Biochemistry Letters,10(7) 2015, Pages: 70 -79

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Establishment of Radioimmunoassay system for Prostate Specific antigen as a diagnostic tool for prostatic diseases

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ARTICLE INFO

Keywords:
Prostate Specific Antigen(PSA), Prostatic diseases , Radioimmunoassay , Radioiodinated PSA tracer, Polyclonal PSA Antibody

Abbreviations
RIA *Radioimmunoassay*
PSA *Prostate specific antigen*
PCa *Prostate cancer*
PEG *Polyethelene glycol*
NRS *Normal rabbit serum*

ABSTRACT

Background: Prostate specific antigen (PSA), a glycoprotein secreted exclusively from epithelial cell lining the prostate gland. The level of PSA in human serum may play a critical role in diagnosis of prostatic diseases. ***Objectives:*** This study was carried out to set up Radioimmunoassay system for PSA estimation in human serum as a diagnostic tool for Prostatic diseases. ***Methods:*** locally produced highly purified PSA was used for preparation of radioimmunoassay components; PSA Polyclonal antibody, radioiodinated PSA Tracer and PSA standards. Optimization and Formulation have been achieved, then validity of this technique confirmed by measuring the quality control parameters; Sensitivity, accuracy, crossreactivity, intra and inter assay precision and method comparison. ***Results:*** we found that; Optimized and Characterized Radioimmunoassay system is the most Sensitive, specific, precise and accurate tool for estimation of prostate specific antigen level in human serum. ***Conclusion:*** Estimation of PSA with locally produced radioimmunoassay system can be used as a diagnostic tool for diagnosis of prostatic diseases.

INTRODUCTION

All men are at some risk for developing prostate cancer, yet there are many men who do not possess correct knowledge about the location and function of this organ that contributes significantly to male development, health, sexual function, and general quality of life [1]. The prostate gland is a secondary sex, exocrine organ that is an integral part of the human male reproductive system [2]. Prostate development begins before birth but rapid growth occurs during puberty in preparation for the production of semen. The prostate remains functional and at adult size as long as androgens are present. As men age they have an increasing chance of developing diseases of the prostate. There are three main diseases of the prostate: prostatitis, benign prostatic hyperplasia, and prostate cancer. Prostate cancer (PCa) is among the most common cancers worldwide and is a leading cause of cancer death in men in the developed world. Prostate-specific antigen (PSA) is a serine protease produced by prostatic epithelial cells, and in the event of prostatic disease, PSA leakage into the bloodstream increases. This characteristic

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makes measurement of plasma PSA an important element in the diagnosis of PCa; the measurement is used as a biomarker to determine the need for further examination [3]. Both normal healthy and neoplastic prostate cells secrete PSA [4] and an increase in the PSA level can at times be attributed to other benign conditions such as acute prostatitis, benign prostatic hyperplasia, and other conditions. Even though the level of PSA expressed on a per cell basis varies, there is no debate on the fact that PSA is consistently expressed in nearly all prostate carcinomas [5]. The absolute value of serum PSA is useful for determining the extent of prostate cancer and assessing the response to prostate cancer treatment; its use as a screening method to detect prostate cancer is common but controversial. In normal healthy males PSA is secreted into prostatic lumen. It is then pumped into the prostatic urethra during ejaculation by means of fibromuscular tissue contractions of the prostate and expelled into seminal fluid. Because PSA is primarily released in prostatic secretions, only very small amounts of PSA are expected to be found circulating in the blood serum of a healthy individual. However, in the presence of prostate cancer, the concentration of PSA in the blood increases significantly [6]. Radioimmunoassay (RIA) is an elegant technique used in the measurement of very low concentrations (typically 10^{-6}-10^{-12} g/ml) of specific compounds in the presence of excess of other materials. RIA is based on the reversible reaction between antigens and antibodies. An antigen is a substance, which stimulates an immune response when injected into an animal, and is usually a high molecular weight compounds such as a protein or polysaccharides. Antibodies are immunoglobulins formed in an animal by the immune response system when an antigen is injected. An antibody normally demonstrates a very high specificity in its reactions for the antigenic compound, which stimulates its formation. It is the specificity that enables an antibody to be used for the selective detection of a particular antigen in the presence of many other materials, which may themselves have antigenic properties [7]. Radioimmunoassay works according to the following principles; the antigen in the specimen and the labeled antigen will compete for the limited binding sites on the antibody. After an equilibration time, a certain amount of the specimen antigen will be bound to the antibody and a certain amount of the labeled antigen will also be bound to the antibody. With increasing concentration of the analyte, less of the radiolabeled antigen is bound to the antiserum. Therefore, a left over antigen (either labeled or unlabeled) will be unbound to the antibody. The next step is to separate and determine the amount of antigen that remained unbound (F) and the amount that became bound to the antibody (B). Since RIA depends on the use of radioactive isotope principle, the main concern will be focused on the F or B of the labeled antigen. From this information, one can determine from a standard curve how much antigen of interest there was in the specimen [8].

