEA supplied reagents. Figure 2 shows the standard curves of the IRMAs constructed using the MAb66+MAb10 combination, MAb2S+MAb10 pair and the MAb2S+MAbJ2 pair. It can be seen that the assay systems are in close correlation with each other.

TABLE II. TESTING OF THE SUITABILITY OF THE IN-HOUSE MAbS FOR CAPTURE

	% B/T (PSA conc. ng/mL)	
MAb used	10 ng/mL	200 ng/mL
MAb66-supplied	4.7	33
MAb19	*5.5*	*33*
MAb2S	*3.3*	*27*
MAb17	9.6	25
MAb18.3	8.5	22
MAb18.1	2.3	19
MAb1B2	0.8	3.6

The antibodies from CGEB, Cuba were also tested for similar purpose. Although none of the MAbs could be used with our in-house antibodies, they could yield a good assay system between themselves and would be an alternate supply of MAb reagents for total PSA assay.

2.5.3.3. ^{125}I-labelled radiotracers

Typically 50% radioiodination yield was realised yielding specific activities around 400–500 kBq/μg (12–15 μCi/μg). Non-specific binding was < 1% and the tracer was stable for at least 30 days when stored at 4° C. However, the radioiodinated PSA prepared for screening the antibody secreting clones using pure PSA from Scripps, USA gave poor yields and poor quality tracer that was very unstable (<10 days). These tracers were used immediately after preparation for screening. A typical elution profile of radioiodinated MAb is given in Figure 3.

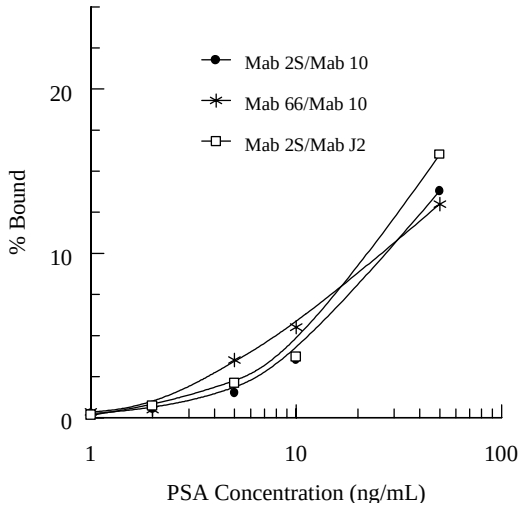

FIG. 2. Comparison of in-house t-PSA IRMAs (MAb2S+MAbJ2 and MAb2S+MAb10) with authentic system (MAb66+MAb10).

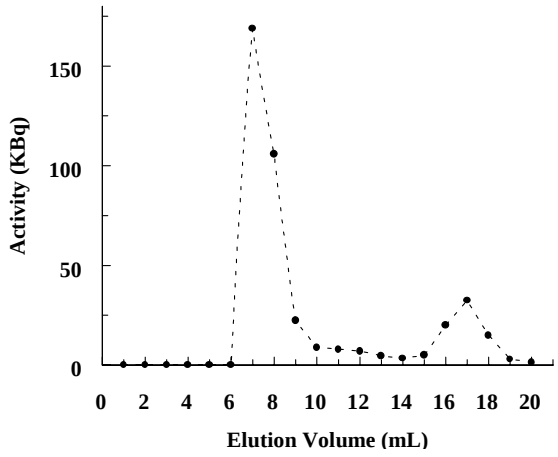

FIG. 3. Typical Elution Pattern of the ^{125}I-MAb on purification through Sephadex G-75 column.

The studies with labelled MAbs to identify a suitable MAb for use as tracer with the in-house MAb2S failed to provide any positive result. None of the in-house MAb tracers bound to the tubes coated with MAb66 significantly. Hence, radioiodinated MAb 10 was used as the tracer for our development studies. To test the retention of immunoreactivity after radioiodination of antibodies, these were reacted with PSA in MAb10 coated tubes. Tracers made from all the antibodies except 1B2 bound to the tubes proving that the radioiodination was not damaging the molecules or hampering their immunoreactivity.

2.5.3.4. Solid matrix for coating capture MAb

The effect of amount of MAb coupled cellulose on the binding at different PSA levels is shown in Table-III. The variation due to the presence of serum is also depicted in the same Table. It was observed that the although MAb coupled magnetic cellulose particles could be used in the assay, the amount needed per tube was high and far more antibody had to be spent compared to the coated tubes. It is presumed that the efficiency was poor, perhaps due to the age of the particles (they formed clumps and settled very rapidly) which has in effect led to the poor economy of the antibodies. The precision is generally better than the coated tubes (<5% C.V. in our assay and ~2% C.V. with IZOTOP kit also based on magnetic cellulose separation). However, the addition of serum had pronounced effect on binding when the magnetic particles were used while in the case of tubes the binding decreased marginally by 1 or 2%.

In the initial studies with tubes, contrary to our expectations, the chemical coupling of antibodies did not give encouraging results. The larger surfaced star tubes also failed to show any advantage over the plain tubes both in passive coating and chemical coupling. Passive coating was successful and gave reproducible results. Hence, in all the optimization experiments, tubes (either plain or star) coated by passive method were used. Table IV illustrates the results obtained with the antibody coating of tubes

It was observed that the amount of MAb used did not alter the standard curve significantly when 1, 2 or 5 μg of MAb was used for coating, when the tubes were freshly coated. This is depicted in Table V. But 0.5 μg of MAb resulted in poorer binding. Glazing with 20% sucrose at the end of all washing, thorough drying and storing in airtight containers after evacuation improved the stability of the coating to a large extent. Unglazed tubes deteriorated progressively and could not be used beyond 2 weeks while glazed tubes could be used for several weeks. With glazing and proper storage, even at 1 μg/tube the tubes performed well and could be used for several weeks. Between the three choices studied for coating the capture antibody, namely, Star tubes, Immulon wells and plain tubes, the Immulon wells gave high precision (<5% C.V. inter assay) and good binding of MAbs and resulted in standard curves very similar to those with Star tubes. But, these suffered from poor stability and difficulties in counting since they have to be broken from the strip at the right site and placed in a numbered tube before counting. The plain tubes exhibited lower precision and poorer stability on storage. Despite glazing with sucrose and proper storage, these could not be used as long as the Star tubes. Moreover, the coating efficiency varied with the Mab used for plain tubes. MAb66 coated well on plain tubes as seen in Table IV while MAb2S fared poorly. Thus finally for both t-PSA and f-PSA assays, Star tubes were used.

2.5.3.5. Optimization and Validation of the total-PSA IRMA

IRMAs could be optimized using the supplied reagents (MAb66+MAb10) and with MAb19 or 2S (in-house MAbs) + MAb10. In each case, assays were developed with the in-house PSA, the supplied PSA and the standards from a commercial kit (IZOTOP, Hungary). On optimization of the reaction temperature, it was found that at 37°C, the binding and the sensitivity were poor. When the assays were carried out at ambient temperature of ~25°C for 3 h and at 4°C for 18 h, the performances were nearly identical. Hence, for further optimization, assays were carried out for 3 h at ambient temperature (25°C) using 100 μL serum sample, 100 μL tracer antibody and 300 μL PBS buffer (pH 7.5), 0.1% BSA. A typical set of results on the tracer amount optimization using MAb10 tracer is shown in Table VI. Based on these results, 15 ng/tube of MAb10 tracer was used in most experiments, to obtain reasonable lowest detection limit and range.

In order to optimise the amount of tracer and have adequate count rate, MabJ2 tracers were prepared at high and low specific activities when MAb2S+MabJ2 based in-house t-PSA IRMA was optimised. Predictably, lower amount of tracer showed better sensitivity. Hence high specific activity tracer (400–500 KBq/μg) was preferred so as to have adequate activity per tube and tracers were used at ~10 ng/tube in the assay. The assays developed at our laboratory had sensitivity of ~0.5–1 ng/mL. Typical standard curves obtained using different standards and different antibody combinations are shown in the figures 4 and 5.

Standard curves obtained with MAb66 and some of the in-house antibodies were similar. MAb2S is depicted here as an example. The standard curves seem reasonably close suggesting usability of the in-house standards in MAb66+MAb10 system as well as in-house MAb2S+MAb10 or MabJ2 systems. However, there were differences in response between the commercial standards we used and the in-house standards as well as the supplied standards. These differences were seen with all capture MAbs; namely MAb66, MAb2S and the commercial kit. This led to a dilemma about the

TABLE III. EFFECT OF THE AMOUNT OF MAb USED ON THE BINDING IN t-PSA IRMA BASED ON MAGNETIC PARTICLES

Amount of MAb* (μg)	% B/T at different PSA Levels (ng/mL)				
	1	2	5	50	100
Binding without serum					
1	1.2		5.0	14.0	
2.5	1.3		6.0	22.0	
5	1.7		7.0	26.0	
Binding with addition of 100 μL PSA free human female serum					
2.5		0.4	1.3	8.6	11.0
7.5		1.2	2.3	9.7	13.4

*This refers to the amount of antibody(MAb 2S) spent and not the amount actually present in the particles; 15 ng/80,000 cpm tracer MAb10, reaction 3 h, R.T.

TABLE IV. COUPLING OF MAb66 TO POLYSTYRENE TUBES

	% B/T			
PSA (ng/mL)	2	10	50	100
2.1.3.2. Plain Tubes				
Physical Adsorption	0.9	2.3	11.7	19.1
Active Coating	Negligible binding in all tubes			
Star Tubes				
Physical Adsorption	0.7	2.0	12.3	18.9
Active Coating	Negligible binding in all tubes			

* Tracer ~ 15 ng/80,000 cpm, 3h reaction, R.T.

TABLE V. EFFECT OF THE AMOUNT OF CAPTURE MAb ON BINDING IN t-PSA IRMA BASED ON COATED TUBES

MAb2S amount	% B/T at different PSA levels (ng/mL)				
μg/tube	1.5	5	10	50	100
0.5	0.4	1.5	3.3	9.4	9.5
1	0.3	3.5	8.6	23.5	25
2	0.6	3.3	7.1	19	22
5	0.8	3.8	8.3	25	27

* Tracer ~ 15 ng/80,000 cpm, 3 h reaction, R.T.

TABLE VI. EFFECT OF THE TRACER AMOUNT ON BINDING IN t-PSA IRMA

^{125}I-MAb10	% B/T at different PSA levels				
ng/tube	1	5	10	50	100
3.2	1.9	12.8	22.8	31.2	33.0
7.5	2.4	11.5	22.0	30.0	30.0
15	1.3	6.1	11.4	24.4	28.0
30	0.5	1.7	4.0	13.2	14.7

In-house standards, MAb 19 capture MAb (2 μg/tube), reaction 3 h, R.T.

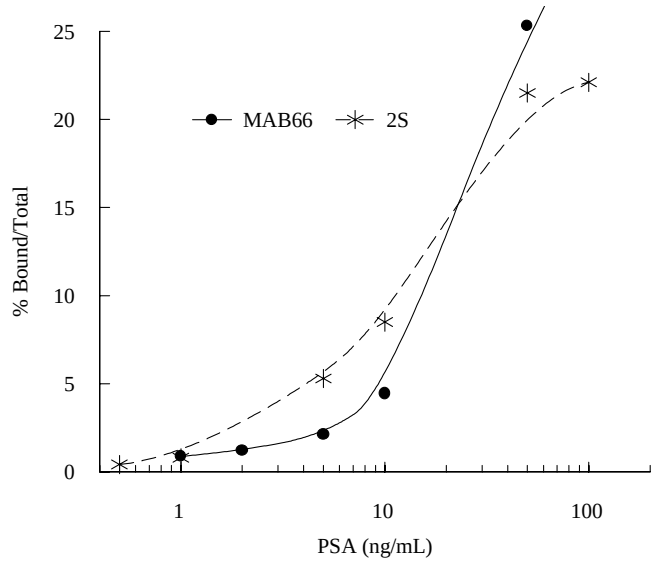

FIG. 4. Comparison of t-PSA IRMA Standard Curves using in-house standards with authentic MAb66 and in-house MAb2S.

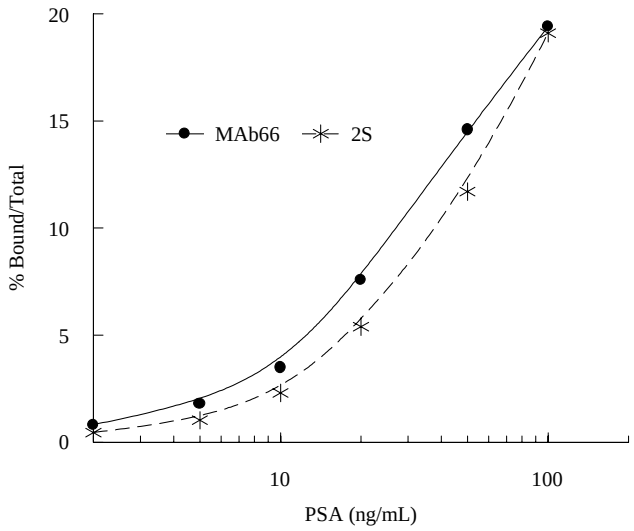

FIG. 5. Comparison of t-PSA IRMA Standard Curves using authentic supplied standards with authentic MAb66 and in-house MAb2S.

standards' actual potency. Hence the supplied standard (Dr. Suresh) was compared with two other commercial kit standards (DPC and DSL). On performing assays with these three sets of standards, it was found that the standards from DPC and DSL were closer to the standards supplied by Dr Suresh and hence in turn our own in-house standards. These results lay emphasis on the need for proper calibration of the standards. Calibration at just one concentration level will be insufficient and any skew in the response at higher PSA concentrations may go unnoticed. The problem of standard preparation, assay calibration, non-matching results between different kits etc. have been addressed by many [41–47]. Although the standard curves for t-PSA IRMA systems using various combinations of matched MAbs were obtained with reasonable efforts, the sample analyses results did not always match with that supplied by the hospital (ELISA method). This was especially so in the case of samples having very high PSA values (which could perhaps be attributed to “hook effect”). Even the standards from different kits did not match perfectly as mentioned above. Table VII lists a set of such analyses as a representative.

TABLE VII. TYPICAL t-PSA VALUES (ng/mL) IN PATIENT SAMPLES OBTAINED USING DIFFERENT ASSAY SYSTEMS

	Hosp. Values	Commercial	Coated tubes, MAb 10 tracer		
Sample no:	ELISA kit	IRMA-mag. Cell.	MAb 66	MAb 19	MAb 2S
	A	B	C	D	E
1	1.1	0.32	2.0	2.0	4.0
2	1.5	1.8	2.0	5.0	3.5
3	2.3	2.8	1.8	2.0	2.2
4	3.0	3.0	2.0	2.0	2.0
5	3.5	5.0	4.0	2.0	5.5
6	7.0	4.0	1.8	2.0	2.0
7	11.5	55.0	60.0	26.0	19.0
8	16.0	24.0	10.0	2.0	3.0
9	34.2	35.0	10.0	8.0	7.5
10	450	17.0	70.0	26.0	36.0

The correlations of the sample values between the various sets (**A** to **E**) is listed below in trems of correlation coefficient 'ν'. Correlation between set A and set B = 0.67 (0.97 barring the samples 7 and 10); between A and C = 0.74; A and D = 0.67; A and E = 0.89. Between B and C = 0.88; B and D = 0.87; B and E = 0.87; C and D = 0.98; C and E = 0.95; D and E = 0.92. Although the correlation with the supplied values (Hospital, ELISA kit) was not very encouraging, barring a few sample results (sample no.s 7 and/or 10), the results correlated reasonably well (ν ~0.8–0.9). Between the IRMA systems developed at our laboratory, using MAb66, MAb2S and MAb19 as capture MAbs, with MAb10 as tracer, the co-relation was very good (0.92–0.98). The IRMA system developed later with MAb2S+MabJ2 tracer combination also correlates well with both MAb66+MAb10 and MAb2S+MAb10 t-PSA IRMAs as seen in Figure 2 earlier. For sample analyses, female serum was found to be suitable non-interfering matrix substitute in standard tubes.

All the IRMAs for t-PSA developed were finally optimised with 3 h reaction at ambient temperature (~25°C) with coated tubes (either plain or star at 1 μg MAb /tube), 100 μL sample volume, 0.05 M phosphate buffer, pH 7.5 with 0.1% BSA, female serum matrix for standards. Generally, the sample values estimated by the developed assays were within 10% of the values estimated in an independent pathology laboratory using CLIA/EIA method and were associated with 4–5% intra-assay C.V. and ~ 10% inter assay C.V. These assays exhibited parallelism of samples with standards and total recovery between 90 to 110% of the added standard values.

2.5.3.6. Optimization of the free-PSA IRMA

In the case of free-PSA IRMA, a suitable matching MAb for use of tracer with any of the in-house MAbs could not be identified and hence the f-PSA IRMA was optimised using the supplied matched MAbs, namely MAb66 and MAb30. On studying the effect of reaction pH on binding it was found that pH 7.5 is best suited among pH 6, 7.5 and 8.5 both in terms of sensitivity and in terms of binding percentage. 0.05 M Phosphate buffer, pH 7.5 with 0.1% BSA was used as the assay buffer.

The effect of amount of capture MAb for coating the tubes is shown in Figure 6. It is seen that both 1 and 2 μg MAb were suitable for coating. Hence for most experiments the star tubes were coated with 1 μg MAb 66.

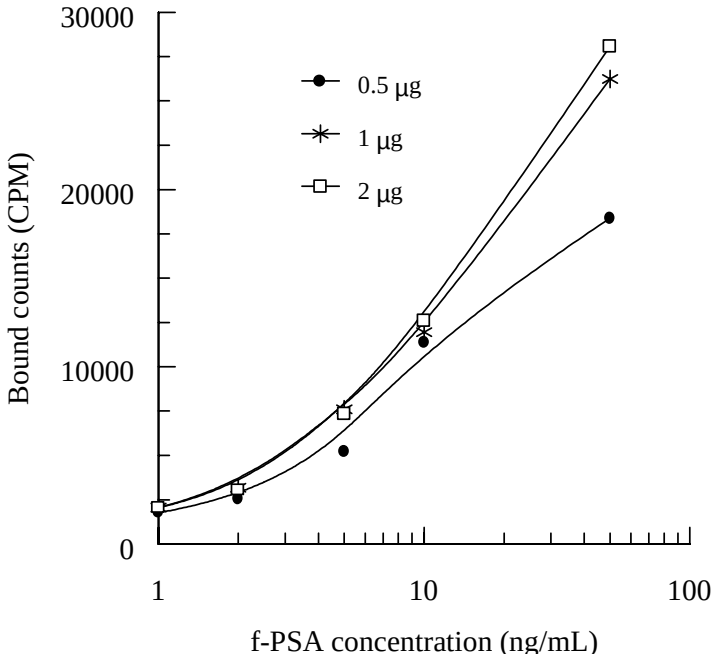

FIG. 6. Effect of the amount of capture MAb66 in the f-PSA IRMA.

