CHAPTER 3

Results

3.1 FLT3 expression on leukemic cell lines

In order to evaluate and compare the expression levels of FLT3, leukemic cell lines were set up for a screening using flow cytometry, Western blot analysis, and RT-PCR assay. FLT3 expression was determined in three independent experiments (n=3) for each cell line. Flow cytometric method for detecting FLT3 protein expression, representative flow cytometry profiles are shown in overlaid histogram (Figure 3.1 A). The results showed that EoL1 cells expressed a prominent degree of FLT3 on cell surfaces with the Δ mean fluorescent intensity value of 5.60±0.72 compared to MV4-11, U937, K562, Molt4, and HL60 cells with the values of 3.53±0.93, 0.66±0.46, 0.59±0.57, 1.00±0.64, and 1.74±0.10, respectively (Figure 3.1 B). The presence of FLT3 protein expression was also compared with the results obtained from Western blotting technique. The FLT3 protein band was 160 kDa and 2 bands of GAPDH protein were 37 and 29 kDa, respectively. Additionally, MV4 and U937 cells showed the different molecular weights and patterns as compared to EoL-1, K562, and Molt4 cell lines due to the difference in cell phenotype. The represented data are shown in Figure 3.2. The data showed that EoL1 and HL60 cells expressed the FLT3 protein levels higher than the other cell lines. However, HL60 cells expressed the highest level of FLT3, while K562 cells showed the lowest level when normalized by using GAPDH protein levels, respectively.

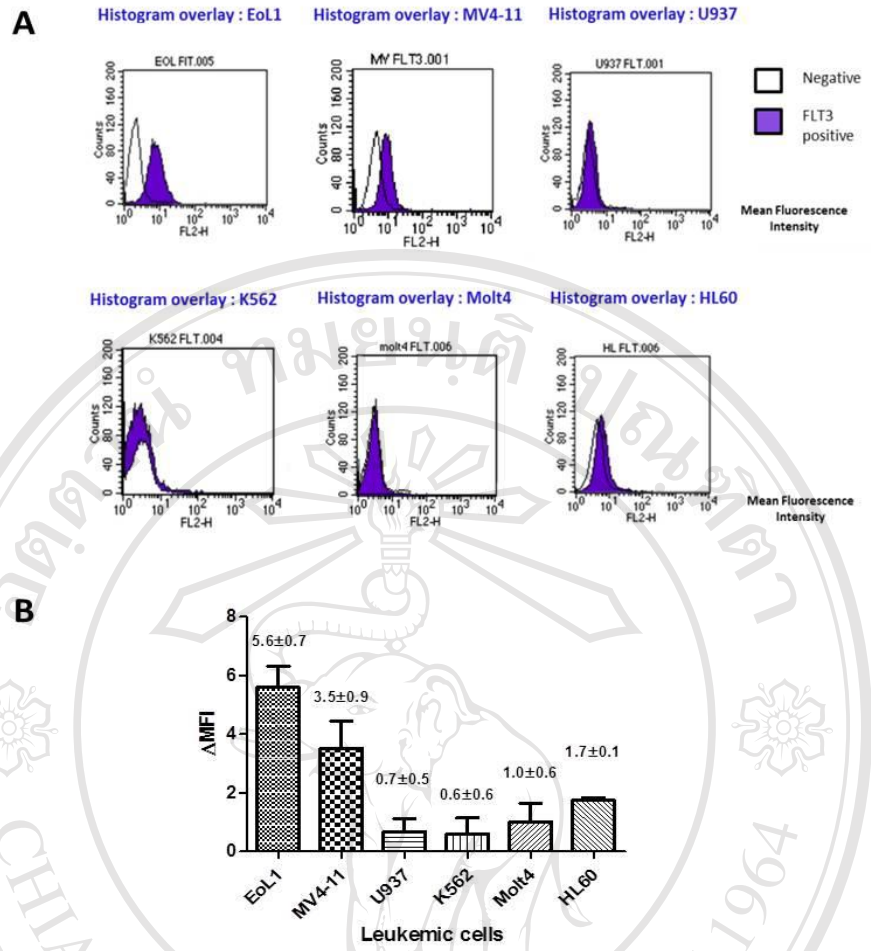

Figure 3.1 Representative flow cytometric profiles of EoL1, MV4-11, U937, K562, Molt4, and HL60 cells incubated with anti-FLT3 antibody.

(A) The data was shown as the overlaid histogram. Filled histograms represent the mean fluorescence intensity of FLT3; open histograms represent the mean fluorescence intensity of negative control.

(B) The Δ mean fluorescent intensity (ΔMFI) and error bars of SD were calculated from 3 independent experiments (n=3).