MATERIALS AND METHODS

(I) Materials:

Highly purified PSA and Polyclonal PSA antibody was obtained from Hot Labs Centre, Atomic Energy Authority. Sodium Iodide–125 (NaI^{125}), Radioactive concentration 3700 M Bq / ml. Half-life 59.9 day, Izotop.

Chemicals: All chemicals used in these experiments were provided from Sigma Chemical Co. of high quality and purity.

(II) Methods:

Preparation of radioiodinated PSA tracer: Radioiodination of PSA with NaI^{125} achieved by Optimized chloramine-T technique. In polystyrene eppendorf tube 10 µl of 0.05 M phosphate buffer pH 6.4, 7µl of PSA solution containing 3.75µg PSA and 500 µ.Ci. NaI^{125} was added then the reaction started by addition of 7 µl containing 35 µg chloramine-T as oxidizing agent, after gentle vortexing for two minutes, the reaction stopped by addition of 7µl containing 70 µg sodium metabisulfite . immediately after stopping the radioiodination reaction, the raction mixture transferred to sephadex G 25 column preequilibrated with assay buffer (0.05 M phosphate buffer pH 7.4 containing 0.9 % NaCl, 0.4 % bovine serum albumin (BSA) and 0.1 % sodium azid). At a flow rate 0.5 ml/min, 0.5 ml fractions collected and counted for radioactivity using the gamma counter as µ. Ci., finally, The first peak that containing radiolabelled PSA separated into aliquots and stored at - 20ºC.

Preparation of PSA standards: Caliberators or standards for PSA prepared using stock solution of PSA (500 µg/ml) and Bovine serum as a matrix concentration from 0.0 to 100 ng /ml prepared, estimated and calibrated using commercial EIA kits(TOSOH AIA 360, USA). These standards used during assay optimization and final standard curve representation.

Radioimmunoassay Optimization: the parameters of radioimmunoassay system such as incubation time , temperature , sample volume, PSA antibody dilution, reaction volume, and separating agents (second antibody dilution, normal rabbit serum dilution and Polyethylene glycol 8000 concentration) have been optimized by keeping all the parameters constant and study focused on each one of them, temperature (4 , 25 and 37ºC),incubation time (1, 2, 3, 6, 12 Hours), sample volume(50,100,200 and 300 µl), reaction volume (300,500 and 1000 µ), second antibody (from 1/10 through 1/100),NRS(1/100,1/200, 1/300 and 1/400) and PEG 8000 concentration(4, 8, 12 %) . the optimum parameter is that who has the highest binding and displacement percent.

Assay Design: following optimization results the radioimmunoassay of PSA can be formulated as the following: in polystyrene test tube add 100 µl sample or standard,100 µl PSA polyclonal antibody at dilution 1/1000 and 100 µl radiolabelled PSA tracer at radioactivity measure approximately 20000 CPM/100 µl. then after gentle vortexing and incubation for three hours at room temperature (25ºC), separating agent added as the following; 100 µl second antibody at dilution 1/40, 100 µl NRS at dilution 1/200 and 500 µl PEG 8000 at concentration 12 %. Again gently vortex the tubes, incubate at RT for 30 minutes, centrifuged at 4ºC and 5000 rpm for 15 minutes the content of the tubes discarded by decantation and after air drying counted for radioactivity cpm.

