Chapter III

Materials and Methods

Materials

1. Chemical and biological reagents

1.1. Two cell lines were received from WHO Collaborating Center of Immunoassay London United Kingdom.

1.2. The commercial kits

- Sigma Immunochemicals's Immunotype™ kit was intended for the rapid, sensitive and specific determination of mouse monoclonal antibody isotypes in culture supernatants.

- Amerlex - M 2nd trimester AFP RIA Kit was intended for the quantitative measurement of alpha-fetoprotein (AFP) in human serum.

1.3. Pool Amniotic fluid were obtained from pregnancy women at 12 week gestation at Department of Medicine, Chulalongkorn hospital.

1.4. Samples were tested for AFP

- Hepatoma, Cirrhosis and liver abscess were obtained from the National Cancer Institute of Thailand.

- Normal sera were obtained from donors at Thai Red Cross, Chulalongkorn Hospital.

2. Chemical reagents

Ammonium sulfate (MERCK, W. Germany)

Affi gel Blue (MERCK, W. Germany)

Ammonium persulfate (MERCK, W. Germany)

Acrylamide (MERCK, W. Germany)

Acetone, AR grade (BDH, England)

Bovine serum albumin (Sigma, USA.)

Barbitone (Sigma, USA.)

Citric acid anhydrous (Sigma, USA.)

Coomassie brilliant blue (Sigma, USA.)

Copper sulphate (Sigma, USA.)

1,1' carbonyldiimidazone (Sigma, USA.)

Cyanogen bromide activated Sepharose 4B (Pharmacia, Sweden)

Ethanolamine (BDH, England)

Formaldehyde (BDH, England)

Folin's Ciocalteau phenol (BDH, England)

Fetal calf serum (GIBCO, USA.)

N,N'-methylene-bis (acrylamide) (MERCK, W. Germany)

Sephadex G100 (Pharmacia, Sweden)

Sephacryl 300S (Pharmacia, Sweden)

Sodium carbonate (Sigma, USA.)

Sodium bicarbonate (Sigma, USA.)

di-Sodium hydrogen phosphate (MERCK, W. Germany)

Sodium dihydrogen phosphate monohydrate (MERCK, W. Germany)

Sodium dodecyl sulfate (Sigma, USA.)

Silver nitrate (Sigma, USA.)

Sodium acetate (MERCK, W. Germany)

Sodium hydroxide (MERCK, W. Germany)

Sodium thiocyanate (MERCK, W. Germany)

Sodium Potassium tartate (MERCK, W. Germany)

Tris base (Sigma, USA.)

Urea (Sigma, USA.)

3. Instruments

CO_2 Incubator model 3164 (Forma Scientific, USA.)

Biological Safety Cabinets Model Nu 425-300 (Nuaire, USA.)

Fraction collector Lambda-Max Model 481 (Waters, USA.)

Chromatography column (Pharmacia, Sweden)

Power supply Model 200/2.0 (Biorad Laboratories, Inc., USA.)

Electrophoresis chamber (Eido, Japan)

Rotator (Leec company, England)

Spectrophotometer Hewlett Model 8452A (Packard, Germany)

High performance liquid chromatography Model 510 (Waters, USA.)

Vortex mixer (Scientific industries, USA.)

Gamma counter RIA star (Packard, A canbera company, USA.)

Automatic refrigerated centrifuge Model SCR 20 BA (Hitachi, Japan)

Micro Mixer (Scientific industries, USA.)

Methods

1. Establishment cell lines

1.1. recloning process

The spleen cells were prepared the day before performing the cloning experiment and suspended at 10^6 cells /ml in complete RPMI medium, then 1 ml of spleen was pipetted into each well of 96 well plates and left overnight in CO_2 gassed incubator. The hybridoma cell lines should be thawed rapidly in 37^0 c water bath,taking care to avoid contact of the water with the seal area of the vials. Then 1 ml of hybridoma cell was plated into 96 wells containing the spleen cells and incubated in a CO_2 gassed incubator for 5-7 days. When a hybrid culture producing antibody was identified and confirm as positive. The log phase hybrid cells in complete RPMI medium were transfered approximately 0.5-1.0 cell/well to 96 well plates containing the spleen cells at 10^6 cells/well. The culture volume needed to give the desired number of cells were planted at 200 ul/well in 96 well plates. About 7 to 14 days, the single clones were examined under an inverted microscope, tested for characterization as described in 3.1 and 3.2. The stable positive clone was selected and expanded to 6 well plates, then to 25 cm^2 flask and finally to 75 cm^2 flask and frozen in liquid nitrogen.