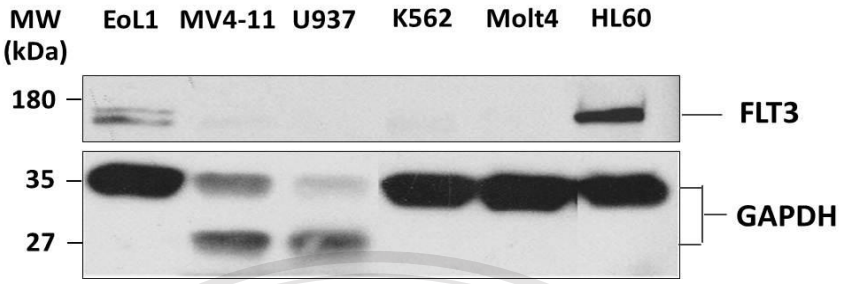

Figure 3.2 The FLT3 protein levels on leukemic cell lines was performed by Western blot analysis.

Protein lysates from leukemic cells were examined by Western blotting. After blotting, membranes were incubated with anti-FLT3 and anti-GAPDH antibodies (housekeeping gene) and then visualized using enhanced chemiluminescence detection. The results showed that HL60 and EoL1 cells expressed the FLT3 protein levels comparing to the other cells.

While EoL1 cells had shown the highest expression of FLT3 by flow cytometry, the results from Western blotting showed that HL60 cells expressed higher levels of FLT3 protein than EoL1 cells. Then the results of Western blotting were verified by separated protein extraction to obtain the membrane protein and cytoplasmic protein. Subsequently, the separated protein lysates were analyzed by Western blotting. The data demonstrated that cytoplasmic FLT3 protein level was higher than membrane FLT3 protein in HL60 cells as shown in Figure 3.3. Thus the highest FLT3 expression on HL60 cells was obtained from both cytoplasmic and membrane proteins by Western blotting due to the whole protein contribution. Therefore, a prominent FLT3 protein's band was observed from the HL60 cells comparing to the other cell lines.

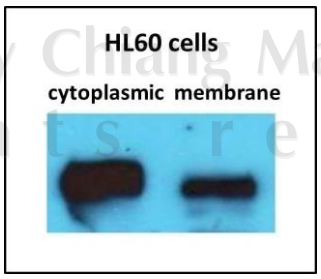

Figure 3.3 The cytoplasmic and membrane FLT3 protein expression by Western blotting on HL60 cell line.

Consequently, EoL1 cells were selected to use as a positive cell control because they presented a prominent expression of FLT3 when using flow cytometry and Western blot analysis, while K562 cells with the lowest degree of FLT3 level was chosen as a negative cell lines for setting the experimental model to study of FLT3 expression on leukemic cells. Moreover, supporting results from RT-PCR assay demonstrated the expression of FLT3 in EoL1 and K562 cells in line with FLT3 protein analysis as shown in Figure 3.4. EoL1 cells showed high level of FLT3 mRNA. However, K562 cells showed very low level. The agarose gel showing a single band of FLT3 (365 bp) and β actin band (247 bp) was obtained using specific primers against FLT3 and β actin, respectively. Then, EoL1 and K562 cells were used as cell lines model for optimization and validation of FLT3 protein levels determination using a flow cytometric method. In addition, the results from RT-PCR demonstrsted that MV4-11 cells showed a shift band of FLT3 above the 366 bp size of the wild-type FLT3 fragment (Figure 3.4). Therefore the FLT3 mutations were identified as bands migrating in MV4-11 cells.

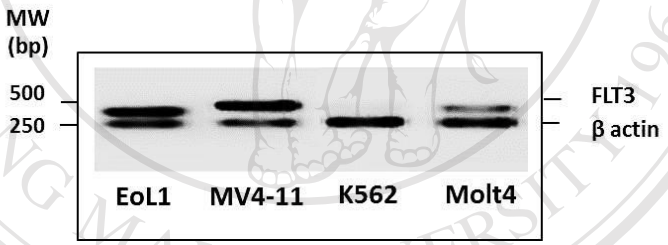

Figure 3.4 FLT3 mRNA levels of leukemic cell lines (EoL1, MV4-11, K562, and Molt4) using RT-PCR assay.

3.2 Development of flow cytometric method

3.2.1 Optimization of staining antibody concentration

EoL1 and K562 cells were selected for using as the positive and negative cell lines model. Optimal antibody concentration was titrated by reacting fixed number of EoL1 cells with the serial dilutions of anti-FLT3 antibody at the concentrations of 0.5, 1, and 2 µg per 100 µl of a staining volume. Samples were prepared in triplicates (n=3) and measured at three independent experiments

(N=3). The highest mean fluorescence intensity signal was obtained from 2 µg of anti FLT3 antibody at 7.48±0.50, followed by 1 and 0.5 µg at 6.69±0.57 and 5.33±0.31, respectively. The significant difference from the control level was shown with all three concentrations of anti-FLT3 antibody. However, when comparing between the concentrations of 1 µg/100 µl and 2 µg/100 µl, Δ mean fluorescence intensity value (ΔMFI was calculated as described in the section of statistical analysis) was slightly increased. Thus, in optimization of a staining antibody concentration, a 1 µg/100 µl of anti FLT3 antibody was selected for the method validation as shown in Figure 3.5.

