Chapter IV

Results and Discussion

1. Establishment cell lines

Two hybridoma cell lines of AFP were established by recloning in 96-well flat-bottomed microliter plates using spleen cell as feeder. This approach yielded the advantage of homogeneous antibody production from unique cell line and could be monitored in the culture supernatant. The cell lines were kept in the liquid nitrogen and could be thawed at any state when the production of antibody was required.

2. Production of monoclonal antibody to AFP

The monoclonal antibodies were produced by propagated the two cell lines and injected the cells into the peritoneal cavity of pristane-primed balb c mice. Two hybridomas grow well and spread in ascites fluid. The ascites fluid from two cell lines were removed, collected and pretreatment for further purification.

3. Characterization of monoclonal antibody from the hybridoma

The supernates of two hybridomas were tested for isotyping using Sigma Immunochemicals' Immuno Type™ kit. The result showed that two hybridomas were IgG_1. The

specificity of the monoclonal antibody was assessed by measuring the appearent response of the binding to human, bovine and rabbit serum albumins. The result was shown in Figure 13, the antibody was highly specific for AFP. Human, bovine and rabbit serum albumins exhibited no cross reactivity when they were assayed at very high concentration (1mg/ml). The association constant (K) for the monoclonal antibody toward the radiolabelled alpha-fetoprotein was estimated from Scatchard plots(Appendix II,4). The association constant (K) of monoclonal anti AFP I and II were 1.55×10^9 and 1.07×10^9 L/mole respectively (Figure 14, 15).

4. Matching of AFP IRMA system using two monoclonal antibodies

The percentage binding (B/T) at standard AFP 320 ng/ml of the labelled antibody I,II and coated antibody I,II was shown in Table 2. The result showed that the use of the coated antibody I and labelled antibody II in AFP IRMA gave the highest percentage binding (45% B/T).Consideration on the K value ,it was obviously seen that the higher K value would provide the high capacity in binding with the antigen ie; the higher K value should be chosen as the captured antibody.Thus,the matching experiment also showed the supportive evidence of the K value applications.

5. Purification of ascites

The precipitate of ascites fluid of monoclonal anti AFP I was purified by DEAE ion exchange chromatography. The IgG fraction of antibody was eluted from the column as shown in Figure 16. The precipitate of ascites fluid of monoclonal anti AFP II was purified by protein A column (Figure 17).

6. Identification of purified antibody by SDS polyacrylamide gel

Comparison of the purified antibody from Ammonium sulfate precipitation, DEAE and protein A was made by SDS PAGE, the resulted bands were shown in Figure 18. The most purified band of monoclonal anti AFP II (lane 7) was from protein A purification. Thus, it was selected as the purification method for the labelling antibody. The purified bands of monoclonal anti AFP I from Ammonium sulfate precipitation (lane 3,4) and DEAE column chromatography (lane 2) were not pure when compared with the ascites fraction (lane 1). Therefore, Ammonium sulfate precipitation should be selected for cellulose coating.

7. Solid phase coated antibody

The couplings of 200 mg activated cellulose and 1 ml of monoclonal anti AFP I solution in 0.05 M Barbitone buffer pH 8, were performed at various concentrations from 0.1-13.1 mg/ml. The result showed that the percentage binding (B/T) of the coupled antibody increased sharply

from the concentration up to 7 mg/ml, and beyond this concentration it appeared to be constant. However, the efficiency of coupling decreased with monoclonal antibody concentration from 45 to 9.38% of available antibody (Figure 19). The solution remaining after coupling should therefore be retained to avoid wastage of reagent particularly if the IgG is of relatively low titer.

The couplings of activated cellulose were performed with monoclonal anti AFP for each of the buffer systems specified (Figure 20). The result showed that maximal coupling occurred at 0.05 M barbitone buffer pH 8, which yielded 30% maximum binding at AFP concentration 320 ng/ml. The comparison of the purified monoclonal anti AFP I by ammonium sulfate purification and DEAE column on cellulose coating were shown in Figure 21. The result showed that no significant change of the binding (B/T) at AFP concentration 0 to 320 ng/ml on coating cellulose with ammonium sulfate purification and DEAE fraction of monoclonal anti AFP I. For this reason, the purification by Ammonium sulfate precipitation was selected for cellulose coating.

8. Labelling of monoclonal antibody as radioisotope tracer

Monoclonal anti AFP II was iodinated to specific activity of 10 uCi/ug with I^{125} using N-Bromosuccinimide method. The results were shown in Figure 22 and Table 3. The level of incorporation of NaI^{125} into monoclonal anti AFP II was 76.6% (Table 3).

The binding of these labels in a sandwich assay was tested at various time intervals to determine the stability of labels at 4° C. The result showed the change in the binding for the labels with time (Figure 23). There was no significant change in zero binding of the 10 and 20 uCi/ug after 5 weeks (Figure 23 and Table 4). After 2 weeks there was a small drop in binding of the 10 uCi/ug label from 33.0 to 23.42%, while the binding of the 20 uCi/ug label started decreasing from 30.53 to 14.47% (Figure 23).

9. Purification of AFP from amniotic fluid for standard material

The purifications of human AFP from amniotic fluid using Sephacryl 300S was shown in Figure 24. The result showed that two peaks of protein and AFP were detected by absorbance at 280 nm. The albumin and AFP should be in the first peak (fraction number 16-26) because of the molecular weight is about 70,000 and the other proteins which is smaller than AFP should be in the second peak (fraction number 34-42). The resulted bands of two peaks were demonstrated by SDS PAGE and silver staining (Figure 25). The elution pattern of AFP and albumin of the first peak (fraction number 16-26) from 8.1 using Affi gel Blue (Figure 26) showed that AFP was eluted out from Affi gel Blue column in fraction number 4-12 which was detected by absorbance at 280 nm. But the albumin which bound with the column was washed out with 1.4 M NaCl in 0.02 M Phosphate

buffer pH 7.4. Then the pooled fraction of AFP from Affi gel Blue was also purified by the protein coupling CNBr activated Sepharose 4B (Affinity column), the elution pattern of AFP was also detected by absorbance at 280 nm (Figure 27). Comparison of purified AFP from Affi gel Blue (8.2) and the protein coupling CNBr activated Sepharose 4B (8.3) were made by SDS PAGE and silver staining (Figure28). The purified AFP from the protein coupling CNBr activated Sepharose 4B was also pure when compared with standard AFP(sigma).The comparison of AFP from CNBr activated Sepharose 4B and the fraction number 2 from Sephacryl 300S with standard human serum albumin and low molecular weight marker protein was shown in Figure 29.The result showed that the AFP purified from CNBr activated Sepharose 4B was pure as same as standard AFP (sigma) with molecular weight about 70,000.It was not found AFP in the fraction number 2 from Sephacryl 300S (Figure 29).

10. Optimization of the assay protocol

10.1. Study on range of standard curve

The result showed that the standard curve yielded exponential curve over the range 0-300 ng/ml reaching a plateau at 1,000 ng/ml. For the AFP concentrations in excess of 1,000 ng/ml, the binding rapidly dropped and reached the background level at 50,000 ng/ml due to hook effect (Figure 30).

10.2. Study on solid phase antibody required for AFP standard curve

The result showed that 2.5 mg/tube of cellulose solid phase anti AFP was sufficient to make the suitable percentage binding (30% B/T) at AFP concentration 320 ng/ml (Figure 31).

10.3. Study on labelled antibody required for AFP standard curve

The result showed that 100,000 cpm of labelled antibody was excess concentration to give the optimal binding at AFP concentration 300 ng/ml (B/T = 28%) and zero AFP (B/T = 0.28%). The higher amount of label (200,000 cpm) made the lower binding at AFP concentration 300 ng/ml (B/T = 22.67%) and higher binding at zero AFP (B/T = 0.4%) (Figure 32, Table 4).

10.4. Study on optimal assay condition

The comparison of the percentage binding (B/T) of the standard curve for the determination of optimal condition in AFP IRMA was shown in figure 33. The result showed that the percentage binding obtained from one step assay with rotating overnight gave the highest binding at AFP concentration 320 ng/ml(B/T = 30%). For two step assay, 3 hours at room temperature in the first and second incubation gave only 20% B/T. Therefore the optimal assay condition at room temperature with rotating overnight was selected for the developed AFP IRMA protocol.

11. Characterization of the developed assay procedure

11.1. Reproducibility

AFP IRMA gave a sensitivity of 0.63 ng/ml (the lowest detection limit) which was determined by measuring the AFP concentration of zero standard from 20 assays. Within assay precision (W.H.O. Immunoassay Programe) was less than 15% from 10 ng/ml,and less than 5% from 30 ng/ml (Figure 34). Between assay precision (W.H.O. Immunoassay Programe) was less than 10% from 10 to 320 ng/ml(Figure35).

11.2. The stability of AFP IRMA kit

The result showed that after 2 days the binding decreased from 15% to 2% and 17% to 11% for the reagent kept at 37° C and room temperature respectively(Figure 36). There was no significant change in the binding after 7 days until 2 months of the reagents kept at 4° C (Figure 36).

11.3. Comparison of self-preparation of AFP IRMA kit vs commercial available AFP kit

The AFP concentrations from 94 samples assayed by both methods were compared using linear regression analysis. The relationship (Figure 37) gave a slope of 0.760 with an intercept of 0.762 ng/ml and regression coefficient of 0.999.

11.4. The expected value of normal subjects

The reference range of AFP was a summary of data obtained by measuring AFP serum samples from normal healthy subjects (Figure 38). From the normal distribution curve,the expected value calculated by using mean + 2SD was found to be 0.68-10.0 ng/ml.

Table 2 Matching of AFP IRMA system using two monoclnal antibodies

Labelled antibody	Solid phase antibody	
	AFP I (%)	AFP II (%)
AFP I	1.8	6.0
AFP II	45.0	0.5

Table 3 Percentage incorporation of labelled antibody.

Content	μCi	% I^{125} Incorporation
Labelled antibody	324	76.6
Free Iodide	99	23.4
Total	423	100

Table 4 The effect of specific activity on labelled antibody stability.

Labelled Age (week)	Zero AFP (%B/T)		320 ng/mL AFP (%B/T)	
	10μCi/μg	20μCi/μg	10μCi/μg	20μCi/μg
0	0.80	0.63	37.51	32.53
2	0.72	0.39	33.00	30.53
3	0.54	0.49	30.93	25.05
4	0.52	-	29.71	-
5	0.72	0.82	28.14	19.30
7	1.07	1.38	25.80	17.56
8	1.16	1.75	23.42	14.47

Table 5 Effect of amount of labelled antibody on AFP IRMA.