1.2. Storage of hybrid in liquid nitrogen

Aliquots of hybridoma cells (10^7 per vial) were resuspended in 1 ml of a mixture of 90% (v/v) fetal calf serum and 10% dimethyl sulphoxide. They should be placed

immediately into -20° C freezer about four hours and transfer the vials to -70° C freezer overnight. Finally they were kept directly into the liquid nitrogen (-196° C). The cell lines were subcultured again every six month.

2. Production of monoclonal antibody to AFP

2.1. Balb-c-mice preparation

Pretreatment of the peritoneal cavity of 5 week old mice were obtained. About 50 balb-c-mice were injected with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) intraperitoneally at least 7 days prior to injecting the cells.

2.2. cell line propagation

The cell lines in complete RPMI medium (AppendixI,1.2) were cultured in CO_2 gassed incubator. The hybridoma cells were collected from the culture medium by centrifugation, the supernatant was poured and suspended the cell with RPMI 1640 to make 10^7 cells/0.5 ml for intraperitoneal injection.

2.3. Ascites production

Approximately 10^7 cells were injected intraperitoneal to each mice previously treated with pristane at least 7 days. About 25 pristane mice were used for each cell line. After 2 weeks, the ascites fluid was removed when the mice show sufficient abdominal swollen. Ascites fluid may be obtained from the same mouse every two days, allowed to clot, clarified by centrifugation and stored frozen for further purification process.

3. Characterization of monoclonal antibody from the hybridoma

3.1. Isotyping of the monoclonal antibody

The supernatants of two hybridoma were collected from culture media and tested for isotyping using Immunochemicals'Immunotype™ kit. A nitro cellulose membrane strip was placed into a test tube containing 2-3 ml of hybridoma supernatant with shaking for 30 minutes. The supernatant was decanted and the strip was washed with 3 ml of PBS-T-BSA for 5 minutes. Then the diluted biotinylated second antibody was poured into the assay tube and incubated for 5 minutes. The solution was decant and washed the strip once in 3 ml of PBS-T-BSA for 5 minutes. The diluted ExtrAvidin-Peroxidase was diluted (1:50) in PBS-T-BSA and poured into the assay tube for 5 minutes. After incubation time, the strip was washed twice with PBS-T-BSA and the substrate solution was prepared by adding substrate chromogen and 2% Hydrogen peroxide in substrate buffer. The substrate solution was then added and incubated until insoluble signal (+) was obtained for the positive control. The results will appear on the strip as an alphabet "IgG" isotype which can be stored as a permanent record by keeping on sheets of filter paper.

3.2. Specificity

The amount of bovine serum albumin (BSA), human serum albumin (HSA) and rabbit serum albumin (RSA) were diluted in PBS to give final concentration of 250 ng, 500 ng, 1 ug, 100 ug and 1 mg. Each concentration of proteins

will be assessed by measuring the appearent response of the binding to monoclonal antiAFP as the protocol in AppendixII

3.3. Estimating the association constants of monoclonal anti AFP I and II

The characterization of association constants of monoclonal antibody was estimated from scatchard plot using liquid phase radioimmunoassay. One hundred microliters of standard AFP ranging from 0-160 ng/ml, together with 50 ul (30,000 cpm) of labelled standard AFP (Appendix II,1) and 50 ul of monoclonal anti AFP I (1:400) or monoclonal anti AFP II (1:16,000) for determination of each association constant of monoclonal anti AFP I and II was incubated with 200 ul assay buffer (0.05 M Phosphate buffer pH 7.4) for 4° C overnight. Then, 5 ug of Goat anti mouse IgG (1:50, Zymed) was added into the assay tubes and incubated at room temperture for 20 minutes. Bound and free fractions were seperated by centrifugation at 3,000 rpm for 40 minutes at 4° C, followed by decanting the supernate. The assay tubes were counted in gamma counter and the curve was plotted between bound/free (B/F) and total bound. The association constants (K) were estimated from this curve in Appendix II,4.