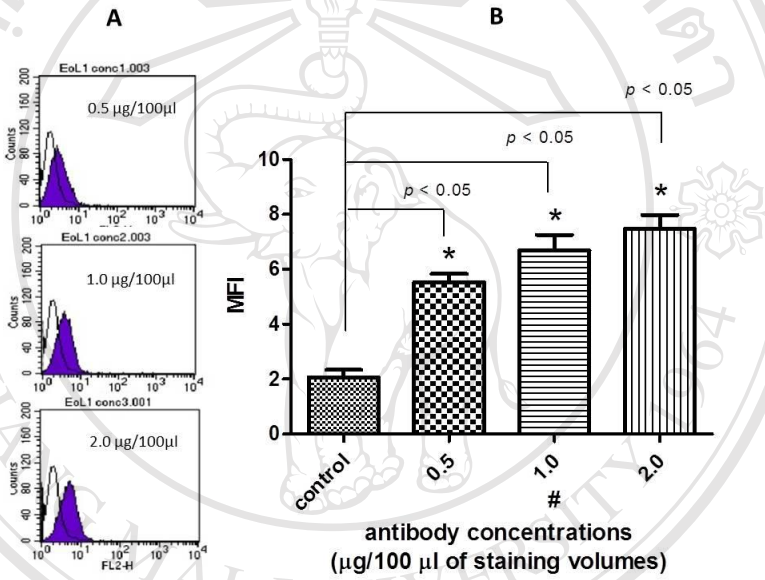

Figure 3.5 Optimization of primary antibody concentration.

(A) Filled histograms represent the mean fluorescence intensity of FLT3; open histograms represent the mean fluorescence intensity of negative control. The data is shown as the histogram overlay of negative control and the cells that were expressed FLT3.

(B) Data from a flow cytometer is shown as the mean fluorescence intensity (MFI) level ± error bars of SD (N=3). Optimal concentration has been marked by a number sign (#).

3.2.2 Optimization of cell concentration

After testing for an optimal anti-FLT3 antibody concentration, suitable cell density was determined to get an appropriated range of cell number in the reaction, to ensure a sufficient antibody for interacting with all expressed FLT3 protein on the cell surface. EoL1 cells, a positive control cell line, were prepared to yield the concentrations of $2.5x10^5$, $5x10^5$, $7.5x10^5$, and $10x10^5$ cells per 100 µl of staining volumes. The 1 µg/100 µl of anti FLT3 antibody, an optimal primary antibody was added and incubated as described in 2.3.1. After that the samples were analyzed using the flow cytometer. The Δ mean fluorescence intensity signals from the cell density of $2.5x10^5$, $5x10^5$, $7.5x10^5$, and $10x10^5$ cells/ml were 4.7±0.22, 5.0±0.09, 5.24±0.49, and 5.25±0.94, respectively. Cell concentration of $2.5x10^5$ cells/ml produced low levels of fluorescence intensity level. The Δ mean fluorescence intensity signals were increased by raising cell concentration except the concentration of $10x10^5$ cells/100 µl showed the saturated point which was started at the cell concentration of $7.5x10^5$ cells/100 µl. The aim of this part is for using in the FLT3 protein analytical method, the middle concentration in the analytical range was chosen to allow a sufficient interaction between FLT3 protein on cell surface and the selected antibody concentration. Therefore, the optimized cell concentration was $5x10^5$ cells/100 µl as shown in Figure 3.6.

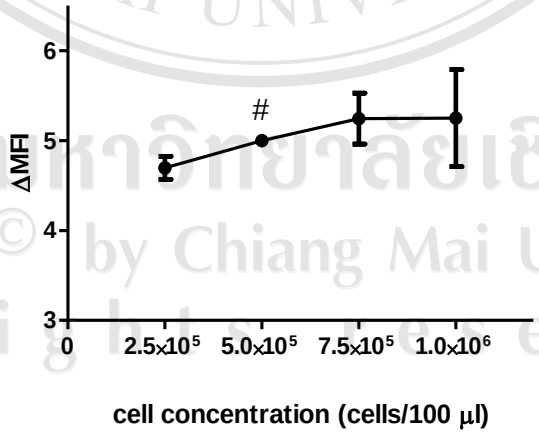

Figure 3.6 Optimization of cell concentration.

The data is shown as the Δ mean fluorescence intensity signals and error bars of SD (N=3). The optimized cell concentration was $5x10^5$ cells/100 µl and has been marked by a number sign (#).

3.2.3 Optimization of staining time

The incubation time was estimated to achieve an optimal staining time. Samples were prepared in triplicates (n=3) and measured at three independent experiments (N=3). The primary antibody reaction times at 15, 30, 45, and 60 min led to difference results, and there are shown in Figure 3.7. The Δ mean fluorescence intensity signals were 3.23±0.46, 3.47±0.46, 4.36±0.40, and 4.44±0.56 in 15, 30, 45, and 60 min of staining time, respectively (Figure 3.8). The incubation time at 15 min produced low level of the Δ mean fluorescence intensity while 60 min reaction was the highest of the Δ mean fluorescence intensity level. However, no difference was noticeable between 45 and 60 min reaction. Therefore, 45 min of antibody reaction was adopted in order to save time.