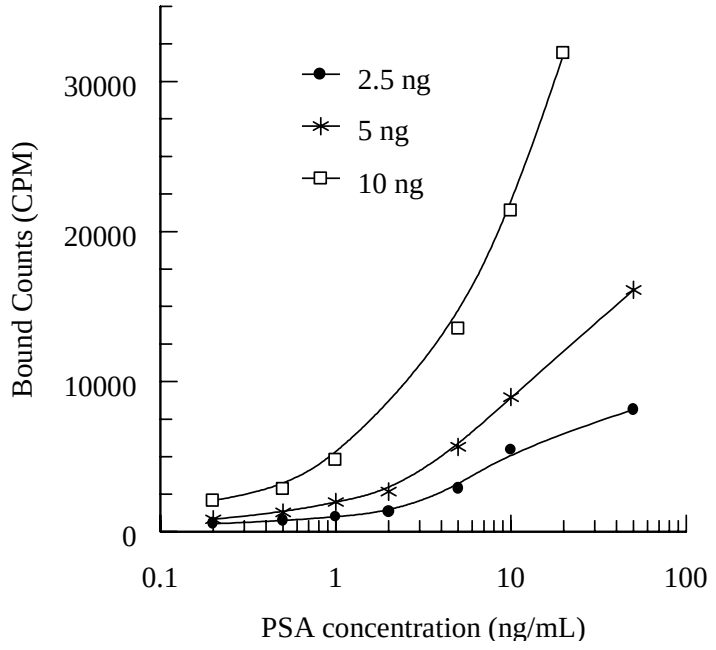

FIG. 7. f-PSA IRMA Standard Curves at different tracer amounts (MAb66+MAb30 tracer).

Tracers of both high and low specific activities were used to optimise the amount of tracer MAb30 added. The results of tracer amount optimization is shown in Figure 7. It was observed that the low end sensitivity did not improve on using low amount of tracer, perhaps due to the inherent limitation imposed by the MAb affinity constant. In this case the low end sensitivity was about 0.5 ng/mL. However, the% binding was better at lower amounts of tracer, and hence high specific activity (~400–500 MBq/µg) tracer was preferred at levels ~ 10 ng/tube.

TABLE VIII. EFFECT OF REACTION TIME AND TEMPERATURE (f-PSA IRMA)

% Binding in the assay at 2 low PSA concentrations					
PSA (ng/mL)	2 h, 37°C	3 h, 37°C	2 h, 25°C	4 h, 25°C	18 h, 4°C
0.1	1.9	3.5	3.6	3.8	3.3
1	2.1	3.5	4.3	4.5	5.8

The effect of reaction temperature and time was studied by estimating the binding at two different PSA levels. The results are depicted as% bound activity in the Table VIII. It was observed that higher temperature did not result in better binding. In 2 h, the binding at room temperature was better than at 37°C. Over night incubation at 4°C resulted in the best sensitivity at the low end and hence was followed.

Since free PSA is known to bind to a variety of proteins and interference from different serum supplements should be expected, the matrix for preparation of standards for assay was one of important issues. A variety of serum supplements were tried based on experience. The performance of the f-PSA IRMA when the various matrices were tried as the serum substitute in the standards is shown in Figure 8. It can be seen that bovine serum, synthetic serum supplement CPSR, a combination of bovine gamma globulin and bovine serum albumin (1.5% BGG + 4.5% BSA) have all yielded very poor sensitivity as well as binding. Female serum was better, but 7.5% BSA in assay buffer was the best among these. The cause for interference in the case of other matrices was not explored.

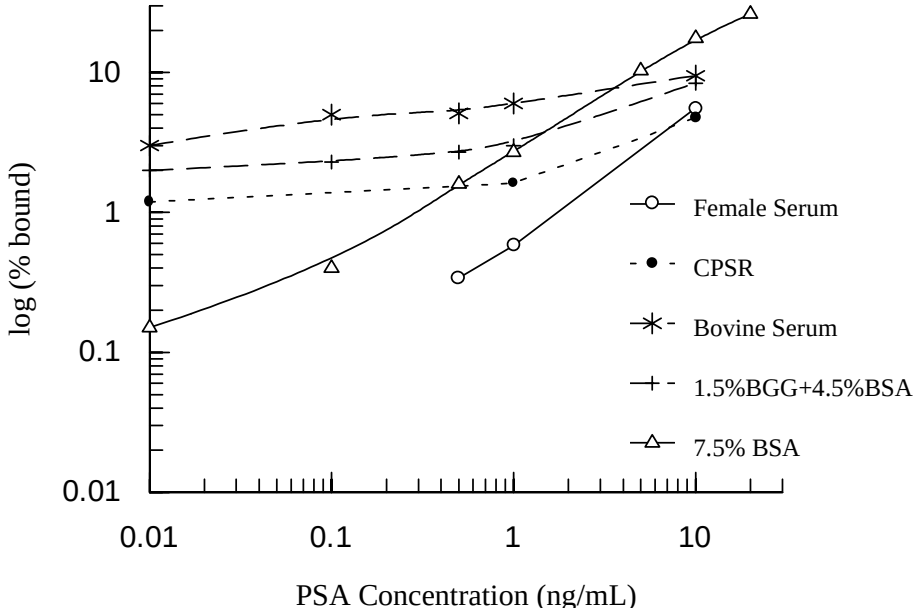

FIG. 8. Effect of standard matrix on the f-PSA IRMA performance.

In the case of free-PSA, the binding of free-PSA to the binding proteins would be a serious problem when pure free PSA is used in standards. Particularly, when the standards are stored in female matrix or when the incubation time is long enough to allow serum protein binding to compete with the immune binding of the assay, there could be errors. Use of 90% PSA-ACT + 10% free PSA is advocated for closeness to the actual situation in serum samples [48]. However, the situation could be non-ideal in pathological samples with very different ratios of bound and free PSA (which would be the real critical samples where f-PSA levels will matter for clinical decisions). Thus, preparation of standards for f-PSA assays is a tricky issue. PSA in female serum that has reached an equilibrium with binding proteins with only a small fraction as f-PSA assay could be perhaps used as standards after

proper calibration. But, here too the reports of f-PSA levels not being stable in patient serum [45,46] and the requirement of analysis immediately after collection of samples, would cause concern in preparation of standards in serum matrix. As an independent check the standards prepared in few matrices were analysed in a pathology laboratory employing CLIA. These results are given in Table IX. It is observed that the values are underestimated in assay buffer alone while over estimated in normal female serum, by the CLIA. Among these, 7.5% BSA appears to be the best supplement yielding values very close to the expected values. Also, as seen in Figure 8, the response is also better than the other matrices. Hence f-PSA standards were prepared in 7.5% BSA in buffer. The selection of the ideal matrix supplement is particularly intriguing in the case of free PSA assay since, on repeated analysis of the same sample in the course of one month with a commercial kit, the estimated values kept varying pointing to the instability of stored samples. Although the instability of serum samples for PSA assays in general has been addressed by many, the situation is more grave in the case of free PSA [49–51].

TABLE IX. MATRIX EFFECT ON STANDARD CONCENTRATION OF f-PSA

Expected value of PSA (ng/mL)	Matrix used		
	Assay buffer (PO_4^{---}, 0.05M, 7.5)	Normal female serum	7.5% BSA in 0.05M PO_4^{---}, 7.5
1.0	0.93	1.28	1.13
10.0	8.71	11.89	11.05

Finally, the free-PSA IRMA developed employed MAb66 for capture (1 or 2 μg/tube in star tubes), MAb30 tracer (at 2–5 ng/tube; 400–500 MBq/μg), standards in PBS with 7.5% BSA, 100 μL samples and was carried out at 4°C for 18 h. This IRMA developed was found to have intra-assay variation of ~5% and inter-assay variation of ~10–12%.

2.5.4. CONCLUSION

The following are the findings as a result of this co-ordinated research project.

1. An IRMA system could be optimized with the supplied key reagents as well as in-house key reagents to measure total PSA in human sera. The system using an in-house MAb2S and commercial matched tracer MabJ2 is envisaged for kit formulation and supply to local users. The developed t-PSA assays had ~ 0.5 ng/mL sensitivity with 5–10% variations.

2. An IRMA system could be optimized with the supplied MAbs to measure free PSA in human sera. The developed f-PSA assays had ~ 0.5 ng/mL sensitivity with 5–12% variations.

3. Standard PSA preparation is an important problem to be addressed as suggested by the discrepancies (both sporadic and regular) in the sample values obtained, particularly in the case of free PSA.

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ANNEX: PSA PURIFICATION FROM SEMINAL PLASMA

Pooled seminal plasma was centrifuged, dialysed against 24 hr against 200 volumes of 0.05 M phosphate-citrate buffer, pH 6.4 with 0.1 mM PMSF (Phenyl Methyl Sulphonyl Fluoride) and centrifuged again at 6000 RPM for 1 h to remove all the particulate matter.

The clear plasma was passed through CM Sephadex column (2.5 × 25 cm) pre-packed and equilibrated with 0.01 M phosphate buffer pH 8.0. The column was washed with equilibrating buffer (10 mL fractions) till O.D was 0.0 at 280 nm.

The column was then eluted with 0.1 M Potassium phosphate, 0.2 M NaCl, pH 6.9 and fractions of 5 mL each were collected till the OD at 280 nm was 0. Protein peak was obtained from 13^{th} to 16^{th} fractions.

The column was then washed with 0.1 M Potassium phosphate + 0. 5 M NaCl, pH 6.9 to elute all the other proteins. 10 mL fractions were collected till O.D was 0.0 at 280 nm. PSA rich fractions were then pooled and the PSA content was estimated. PSA was precipitated by 30 –75% ammonium sulphate fractionation, dissolved in 0.01M Tris HCl buffer pH 7.8 and dialysed against 3 × 2 litres of the same buffer in 24 h. The dialysate was centrifuged and the supernatant was retained.

This preparation was then loaded on a Cibacron blue affigel column (5mL, 1.6 × 10 cm) and equilibrated with 0.02 M Tris, 0.075M NaCl till O.D was 0.035 at 280nm. The unwanted proteins were washed away progressively with 0.02 M Tris-NaCl buffers, pH 8 with increasing NaCl concentrations –0.1, 0.3 and 0.5 M NaCl till O.D was 0.0 at 280 nm after each washing. PSA was finally eluted with 0.02 M Tris-0.75 M NaCl, pH 8 buffer till the OD at 280 nm reached 0. The PSA content after each stage was estimated.

REFERENCES

[1] OESTERLING, J.E., PSA: a critical assessment of the most useful marker for adenocarcinoma of the prostate, J. of Urol. **145** (1991) 907.

[2] BANGMA, C.H., BLIJENBERG, B.G., SCHRODER, F.H., Prostate-specific antigen: Its clinical use and applications in screening for prostate cancer, Scandinavian Journal of Clinical and Laboratory Investigation **55**-SUPPL. 221 (1995) 35–44.

[3] WOOLF, S.H., Screening for prostate cancer with prostate-specific antigen: An examination of the evidence, New England Journal of Medicine **333** (1995) 1401–1405.

[4] IMAI, K., ICHINOSE, Y., KUBOTA, Y., YAMANAKA, H., SATO, J., Diagnostic significance of prostate specific antigen and the development of a mass screening system for prostate cancer, Journal of Urology **154** (1995) 1085–1089.

[5] GARNICK, M.B., FAIR, W.R., Prostate cancer: Emerging concepts: Part I & II, Annals of Internal Medicine **125** (1996) 118–125; 205–211.

[6] FILELLA, X., MOLINA, R., BALLEST, A.M., GIL, M.J., ALLEPUZ, C., et al., Value of PSA in the detection of prostate cancer in patients with urological symptoms. Results of a Multicentre Study, European Journal of Cancer **32A** (1996) 1125–1128.

[7] WOLFF, J.M., BARES, R., JUNG, P.K., BUELL, U., JAKSE, G., PSA as a marker of bone metastasis in patients with prostate cancer, Urologia Internationalis **56** (1996) 169–173.

[8] GLEAVE, M.E., COUPLAND, D., DRACHENBERG, D., COHEN, L., KWONG, S., et al., Ability of serum prostate-specific antigen levels to predict normal bone scans in patients with newly diagnosed prostate cancer, Urology **47** (1996) 708–712.

[9] REISSIGL, A., BARTSCH, G., PSA as a screening test, Urol. Clin. of N. Amer. **24** (1997) 315–21.

[10] BECKER, C., LILJA, H., Individual prostate-specific antigen (PSA) forms as prostate tumor markers, Clin. Chem-Acta. **257** (1997) 117–32.

[11] ROSS, K.S., CARTER, H.B., PEARSON, J.D., GUESS, H.A., Comparative efficiency of prostate-specific antigen screening strategies for prostate cancer detection, JAMA **284** (2000) 1399–405.
[12] PRESTIGIACOMO, A.F, STAMEY, T, A, Clinical usefulness of free and complexes PSA, Scandinavian Journal of Clinical and Laboratory Investigation **55**-SUPPL. **221** (1995) 32–34.
[13] FROSCHERMAIER, S.E., PILARSKY, C.P., WIRTH, M.P., Clinical significance of the determination of noncomplexed prostate-specific antigen as a marker for prostate carcinoma, Urology **47** (1996) 525–528.
[14] REISSIGL, A., KLOCKER, H., POINTNER, J., et al., Usefulness of the ratio free/total PSA in addition to total PSA levels in prostate cancer screening. Urology **48** (1996) 62–6.
[15] DEMURA, T., SHINOHARA, N., TANAKA, M., ENAMI, N., CHIBA, H., et al., The proportion of free to total prostate specific antigen: A method of detecting prostate carcinoma, Cancer **77** (1996) 1137–1143.
[16] ELGAMAL, A.A.A., CORNILLIE, F.J., VAN-POPPEL, H.P., WAN-DE-VOORDE, W.M., MCCABE, R., et al., Free-to-total prostate specific antigen ratio as a single test for detection of significant stage T1c prostate cancer, J. of Urology **156** (1996) 1042–49.
[17] LERNER, S.E., JACOBSEN, S.J., LILJA, H., BERGSTRALH, E.J. RANSOM, J., et al., Free, complexed, and total serum prostate-specific antigen concentrations and their proportions in predicting stage, grade and deoxyribonucleic acid ploidy in patients with adenocarcinoma of the prostate, Urology **48** (1996) 240–248.
[18] MOROTE, J., RAVENTOS, C.X., LORENTE, J.A., LOPEZ-PACIOS, M.A., ENCABO, G., et al., Measurement of free PSA in the diagnosis and staging of prostate cancer, Int. J. Cancer **71** (1997) 756–9.
[19] BANGMA, C.H., RIETBERGEN, J.B., KRANSE, R., BLIJENBERG, B.G., PETTERSON, K., et al., The free-to-total prostate specific antigen ratio improves the specificity of prostate specific antigen in screening for prostate cancer in the general population [see comments], J. Urol. **157** (1997) 2191–6; Comment in: 2197–8.
[20] REISSIGL, A., KLOCKER, H., POINTNER, J., ENNEMOSER, O., FALK, M., et al., Improvement of prostate cancer screening by determination of the ratio free/total PSA in addition to PSA levels, Prostate **30** (1997) 243–7.
[21] RICCARDO, B., ALBERINO, D., FABRIZIO, T., GINO, C., SIMONE, A., et al., Free to total prostatic specific antigen ratio as a new diagnostic tool in prostatic carcinoma, Anticancer-Res. **17** (1997) 1297–301.
[22] BIJORK T., LILJA, H., et al., The prognostic value of different forms of prostate specific antigen and their ratio in patients with prostate cancer, BJU-Int. **84** (1999) 1021**–7**.
[23] AKDAS, A., CEVIK, I., TARCAN, T., TURKERI, L., DALAMAN, G., et al., The role of free prostate-specific antigen in the diagnosis of prostate cancer, Br-J-Urol. **79** (1997) 920–3.
[24] NAGASAKI, H., WATANABE, M., KOMATSU, N., KANEKO, T., DUBE, J.Y., et al., Epitope analysis of a prostate-specific antigen (PSA) C-terminal-specific monoclonal antibody and new aspects for the discrepancy between equimolar and skewed PSA assays, Clin-Chem. **45** (1999) 486–96.
[25] SUMI, S., UMEDA, H., KOGA, F., YANO, M., IMAI, T., et al., Determination of free type and complex type prostate-specific antigen (PSA): Differences in immunorecognition by Delfia PSA, ACS-PSA and Eiken PA kits, Acta Urologica Japonica **42** (1996) 279–284.
[26] JUNG, K., LEIN, M., SCHNORR, D., BRUX, B., HENKE, W., et al., Comparison between equimolar- and skewed-response assays of prostate specific antigen: is there an influence on the clinical significance when measuring total serum prostate specific antigen? Ann-Clin-Biochem **33** (1996) 209–14.
[27] TEWARI, P.C., BLUESTEIN, B.I., Multiple forms of prostate specific antigen and the influences of immunoassay design on their measurement in patient serum, Journal of Clinical Ligand Assay **18** (1995) 186–196.

[28] GRAVES, H.C., Issues on standardization of immunoassays for prostate-specific antigen: a review, Clin. Invest-Med. **16** (1993) 415–24.
[29] GRAVES H.C., Standardization of immunoassays for PSA: a problem of PSA or a problem of assay design?, Cancer **72** (1993) 3141–4.
[30] STAMEY, T.A., PRESTIGIACOMA, A.F., CHEN, Z., Standardization of immunoassays for PSA: A different view on experimental observations, Cancer **74** (1994) 1662–6.
[31] MURPHY, G.P., The second Stanford conference on international standardization of PSA assays, Cancer **75** (1995) 122–9.
[32] STAMEY, T.A., Progress in standardization of immunoassays for prostate-specific antigen, Urol-Clin-North-Am. **24** (1997) 269–73.
[33] SEMJONOW, A., BRANDT, B., OBERPENNING, F., HERTLE, L., Discrepancies in assays impair the interpretation of prostate-specific antigen, Urologe Ausgabe A **34** (1995) 303–315.
[34] BRAWER, M.K., DAUM, P., PETTEWAY, J.C., WENER, M.H., Assay variability in serum prostate-specific antigen determination, Prostate **27** (1995) 1–6.
[35] VAN-DUIJNHOVEN, H.L.P., PEQUERIAUX, N.C.V., VAN-ZON, J.P.H.M., BLANKENSTEIN, M.A., Large discrepancy between prostate-specific antigen results from different assays during longitudinal follow-up of a prostate cancer patient, Clinical Chemistry **42** (1996) 637–641.
[36] NARDONE, D.A., Problems with interpreting results of PSA [letter] J-Gen-Intern-Med. **12** (1997) 200, discussion 200–1.
[37] WANG, M.C., VALENZUELLA, L.A., MURPHY, G.P., CHU, T.M., A simplified purification procedure for human prostate specific antigen, Oncology **39** (1982) 1–5.
[38] RUCIANO, D., BERARDI, A., CECCARINI, C., TERRANA, B., Concomitant purification of prostate carcinoma tumour markers from human seminal fluid under nondenaturing conditions, Clin. Chem. **34** (1988) 2528–32.
[39] KOHLER, G., MILSTEIN, C., Continuous culture of fused cells secreting antibody of predefined specificity, Nature **256** (1975) 495–7.
[40] GROTH, F., SCHEIDEGGER, D., Production of monoclonal antibodies: Strategy and Tactics, J., Immunological Methods **35** (1980) 1–21.
[41] PRESTIGIACOMO, A.F., CHEN, Z., STAMEY, T.A., A universal calibrator for prostate specific antigen (PSA), Scandinavian Journal of Clinical and Laboratory Investigation **55** SUPPL. 221 (1995) 57–59.
[42] SOKOLOFF, R.L., WOLFERT, R.L., RITTENHOUSE, H.G., Standardization of PSA Immunoassays: Proposals and practical limitations, Journal of Clinical Ligand Assay **18** (1995) 86–92.
[43] BELANGER, A., et al., Molecular mass and carbohydrate structure of prostate specific antigen: Studies for establishment of an International PSA Standard, Prostate **27** (1995)187–197.
[44] CHEN, Z., PRESTIGIACOMA, A., STAMEY, T.A., Purification and characterization of prostate-specific antigen (PSA) complexed to alpha-1-antichymotrypsin: Potential reference material for international standardization of PSA immunoassays, Clinical Chemistry **41** (1995) 1273–1282.
[45] STENMAN, U.H., LEINONEN, J., ZHANG, W.,M., Standardization of PSA determinations, Scandinavian Journal of Clinical and Laboratory Investigation **55**-SUPPL. **221** (1995) 45–51.
[46] STENMAN, U.H., LEINONEN, J., ZHANG, W.M., Problems in the determination of prostate specific antigen, Eur-J-Clin-Chem-Clin-Biochem,. **34** (1996) 735–40.
[47] WENER, M.H., DAUM, P.R., BRAWER, M.K., Variation in measurement of prostate-specific antigen: Importance of method and lot variability, Clinical Chemistry **41** (1995) 1730–7.
[48] OESTERLING, J.E., et al., Free, complexed and total PSA: The establishment of appropriate reference ranges for their concentrations and ratios. Journal of Urology **154** (1995) 1090–1095.