Calculation of the assay output results: now we have total activity count added to each tube and the bounded activity that measured after decantation. The data of the radioimmunoassay can be represented as maximum binding B0 for zero standard tube. And sample binding Bs.

Standard curve representation: standard curve for PSA RIA represented by measuring the B/B0 value for each standard value from zero to 100 ng/ml.

Validation of PSA radioimmunoassay system: assay validation achieved by quality control measures; cross-reactivity with related tumor markers such AFP, CEA and CA19.9, sensitivity of the assay, inrassay and interassay precision, accuracy (recovery and dilution) and finally method comparison with commercial kits.

RESULTS

Radioiodination of PSA: PSA labeling by Chloramine T technique has been optimized for Ch-T concentration (35µg), pH(6.4) ,concentration of PSA(3.75µg) and inreaction Time(2 min.), at these conditions, the iodination yield obtained was 53.8±0.5 % and specific Activity 64.57 µ. Ci. / µgPSA as shown in Figure 1, the purity of produced tracer was measured by paper electrophoresis and the radiochemical purity of the tracer calculated to be 98.8±0.5 % as shown in figure 2.

Preparation of PSA standards: PSA standards prepared in bovine serum matrix compared with the commercial EIA Kits TOSOH AIA 360, the correlation coefficient "r" between EIA standards and locally prepared standards was measures statistically to be 0.999 as shown in figure 3.

Radioimmunoassay Optimization: the result of optimization for each assay parameter can be summarized as the following: the optimum reaction time was 3 hours with binding and displacement percent equal to 43.7±0.5 % and 62.7±1.5 % respectively as shown in table 1. The optimum temperature for PSA RIA was 25°C with binding and displacement percent equal to 45.9±0.5 % and 60.5±1.5 % respectively as shown in table 2. The optimum Sample volume for PSA RIA was 100 µl with binding and displacement percent equal to 46.0±0.5 % and 69.3±1.5 % respectively as shown in table 3. The optimum dilution for second antibody used in separation process for PSA RIA was 1/50 with binding and displacement percent equal to 43.6±0.5 % and 64.2±1.5 % respectively as shown in table 4. The optimum dilution for NRS used in separation process for PSA RIA was 1/200 with binding and displacement percent equal to 47.9±0.5 % and 62.4±1.5 % respectively as shown in table 5. The optimum PEG8000 concentration for PSA RIA was 12% with binding and displacement percent equal to 47.0±0.5 % and 56.2±1.5 % respectively as shown in table 6. And finally the total reaction volume for PSA RIA was 300 µl with binding and displacement percent equal to 47.5±0.5 % and 70.0±1.5 % respectively as shown in table 7.

Standard curve for PSA RIA: from the result of standard curve that is represented on logit log graphitic sheet as show in figure 4 we can find that the concentration of PSA Inversely proportional to the radioactivity bounded to the antibody.

Radioimmunoassay validation: quality control results for PSA RIA assured by statistical measures for the result obtained by each parameter as the following; the sensitivity of PSA RIA measured by assaying twenty replicate of zero standard, the mean of the tubes and standard deviation calculated and the result of mean±2SD represented as B/B0 % equal to 95 % interploted on the PSA RIA standard curve, the corresponding concentration 0f 0.29 ng/ml can be considered as the sensitivity value of PSA RIA, as shown in table 8.the specificity or crossreactivity of PSA RIA measured by replacing PSA standards by Crossreactant standards the result of crossreactivity measured by dividing the concentration of PSA by the concentration of crossreactant at B/B0 equal to 50% of PSA standards, the result of crossreactivity for all crossreactants measured to be less than 0.2 ng/ml as show in table 9. Precision both intraassay and interassay results obtained by repeated within run and between runs assaying of pooled three samples with low, moderate and high PSA values, the result of presision calculated using mean and standard deviation values as shown in table 10 the coeffetient of variation CV value ranged from 4.1±0.2 to 6.9±0.2 % . also the accuracy measurement achived by two methods, the first one recovery test where samples with known PSA values added to three pooled samples used after assaying the expected and observed results calculated and the recovery percent measured to be from 95.1±0.5 % to 105.6±0.5 % as shown in table 11, the second accuracy test, the dilution test achieved by serial dilution of the same three pooled samples in recovery test and then the results of expected and observed values calculated then the recovery percent of dilution test measured to be from 93.3±0.5 to 108.3±0.5 % as shown in table 12. Finally the comparison of the results of 20 samples tested by our PSA RIA technique and other commercial EIA TOSOH technique revealed that the correlation coefficient between them measured statistically to be r = 0.999 as shown in figure 4.