4. Purification of ascites

4.1. Ammonium sulfate precipitation

One hundred grams of ammonium sulfate was dissolved in 1 litre distilled water at 50° C. It was allowed to cool at room temperature and adjusted pH to 7.2

using 2N H_2SO_4. The ammonium sulfate solution was added to the mouse ascites to give a final concentration of 50% saturated (50 volumes saturated ammonium sulfate + 50 volumes ascites) and stirred at room temperature for 30 minutes. The precipitate was collected by centrifugation (3000 rpm for 15 minutes) and washed once in 50% saturated ammonium sulfate. The precipitate of monoclonal anti AFP I,II were redissolved in 0.07 M phosphate buffer pH 6.3 for further purification using DEAE column and 0.05 M phosphate buffer pH 7.4 for labelling to the original volume, and reprecipitated at 45% saturated ammonium sulfate. It was washed once in 45% ammonium sulfate, redissolved in a minimal volume of buffer as required and dialysed at 4° C for 2 days against two changes of buffer.

4.2. DEAE ion exchange chromatography

The DE 52 (Diethylaminoethyl cellulose) was equilibrated with phosphate buffer 0.07 M pH 6.3, packed into a column 3x21 cm(and washed with the same buffer at room temperature. When the DEAE column and sample were fully equilibrated, 100 mg of the precipitate of monoclonal anti AFP I was applied to the column and eluted with 0.07 M phosphate buffer pH 6.3 at room temperature. Two ml fractions were collected and monitored by UV absorbance at 280 nm. The early fractions of the first peak were pooled and dialysed against 0.05 M Phosphate buffer pH 7.4 (for storage). The immunoglobulin was eluted by the starting buffer and the impurities were bound and eluted by the 0.1 M phosphate buffer pH 7.4.

4.3. Affinity separation on Protein-A-Sepharose

The swollen gel (Protein A-Sepharose CL-4B) was packed into the column and washed with 5 ml 0.1 M phosphate buffer, 2 ml citric acid solution and 5 ml 0.1 M phosphate buffer. The pH of the ascites of monoclonal anti AFP II was adjusted to 8.0 by adding tris base and 1 ml 0.1 M phosphate buffer pH8.0, mixed and applied to the Protein A column (0.9 x 15 cm). The 0.5 ml fractions were collected and monitored by UV absorbance at 280 nm. The column was washed with 20 ml 0.1 M phosphate buffer and eluted with 15 ml citrate buffer pH 6.0. Sixty microliters of 2 M tris was placed in the next 20 empty fraction collector tubes for eluting with 10 ml citrate buffer pH 4.5 and followed by 10 ml citrate buffer pH 3.5. The column was then washed with 2 ml citric acid solution and 5 ml 0.1 M phosphate buffer. The eluted fractions were dialysed against 0.05 M phosphate buffer pH 7.4.

5. Identification of purified antibody by SDS polyacrylamide gel

A separation gel mixture (Appendix I,4.5.1) was prepared and poured in the gel slab, then overlaid by distilled water and allowed to polymerize for about 30 minutes. Then the water phase was removed and a mixture for the stacking gel (Appendix I,4.5.2) was poured over the lower gel. The comb was inserted on the top and the gel was allowed to polymerize about 20-30 minutes. Thirty microliters of each sample from 4.1, 4.2, 4.3 and ascites

in sample buffer (Appendix I,4.4) were heated in boiling water bath for 2 minutes. The samples were loaded on top of the stacking gel and overlay with running buffer. Electrophoresis was carried out at 100 volt, 20 mA constant current until stacking dye reached the bottom of the gel. The gel was stained in coomassie blue (Appendix I, 4.7) for at least 1 hr and destained with several changes of the destaining solution (Appendix I, 4.8) overnight.

6. Solid phase coated antibody

6.1. Activation of cellulose

Five grams of sigma cell (micro crystalline cellulose) was weighed into a 50 ml conical flask fitted with a ground glass stopper, then 1,1-Carbonyldiimidazole (CDI) 0.61 gm in 25 ml acetone was added and the reaction proceeded at room temperature for 1 hour, with vigorous stirring. The activated, imidazole-carbamate cellulose was then filtered through a glass microfibre filtre (Whatman GF/A) and washed three times with 100 ml acetone. The activated cellulose was then allowed to air dry and used immediately.