[49] PIIRONEN, T., PETTERSSON, K., SUONPAA, M, STENMAN, U.H., OESTERLING, J.E. et al., In vitro stability of free PSA and PSA complexed to alpha 1-antichymotrypsin in blood samples, Urology **48** 6A Suppl. (1996) 81–7.
[50] WOODRUM, D., FRENCH, C., SHAMEL, L.B., Stability of free prostate-specific antigen in serum samples under a variety of sample collection and sample storage conditions, Urology, **48** 6A Suppl. (1996) 33–9.
[51] LEINIONIN, J., STENMAN, U.H., Reduced stability of prostate specific antigen after long term storage of serum at –20°C, Tumor Biol. **21** (2000) 46–53.

2.6. ISLAMIC REPUBLIC OF IRAN

Title of the Project:	Development of simple Immunoradiometric Assay Kits For Measurement of Prostate-Specific Antigen (Total and Free)
Chief Scientific Investigator:	R. Najafi
Scientific Co-Investigator:	T.Hadizad, P.Zarsav, M.Pourabdi, M.Moharamzadeh, and B.Mahdiani

Abstract. Immunoradiometric assays for the measurement of total and free PSA in human serum were developed in our laboratory using the matched pairs of antibodies and purified PSA supplied. The Avidin-Biotin high affinity binding technology was used for immobilizing antibodies on beads/tubes. Different reaction parameters such as coating procedures, incubation time, sample volume etc. were optimized for both free and total PSA assays using beads as well as tubes from different sources. In brief, the optimized assays for total and free PSA were reliable and simple. The results on development of immunoradiometric assays for the measurement of total and free PSA are summarized in this report.

2.6.1. INTRODUCTION

Sandwich immunoradiometric assay (IRMA) method using tubes or beads as solid phase, would be ideal for measurement of total and free PSA in serum. We utilised the matched pair of monoclonal antibodies against the appropriate epitopes on PSA for development of IRMAs for total- and free-PSA. In brief, antibodies that bind to epitopes that are removed from ACT binding area were used as capture and tracer antibodies in the case of t-PSA while a monoclonal antibody against a determinant on PSA that is involved in binding to ACT was used as tracer in f-PSA assay. For immobilizing capture antibodies on the wall of tube or bead, we used avidin-biotin system to enhance coating efficiency and sensitivity of the assay. In this procedure, avidin was coupled to the beads or tubes and the capture antibody was biotinylated. After a careful study of various reaction parameters, sandwich IRMAs were optimised at room temperature for both t- and f-PSA.

2.6.2. MATERIALS

Matched pairs of monoclonal antibodies for PSA, namely, MAb66 (capture antibody), MAb30 (tracer antibody for t-PSA), MAb10 (tracer antibody for f-PSA) and semipurified (70% purity) PSA were obtained from Dr.M.R. Suresh, Faculty of pharmacy, University of Alberta, Canada. Biotinamidocaproate-N-hydroxy succinimide ester, avidin from egg white, glycine, BSA and dimethyl formamide were purchased from M/s.Sigma Chemical Co. Glutaraldehyde was from M/s. E.Merck and other chemicals were purchased from M/s. Fluka and BDH. Rabbit IgG was prepared in our laboratory. Polystyrene beads (8mm dia. specfinish) were from Kramel Bioteck; UK. Polystyrene tubes for antibody coating were purchased from four companies M/s. Nunc in Denmark ; M/s. Western in Germany, M/s. Griena in Germany and M/s. Haghighat in Islamic Republic of Iran (local company). ^{125}I was from M/s. Amersham, UK.

2.6.3. METHODS

2.6.3.1. Coating of Beads and tubes with avidin

Beads and tubes were coated with avidin following the reported procedure [3, 4]. Avidinated beads were prepared by both passive simple coating procedure and active coating method. In both methods, avidin was used at various concentrations such as 10, 20, 30, 40, 50, 60, 70 and 80 μg/mL. After avindination, the beads were tested for their binding capacities with ^{125}I-biotinylated IgG solution.

In the optimized procedure, the beads were immersed in 0.2% BSA in 0.1 M bicarbonate buffer or 0.05 M phosphate buffer for 3–4 hours, rinsed with distilled water twice and placed in 2% glutaraldehyde in distilled water overnight. They were then washed ten times with distilled water and incubated in avidin solution 40 μg/mL in phosphate buffer for two hours at room temperature, washed thrice with distilled water, glazed with 0.5% BSA and 2.5% sucrose and finally dried. The beads were sealed in a plastic container and stored at –20°C. Tubes were coated following the same procedure using 300 μL of each solutions (mentioned above) in each tube.

2.6.3.2. Lowry method for avidin determination

In order to assess the quantity of avidin that has been consumed for coating, we determined the concentration of used avidin solution following coating procedure by Lowry method. In this method, Cupric ions in alkaline tartarate solution react with peptide bonds. Addition of Folin's agent develops a purple-blue complex the absorbance of which at 640 nm, is proportional to the total protein concentration.

The following reagents were used in the Lowry procedure:

a. Sodium Carbonate, 3% in 0.2 M Sodium Hydroxide
b. Sodium Potassium Tartarate, 4% in D.D. Water
c. Cupric Sulfate, 2% in D.D. Water
d. Folin reagent.

Working Solution A is prepared by addition of 1 mL Cupric Sulfate solution and 1 mL NaK Tartrate to 48 mL of sodium carbonate solution.

Standard solutions of avidin of 0, 20, 40 and 60 μg/mL concentration were prepared for construction of the standard curve. 1mL of standards or samples were added in individual tubes to 1 mL of working solution A and incubated for 10 minutes at room temperature. Then, 50 μL Folin's reagent was added, incubated for 25 minutes at room tempreture and the absorbance at 640 nm was noted. The absorbance was plotted against avidin concentrations for standards and this curve was used for estimating the avidin content in the unknown samples.

2.6.3.3. Biotinylation of capture antibody

The capture antibody was biotinylated by following published methods [3,5,6]. In brief, 1 mg of the capture MAb was dissolved in 0.5 mL 0.1 M bicarbonate buffer to which 10 μL of biotin solution (1 mg/mL in DMF) was added, stirred and incubated for 2 hours at room temperature. Another aliquot of 10 μL biotin solution was then added and incubated further for 2 hours at room temperature. At the end of incubation, the reaction mixture was dialyzed against phosphate buffer overnight with three changes of buffer in order to remove the unreacted biotin. The dialysed biotinylated MAb was finally diluted with 50 mL of assay buffer containing 900 mg of NaCl, 300 mg of BSA, 1 mL of normal mouse serum, 500 mg of neomycin and 8.5 mg of chloramphenicol and stored at 4°C.

2.6.3.4. Radiolabelling of monoclonal antibodies

Both the tracer MAbs for use in t-PSA and f-PSA assays were labeled with ^{125}I using Chloramine-T as the oxidant. The radioiodination was carried out using appropriate amounts of MAbs and radioiodine in order to finally obtain specific activities of ~10 μCi/μg. The ^{125}I-MAbs were purified by gel filtration using sephadex-G-25 and stored at –20°C in aliquots.

2.6.3.5. Preparation of PSA Standards

The PSA standards were prepared as follows :

a. To the supplied vial containing 1.3 mg of semipurified PSA (70% purity) in lyophilized form, 22.75 mL phosphate buffer 0.05 M was added to obtain the stock standard of PSA. In this solution, the concentration of pure PSA is expected to be 40 μg/mL. Aliquots of 1 mL were dispensed in vials and stored frozen at –70°C.
b. 9 mL of delipidized female serum free of PSA was added to 1 mL of the above PSA stock standard to obtain a concentration of ~ 4000 ng/mL. This was named Standard A.
c. Three more PSA standards, namely B, C and D of concentrations 200 ng/mL, 100 ng/mL and 40 ng/mL were also prepared by addition of appropriate amounts of female serum to standard A (20, 40 and 100 folds dilution respectively). These standards were assayed using a commercial t-PSA kit and the actual concentrations of PSA obtained were used to recalibrate Standard A. Based on this calibration, fresh lots of Standard A with exactly 4000 ng/mL t-PSA, were then prepared from the stock standard by dilution with female serum. The f-PSA level of Standard A was also then estimated using a commercial f-PSA kit.
d. The calibrated Standard A was then used to prepare working standards of total and free PSA. Typically, concentrations of 0.5, 1, 5, 10, 50 and 100 ng/mL were used for t-PSA and 0.5, 1, 2.5, 5, 10 and 30 ng\mL were used for f-PSA.

2.6.3.6. Optimization of immunoassays

In order to develop a simple and reproducible assay format with adequate sensitivity for total and free PSA, the following aspects were studied.

i. *Effect of concentration of biotinylated antibodies on coupling with the avidin coated beads or tubes:* Standard curves were set up using 20 μL standards of 0, 2.5, 125 and 315 ng/mL t-PSA, 100 μL assay buffer (0.05 M phosphate buffer + 1% gamma globulin), 100 μL biotinylated capture antibody at various concentrations such as 0.05, 0.1, 0.25, 0.5, 1.5, 2.5, 5 and 10 μg/mL, 100 μL ^{125}I-MAb10 (^{125}I-MAb30 in a separate assay for f-PSA) and one avidinated bead. The tubes were incubated under gentle rotation for 4 hours at room temperature. The beads were washed with wash buffer thrice and counted.

ii. *Effect of using mixed antibodies (biotinylated and tracer) as a single reagent:* In order to reduce the number of pipeting steps and simplify the assay procedure, use of mixed antibodies at optimal concentrations was tested and compared with the addition of individual reagents

iii. *Studies of factors to decrease non-specific binding:* Various parameters such as use of streptavidin instead of avidin for coating, use of BYCOA as the blocking agent in place of BSA, removal of possible impurities in the tracer by use of BSA coated beads to pre-treat the tracer etc, were studied to see if the NSB could be reduced.

iv. *Effect of various factors such as the volume of standards/samples used in the assay, incubation condition, time etc. on the standard curve*

v. *Comparison of different types of tubes for antibody immobilization and assay performance*

vi. *Assay validation and clinical evaluation :* The t-PSA and f-PSA assays developed were validated by estimation of the intra- and inter-assay variations, parallelism testing, recovery of added standards in the samples and comparison of sample analyses using the in-house kits and commercial kits.

The final optimized assay protocol is as follows : Add 50 μL standard or sample of total PSA (in the case of free PSA 100 μL standard or sample) and 200 μL mixed antibodies (capture and tracer) into avidin coated tube, mix and incubate at room temperature for 2–4 hours, wash the tubes (or beads) and assay for radioactivity.

2.6.4. RESULTS AND DISCUSSION

2.6.4.1. Effect of avidin concentration on immobilization

Avidinated beads were prepared by both passive simple coating procedure and active coating method as described earlier. In both methods, avidin was used at various concentrations such as 10, 20, 30, 40, 50, 60, 70 and 80 μg/mL. After avindination, the beads were placed in assay tubes containing ^{125}I-biotinylated IgG solution of proper specific activity and concentration. The tubes were incubated for 2 hours at room temperature, washed and counted. It was observed that a maximum of 72% of ^{125}I-biotin-IgG bound to the beads when 60 μg/mL avidin was used in passive coating procedure. On the other hand, in active method, the maximum adsorbed ^{125}I-biotin-IgG was 83% at 40 μg/mL avidin. Hence active method was preferred. However, since avidin as a biochemical is relatively expensive it was felt that using avidin at 40 μg/mL concentration for coating thousands of tubes would not be economical. Hence, it was decided to determine the concentration of the remaining unbound avidin in the solution left after coating, in order to salvage it. By doing several experiments, we realised that only 30% avidin is consumed for coating the beads and 70% avidin remained in the solution. It was decided to reuse the avidin solution after the first use by addition of 12 μg/mL of avidin to the used solution and use it afresh for coating. The results obtained following this procedure were satisfactory.It is recommended to use sterilized vessels for preparation of avidin solution and keeping it in deep freeze in order to prevent micro organism growth in the solution.

2.6.4.2. Effect of biotinylated capture antibody concentration on the reaction with avidinated beads and sandwich complex formation

The results of the experiment on variation of biotinylated capture MAb66 on the assay performance are shown in Tables I and II. It is evident from these tables that concentration between1 to 2.5 μg/mL of biotinylated capture MAb66 is suitable for sandwich form of t-PSA IRMA. Use of greater concentrations of biotinylated MAb would be waste of materials as it attains the saturated state.

TABLE I. EFFECT BIOTINYLATED CAPTURE ANTIBODY CONCENTRATION (t-PSA)

Biotinylated MAb66 (μg/mL)	Binding (CPM) (^{125}I-MAb10 total 60810 CPM) PSA Concentration(ng/mL)			
	0	2.5	12.5	315
0.05	89	131	129	132
0.1	135	83	136	206
0.25	115	162	259	261
0.5	162	157	882	1343
1.0	99	166	2058	3252
1.5	109	198	2567	3915
2.5	116	213	3031	4794
5.0	134	235	3246	5557
10.0	130	232	3012	4669

2.6.4.3. Effect of mixing the biotinylated and iodinated antibodies to use as a single reagent

In order to increase sensitivity and simplicity of the assay decided to mix the biotinylated antibody (100 μg/mL) with the tracer antibody solution and adjusted the concentrations of both antibodies to the desired amount (e.g. 2.5 μg/mL capture antibody, 60.000 CPM for tracer antibody). Thus the developed assay would consist of adding only appropriate volumes of PSA standards (total or free) and mixed antibodies solution into coated tube, shaking and incubating. On using different volumes (100 to 300 μL) of mixed antibodies it was found that the binding activities enhanced by increasing volume of the above solution. But because of increasing NSB associated with this increase in binding, we preferred to apply only 200 μL of the mixed antibodies solution.

TABLE II. EFFECT BIOTINYLATED CAPTURE ANTIBODY CONCENTRATION (f-PSA)

Biotinylated MAb66 (μg/mL)	Binding (CPM) (^{125}I-MAb30 total 71155 CPM) PSA Concentration(ng/mL)			
	0	2.5	12.5	315
1.0	66	124	8156	14145
1.5	55	190	9674	18208
2.5	64	182	11964	19933
5.0	91	204	9590	20305
10.0	59	128	9768	19208

2.6.4.4. Effect of specific activity of tracers on the standard curves

Tracers for t-PSA and f-PSA assays were prepared at various specific activities such as 5, 10, 20, 40 and 80 μCi/μg. Standard curves were then set up using these tracers and using the earlier mentioned assay protocols. It was realized that the specific activity of ~10 μCi/μg was optimum for both t-PSA as well as f-PSA to obtain good standard curves.

2.6.4.5. Studies on factors to reduce non-specific binding

The Avidin-Biotin system is generally associated with a high Non-specific Binding. In this regard we investigated some effective parameters hoping to reduce NSB to the extent possible.

First of all, we used streptavidin instead of avidin for coating beads and compared with avidin coated beads. It was found that although the NSB decreased, the% maximum binding also decreased significantly and the%C.V. also increased.

In order to test if the type of blocking agent used had a role in determining the NSB, ByCOA (Hydrolysed gelatin) was used as blocking agent and compared with BSA that has been used for blocking. The results are tabulated in Table III and it can be seen that ByCOA resulted in lower NSB and the assay had better sensitivity.

In order to investigate the effect of probable impurities in the tracer solution that may bind non-specifically to the coated tubes, we added BSA coated beads (for preparation of BSA coated beads, we immersed beads into 0.2% BSA in PBS, pH 7.4 for at least 3 h, washed and dried) into eluted tracer solution and incubated for 3–4 h at 4°C. The BSA coated beads were then removed and this treated tracer was used in the assay. Table IV compares the results of the assays obtained using tracers with and without treatment of BSA beads and it is seen that NSB is reduced with the treatment of tracer with BSA coated beads. This would help to increase the sensitivity of the assay marginally.

TABLE III. EFFECT OF USE OF HYDROLYSED GELATIN FOR BLOCKING

PSA (ng/mL)	Bound Counts (mean CPM)	
	BSA blocked	BYCOA blocked
0.0	338	255
0.3	240	320
0.6	305	416
4.0	688	757
8.0	1627	1823
40.0	5619	6433
90.0	10556	11818
180.0	15908	173175
Maximum binding	34.6%	37.6%

The effect of amount of tracer used on the NSB was studied and the results are shown in Table V. As expected, with increase in total activity of tracer used, the NSB also increased.

2.6.4.6. Effect of standards volume on the standard curve

The effect of standard volume on the performance of assay is depicted in Table VI. Since f-PSA is present in low concentrations, the bound counts were lower than that for t-PSA. The effect of standard volume on the assay performance was studied by varying the volume of standards used from 10 to 100 µL for both f-PSA and t-PSA assays.

It can be seen from the results indicated in Table VI, that use of 100 µL of standards for f-PSA (MAb30 tracer) assay yielded better binding values while in the case of t-PSA assay (MAb10 tracer), 50 µL seems adequate as higher volumes exhibit saturation and could lead to hook effect.

2.6.4.7. Effect of incubation time and rotation

The dependence of labelled sandwich complex formation on incubation time and rotation at room temperature was investigated. Incubation periods of 2, 3, 4 and 18 h with and without rotation were studied for both t-PSA and f-PSA assays following the reaction protocols detailed above. Tables VII and VIII list the results obtained.

TABLE IV. EFFECT OF IMMERSION OF BSA COATED BEADS IN TRACERS

PSA (ng/mL)	Bound Counts (mean CPM)	
	Tracer treated with BSA bead	Untreated tracer
0.0	159	365
0.3	175	406
0.6	276	472
4.0	609	668
8.0	1142	1079
40.0	3529	4427
90.0	7736	8643
180.0	11593	12433
Maximum binding	22.78%	24.55%

It is evident that with increasing incubation time and rotation the binding increases. The results for 18 h incubation were approximately the same as that for 4 h incubation time. Therefore we chose 4 h incubation with rotation as optimum as the variations were also lower. However, 2 hour incubation without rotation also resulted in low variations and standard curves that were parallel to that of 4 h with rotation. This was also hence acceptable though the binding was lower.