DISCUSSION:

Radioimmunoassay (RIA) is known as the most recognized sensitive microanalytical technique for the determination of the very low concentration of a wide range of substances of medical, veterinary and biological interest. RIA employs principles of immunology thus making the reaction very specific, and uses radioactive substances which can be counted with great sensitivity and accuracy. No other analytical method can reach the low detection limits of assayed analytes attained by RIA related techniques. [7].

The presence of PSA has been demonstrated in normal, benign hyperplastic, and malignant prostatic tissue, in metastatic prostatic carcinoma and also in prostatic fluid as well as seminal plasma [9]. Elevated serum PSA levels have been reported in patients with prostatitis , benign prostatic hypertrophy and prostate cancer , [10],[11]. PSA is not present, however, in any other tissue from men, nor is it produced by cancers of lung, colon, rectum, stomach, pancreas or thyroid[12]. PSA's unique tissue specificity is what make it significant as a tumor marker[13] .

From the result of standard curve that is represented on logit log graphitic sheet as show in figure 4 we can report that the radioimmunoassay of PSA is a competitive assay where both the cold antigen (PSA in the serum sample or standard) compete with the radiolabelled antigen PSAI^{125} for binding to the constant active sites on the PSA polyclonal antibody. So, with increasing the PSA level the binding of PSAI^{125} decrease and the Radioactivity decrease and the concentration of PSA Inversely proportional to the radioactivity bounded to the PSA antibody.

Our results revealed that, the PSA RIA is sensitive, specific, presize and accurate technique for estimation of PSA values in human serum to be used as a diagnostic tool for prostatic diseases prostatitis, benign prostatic hyperplasia and prostate cancer in a hope to cure the patients in early stage and avoid prolongation of the disease and metastasis to other organs.

The results of sensitivity reported in our work for PSA RIA was 0.29 ng/ml was in a good agreement with the results obtained by **zhong et al., 1996** [14] that reported sensitivity value of PSA RIA as 0.5 ng/ml.

The results of specificity reported in our work revealed that there is no crossreactivity in PSA RIA with other related tumor markers so; our locally produced polyclonal antibody is highly specific for PSA.

The results of precision of our work, CV value ranged from 4.1±0.2 to 6.9±0.2 % are in a good agreement with numerous studies **Pillai and Bhandarkar, 1998**[15] which stated that the intra-assay coefficient of variation (CV) should be less than 10%, while in case of inter-assay, they reported that the CV of the measured ligand concentration should be less than 15%.

The results of accuracy testing reported in our work were in a good agreement with **Pillai and Bhandarker (1998)** [15] results**,** the recovery of an assay should be 100+15%. From The recovery data of the present study for PSA we can be observe that recovery percent ranged from (95.1% to 105.6 %) in recovery test of accuracy and from (93.3% to 108.3 %) in dilution test for accuracy, we can now report that the PSA RIA is an accurate method for estimation of PSA level in human serum.

When this optimized radioimmunoassay of prostate specific antigen compared with other commercial Kits, the result of correlation coefficient ‘r’=0.999 obtained was accepted, and now it can be used by clinical laboratories for estimation of PSA in patients with prostatic diseases.

Conclusion:

In conclusion, Optimized Radioimmunoassay for prostate specific antigen is a cheap, sensitive, specific, precise and accurate method can be used for diagnosis of prostatic diseases.

Acknowledgment

The authors thank everyone contribute this work at labeled compound department, Egyptian atomic energy authority and chemistry department, Faculty of science Zagazig Univeristy.