6.2. Coupling of monoclonal anti AFP with activated cellulose

Five grams activated cellulose was weighed into a polystyrene tube, 200 mg of monoclonal anti AFP I (4.1), in 25 ml 0.05 M barbitone pH 8 was added and rotated end over end for 18 hours at room temperature. The protein remaining in solution was recovered by centrifugation and

the immunoadsorbent was washed repeatedly with 10 ml aliquots of the following buffers series and collected by centrifugation :

1. 0.5 M bicarbonate buffer pH 8, rotate 20 min
2. Repeat as no.1
3. 0.1 M acetate buffer pH 4, rotate 60 min
4. 0.1 M acetate buffer pH 4, sonicate for 30 s rotate 16-20 hr
5. 0.05 M PB pH 7.4 with 0.1% BSA rotate 20 min
6. repeat as no.5

The solid-phase antibody could then be stored at 4°C until required.

6.3. Determination of coupled monoclonal anti AFP I

Monoclonal anti AFP coupled to the activated cellulose could be estimated by determining the difference of the protein concentration before coupling and the protein recovered post coupling. Protein was determined by Lowry's method using the normal Immunoglobulin G as a protein standard. The calibration curve was prepared by making a series of normal Immunoglobulin G standard solution at concentration 20,40,80,200 and400 ug/ml using phosphate buffer saline. Two milliliters of solution C (Appendix I, 6.3) was added to each tube. After mixing and standing at room temperature for 15 minutes, 0.2 ml of solution D (Appendix I,6.4) was added and mixed immediately. The tubes were left for 30 minutes and the absorbance was read at 540 nm against zero standard. The absorbance VS standard protein concentration was plotted as

the calibration curve and the unknown concentrations were read off from this curve.

7. Labelling of monoclonal antibody as radioisotope tracer

7.1. Iodination of monoclonal antibody by N-Bromosuccinimide

Monoclonal anti AFP II purified by ammonium sulfate precipitation (4.1),then followed by Protein-A sepharose column chromatrography as in 4.3 was used for iodination. Five microliters of NaI^{125} (0.5 mCi) was added into an eppendoff tube containing 30 ug of monoclonal anti AFP II in 20 ul 0.5 M phosphate buffer pH 7.4. Then 5 ul of N-Bromosuccinimide 200 ug/ml was also added.The vial was mixed 20 sec and the reaction was stopped with 150 ul 0.05 M Phosphate buffer pH 7.4. The mixture was injected into High performance liquid chromatography (HPLC) using 7.8 mm (ID) x 30 cm (L)Protein Pak 125, at a flow rate 1 ml/min with 0.05 M phosphate buffer pH 7.4 as a mobile phase in isocratic system. The fractions of labelled antibody were collected and pooled in 0.05 M phosphate buffer pH 7.4 containing 0.5 % BSA.

7.2. Matching of AFP IRMA system using two monoclonal antibodies

Monoclonal anti AFP I and II were coated and iodinated by the method described in 6.2 and 7.1. Each 100 ul of coated antibody I and II was added into 50 ul of labelled antibody I and II at the concentration of AFP 0 and 320 ng/ml.The tubes were rotated overnight and washed

with the wash buffer twice by centrifugation (3000 rpm, 15 min). Monoclonal antibodies were selected for coating and iodination from the percentage binding (B/T).

7.3. The effect of specific radioactivity on label stability

Monoclonal anti AFP II was iodinated by N-Bromosuccinimide to yield products with specific radioactivity of 10 and 20 uCi/ug. 30 and 15 ug of monoclonol anti AFP II for specific radioactivity 10 and 20 uCi/ug respectively, were added into each vial containing 20 ul 0.5 M phosphate buffer pH 7.4. 5 ul of NaI^{125}(0.5 mCi) and 5 ul of N-Bromosuccinimide (200 ug/ml) was also added into each vial. The reaction mixture was mixed for 20 sec, and injected to HPLC using 7.8 mm (ID) x 30 cm (L)Protein Pak 125, at flow rate 1 ml/min with 0.05 M phosphate buffer pH 7.4 (mobile phase) in isocratic system. The binding of these labels in a sandwich assay for AFP (Appendix II,3.1) concentration ranging from 0 to 320 ng/ml was tested at various time intervals ie: 1,3,5,7 and 8 weeks to determine the stability of the labels at 4° C.

