# CHAPTER 2

# EXPERIMENTAL

## 2.1 Materials and apparatus

1. Peristaltic pump (Alitea, USA).
2. Three-way solenoid valve (Cole-Parmer, USA)
3. Spectronic 21 (Spectronic instrument, USA)
4. Flow through cell 1 cm path length, 8 μL (Starna Scientific Ltd., UK)
5. BASIC Stamp Microcontroller (Parallax, Inc., USA)
6. Computer software (StampDAQ, Parallax, Inc., USA)
7. eDAQ Chart 5 (Apache Software Foundation, USA)
8. Computer
9. Micropipette 10 μL, 100 μL, 1000 μL (Eppendorf, Germany)
10. Stopwatch (Citizen, Thailand)
11. Syringe (Nipro Co. Ltd., Thailand)
12. Microcentrifuge tube (Axygen Scientific Imc., USA)
13. Polytetrafluoroethylene (PTFE) tubing (1/16”OD, 0.03”ID)(Upchurch, USA)
14. Barbed Fitting T connector (Cole-Parmer, USA)
15. Water bath (Memmert, Germany)

**2.2 Reagents**

1. L-Ascorbic acid : $C_6H_8O_6$ (Ajax, Australia)
2. Saponin from Quillaja bark (Cat. No. S4521, Cas:8047-15-2, Sigma, USA)
3. Tris [hydroxymethyl] aminomethane : TRIZMA® BASE (Cat. No. T-1503, Lot:041K5423, Sigma, USA)
4. 2,6 – dichlorophenolindophenol sodium salt hydrate (DCIP) : $C_{12}H_6Cl_2NNaO_2$ (Fluka, Switzerland)
5. Ethylenediaminetetraacetic acid (EDTA) : $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$ (Aldrich, USA)
6. Hydrochloric acid : HCl (J.T. Baker, USA)

**2.3 Preparation of standard solution and reagent**

2.3.1 Diluent solution

Tris base 4.36 g, EDTA 2.68 g and saponin 0.05 g were dissolved in 400 mL DI water and the pH was adjusted to 7.5 with 4 M HCl. Then the volume was brought up to 500 mL with DI water. This solution was used as a diluent of blood samples and a solvent for preparation of DCIP solution.



2.3.2 DCIP solution

In batch standard method as suggested by the Thalassemia Research Laboratory, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University, DCIP solution was prepared at 0.19 mM by dissolving 0.0055 g of DCIP in the diluent

solution and making up final volume to 100 mL. Then 5 mL of this DCIP solution was mixed with 0.02 mL packed red cells.

In FI system, only small volume of DCIP solution was used because of the difference in solution mixing process of the FI system as compared to the batch method. Therefore, the concentration of DCIP was changed based on calculation from the ratio of blood sample and DCIP solution used in the conventional method ($9.5\times10^{-7}$ mole DCIP: 0.02 mL packed red cells). The effect of amount of DCIP compared to red cell was studied by using various ratios of DCIP:red cell in the FI system.

2.3.3 DCIP-Clearing solution

DCIP-clearing solution in batch method was prepared at 0.125 M by dissolving 2.20 g of L-ascorbic acid in 100 mL of DI water. In FI system, the concentration of DCIP-clearing solution was again changed based on the concentration of DCIP. L-ascorbic acid 0.53 g was dissolved in 100 mL of DI water to yield the concentration of 0.03 M.



2.3.4 Blood sample

Samples were collected in the vacutainer tube containing EDTA that prevents red cells from coagulation. Next, they were centrifuged at 2500 rpm for 10 min to separate into two layers. The serum (upper layer) was discarded and the packed red cells (lower layer) were kept for further experiments. Blood samples could be kept for 2-4 weeks. In batch method, packed red cells were used directly.

However, in an optimization experiment of the FI system, five of negative and positive samples were pooled together to ensure the sufficient amount of the same samples during various optimizations. Then 50 μL of the pooled blood sample packed red cell was mixed with 450 μL of the diluent solution pH 7.5 in a microcentrifuge tube (~ 10 fold dilution) before being injected into the FI system.