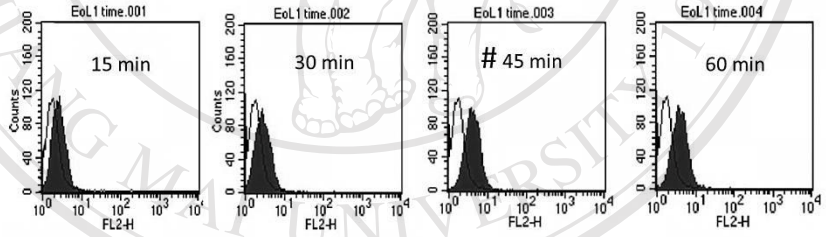

Figure 3.7 Histogram overlay of optimal staining time.

Filled histograms represent the mean fluorescence intensity of FLT3; open histograms represent the mean fluorescence intensity of negative control. For the above reported tests, the optimal conditions have been marked by a number sign (#). The data are representative of one experiment.

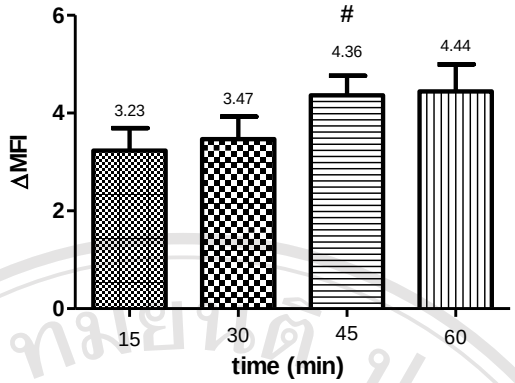

Figure 3.8 Variation of the primary antibody reaction time.

The Δ mean fluorescence intensity (ΔMFI) was calculated as described in the section of statistical analysis. Optimized staining time has been marked by a number sign (#). The results are shown as the ΔMFI and error bars of SD (N=3).

3.3 Validation of flow cytometry

After testing for optimal primary concentration, staining time, and number of cells by flow cytometer, the validation was examined with respect to calibration curve, linearity and range, precision, accuracy, lower limit of quantification and stability.

3.3.1 Calibration curve

EoL1 cells were used as a references standard, and K562 cells were a negative control. Mixture dilutions of EoL1 and K562 cells were freshly prepared with different ratio and they were serially diluted to yield six standard solutions of 20, 40, 60, 80, 100, and 120% of EoL1 cells (the optimal number of cells was $5x10^5$ cells). This was done in triplicate (n=3) and measured for six independent experiments (N=6). The Δ mean fluorescence intensity signals from the assay were plotted with serial dilution to generate a calibration curve. The results are indicated in Figure 3.9. The percentage coefficient of variation (%CV) as [(SD/average) x 100] was estimated for accepted criteria of calibration curve. The data showed that %CV at each concentration was less than 20% in all cases. Moreover, QC levels were prepared at 25, 50, and 75% of EoL1 cells for low, medium, and high concentrations, respectively. They were used to assess the abilities of the assay and would be performed for each validation run.

3.3.2 Linearity and range

The calibration curve was done in triplicate and repeated for six independent experiments then the linearity and range were estimated. In the range of linearity, the correlation and regression analyses showed a strongly linear correlation between the Δ mean fluorescence intensity from the assay by flow cytometer and serial dilution of positive cell line (EoL1) that can be represented of FLT3 levels. The range of the method was between 20 to 120% of EoL1 cell concentration. The linearity of assay was $y = 0.4027X + 2.73$ and $r^2 = 0.997$ (Figure 3.9).

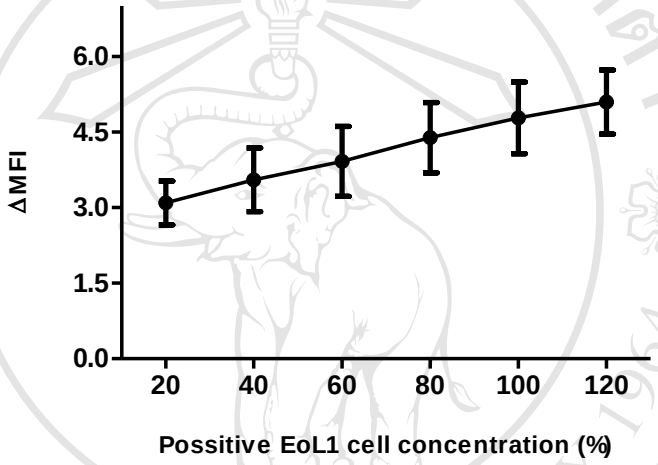

Figure 3.9 Calibration curve of flow cytometric analysis.

Serial dilutions of EoL1 and K562 cells with different ratio were prepared in PBS for creating a calibration curve. Samples were prepared in triplicates and measured at 6 independent experiments (n=6). The Δ mean fluorescence intensity (ΔMFI) was calculated as described in the section of statistical analysis.

3.3.3 Specificity

In bioanalytical method validation, specificity could be defined by how well the antibody recognizes the antigenic target. For high specificity of flow cytometric method, anti-FLT3 monoclonal antibody was used in this study. Moreover, EoL1 and K562 cells (positive and negative control) were determined in all experimental runs. In this study, the constant results of FLT3 protein levels

on EoL1 cells were the highest levels (ΔMFI=4.70±0.7) while K562 cells were very low levels (ΔMFI=0.59±0.5) in all validation experiments.