TABLE V. EFFECT OF TRACER CONCENTRATION (TOTAL COUNTS) ON NSB

Free PSA		Total PSA	
Total count (CPM)	NSB (CPM)	Total count (CPM)	NSB (CPM)
88000	712	95000	293
66000	577	71000	297
47000	491	58000	163

TABLE VI. EFFECT OF TRACER VOLUME ON THE STANDARD CURVE

	Total PSA assay — B/T%				Free PSA assay — B/T%			
	MAb10 tracer volume (µL)				MAb30 tracer volume(µL)			
PSA(ng/mL)	10	20	50	100	10	20	50	100
0.0	0.3	0.3	0.2	0.1	0.6	0.5	0.4	0.3
2.5	0.6	1.0	0.8	0.8	1.2	1.4	1.5	1.5
10.0	1.1	1.2	1.4	2.0	2.0	2.7	5.4	5.8
30.0	2.4	2.8	5.0	7.1	3.0	5.3	9.2	9.5
60.0	4.3	6.5	10.7	13.0	4.9	8.0	13.9	14.0
120.0	6.8	11.0	15.7	17.0	12.0	16.0	17.9	17.4
360.0	16.0	26.0	27.2	20.0				

TABLE VII : EFFECT OF INCUBATION TIME AND ROTATING ON THE STANDARD CURVE TOTAL-PSA ASSAY

	2 hours, room temperature				4 hours room temperature			
	without rotation		with rotation		without rotation		with rotation	
PSA (ng/mL)	Mean CPM	% C.V.	Mean CPM	% C.V.	Mean CPM	% C.V.	Mean CPM	% C.V.
0.0	245	16.7	238	55.7	435	52	340	12.1
2.5	327	8.6	404	10,0	574	7.1	728	9.1
10.0	485	3.6	634	8.6	1057	6.3	1552	6.3
30.0	968	0.7	1512	1.9	2495	0.6	3188	3.1
60.0	1615	0.7	3056	6.2	4452	1.7	5847	4.1
120.0	2528	2.1	4694	12.2	6049	3.1	8228	4.7
360.0	5211	0.3	9921	2.0	9815	0.8	13589	0.2
Total	62922		62948		63508		63375	
Max. B/T	9.5%		18.6%		17.9%		25.2%	

2.6.4.8. Comparison of different types of tubes for use in the assays as the capture matrix

After optimizing of coated beads and obtaining acceptable results for total and free PSA assays, various polystyrene tubes were tested for antibody coating. Polypropylene and polystyrene tubes from established manufacturers as well as local manufacturers were coated with avidin using the same coating procedure as for beads by adding 300 µL avidin solution (40 µg/mL) per tube. Table IX lists the results obtained using Nunc tubes specifically meant for coating and the local polypropylene tubes

specially made for immunoassays. Although the mean binding values were similar for both Nunc and the local tubes, better sensitivity and precision could be achieved with Nunc tubes than with local tubes. The high% C.V. is perhaps the main reason for this and probably by optimizing some factors such as increasing coating surface, one could achieve better precision with locally available inexpensive polypropylene tubes. Figure 1 shows the results of the above assays. Table X lists the values of the three BioRad QC samples obtained using Nunc tubes and local tubes. Both methods have yielded results within the range obtained using DSL kits. However, the values obtained using local tubes do not tally with that using Nunc tubes. This could perhaps be again attributed to the variations observed in the local tubes.

TABLE VIII. EFFECT OF INCUBATION TIME AND ROTATING ON THE STANDARD CURVE-FREE-PSA ASSAY

	2 hours, room temperature				4 hours room temperature			
	without rotation		with rotation		without rotation		with rotation	
PSA (ng/mL)	Mean CPM	% C.V.	Mean CPM	% C.V.	Mean CPM	% C.V.	Mean CPM	% C.V.
0.0	67	1.1	112	40	99	12.2	105	22.9
2.5	158	0.9	227	6.9	270	1.3	315	3.1
10.0	356	2.8	555	14.3	638	10.7	558	8.5
30.0	958	8.8	2135	3.5	2294	8.3	2971	2.0
60.0	2266	4.1	5055	5.9	5110	4.3	8270	1.4
120.0	4277	4.8	9385	7.2	8660	9.6	13061	8.7
360.0	9480	4.4	21411	0.2	16066	1.0	25040	6.5
Total	83652		83778		63612		82995	
Max. B/T	13.0%		30.5%		22.9%		36.1%	

TABLE IX. COMPARISON OF NUNC AND LOCAL TUBES FOR USE IN ASSAYS

PSA(ng/mL)	Nunc tubes		Local tubes	
	Mean CPM	% C.V.	Mean CPM	% C.V.
0.0	134	6.4	118	25.2
0.5	165	5.6	93	28.9
1.0	240	4.4	138	28.7
2.5	409	6.7	386	25.4
5.0	1045	7.0	865	7.4
25.0	5783	5.3	5486	14.7
50.0	10672	3.9	10871	5.3
125.0	19175	1.2	18797	3.7
250.0	23695	2.8	23964	4.8
Total Counts	49329		49004	
Max.% B/T	57.3		58.4	
QC 1	271	9.1	251	42
QC 2	726	2.9	883	31
QC 3	8859	1.4	5097	12.6

TABLE X. QC SAMPLE ANALYSIS: COMPARISON OF NUNC AND LOCAL TUBES

QC range* (ng/mL)	Value obtained with Nunc tubes	Value obtained using local tubes
Level 1 (1.09–1.5)	1.21 ng/mL	1.69 ng/mL
Level 2 (3.5–4.8)	3.67 ng/mL	5.08 ng/mL
Level 3 (23.4–38.4)	38.2 ng/mL	23.5 ng/mL

* Range of PSA value in QC samples analysed by BioRad with DSL kit as part of their QC programme.

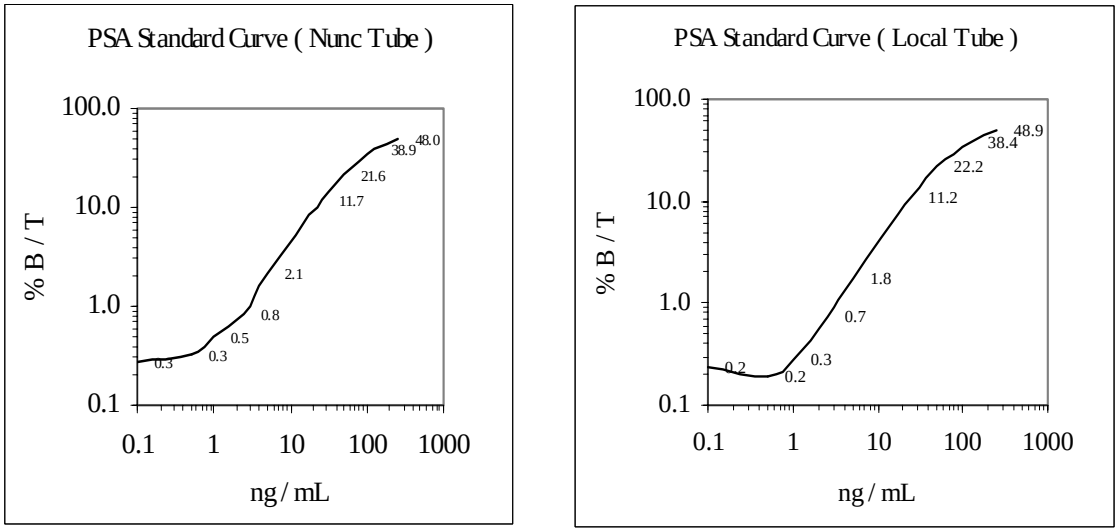

Figure 1. Total PSA IRMA standard curves with avidinated Nunc and Local tubes

2.6.4.9. Assay Validation and Clinical Evaluation

Intra-assay and Inter-assay variations were estimated for both total and free PSA IRMAs developed using samples at three different concentrations, namely, low, medium and high levels of PSA. These results are tabulated in the Table XI. It is seen that the variations are generally in the acceptable range for both assays.

Known amounts of human PSA were added to three serum samples and assayed to determine the recovery in both t-PSA as well as f-PSA assays. Table XII lists the results obtained and it is seen that the% recovery ranged between 89.5% to 103%.

TABLE XI. ASSAY VARIATIONS

Total-PSA Assay						
	Intra assay (n=15)			Inter assay (n=10)		
Sample Number	1	2	3	1	2	3
Mean (ng/mL)	0.7	3.8	31.1	1.8	4.5	34.9
% C.V.	12.4	6.3	2.5	13.1	7.3	6.2
Free-PSA Assay						
	Intra assay (n=15)			Inter assay (n=5)		
Sample Number	1	2	3	1	2	3
Mean (ng/mL)	1.9	4.1	10.4	0.6	1.6	10.5
% C.V.	10.4	12.5	3.3	6.7	19.8	4.8

TABLE XII. RECOVERY TEST

PSA in sample (ng/mL)	PSA Added (ng/mL)	Expected PSA (ng/mL)	Observed PSA (ng/mL)	% Recovery
Total PSA Assay				
0.35	2.5	2.85	2.55	89.5
1.89	5.0	6.89	6.40	92.9
15.54	25.0	40.54	41.75	103
Free PSA Assay				
0.24	10.0	10.24	9.98	97.5
2.05	5.0	7.05	6.85	97.2
4.5	10.0	14.5	15.0	103

TABLE XIII. PARALLELISM TEST

Sample	Dilution	Expected PSA Value (ng/mL)	Observed PSA Value (ng/mL)	% Recovery
Total PSA Assay				
1	Undiluted		31.47	
	2 ×	15.73	15.28	97.1
	4 ×	7.87	6.98	89.5
	8 ×	3.93	3.86	99
2	Undiluted		24.33	
	2 ×	12.16	12.55	102.8
	4 ×	6.08	5.16	84.6
	8 ×	3.04	3.32	110.6
3	Undiluted		20.26	
	2 ×	10.13	9.04	89.2
	4 ×	5.06	4.81	96.2
	8 ×	2.53	2.74	109
Free PSA Assay				
1	Undiluted		24.26	
	2 ×	12.13	12.06	99.4
	4 ×	6.06	6.14	100.6
	8 ×	3.03	3.15	105
2	Undiluted		21.62	
	2 ×	10.81	12.10	112
	4 ×	5.40	5.93	109.8
	8 ×	2.70	2.65	98.1
3	Undiluted		19.25	
	2 ×	9.62	9.61	100
	4 ×	4.81	4.48	93.3
	8 ×	2.40	2.54	105.4

Parallelism was tested by diluting three serum samples with PSA-free serum and estimating the f-PSA and t-PSA values using the developed kits. Table XIII shows the results of these tests and it is seen that both free-PSA as well as total-PSA procedures yield values between 85 to 111% of expected values.

Clinical evaluation of the developed assay procedures was done by assaying clinical samples by the in-house kits as well as commercial kits. 60 male samples were assayed for total PSA levels and 20 male samples for free PSA levels. These samples were from patients with doubtful clinical diagnosis. Good correlation was observed for both the in-house kits. For the t-PSA IRMA, correlation coefficient r = 0.9944 and Y= 0.6794 + 0.9727 X and for f-PSA IRMA, r= 0.9734 and Y= –1.2108+1.1502 X. These are depicted in Figure 2. In conclusion, IRMA procedures for both total and free PSA were satisfactorily developed, optimised and validated at our laboratory.

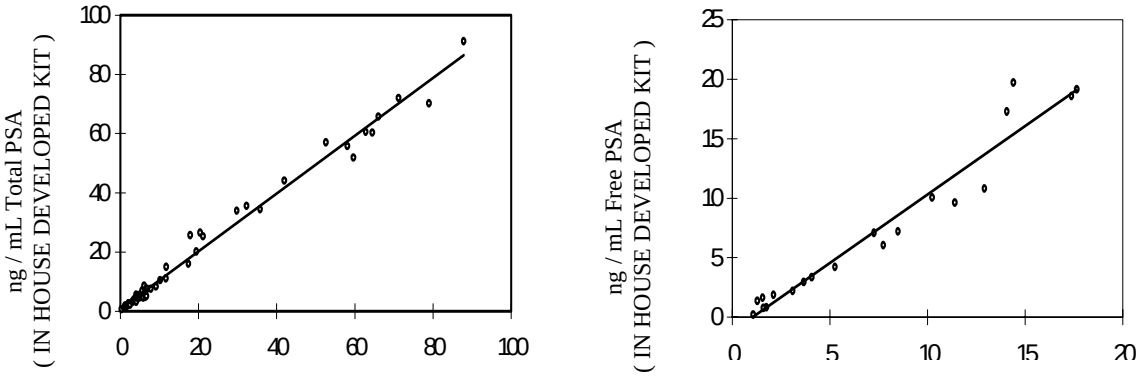

Figure 2. Correlation of PSA (total and free) levels in patient samples obtained using the developed in-house kits and commercial kits.

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REFERENCES

[1] INTERNATIONAL ATOMIC ENERGY AGENCY, Summary report of the First Research Co-ordination Meeting of the CRP on Development of Kits for Radioimmunometric Assay for Tumour Markers, Vienna (1997).

[2] HANS, L., Significance of different molecular forms of serum PSA, Urologic Clinics of North America, **20** No.4 (1993).

[3] JYOTSNA, N., SINGH, Y., CHOUTHKANTHIMAR, V., PARADKAR, S.H., SIVA PRASAD, N., Development of simple immunoradiometric assay using avidin coupled to polystyrene beads as a common solid phase. International symposium of modern trends in radiopharmaceuticals of diagnosis and therapy, IAEA, Portugal (1998).

[4] DIAMANDIS, E.P., CHRISTOPOULOS, T.K., The Biotin-(Strept) Avidin systems : Principles and Application in Biotechnology, Clin.Chem. **37** (1991).

[5] ROSARIA. P., YOU, H.&W., Coupling of monoclonal antibody with biotin, Methods in Molecular Biology, Monoclonal antibody protocols (W.C. Davis, Ed.) Humana Press, Inc., Totowa, NJ, Vol. 45.

[6] ODELL, W.D., GRIFFIN, J., ZAHRADNIK, R., Two monoclonal antibody sandwich type assay for thyrotropin, with use of an avidin-biotin technique, Clin. Chem. **32** (1986).

2.7. THAILAND

Title of the Project: Development of Kits for Serum PSA Monitoring

Chief Scientific Investigator: Prapaipit Suprarop

Scientific Co-Investigator: V. Tanjoy, T. Saraneeyatham, R. Rojanaurai

Abstract. Solid phase two-site immunoradiometric assays were developed for total PSA and free PSA in human serum using the matched pairs of monoclonal antibodies for PSA, supplied by University of Alberta, Canada. In the case of total PSA, the capture MAb was immobilised on both magnetic particles as well as polypropylene tubes. The IRMAs developed using these were very similar and comparable in their performances. The optimised assays involved two-step incubations (0.5 × 0.5 h for magnetic particles and 2 × 2 h for coated tube) at ambient temperature, had the working range of 0 to 200 ng/mL PSA and sensitivity of 0.38 ng/mL (magnetic particles method) and 0.05 ng/mL (coated tubes method). The assay exhibited good inter and intra assay precision, recovery and accuracy. The patient sample values analysed by the in-house kits and CIS bio international kit show good correlation with correlation coefficient of r = 0.9693 and CIS kit value = 0.80 × OAEP kit value + 0.8034 (n=83) for magnetic particle based method and r = 0.9853, CIS kit value = 1.10 × OAEP kit value – 0.4795 (n=63) for coated tubes method. In the case of free-PSA, the assay was optimised using polypropylene tubes as the solid phase. The assay involved a single step overnight incubation at ambient temperature and exhibited good sensitivity (0.06 ng/mL), and precision (8.24–12.04% for inter assay and 4.41–7.73% for intra-assay%C.V.). The developed f-PSA assay shows promising results, but needs further clinical assessment.

2.7.1. INTRODUCTION

Human prostate-specific antigen (PSA), a serine protease, a single chain glycoprotein with a molecular weight of approximately 34,000 daltons containing 7% carbohydrate by weight is immunologically specific for prostatic tissue. It is present in normal, benign hyperplastic and malignant prostatic tissue, in metastatic prostatic carcinoma, in prostatic fluid and seminal plasma. PSA is neither present in other normal tissues of men, nor is it produced by other cancers such as cancers of breast, lung, colon, rectum, stomach, pancreas or thyroid. Besides, it is functionally and immunologically different from prostatic acid phosphatase (PAP). Elevated serum PSA concentrations have been reported in patients with prostate cancer, benign prostatic hypertrophy, or inflammatory conditions of other adjacent genitourinary tissues, but not in healthy men, men with non-prostatic carcinoma, healthy women, or women with cancer. Reports suggest that serum PSA is one of the most useful tumour markers in oncology and could serve as an accurate marker for assessing response to treatment in patients with prostatic cancer. Therefore, measurement of serum PSA concentrations is an important tool in monitoring patients with prostatic cancer and in determining the potential and actual effectiveness of surgery or other therapies. Recent studies also indicate that PSA measurements can enhance early prostate cancer detection when combined with digital rectal examination (DRE).

There are several forms of PSA in the serum. Most of the measurable PSA in serum is complexed with the protease inhibitor alpha-1 antichymotrypsin (PSA-ACT). PSA is also found in a free form, not complexed to antichymotrypsin. Measurement of PSA alone is unable to distinguish benign prostatic hypertrophy (BPH) from cancer. Determination of the percentage of each of the forms of PSA may help in distinguishing benign from malignant conditions. The ratio of free to total PSA is useful in the differentiation of prostate cancer from benign conditions. This is especially true for the population of men with PSA values between 4.1–10 ng/mL. It is in this range that determination of the free to total PSA ratio may help distinguishing those patients most likely to have BPH. Patients with prostate cancer generally have a lower ratio of free to total PSA than patients with benign prostatic hypertrophy. This ratio may also be used to aid in the decision of whether a patient should undergo prostatic biopsy. Free PSA has also been reported to be of value in patients with total PSA levels of 2.6–4.0 ng/mL. Although sufficient data is not yet available to establish definitive cut-off values, most assign a cut-off of less than 20–25% to detect 90–95% of prostate cancers. Levels

above 20–25% are more likely to be associated with benign conditions and therefore prostatic biopsy is required for the diagnosis of cancer.

In order to measure free/total ratio of PSA levels, it is important to assay both forms of PSA concurrently with compatible methods, due to the variations in the commercial kits for t-PSA. ARUP uses the Hybritech Tandem-MP Free PSA Assay. Our aim was to develop a rapid, simple, and direct immunoradiometric assays for total and free serum PSA. Here we describe the development, optimization and validation of IRMAs for total PSA using both magnetic particles and polypropylene tubes as solid phase and for free PSA using tubes. The reagents such as matched pairs of monoclonal antibodies against PSA and standard PSA were supplied by Dr. M. Suresh, University of Alberta, Canada.

2.7.2. MATERIALS

Standards: Purified PSA were obtained from University of Alberta, Canada.
Antibody: Anti-PSA MAbs, MAb66 for coating and MAb10 and MAb30 for tracer preparation were obtained from University of Alberta,Canada.
Magnetic particles: Magnetizable cellulose particles were purchased from M/s. Scipac, UK.
Reagents: 1,1' carbonyldiimidazole (CDI) was purchased from M/s.Sigma Chemical Co., $Na^{125}I$ was obtained from M/s. Amersham Pharmacia Biotech, UK. All the solvents and other reagents were purchased from M/s. BDH Chemicals, Ltd.
Clinical Specimen: Patients'samples with PSA concentrations were obtained from Chulalongkorn Hospital, Bangkok.

2.7.3. METHODS

2.7.3.1. Preparation of radioiodinated anti-PSA monoclonal antibodies

The MAbs to be used as tracers, namely MAb10 and MAb30 for total and free PSA assays respectively, were labelled with radioiodine using chloramine-T. The method of *Alan Johnstone and Robin Thorpe* was followed with slight modification that the purification was carried out by HPLC system. The details of this method are given in Annex I. The radioiodinated MAb had specific activities ranging between 15 and 20 μCi/μg in the case of MAb10 and 35–40 μCi/μg in the case of MAb30. The tracers were of 94% purity and had a shelf life of about 6 weeks. The tracers were diluted in assay buffer and used at 50,000 CPM per tube for t-PSA based on magnetic particles, at 100,000 CPM for t-PSA based on coated tubes and 100,000 CPM/tube for f-PSA.