**2.4 Method of the conventional DCIP precipitation**

The procedure of conventional DCIP precipitation is described as follows. A 20 μL of packed red cell was added to 5 mL of DCIP solution (0.19 mM) in a test tube. The test tube was then sealed with parafilm and the solutions were mixed thoroughly by shaking. Then, the mixture was incubated at 40°C for 25 minute in a water bath. Next, ascorbic acid solution 0.125M, 100 μL was added to reduce blue color of DCIP to colorless. Conventionally, turbidity was observed by bare eyes. A piece of printed paper may be placed behind the tubes to aid the observation.

In this study, the observation process was modified using a spectrophotometer. The change in turbidity, observed as transmittance signal, was monitored at 600 nm. The sample with the presence of hemoglobin E was more turbid than the normal sample without hemoglobin E. Therefore, transmittance signal of a hemoglobin E sample was lower than a normal sample.

**2.5 Manifold and operation step of FI-DCIP system**

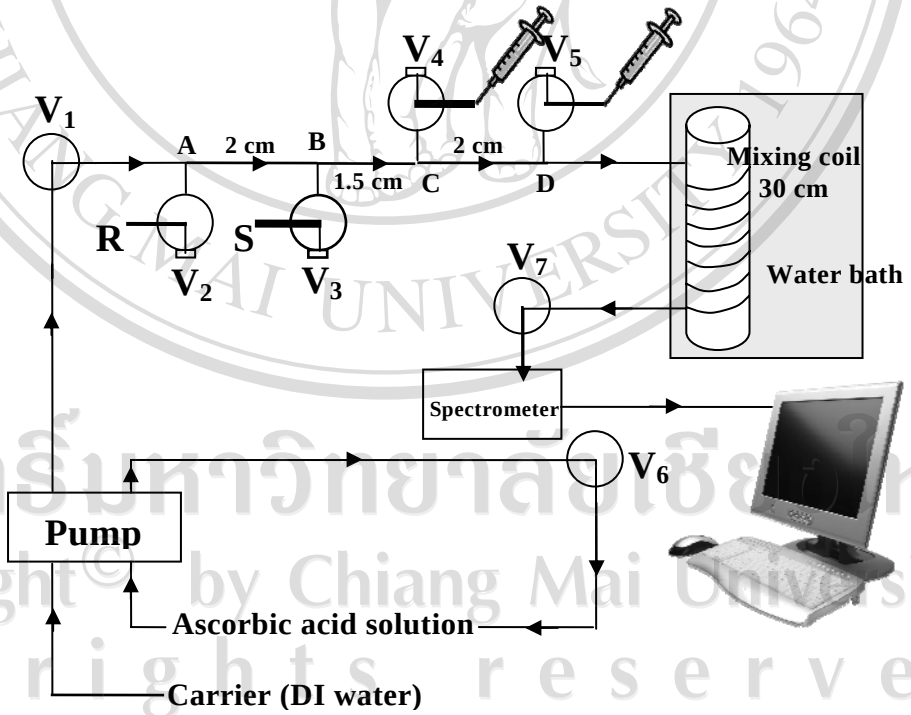
In modified FI-DCIP precipitation system, sample and reagent were handled in small tubings using pumps with constant flow rate. Mixing step, incubation step, and detection step were done simultaneously. The operation involved sample preparation and detection system as described here. Packed red cells were diluted ten times in diluent solution before starting the test. The process of oxidization of unstable hemoglobins with DCIP was the same as in batch method. Hemoglobin E is oxidized faster than any other types of hemoglobin at the optimum temperature and time. The turbidity of hemoglobin E solution blocked the light path showing low transmittance. Higher transmittance was observed from normal hemoglobin solution because there was no precipitation.

The manifold of flow injection-dichlorophenolindophenol precipitation (FI-DCIP) system used in this study is as shown in Figure 2.1(a). Blood sample and DCIP solution were introduced into the carrier stream by mean of hydrodynamic injection (see detail in Figure 2.1 (d)), which controlled by the microcontroller (BASIC stamp, Parallax, Inc., USA). Then the blood sample and DCIP solution zones were mixed while passing into the mixing coil which was located in a water bath whose temperature was set at the required temperature. The mixture was stopped in the water bath for a period of time. After that, the blue color of DCIP was reduced by adding the clearing solution. The turbidity that occurred during reaction of unstable hemoglobin with DCIP was monitored as transmittance at 600 nm. Finally, the difference of signal between hemoglobin E sample and normal hemoglobin sample was compared. The transmittance was

converted to voltage and recorded with a computer software (StampDAQ, Parallax, Inc., USA). The height of signal were calculated using the eDAQ Chart 5 software (Apache Software Foundation, USA).