AFP (ng/mL)	30995 cpm/tube (%B/T)	61950 cpm/tube (%B/T)	119559 cpm/tube (%B/T)	250684 cpm/tube (%B/T)
0	0.30	0.31	0.28	0.40
9.37	2.90	2.54	2.27	1.61
18.75	5.00	4.22	4.10	3.00
37.50	8.055	7.15	6.61	4.95
75.00	14.05	14.13	11.29	8.39
150.00	21.40	21.10	19.00	13.92
300.00	27.82	27.22	28.00	22.67

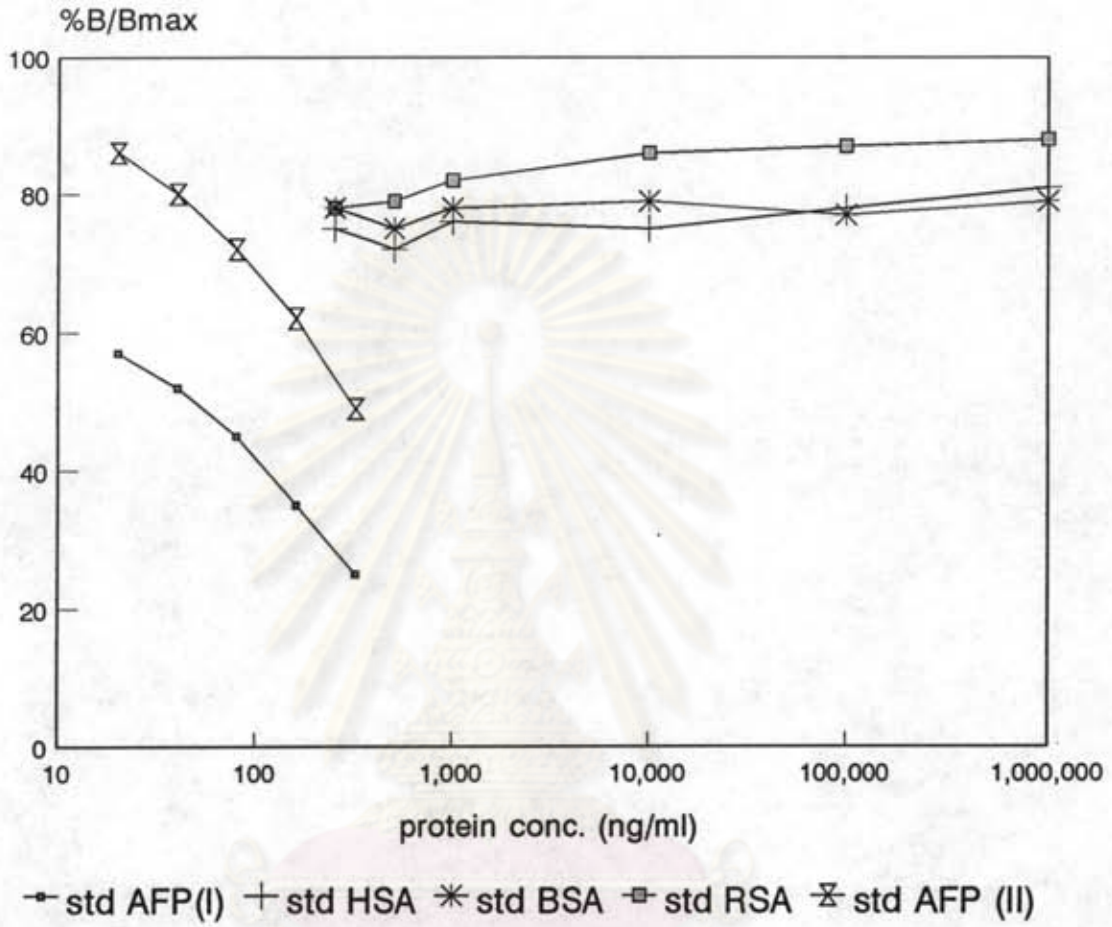

Figure 13 Specificity of monoclonal anti AFP with standard AFP, human serum albumin (HSA), bovine serum albumin (BSA) and rabbit serum albumin (RSA)

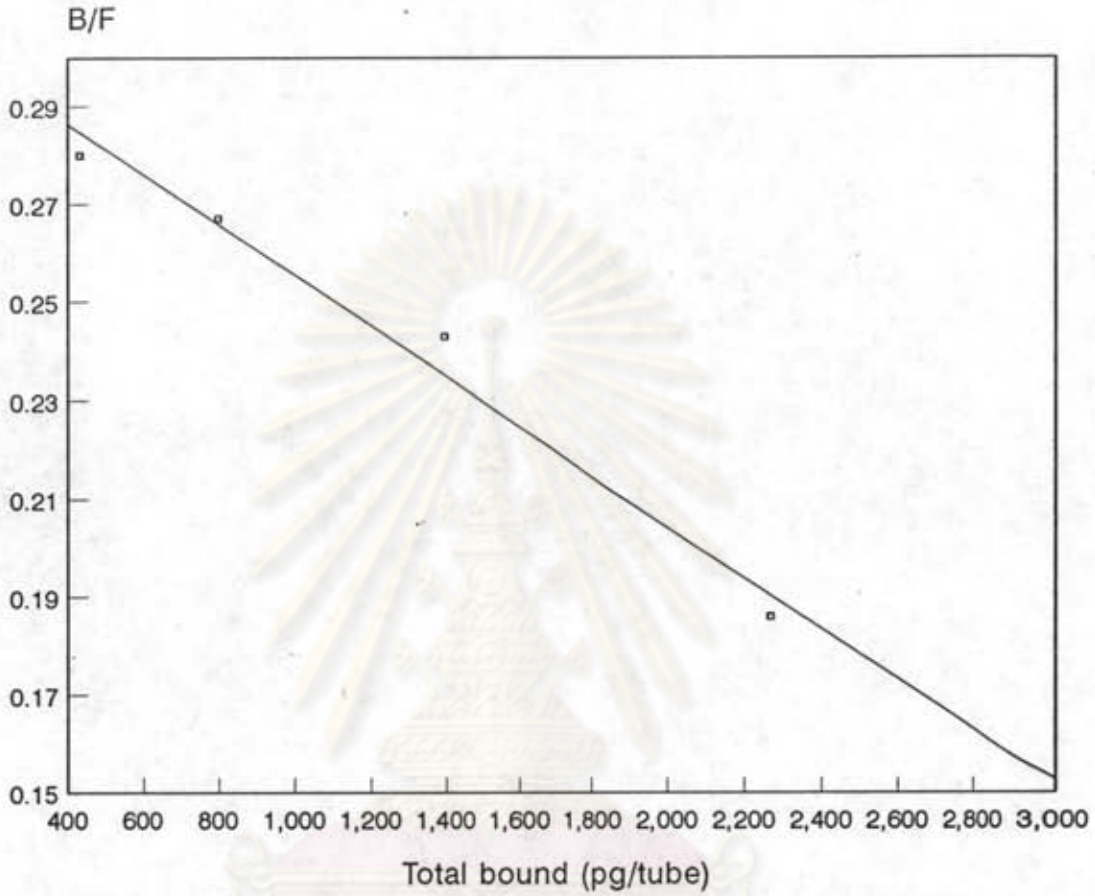

Figure 14 Scatchard plot for solid phase monoclonal anti AFP I
$K = 1.55 \times 10^{9}$ L/mole

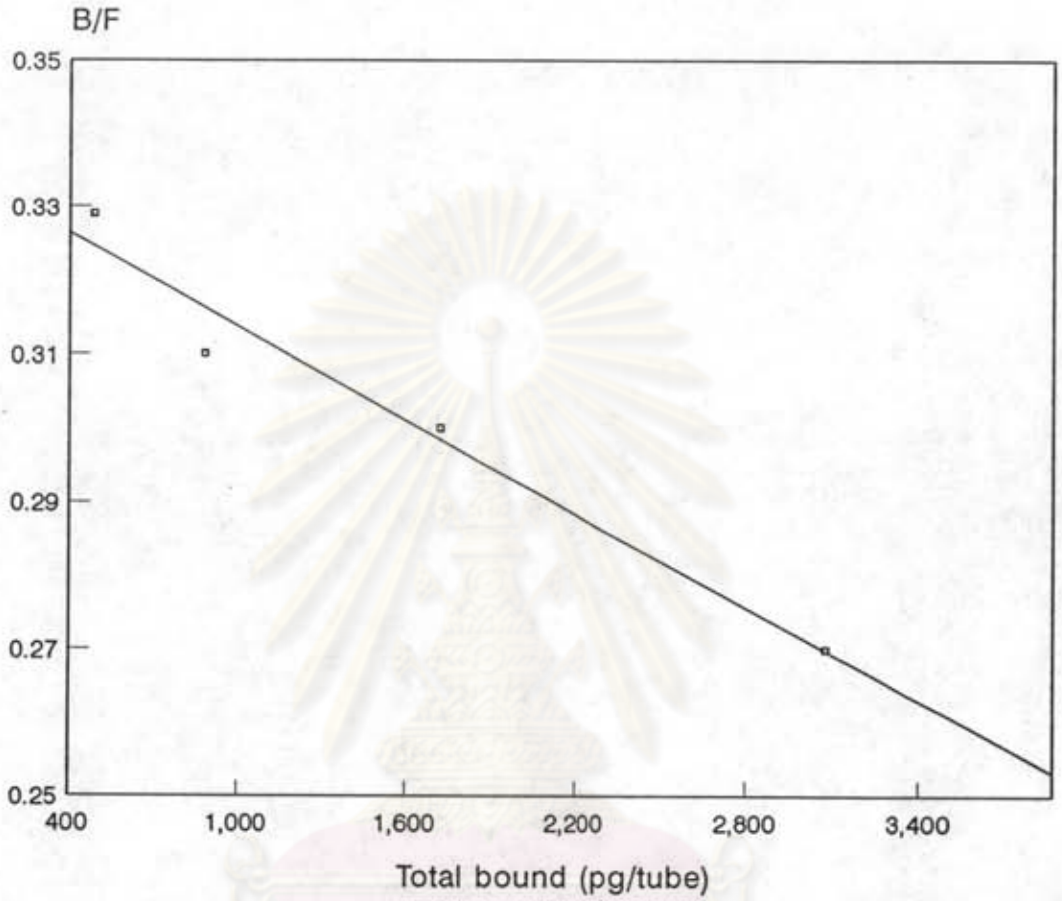

Figure 15 Scatchard plot for solid phase monoclonal anti AFP II
$K = 1.07 \times 10^{9}$ L/mole

