CHAPTER 3

RESULTS AND DISCUSSION

3.1 Operational step and signal profile of negative and positive samples

Blood sample and DCIP solution were introduced and mixed while passing into the mixing zone. The mixture was stopped in the water bath for a period of time. Then the reaction zone was moved into the detector. The turbidity that occurs during precipitation reaction of unstable hemoglobin with DCIP solution was monitored through the change in light transmittance.

Example of signal profiles of a negative sample and a positive sample obtained from the proposed system is illustrated in Figure 3.1. Transmittance was recorded as electrical signal and therefore it was shown as voltage. Hemoglobin E was oxidized and precipitated faster and at higher extent than normal hemoglobin. The precipitate blocked the light path showing low transmittance. Therefore, the peak height of the positive sample is higher than that of the negative sample. In both cases, a little bump at the end of peak signal was observed. This probably due to the absorbance of the blue DCIP solution remaining in the line of A to B zone, in Figure 2.1 (b). After the reaction zone flowed out of the flow through cell, baseline signal was changed which probably due to some precipitate and red cells from the reaction remained in flow through cell or the DCIP color may be adhered on the wall of the flow through cell. Increasing the flow rate (4.8 ml/min) helps to remove these residues. Then, slightly baseline shifting was further corrected by adjusting the

spectronic 21 to zero before the next run. After all the experiment was done each day, the system and flow through cell was washed by flowing 10 % of hydrogen peroxide through the system for 5 min, following by DI water and air in the last step.

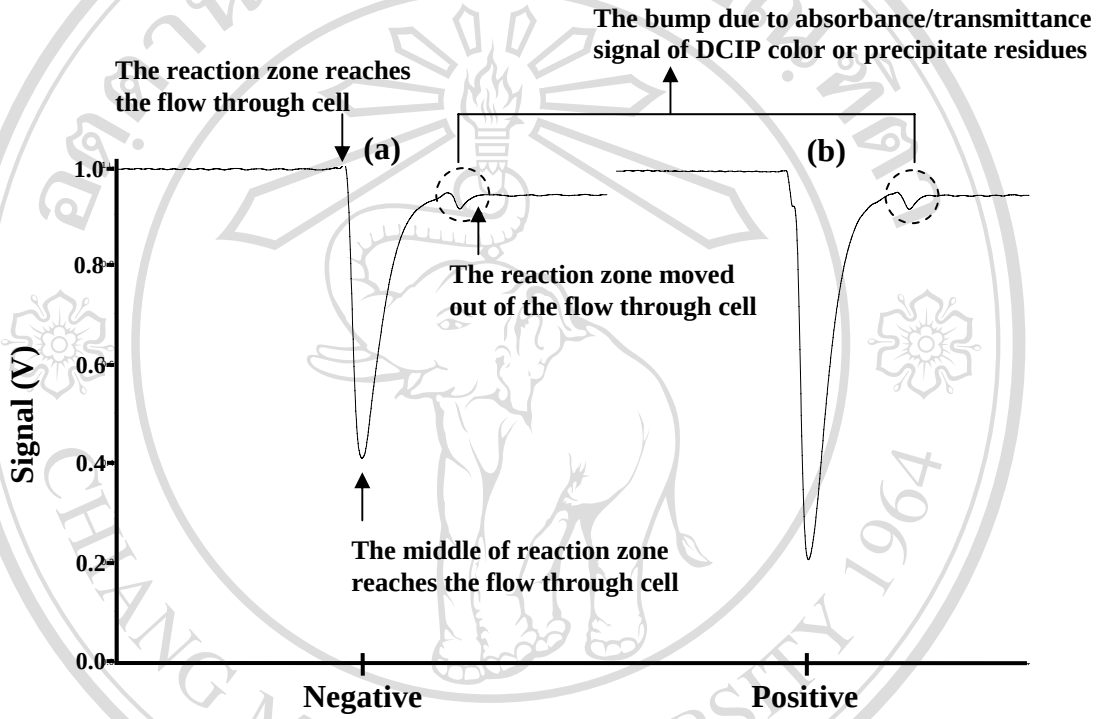

Figure 3.1 The profile of negative and positive samples obtained from the proposed FI-DCIP system (a) Negative sample (healthy sample) (b) Positive sample (hemoglobin E sample)

3.2 Optimization

3.2.1 Wavelength

The purpose of this preliminary study is to find the suitable detction wavelength using batch method to differentiate negative from positive sample. In conventional method, translation of the result was done with naked eyes which was prone to human error. It was expected that when packed red cell was incubated with DCIP solution at the optimum temperature and time, the exposed sulfhydryl (-SH) group (from cysteine) of hemoglobin E was oxidized and precipitated in cystien form as shown in Figure 3.2.