8. The system of solid phase coating

8.1. The effect of monoclonal anti AFP I concentration in the coating solution

Monoclonal anti-AFP I was purified by ammonium sulfate precipitation in 4.1 and coated on cellulose by the method described in 6.1 and 6.2. To optimise the coating conditions, 200 mg Sigma cell was coated with 8 mg of

monoclonal anti-AFP I solution of various concentrations ie: 0.1, 0.5, 1, 3, 6.5 and 13.1 mg/ml. Then, aliquots of 50 ul of coated cellulose were tested for binding to I^{125} - AFP II (100,000 cpm) at the concentration of AFP 0 and 320 ng/ml. The tubes were rotated overnight and washed with the wash buffer twice. The curve was plotted between the percentage binding (B/T) and the concentrations of monoclonal anti AFP I (mg/ml). The optimal concentration of monoclonal antibody in the coating solution was then read off from this curve.

8.2. The effect of buffer on cellulose coating

Three various buffer solutions ie: 0.05 M barbitone buffer pH 8.0, 0.05 M bicarbonate buffer pH 8.0 and 0.05 M phosphate buffer pH 7.4 were prepared to make a coating solution of 8 mg/ml protein concentration. Two hundred milligrams of activated cellulose was added to each preparation and rotated end over end for 18 hr at room temperature. Then,the cellulose was collected by centrifugation and followed by triple washing with the procedure in 6.2. Fifty microliters of the product was tested for binding with 50 ul (100,000 cpm) I^{125}-AFP II at AFP concentration ranging of 0 and 320 ng/ml. The curve was plotted between the percentage binding(B/T) and the concentration of AFP (ng/ml). Then,the optimal buffer system was selected from this curve.

8.3. Comparison of purified monoclonal anti AFP I by ammonium sulfate cut and DEAE column on cellulose coating

The purified monoclonal anti AFP I by ammonium sulfate preparation (4.1) and DEAE column (4.2) were coated on cellulose by the method as described in 6.1 and 6.2. Each 200 mg Sigma cell was coated with 8 mg of ammonium sulfate precipitation and DEAE fraction of monoclonal anti AFP I concentration 7 and 14 mg/ml. Each 50 ul of coated cellulose was tested for binding with labelled anti AFP II at AFP concentration 0 to 320 ng/ml. The curve was plotted between the percentage binding(B/T) and concentration of AFP (ng/ml). The effect of different purification procedures was then shown from this curve.

9. Purification of AFP from amniotic fluid for standard material

9.1. Purification of human AFP using Sephacryl 300S column chromatography

The Sephacryl 300S was packed into a column (0.9x 30 cm) and equilibrated with 0.02 M PB pH 7.4. One milliliter of amniotic fluid was loaded to the column and eluted with 0.02 M PB at a flow rate 10 ml/hr. Then 1 ml fractions were collected for 60 tubes. The absorbance of each fractions were measured at 280 nm. The fraction number 16-26 (peak 1) and 34-42 (peak 2) were pooled and collected for determination of AFP with SDS PAGE and silver staining (Appendix II,2). Then, the desired fraction was collected for further purification in 8.2.

9.2. Purification of human AFP using Affi gel Blue

The Affi gel Blue column (1 x 50 cm) was prepared with 36 ml of Cibacron Blue F3 GA gel (Bio-rad) and prewashed with 100 ml of 0.02 M phosphate buffer pH 7.4. Ten milliliters of the pooled fraction number 16-26 from 9.1 was applied to the column. The column was then eluted with 60 ml of 0.02 M phosphate buffer pH 7.4 and the 2 ml fractions were collected for 30 tubes. The absorbance of each fractions were also measured at 280 nm. Each fraction number 1,4,8 and 12 were tested for AFP using SDS PAGE and silver staining (Appendix II,2). The eluates from fraction number 4-12 were pooled and collected for further purification in 8.3. The column was then regenerated with 1.4 M NaCl in 0.02 M PB.

9.3. Purification of human AFP using Affinity chromatography on CNBr activated Sepharose 4B

9.3.1. Preparation of CNBr activated Sepharose 4B

One gram of freeze dried cyanogen bromide activated Sepharose 4B was allowed to swell in 25 ml of 1 mM HCl. After swelling, the activated Sepharose was washed with 200 ml 1 mM HCl, filtered through a sintered glass funnel and suspended in 5 ml 0.1 M Bicarbonate buffer containing 0.5 M NaCl pH 8.3 (coupling buffer).