The detail operation steps of the system are as follows, the DI water was propelled through the flow through cell to adjust the baseline with the constant flow rate (solid line). At the same time, ascorbic acid solution was flowed continuously because this system use a single pump to control two lines of reagents, see Figure 2.1 (a). By switching V1 and V7, the flow direction of the

**(a)**



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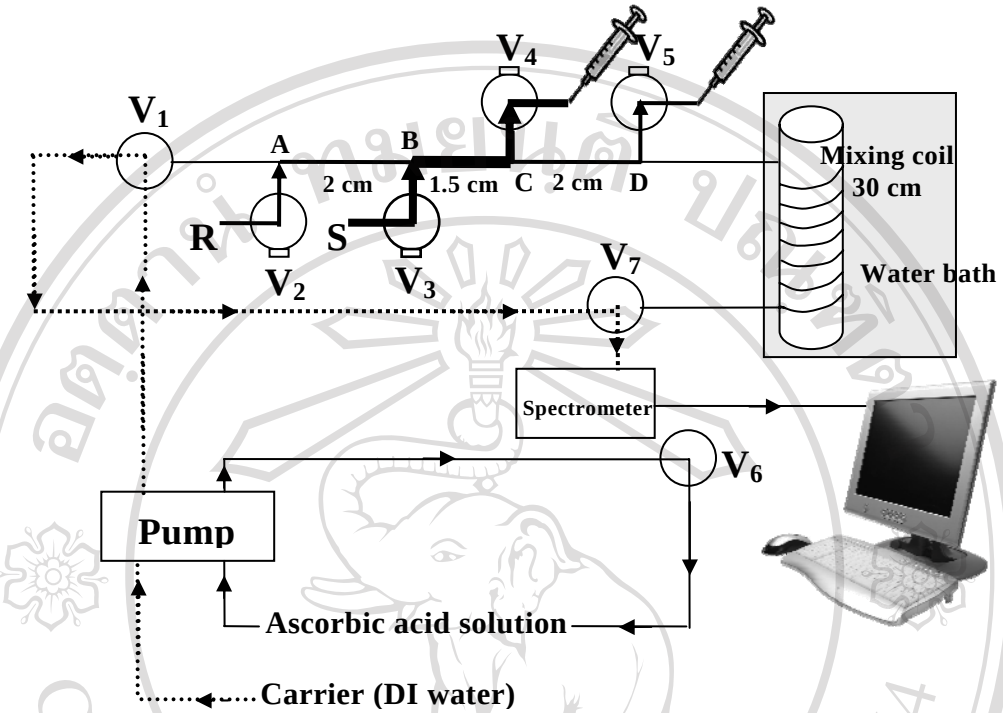
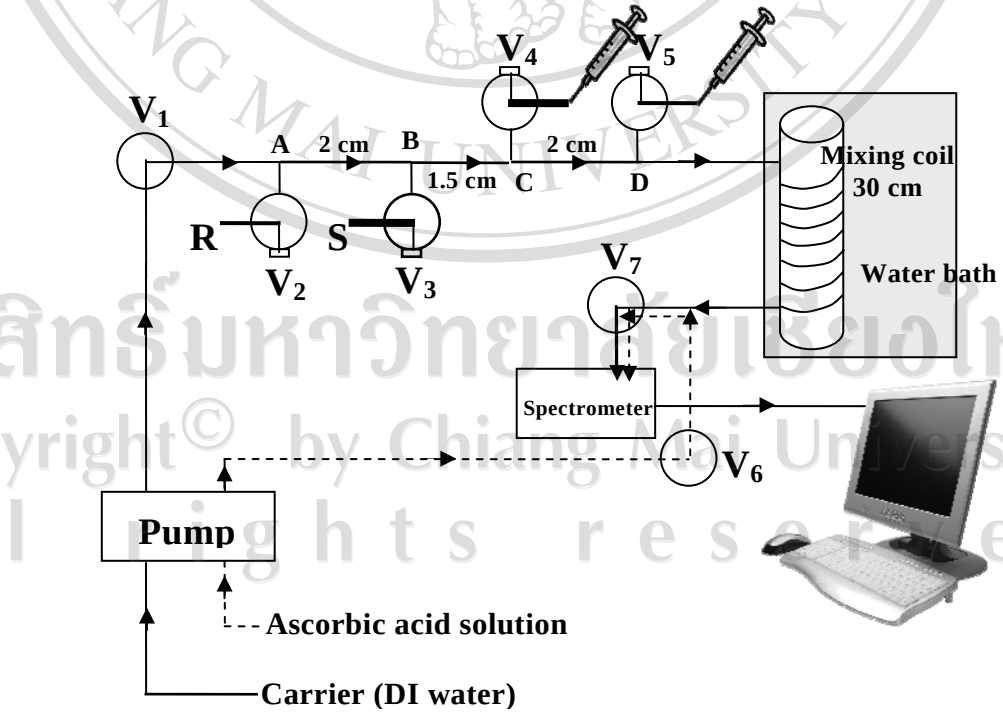