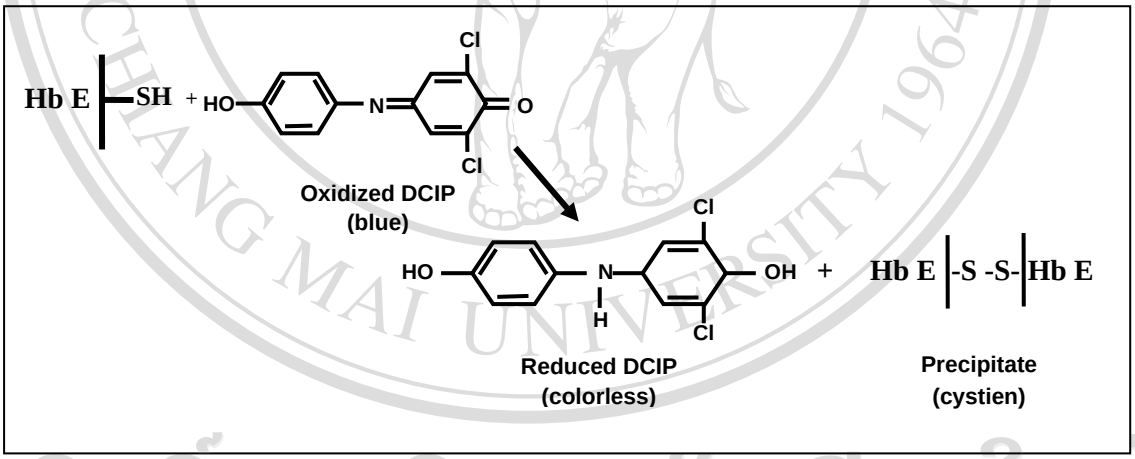

Figure 3.2 Sulfhydryl (-SH) group (from cysteine) of hemoglobin E is oxidized and precipitated (cystien form).

Oxidized form of DCIP (blue color) can oxidize –SH group by receiving H^+ from hemoglobin E and change itself to be a reduced form of DCIP (colorless). The precipitates that occur from S-S bonding of hemoglobin E make the DCIP solution

cloudy. However, the excess of oxidized form of DCIP (blue color) still cause the solution to be in dark blue color. Monitoring of the turbidity by a spectrophotometer should be more accurate and precise than human eyes. Packed red cells sample has red color and also has turbidity due to the precipitation of unstable hemoglobin. The wavelength that the change in turbidity plays more important role than the red color of packed red cells was sought. The suitable wavelength was investigated in the range 380-800 nm. The spectra of negative and positive samples from the reaction of packed red cell with DCIP solution were monitored with manual scan of the spectrophotometer (spectronic 21) after stopping the reaction with ascorbic acid solution as shown in Figure 3.3