Table (1): Optimization of incubation time for RIA of PSA.

STD (ng/ml)	Inc. Time	Binding Percentage (B%)				
		1hr	2hr	3hr	6hr	12hr
0		35.3	39.6	43.7	43.6	42.8
10		32.9	35.4	36.7	39.0	39.0
100		16.2	17.1	16.1	19.2	24.8
Displacement %		54.1	56.8	**62.7**	56.0	42.0

Table (2): Optimization of incubation temperature for RIA of PSA.

STD (ng/ml)	Temp.	Binding Percentage (B%)		
		4^oC	37^oC	RT (25^oC)
0		45.1	37.8	45.9
10		35.2	32.0	38.1
100		19.4	17.4	18.1
Displacement %		57.1	53.9	**60.5**

Table (3): Optimization of sample volume for RIA of PSA

STD (ng/ml)	Sample volume	Binding Percentage (B%)				
		25(µL)	50(µL)	100(µL)	200(µL)	300(µL)
0		43.7	45.5	46.0	38.8	37.5
10		40.1	42.7	43.9	28.1	26.5
100		30.9	21.0	14.1	15.9	16.2
Displacement %		29.2	53.8	**69.3**	59.0	56.8

Table (4): Effect of Second antibody dilution on the liquid-phase RIA system for PSA

STD ng/ml	Dilution of 2^{nd}Ab	Binding Percentage (B%)						
		1:10	1:20	1:30	1:40	1:50	1:80	1:100
0		46.6	44.1	41.1	41.7	43.6	31.8	27.9
10		43.8	41.0	34.7	34.2	38.0	28.8	18.8
100		19.8	17.7	16.3	16.0	15.6	12.7	11.6
NSB		3.0	3.4	2.8	2.7	2.5	2.9	2.6
Displacement %		57.6	59.8	60.4	61.7	**64.2**	60.0	58.3

Table (5): Effect of NRS dilution on the liquid-phase RIA system for PSA

STD (ng/ml)	Dilution of NRS	Binding Percentage (B%)			
		1:50	1:100	1:200	1:400
0		47.4	47.6	**47.9**	45.7
10		46.6	45.1	46.8	34.6
100		18.9	18.4	18.0	18.8
NSB		3.0	2.7	2.7	2.9
Displacement %		60.1	61.3	**62.4**	59.0

Table (6): Effect of PEG8000 concentration on the liquid-phase RIA system for PSA

STD (ng/ml)	PEG %	Binding Percentage (B%)		
		4 %	8 %	12 %
0		35.2	44.1	**47.0**
10		32.5	38.6	40.5
100		17.5	21.5	20.6
NSB		2.1	2.1	2.1
Displacement %		50.2	51.3	**56.2**

Table (7): Effect of reaction volume on the liquid-phase RIA system for PSA.

STD (ng/ml)	Vol.(ml)	Binding Percentage (B%)		
		300 µl	500 µl	1000 µl
0		**47.5**	44.3	32.6
10		38.6	35.2	28.5
100		14.3	19.2	17.4
Displacement %		**70.0**	56.7	46.6

Table (8): The sensitivity of PSA RIA.

cpm (mean –SD)	cpm (mean – 2SD)	B/B_o %	Apparent concentration (ng/ml)	Approximate sensitivity (ng/ml)
10284–120	10043	94.97	0.29	0.29

Table (9): Cross reactivity of PSA RIA.

Related markers	% cross-reactivity
PSA	100
AFP	ND
CEA	ND
CA19.9	ND

Table (10): Precision profile for PSA RIA.

