APPENDICES

APPENDIX A

Reagent Preparations

I. Reagents for Alkaline phosphatase activity

1. 2-amino 2-methyl 1-propanol (AMP) buffer pH 10.5

Add 116 ml of AMP to 600 ml of free CO_2 distilled water mix and adjust the pH to 10.5 with 6 M HCl and then make up to 1 liter with distilled water.

2. Magnesium chloride (1.5 mmol/L)

Dissolve 20.3 gm $MgCl_2\,6H_2O$ in 100 ml reagent grade water.

3. 0.67 M *p*- nitrophenyl phosphate substrate solution

Dissolve 0.8 g *p*- nitrophenyl phosphate substrate (PNPP) in 10 ml 1.5 mmol/L $MgCl_2$

II. Reagents for preparation of ALP isoenzyme from tissues

1. Homoginized buffer: 100 mM Tris-HCl (pH 7.6) countaining 100 mM NaCl, 1 mM $MgCl_2$, 0.02 mM $ZnSO_4$

Tris	3.03	g
NaCl	5.85	g
$MgCl_2 6H_2O$	0.05	g
$ZnSO_4 7H_2O$	1.44	g

III. Reagents for electrophoresis on polyacrylamide gel

1. Polyacrylamide gel electrophoresis for separated ALP isoenzymes

1.1 Separating gel buffer stock (1.5 M Tris-HCl pH 8.8)

Dissolved 27.2 g Trisma base in approximately 100 ml deionized distilled water. Adjusted to pH 8.8 with HCl. Made to 150 mL with deionized distilled water and stored at 4°C

1.2 Stacking gel buffer stock (0.5M Tris-HCl pH 6.8)

Dissolved 6 g Trisma base in approximately 60 ml deionized distilled water. Adjusted to pH 6.8 with HCl. Made to 100 mL with deionized distilled water and store at 4°C

1.3 Sample buffer

Deionized distilled water	4.8	mL
0.5M tris-HCl, pH 6.8	1.0	mL
Glycerol	2.0	mL
0.5% bromphenol blue	0.2	mL

Total volume 8.0 mL mixed and stored at -20°C

1.4 30% Acrylamide stock solution

Acrylamide	146.0	g
N'N-bis-methylene-acrylamide (Bis)	4.0	g

Dissolved in about 350 mL deionized distilled water then adjusts to 500 mL with deionized distilled water. Filter and stored at 4°C in the dark.

1.5 20% Triton X-100

Dissolved Triton X-100 20 g made to 100 ml with deionized distilled water.

1.6 0.375M Tris-boric acid buffer pH 9.0

Dissolved 45.5 g of Trisma base in 900 mL of deionized distilled water. Adjusted to pH 9.0 with boric acid solution (dissolved 15.5 g boric acid with deionized distilled water 100 ml). Made to 1000 mL with deionized distilled water and strored at 4°C.

1.7 Staining solution (NBT/BIP)

3-indoxyl phosphate p-toluidine salt	20	mg
Nitroblue tetrazolium	10	mg
2-amino-2-metyl-1,3-propanediol (AMP)	4.47	g
Magnesium sulfate	6	g

Dissolved in deionized distilled water approximately 30 ml, adjust pH to 10.1. Made to 50 mL with deionized distilled water.

1.8 10% ammonium persulfate

Dissolved 0.1 g of ammonium persulfate with 1 mL of deionized distilled water.

2. SDS-PAGE for immunobloting

2.1 Sample buffer (SDS-reducing buffer : 0.125M Tris HCl, 4% SDS, 20% v/v glycerol, 0.02% bromphenol blue pH 6.8)

Stacking gel buffer stock	2.5	mL
SDS	0.8	g
Glycerol	2	mL

Made to 10 mL with deionized distilled water. Added 50 µL beta-mercaptoethanol to 950 µL of SDS-reducing sample buffer prior to use. Mixed equal volume of sample buffer and heat 95°C for 5 min.

2.2 10X Electrode (running) buffer pH 8.3

Tris base	30.3	g
Glycine	144.0	g
SDS	10.0	g

Dissolved and adjust to 1,000 ml with deionized distilled water. Do not adjust pH with acid or base.

2.3 1X Electrode (running) buffer pH 8.3

To make 1 liter of 1X electrophoresis buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3) diluted 100 ml of 10X electrode buffer with 900 ml deionized distilled water.

2.4 Protein staining (Coomassie Blue)

2.4.1 Coomassie Blue staining solution

Coomassie Brilliant Blue R250	0.125	g
Methanol	200	mL
Acetic acid	35	ml

Mixed and adjusted volume to 500 ml with deionized distilled water. Stored at room temperature.

2.4.2 Destain solution I

Methanol	200	mL
Acetic acid	70	ml

Mixed and adjusted volume to 500 ml with deionized distilled water. Stored at room temperature.

2.4.3 Destain solution II

Acetic acid	70	ml
Methanol	50	ml

Mixed and adjusted volume to 500 ml with deionized distilled water. Stored in room temperature.

IV. Reagent and buffer for Western blot analysis

1. Electrotransfer of the separated protein into the blotting paper

a. Transfer buffer (Chill before use)

Tris base	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolved in 750 ml distilled water. Added 200 ml of methanol. Bring to 1 liter with deionized distilled water. Do not adjust the pH, which should be between 8.2 and 8.4.

2. Labeling of the transferred proteins with antibodies

a. 10X TBS-Tween buffer pH 7.5

Tris base	24.2	g
NaCl	80	g

Dissolved with deionized distilled water approximately 800 ml, adjusted pH to 7.5 and filled deionized distilled water to volume 1000 ml. After that added 10 ml of Tween-20, mixed and stored at 4°C.

b. 1X TBS-Tween buffer pH 7.5

To make 1 liter of 1X TBS-Tween buffer pH 7.5 diluted 100 ml of 10X TBS-Tween buffer ph 7.5 with 900 ml deionized distilled water.

c. Blocking buffer (5% skimmed milk in 1X TBS-Tween buffer pH 7.5)

Dissolved 5 g of skimmed milk in 100 ml 1X TBS-Tween buffer pH 7.5.