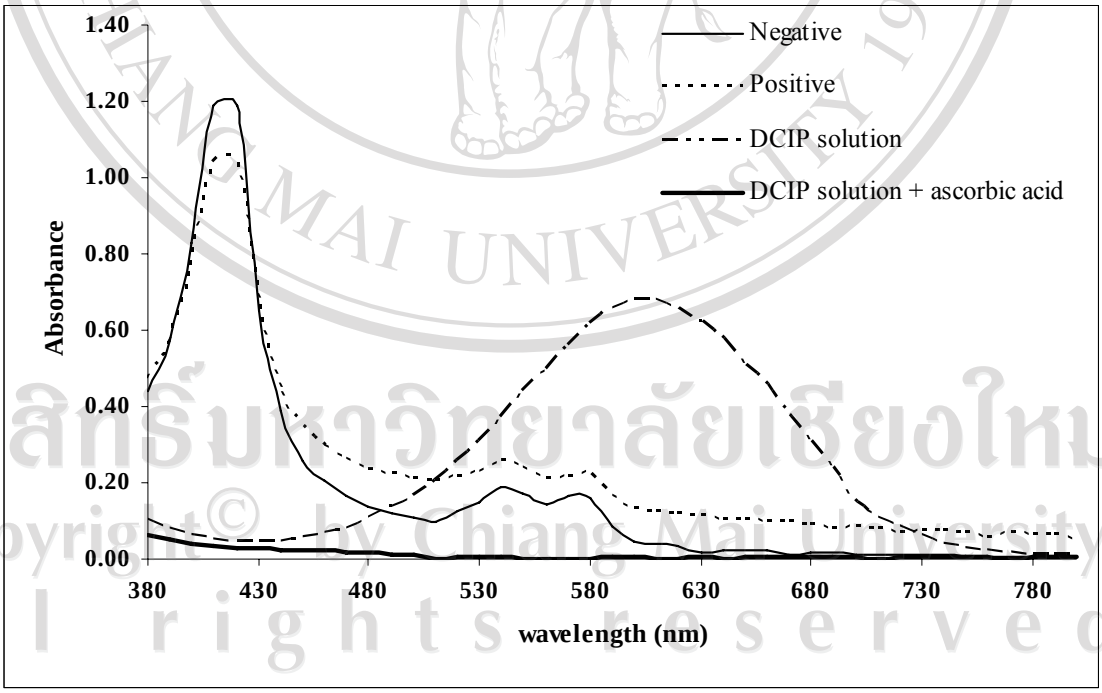

Figure 3.3 The absorbance spectra of a negative sample and a positive sample after mixing with DCIP solution and ascorbic acid solution.

From the result, the absorbance of positive sample is higher than negative sample in the range 440-800 nm because the positive sample (with high precipitation) should block the light more than negative sample (without precipitation). However the wavelengths in the range of 380-430, may be unsuitable wavelength due to the opposite results were observed. The highest different of absorbance can be obtained at 500 and 600 nm. The absorbance at 500 nm was not a choice because it is the same position of the absorbance of red color of packed red cell which may interfere the measurement. The concentration of particle (precipitate) in solution has an effect to the intensity of directionally transmitted light. If the concentration of particle in solution increases the intensity of transmitted light will be decreased [44]. The maximum absorbance of DCIP solution is at wavelength 600 nm. After adding ascorbic acid solution, the absorbance at 600 nm was disappeared. In addition, negative and positive samples could be differentiated clearly at 600 nm. Thus, the wavelength at 600 nm was chosen for the FI-DCIP precipitation system.

3.2.2 Mixing coil length

The change of mixing coil length affects the difference of peak height between positive and negative samples due to the difference in effectiveness of mixing process of blood sample with DCIP solution. In this research, the mixing coil lengths of 13, 25, 30 and 35 cm were investigated. The results are shown in the Figure 3.4.

