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Development of Kits for Total PSA monitoring

R.C. No. 9822/RO

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Development of Kits for total serum PSA monitoring
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1. Preparation of iodinated tracers for Total PSA.
2. Preparation of standards for Total PSA.
3. Preparation of magnetizable particles captured PSA mAb.
4. Assay optimization.
5. Assay validation.

The work has been carried out using supplies from Alberta.
Tracer : monoclonal anti PSA lot 10.
Captured Ab : monoclonal anti PSA lot 66.
Standard PSA (semi-purified).

1. Preparation of iodinated tracers for Total PSA.

1.1 Iodination
1.2 Purification
1.3 Assessment
1.3.1 Purity
1.3.2 Specific activity
1.3.3 Stability

1.1 Iodination of mAb PSA by Chloramin T method.

Radioiodination of monoclonal anti PSA for Total PSA measurement was carried out using Alberta supplies. The following protocol is followed :

Add to the vial containing
10 µl (10 µg) of mAb PSA
10 µl 0.5 M Phosphate buffer pH 7.4
2 µl (0.2 mCi) NaI^{125}
10 µl (1 mg/ml) CT
Mix 20 seconds.
Then add
10 µl (1 mg/ml) $Na_2S_2O_5$
100 µl (10 mg/ml) KI
Transfer to column for Purification

1.2 Purification of radioiodinated mAb PSA

HPLC system :

Column	Biosep Sec-3000 size 30 x 0.78 cm.
Detector	Gamma detector
Mobile phase	0.1 M Phosphate buffer pH 7.4
Flow rate	0.6 - 0.8 ml/min

Collect 60 fractions (30 second per fraction) and count each of the collected fraction for 1 second, calculate the % of radioactivity of PSA mAb peak.

For pooling, select the fractions corresponding to PSA mAb and dilute to give a radioactive concentration of 20µCi/ml with 0.1M Phosphate buffer pH 7.4 containing 1% BSA.

1.3 Assessment

1.3.1 Purity of radiolabelled mAb PSA was checked by paper electrophoresis. About 10 µl of radiolabelled PSA was loaded onto the paper and electrophoresis was carried out using 10 volt/cm for approximately 60 minutes. Percent purity was calculated using the formula ;

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

1.3.2 Determination of Specific Activity

$$\text{Specific Activity } (\mu Ci/\mu g) = \frac{\% \text{ Iodination Yield} \times \text{Total Activity of Iodine}^{125} (\mu Ci)}{\text{Total amount of antibody } (\mu g)}$$

1.3.3 Stability of radiolabelled mAb PSA was studied under storage conditions: liquid form (4° C) or lyophilized form (4° C) over a period of two months.

Two mAb PSA (Lot 10 and 30) were labelled with ^{125}I. The radiolabelling results were listed in Table 1 and Figure 1. Both of them were used as tracer in PSA IRMA. The results as seen in table 2 shown that mAb PSA lot 10 and capture antibody lot 66 formed good sandwich partners. This pair have been choosen for use in PSA IRMA.

Table 1 Radiolabelled mAb PSA using chloramine-T method

	Monoclonal antibody	
	PSA 10	PSA 30
% Yield	73.0	71.0
% Purity	94.0	-
Specific activity	14.6	13.8

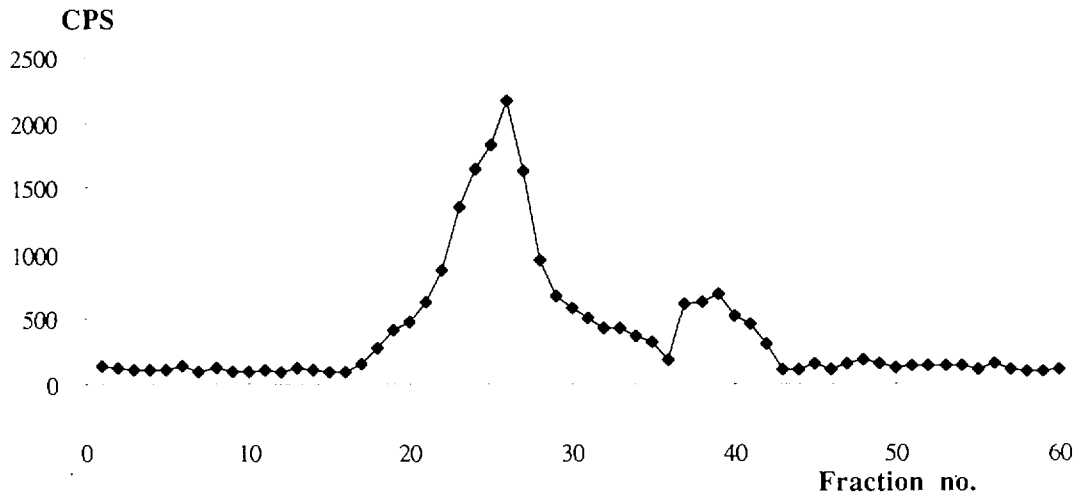

Figure 1 Elution profile of radiolabelled mAb PSA using CT method

Table 2 Matching of PSA monoclonal antibody (lot 10 and lot 30) with mAb coated magnetic (lot 66)

