CHAPTER II

Material and Method

1. Instrument

- Autopipette (Gilson 1-100 ul, Code J-82-11286)
- Balance (Precisa, model SKP 0536-26)
- Calibrating viewer (Transidyne General, model2743)
- Centrifuge (Clay-Adam ; Cat No. 0102)
- ELISA-reader (Titertek Multiskan, model 1983, type 310 C)
- Freezer (Warrant)
- Hot plate and stirrer (Thermolyne, Nuova II, model 221-7001)
- Microtiter plate for ELISA, flat shape (Nunc)
- Multipipette (Eppendorf, code 4780)
- pH meter (corning, model 10)
- Power supply and chamber (Gelman, model 3820)
- Precipitin reader (Shandon, Cat no. 2753)
- Water bath (Precision, Thelco, model 84)

2. Reagent

2.1 Commercial reagent

- Anti-Liver Ferritin (Dakopaks, A 133. Lot No.082 B)
- Anti-Liver Ferritin Conjugated Peroxidase (Dakopaks, P145, lot No. 053 A) (peroxidase conjugated antiferritin)
- Agarose (Indubiose, lot No. 1270)
- Chicken Serum (Flow lab. lot No. 29-501-49)
- Hydrogen Peroxide (Merke)
- O-phenylenediamine Dihydrochloride (Sigma, Lot No.82 F1004)
- Standard Liver Ferritin (Dakopaks, Lot No. 064)
- Tween-20 (Sigma)

2.2 Prepared solution

- Amido black staining solution
- Barbitone buffer
- Chromogen diluent solution
- Chromogen solution
- Coating buffer
- Conjugate diluent solution

- Normal saline solution
- Phosphate buffer saline
- Serum diluent
- Sodium citrate solution
- Veronal buffer
- Washing buffer

3. Samples

3.1 Sera from Lung Cancer Patients

The patients under study were lung cancer patients from The National Cancer Institute, Chulalongkorn Hospital and Siriraj Hospital. Samples were divided into 3 groups.

The first group of sera was obtained from 31 males (39-71 yrs., $\overline{X}$ = 59.2 yrs.) and 3 females whose ages were 54, 61 and 70 years. All patients were non-metastatic lung cancer and have not been treated by any cancer therapy method.

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The second group of sera was collected from 19 male patients (38-72 yrs., $\overline{X}$ = 58.3 yrs.) and 2 females whose ages were 21 and 52 years. This group of sera was collected from the non-metastasis and during treatment.

The last group, was collected from 13 males (45-85 yrs., $\overline{X}$ = 66.3 yrs) and one female whose age was 65 years. All patients had metastatic cancer from lung.

3.2 Sera from other Lung Diseases

The sera from non-cancer lung diseases were divided into 4 groups.

The first group of sera was collected from 28 males (18-71 yrs., $\overline{X}$ = 39.8 yrs.) and 8 females (18-70 yrs., $\overline{X}$ = 37.1 yrs.) which were obtained before treatment.

The second group of sera was collected from 28 males (19-70 yrs., $\overline{X}$ = 35.8 yrs) and 12 females (20-64 yrs., and $\overline{X}$ = 33.4 yrs.) which were obtained from patients after having been treated for 1 course of chemotherapy.

Both groups were diagnosed as tuberculosis by their signs, symptoms, bacteriological examination, chest X-rays and chemotherapeutic diagnosis by chest specialists of Lung Division, Medical Department (Yosse, Bangkok).

The third group of sera, non-cancer lung disease was collected from 21 males (35-77 yrs., and $\overline{X}$ = 57.4 yrs.) and 2 females whose ages were 56 and 64 years. The symptoms of all these patients were similar to cancer but their pathological examination were inflammation. This group of sera was obtained from Chulalongkorn Hospital.

The last group of sera, other lung disease was collected from 7 male pneumonitis patients (20-66 yrs., $\overline{X}$ = 44 yrs.) and 5 females (27-66 yrs, $\overline{X}$ = 52.4 yrs.). This group of sera was obtained from Lung Division Medical Department (Yosse).

3.3 Sera of Liver Cancer Patients

Sera were collected from 25 liver cancer (hepatocellular carcinoma) who were diagnosed as carcinoma of liver and were confirmed by pathological examination. Age range of patients was 34-69 years and the mean was 53.4 years, only one was female, age was 52 years. All sera were collected from Chulalongkorn Hospital.