3.3.4 Precision

Precision is a measurement of the ability of the method to generate reproducible results. QC levels were used for assessing the ability of the assay to measure the biomarker of interest for precision experiments. QC levels were prepared at 25, 50, and 75% of EoL1 (the optimal number of cells was $5x10^5$ cells) for low, medium and high concentration, respectively. Each run of the assay was performed on 3 separated occasions and assessed by 3 replicates of QC samples at each concentration within the same day (intra-assay repeatability) and on 6 independent experiments (inter-day variation). The precision is expressed as percentage coefficient of variation (%CV). In order to calculate the precision, the average, standard deviation (SD) and %CV were determined.

The intra-days precision at 3 QC levels concentrations of EoL1 for 25, 50, and 75% were 3.35, 3.36, and 5.59, respectively. For the inter-days precision, the percentage CV values were 11.07, 14.02, and 14.47 for 25, 50, and 75% of EoL1 concentrations, respectively. The pooled repeatability and inter-days precision were 4.1 and 15.25, respectively. The percentage CV levels of intra-day and inter-day precision were less than 20% in all cases. The results are indicated in Table 3.1.

Table 3.1 Intra-day and inter-day precision.

QCs levels	Intra-day precision		Inter-day precision	
(%EoL1)	ΔMFI ± SD	%CV	ΔMFI ± SD	%CV
25	3.03 ± 0.10	3.35	3.18 ± 0.35	11.07
50	3.88 ± 0.13	3.36	3.84 ± 0.54	14.02
75	4.39 ± 0.25	5.59	4.20 ± 0.61	14.47

The Δ mean fluorescence intensity (ΔMFI) was calculated as described in the section of statistical analysis. Each run of the assay was performed on triplicates (n=3) of QC samples at each concentration within the same day (intra-assay repeatability) and on 6 independent experiments (inter-day variation).

3.3.5 Accuracy

The accuracy is assessed by the methodological recovery. Three QC levels (25, 50, and 75% of EoL1 cell concentrations) were used to determine the ability of the accuracy assay. The recovery of the method was calculated by comparing the determined concentration of spiked samples to the theoretical concentrations from a calibration curve. The percentage mean recovery for each QC level was calculated and reported with its standard deviation. The percentage recoveries of intra-day for QCs level at 25, 50, and 75% of EoL1 cell concentrations were 97.74±3.27, 106.39±3.58, and 104.54±5.83%, respectively. For the percentage recoveries of inter-day assay, their percentage recoveries were 99.85±7.76, 103.36±5.99, and 98.64±5.88%, respectively. The results are indicated in Table 3.2. The percentage mean recoveries for all concentration of QC levels were between 80 to 120%.

In addition, the percentage relative error (RE) was reported. The relative error was calculated by absolute error of the determined concentration and the theoretical concentrations from a calibration curve, and divided by the theoretical value. The percentage RE is the relative error expressed in terms of per 100. The data are shown in Table 3.2. The percentage RE of intra-day for QCs levels at 25, 50, and 75% of EoL1 cell concentrations were 2.69±2.74, 6.39±3.58, and 4.84±5.45%, respectively. For the inter-day assay, percentage RE were 5.46±5.36, 5.52±3.94, and 4.18±4.24%, respectively. The percentage RE values of inter-day precision at each run were less than 15% in all cases.

Table 3.2 The percentage recovery and percentage relative error of intra-day and inter-day assay.

QCs levels	Intra-day		Inter-day	
(%EoL1)	%recovery ± SD	%RE ± SD	%recovery ± SD	%RE ± SD
25	97.74 ± 3.27	2.69±2.74	99.85 ± 7.76	5.46±5.36
50	106.39 ± 3.58	6.39±3.58	103.36 ± 5.99	5.52±3.94
75	104.54 ± 5.83	4.84±5.45	98.64 ± 5.88	4.18±4.24

Each assay was performed on triplicates of QC levels at each concentration within the same day (intra-day assay) and on 6 independent days (inter-day variation).

3.3.6 Sensitivity or lower limits of quantification

The validation experiment established the working range defined by a calibration curve, whereby the sensitivity of the method based on the lower limits of quantification (LLOQ). The lowest concentration of EoL1 cells can be quantitatively determined with acceptable precision and accuracy. The lower limit of quantification was found to be 10% of EoL1 cell concentration. Each run of the assay was performed on triplicates for 6 independents experiments. The percentage CV value of precision was 14.93%, and their percentage CV values at each run were less than 15% in all cases. For acceptable accuracy assay, the percentage recovery and RE values were determined and reported with SD. The percentage recovery assay was 99.67±13.71% and the percentage RE was 10.66±8.23%.