APPENDIX B

Protocols for ABO Blood Grouping and ABH Secretion Determination

I. Protocol of ABO Blood Grouping

(Bhatia, 1997; Bharucha & Chouhan, 1990)
(http://www.w3.whosea.org/bct/pdf/sop/13.pdf)

RBC Testing

1. Label tubes with test identification

2. Prepare cell suspension for cells being tested

3. Place two drops of anti-A, anti-B and anti-AB reagent in the labeled tubes

4. Add to each tube one drop of a 2 - 5% cell suspension (in normal saline, serum or plasma) of the red cells to be tested

5. Mix the contents of the tubes gently and incubate at room temperature for 15 minutes

Serum Testing

1. Label tubes with test identification
2. Add 2 drops of test serum in all tubes in the corresponding column
3. Add 1 drop of 2% pooled A red cell suspension in tube labeled Ac
4. Add 1 drop of 2% pooled B red cell suspension in tube labeled Bc
5. Add 1 drop of 2% pooled O red cell suspension in tube labeled Oc
6. Mix the contents of the tubes gently and incubate the test for minimum 15 minutes at room temperature
7. Centrifuge all tubes at 1000 rpm for 5 seconds
8. Gently resuspend the red cell button & examine for agglutination

Interpretation

1. Agglutination in any tube of RBC tests and agglutination or haemolysis in serum test constitutes a positive test result. The expected agglutination reactions for positive tests are 3+ to 4+

2. A smooth suspension of RBCs after resuspension of RBC button is a negative test result. All negative results must be verified under microscope. Cells should be separate without any clumping

3. The interpretation of ABO group is as follows:

Cell Typing			Serum Typing			Interpretation of ABO blood group
Anti-A	Anti-B	Anti-AB	Ac	Bc	Oc	
C	-	C	-	C/L	-	A
-	C	C	C/L	-	-	B
C	C	C	-	-	-	AB
-	-	-	+	+	-	O

C = Clumps, L = Lysis

II. Protocol for Determination of ABH secretion
(http://www.matcmadison.edu/is/hhps/mlt/mljensen/BloodBlank/Lab_Manual/determinigg_secretor_.htm)

1. Collect 2 to 3 ml saliva in a clean 16 x 100 mm tube
2. Place in a boiling water bath for 10 minutes. This inactivates enzymes that might otherwise destroy blood group substances.
3. Allow to cool briefly, then transfer to a 12 x 75 mm tube
4. Centrifuge at least 5 minutes
5. Label three 12 x 75 mm test tubes: A TEST, B TEST and H TEST
6. Label three more tubes: A CONTROL, B CONTROL and H CONTROL (These are dilution controls to ensure the anti-sera was not diluted beyond its capacity to agglutinate)
7. Add one drop of the appropriate dilute antiserum to each tube:
   -one drop dilute anti-A to the A test and A control
   -one drop dilute anti-B to the B test and B control
   -one drop anti-H to the H test and H control
8. To each TEST tube, add one drop of clear saliva
9. To each CONTROL tube, add one drop of saline
10. Mix and incubate at room temperature 10 minutes
11. Add one drop of the appropriate reagent red cells to each tube:
    -A cells for the A test and control
    -B cells for the B test and control
    -O cells for the H test and control
12. Mix and incubate at room temperature 10 minutes
13. Centrifuge all tubes at 1000 rpm for 5 seconds

14. Using the lighted agglutination viewer, read, grade and record the reactions

15. The CONTROL tube should have agglutination for the test to be valid

Interpretation

1. Agglutination in all of the patient TEST tubes indicates a negative result for secretor status
2. If any one of the patient TEST tubes is not agglutinated, this indicates a positive test for secretor status, and the tube showing the non-agglutination should indicate the ABO type

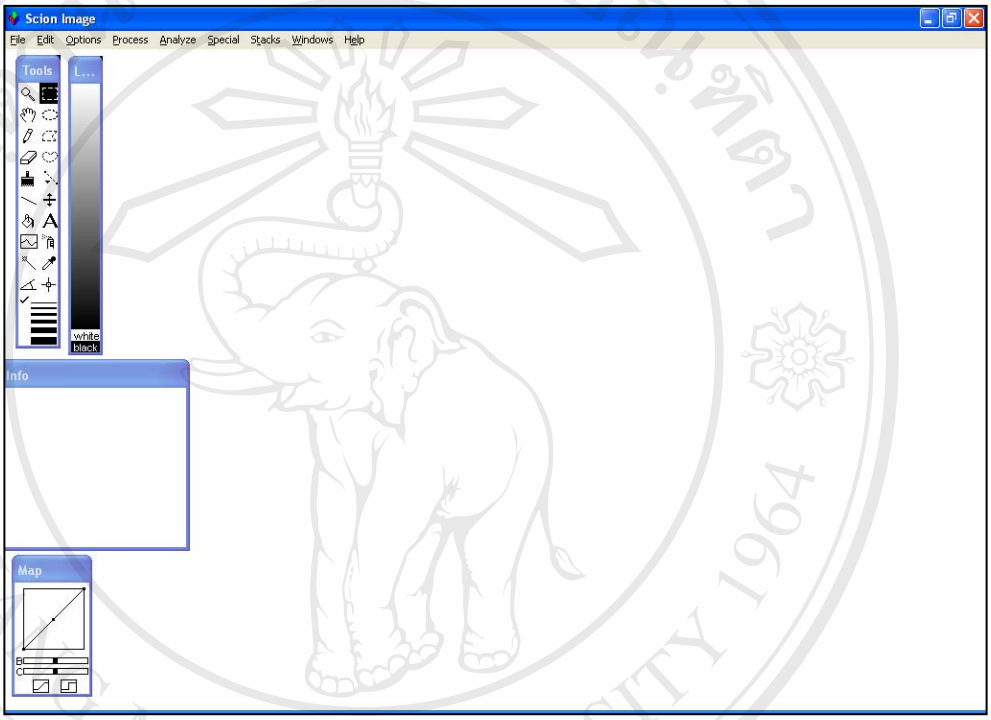
APPENDIX C

Scanning of ALP Isoenzyme Bands and Images Processing by Scion Image Program

Scion Image is an image processing and analysis program for the PC. It is based on the popular NIH *Image* on the Macintosh platform. It can acquire, display, edit, enhance, analyze and animate images. It reads and writes TIFF and BMP files, providing compatibility with many other applications, including programs for scanning, processing, editing, publishing and analyzing images. It supports many standard image processing functions, including contrast enhancement, density profiling, smoothing, sharpening, edge detection, median filtering, and spatial convolution with user defined kernels. *Scion Image* for Windows can be used to measure area, mean, centroid, perimeter, etc. of user defined regions of interest. It also performs automated particle analysis and provides tools for measuring path lengths and angles. Spatial calibration is supported to provide real world area and length measurements. Density calibration can be done against radiation or optical density standards using user specified units. Results can be printed, exported to text files, or copied to the Clipboard. The following is one possible procedure for using *Scion Image* to analyze a one dimensional electrophoretic gel. It also demonstrates some of the less obvious features in *Scion Image*, and also a few shortcuts.