Samples	Intra-assay			Inter-assay		
	Mean (ng/ml)	SD (ng/ml)	CV %	Mean (ng/ml)	SD (ng/ml)	CV %
1	19.8	1.2	6.0	20.2	1.4	6.9

Biochemistry letters, 1(1) 2015, Pages: 1-11

2	37.5	2.3	6.1	36.1	1.8	4.9
3	47.4	2.2	4.6	46.3	1.9	4.1

Table (11): Recovery test for PSA RIA:

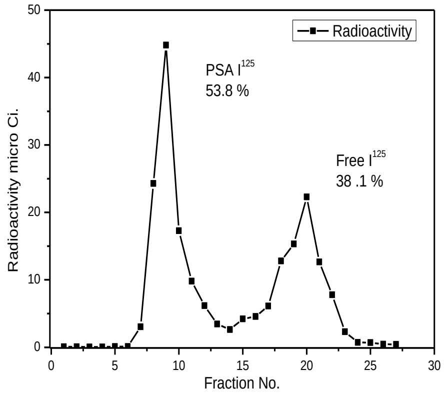
Sample	PSA (ng/ml)	Added PSA (ng/ml)	Expected (E)	Observed (O)	Recovery % (O/E)
1	10.1	2.3	6.2	5.9	95.1
		24.8	17.4	18.0	103.4
		50.0	30.0	31.7	105.6
2	28.1	2.3	15.2	14.8	97.3
		24.8	26.4	27.2	103.0
		50.0	39.0	40.9	104.8
3	50.0	2.3	26.1	26.9	103.0
		24.8	37.4	38.0	101.6
		50.0	50	52.2	104.4

Table (12): Dilution test for PSA RIA.

Sample	PSA (ng/ml)	Dil.	Expected (E)	Observed (O)	Recovery% (O/E)
1	10.1	1/2	5.0	5.2	104.0
		1/4	2.5	2.6	104.0
		1/8	1.2	1.3	108.3
		1/16	0.6	0.6	100.0
		1/32	0.3	0.3	100.0
2	28.1	1/2	14.0	14.2	101.4
		1/4	7.0	6.9	98.5
		1/8	3.5	3.4	97.1
		1/16	1.7	1.6	94.1
		1/32	0.9	0.9	100.0
3	50.0	1/2	25.0	24.6	98.4
		1/4	12.5	12.2	97.6
		1/8	6.2	6.0	96.7
		1/16	3.1	3.2	103.2
		1/32	1.5	1.4	93.3

Biochemistry letters, 1(1) 2015, Pages: 1-11

Figure(1): the radioiodination yield for PSA



Figure(2) the radiochemical purity of PSA tracer

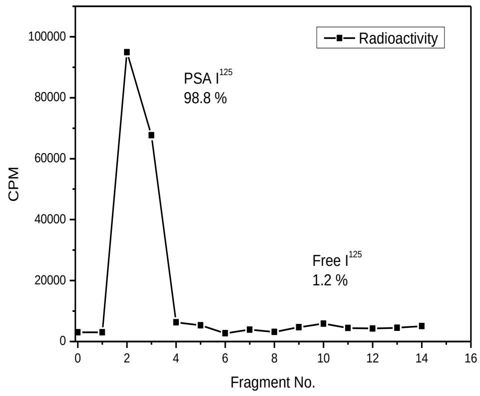
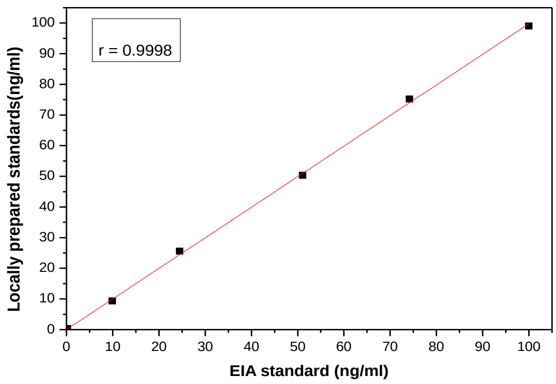
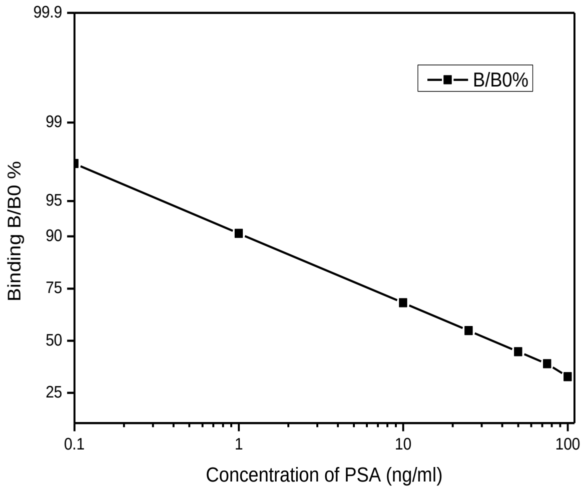


Figure (3): Linear regression equation and correlation coefficient "r" between EIA standards and locally prepared standards in bovine serum.