2.7.3.2. Preparation of PSA standards

Total PSA standards were prepared by addition of semi-purified PSA (University of Alberta) to screened normal female serum to a final concentration of 200 ng/mL. This stock standard was diluted to the following concentrations: 0.5, 2, 10, 25, 50 and 100 ng/mL. These working standards were calibrated using commercial standard (CIS bio international).

For free-PSA assay, the standards were prepared by reconstitution of purified PSA in distilled water and dilution in artificial serum (5% BSA in 50 mM phosphate buffer) to give a range of working standards from 0–20 ng/mL. Aliquots of standards were stored at –20°C.

2.7.3.3. Preparation of monoclonal antibody immobilized on magnetic particles

In order to use magnetic particles as the solid phase for capture, MAb66 was coupled to activated magnetic particles at a ratio of 1:4 followed by various washing steps to remove excess antibodies. The details are given in Annex II. The concentration of MAb coated magnetic particles used in the assay is 0.5 mg/tube (10 mg/mL;equivalent to ~0.3 μg MAb66).

2.7.3.4. Preparation of monoclonal antibody coated tubes

Polypropylene tubes (12 × 75 mm) were coated with MAb66 for use as the solid phase capture antibody. In t-PSA assay, the tubes were coated with 1 µg of the MAb and for f-PSA assay with 0.5 µg MAb. Typically, 400 µL of MAb66 in 50 mM sodium hydrogen carbonate buffer, pH 8.0, was added per tube (2.5 µg/mL in the case of t-PSA and 1.25 µg/mL for f-PSA). The tubes were incubated at 4°C for 18–24 hrs and rinsed twice with 0.1 M phosphate buffer. The coated tubes were finally blocked with 500 µL 1% BSA at 4°C for 2 h., rinsed twice with 0.1M phosphate buffer and glazed with 500 µL of 2% sucrose.

2.7.3.5. IRMA of total-PSA based on magnetic particles

The assays were optimized and validated using both coated tubes as well as magnetic particles. The t-PSA assays were of two steps and were carried out in glass tubes. Typically, 50 µL of standards or samples were added to the tubes followed by 50 µL MAb66 coupled magnetic particles and 100 µl assay buffer. The tubes were mixed gently on a vortex mixer and incubated at room temperature by rotating continuously on rotary mixer for 30 minutes. The tubes were placed on a magnetic rack for a few minutes to hold the particles as a pellet and washed twice with 1 mL of 0.1% Triton X-100 in 50 mM phosphate buffer pH 7.4 and decanted. 200 µL of ^{125}I-MAb10 tracer (50,000cpm) was then added to each tube and incubated for 30 minutes on the rotator. The tubes were washed as before with the aid of the magnetic rack and the pellets of magnetic particles were counted in a gamma counter.

The typical protocol is depicted below.

Set up the assay tubes in duplicates. Add the following reagents:

Standard/Samples	50 µL
Magnetic particles coupled to anti-PSA MAb	50 µL (10 mg/mL)
Assay buffer	100 µL

Vortex the tubes and place on a rotary mixer for 0.5 h. at RT

Add 1 mL washing solution (0.1% Triton X-100 in 50 mM phosphate buffer, pH 7.4), vortex well and separate on magnet (10 minutes).

Decant the supernatant and repeat washing step.

Add 200 µL ^{125}I-MAb10 tracer and incubate for 0.5 hour at R.T. on the rotator.

Repeat washing step.

Count the bound fraction in the tubes for 60 seconds.

2.7.3.6. IRMA of total-PSA based on coated tubes

In order to avoid hook effect, a two-step IRMA was developed using the supplied matched pair of high affinity monoclonal antibodies. The standards or samples were incubated with the coated MAb66 for 2 hours following which the radiolabelled ^{125}I-MAb10 was added and incubated for another 2 hours at ambient temperature. At the end of incubation, the contents of the tubes were removed, the tubes were washed twice and counted in the gamma counter. The typical protocol is shown below.

Set up the coated tubes in duplicates and add the following reagents.

Standard/Samples	50 µL
Assay buffer	250 µL

Vortex tubes and place on rotary mixer for 2 hours at RT

Add 1 mL washing solution and vortex well.

Aspirate the supernatant and repeat the washing step.

Add 300 µL tracer and incubate for 2 hours at RT. on the rotator.

Repeat washing step.

Count the bound fraction for 60 seconds.

2.7.3.7. IRMA of free PSA

Polypropylene tubes coated with MAb66 were used for the IRMA of free PSA. ^{125}I-MAb30 was used as the tracer.

The typical protocol is shown below.

Set up the coated tubes in duplicates. Add the following reagents.

Standard/Samples/QC	200 µL
^{125}I-MAb30 Tracer	300 µL

Vortex tubes and place on a rotary mixer for 16–18 hours at R.T.

Add 1 mL washing solution (0.1% Triton X-100 in 50 mM phosphate buffer, pH 7.4) and vortex well.

Decant the supernatant and repeat the washing step.

Count the bound fraction in the tubes for 60 seconds.

2.7.4. RESULTS

2.7.4.1. Total PSA IRMA based on Magnetic Particles

2.7.4.1.1. Assay Performance

The results obtained following the typical protocol for IRMA of t-PSA are tabulated in Table I and depicted in Figure 1.

2.7.4.1.2. Detection Limit

The minimum detection limit of the developed IRMA for total PSA using MAb coupled to magnetic particles was estimated to be 0.38 ng/mL.

TABLE I. RESULTS OF TOTAL PSA IRMA BASED ON MAGNETIC PARTICLES

PSA concentration (ng/mL)	Mean Count rates (CPM)
0	187
1.3	449
6.8	1181
16	2453
30	5326
60	12243
100	17757
200	25620

2.7.4.1.3. Recovery of added PSA

Two individual patients' sera having PSA level between 1 to10 ng/mL were used for testing the recovery of added standards in the assay. Four different concentrations of PSA, namely 5, 10, 20 and 30 ng/mL, were then added to aliquots of each patients' serum and analysed for total PSA content and the recovery of added standard was calculated. The recovery ranged from 96.6 to 111.1% with a mean of 102.6%.

2.7.4.1.4. Parallelism Testing

Two sera with PSA concentrations above 100 ng/mL were serially diluted and analysed in the t-PSA IRMA developed. These values on plotting gave a response curve parallel to the standard curve as depicted in Figure 2. This suggests identity of behaviour between serum samples and the standard matrix in the t-PSA IRMA developed using magnetic particles.

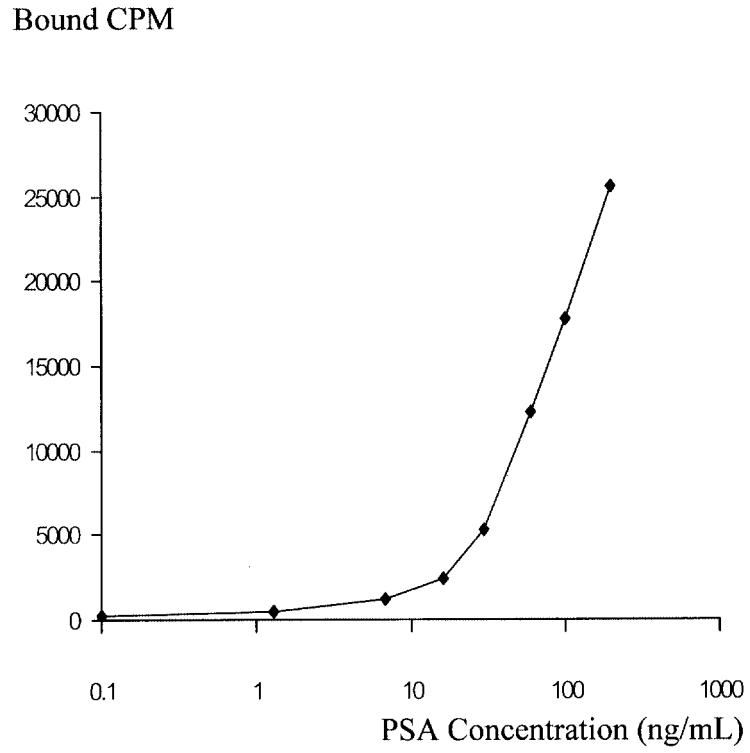

FIG. 1. Typical Standard Curve of t-PSA IRMA based on Magnetic Particles

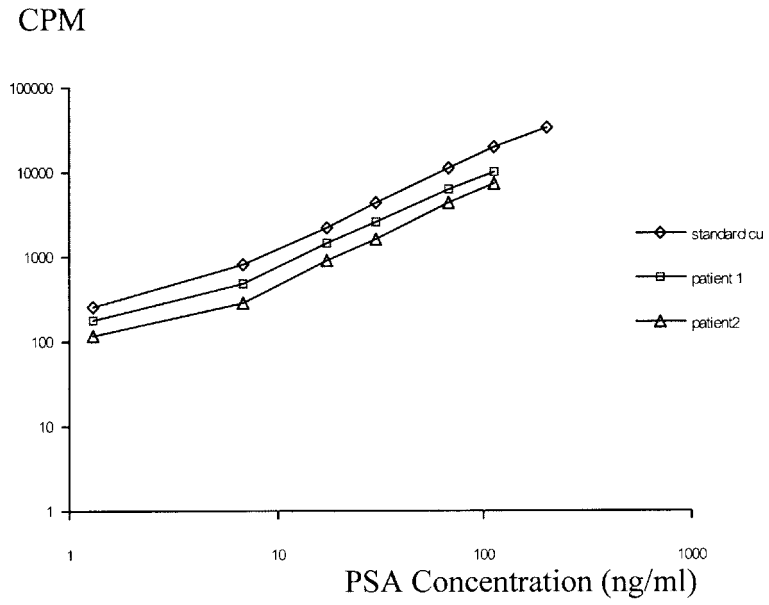

FIG. 2. Parallelism between samples (79.57 and 112.79 ng/mL) and standards exhibited by the in-house IRMA system for t-PSA

2.7.4.1.5. Assay Precision

The assay precision was estimated by analysing three serum samples 20 times in one assay on 20 consecutive days. The within assay and between-assay coefficients of variation were 3.8–15.9% and 5.9–14.6%, respectively. Precision data was obtained over the range of 1.28–23.96 ng/mL (Table II).

TABLE II. PRECISION OF THE t-PSA ASSAY BASED ON MAGNETIC PARTICLES

Within-assay precision			
Sample	Number of replicates	Mean Value (ng/mL)	CV (%)
1	20	1.44	15.9
2	20	4.16	3.8
3	20	23.03	4.2
Between-assay precision			
Sample	Number of duplicate determination	Mean Value(ng/mL)	CV(%)
1	10	1.28	14.6
2	10	3.88	9.7
3	10	23.96	5.9

2.7.4.1.6. Comparison with commercial kit

Clinical samples were obtained from Chulalongkorn Hospital with the value of each sample analysed by IRMA Kits from Cis Bio International. PSA levels in these patient sera were then determined by the in-house IRMA system. Results were compared by regression analysis. The regression equation was Y (CIS Kit) = 0.7989 X (In-house Kit) + 0.8034 (n=83). The correlation coefficient (r = 0.9693) reflects very good agreement between the two kits.(Fig.3)

2.7.4.2. Total PSA IRMA based on Coated Tubes

2.7.4.2.1. Assay Performance

The results obtained following the typical protocol for coated tube IRMA of t-PSA are tabulated in Table III and depicted in Figure 4.

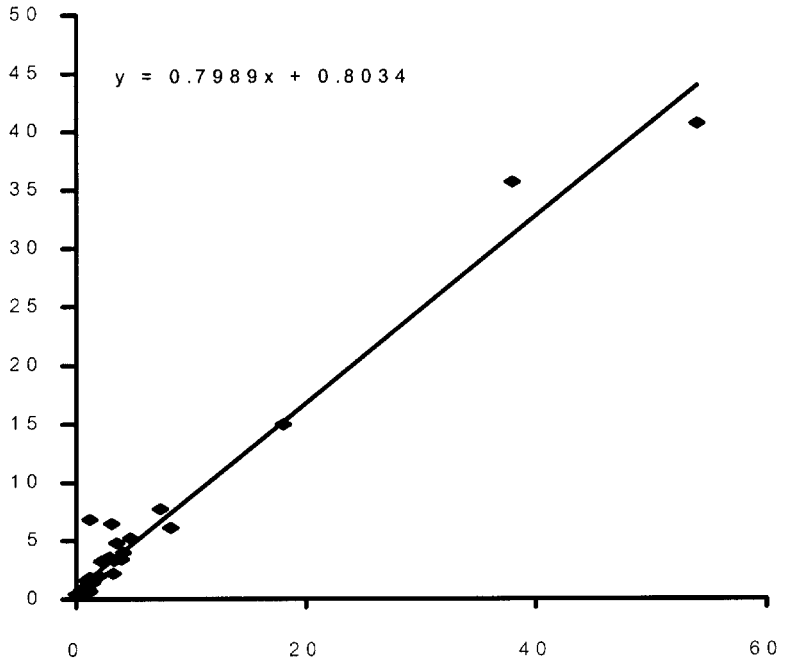

FIG. 3. Correlation between Commercial Kit and OAEP kit for t-PSA (magnetic particles)(n=83).

TABLE III. RESULTS OF TOTAL PSA IRMA BASED ON COATED TUBES

PSA concentration (ng/mL)	Mean count rates (CPM)
0	104
2	737
6.8	4289
15	9581
28	22425
54	39891
90	48513
180	60946

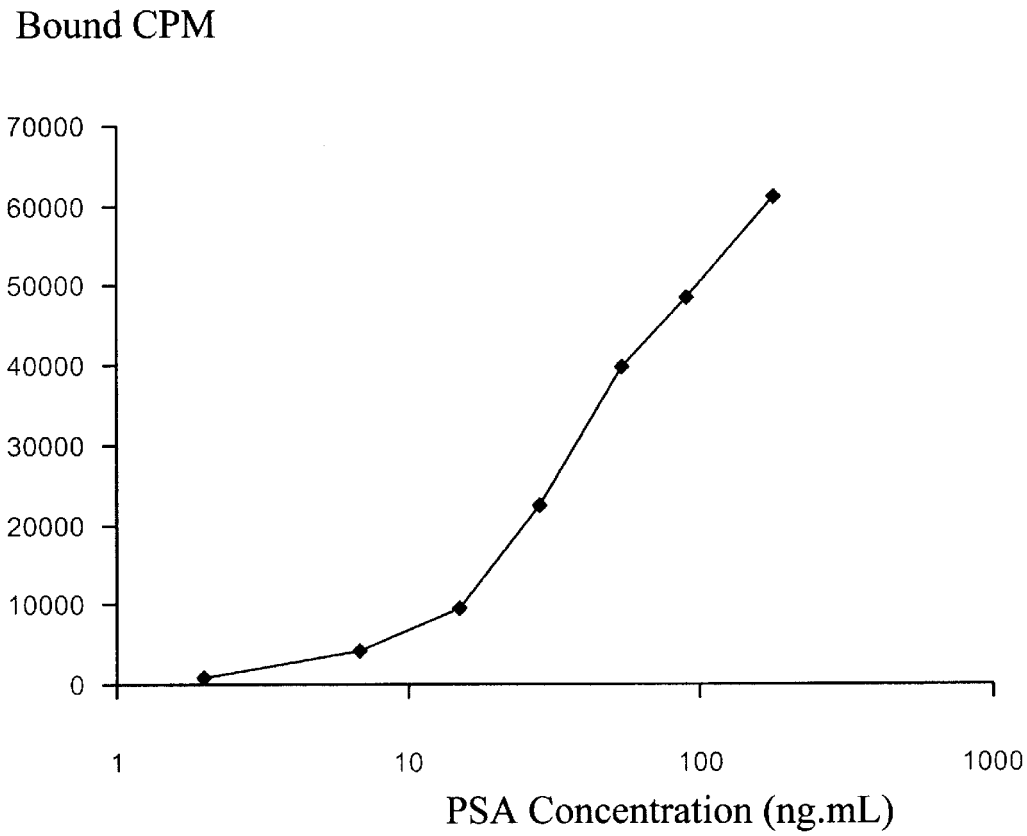

FIG. 4. Typical Standard Curve of t-PSA IRMA based on Coated Tubes.

2.7.4.2.2. Detection Limit

The minimum detection limit of the developed IRMA for total PSA using MAb coated on polypropylene tubes was estimated to be 0.05 ng/mL.

2.7.4.2.3. Recovery of added PSA

Three serum samples (PSA concentration 1.56, 3.45 and 5.02 ng/mL) were assayed in duplicate with and without the addition of PSA (5, 10, 20 and 50 ng/mL). The recovery of added PSA was found to be between 96.30 and 103.95%.

2.7.4.2.4. Parallelism Test

Two sera with PSA concentrations of 79.57 and 112.79 ng/mL were serially diluted 1:2, 1:4, 1:8, 1:16 and 1:32 folds with 'Zero" standard and analysed in the t-PSA IRMA developed. These values on plotting gave a response curve parallel to the standard curve as depicted in Figure 5. This suggests that the dilution did not affect the assay results and serum samples and the standards behaved identically in the t-PSA IRMA developed using coated tubes.

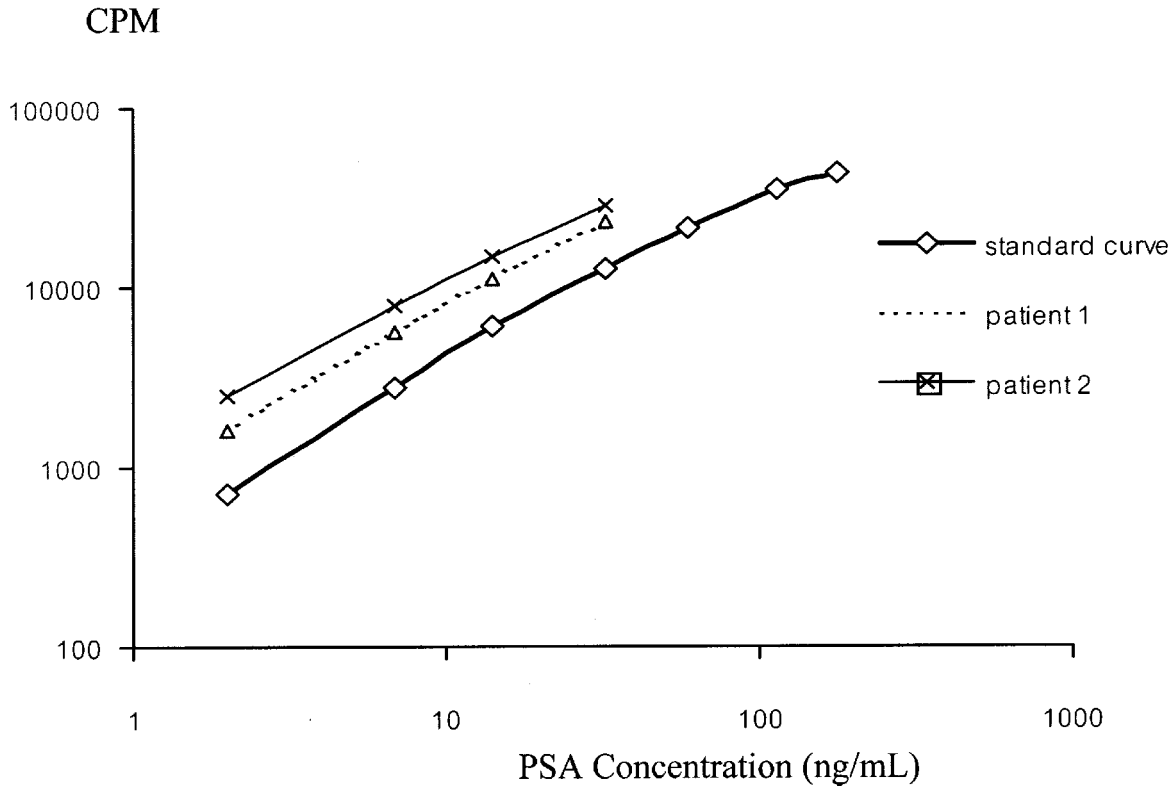

FIG. 5.