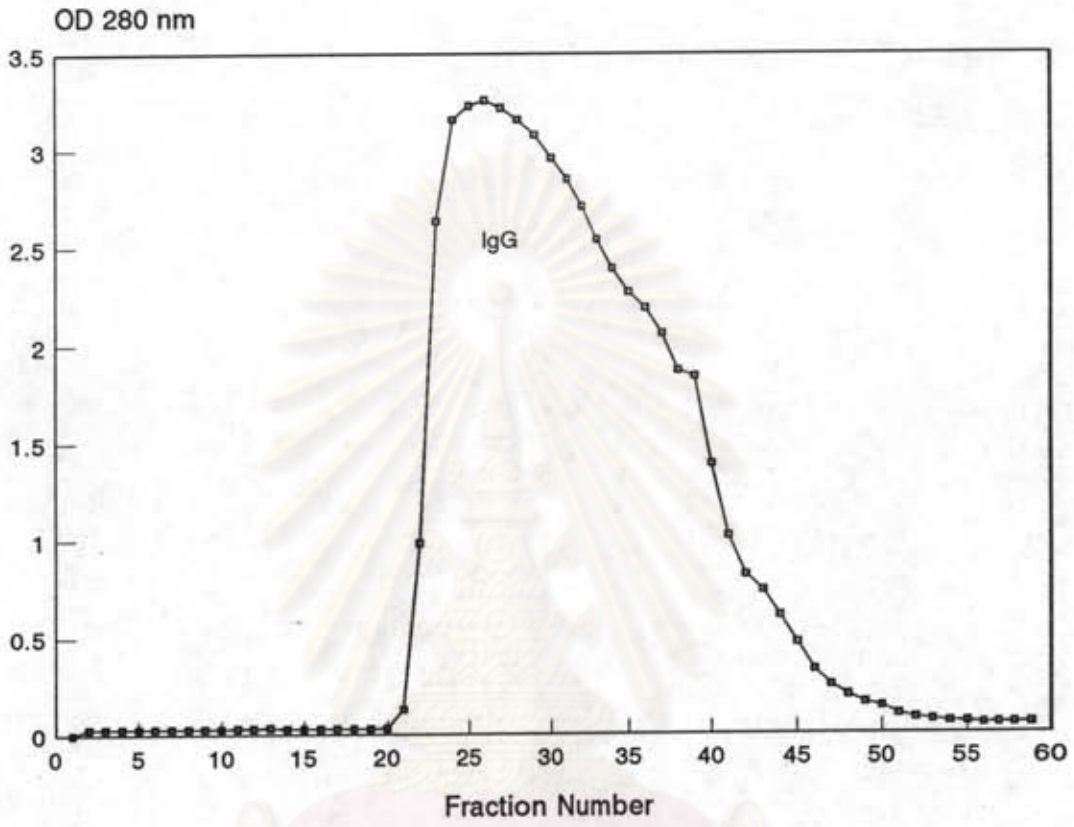

Figure 16 The elution pattern of ammonium sulfate cut ascites fluid I from DEAE column

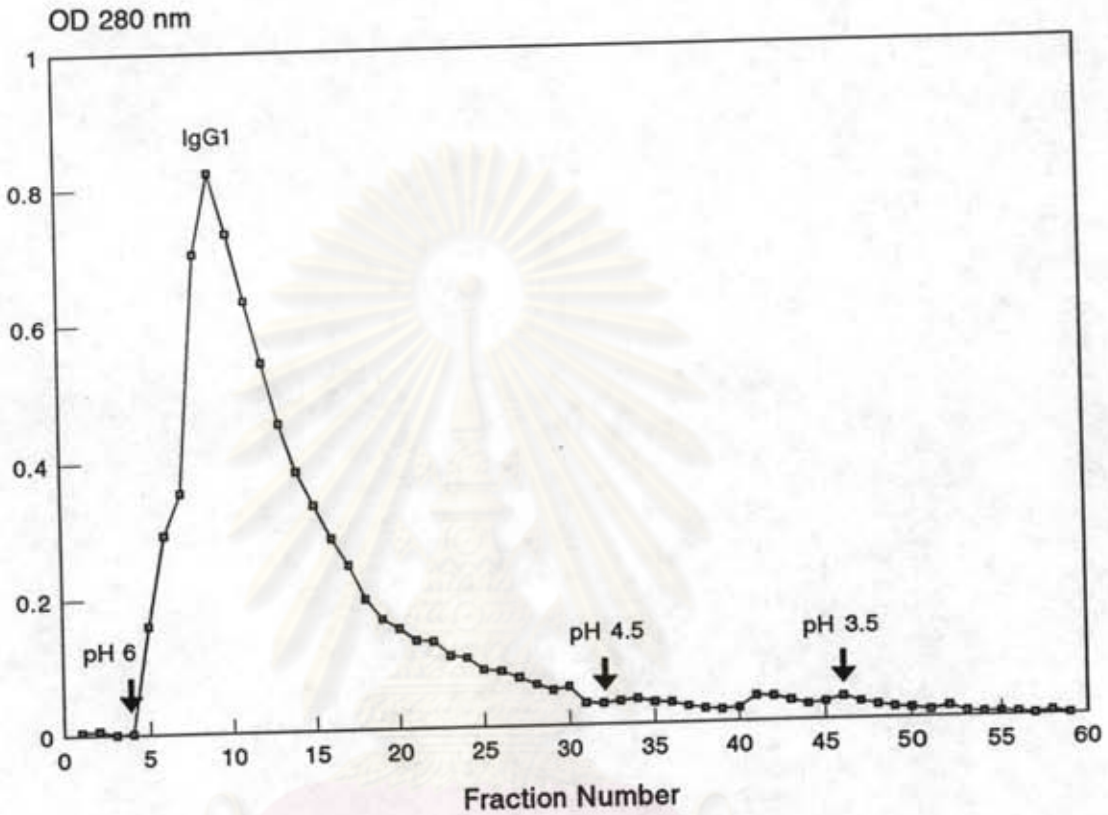

Figure 17 The elution pattern of ammonium sulfate cut ascites fluid II from protein A

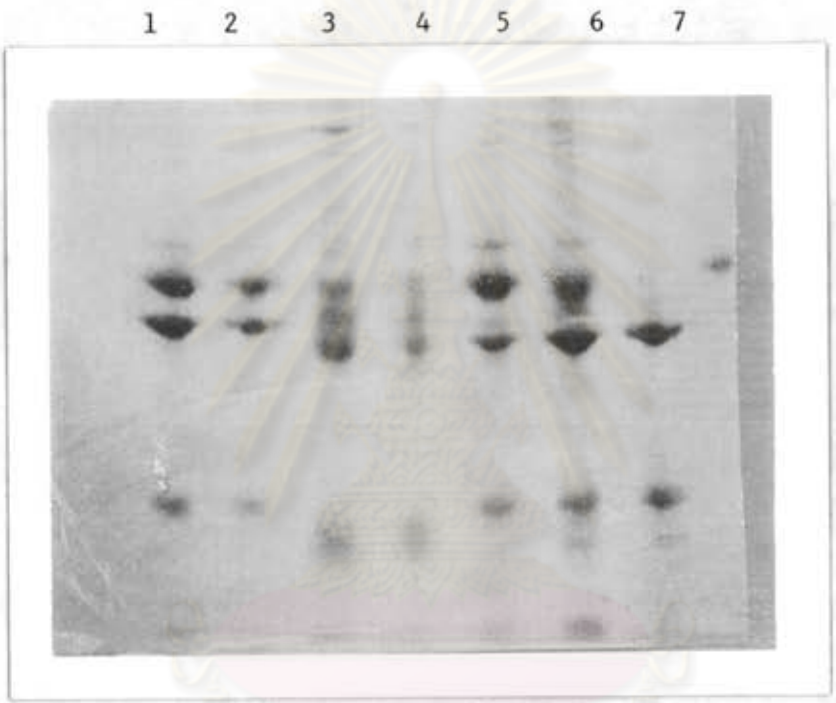

Figure 18 SDS PAGE of ascites fluid I and II purified by various methods

1 = Ascites fluid I , 2 = DEAE column chromatography
3, 4 = Ammonium sulfate precipitation (I)
5 = Ascites fluid II , 6 = Ammonium sulfate precipitation (II)
7 = Protein A purification