3.4 Sera of other Cancers

Sera were collected from 15 males and 13 females cancer patients who were diagnosed as different kinds of cancers such as cancers of gastro-intestinal tract, esophagus, cervix, breast and others. All was confirmed by pathological examination. This group of sera was collected from The National Cancer Institute(Thailand).

3.5 Normal sera control

Normal sera were collected from 56 males (20-72 yrs., $\overline{X}$ = 50.5 yrs.) and 41 females (22-67 yrs., $\overline{X}$ = 45.5 yrs.). Sera were obtained from normal individuals among the check-up individuals at The National Cancer Institute, Blood donors of The National Blood Bank of Thai Red Cross Association and volunteers.

4. Methods

4.1 Serum Collection

5-8 ml of blood was drawn from basilic vein of each individual and was leaved at room temperature for 1 hour until coagulated and kept at 4°C for 1-2 hours. Serum was seperated by centrifugation at 1,500 - 2,000 rpm. for 10 minutes. Then 2 - 4 ml of serum was collected by pastuer pipette with rubber bulb and kept at -20°C until used.

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4.2 Determination of Serum Ferritin

4.2.1 Enzyme Linked Immunoadsorbent Assay (ELISA)

4.2.1.1 Conditions and Quality Control of the Test

The suitable conditions of the ELISA technique for serum ferritin level examination was found out for comparison with Anderson and Kelly's method (94). And the method was modified as the result shown in Figures 10 to 15 and will be detailed in the Result. The modified ELISA technique for serum ferritin level was compared with the Radioimmuno Assay using Gammadab kit (as shown in Figure 19) and the precision test was also checked which included both within assay and between assay (n = 20) by using 3 levels of serum ferritin (low, medium, and high level) as shown in Figures 16 to 18 and Table 4.

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4.2.1.2 The modified Anderson and Kelly's ELISA technique

The tested ELISA technique was modified from Anderson and Kelly's technique (Figure 7). The wells of microtitration plate were coated with 50 μl of diluted ferritin antiserum, and left overnight at 4°C. The plate was then emptied by inversion and tapped dry on adsorbent paper. All washing steps were carried out by filling the wells with wash buffer dispersed from a wash

bottle, emptying and tapping dry. This wash was repeated three times. Tested sera were diluted 1:5 (or more, if serum ferritin level is high) with serum diluent and 50 µl of diluted serum or standard were applied in duplicate to the wells. For balancing of reaction time in each step of the reactions, the pattern of sample, control and standard wells were arranged as shown in Figure 9. Then, The plate was incubated at 37°C (water bath) for 15 minutes, washed three times with wash buffer followed by addition of 50 µl peroxidase conjugated antiferritin. The plate was incubated for 15 minutes at 37°C (water bath), washed three times with wash buffer. 150 µl of chromogen solution was applied to each well and the reaction allowed to proceed for 20 minutes in dark condition before being stopped by the addition of 50 µl sulphuric acid (4 mol/L). The absorbance of the contents in each well which relates with serum ferritin concentration was measured at 492 nm. A dose response curve was plotted on Semi-log paper. The processes were shown in Figure 8.