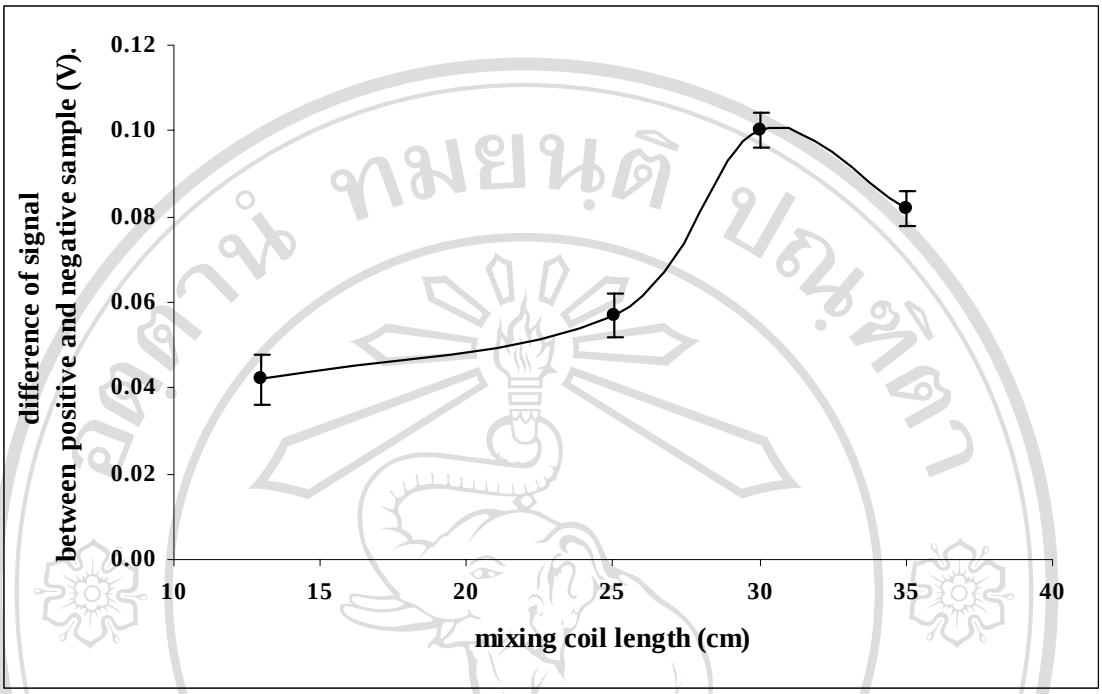

Figure 3.4 Effect of the mixing coil length on the analytical signal

This result indicated that the mixing coil length of 13 cm gave a small difference of peak height, which may be resulted from the ineffective mixing in a short distance mixing coil. Similarly, the decrease in the difference of peak height was also observed in the long mixing coil of 35 cm which may be due to the high dispersion effect of analytical zone. For future experiments, the mixing coil length of 30 cm was selected because it yielded the biggest differences between positive and negative samples.

3.2.3 Concentration of DCIP solution

In this experiment, the concentrations of DCIP were studied in the range of 1.75, 3.50, 4.60, 7.00 and 8.75 mM. Each concentration of DCIP solution 80 μL was mixed with 30 μL red cells to yield more than the ratio as used in the conventional method. The precipitation of unstable hemoglobin did not occur to the extent that could be observed if the concentration of DCIP was inadequate. However, the excess amount of DCIP solution was unnecessary and may cause normal hemoglobin to precipitate as well, therefore differentiation of normal hemoglobin and hemoglobin E might not be possible.

As expected, the result in Figure 3.5 shows that a small difference of peak height was observed when a low DCIP concentration was used. This was due to insufficient amount of DCIP to precipitate hemoglobin E. At the concentration of