**(c)**



**(d)**

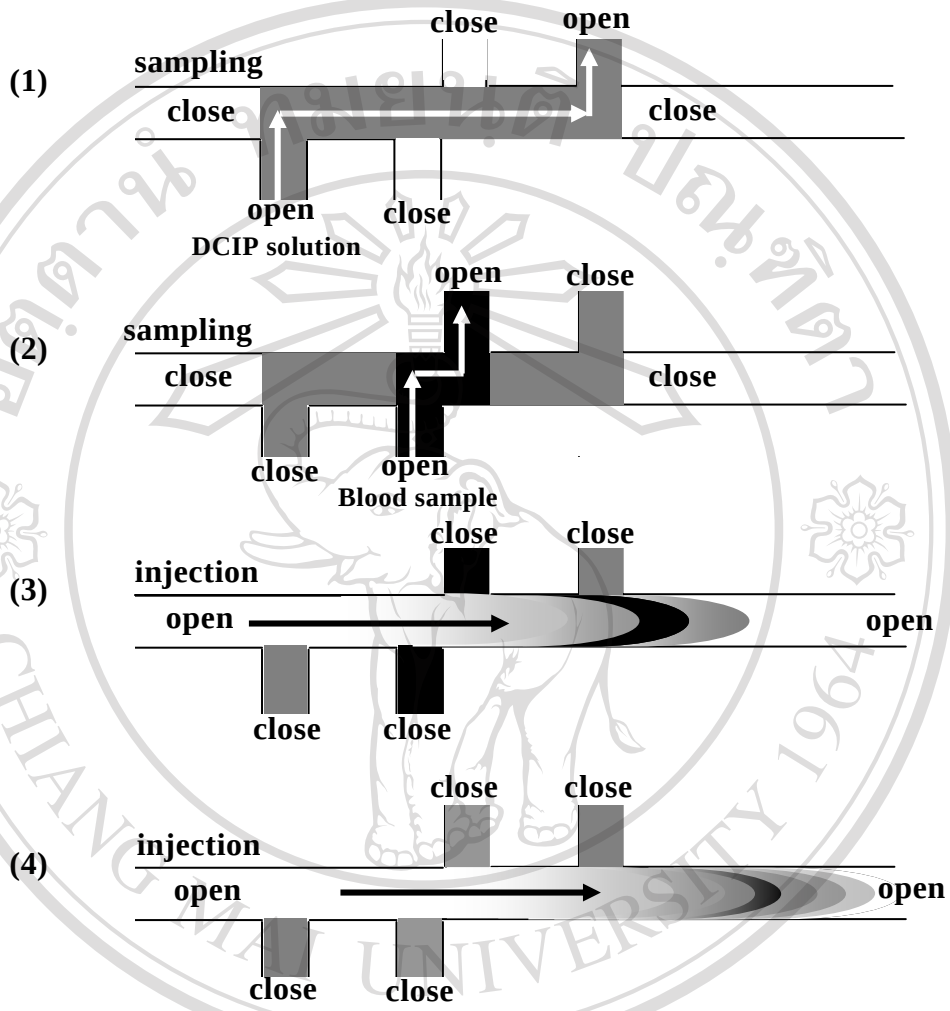



**Figure 2.1** (a)-(c) The FI-DCIP precipitation system for hemoglobin E screening, V1-V7 are three-way solenoid valves; R is reagent (DCIP solution); S is sample. The distance from A to B and from C to D are DCIP reagent zones. The distance from B to C is sample zone. The total distance from A to D is the sample-reagent mixture zone that was sent in to the detector. (d) Hydrodynamic load/injection step.