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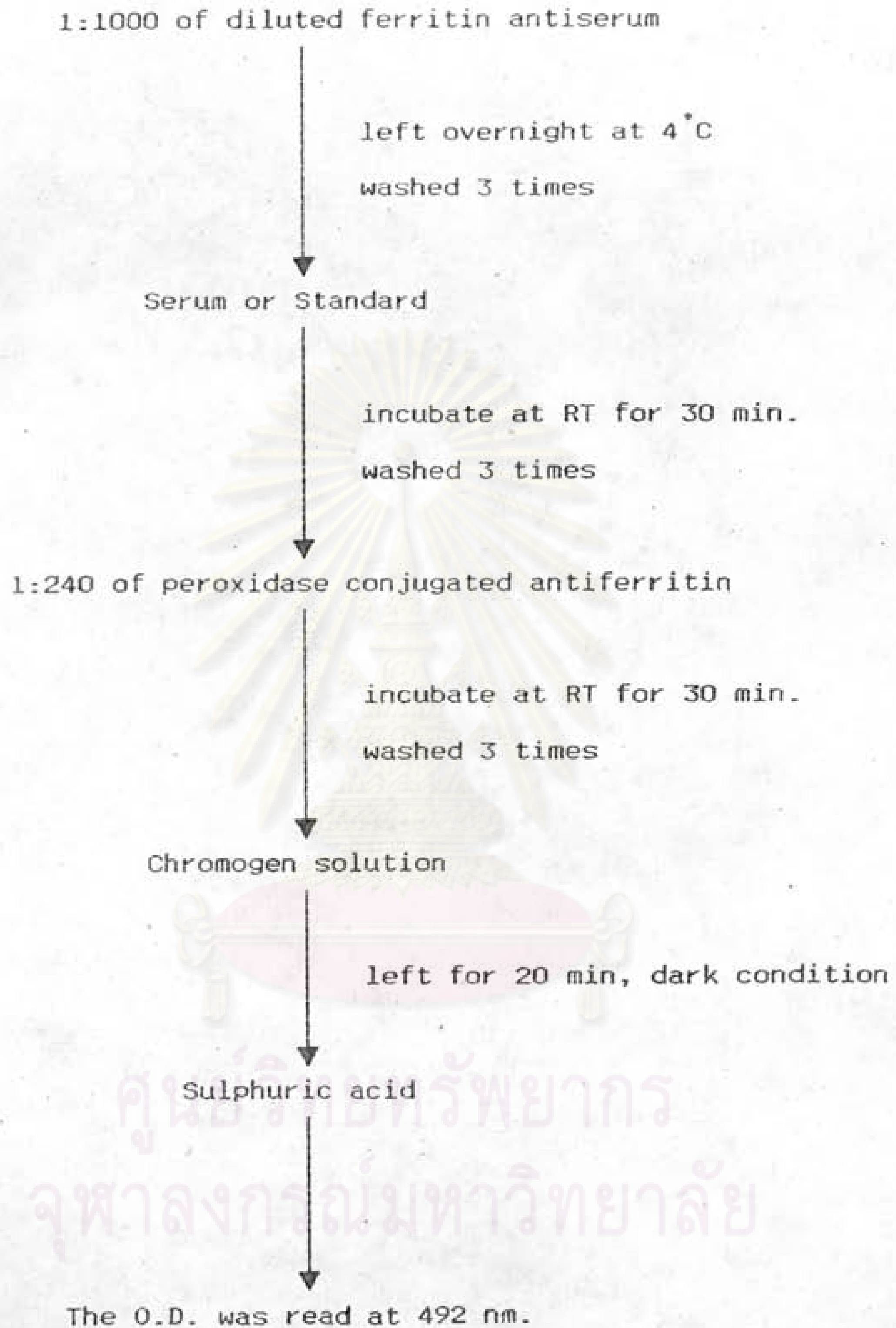

Figure 7. Procedure steps of Anderson and Kelly's ELISA technique.