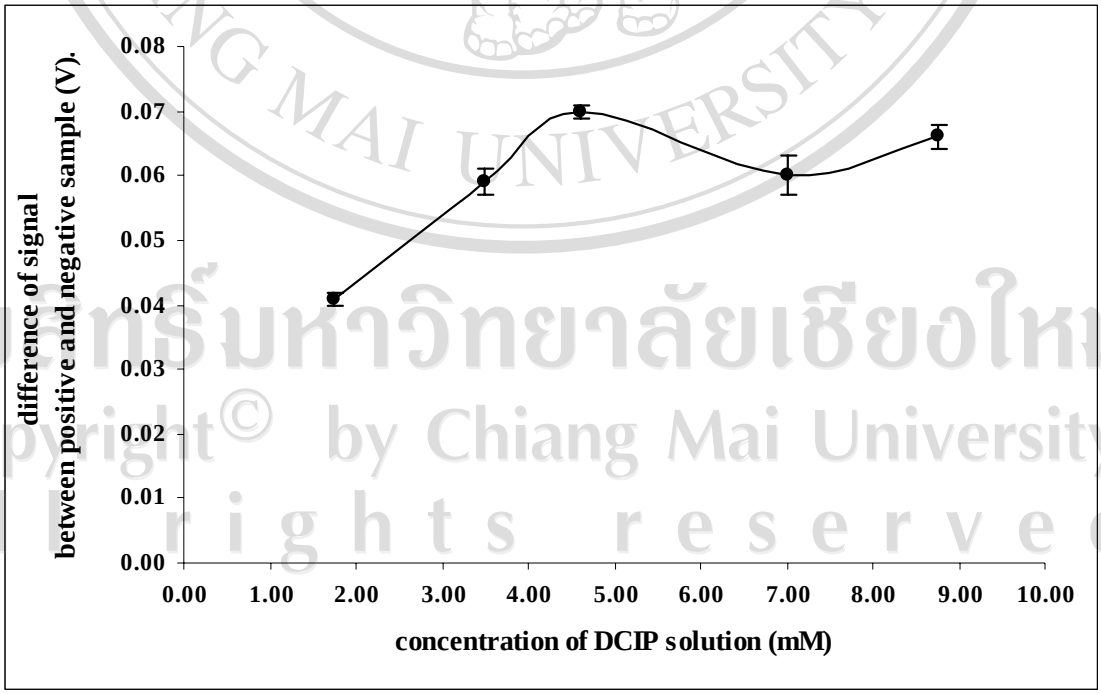

Figure 3.5 Effect of the DCIP solution concentration on the analytical signal

DCIP higher than 4.6 mM, signal difference were insignificantly changed. The reason that the difference of signal was not changed with the increased DCIP concentration more than 4.6 mM may be due to the limited amount of hemoglobin E. In this work, the DCIP concentration of 4.6 mM was selected for further experiments.

3.2.4 Incubation temperature and incubation time

The aim is to reduce incubation time as much as possible to shorter the total analysis time and avoid temperature fluctuation of water bath during a long incubation period. It is likely to require higher temperature than that used in the batch process if shorter incubation time was to be used. The effects of incubation temperature and incubation time were studied by comparing the difference of peak height at 40 °C, 50 °C and also 60 °C for 1, 3 and 5 min.

From the result in the Figure 3.6, it was found that the difference of signal was affected by incubation temperature. At the 40 °C, the difference of signal was increased when using longer incubation time. The same trend was found at 50 °C, with the better signal differences. The difference of signal at 50 °C for 3 min was the highest. This may be because only hemoglobin E was precipitated while normal hemoglobin was stable at this condition.

The result indicated that 60 °C was unsuitable incubation temperature. This might be because both normal hemoglobin and hemoglobin E were degraded and precipitated at this high temperature. Therefore, the peak heights of positive and negative sample was not different. It can be concluded that the mixture of DCIP solution with blood sample should be incubated in the water bath at 50 °C for 3 min.