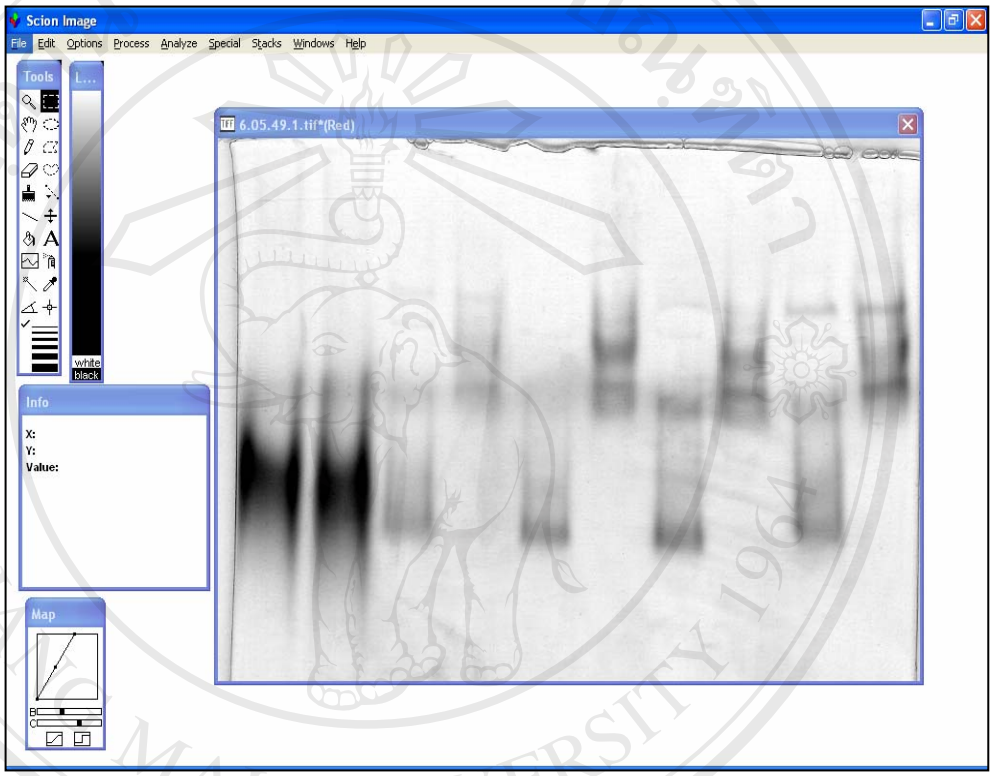
Determination of ALP isoenzyme activity on PAGE and Western blot analysis using a Scion image program (The program can be downloading freely from online-internet)

1. The below figure shows the first page of program Scion image for opening the image files and analyzing.



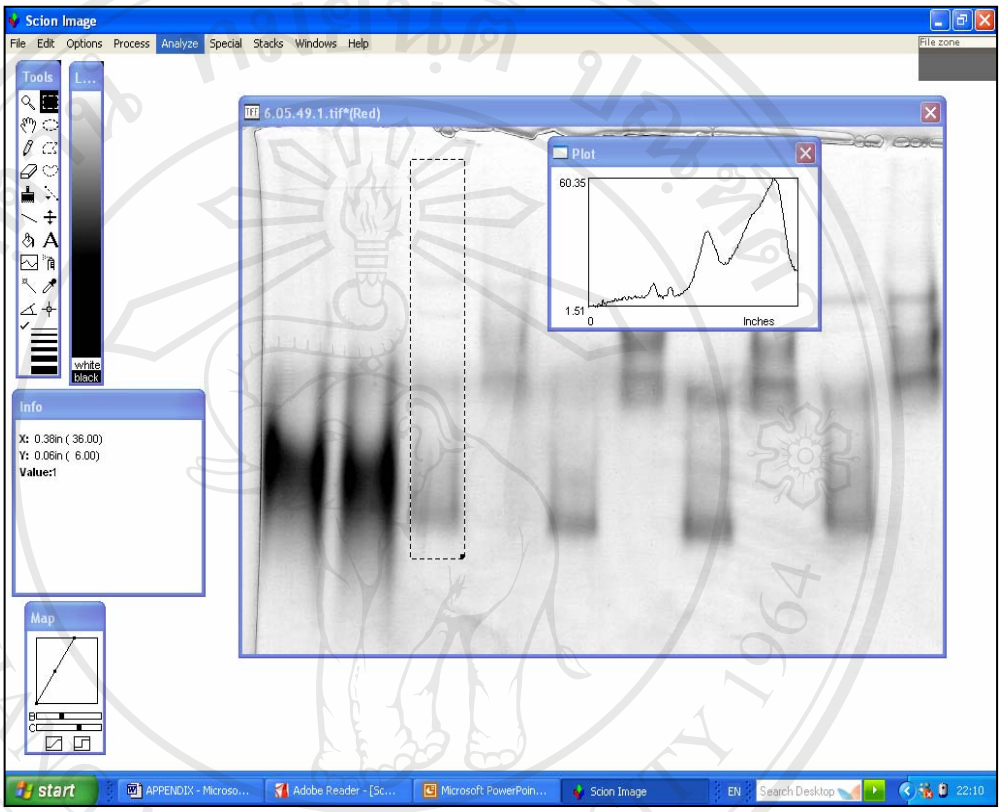
2. The example; Analyzing of ALP isoenzymes in serum

- Click chooses a picture of interest from the file and opening on the Scion image box. (The picture file must be TIFF.)



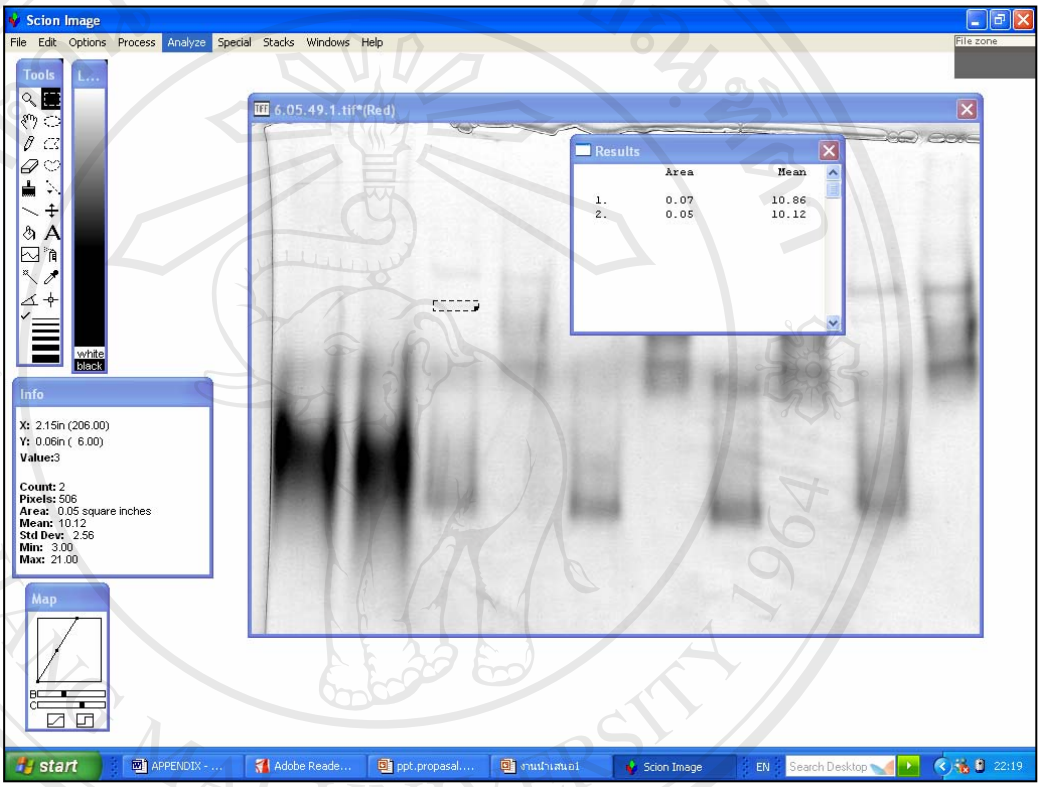
3. Select the interested lane and mark, then click analyze on the menu.