Capture antibody	Tracer antibody	PSA (ng/ml)		
		0	0.87	120
Lot 66	**Lot 10**	**0.49**	**0.60**	**21.97**
Lot 66	Lot 30	0.57	0.69	5.84

2. Preparation of standards for Total PSA.

Total PSA standards are prepared by adding semi-purified PSA (Alberta concentration 70% (w/w)) in distillated water to screened normal female serum to a final concentration of 120 ng/ml. Then do double dilutions to the following dose levels ; 60, 30, 15, 7.5, 3.75, 1.88, 0.94, 0.46 ng/ml. These working standards were tested with commercial standard (Netria, UK. and Fitzgerald Inc., USA.) for the estimated concentrations.

3. Preparation of magnetizable particles captured monoclonal anti PSA.

3.1 Activation of Magnetic cellulose

Magnetic particles (SCIPAC, UK.) were dispersed by gentle mixing on the rotator for 30 minutes at room temperature. 20 ml (1gm) suspension was immediately pipetted out. Particles were sedimented on magnetic block and supernatant was aspirated. Then wash the particles with 20%, 60% and 100% acetone respectively. 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.

3.2 Coupling of activated magnetic cellulose to antibody.

The particles were resuspended in 10 ml of the Borate buffer with ~~6.25~~ 0.625 mg anti PSA (Alberta 66). The reactants were mixed gently by rolling 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 for 1 hours.
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.

3.3 Choice of coupling condition

3.3.1 Comparison of different types of magnetic particles.

Two types of magnetic particles, M 104 and M 108 (SCIPAC; UK.) were compared in PSA IRMA. A monoclonal anti-PSA (PSA 66; Alberta) was immobilized on the two types of particles using above protocols. The results summarized in Table 3 demonstrated that both types were found to be suitable. The best results, lower (B_0) and higher (B_{120}), were achieved by M 104.

Table 3 Comparison of two types of magnetic particles in PSA IRMA
Immobilized Ab: monoclonal anti-PSA, Alberta PSA 66
Labelled Ab: monoclonal anti-PSA, Alberta PSA 10

PSA	Binding (CPM)	
(ng/ml)	**M 104**	M 108
0	**170.4**	129.0
0.46	**243.3**	289.0
120	**8062.7**	6975.5
Total	**29351**	29351
% B_0/T	**0.58**	0.44
% B_{120}/T	**27.47**	23.77

3.3.2 Choice of coupling ratio.

The various amounts of monoclonal anti-PSA were coupled to the magnetic particles using the same coupling method and condition. Table 4 and Figure 2 shows that the ideal amount of antibody to be coupled per gram of magnetic particle was found to be 1:4 at 0.5 mg per tube.

Table 4 Determination of the amount of antibody used in the coupling reaction per gram of magnetic particles

PSA	Binding (CPM)		
(ng/ml)	1:3	**1:4**	1:6
0	170.4	**136.2**	179.4
0.46	199.8	**157.7**	228.5
120	8062.7	**7264.1**	5353.6
Total	29351	**29351**	29351
% B_0/T	0.58	**0.46**	0.61
% B_{120}/T	27.47	**24.75**	18.24

4. Assay optimization

In order to optimize the assay performance, the effect of various factors (such as, incubation time, incubation temperature, amount of magnetic particles in the tubes, amount of labelled antibody, etc.) on the standard curve and on the levels of the control serum was evaluated. The general assay protocol is as follows:

Add 50 µl standards or samples
50 µl ^{125}I-MAb PSA
100 µl assay buffer (0.05 M Phosphate, pH 7.4 containing 0.1% Azide, 0.2% Triton X-100 and 1% BSA)
50 µl anti-PSA coated magnetic particles

Mix the reactants and incubate at room temperature for 1, 1.5, 2 or 18 hours.