Biochemistry letters, 1(1) 2015, Pages: 1-11

References:

1-Winterich, J. A., Grzywacz, J. G., Quandt, S. A., Clark, P. E., Miller D. P., Acuña, J., Arcury, T. A., (2009): Men's knowledge and beliefs about prostate cancer: Education, race, and screening status. Ethnicity & Disease, 19(2): 199-203.

2-Lang, S., Frame, F., Collins, A., (2009): Prostate cancer stem cells. Journal of Pathology, 217, 299–306.

3- Orsted D., Nordestgaard B. G., Jensen G. B., Schnohr P., Bojesen S.E., (2012): Prostate-Specific Antigen and Long-Term Prediction of Prostate Cancer Incidence and Mortality in the General Population. Eurpean Urology, 61 :865 – 874.

4- Vickers, A.J., Ulmert, D., Serio, A. M., Björk, T., Scardino, P. T., Eastham, J. A., Lilja H., (2007): The predictive value of prostate cancer biomarkers depends on age and time to diagnosis: towards a biologically-based screening strategy. International Journal of Cancer, 121(10): 2212-2217.

5- Stamey, T. A., Kabalin, J. N., McNeal, J. E., Johnstone, I. M., Freiha, F., Redwine, E. A., Yang, N., (1989): Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. II. Radical prostatectomy treated patients. Journal of Urology, 141(5): 1076-1083.

6- Kulasingam, V., Diamandis, E. P., Vega, C. P., (2008): Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nature Clinical Practice Oncology.; Retrieved from;
http://www.medscape.org/viewarticle/578950
http://img.medscape.com/fullsize/migrated/editorial/journalcme/2008/17049/kulasingam.fig1.gif

7 – Daci, B., Malja, S. Bylyku, E., (2003): Fifth General Conference of Balkan Physical Union. Vrnaeka Banja, Serbia and Montenegra, 2176 - 2193.

8 – Saha, G. B., (1994): In: In-vitro and in vivo non imaging tests. In: Fundamental of Nuclear Pharmacy, 3rd. ed., 219 - 221.

9 – Nadji, M., Tabei, S.Z., Castro, A., Chu, T.M., Murphy, G.P., Wang, M.C., et al.,(1981): Prostatic specific antigen: An immunohistologic marker for prostatic neoplasms. Cancer, 1229–1232.

10 – Papsidero, L.D., Wang, M.C., Valenzuela, L.A., Murphy, G.P., Chu, T.M.,(1980): A prostate antigen in sera of prostatic cancer patients. Cancer Res., 40:4828.

11 – Kuriyama, M., et al, (1980). Quantitation of prostate-specific antigen in serum by a sensetive Enzyme Immunoassay. Cancer Res. 40:4658 .

12 - Frankel, A.G., et al, (1982). Monoclonal antibodies to a Human prostate antigen. Cancer Res. , 42:3714 .

13 – Wang, MC, Papsidero, LD, Kuriyama, M, Valenzuela, LA, Murphy, GP, Chu, TM., (1981): Prostatic antigen: a new potential marker for prostatic cancer. Prostate , 2:89-96.

14 – ZHONG, W., CHEN, L., WANG, R.,(1996): Human prostate specific antigen(hPSA) purification and establishment of hPSA Radioimmunoassay . Journal of Radioanalytical and Nuclear Chemistry, Articles, Vol. 206,No. 2 227-232.

15 - Pillai, M. R. A. and Bhandarkar, S. D, (1998): In Radioimmunoassay, Principles and Practice. 3 rd (edn), Isotope Division Bhabha Atomic Research Center Trombay, Mumbai India, 40 - 98.