   Step1. Choose the “plot lanes” Lanes to generate the lane profile plots.

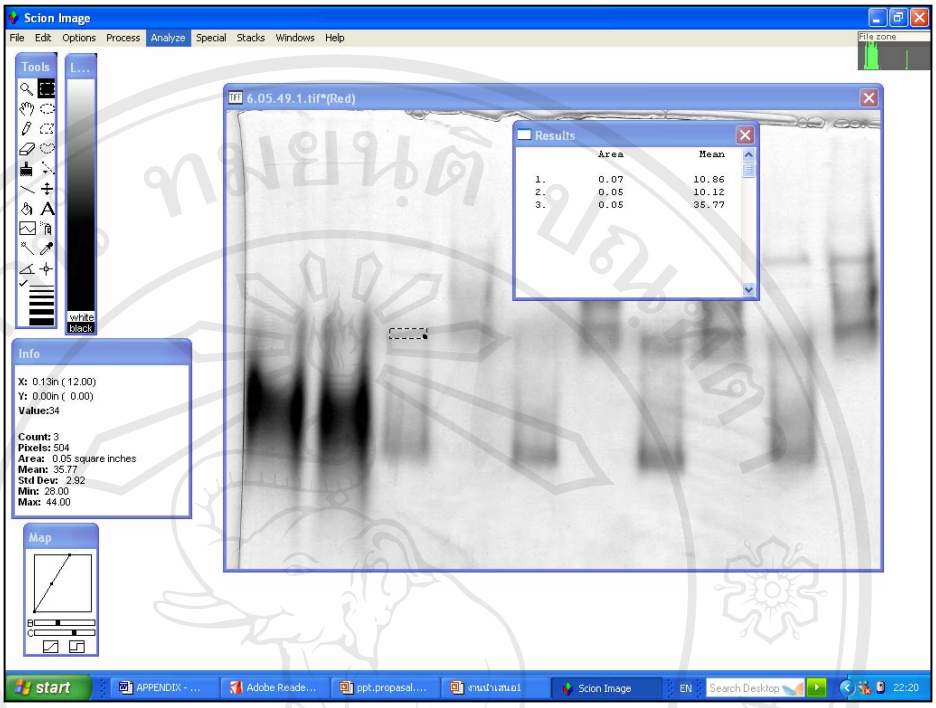


Step2. Crop on each band (HIAP1, HIAP2, NIAP and TNALP isoenzymes, respectively) in the same lane. Measure the areas of the peaks by clicking on result menus and select measure.