Separate magnetic particles on magnetic separator, decant the supernatant

Wash the particles with 1 ml wash buffer (0.05 M Phosphate buffer containing 0.1% Azide and 0.2% Triton X-100)

Count the radioactivity in the tubes.

4.1 Incubation time

Using the above protocol the reaction mixture was incubated under gentle shaking for 1, 1.5, 2 and 18 hours. The solid phase was separated as described and the tubes were counted, the count rates are listed in Table 5.

Table 5 Count rates of standards as a function of incubation time

PSA	Binding (CPM)			
(ng/ml)	1 hour	**1.5 hours**	2 hours	18 hours
0	464.5	**410.9**	475.9	525.9
2.2	837.0	**732.3**	880.1	1060.9
11	1921.0	**2133.2**	2311.7	3456.6
27.5	4008.9	**3814.4**	4518.8	6445.3
55	6664.0	**6538.7**	7179.9	9003.2
110	9322.9	**9551.6**	9560.6	10638.2
275	10053.9	**11327.7**	11118.0	13094.0
550	11218.5	**11619.3**	11570.7	14670.3
Total (CPM)	45533.7	**45533.7**	45533.7	45533.7
% B_0/T	1.01	**0.90**	1.04	1.16
% B_{550}/T	24.53	**25.52**	25.47	32.40

4.2 Incubation temperature

50 µl Standard or control serum, 50 µl ^{125}I labelled anti PSA, 100 µl assay buffer and 50 µl magnetic particles was incubated at 37 °C (water bath), room temperature under conditions: rotation and without rotation for 1.5 hours. The solid was separated and counted, the results are shown below. It is worth mentioning that the binding was considerbly higher in the case of shaken incubation mixture as compared to the unshaken one.

Table 6 Count rates of standards as a function of incubation temperature

PSA	37 °C	Room temperature	
(ng/ml)	without rotation	with rotation	without rotation
0	205	218	180
0.5	243	362	206
500	8415	14498	6704
Total (CPM)	47401	47401	47401
% B_0/T	0.43	0.46	0.38
% B_{500}/T	17.75	30.74	14.27

4.3 Titration of immobilized antibodies

Various amount (1, 0.5, 0.25 mg/tube) of magnetic particles captured antibodies were incubated with radiolabelled anti PSA. At the end of incubation period, the bound fraction was separated using magnetic separator and wash with wash buffer. The pellets of bound fraction were then counted and % B/T was calculated and plotted.

Table 7 and Figure 3 shows that the bound radioactivity increase with increasing amount of magnetic per tube. Nevertheless,with 1.0 mg/tube , the 0-0.23 ng/ml standards are almost indistinguishable. The optimum amount of magnetic per tube proved to be 0.5 mg/tube.

Table 7 Count rates of standards as a function of the amount of magnetic particles per tube

PSA	Binding (CPM)		
(ng/ml)	1 mg/tube	**0.5 mg/tube**	0.25 mg/tube
0	248.5	**309.4**	243.7
0.23	270.0	**474.7**	422.6
120	17918.3	**13958.6**	10046.4
Total (CPM)	47684	**47684**	47684
% B_0/T	0.52	**0.65**	0.51
% B_{120}/T	37.58	**29.27**	21.07

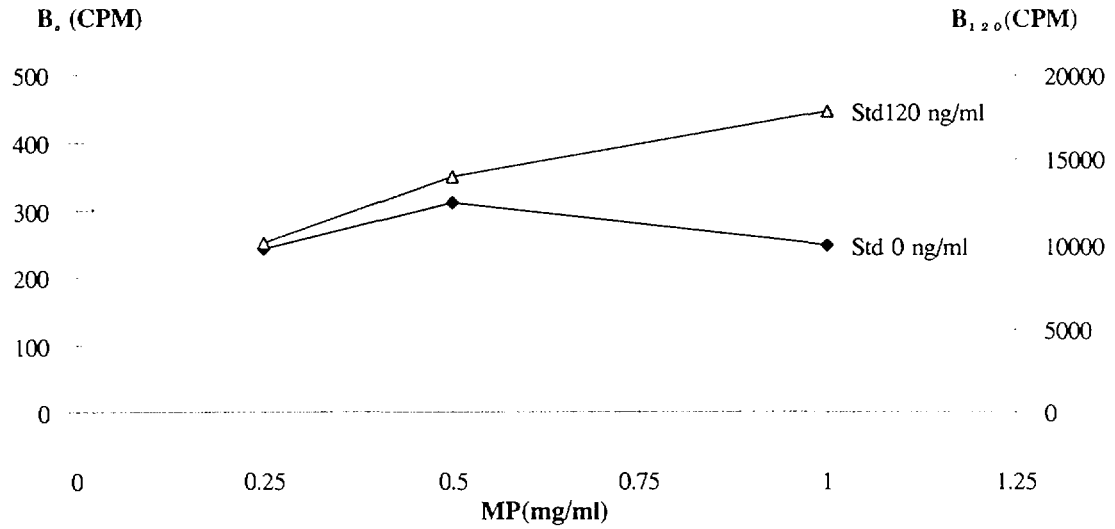

Figure 3 Determination of the amount of magnetic particles per tube to be used in IRMA PSA