2.7.4.2.5. Assay Precision

The assay precision was estimated by analysing three serum samples 20 times in one assay on 20 consecutive days. The within assay and between-assay coefficients of variation were 4.27–8.0% and 8.0–12.30%, respectively. Precision data was obtained over the range of 2.7–22.0 ng/mL (Table IV).

2.7.4.2.6. Comparison with commercial kit

Clinical samples were obtained from Chulalongkorn Hospital with the value of each sample analysed by IRMA Kits from Cis Bio International. PSA levels in these patient sera were then determined by the in-house IRMA system. Results were compared by regression analysis. The regression equation was Y (CIS Kit) = 1.10 X (In-house Kit) – 0.4795 (n=63). The correlation coefficient (r = 0.9853) reflects very good agreement between the two kits. (Fig. 6).

TABLE IV. PRECISION.OF THE t-PSA ASSAY BASED ON COATED TUBES

Within-assay precision

Sample	Number of replicates	Mean Value (ng/mL)	CV (%)
1	20	2.7	6.3
2	20	4.5	8.0
3	20	22.0	4.3

Between-assay precision

Sample	Number of duplicate Determination	Mean Value (ng/mL)	CV (%)
1	10	2.7	8.0
2	10	4.9	12.3
3	10	21.4	10.9

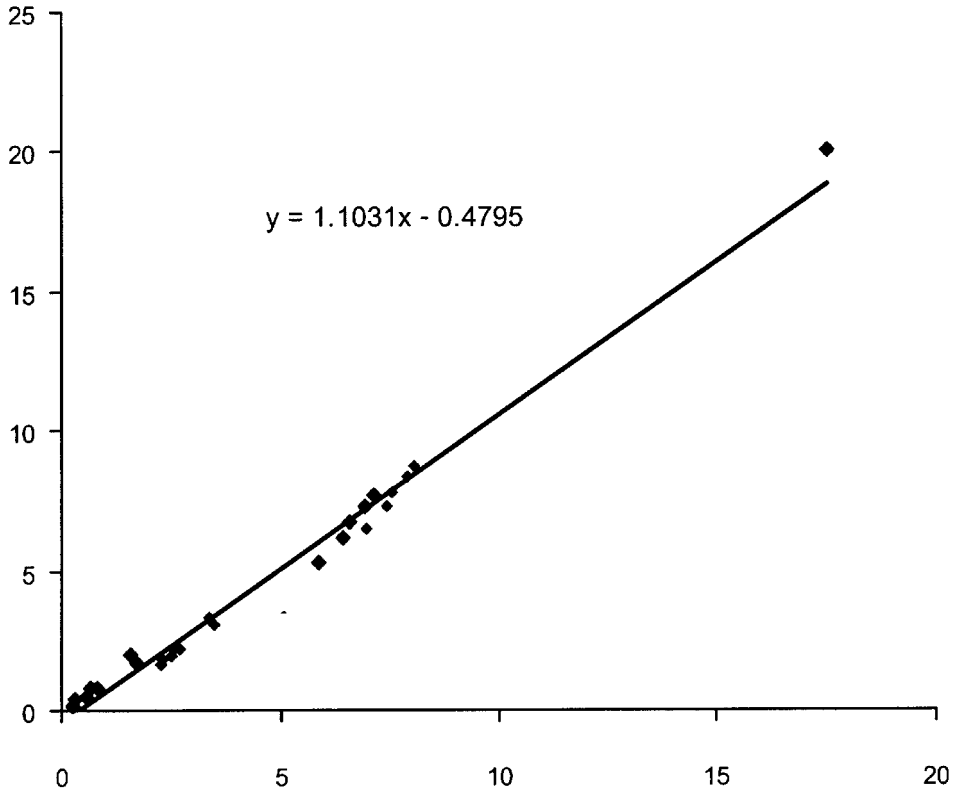

FIG. 6. Correlation between Commercial Kit and OAEP kit for t-PSA (coated tubes).

2.7.4.3. Free PSA IRMA based on Coated Tubes

2.7.4.3.1. Assay Performance

The results obtained following the typical protocol for IRMA of f-PSA are tabulated in Table V and depicted in Figure 7.

TABLE V. RESULTS OF FREE PSA IRMA BASED ON COATED TUBES

PSA concentration (ng/mL)	Mean Count Rate (CPM)
0	317
0.25	447.6
0.5	679.4
1	1006.5
2	2133
5	5425.3
10	9711.9
20	18765.9

2.7.4.3.2. Detection Limit

The minimum detection limit of the developed IRMA for free PSA using MAb coated on polypropylene tubes was estimated to be 0.06 ng/mL.

2.7.4.3.3. Recovery of added PSA

Two serum samples (PSA concentration 0.07 and 0.08 ng/mL) were assayed in duplicate with and without the addition of PSA (0.15, 0.4, 0.9, 4.9 and 9.9 ng/mL). The recovery of added PSA was found to be between 74.3 and 126.5%.

Note: The recovery experiment was done in real patient samples without sample manipulation to prevent the complexation of the PSA and anti-chymotrypsin. The results obtained were not satisfactory perhaps due to the presence of binding substance in serum samples and the dissociation of PSA complex.

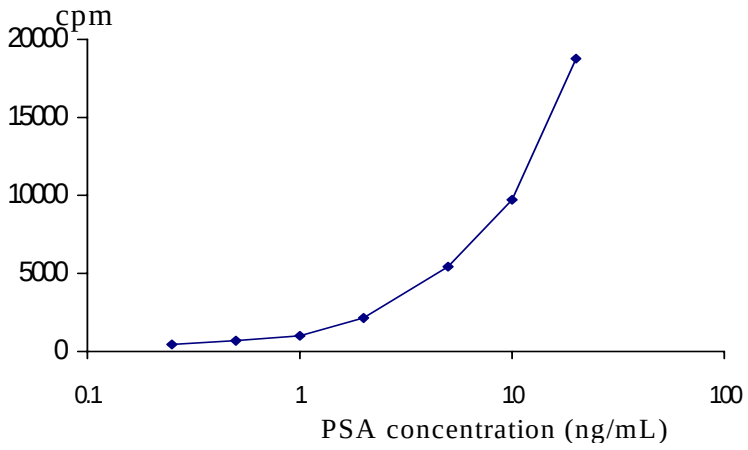

FIG. 7. Typical Standard Curve of f-PSA IRMA based on Coated Tubes.

2.7.4.3.4. Parallelism Testing

Two sera with PSA concentrations of 8.30 and 9.12 ng/mL were serially diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 folds with 'Zero" standard and analysed in the f-PSA IRMA developed. These values on plotting gave a response curve parallel to the standard curve as depicted in Figure 8. This suggests that the dilution did not affect the assay results and serum samples and the standards behaved identically in the f-PSA IRMA developed using coated tubes.

2.7.4.3.5. Assay Precision

The assay precision was estimated by analysing three serum samples 20 times in one assay on 20 consecutive days. The within assay and between assay coefficients of variation were 4.41–7.73% and 8.24–12.04% respectively. Precision data were obtained over the range of 0.65–6.19 ng/mL serum PSA. (Table VI)

TABLE VI. PRECISION.OF THE f-PSA ASSAY BASED ON COATED TUBES

Within-assay precision

Sample	Number of replicate	Mean Value (ng/mL)	CV (%)
1	20	0.68	4.41
2	20	2.66	6.05
3	20	5.95	7.73

Between-assay precision

Sample	Number of duplicate Determination	Mean Value (ng/mL)	CV (%)
1	20	0.65	9.23
2	20	2.74	12.04
3	20	6.19	8.24

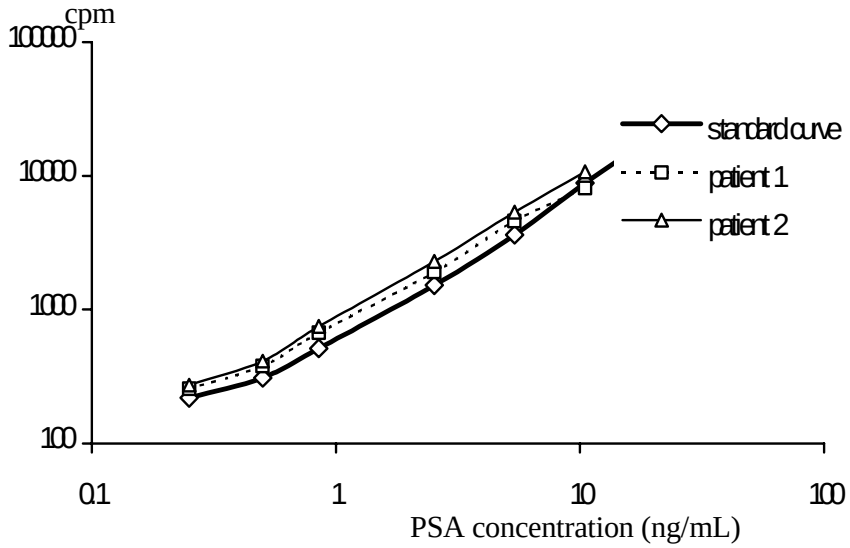

FIG. 8. Standard curves of f-PSA IRMA based on Coated Tubes; Parallelism between samples and standards.

2.7.5. CONCLUSIONS

IRMA procedures for total as well as free PSA measurement in human sera could be optimised and kits formulated using appropriately matched MAbs supplied by University of Alberta, Edmonton, Canada.

Two kinds of IRMA kits were developed for total PSA. Both systems are shown to be rapid, reliable and have good sensitivity, precision and working range. All essential parameters are given below to compare their features and performance.

	Magnetic Particles	Coated tubes
Parameters of the procedure		
Sample volume	50 µL	50 µL
Incubation	0.5 h + 0.5 h at RT	2 h + 2 h at RT
Standard range	0–200 ng/mL	0–180 ng/mL
Amount of antibody per assay tube	0.3 µg	1.0 µg
Test characteristic		
Sensitivity	0.38 ng/mL	0.05 ng/mL
Recovery	96.6–111.1%	81.4–107.7%
Precision		
Within-assay	4.2–15.9%	4.3–8.0%
Between-assay	5.6–14.6%	8.0–12.3%
Correlation with commercial kit		
Regression equation	y = 0.7989x + 0.8034	y = 1.1031x–0.4795
Correlation coefficient	0.9693	0.9853

(x = In-house method and y = Reference method (CIS bio international))

The free PSA assay developed is based on coated tubes and involves one-step overnight incubation at ambient temperature. The optimization and validation of the f-PSA IRMA yielded a kit which allows the measurement of f-PSA in 200 µL samples at concentrations ranging from 0–20 ng/mL with a sensitivity of 0.06 ng/mL. The assay exhibited good precision (8.24–12.04% for inter-assay and 4.41–7.73% intra-assay) and reasonable recovery and accuracy.

These systems developed at the OAEP are simple, rapid and inexpensive. Its introduction will lead to a routine clinical service with increased laboratory efficiency while better patient management could be obtained without the penalty of increased costs. Determination of free/total PSA ratio may help to distinguish benign prostatic hypertrophy (BPH) from cancer.

BIBLIOGRAPHY

CHAN, D.W., “PSA as a marker for prostatic cancer”, Laboratory Management. (1988) 35–39.

PARTIN, A.W., OESTERLING J.E., The clinical usefulness of prostate specific antigen: update 1994 (Review Article), J. Urol. **152** (1994) 1358–1368.

ARMBRUSTER, D. A., Prostate-Specific Antigen: Biochemistry, Analytical Methods, and Clinical Application (Review), Clin. Chem. **39** (1993) 181–195.

OESTERLING J.E., Prostate Specific Antigen: A critical assessment of the most useful tumour marker for adenocarcinoma of the prostate (Review Article), J. Urol. **145** (1991) 907–923.

SEAMONDS, B., et al., Evaluation of prostate-specific antigen and prostatic cancer markers, Urology **28** (1986) 472–479.

HOWARD, C. B., et al., Ultrasensitive Radioimmunoassay of Prostate-Specific Antigen, Clin. Chem. **38** (1992) 735–742.

JOHNSTONE, A., THORPE, R., “Radiolabelling techniques”, Immunochemistry in practice (second edition), Blackwell Scientific Publications, London (1987) 113–121.

PATERSON, K.,et al.,Free and complexed Prostate Specific Antigen(PSA): *In vitro* stability, Epitope Map, and Development of Immunofluorometric Assays for Specific and Sensitive Detection of Free PSA and PSA-antichymotrypsin Complex, Clin Chem. 41/10(1995) 1480–1488.

KLEE, G.G., et al., Development of a highly sensitive immunochemiluminometric assay for prostate-specific antigen, Urology **44** (1994) 76–82.

BANGMA, C.H., et al., The free-to-total prostate-specific antigen ratio improves the specificity of prostate-specific antigen in screening for prostate cancer in the general population, J. Urol., **157** (1997)2191-2196.

SURESH, M.R., "Cancer markers", The Immunoassay Handbook (WILD, D., Ed.), Stockton Press, New York (1994) 452–453.

JOHNSTONE, A., THORPE, R., "Radiolabelling techniques", Immunochemistry in Practice (second edition), Blackwell Scientific Publications, London (1987) 113–121.

PATERSON,K.,et al.,Free and complexed Prostate Specific Antigen(PSA): *In vitro* stability, Epitope Map, and Development of Immunofluorometric Assays for Specific and Sensitive Detection of Free PSA and PSA-antichymotrypsin Complex, Clin Chem. 41/10(1995) 1480–1488.
http://www.arup-lab.com/guides/clt/tests/clt_p40.htmL

ANNEX I

IODINATION OF ANTI PSA MONOCLONAL ANTIBODY BY CHLORAMINE T

Method

The anti-PSA MAbs (MAb10 or MAb30) were labelled with radioiodine by chloramine-T method of Alan Johnstone & Robin Thorpe with slight modification that the purification was carried out by HPLC system. The details of this method are given below.

500 μCi $Na^{125}I$ was added to 20 μL (20 μg) MAb10 (Total PSA) or MAb30 (Free PSA), 5 μl 0.5 M phosphate buffer, 10 μg chloramine T (10 μL in 0.05 M phosphate buffer). The mixture was mixed by vortex mixer for 20 sec. Then 10 μg sodium metabisulphite (10 μL in 0.05 M phosphate buffer) was added to the reaction tube followed by addition of potassium iodide (100 μl, 10 mg/mL). The mixture was then applied to the HPLC column and eluted under the following conditions.

Column ; Biosep sec-S3000 size 300 × 7.80 mm
Detector ; Gamma detector
Mobile phase ; 0.1 M phosphate buffer pH 7.4
Flow rate ; 0.6 mL/min

60 fractions (30 sec. per fraction) were collected and monitored for the activity using gamma counter. PSA tracer is eluted in the first peak and free iodide component in the second peak (Fig. 9). The fractions corresponding to the PSA peak were pooled and diluted to give a radioactive concentration of 20 μCi/mL with 0.1 M phosphate buffer, pH 7.4 containing 1% BSA and stored at 4°C till use.

Assessment

1. Purity of radiolabelled MAb was checked by paper electrophoresis. About 10 μL of radiolabelled MAb was loaded onto the paper and electrophoresis was carried out using a potential of 10 Volt/cm for approximately 60 minutes. The purity (%) was calculated as,

$$\% \text{ Purity} = \frac{\text{Peak count (CPS)} \times 100}{\text{Total count (CPS)}}$$

2. The specific activity of the radioiodinated MAb was calculated as,

$$\text{Specific activity (μCi/μg)} = \frac{\text{\% Iodination yield} \times \text{Total activity of } ^{125}\text{I (μCi) applied}}{\text{Total amount of antibody (μg)}}$$

3. Stability of the radiolabelled MAb was studied over a period of 8 weeks. The results are shown in Tables VII and Fig. 10.

Typically, yield of ~ 71%, purity of ~ 94% and specific activity of 17.5 μCi/μg were achieved.

TABLE VII. STABILITY OF RADIOLABELLED ANTI PSA MAB

Week	1	2	3	4	5	6	7	8
% B/T min.	0.17	0.15	0.18	0.14	0.16	0.16	0.18	0.11
% B/T max.	56.42	54.34	47.12	45.31	46.25	43.65	41.83	39.15

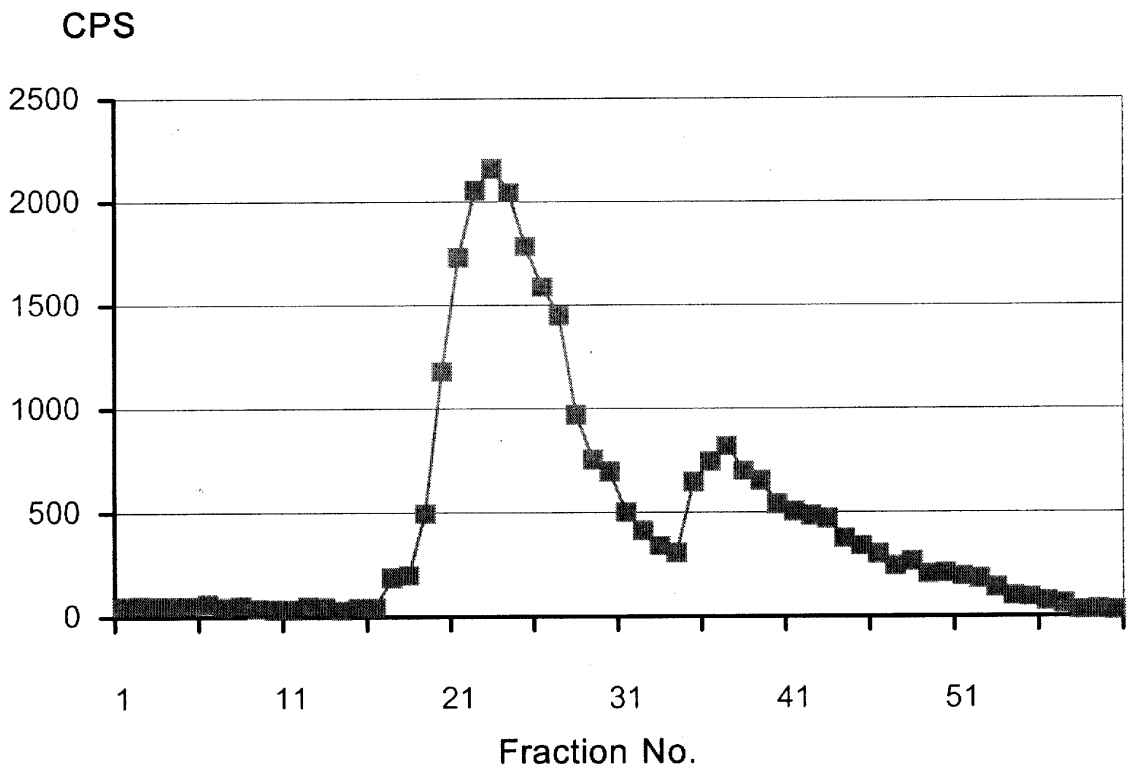

FIG.9. HPLC Elution Pattern of the Radioiodinated Mab.

