APPENDICES

APPENDIX A

1. BASIC Stamp microcontroller for controlling the solenoid valve in the proposed FI based system

1.1 Display of BASIC Stamp that command the program by the Parallax PBASIC language

2. The Parallax PBASIC language for controlling the solenoid valve to vary the mixing time, incubation time, DCIP-clearing solution time and detection time.

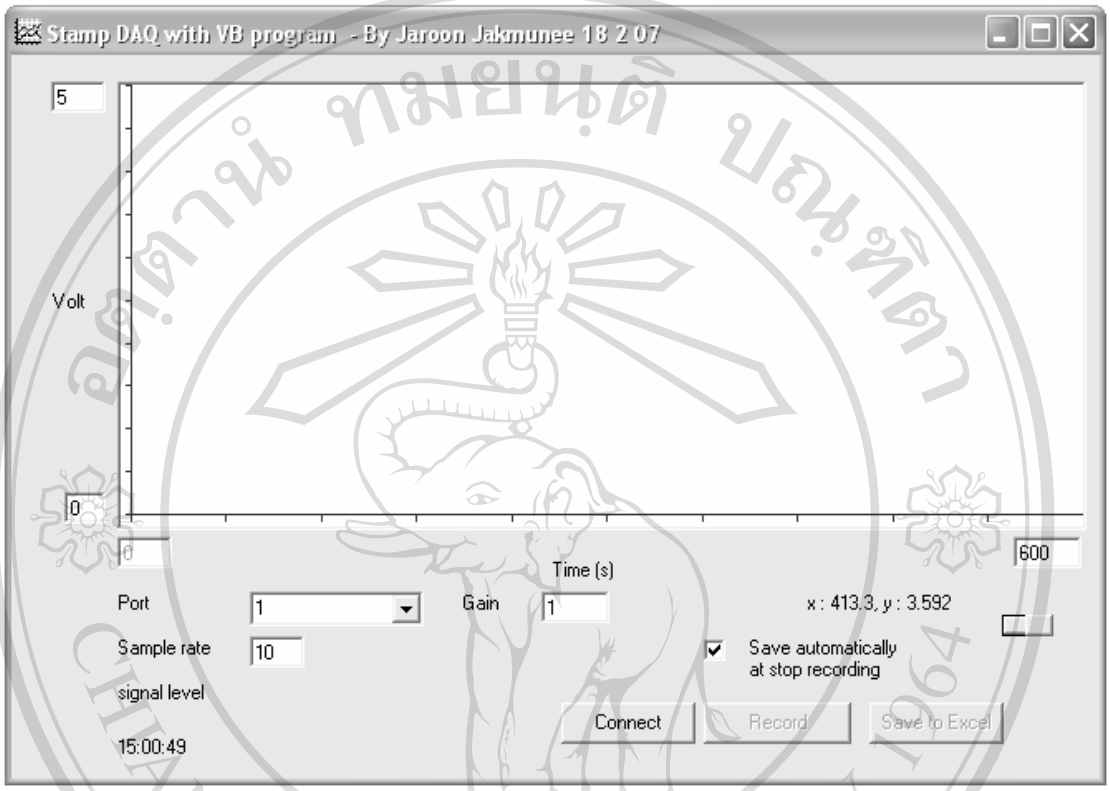
```
'***************** StaMP Control of hydrodynamic SIA
********************
'This program is created by Jaroon Jakmunee for control of
hydrodynamic SIA
'system by control switching of solenoid valves at different timing

'21/11/04-Jaroon Jakmunee
'29/9/07-modify by JJ => filename: thalassemia type III-r.bsx

'***************** StampDAQ - define constant ***************
'Port of stamp for control of various valve


Input 8             'set port 8as input port
V1    CON   0       'output port to control each valve
V2    CON   1
V3    CON   2
V4    CON   3
V5    CON   4
V6    CON   5
V7    CON   6
LED   CON   14


'Time in seconds for switch on each valve
V1_On  CON   5000          'clean line
V2_On  CON   5000          'load R1
V3_On  CON   5000          'load R2
V4_On  CON   5000          'load S
V5_On  CON   5000          'flow to detector
V6_On  CON   5000          '
V7_On CON    5000

'***************Variable for Next step of program***************

DelayT      VAR   Word   'a time period before sampling next data
sPin        CON   16     'Serial Pin - P16, Programming port
Baud        CON   240    'use 84 for BS2: Baud mode for a rate of 9600, 8-N-1
                         'BS2P, BS2SX use 240 for 9600, 8-N-1

'***********************Start program*********************************

'Set initial status of port to OFF
LOW V1
LOW V2
LOW V3
LOW V4
```

```
LOW V5
LOW V6
LOW V7

High LED
pause 5000

'test turn on solenoid - at start up
Low  LED    'off LED
High V1
pause 1000
Low  V1
High V2
pause 1000
Low  V2
High V3
pause 1000
Low  V3
High V4
pause 1000
Low  V4
High V5
pause 1000
Low  V5
High V6
pause 1000
Low  V6
High V7
pause 1000
Low  V7
High LED 'turn on LED

Main:
      'check for key press
      'if IN8=0 then Start_Cycle_test: 'if P8=0->key press
      if IN8=0 then Start_Cycle:
      High  LED  'turn off LED
      PAUSE 500 'wait 500 ms
      Low   LED
      PAUSE 250
GOTO Main

Start_Cycle_test:
      Low   LED  'turn off LED
      'debug "start run", cr      'show start
      High  V7
      pause 5000
      Low   V7
      High  LED
GOTO Main
```

```
Start_Cycle:
'Start cycle I
      Low   LED  'turn off LED

      'High       V7     'test switch V7   =for checking program
      'pause      1000
      'Low  V7

'Fill reagent and sample

      High  V1
      High  V7

      High  V2           'turn on load dcip
      High  V5           'load dcip
      High  LED
      pause 5000

      Low   LED
      Low   V2           'turn off load dcip
      Low   V5
      pause 2000

      High  V3
      High  V4
      High  LED          'turn on load sample
      pause 10000        'load sample

      Low   LED
      Low   V4           'turn off load sample
      Low   V3


'Push to water bath
      Low   V1
      Low   V7
      pause 3600         'mixing time

'Stopped in water bath
      High  V1
      High  V7
      pause 60000        'incubation time
      pause 60000
      pause 60000

'inject ascorbic acid and push to detector
      Low   V1
      Low   V7


      High  V6
      pause 28000
      Low   V6           'DCIP-clearing solution time
```

```
        High  LED           'detection time
        pause 60000

GOTO Main
```