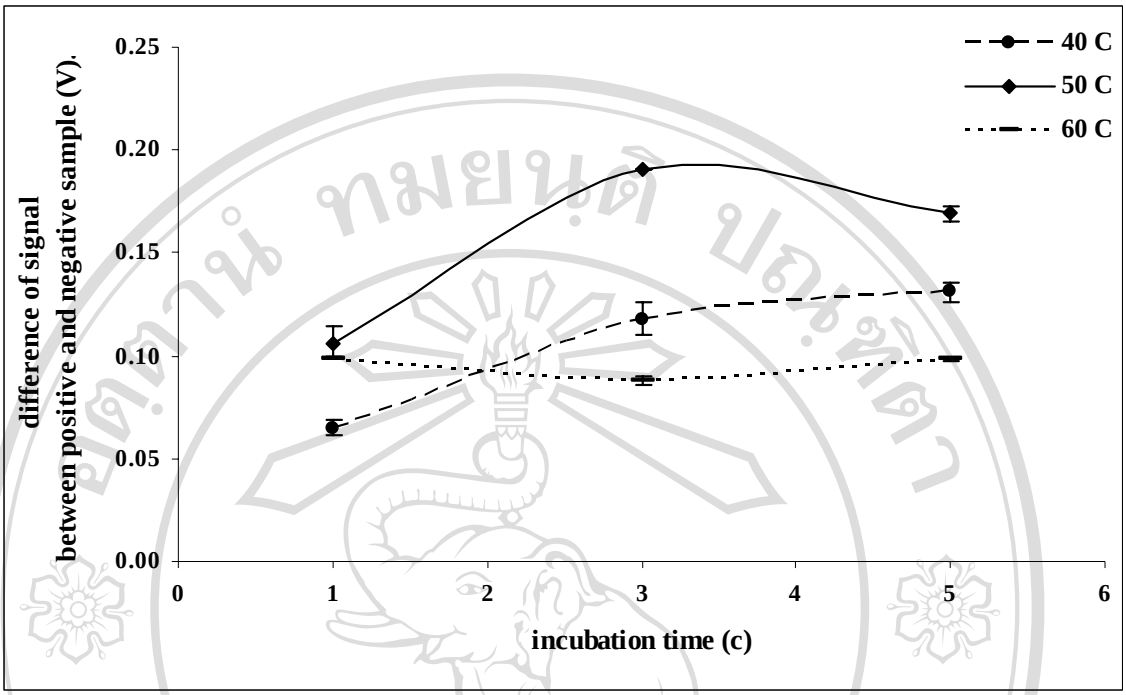

Figure 3.6 Effect of the incubation time and incubation temperature on the analytical signal

3.3 Within-run precision

Within-run precision or repeatability was determined by analyzing a packed red cells sample repeatedly 10 times within a day. This study was done with pooled samples (from 5 subjects). The percentages of relative standard deviation (%RSD) were 3.71 for negative sample and 1.82 for positive sample, see detail in Table 3.1.

Table 3.1 Within-run precision of the FI-DCIP system for positive and negative sample.

No. of Run	Peak height (volt) Negative sample	Peak height (volt) Positive sample
1	0.600	0.751
2	0.574	0.785
3	0.578	0.786
4	0.626	0.778
5	0.626	0.803
6	0.629	0.772
7	0.610	0.784
8	0.593	0.773
9	0.627	0.797
10	0.638	0.778
Average	**0.610**	**0.781**
SD	**0.023**	**0.014**
%RSD	**3.71**	**1.82**

3.4 Between-run precision

Between-run precision or reproducibility was determined in 3 different days (1, 2 and 3 days apart). Number of studied days limited by the packed red cell storage time period which should not be longer than 2-4 weeks as suggested by the Thalassemia Laboratory Maharaj Nakorn Chiang Mai Hospital. The percentages of relative standard deviation (%RSD) were 2.45 for negative sample and 3.49 for positive sample, see detail in Table 3.2.

Note: The blood samples used in this study were obtained from Thalassemia Laboratory Maharaj Nakorn Chiang Mai Hospital. The age of blood sample is about 1-2 weeks before used in this study.

Table 3.2 Between-run precision of the FI-DCIP system for positive and negative samples

Day	Peak height (volt) Negative sample	Peak height (volt) Positive sample
1	0.619	0.789
2	0.638	0.747
3	0.650	0.798
Average	**0.636**	**0.778**
SD	**0.016**	**0.027**
%RSD	**2.45**	**3.49**

3.5 Operational conditions for the FI-DCIP precipitation system

Table 3.3 summarizes the selected conditions from the previously described experiments for screening of hemoglobin E using the FI-DCIP system.

Table 3.3 The selected operational conditions of the FI-DCIP precipitation system for hemoglobin E screening

Parameters	Conditions
Wavelength	600 nm
Mixing coil length	30 cm
Concentration of DCIP solution	4.6 mM
Incubation temperature	50 °C
Incubation time	3 min
Volume of DCIP solution	80 µL
Volume of packed red cell using to react with DCIP solution	30 µL (10 fold dilution with diluent solution)
Total volume of packed red cell using to inject into the system	100 µL (10 fold dilution with diluent solution)
Concentration of ascorbic acid solution	0.03 M
Volume of ascorbic acid solution	0.745 mL
Flow rate of DCIP solution	2.0 mL/min
Flow rate of ascorbic acid solution	1.6 mL/min
Total flow rate	3.6 mL/min
Sample Throughput	12 / h

3.6 Evaluation of the FI-DCIP precipitation system

The FI-DCIP precipitation system was developed for hemoglobin E screening. In this work, 50 normal samples and 50 hemoglobin E patient samples were examined. Sample No. 1-50 are negative samples and sample No. 51-100 are positive samples as verified by a conventional ion exchange microcolumn techniques carried out at the Thalassemia Research Laboratories, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University. The peak heights obtained from the proposed FI-DCIP precipitation system are shown in Figure 3.7. It was found that the peak heights of all positive samples are higher than 0.67 V. This is an estimated cut-off value for hemoglobin E screening of the proposed FI system. This cut off value (0.67 V) gave both sensitivity and specificity at 1.0 or 100%, see Table 3.4. However, if the cut off value is 0.70 V, it gave the higher gap between positive and negative result. It can differentiate positive/negative easier than 0.67 V. Nevertheless, this 0.70 V cut off value gave a false negative. Determination of the cut off value should be done using more number of samples and the help of chemometrics.