carrier stream (DI water) was changed as shown in dash line (·····), see Figure 2.1 (b). Then DCIP solution and blood sample were introduced into the system by syringe aspiration via V2-V5 and V3-V4, respectively. The red blood cell zone was sandwiched between DCIP zones. This should help to promote mixing when they were moved into the incubation zone. After that, flow was resumed by switching V1 and V7 to the injection mode (solid line), causing the sample zone (A-D) to flow into the water bath. The mixture was stopped for a fixed period of time in the mixing zone. Next, flow was resumed by switching on V1 and V7 again (solid line). The V6 was also switched at the same time to push ascorbic acid solution to merge into the line of the blood DCIP mixture ( — — — ), see Figure 2.1 (c). The blue color of DCIP was reduced to colorless. Switching of the valves V1, V6, V7 at the same time increased total flow rate of the system. The cloudiness solution zone was flowed into the detector. The change of transmittance was automatically recorded at 600 nm. The instrumental set up is shown in Figure 2.2.

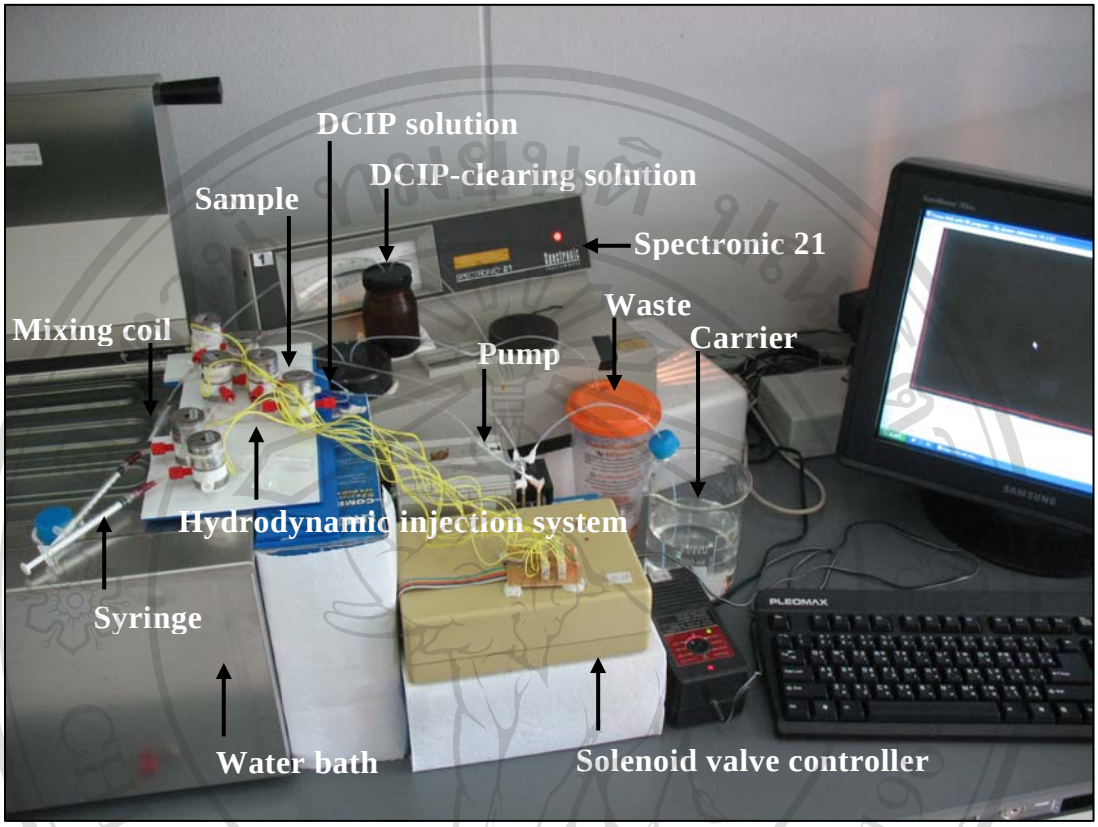



**Figure 2.2** Instrumental set up for screening hemoglobin E

In this study, the hydrodynamic injection system was employed to avoid possibility of cell breaking due to the mechanical force of switching a normal six-port injection valve as shown in Figure 2.1(b). It is important to ensure that the only cause of hemoglobin E precipitation comes from reaction between DCIP with the exposed sulfhydryl group of hemoglobin E. Because cell breaking by other causes would also make the solution turbid and would interfere the result from precipitation. See Figure 2.1(a) and detail operation, the sample solution near the V3-V4 may also cause some cells to break but this should not interfere the

measurement because the sample zone that is mixed with the DCIP solution is zone B-C which located far away from V3 and V4 valves. In addition, the possibility of cell blocking the solenoid valve should be minute as compared to using a normal six-port valve.