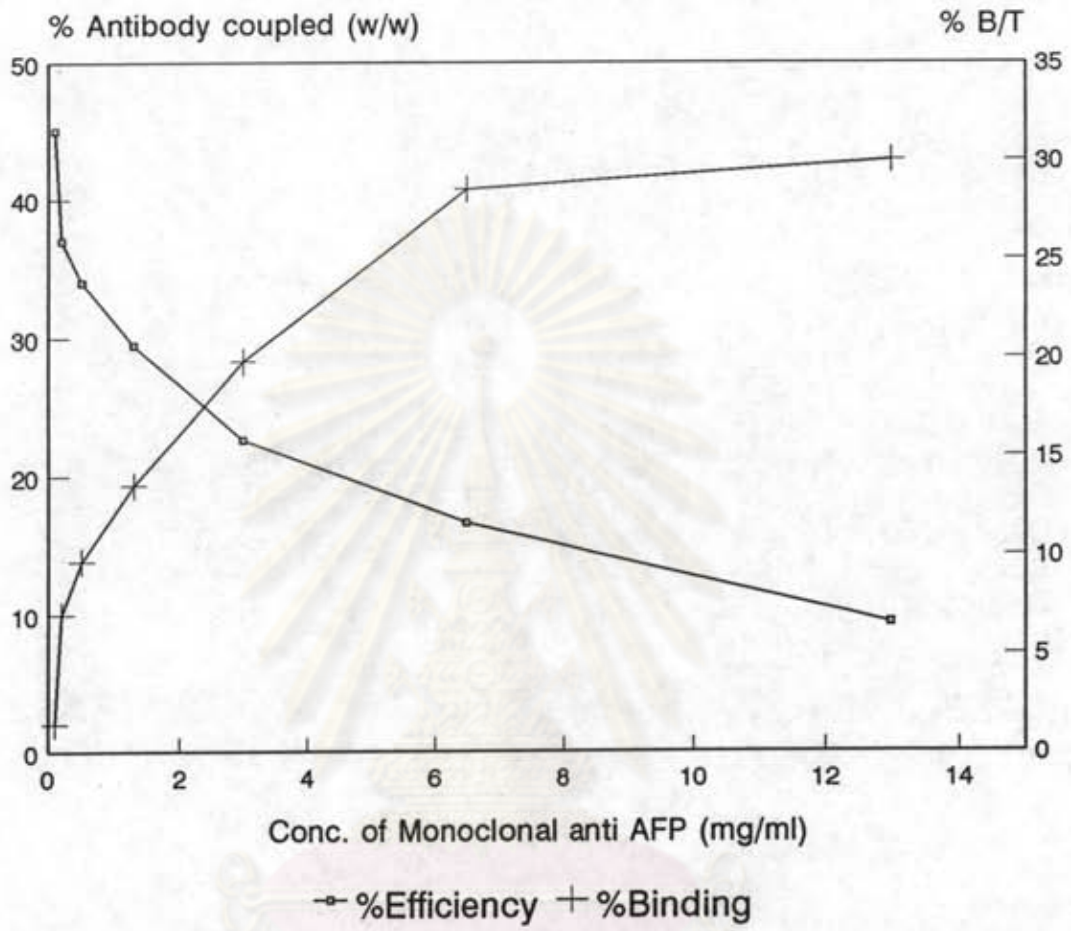

Figure 19 Coupling of monoclonal anti AFP I with activated cellulose

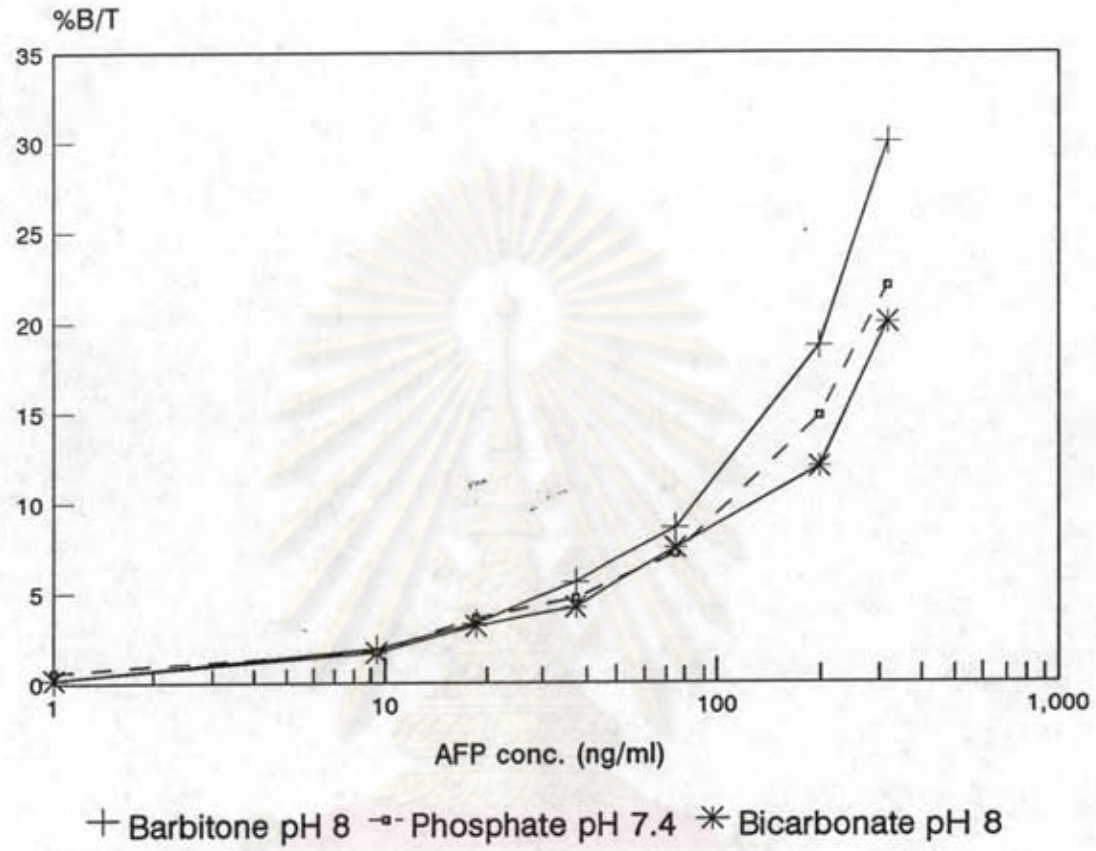

Figure 20 The effect of buffer on cellulose coating

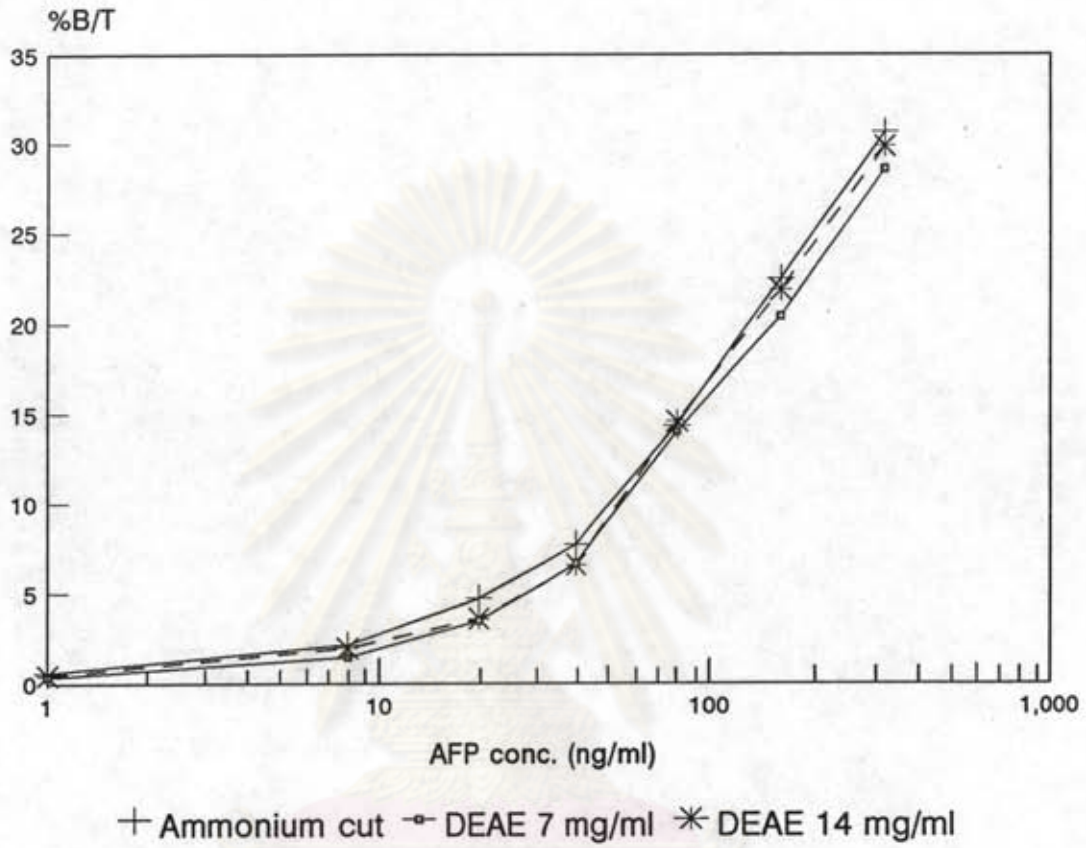

Figure 21 Comparison of DEAE and ammonium sulfate precipitate of monoclonal anti AFP I on cellulose coating