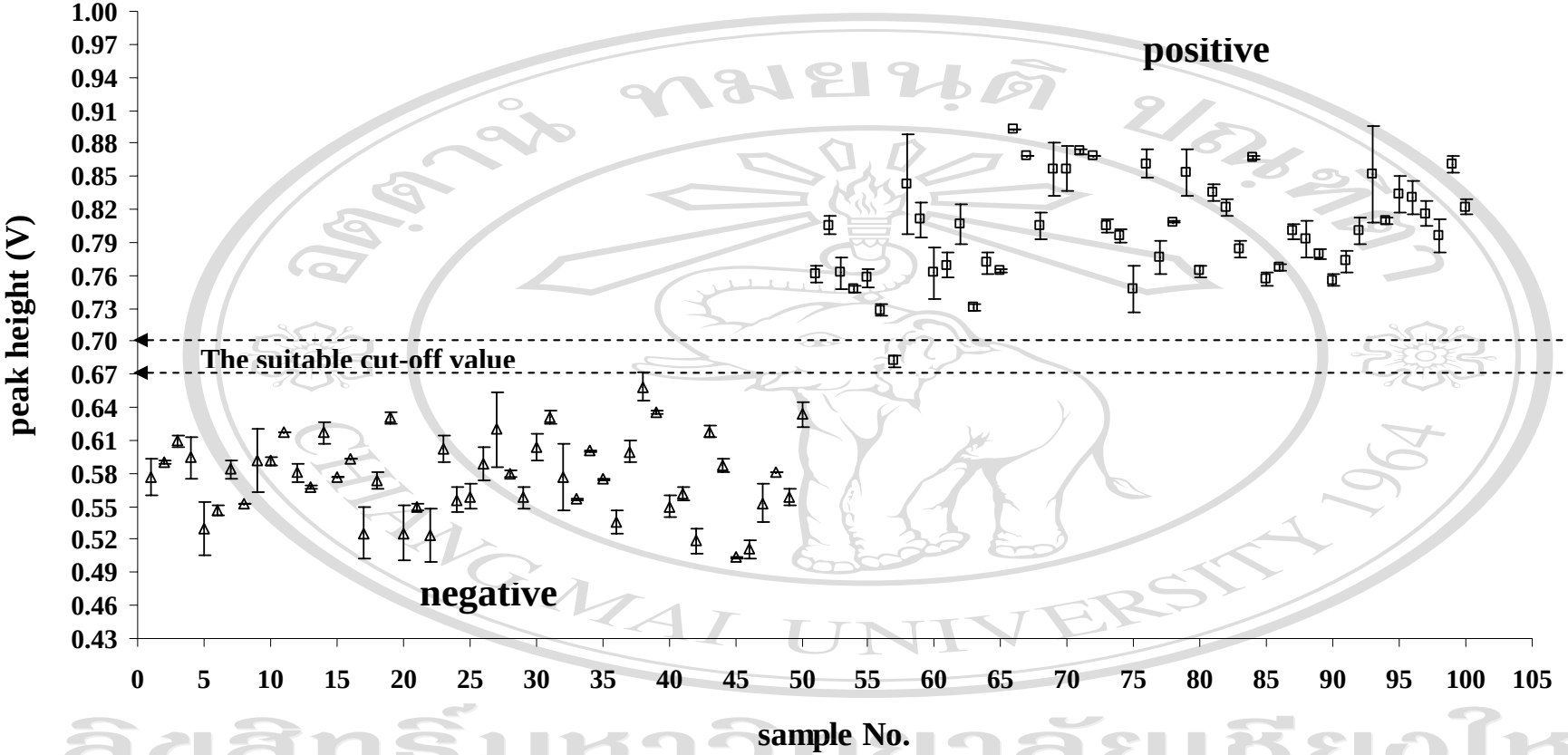

Figure 3.7 Comparison the peak heights obtained from negative and positive samples and estimation of the suitable cut-off value for hemoglobin E screening using the proposed FI-DCIP system.(Δ = negative samples, □= positive samples)

The peak heights of normal and hemoglobin E samples obtained from the FI-DCIP precipitation system were compared as presented in Figure 3.8 (see detail in APPENDIX B). The average peak height of positive and negative sample groups (Mean and S.D.) are significantly different ($p \leq 0.05$).