3. Display of StampDAQ for recording the data



APPENDIX B

1. Ratio of Blood sample and DCIP concentration

In conventional method

Packed red cell	20	μL	:	DCIP 5000	μL
				(0.19 mM)	
				n = CV/1000	
				n = (0.19 mM×5000μL)/1000	
Packed red cell	20	μL	:	DCIP 0.00095	mmole
∴Packed red cell	1	μL	:	DCIP 0.000048	mmole

In FI-DCIP precipitation system

Packed red cell	30	μL	:	DCIP 80	μL
(~ 10 fold dilution)					
Undiluted packed red cell	3μL		:	DCIP 80	μL
				DCIP 3×0.000048	mmole
				DCIP 0.000144	mmole
				n = CV/1000	
				C = 1.75 mM	
∴ Packed red cell	30	μL	:	DCIP solution 80	μL
(~10 fold dilution)				(1.75 mM)	

Vary concentration

1.75×1.0	=	1.75	mM
1.75×2.0	=	3.50	mM
1.75×2.6	=	4.60	mM
1.75×4.0	=	7.00	mM
1.75×5.0	=	8.75	mM

2. Comparison of the difference of the average peak heights of the two groups

F-test for the comparison of standard deviation

Standard deviations of two data groups were compared using F-test. The ratio of two variances was calculated (*see in equation (1)*)

Null Hypothesis, $H_0 : S^2_{pos} / S^2_{neg} = 1$

$H_1 : S^2_{pos} / S^2_{neg} > 1$

Test Statistic

$$F = S^2_{pos} / S^2_{neg} \qquad \textit{equation (1)}$$

Where

S^2_{pos} = variance, is the squared of the standard deviation of positive sample

S^2_{neg} = variance, is the squared of the standard deviation of negative sample

t-test for the comparison of average peak heights of the two groups

The standard deviations of the two groups are in significantly different. The comparison was continued with t-test when the number of sample less than 100. The t-test that compare the different between independent sample means ($\bar{x}_{pos}$ and $\bar{x}_{neg}$) with equal variance (*see equation (2)*) should be used. An approximate method in these conditions is given below:

Null Hypothesis, $H_0 : \mu_{pos} - \mu_{neg} = 0$

$H_1 : \mu_{pos} - \mu_{neg} \neq 0$

Test Statistic

$$t = \frac{(\bar{X}_{pos} - \bar{X}_{neg})}{[(S_{pos}^2/n_{pos}) + (S_{neg}^2/n_{neg})]^{1/2}} \qquad \textit{equation (2)}$$

where

$\bar{x}_{pos}$ = positive sample mean

$\bar{x}_{neg}$ = negative sample mean

s^2_{pos} = variance, is the squared of the standard deviation of positive sample

s^2_{neg} = variance, is the squared of the standard deviation of negative sample

n_{pos} = the number of positive sample observation (sample size)

n_{neg} = the number of negative sample observation (sample size)

In this research, two sample t-test was used for comparison of the difference of peak height mean in positive sample and negative sample from the proposed FI-DCIP precipitation system. It demonstrates significant difference (the null hypothesis (H_0) was rejected). The result was calculated as shown in table B.

Table B The results from calculate of F-test and t-test

	Positive sample	**Negative sample**
Sample size; n	50	50
Sample mean; $\bar{x}$	0.802	0.577
Standard Deviation; S	0.045	0.036
Variance; S^2	0.00202	0.00129
$F = S^2_{pos} / S^2_{neg}$	1.562	
$F_{critical}$ when ν_1=49, ν_2=49 at p = 0.05	1.618	
$t = \frac{(\bar{X}_{pos} - \bar{X}_{neg})}{[(S_{pos}^2/n_{pos}) + (S_{neg}^2/n_{neg})]^{1/2}}$	27.608	
$t_{critical}$ when ν_1=49, ν_2=49 at p = 0.05	1.900	

The calculated value of F (= 1.562) is less than the critical value (F=1.618), so there was no significant difference between the two variances at the 95% probability level. The t-test is suitable for comparison of the difference of peak heights in positive samples and negative samples from the FI-DCIP precipitation system

The critical value is t = 1.90 (P=0.05). The observed value of |t| (=27.608) which is greater than the critical value so the null hypothesis (H_0) is rejected. There is sufficient evidence showing that the peak height of positive samples and negative samples from the proposed FI-DCIP precipitation system are significantly different.

APPENDIX C

Thalassemia Laboratory

Department of Pediatrics, Faculty of medicine,

Chiang Mai University, Chiang Mai 50200, Thailand

Hemoglobin E Screening test (ion exchange microcolumn)

principle

Hemoglobin E and hemoglobin A_2 are co-eluted from an ion exchange resin (DEAE Sephadex A 50) column. The ratio of hemoglobin E more than 10% causes the eluate reddish. In this separation condition, other hemoglobins for examples, Hb A, Hb F, Hb Bart's and Hb H still remain in the column

Sample

Packed red cell 30 μL

Reagent

1. Resin: anion exchanger DEAE Sephadex A 50

2. Stock buffer: 1.0 M Tris-HCl, pH 9

Tris	121.14	g
Distilled water	800	mL

Adjust pH to 9.0 with HCl 4 M and then volume was made up to 1000 mL

3. Working Buffer:

Stock buffer	50	mL
KCN	100	mg
Distilled water	1000	mL

Adjust pH to 8.5 with HCl 4 M

4. Hb E-buffer: 0.05 M Tris-HCl / KCN, pH 8.2:

Stock buffer	50	mL
KCN	100	mg
Distilled water	1000	mL

Adjust pH to 8.2 with HCl 4 M

(Stock and Hb E-buffer kept at 4°C can be used within 6 months)

Resin DEAE Sephadex A 50 preparation

A 10 g of DEAE Sephadex A 50 was added to 500 mL of Hb E-buffer and allowed to stand at room temperature for 1-2 days before washing with working buffer 2-3 times. Next, pH of DEAE Sephadex solution was adjusted to 8.5 with HCl 4 M

Column preparation

A syringe column (3 cc. disposable syringe) was placed on the stand. A small cotton was loosely plugged at the tapered end. The column was filled with the prepared DEAE Sephadex A 50. The settled resin should be 3 cc in height. Then the 10 cc disposable syringe column was placed above a 20 cc test tube (see in figure C).