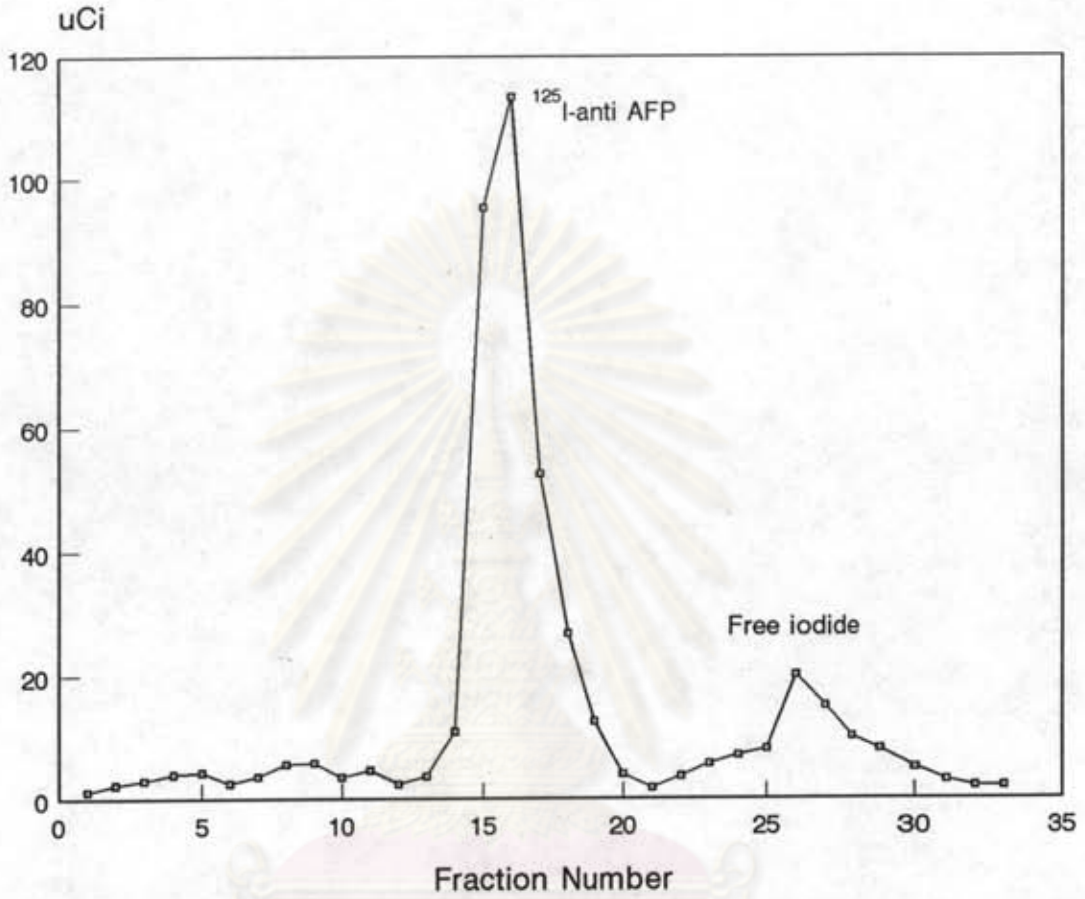

Figure 22 Iodination of monoclonal anti AFP II using N-Bromosuccinimide method

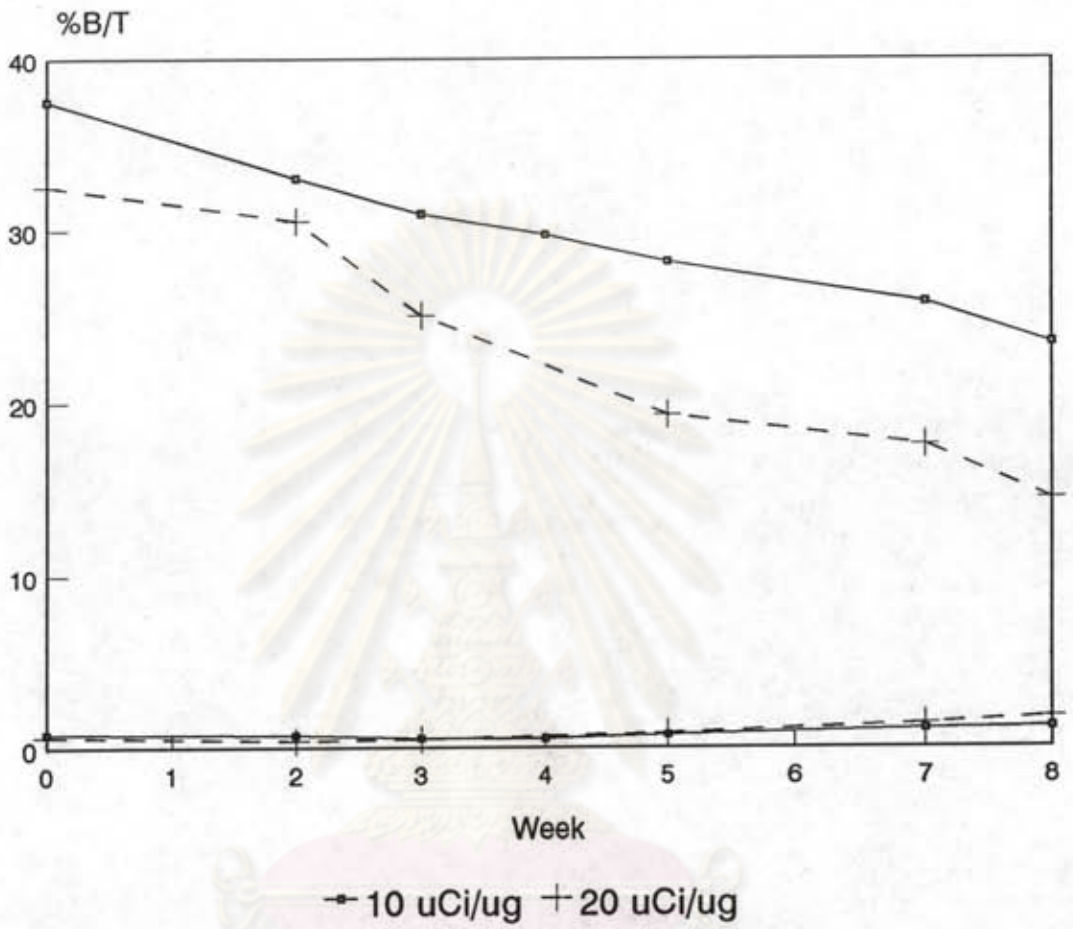

Figure 23 The effect of specific radioactivity on label stability

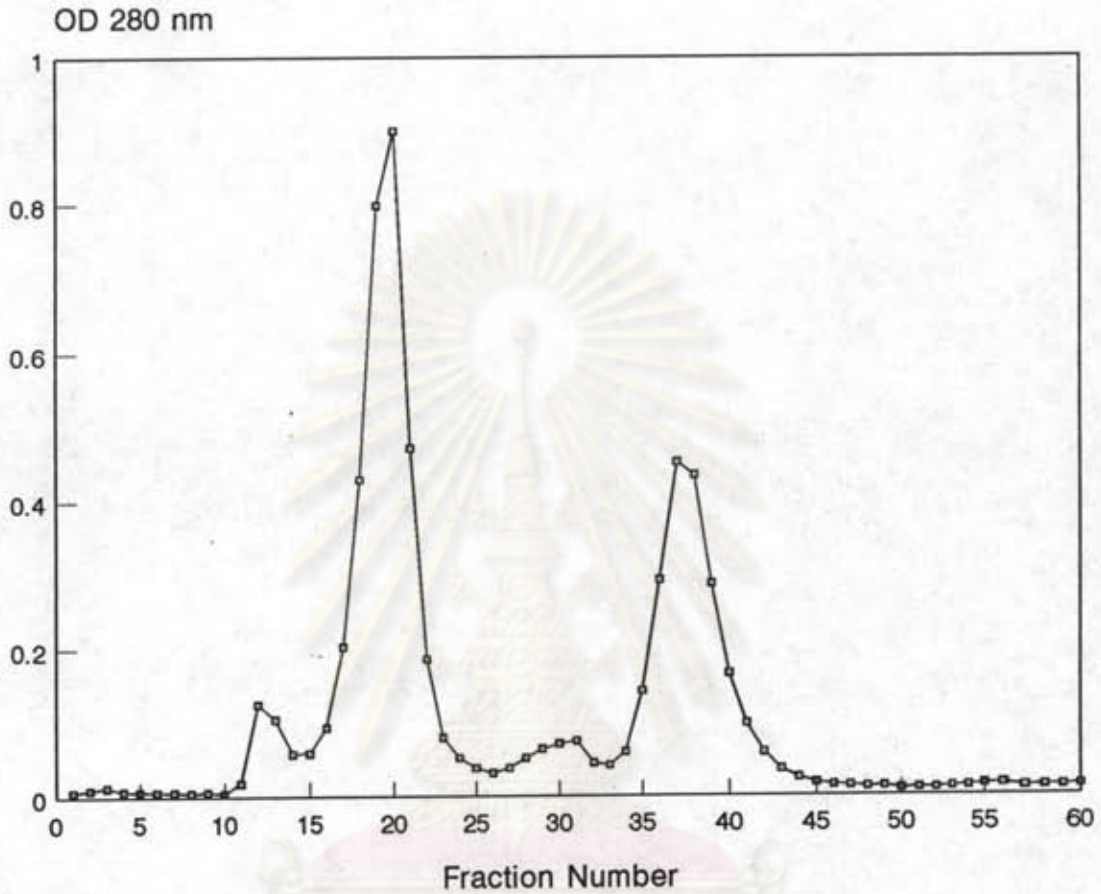

Figure 24 Purification of AFP from amniotic fluid using Sephacryl 300S column chromatography

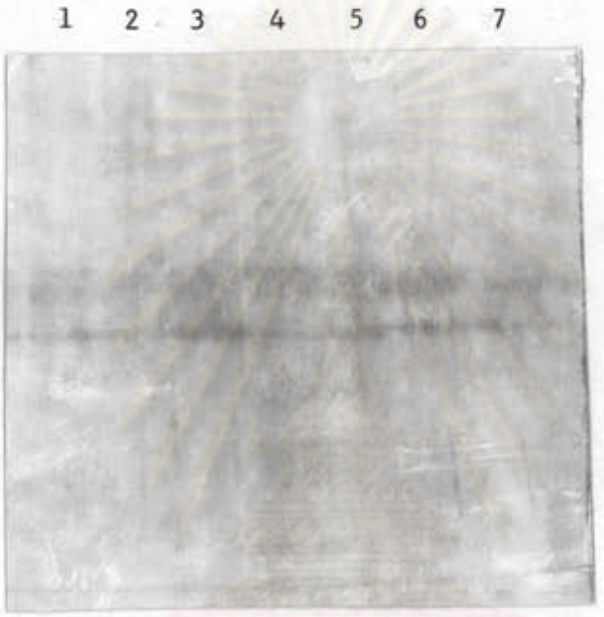

Figure 25 SDS PAGE and silver staining of AFP and the protein purified from Sephacryl 300S

1, 2, 3 = Standard AFP
4 = Standard marker
5 = Fraction number 1 (Sephacryl 300S)
6, 7 = Fraction number 2 (Sephacryl 300S)

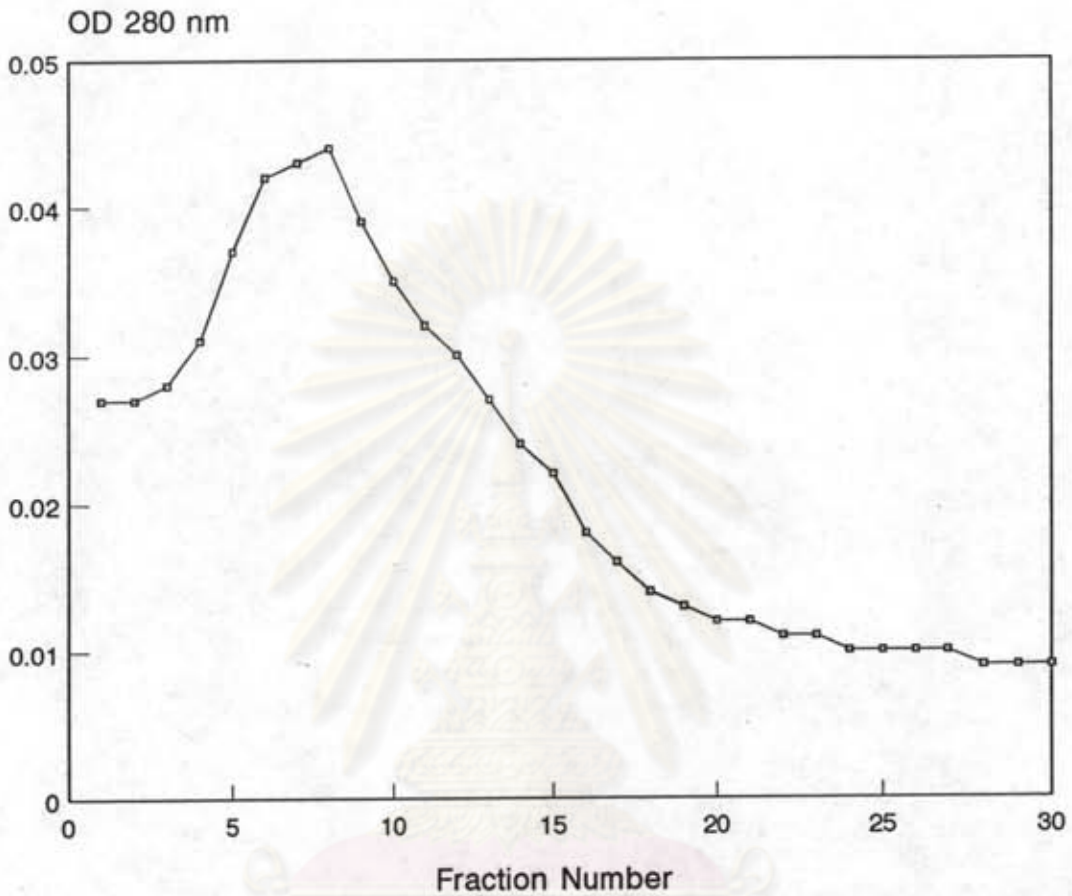

Figure 26 Purification of AFP from amniotic fluid using Affi gel Blue column chromatography

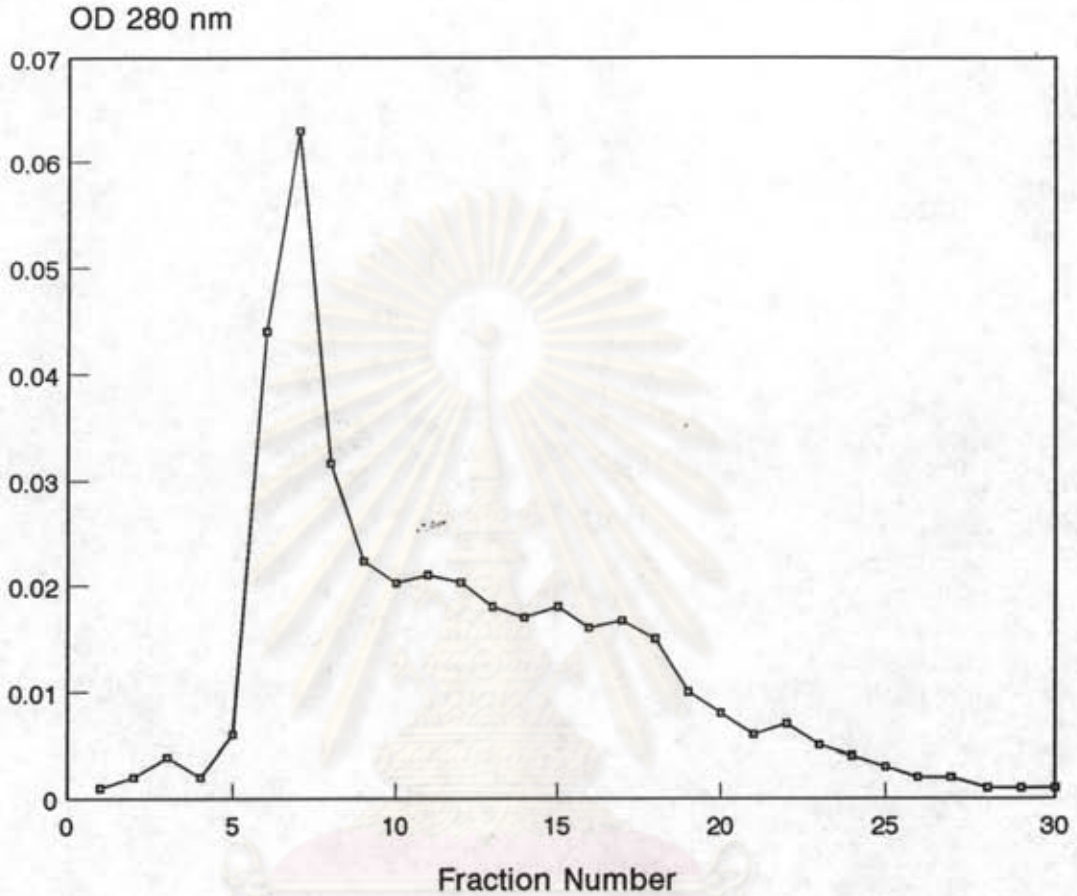

Figure 27 Purification of AFP from amniotic fluid using CNBr activated Sepharose 4B