4.4 Titration of radiolabelled anti PSA

Various activity (60,000, 45,000, 30,000 CPM per assay tube) of ^{125}I-monoclonal anti-PSA were incubated with immobilized antibodies. After incubation, bound fraction were counted and %B/T was calculated. Table 8 indicates that the amount of radiolabelled PSA 45,000 cpm were needed per assay tube.

Table 8 Count rates of standards as a function of the activity of radiolabelled anti PSA

PSA	Binding (CPM)		
(ng/ml)	60,000 CPM	**45,000 CPM**	30,000 CPM
0	223.0	**193.6**	169.5
0.23	280.4	**215.0**	206.5
120	13702.7	**10882.0**	6760.2
Total (CPM)	59850	**59850**	59850
% B_0/T	0.37	**0.44**	0.61
% B_{120}/T	22.90	**24.82**	24.18

4.5 Effect of washing the magnetic particles after sedimentation

Using the general assay protocol the reaction mixture was incubated under gentle shaking for 1.5 hours . At the end of incubation, the pellets of bound fraction were separated by magnetic separator plate and counted. These pellets were washed with wash buffer for one and two times. The effect of washing is evident from the data of table 8. The early part of the standard curvve, which is drastically distorted when no washing step is included in the assay protocol, improves significantly with washing and so does the low end sensitivity.

Table 8 Effect of washing the magnetic particles

Std. PSA	Binding (CPM)		
(ng/ml)	No washing	1 wash	**2 wash**
0	5056	704	**411**
2.2	5880	1248	**732**
11	7667	3076	**2133**
27.5	9727	5389	**3814**
55	13733	8883	**6538**
110	17280	12409	**9551**
275	19393	14301	**11357**
550	19458	15026	**11619**
Total (CPM)	45588	45588	**45588**
% B_0/T	11.09	1.54	**0.90**
% B_{550}/T	42.68	32.89	**25.52**

4.6 One and Two steps Incubation

This was investigated by setting-up standard curve using the following protocol. The count rates of the tubes were listed in table 9 indicated that the suitable protocol for PSA IRMA was two step assay.

One step method:	**Two step method**
50 µl Standard or sample	50 µl Standard or sample
50 µl ^{125}I-MAb PSA	50 µl anti-PSA coated magnetic particles
100 µl assay buffer	100 µl assay buffer
50 µl anti-PSA coated magnetic particles	Mix and incubate at RT for 1 hour
	Wash and separate
	200 µl ^{125}I-MAb PSA
Mix and incubate at RT for 1.5 hours	Mix and incubate at RT for 1 hour

Separate magnetic particles on magnetic separator, decant the supernatant
Wash the particles with 1 ml wash buffer
Count the radioactivity in the tubes.

Table 9 Count rates of standards as a function of one and two steps incubation

PSA (ng/ml)	Binding (CPM)	
	One step	**Two step**
0	494	**375**
2.2	1004	**787**
11	3213	**2219**
27.5	6634	**5386**
55	11292	**10159**
110	15747	**17332**
275	17466	**27553**
550	17997	**29844**
Total (CPM)	47003	**47003**
% B_0/T	1.05	**0.82**
% B_{550}/T	38.29	**64.99**

5. Assay Validation

A developed assay protocol required validation to establish suitability before proceeding to practical application. In broad terms validation should carried out by the following ways:

5.1 Recovery and dilution test

5.2 Intra and inter-assay precision

5.3 Sensitivity test

5.4 Specificity test

6. Summary

The development of kits for Total PSA assay has shown promising results. All essential components of the assay were prepared with reproducibility and used to optimize the assay.

By choosing two steps method, we could avoid the hook effect and obtain satisfactory Q.C. parameters of the standard curve i.e. blank = 0.8%, maximum binding = 65%. If reference material for calibration of the standard is agreed upon, the validation could then be carried out with total confidence. Our final goal is to reduce the step of incubation to just one step with no interference from hook effect.