9.3.2. Coupling monoclonal anti AFP to the activated Sepharose 4B

Ninety-two milligrams of monoclonal anti AFP I (4.1) was added to 4 g CNBr activated Sepharose

suspension. The slurry was incubated on a rotator mixing at 4° C overnight. The excess antibody was washed away with coupling buffer and blocked any remaining active groups with 1 M ethanolamine pH 9 for 2 hr at room temperature. The product was then washed three times with the following series of buffers. Each series consisted of a wash with 0.1M acetate buffer pH 4 containing 0.5 M NaCl (1:2), followed by 0.1M tris buffer pH 8 containing 0.5M NaCl(1:2). The product was stored at 4° C.

9.3.3. Isolation of human AFP

After the protein coupling CNBr activated Sepharose 4B column (0.9x 15 cm) was equilibrated with 0.02 M PB pH 7.4,the pool fraction from 9.2 was passed through the column. Non-adsorbing proteins were eluted with 20 ml of 0.02 M PB, then followed with 8 M urea in 0.02 M PB pH 7.4. The elution of AFP was monitored by UV absorption at 280 nm,SDS PAGE and silver staining (Appendix II,2). The AFP-containing fractions were pooled, dialysed against 0.02 M PB and lyophilized. The column was regenerated by washing with 10 ml of 8 M urea, followed by 0.02 M PB and could be used at least 10 times to purify AFP.

9.4. Characterization of purification using SDS PAGE

The samples from 8.1, 8.2, 8.3, standard AFP (sigma) and human serum albumin (HSA) were prepared according to the procedure number 2 (Appendix II).

10. Optimization of the assay protocol

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

At the concentration of AFP ranging from 0 to 320 ng/ml, 100 ul of various dilution of cellulose anti AFP : 1:2, 1:4, 1:8 and 1:16 was mixed with 50 ul of the labelled anti AFP (100,000 cpm/tube).Then the assay buffer were added to make 500 ul of incubation volume and the tubes were rotated at room temperature overnight. The solid phase suspension was then washed twice with wash buffer by centrifugation (3000 rpm,15 minutes). Then, each assay tube was counted for 1 minute in the gamma counter.

10.2. Study on range of standard curve

The various concentration of standard AFP ranging from 0 ng/ml to 50 ug/ml; were tested by pipetting 20 ul of each standard solution (Appendix II,5)into each tube to make a calibration curve. The labeled anti AFP (100,000 cpm/tube),100 ul of cellulose anti-AFP 200 ul of and assay buffer (0.1% BSA in 0.05 M PB) were added simultaneously. The tubes were rotated at room temperature overnight, and then washed with a wash buffer (0.4% Tween 20 in 0.05 M PB) twice by centrifugation (3000 rpm, 15 min) and its radioactivity was measured by a gamma counter.

10.3. Study on labelled antibody required for AFP standard curve

Fifty microliters of the amount of labelled anti AFP varied from 35,000, 50,000 to 100,000 cpm was added in each assay tube containing 100 ul of solid phase antibody

and 20 ul of standard AFP ranging from 0-320 ng/ml. The assay tubes were rotated overnight at room temperature. After incubation,the tubes were washed with a wash buffer twice by centrifugation (3000 rpm,15 minutes) and the radioactivity was measured by a gamma counter.

10.4. Study on optimal assay condition

The AFP IRMA has been performed with a variety of different conditions to determine the method that is most convenient to perform with the best sensitivity. For the one step method the sample/standard, cellulose antibody and labelled antibody were all mixed together, as described in the detailed protocol in Appendix II,6.

For the two step method sample/standard and labelled antibody were incubated in the first step and then cellulose antibody was incubated seperately in a second step at various conditions (Appendix II,6).

11. Characterization of the developed assay procedure

11.1. Reproducibility of cellulose solid AFP IRMA

11.1.1. To estimate within-assay reproducibility these commercial control sera (Kodak, Clinical diagnostics) were assayed in a minimum of 10 replicates.

11.1.2. To determine between-assay reproducibility a total of 15 assays were performed using different batches of the developed AFP IRMA, over the shelf-life of the product.

11.2. The stability of AFP IRMA kit

The cellulose solid phase AFP IRMA kits were stored at various conditions and time ie : room temperature, 37° C and 4° C and the storage time up to 2 months. The AFP IRMA was performed at each condition.

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

Thirty patient samples from National Cancer Institute of Thailand and 64 normal human samples from Thai Red Cross were estimated for AFP using Amerlex AFP RIA kit and the developed AFP IRMA.

11.4. The expected value of normal subjects

The distribution of AFP values was determined for 387 specimens obtained from Thai Red Cross. AFP measurements were obtained from 387 normal subjects using the developed assay.