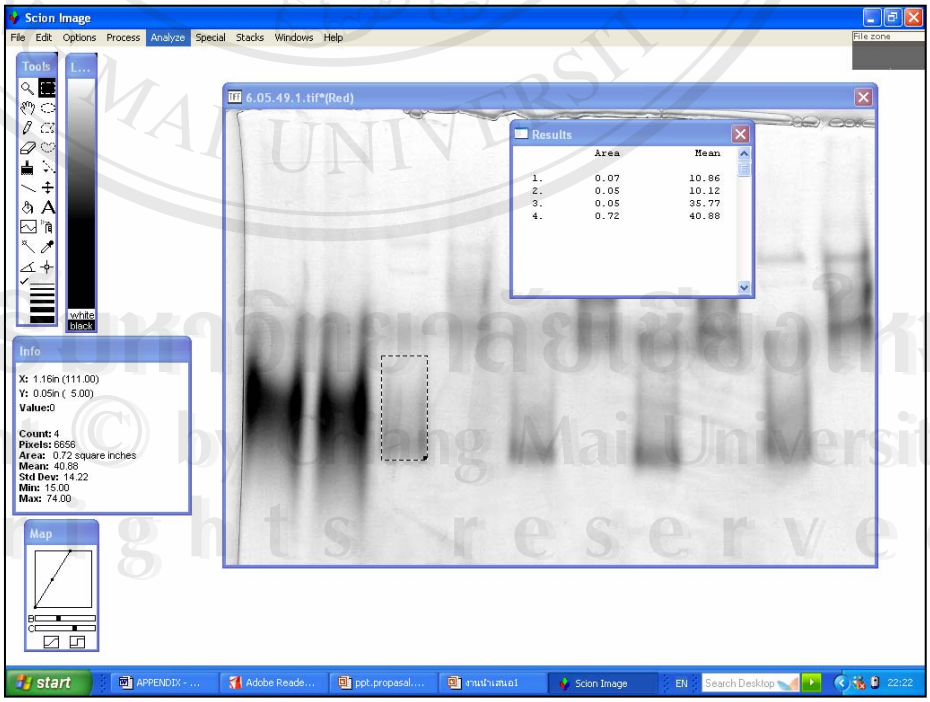
2.1 The first band



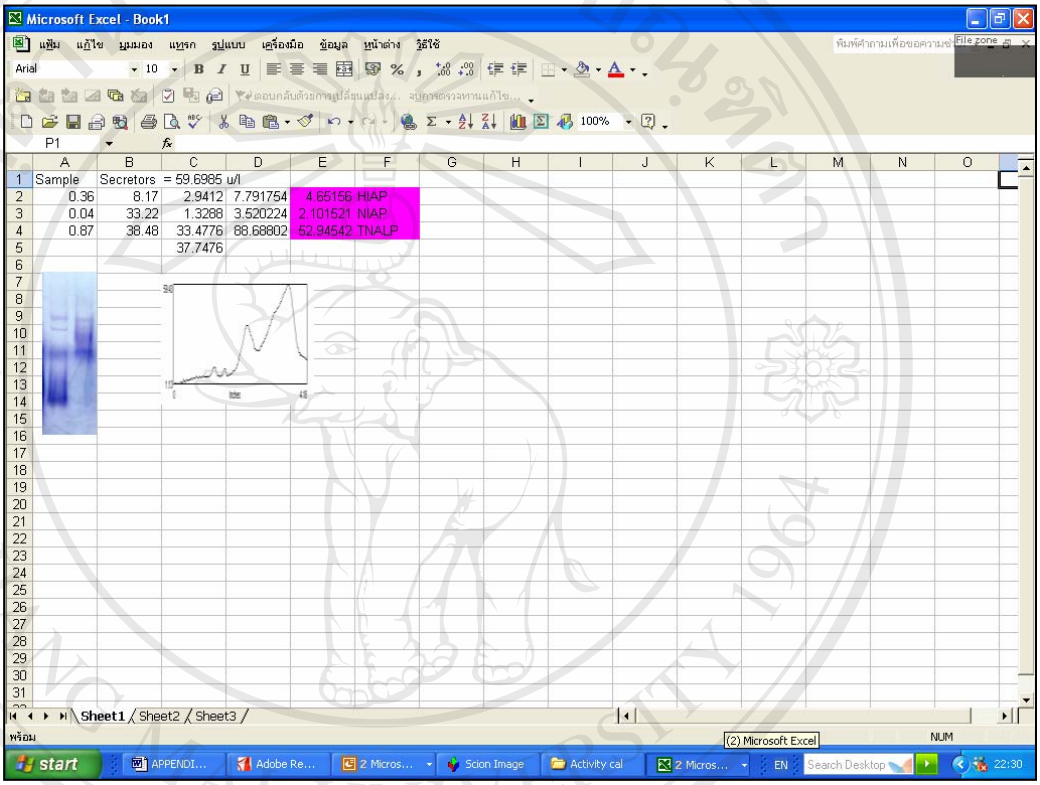
2.2 The second band



2.3 The third band



Step 3: After the plot profile and calculated intensity appeared, export all to the excel program for calculation of ALP activities.



4. Calculation of ALP isoenzymes:

$$\text{Area} \times \text{Mean} = \text{Density (inches/pixels)}$$

$$\text{Percentages of each fractions} = \frac{\text{SUM of total fractions} \times \text{Density}}{100}$$

$$\text{Activity (U/L)} = \frac{\text{\% of fraction} \times \text{total ALP activity (U/L) by AMP method}}{100}$$

APPENDIX D

Application of Mean Reference Value for Monitoring of Intestinal Diseases

Application of using results from this study in interpretation of patients' data (EC) Performa 1 No.063/2004.

1. Blood Group Typing of Patients

. In this study, 13 serum specimens of patients with intestinal disease (colon and rectal cancer) collected at Endoscopy room, Department of Medicine, Faculty of Medicine, were identified as 6 colon cancer (1 of blood group A and 5 of blood group B) and 7 rectal cancer (2 of blood group O and the rest was no data). All serum specimens were aliquoted and stored at -20°C and used further for determination of liver function test, total ALP activity, IAP activity and IAP isoforms, respectively.

2. Screening of Liver Disease on Patient specimen by Determination of the Liver Enzymes in Serum Specimens

The means of ALT and AST activities in patient sera (N=13) were 23.0 ± 10.51 U/L (Mean ± SD) and 27.6 ± 6.08 U/L (Mean ± SD), respectively. The ALT activity and AST activity in all sera of patients were within reference range. The patients were concluded to be free from liver disease.

3. Total ALP Activity Measured in Patients Serum

Total ALP activity in patient's serum was measured in a double beam UV-2450 Spectrophotometer. The mean ± SD of total ALP activity in patient serum (N= 13) were 106.3 ± 47.17 U/L. The difference between the mean of total ALP activity in normal and patient serum was statistically significant at $p < 0.01$. The difference between the means of total ALP activity in normal sera of B or O secretor and non-secretors or patient sera were statistically significant at $p < 0.01$.

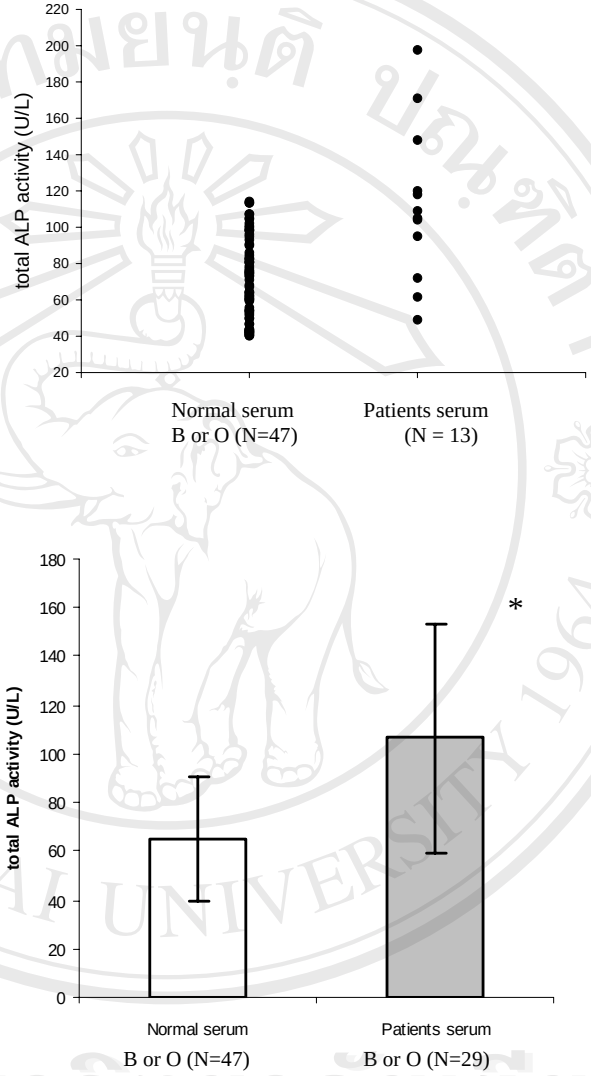


Figure 23. Total ALP activity of patients' sera as compared with the reference value determined in this study.
Upper: The distribution of total ALP activity in normal serum of B or O blood group and patients' sera.
Lower: The comparison of the mean and standard deviation of total ALP activity in both groups, * the difference of mean was significant at $p < 0.01$.

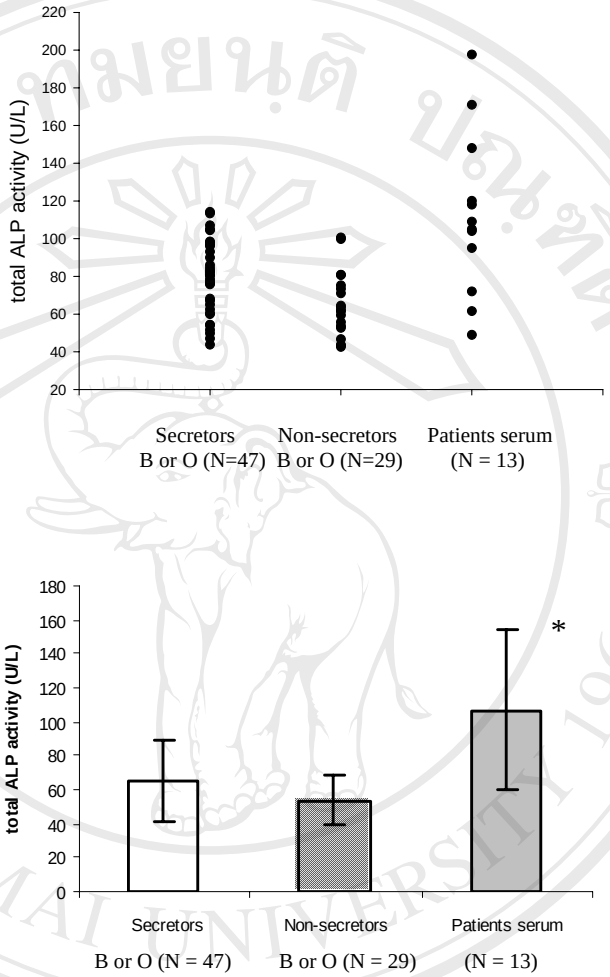


Figure 24. Total ALP activity of patients' sera as compared with the reference value determined in this study.
Upper: The distribution of total ALP activity in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the mean and standard deviation of total ALP activity in different groups, * the difference of mean was significant at $p < 0.01$.