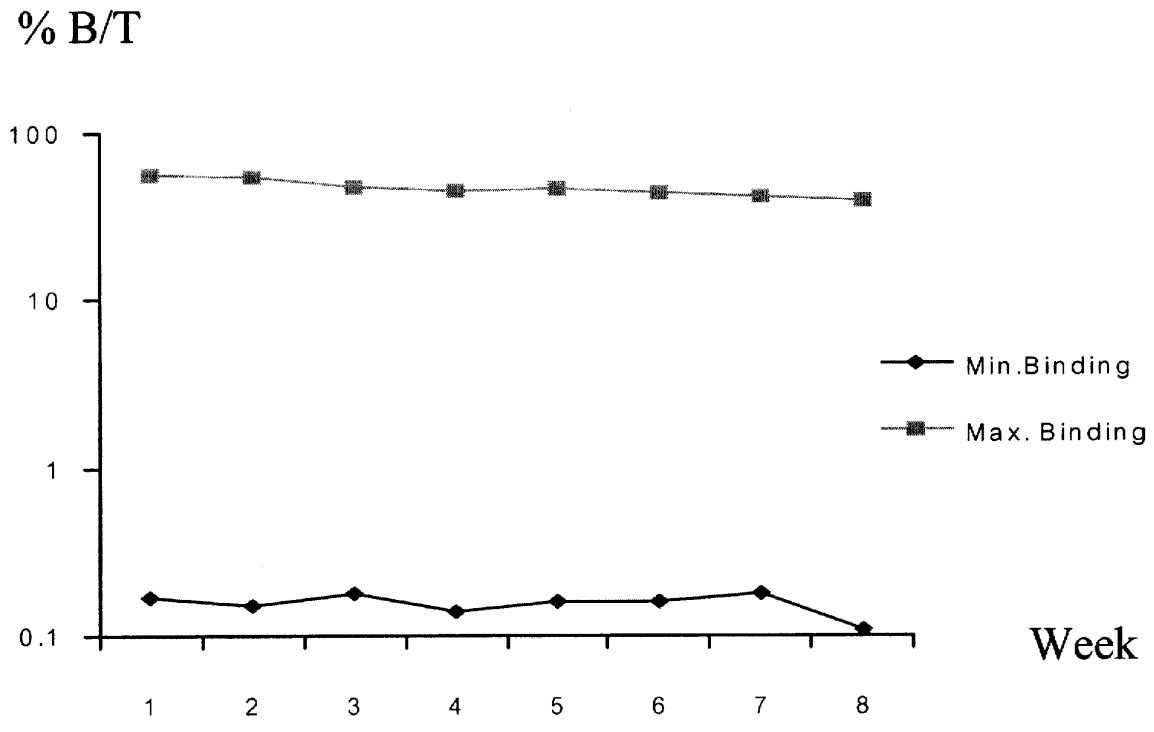

FIG.10. Stability of the Radioiodinated Mab.

ANNEX II

PREPARATION OF MAGNETIZABLE PARTICLES COUPLED TO ANTI PSA MONOCLONAL ANTIBODIES

1. Activation of magnetic cellulose

Magnetic particles M104 (SCIPAC,UK) were dispersed by gentle mixing on the rotator for 30 minutes at room temperature. 20 mL (1 gm) suspension was immediately pipetted out. Particles were sedimented on magnetic block and supernatant was aspirated. The particles were then washed with 20%, 60% and 100% acetone sequentially. Finally the volume was adjusted with acetone to 10 mL and 250 mg 1,1' — carbonyldiimidazole was added. The suspension was mixed gently for 1 hour at room temperature. The activated particles were washed sequentially with 100%, 60% and 20% acetone and finally with distilled water.

2. Coupling of antibody to activated magnetic cellulose

The particles were resuspended in 10 mL of borate buffer with 0.625 mg anti PSA MAb66 (University of Alberta). The reactants were mixed gently on the roller mixer at room temperature for 36–48 hours. Particles were washed with following solutions, mixing 20 minutes each time.

1. Triple wash with sodium bicarbonate, 0.5 M, pH 8.0
2. Sodium acetate, 0.1 M, pH 4.0, rotate to mix for1 hour.
3. Sodium acetate, 0.1 M, pH 4.0, rotate to mix overnight.
4. Triple wash with phosphate buffer, 0.05 M, pH 7.4, containing 0.1% sodium azide.
5. Finally suspend the adsorbent in 20 mL phosphate buffer 0.05 M, pH 7.4 and store at 2–8°C until required. The final concentration is 50 mg/mL.

2.8. URUGUAY

Title of the Project:	Development of Immunometric assays for Free and Total Serum PSA
Chief Scientific Investigator:	Ana Robles
Scientific Co-Investigators:	H. Balter, A. Lopez, Z. Gonçalvez and J. Berbejillo

Abstract. Total and free PSA immunoradiometric assays were developed using the appropriate matched pair of monoclonal antibodies supplied in the project. Anti-PSA MAb recognizing the intact molecule (MAb66) was used as the capture antibody by immobilization to polystyrene tube walls. Another MAb that recognises the intact molecule in an equimolar way (Mab M10) was used as the tracer MAb for total PSA assay. Free PSA assay used as tracer a MAb that recognises the ACT binding site of the PSA molecule (Mab M30). By means of these assays, normal ranges and minimum detectable doses were assessed. Inter-assay reproducibility was determined by means of control sera at 3 levels of PSA concentrations. The specificity of the antibodies employed for the PSA-ACT complex was ascertained. MAb tracers could be prepared in high purity, reasonable specific activity and yields. The in-house assays optimised with respect to the various reaction parameters exhibited the minimum detectable dose of 0.5 ng/mL (CV 70%) for total PSA and 0.2 ng/mL for free PSA (CV 35%). The correlation of the sample values of patient samples estimated by the in-house assays and commercial reference assay were very good in the case of total PSA (in year 1999 r^2=0.905; n=55 and in year 2000 r^2=0.935, n=43) and moderate in the case of free PSA (in year 1999 r^2=0.812; n=55 and in year 2000 r^2=0.59 n=30). For the samples evaluated, the free/total ratio was 0.2±0.2 (n=128). Of these, 34% samples fell in the range 0–4 ng/mL of tPSA, 32% in the range of 4–10 ng/mL, 16% in the range of 10–20 ng/mL and 17% over 20 ng/mL. Correlation of sample values of prostate cancer patients undergoing pain relief therapy with ^{188}Re at the university hospital with a reference commercial system is being carried out. In summary, with the bulk reagents provided by the IAEA and the strategy developed in our institution we have optimised unique IRMA systems for total and free PSA measurements. These employ a unique solid matrix coated with a capture antibody, a unique set of standards with two sets of calibration values for the two analytes evaluated and two different tracers.

2.8.1. INTRODUCTION

Prostate Specific Antigen, molecular weight ~34 kDa, is secreted into the prostatic plasma semen in milligram amounts while it is present in much lower amounts in blood. There are two biologically active species of PSA, the free molecule and PSA bound to serum proteins mainly anti-chymotrypsin (ACT). PSA-ACT complex accounts for >80% of the total PSA. Prostatic cancer produces aberrant amounts of PSA while in benign hyperplasia the ratio of free to total PSA is kept within a preset range. Thus the measurement of total PSA and free unbound PSA could aid in the differentiation of benign hyperplasia from prostatic cancer.

Immunometric assays are generally used for measurement of such analytes. In the case of PSA, the assays will need to be carefully designed with the right pair of antibodies that recognise the appropriate epitopes on the PSA molecule so that the total PSA assay is equimolar. Many commercial assays overestimate free PSA basically because of the changes in the complex form with time, and hence result in poor clinical specificity for discrimination between the two pathologies.

It has been observed that the free unbound PSA is very stable even in high saline buffer. But for maintaining the identity of matrix between the patient sample and the standard used, the use of serum matrix could cause time dependant changes in the amounts of free form. Generally, the potency is lowered. Kinetic study of this PSA-ACT complex must be carried out to use this species as reference material. Thus short incubation times with sample, addition of matrix buffer on the reaction vial instead of preparing the standard in this matrix have been devised to overcome this species variation.

The objective of the present work was to optimise a combined system to evaluate PSA in its two major biologically active forms, namely total PSA and free PSA. We have developed assays for total and free PSA using the matched pairs of reagents and PSA supplied as a part of the project. These assay systems were validated by comparison with commercial assays. Patients from a reference population and others from pain relief therapy with ^{188}Re were evaluated using these systems.

2.8.2. MATERIALS AND METHODS

Matched pairs of anti-PSA MAbs MAb66 (capture MAb-3 batches of 5 mg enough for 5000 tubes each), MAb10 (tracer MAb for t-PSA, with equimolar response — 2 batches of 0.5 mg each), MAb30 (tracer MAb for f-PSA, recognizing ACT binding site of PSA molecule. — 2 batches of 0.5 mg each), PSA (1.3 mg 70% purity calibrated against a reference gold standard) were supplied by Dr M.Suresh, Univ. of Alberta, Canada.

^{125}I-NaI was purchased from CIS (France). NUNC Maxisorb star tubes 12×75 mm were used for coating the MAbs. Q.C samples were from BIORAD and some were in-house prepared. Commercial calibrators for standard PSA were purchased to check the potency.

0.1 M bicarbonate buffer, pH 9.4–9.6 was used for coating the tubes; 0.05 M phosphate buffer, pH 7.5, with 0.2% BSA, 0.16% NaCl and 0.01% Sodium azide was used as the working assay buffer; 0.05 M phosphate buffer, pH 7.5 with 0.05% Tween-20 was the washing buffer.

2.8.2.1. Reconstitution of reagents

Capture MAbs received in the lyophilized form were reconstituted in (1:1) Glycerol/Saline. For radioiodination, the tracer MAbs were reconstituted in saline and used at 10–20 µg in 5–10 µL. The standard PSA stock solution was prepared by dissolving the content of the vial to a final concentration of 1 mg/mL in sterile saline. The solution was accurately dispensed in fractions of 0.1 mL and kept at –30°C in a tightly closed container. Working standard solutions of potencies from 0.01ng/mL to 122 ng/mL were prepared from this stock solution in 0.05 M phosphate buffer with BSA 10% and 0.05% sodium azide valid for at least 6 months.

2.8.2.2. Labeling with ^{125}I

Monoclonal antibodies (MAb10 / MAb30), 10 µg in 10µL, was placed in an eppendorf conical tube with cap (1.5 mL capacity) with 10µL of 0.5 M phosphate buffer. 18.5 MBq (500 µCi) of ^{125}I (as NaI in 1–5 µL of 0.01 M NaOH) was added to this tube followed by 1.2 µg of chloramine-T, the oxidant. After approximately 1 minute of reaction during which the mixture was vortex mixed several times, the mixture was loaded on a short PD-10 column for purification of the tracer by gel chromatography. MAb66 was also labelled with ^{125}I for use as tracer to optimise the coating procedure. 30 µg of MAb66 and 10 MBq of ^{125}I were used for labelling.

An aliquot of the radioiodination reaction mixture was used for estimation of the radioiodination yield. In brief, a small aliquot of the reaction mixture was placed in a vial containing 0.1 mL of BSA (50 mg/mL); 0.9 mL Trichloroacetic acid (20%) was added to this, vortex mixed and centrifuged for 3 minutes at 3000 rpm. The supernatant and precipitate were separated and counted in a well counter. The percentage activity in the precipitate was taken as the radioiodination yield.

2.8.2.3. Immobilization of capture MAb on the solid phase

MAb66 was diluted to a concentration of 5 µg/mL in coating buffer and 0.2 mL (1 µg/tube) of this solution was added into the NUNC Maxi-sorb star tubes. The tubes were incubated overnight at room temperature and the solution was aspirated. The surface was then treated with BSA to block any adsorbing site and glazed with sucrose. Washing with buffer or non-ionic detergent was avoided.

2.8.2.3.1. Optimization and evaluation of the coating procedure by radiometric method

In order to evaluate the amount of MAb actually tagged to the solid phase, MAb66 labelled with ^{125}I was used as the tracer. The coating solution of the MAb at 5 µg/mL was prepared with a small amount of tracer (approximately 300000 cpm/mL, Specific activity 60000 cpm/µg). Tubes were coated with MAb solution under various conditions. At the end of the incubation, the content of the tubes was aspirated and washed as usual. The percent of activity retained in the tubes was used to calculate the amount of MAb tagged to the wall.

2.8.2.4. Optimization of the assays

The immunoradiometric assays for total and free PSA were optimized with respect to various parameters such as reaction time, temperature, sample volume, standard matrix, rotation during reaction and wash buffer. In the optimized procedure, tubes were set in duplicate, into which 200 µL of working buffer, standard PSA, samples and control sera were added. The tubes were rotated for 2 hours at room temperature after which the contents of the tubes were aspirated. The tubes were washed twice with wash buffer taking care to avoid foaming. Tracer MAb10-^{125}I or MAb30-^{125}I was added equivalent to 2400 Bq in 200 µL and left under rotation overnight at room temperature. After measurement of total activity, the contents of the tubes were aspirated and washed twice. Activity in the tubes was measured for 1 minute in a well-type gamma counter.

2.8.2.4.1. Validation of the in-house PSA standards using commercial calibrators

Standard PSA was prepared in-house from seminal plasma containing free form of PSA. These standards were assayed in parallel with other commercial PSA preparations from CIS, NETRIA and DPC. The values obtained were correlated by simple linear regression.

2.8.2.5. Shelf life of the primary reagents

The radiolabeled tracers, PSA calibrator as well as coated tubes were studied for their shelf life under various storage conditions. Storage conditions and matrix were selected to obtain the maximum storage stability and the best shelf life possible.

2.8.2.6. Analysis of QC samples and patients sera

Three levels of quality control samples were included in all assays. Patient samples from the university hospital were measured with these assays.

2.8.3. RESULTS

2.8.3.1. Radiolabeling

The specific activity of the MAb tracers was in the range of 106±65 MBq/nM with an incorporation of 1.3±0.5 atoms of ^{125}I per molecule of MAb. Labelling yields were 44±16% as estimated by trichloroacetic acid non-specific precipitation. The purification yields with short PD-10 columns were 46±25%.

2.8.3.2. Immobilization of the capture MAbs in tubes

2.8.3.2.1. Radiometric optimization of solid phase coating of MAb66

Table I presents the results from the radiometric studies using ^{125}I-MAb66. The amount of MAb bound to the tubes under various conditions of coating are given here.

TABLE I. OPTIMIZATION OF THE COATING PROCEDURE

Reaction conditions	Amount of MAb66 (µg/tube)	SD	CV%
Initial amount taken	0.979	0.008	0.8
1 hr, RT, no rotation	0.051	0.006	11.3
1 hr, RT, with rotation	0.053	0.004	8.3
1 hr, 37°C, no rotation	0.048	0.002	4.2
Overnight, RT, no rotation	0.054	0.003	5.2
Overnight, RT, with rotation	0.075	0.009	12.3

TABLE II. STABILITY OF THE COATED TUBES — BINDING STUDIES

Tracer used	MAb10-^{125}I - total PSA		MAb30-^{125}I- free PSA	
	% Binding to solid matrix coated with MAb66 at 1µg/piece			
PSA Conc. (ng/mL)	Tubes	Beads	Tubes	Beads
0	0.4	0.5	0.8	1.3
0.12	0.5	0.5	0.8	1.4
0.5	0.7	0.5	1.2	1.4
8.1	5.6	1.0	5.3	2.5
65	12.7	2.2	8.3	2.2

TABLE III. ASSAY BINDING AT VARIOUS REACTION CONDITIONS OF t-PSA IRMA

	Reaction Conditions					
	1 h, RT	1 h, 37°C	ON+RT + rotation	ON+RT	ON+RT+ rotation	ON+RT
Std. Vol. (µL)	50	50	10	25	50	50
Total PSA (ng/mL)						
0	0.4	0.4	0.5	0.5	0.5	0.5
0.12	0.5	0.5	0.5	0.5	0.6	0.6
1.9	2.7	2.5	1.2	2.2	3.4	3.7
61	12.4	15.8	23.4	31.7	27.4	29.3

2.8.3.2.2. Stability of the MAb66 coated tubes

Table II shows the performance of coated tubes stored for three months at –20°C in comparison with a fresh lot of activated beads prepared following the optimised coating procedure. The percentage binding at various PSA concentrations were estimated using tubes and beads. Both free and total PSA assay tracers were used for this evaluation.

2.8.3.3. Optimization of the assays

The results of total PSA IRMA employing MAb10-^{125}I tracer under various reaction conditions such as reaction time, temperature and modality are depicted in Table III. The table lists the binding% of the tracer.

Good response is seen with overnight incubation at room temperature. The sample volume and rotation during incubation do not seem to make much difference in the assay performance. However, below 25 µL sample volume, the response starts falling. Use of 50 or 25 µL sample/standard,

overnight incubation with or without rotation have yielded the best results in this study. In the optimised t-PSA IRMA, PSA standards at concentrations of 0, 0.15. 2 and 64 ng/mL were used.

Table IV shows data for free PSA IRMA, developed in-house using MAb30 as tracer. In this case it is clearly seen that, overnight incubation yields better standard curves while rotation and sample volume have little effect on the assay performance. Similarly to total PSA, 10 µL sample volume gave reduced binding. Use of 50 or 25 µL sample and standard volumes as well as overnight incubation with or without rotation have yielded the best results.

TABLE IV. ASSAY BINDING AT VARIOUS REACTION CONDITIONS OF f-PSA IRMA.

	Reaction conditions					
	1 h, RT	1 h, 37°C	ON+RT + rotation	ON+RT	ON+RT+ rotation	ON+RT
Std. Vol. (µL)	50	50	10	25	50	50
Total PSA (ng/mL)	% Binding of MAb30-^{125}I to solid matrix					
0	0.6	0.7	1.6	1.3	1.3	0.8
0.12	0.6	0.8	1.6	1.5	1.3	1.0
1.9	1.9	2.1	2.4	3.4	5.1	4.5
61	10.6	16.6	21.7	30.1	29.0	29.0

2.8.3.3.1. Calibration of the PSA standard

Free PSA from seminal plasma was used as the standard. Consensus values were obtained for total and free PSA standard preparations on calibration using standards from commercial sources such as DPC and NETRIA. Figure 1 presents various calibration curves for total PSA. All the standard curves are close and have similar slopes. Particularly, the regression lines for the in-house standard PSA preparation supplied by Dr. Suresh (CIN, Lot 09/2000), NETRIA and DPC, with slopes and correlation coefficients represented in italics in Figure 1 are nearly coincident. This shows that the standards for total PSA prepared from seminal plasma is similar to the commercial PSA standards supplied by NETRIA.

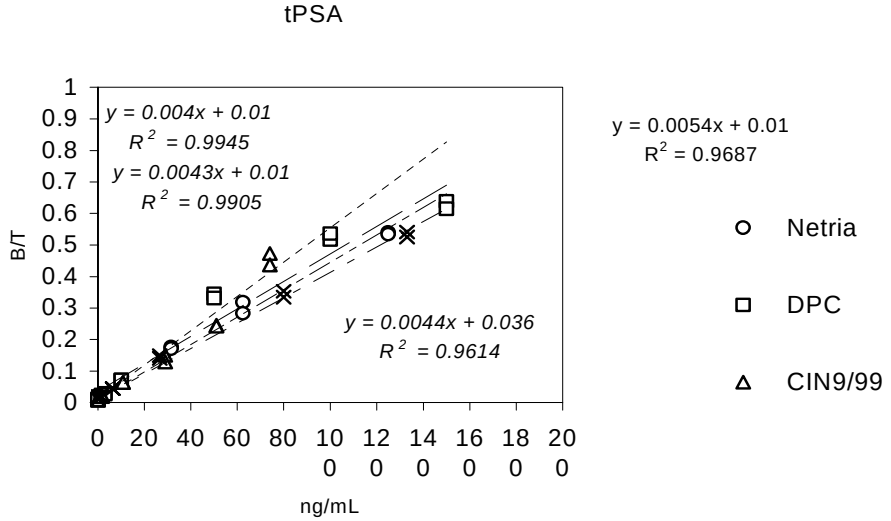

FIG. 1. Comparison and calibration of in-house PSA standards with commercial standards from NETRIA using the developed IRMA for total PSA (MAb10-^{125}I tracer, MAb66 capture).