**2.6 Optimization**

Various parameters are optimized to suit the operation of the flow based technique. Some parameters (detection wavelength, incubation temperature, concentration of DCIP, volume of packed red cells) were optimized in batch method because it was important to know the effect of certain parameters before setting up a suitable FI-DCIP precipitation system. All experiments in this section employed pooled blood samples.

2.6.1 Wavelength

The suitable wavelength was sought by comparing the difference of absorbance between a negative sample and a positive sample by batch method following the standard method obtained from Maharaj Nakorn Chiang Mai Hospital. Blood sample 20 μL (10 fold dilution) was mixed with 5 mL, 0.19 mM DCIP solution in the test tube and incubated at 40 °C for 25 min before adding 100 μL, 0.125 M ascorbic acid solution to reduce blue color of DCIP. The change in the absorbance was monitored by a spectrophotometer (spectronic 21, Spectronic instrument, USA) in the range of 380-800 nm.

2.6.2 Mixing coil length

Various mixing coil lengths (13, 25, 30 and 35 cm.) located in a water bath were tested for effective mixing of blood sample with DCIP solution. DCIP solution 4.6 mM, 80 μL was used for mixing with 30 μL of 1:10 dilution of blood sample and they were incubated at 40°C for 5 min. Then ascorbic acid solution 0.03 M was added to reduce blue color of DCIP solution before measuring transmittance signal by a spectrophotometer. Peak height of pooled negative samples and pooled positive samples were compared. The suitable mixing coil length should offered the highest in the difference of peak height of negative and positive samples.

2.6.3 Concentration of DCIP solution

In this study, the optimum concentration of DCIP solution was determined by varying its concentration in the range of 1.75 to 8.75 mM. This range of concentration was selected from the calculation of blood and DCIP solution ratio in conventional method which is 5000 μL 0.19 mM DCIP : 20 μL red blood cells. By taking into account dilution and dispersion in the flow system, concentration of DCIP solution is expected to be higher than the conventional method for the precipitation of hemoglobin E to occur. Therefore, concentration of DCIP solution at 1, 2, 2.6, 4 and 5 times of the conventional method were tested which corresponded to 1.75, 3.50, 4.60, 7.00 and 8.75 mM (see Appendix B). The signal (peak height) when using various concentrations of DCIP solutions were

compared and the one that yielded the highest difference of peak height between positive and negative sample was selected for further experiments.

2.6.4 Incubation temperature and incubation time

The effect of incubation temperature and incubation time were studied by comparing the precipitation done at 40°C, 50°C and also 60°C for 1, 3 and 5 min. The DCIP solution 4.6 mM 80 μL, selected from the optimization, was mixed with blood sample (1:10 dilution, 30 μL). Then 0.3 mL of 0.03 M ascorbic acid solution was added. Differences of peak heights between positive and negative samples of each condition were investigated and compared.

**2.7 Within-run precision**

The FI-DCIP precipitation system was operated under the optimum condition using a pooled positive sample and a pooled negative sample (5 samples each) repeatedly 10 times within one day. The percentage of relative standard deviation (%RSD) was calculated.

**2.8 Between-run precision**

The FI-DCIP precipitation system was operated under the optimum condition. Peak heights of a pooled positive and a pooled negative sample were investigated in 3 different days (1, 2, and 3days). The percentage of relative standard deviation (%RSD) was calculated.

**2.9 Evaluation of FI-DCIP precipitation system and estimation of cut-off level to predict for hemoglobin E**

Analysis of 50 normal samples and 50 hemoglobin E patient samples were performed using the developed FI-DCIP system with operational conditions selected from optimization. The results obtained from the FI-DCIP system were compared to those obtained from the conventional ion exchange microcolumn standard method (see detail in Appendix C) which was performed at the Thalassemia Laboratory, Maharaj Nakorn Chiang Mai Hospital. The average peak heights of the normal group and hemoglobin E group were compared.