4. IAP Activities and Characteristic of IAP isoforms separated by PAGE in Patient Serum

The mean of TIAP activity in sera of patients was 3.7 ± 1.21 U/L (Mean ± SD). The differences between the mean of TIAP activity in sera of patients and B or O secretors or non-secretors were statistically significant at $p < 0.0001$ and $p < 0.001$, respectively. The mean of NIAP activity in patient sera was 3.7 ± 1.21 U/L (Mean ± SD). It was significantly higher than the means of NIAP activity in sera of B or O secretors or non-secretors ($p < 0.01$ and $p < 0.01$, respectively). The mean of TNAP activity in sera of patients was 95.4 ± 35.14 U/L (Mean ± SD). The differences between the means of TNAP activity in sera of patients and B or O secretors or non-secretors were also statistically significant at $p < 0.01$ and $p < 0.01$, respectively.

Figure 25 shows the Scion image scanning of ALP isoenzymes in patient sera separated by PAGE method. The distributions and comparison of Mean ± SD data of TIAP, NIAP and TNAP activities were shown in Figure 25, 26 and 27, respectively.

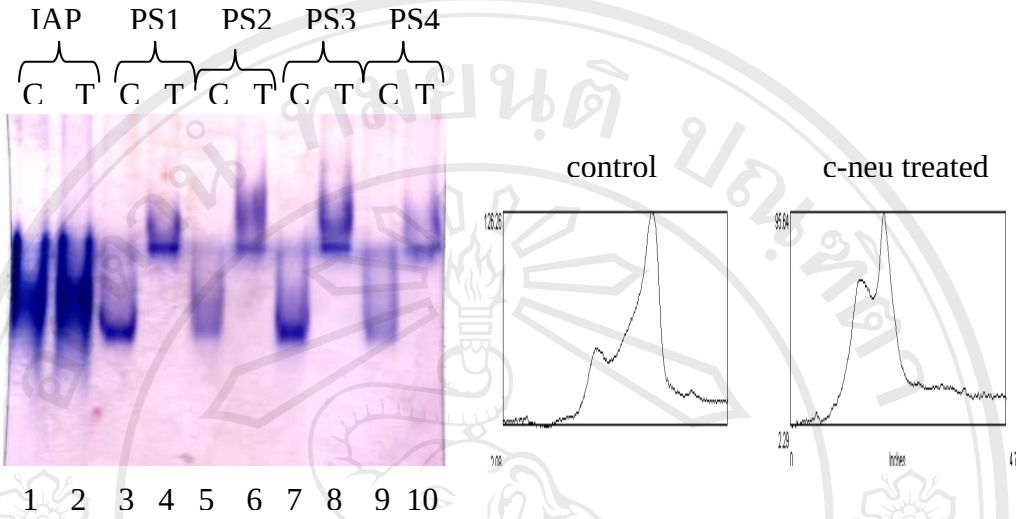


Figure 25. The ALP isoenzymes in patients' sera separated by PAGE method. Lane 1, 2: IAP standard control (C-neu untreated) and C-neu treated, Lane 3-10: patients' serum untreated and C-neu treated, respectively.

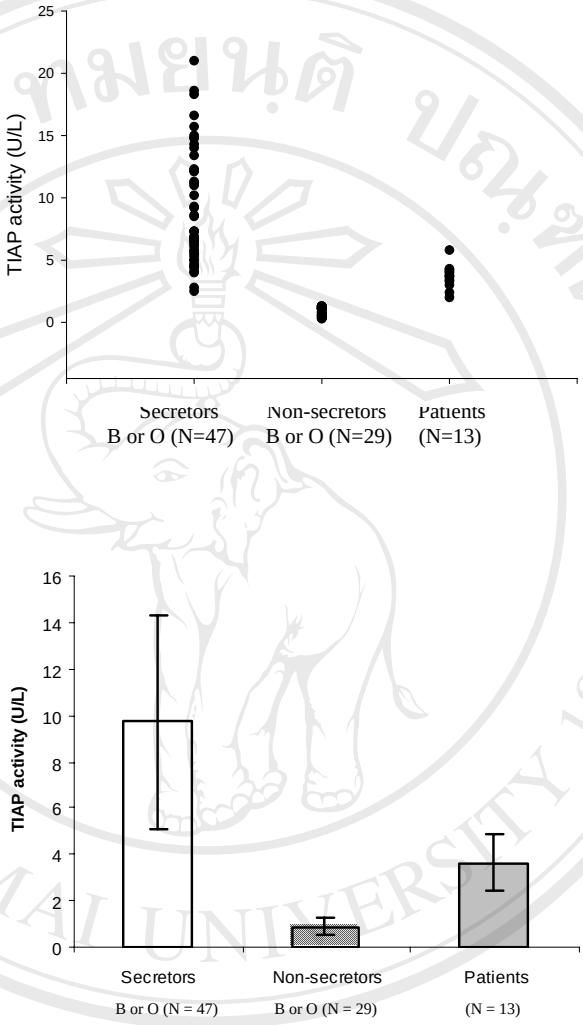


Figure 26. TIAP activity of patients' sera as compared with the reference value determined in this study (PAGE method).
Upper: The distribution of TIAP activity in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the mean and standard deviation of TIAP activity in different groups.