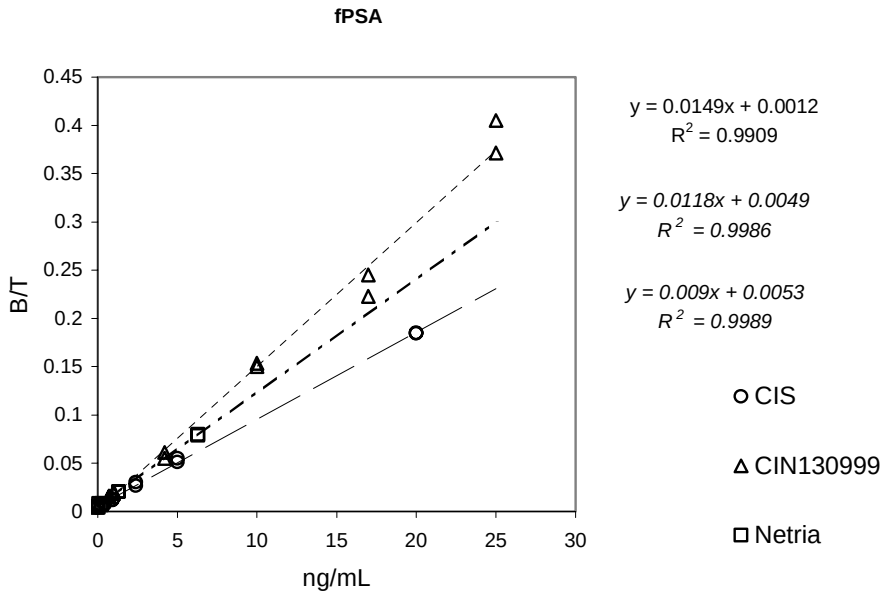

FIG. 2. Comparison and calibration of in-house f-PSA standards with commercial standards (CIS and NETRIA) using the developed IRMA for free PSA (MAb30-^{125}I tracer, MAb66 capture).

Figure 2 presents similar calibration curves for free PSA assay using MAb30-^{125}I tracer. The reference preparation (CIN) represents the free PSA present in approximately the same ratio normally present in blood. The in-house assay (CIN) standard curve regression line shows good correlation coefficient (r2=0.9909) and the slope (0.0149) is similar to the NETRIA standard curve (correlation coefficient r^2=0.9986 and slope 0.0118) in the low end region. However, at higher standard ranges there appears to be some variation.

2.8.3.4. Analysis of QC samples and patients' sera

A set of three quality control samples was used along with the analysis of samples from patients. Samples from the university hospital that had been assayed by automatic enzyme immunoassay (EIA) both for total and free PSA, were chosen to be assayed by our in-house IRMAs. These samples were of high risk patients. Initially, in the year 1999, only those samples which had been already analysed for both free and total PSA were chosen. Later, in 2000, all the samples stored at the Center were assayed by our system. Some samples, with values of total PSA below 2.5 ng/mL and samples with more than 10 ng/mL had not been evaluated for free PSA levels by EIA. We analysed total and free PSA in all of them and the average ratio was estimated by the slope of the regression line of t-PSA vs. f-PSA levels. Figures 3 and 4 give the correlation of total and free PSA values obtained in patient samples assayed by the developed in-house IRMA systems with the reference assay.

Although, the data is not very large in number, it is seen that the in-house assay for total PSA (Fig. 3) correlates well (correlation coefficient r^2= 0.935) with the reference commercial ELISA values, independently analysed at the hospital. The correlation between free PSA values estimated by the in-house system and the reference system (correlation coefficient r^2=0.59) is not as good as in the case of total PSA. This is perhaps due to a time lag between the evaluation at the University Hospital and our analysis. It is observed and reported by many practicing laboratories that free PSA levels are unstable in human serum, unlike in semen. It is hence very likely that the samples on storage have changed with respect to free PSA concentration and hence given lower values in our system. Only about 50% of the free PSA seems to be present in the free form on storage. Since the total PSA values correlate very well it could be inferred that the free form is perhaps getting complexed on storage.

Figure 5 shows the relationship between the total and free PSA values evaluated in a selected population with in-house reagents from CIN. It is seen that the values are scattered to an extent around the tendency line. However, the slope of the tendency line gives a mean ratio of 0.2 which is close to the expected ratio for free to total PSA levels in normal population.

The samples were segregated with respect to their f/t-PSA ratios and the levels of t-PSA within each group of f/t range was further analysed. Figure 6 shows the distribution of values of t-PSA for the various f/t ratio ranges in the form of a histogram. It is seen that most samples had the f/t ratio between 0 and 0.2. In this group, the distribution of t-PSA value was mostly in the lower regions of 0–4 and 4–10 ng/mL, as expected. However, significant fractions of samples did show values in the higher zones of 10–20, 20–100 and >100 ng/mL, which may need further clinical interpretation. One of the reasons could be the nature of samples, which are from high risk patients already visiting the hospital. Some patients were on treatment too for prostate cancer. On the other hand, in the f/t ratio range >0.2, the t-PSA levels are mostly within the 0–4 and 4–10 ng/mL ranges.

Figure 7 presents the QC performance for in-house total PSA IRMA at three concentration levels in the various assays carried out during the period involved. Figure 8 shows similar results for the in-house free PSA IRMA. It can be seen that in both the cases, at high PSA levels the QC values are within reasonable limits while at low PSA levels the variations are high.

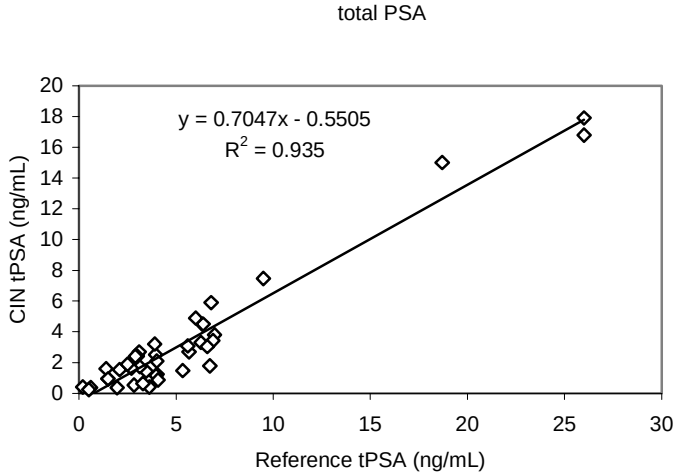

FIG. 3. Correlation of the total PSA values in patient samples: in-house IRMA with ref. ELISA.

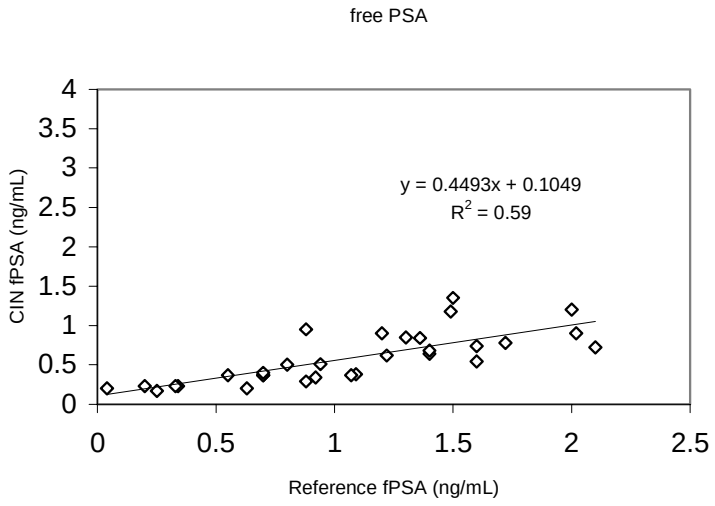

FIG. 4. Correlation of free PSA values in patient samples: in-house IRMA with ref. ELISA.

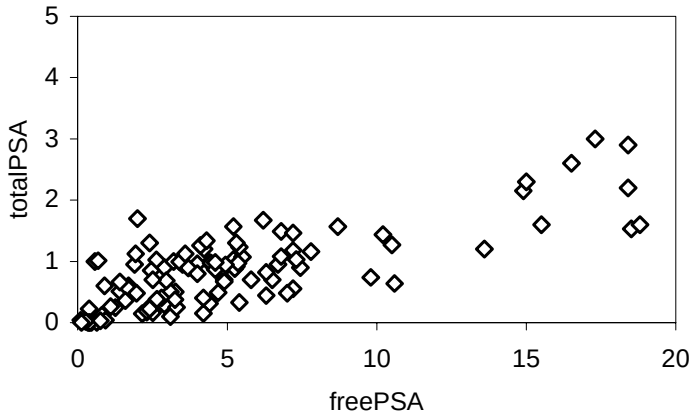

FIG. 5. Total and free PSA values in normal population, as measured by the in-house IRMA systems

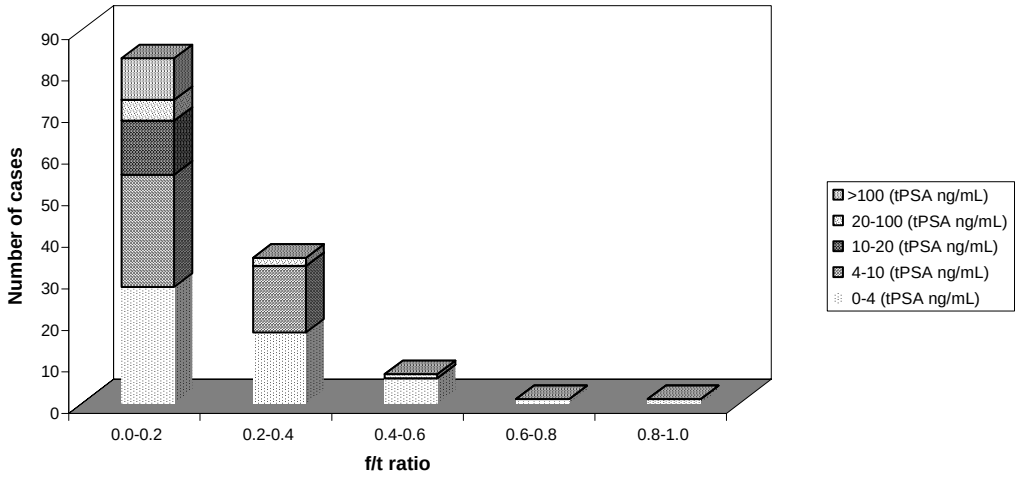

FIG. 6. Distribution of t-PSA levels in population at various f/t PSA ratio ranges.

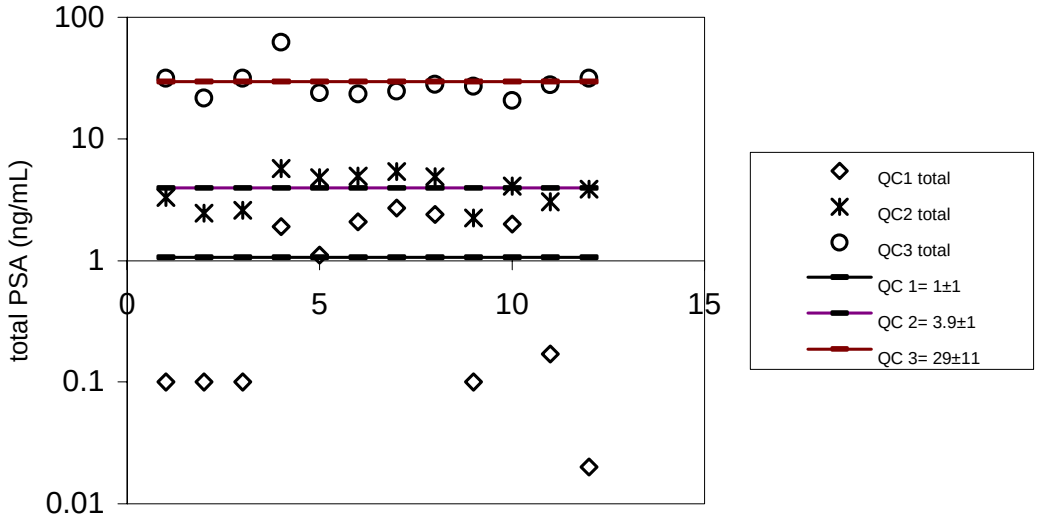

FIG. 7. In-house t-PSA IRMA performance depicted by inter assay QC analysis.

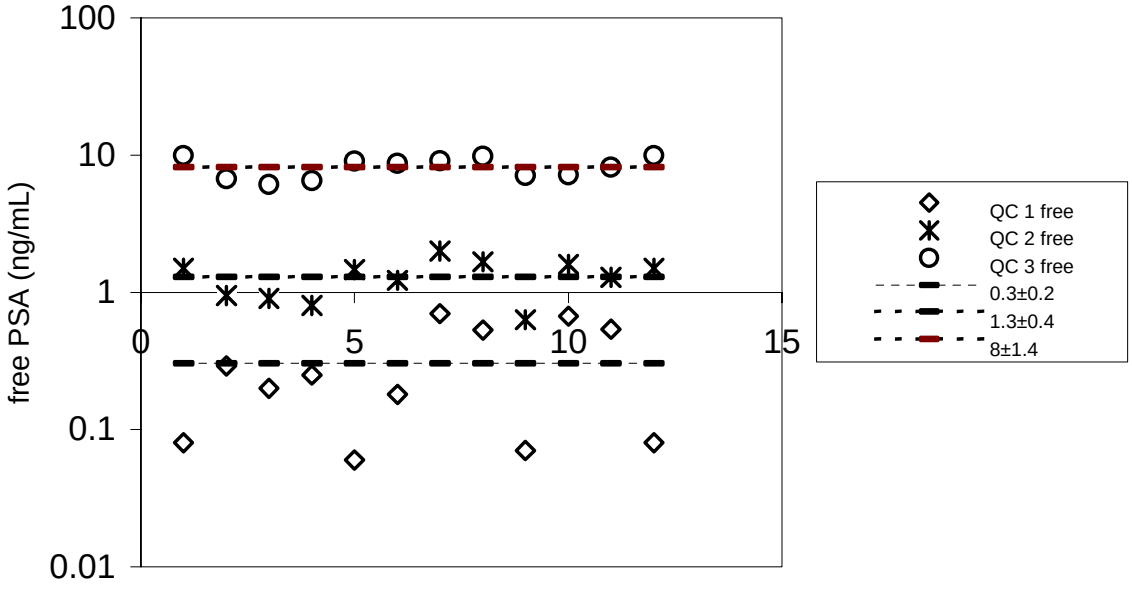

FIG. 8. In-house f-PSA IRMA performance depicted by inter assay QC analysis.

2.8.4. CONCLUSIONS

From the analytical observation of the results presented here, it may be concluded that:

i. A combined IRMA system that is robust although still lacking sensitivity in the low range of PSA levels has been optimised for total and free PSA measurement. In brief, the assays were of 2 steps in order to achieve low end sensitivity. The performance in terms of maximum binding capacity was 35% at 2.2 nM of total PSA concentration and 30% at 0.74 nM free unbound PSA concentration. The minimum detectable dose was 0.5 ng/mL (CV 70%) for total PSA and 0.2 ng/mL for free PSA (CV 35%).
The QC sera values were as follows:
QC1: 1±1.0 ng/mL t-PSA; 0.3±0.2 ng/mL f-PSA;
QC2: 3.9±1.2 ng/mL t-PSA; 1.3±0.4 ng/mL f-PSA
QC3: 29±11 ng/mL t-PSA; 8.0±1.4 ng/mL f-PSA

The developed t-PSA IRMA compared well with the commercial reference assay while in the case of f-PSA IRMA, further refinement is needed.

For the population studied in 1999, the results of correlation between a reference system and our assay was:
For total PSA: y(CIN)=0.54(Reference)-0.3 (r^2=0.905 n=55)
For free PSA: y(CIN)= 0.36(Reference)+0.11 (r^2=0.812 n=55)
For the population studied in 2000
For total PSA: y(CIN)= 0.705(Reference)-0.55 (r^2=0.935, n=43)
For free PSA: y(CIN)= 0.449 (Reference)+0.1 (r^2=0.59 n=30)

ii. Tracers for the IRMAs, MAb10 (t-PSA) and MAb30 (f-PSA) could be successfully prepared at specific activities of 59 and 77 kBq/pM respectively with mean iodine incorporation of 0.7 and 0,9 respectively.

iii. The capture MAb could be successfully immobilised on the polystyrene tubes and stored for at least three months at –20°C without loss of potency.

iv. PSA standards could be prepared from seminal plasma. These standards proved to be usable in the in-house assays when calibrated against commercial reference standards. These standards were very stable even in liquid form and could be stored for very long periods at 4 to 10°C. Even at room temperature, they were found to be stable for months together, except in the low end region. However, the matrix effect on the standards and their potency in free PSA assays, is an important issue not yet addressed satisfactorily.

v. Better understanding of the effect of matrix on assay performance problems is needed, particularly in the free PSA assay. In our system, the use of two incubation steps along with the use of very low sample volume has made it possible to reduce the strong adsorption of tracer to the solid phase at 0 PSA level (zero binding). This has enormous effect on the sensitivity of the assay. Methods to reduce non specific binding/zero binding need to be devised. The importance of increasing the assay sensitivity was evident from the Quality control analysis wherein the% CV at low QC sample values were too high and could cause rejection in clinics.

vi. Patient sample analyses gave an insight into the variations in f/t PSA ratios and t-PSA levels. At f/t ratio 0.2±0.2 (n=128), 34% of the samples were in the range of 0–4 ng/mL of t-PSA, 32% in the range 4–10 ng/mL, 16% in the range 10–20 ng/mL and 17% over 20 ng/mL. Correlation with a reference commercial system is being held through assay of samples from a risk population at the university hospital and cancer patients under pain relief therapy with ^{188}Re.

vii. Finally, it was realised that the reagent preparation following the recommended steps strictly was necessary to obtain good quality stable reagents. Similarly carrying out the assay with strict adherence to the recommended protocol and good pipetting were essential to realise the maximum sensitivity in the assays.

ABBREVIATIONS

ACT	ζ-antichymotrypsin
BGG	bovine gamma globulin
Bmax	maximum binding
Bq	bequerel = 1 disintegration per second
BSA	bovine serum albumin
Ci	curie = 37 Giga Bq
CLIA	chemiluminescent immunoassay
CPM	counts per minute
CV	coefficient of variation
DMEM	dulbeccos modified eagle medium
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
f/t	free PSA to total PSA ratio
FCS	fetal calf serum
f-PSA	free-PSA, unbound to any other protein
IAEA	International Atomic Energy Agency
IRMA	immunoradiometricassay
ITLC-SG	instant thin layer chromatography silicagel
kDa	kilo-Dalton
MAb	monoclonal antibody
NSB	non-specific binding
PBS	phosphate buffered saline; 0.05 M phosphate, pH 7.5, 0.015 M NaCl
PBSX	phosphate buffer saline, Triton-X–100
PSA	prostate specific antigen
PSA-ACT	bound complex form of PSA with ζ_1-ACT
QC	quality control
RCP	radiochemical purity
RIA	radioimmunoassay
s.d.	standard deviation
t-PSA	total-PSA (PSA bound to proteins, i.e. ζ-ACT, ζ_2-macroglobulin, etc., and unbound PSA)

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