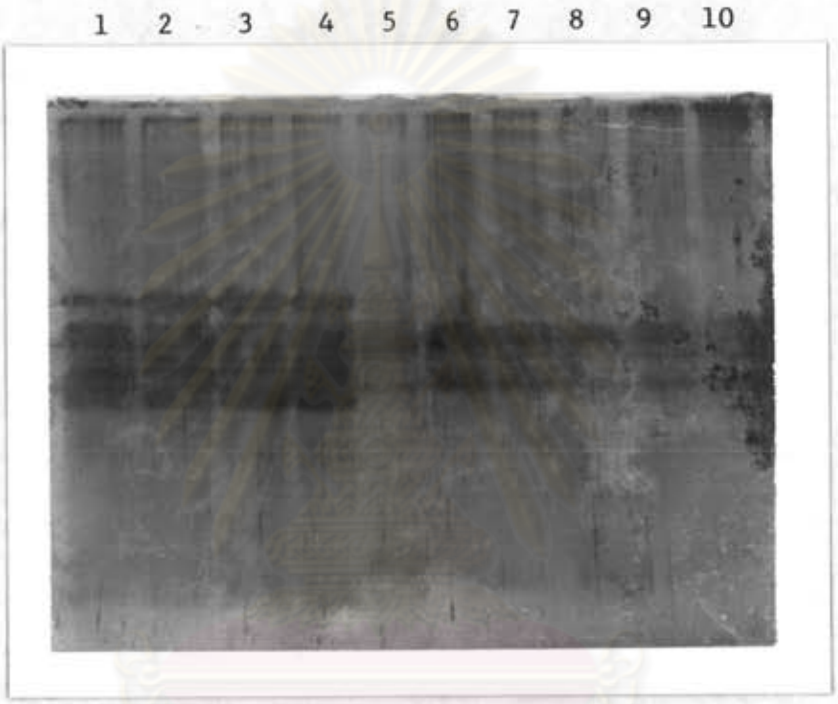

Figure 28 SDS PAGE of AFP purified by various methods

1, 2, 3, 4 = Affi gel Blue
5, 6, 7, 8 = CNBr activated Sepharose 4B
9, 10 = Standard AFP (Sigma)

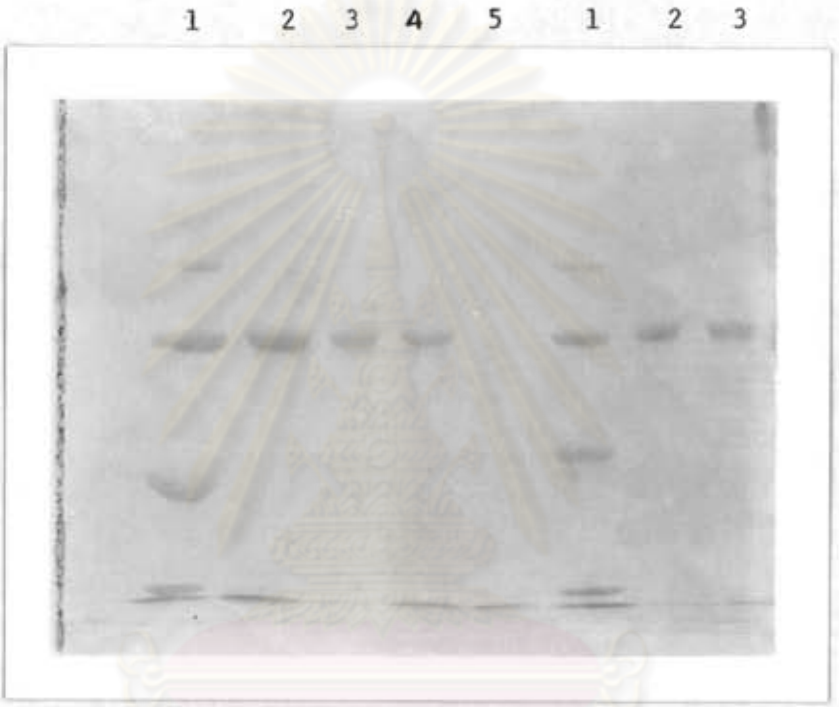

Figure 29 SDS PAGE of various proteins

1 = Low molecular weight marker
2 = Standard human serum albumin
3 = Standard AFP (Sigma)
4 = AFP (CNBr activated Sepharose 4B)
5 = Fraction number 2 (Sephacryl 300S)

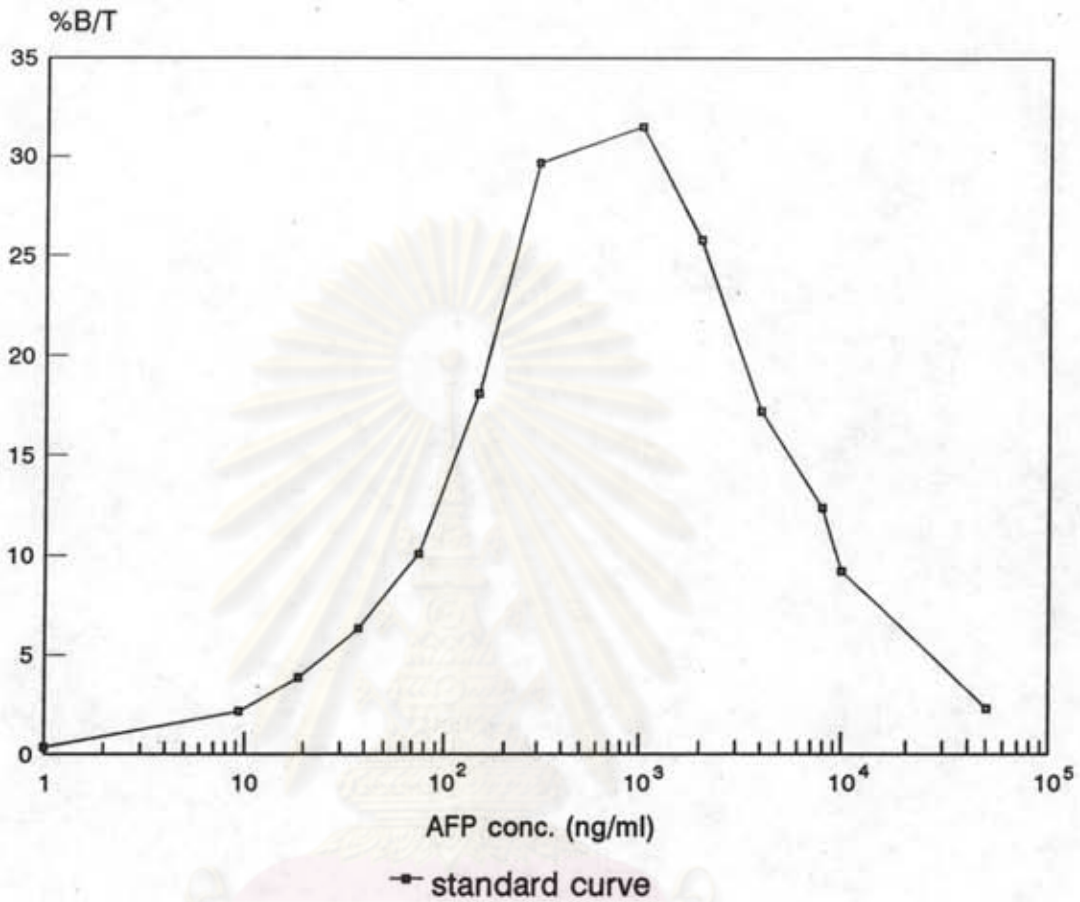

Figure 30 Extended standard curve (0-50,000 ng/ml)