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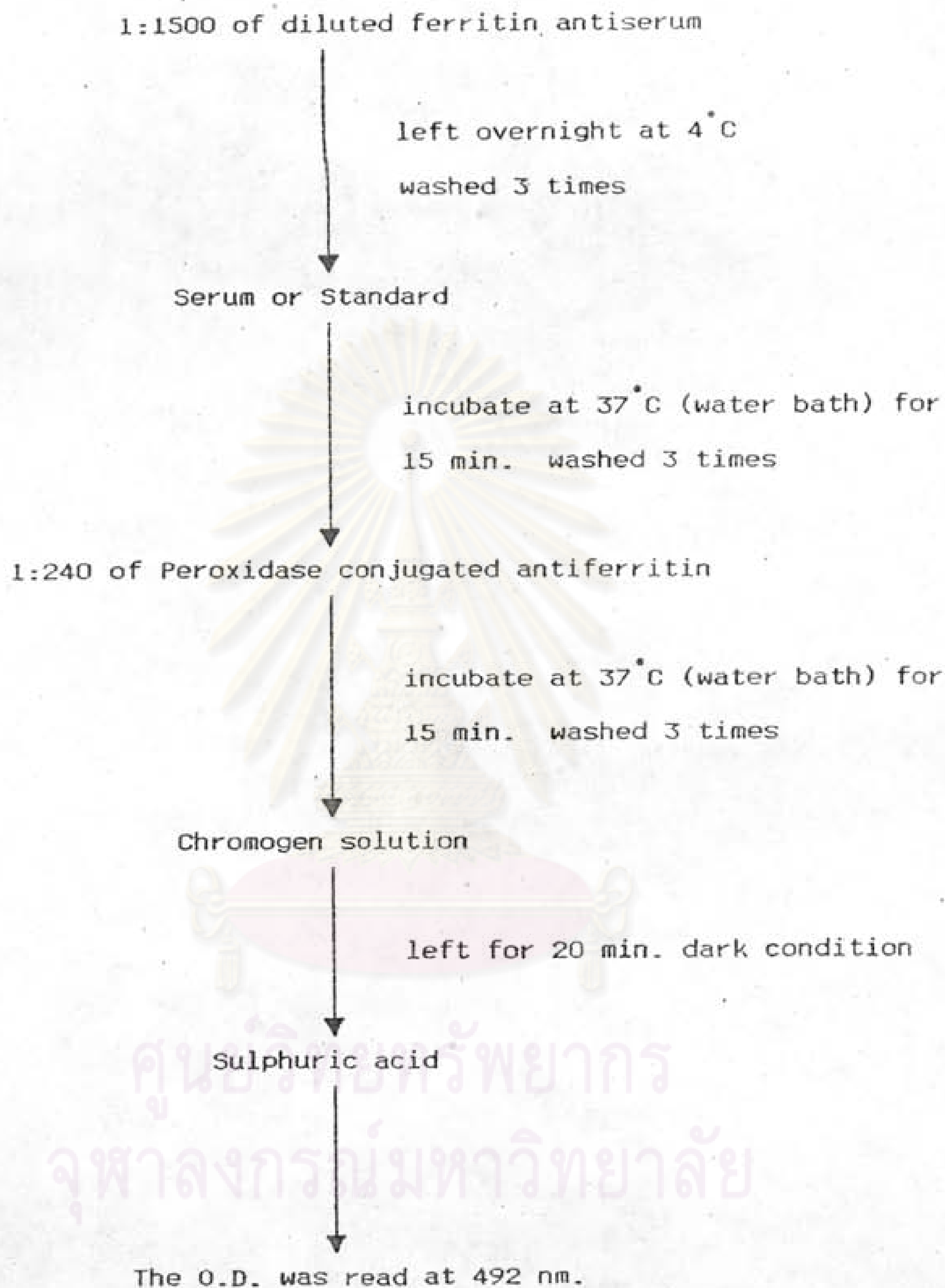

Figure 8. Procedure steps of modified Anderson and Kelly's ELISA technique.