3.3.7 Cell stability after fluorescence staining

Stability testing defines the length of times the sample is suitable for analysis. The stability of an analyte in a given matrix under specific conditions should be determined to identify method stability. QC samples are used to assess the stability of the assay. QC levels were prepared at 25, 50, and 75% of EoL1 cell concentration for low, medium and high concentrations, respectively. They

were stored in 2-8°C for waiting to analyze. Each run of the assay was performed on day 0, 1, 2, 7, and 14. The stability was monitored by percentage coefficient of variation (%CV). The percentage CVs were less than 10% in all cases, and the data are demonstrated in Table 3.3.

Table 3.3 The percentage coefficient of variation (%CV) of QC levels on day 0, 1, 2, 7 and 14.

QC levels	Percentage coefficient of variation (%CV)				
	Day 0	Day 1	Day 2	Day 7	Day 14
25%	2.50	6.03	3.82	6.53	4.20
50%	5.79	6.75	2.31	1.49	4.31
75%	4.73	3.70	3.79	5.60	3.21

The percentage CVs of QC samples were 5.90, 7.77, and 5.85 for 25, 50, and 75% of EoL1 cell concentrations, respectively. The results are indicated in Table 3.4. The sample was suitable for analysis within two weeks under temperature of 2-8°C in the assay matrix.

Table 3.4 The Δ mean fluorescence intensity (ΔMFI) and percentage coefficient of variation (%CV) of QC levels in stability assay.

QCs level	The pooled value	
(%EoL1)	ΔMFI ± SD	%CV
25	3.07±0.18	5.90
50	3.79±0.29	7.77
75	4.05±0.24	5.85

QC levels were measured on day 0, 1, 2, 7 and 14. The percentage CVs were shown as the pooled value.

3.4 Application for detecting expression of FLT3 on leukemic cells

After optimization and validation of flow cytometry, 21 bone marrows including AML (n=11) and ALL (n=10) were analyzed to detect the expression of FLT3. Two methods; flow cytometry and Western blot analysis were included in the determination

of FLT3 protein expression in each sample. Different principles and techniques of these two methods were employed to verify each other and EoL1 cells were used as the positive control. Optimized protocol of flow cytometry was applied to analyze the expression of FLT3 protein level on leukemic cells which obtained from leukemia patients. The overlaid histograms were shown in Figure 3.10.