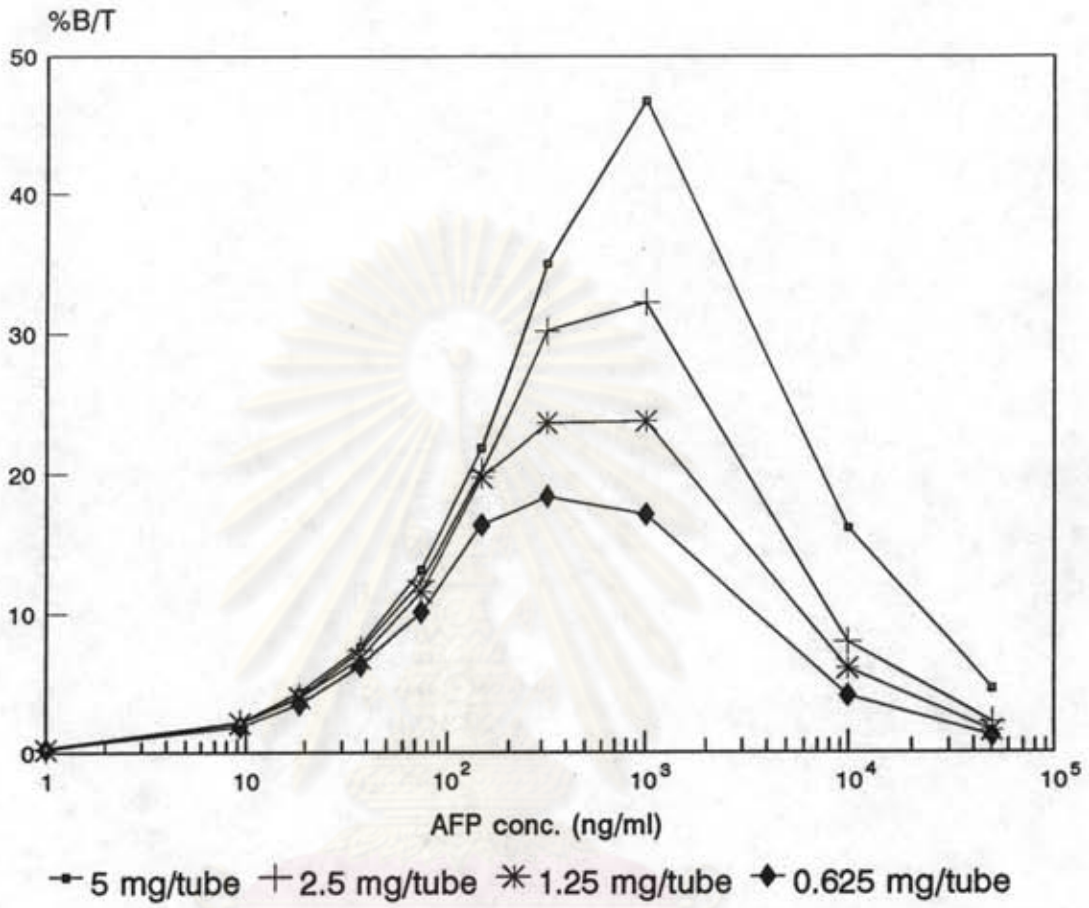

Figure 31 The amount of antibody coated cellulose required for IRMA

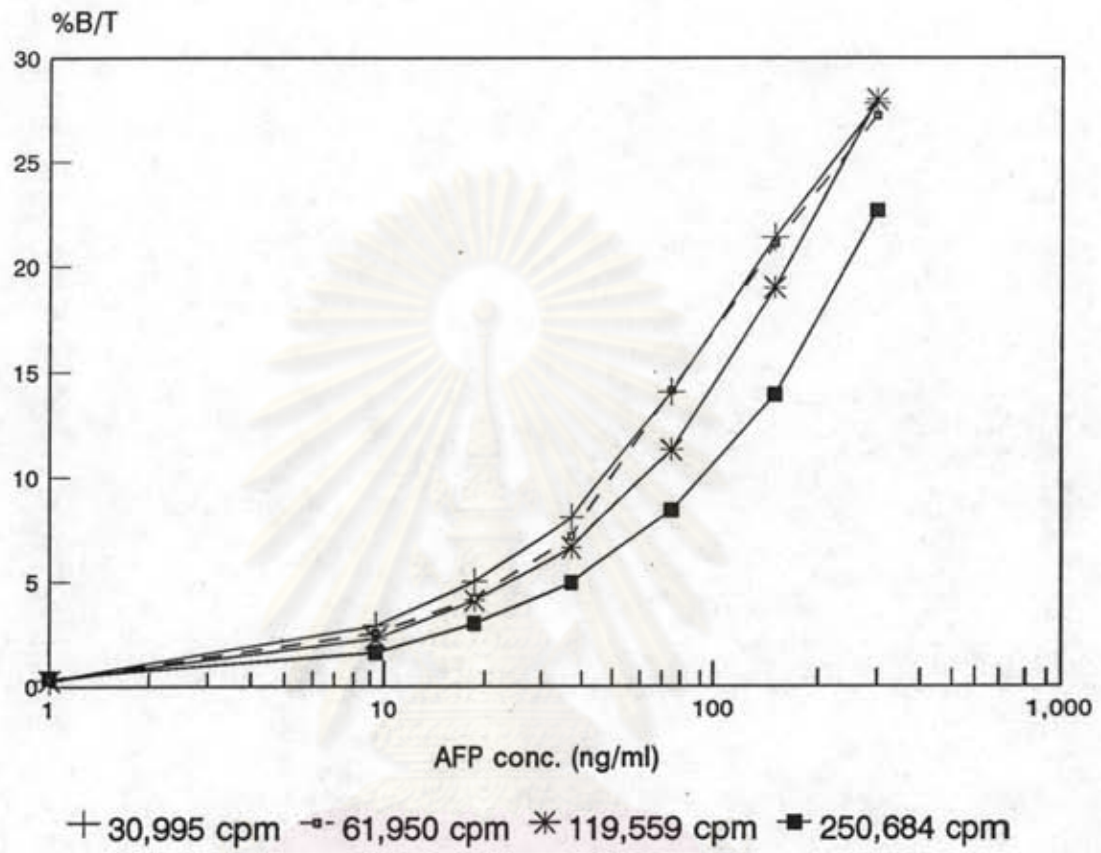

Figure 32 The amount of labelled antibody required for AFP standard curve

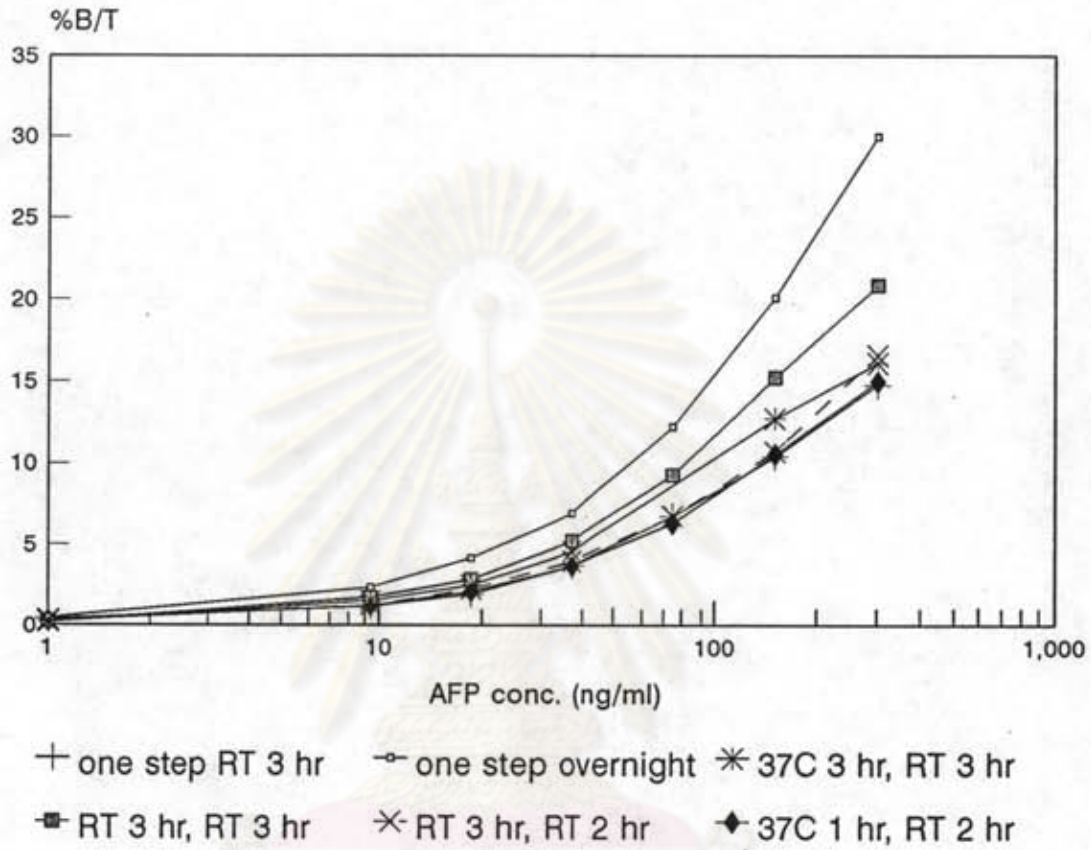

Figure 33 Response curves with different incubation times

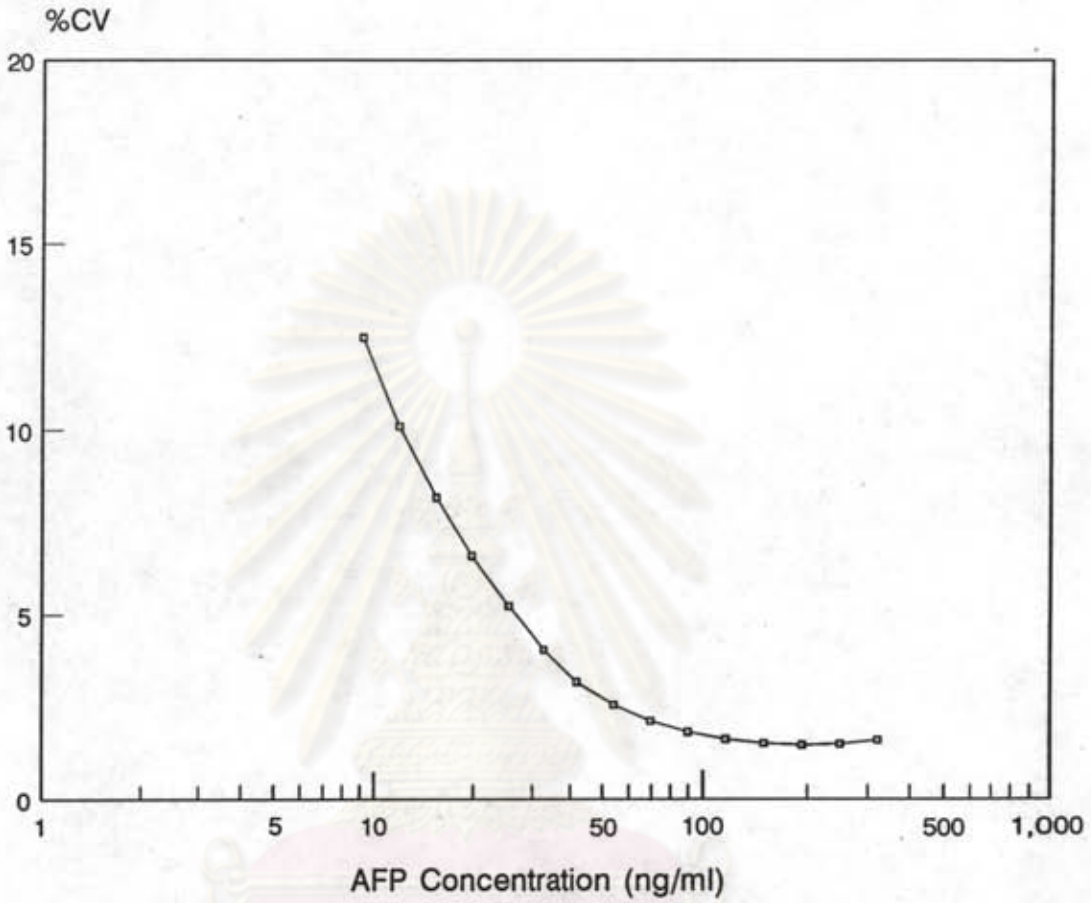

Figure 34 Within-assay Precision Profile of AFP IRMA

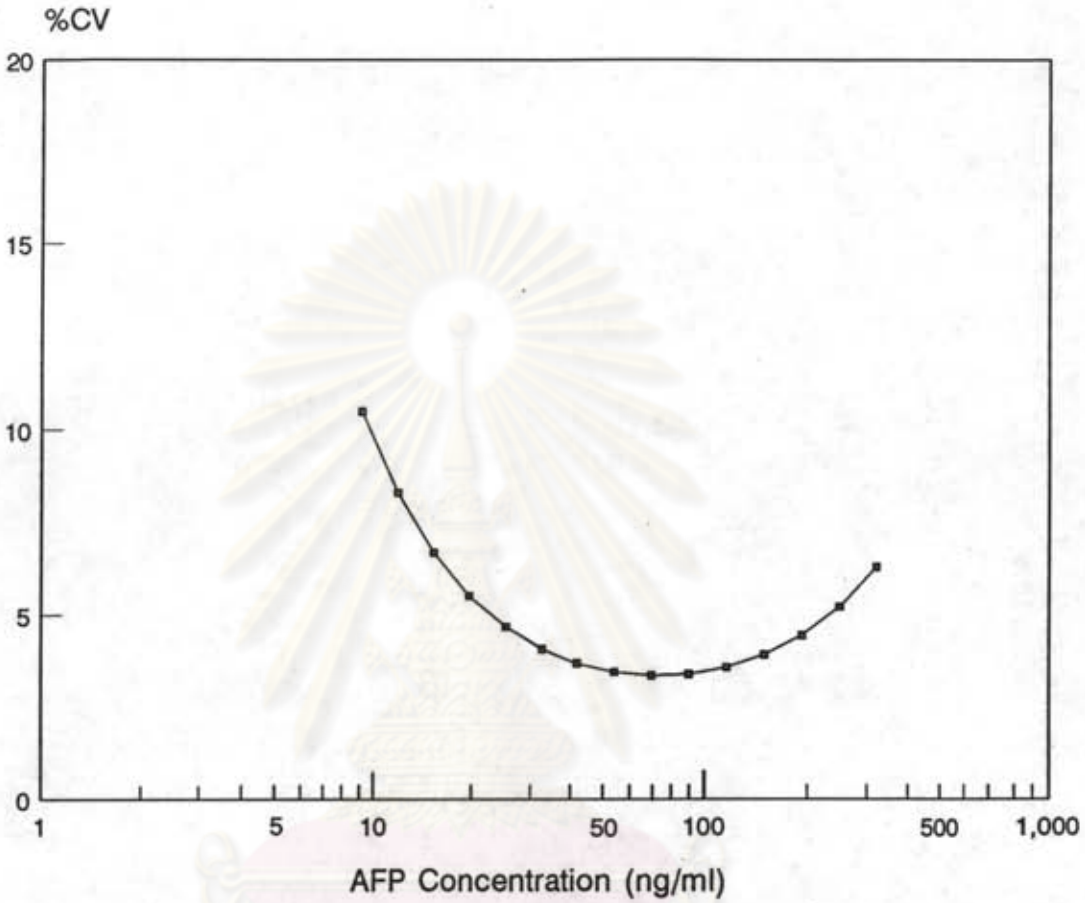