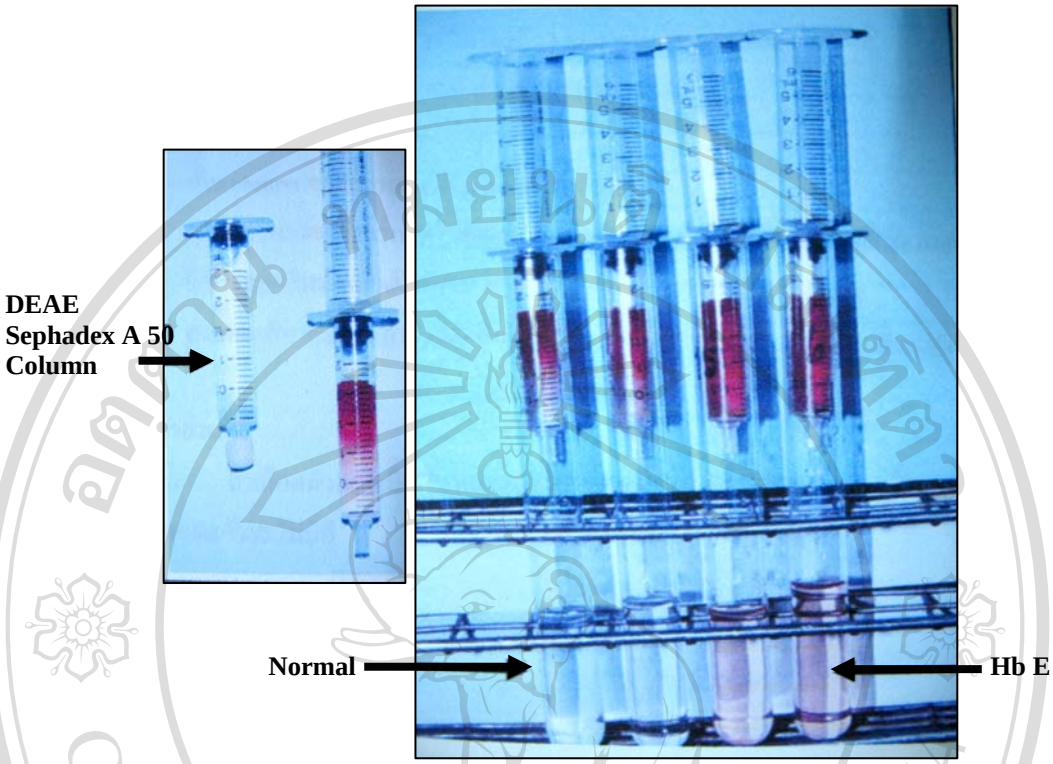


Figure C. Ion exchange microcolumn Chromatography for screening hemoglobin E

Method

1. 30 μL of packed red cell and 10 mL of Hb E-buffer were mixed and allowed to stand at room temperature for 10 min.

2. The mixture of blood and buffer was added into the syringe reservoir

3. The eluate was collected in the test tube.

Result

When there is hemoglobin E in a blood sample, the eluted solution would be red; in contrast, with a negative result the solution remains colorless (because of no hemoglobin eluted out of the column).

APPENDIX D

Glossary

Gestational age	The age of an embryo or fetus (or newborn infant) from the first day of the woman's last menstrual period (LMP). This standard system of counting the progression of pregnancy starts approximately two weeks before fertilization takes place; it does not in itself constitute the beginning of pregnancy
Globin	Globular proteins, or globin are one of the two main protein classes, comprising "globe"-like proteins that are more or less soluble in aqueous solutions (where they form colloidal solutions). This main characteristic helps distinguishing them from fibrous proteins (the other class), which are practically insoluble.
Hemoglobinopathy	A kind of genetic defect that results in abnormal structure of one of the globin chains of the hemoglobin molecule. Common haemoglobinopathies include sickle-cell disease and thalassemia.