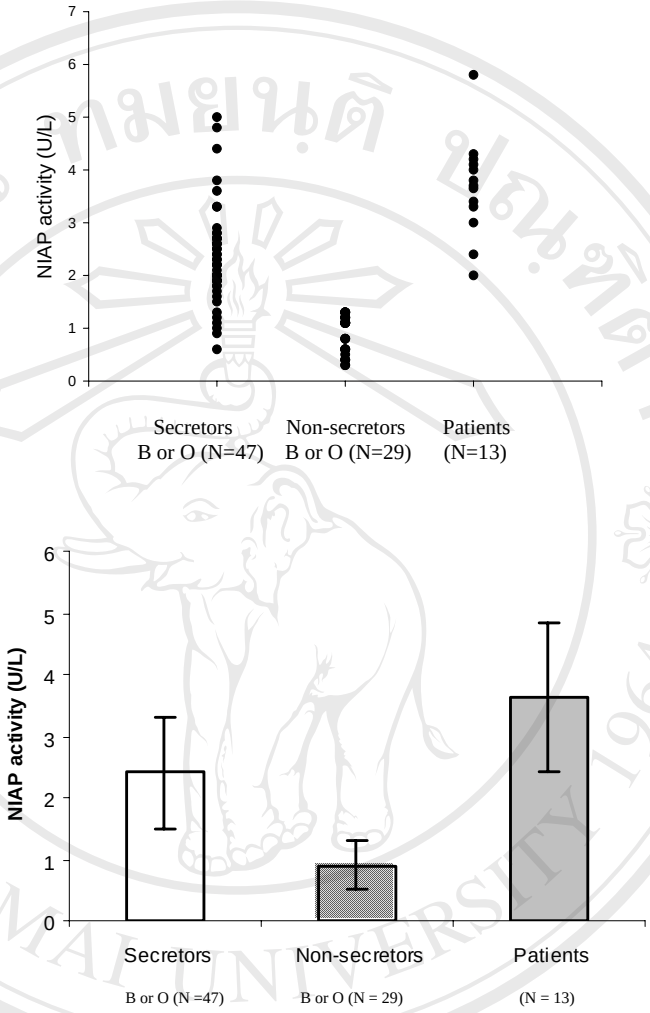


Figure 27. NIAP activity of patients' sera as compared with the reference value determined in this study (PAGE method).
Upper: The distribution of NIAP activity in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the mean and standard deviation of NIAP activity in different groups.

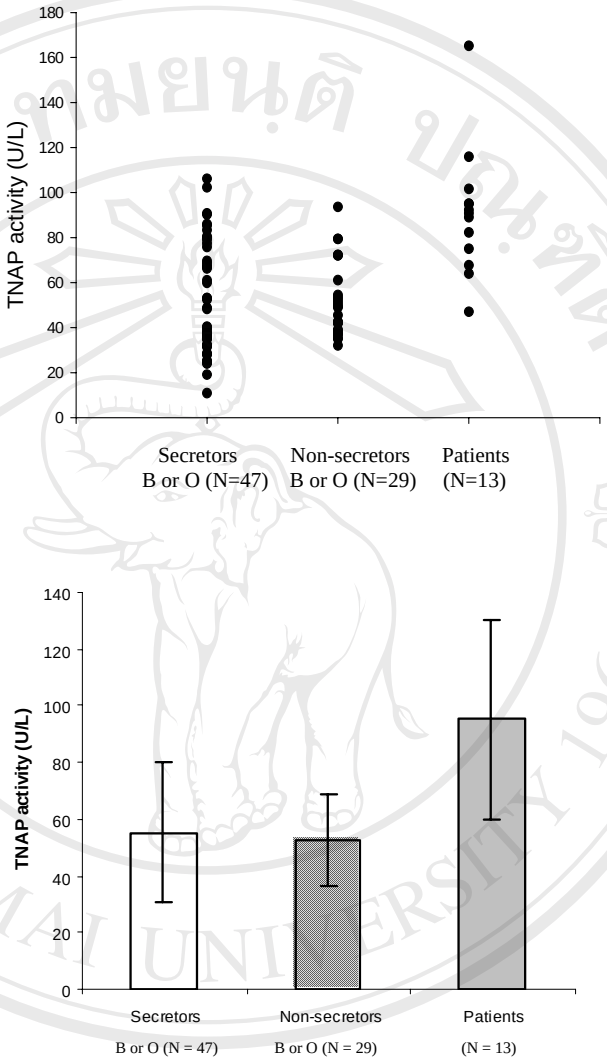


Figure 28. TNAP activity of patients' sera as compared with the reference value determined in this study (PAGE method).
Upper: The distribution of TNAP activity in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the means and standard deviations of TNAP activity in different group.

5. Molecular mass determination and confirmation of IAP isoenzyme detection by Western blot analysis

The comparison in of NIAP and HIAP fractions in normal sera of B or O secretors and non-secretors with those found in patient sera were demonstrated in Figure 28. In patients' serum, the NIAP and HIAP fractions were found higher than those of B or O secretors and non-secretors. The molecular mass of IAP isoforms determined by Western blot analysis were resembled the molecular mass of IAP isoforms as detected in sera of B or O secretors and non-secretors.

The distributions and comparison of mean ± SD data of NIAP, HIAP and HIAP/NIAP were shown in Figure 29, 30 and 31.

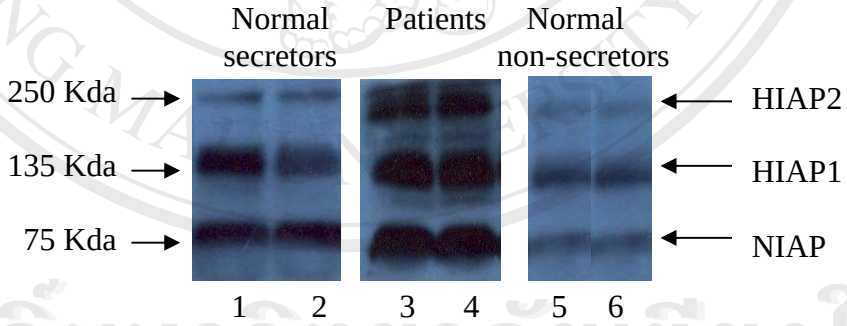


Figure 29. Molecular masses of three IAP isoforms in sera of B or O secretors, non-secretors and patient sera by Western blot analysis. Lane 1&2 normal serum of B or O secretors, Lane 3&4 serum of patients with intestinal disease and Lane 5&6 normal serum of B or O non-secretors.

Table 13 is the summary of the IAP isoform fractions in sera of B or O secretors, B or O non-secretors and patients with intestinal disease. The value of HIAP/NIAP ratio of patients was statistically different from that of B or O secretors and non-secretors at $p < 0.0001$ and $p < 0.0001$, respectively.

Table 13. The fractions of IAP isoenzyme in normal sera of B or O secretors, B or O non-secretors and serum of patients with intestinal disease

Subjects	NIAP fraction Mean ± SD	p	HIAP fraction Mean ± SD	p	HIAP/NIAP ratios Mean ± SD	p	Detected range of HIAP/NIAP ratios
B or O secretors (N = 47)	57.3 ± 9.60	NS	74.2 ± 12.57	< 0.0001	1.3 ± 0.22	< 0.0001	1.01 – 1.88
B or O non-secretors (N = 29)	32.8 ± 5.42	< 0.0001	25.1 ± 3.38	< 0.0001	0.8 ± 0.12	< 0.0001	0.30 – 0.92
Patients (N = 13)	63.9 ± 14.06		137.7 ± 27.76		2.2 ± 0.83		1.41 – 3.83