Figure 35 Between-assay Precision Profile of AFP IRMA

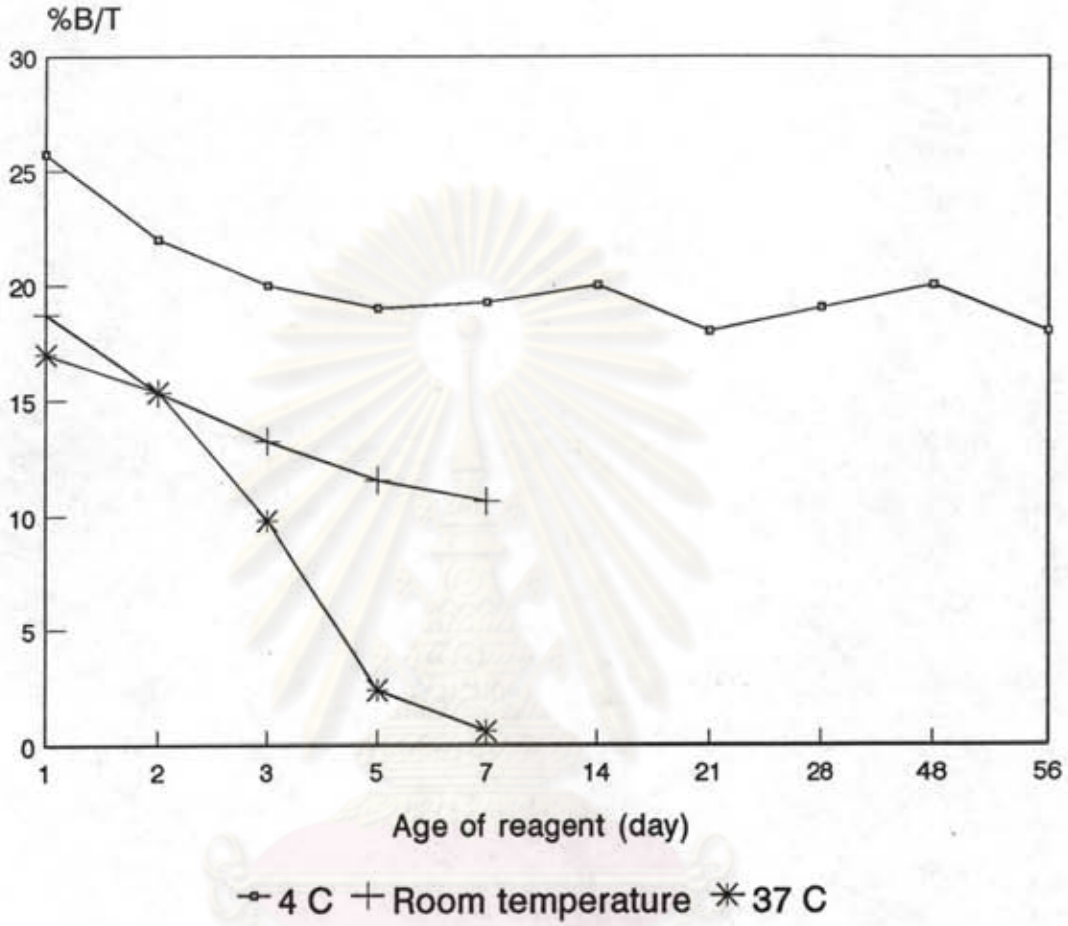

Figure 36 The stability test of AFP IRMA kit

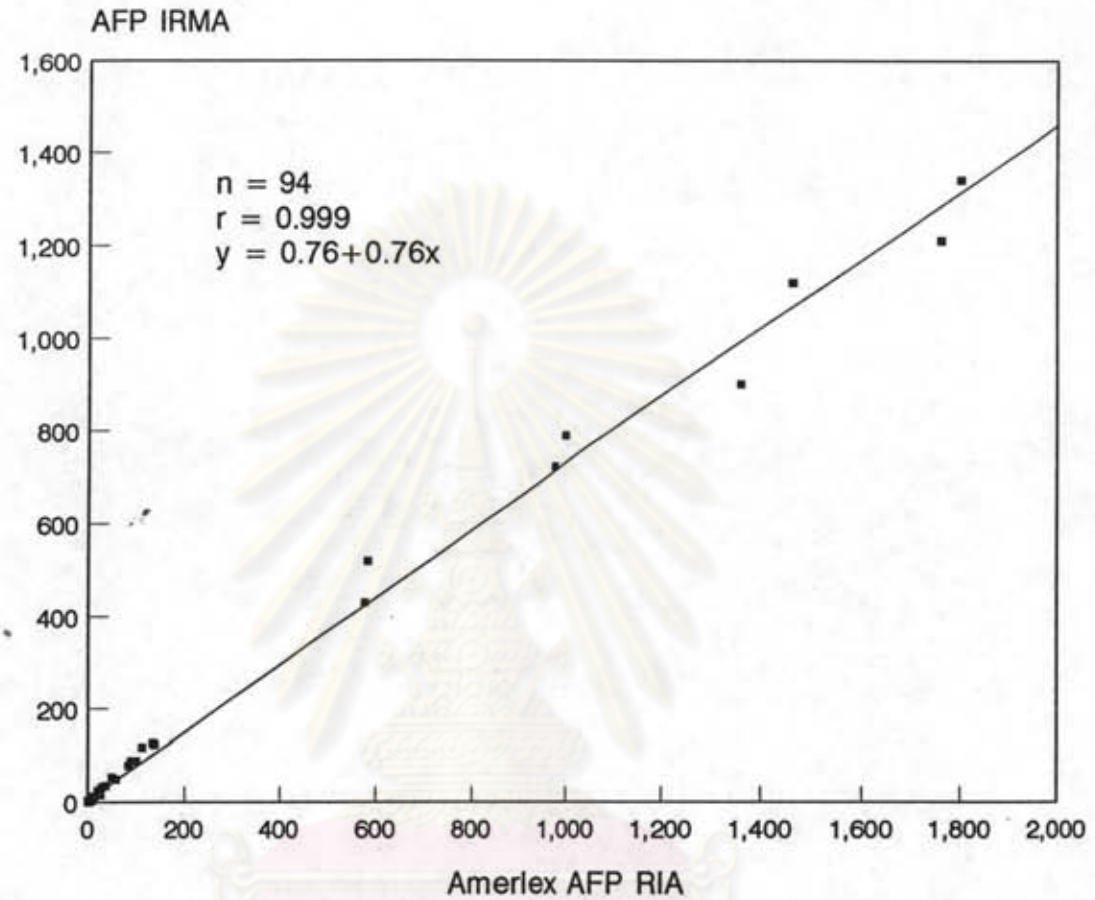

Figure 37 Correlation between AFP IRMA kit and Amerlex AFP RIA for the determination of AFP in 94 sera

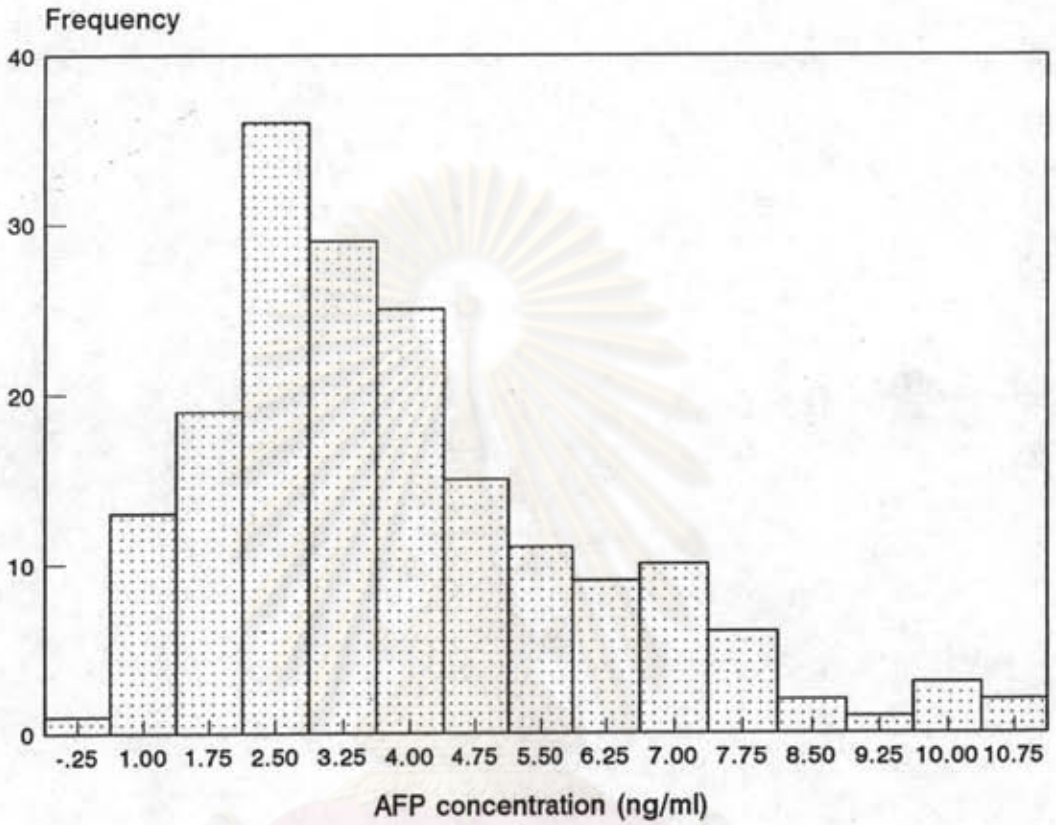

Figure 38 Normal distribution of AFP concentration of normal subjects mean = 4.16 ng/ml, mean ± 2SD = 0.68-10.00 ng/ml