MCH	The mean corpuscular hemoglobin, or "mean cell hemoglobin" (MCH), is the average mass of hemoglobin per red blood cell in a sample of blood. It is reported as part of a standard complete blood count.
MCV	The mean corpuscular volume, or MCV, is a measure of the average red blood cell volume (i.e. size) that is reported as part of a standard complete blood count.
Postnatal age	The period beginning immediately after the birth of a child and extending for about six weeks. The period is sometimes incorrectly called the postpartum period, which refers to the mother.
RBC	Red blood cell indices are blood tests that provide information about the hemoglobin content and size of red blood cells. Abnormal values indicate the presence of anemia and which type of anemia it is.
Splenomegaly	An enlargement of the spleen, which usually lies in the left upper quadrant of the human abdomen.

Thalassemia	An inherited autosomal recessive blood disease. In thalassemia, the genetic defect results in reduced rate of synthesis of one of the globin chains that make up hemoglobin. Reduced synthesis of one of the globin chains can cause the formation of abnormal haemoglobin molecules, and this in turn causes the anemia which is the characteristic presenting symptom of the thalassemias.

CURRICULUM VITAE

NAME Miss Warisara Khotchasit

DATE OF BIRTH July 13, 1983

ACADEMIC STATUS

2004 B.S. (Chemistry), Naresuan University

2005 Certificate in Education (Cert.in Ed.)

2008 M.S. (Chemistry), Chiang Mai University

SCHOLARSHIPS

2001-2005 Full support from The Promotion Project for Teacher Production in Sciences and Mathematics (The Institute for the Promotion of Teaching Science and Technology)

2006-2008 Full support from the Center of Excellent for innovation in Chemistry: (PERCH-CIC)

PRESENTATION

2007 S. K. Hartwell, S. Khonyoung, W. Khotchasit, P. Kongtawelert, J. Jakmunee, S. Lapanantnopphakhun and K. Grudpan, Immobilization of some Biomarkers on Glass Cappillary for Development of Flow Based Immunoassay (Poster Presentation), The 6th Annual Symposium on TRF Senior Research Scholar and Research Group on Innovation on Analytical Instrumentation CHE, 16 August 2007, Chiang Mai University, Chiang Mai, Thailand.

2007 W. Khotchasit, W. Sripaoraya, S. Kerdphon, T. Sanguansermsri, J. Jakmunee, S. Lapanantnoppakhun, K. Grudpan, and S. K. Hartwell, Flow Based Techniques for Screening of HbE Using Ion-exchange Column and DCIP Precipitation (Oral and Poster Presentation), International Symposium on Flow-Based Analysis VII, 16-18 December 2007, Sirinart Garden Hotel, Chiang Mai, Thailand.

2008 S. K. Hartwell, B. Srisawang, S. Khonyoung, W. Khotchasit, W. Sripaoraya, S. Kerdphon, J. Jakmunee, S. Lapanantnoppakhun, T. Sangunsermsri and K. Grudpan, Flow Injection with Spectrophotometric Detection for Automatic Screening of Thalassemia (Poster Presentation), Symposium for Younger Generation Researchers, 29 August 2008, Chiang Mai University, Chiang Mai, Thailand.

2008 W. Khotchasit, S. Kerdphon, T. sanguansermsri, J. Jakmunee, S. Lapanantnoppakhun, S. K. Hartwell and K. Grudpan, Development of a Flow-Based Dichlorophenolindophenol Precipitation System for Screening of Hemoglobin E (Poster Presentation), The 15th International Conference on Flow Injection Analysis (ICFIA2008), 28 September – 3 October 2008, Nagoya, Japan.

THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Hemoglobin E is the important hemoglobinopathies the needs to be monitored for the public health in Thailand. The effective and economical techniques for screening of hemoglobin E are very important. Screening of hemoglobin E will help to reduce the number of samples needed to be tested using expensive method and will also help to prevent and control spreading of hemoglobin E. This work aim to apply the flow based injection technique for hemoglobin E screening on the basis of reaction between dichlorophenolindophenol with unstable hemoglobin. Hemoglobin E can be precipitated easily and rapidly as compared to normal hemoglobin at the optimum condition. The advantages of flow-based dichlorophenolindophenol precipitation (FI-DCIP precipitation) include more automated operation, shorter analysis time, reduce risk of direct contact of blood sample and lower amount of blood sample consumption as compared to the conventional dichlorophenolindophenol precipitation test. The system should be useful for routine blood screening of hemoglobin E.