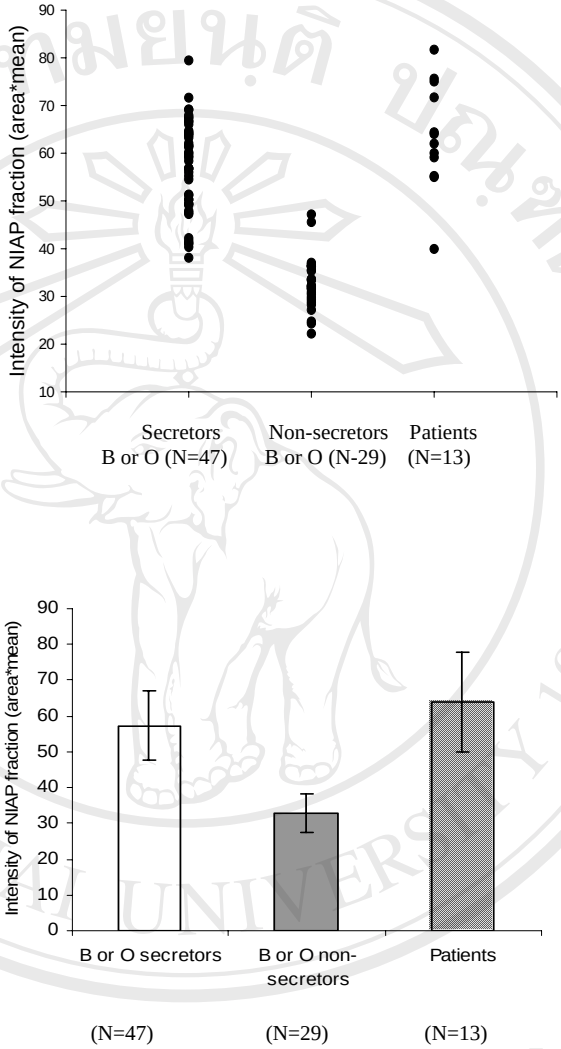


Figure 30. The intensity of NIAP fractions in patients' sera as compared with the mean reference value determined in this study.
Upper: The distribution of intensity of NIAP fraction in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the means and standard deviations of intensity of NIAP fractions in different groups.

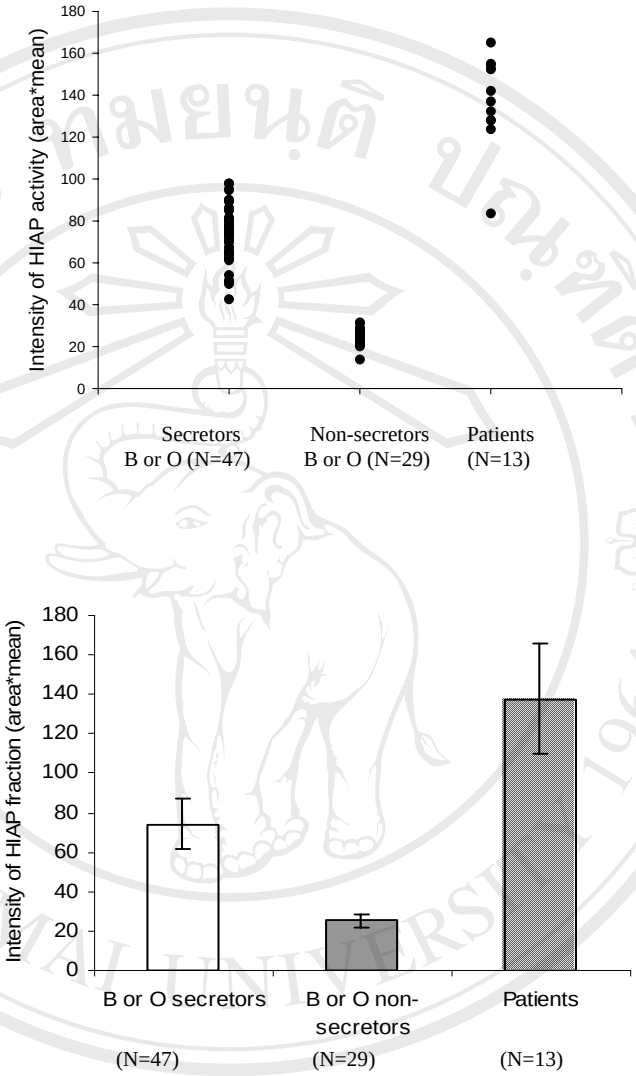


Figure 31. The intensity of HIAP fractions in patients' sera as compared with the mean reference value determined in this study.
Upper: The distribution of intensity of HIAP fraction in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the means and standard deviations of intensity of HIAP fractions in different groups.

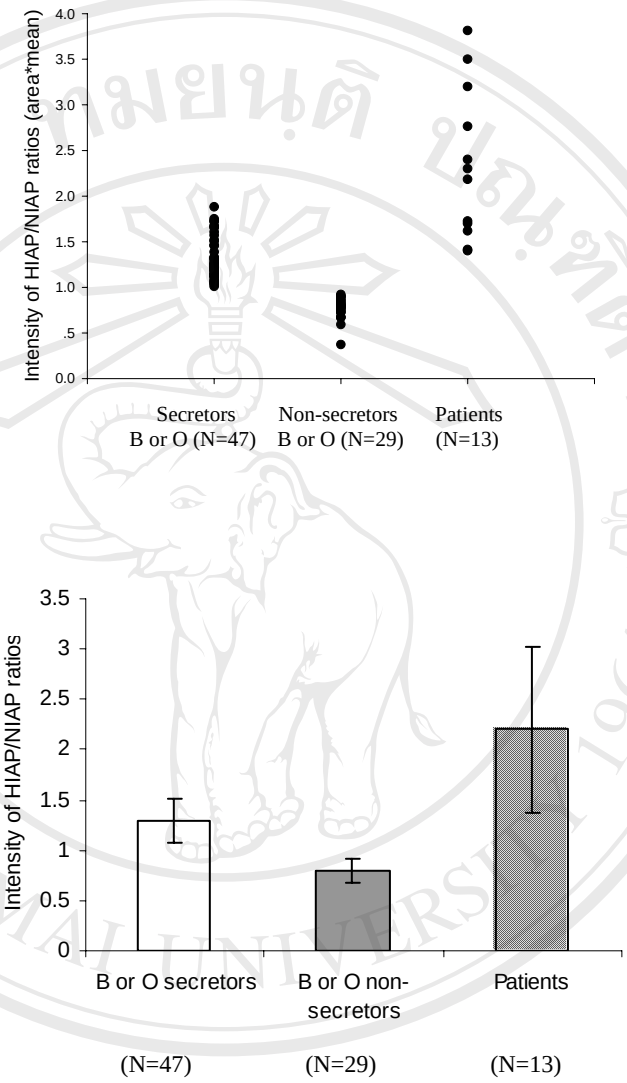


Figure 32. The intensity of HIAP/NIAP ratios of patients' sera as compared with the mean reference value determined in this study.
Upper: The distribution of intensity of HIAP/NIAP ratios in normal serum of B or O secretors, B or O non-secretors and patient sera.
Lower: The comparison of the means and standard deviations of intensity of HIAP/NIAP ratios in different groups.

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