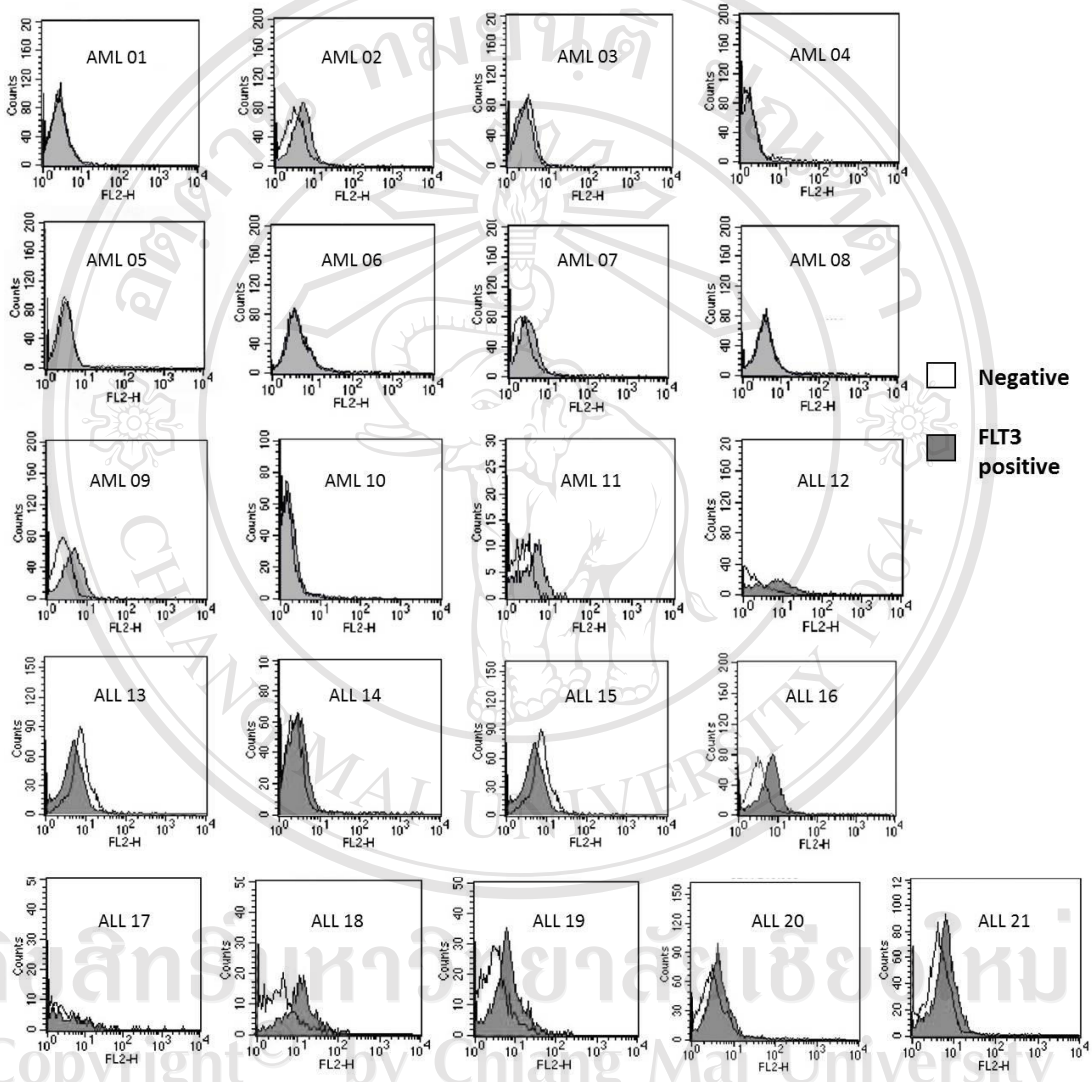

Figure 3.10 The histogram overlay of the expression of FLT3 protein on leukemic cells

The leukemic cell pellets ($5x10^5$ cells) were incubated with the optimal anti-FLT3 antibody concentration for 45 min. The data were shown as the histogram overlay of negative control and the cell that were reacted with anti-FLT3 antibody on each sample

in one experiment. Filled histograms represent the mean fluorescence intensity of FLT3; open histograms represent the mean fluorescence intensity of negative control.

Western blot analysis was used for determining FLT3 expression for supporting the results from the flow cytometry. The percentage relative FLT3 levels in both flow cytometry and Western blotting were calculated and compared to the percentage FLT3 expression of EoL1 cells as positive control. The results of flow cytometry and Western blotting were shown in Figure 3.11. Interestingly, FLT3 protein expression which measured by both methods were correlated in the same direction in the cases which expressed high FLT3 protein level. However in Western blotting, the FLT3 protein expression could not detect in many cases of patient samples while positive and negative cell lines showed the expected results. Despite the experiment of Western blotting was repeated and optimized the conditions of analysis for FLT3 protein detection on patient samples, the results were not effective.

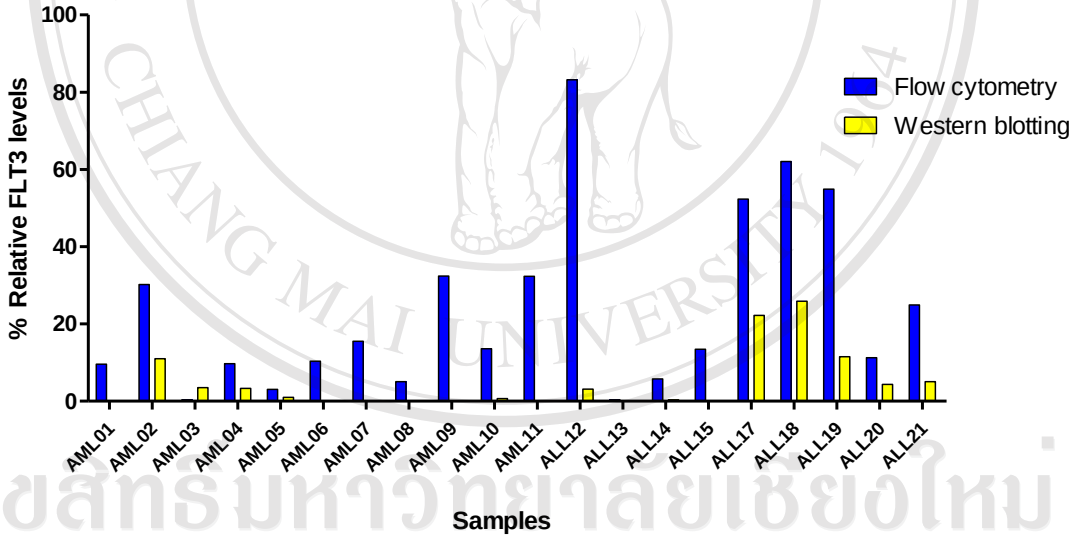

Figure 3.11 The expression of FLT3 protein in leukemic cells obtained from leukemia patient's samples using flow cytometry and Western blotting.

FLT3 expressions were assessed by the percentages of relative FLT3 protein levels which were calculated as compared to EoL1 cells (positive control) in both methods. Blue bars represent the % relative FLT3 levels using flow cytometry; yellow bars represent the % relative FLT3 levels using Western blotting.

After Western blot analysis was not successful to measure FLT3 protein expression in the leukemia patient's specimens, the expression of *FLT3* gene in some cases of samples were examined using RT-PCR assay. EoL1 and K562 cells were also used as positive and negative control, respectively. Moreover, MV4-11 cells were used to identify FLT3 mutations (FLT3-ITD). The results showed that the samples including AML02; AML05; AML09; AML11 and ALL15 had high levels of FLT3 mRNAs. The data are represented in Figure 3.12.