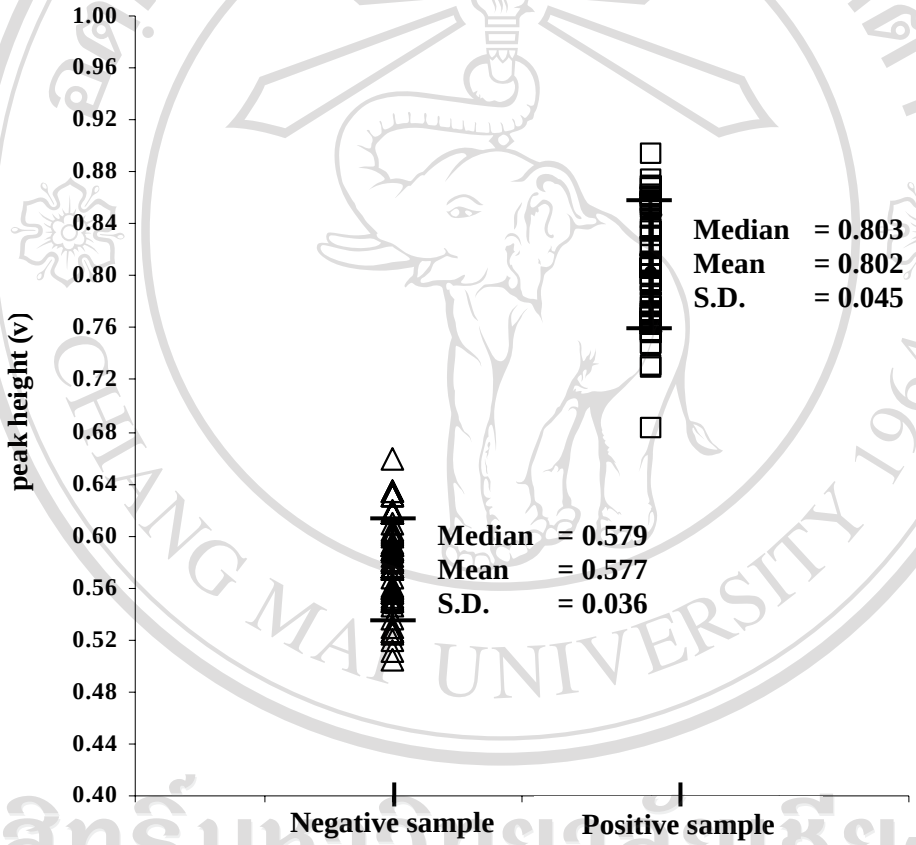

Figure 3.8 Distribution of peak heights in positive and negative samples obtained from the proposed FI-DCIP system (△= negative samples, □= positive samples)

Table 3.4 Summarization of the numbers of true and false responses and the sensitivity and specificity of the system when 0.67 volt of peak height is chosen as a cut-off value

Sample group	No. of sample	TP	FN	TN	FP	SV	SP	Prob.positive
Normal	50	-	-	50	0	-	1.00	-
Hemoglobin E	50	50	0	-	-	1.00	-	1.00

TP is true positive or the number of diseased patients that test positive

FN is false negative or the number of diseased patients that test negative

TN is true negative or the number of nondiseased patients that test negative

FP is false positive or the number of nondiseased patients that test positive

SV is sensitivity or $\frac{number\ of\ diseased\ patients\ with\ positive\ test}{number\ of\ diseased\ patients} = \frac{TP}{TP+FN}$

SP is specificity or $\frac{number\ of\ nondiseased\ patients\ with\ negative\ test}{number\ of\ nondiseased\ patients} = \frac{TN}{TN+FP}$

Prob. positive is probability of disease if the test is positive = $\frac{TP}{TP+FP}$