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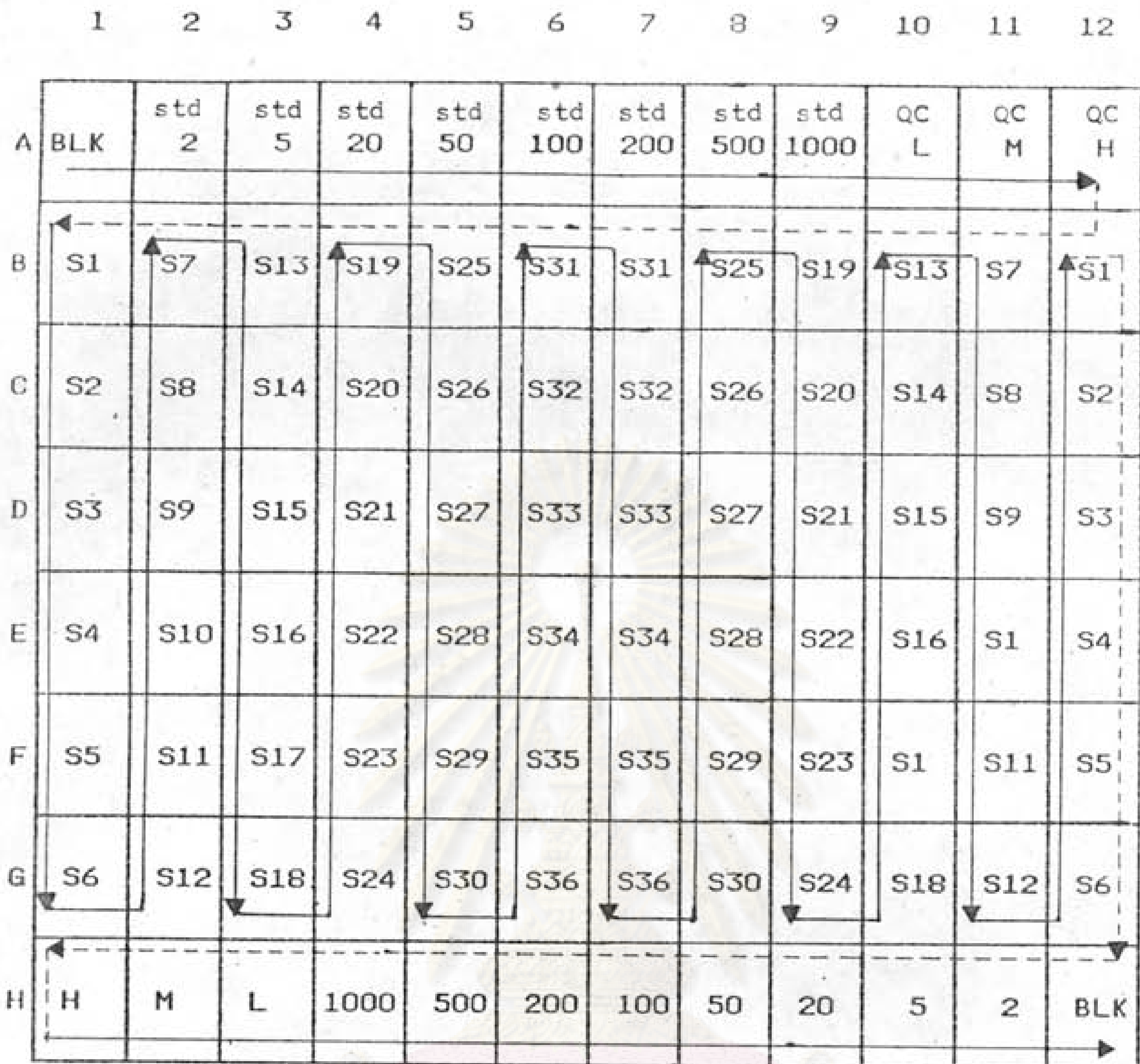

<u>Figure 9</u> Diagram of ELISA plate showing pattern for applying sample, standard, and serum control. Note that, each sample was duplicated. the ⟶ and ---▸ were the direction for applying the sulution in each step.

(QC = Quality control with H, M, L = high, medium, low, Std = standard liver ferritin were varied from 2 to 1000, BLK = Blank, S = sample with the number of each sample).

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4.2.2 Radial Immunodiffusion Technique(RID)

Melted 1 % agarose in barbitone buffer, kept at 56°C. Then, ferritin antiserum was added to agarose until the dilution was 1:600 and mixed well. And, the mixed antiserum and agarose was laid onto a precoated microscopic slide standing on a levelled surface and allowed to set. After the agarose had set, a gel punch was used to cut 4-5 wells per slide (Figure 22). The well was 3 mm. in diameter and then, removed the agarose plug with a pastuer pipette attached to vacuum pump, and filled each well with tested serum or standard ferritin. The slides were placed in a humid chamber at room temperature (RT) for 48 hours. Observed and measured the precipitin ring of Ag-Ab reaction with calibrating viewer. The serum ferritin level was calculated by comparing the diameter size of precipitin ring with standard curve (95,96).

4.2.3 Counter Immunoelectrophoresis (CIEP)

Melted 1% agarose in veronal buffer was prepared, 3 ml of the agarose were laid onto a precoated slide standing on a levelled surface and allowed to set. After the agarose has set, the 8 twin pair wells per slide were punched in the agarose by a gel punch (Figure 24). The well was 3 mm. in diameter. Then, the agarose plug was removed by a pastuer pipette attached to vacuum pump. Anti-liver ferritin was placed in the anodal well and serum

samples or standard ferritin in the cathodal well. After electrophoresed at 100 volts for 45 minutes. The precipitin line of Ag-Ab reaction was observed and stained with 0.1% Amido black B. The presence of precipitin line indicated high level of ferritin in serum (95,96).

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