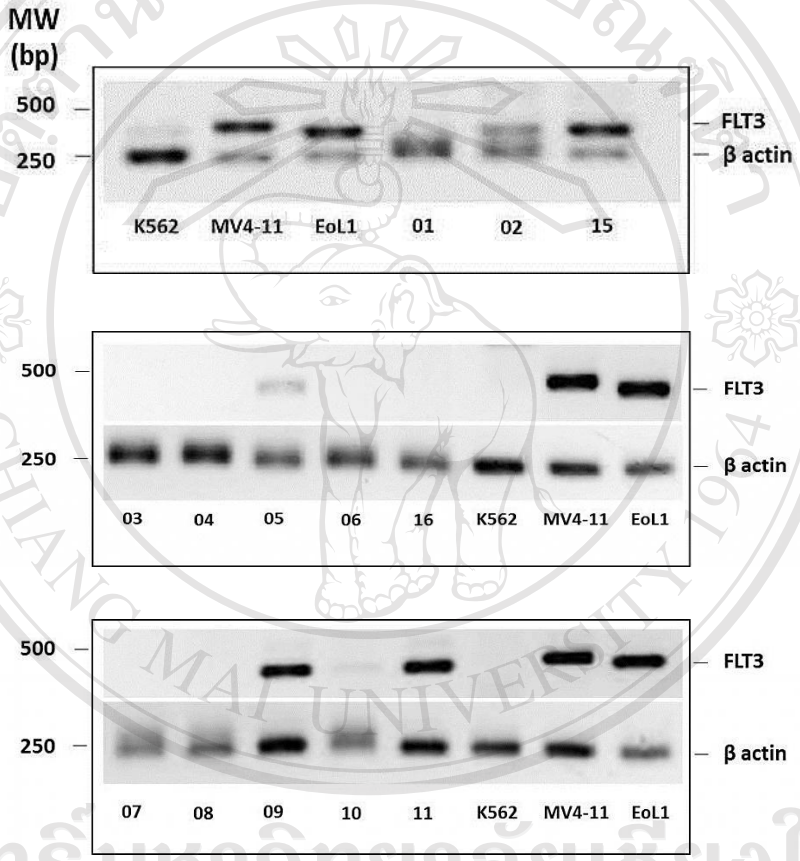

Figure 3.12 The levels of FLT3 mRNAs in leukemic cells using RT-PCR

The RT-PCR products were analyzed by agarose gel electrophoresis. The samples including AML01-11, ALL15-16, K562, and MV4-11, and EoL1 cells were analyzed to evaluate FLT3 mRNA levels. The FLT3 wild type, FLT3 mutant type (FLT3-ITD), and β actin bands showed at 366, >366, and 247 bp, respectively.

Moreover, the results of 12 leukemia patient's samples which were detected for FLT3 protein levels by flow cytometry and Western blotting were compared to RT-PCR

as shown in Figure 3.13. The results from RT-PCR assay demonstrated that all patient samples which had expressed the high levels of FLT3 protein correlated to the FLT3 mRNA levels. Nevertheless, some cases of samples were shown levels of FLT3 mRNA in different correlation.

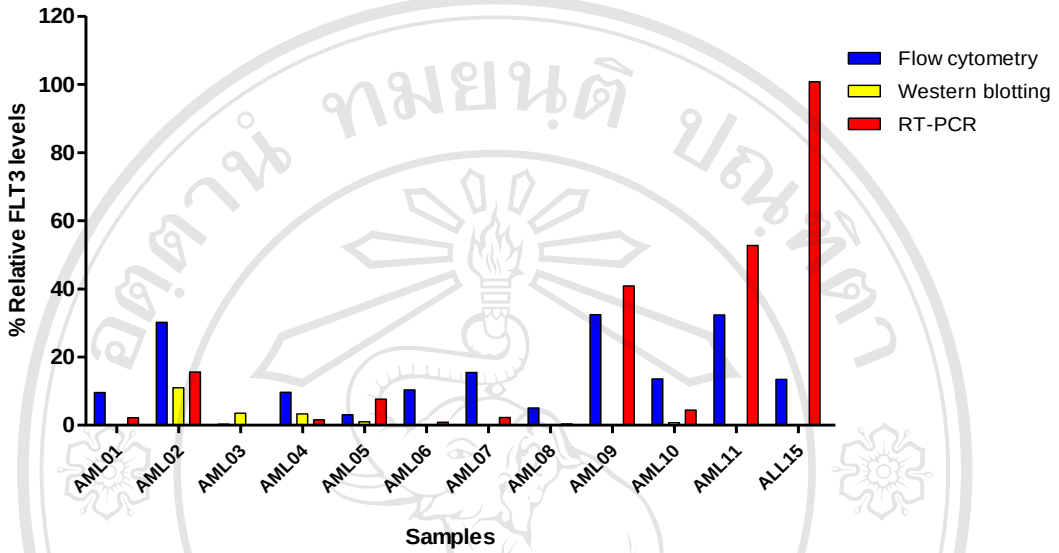

Figure 3.13 The percentages of relative FLT3 levels in leukemic cells obtained from leukemia patient's samples were detected by flow cytometry, Western blotting, and RT-PCR.

FLT3 expressions were assessed by the percentages relative FLT3 levels which were calculated as compared to EoL1 cells as positive control. Blue bars represent the % relative FLT3 levels by using flow cytometry; yellow bars represent the % relative FLT3 levels by using Western blotting and red bars represent the % relative FLT3 levels by using RT-PCR.