CHAPTER 2

Materials and Methods

2.1 Chemicals and reagents

The details concerning chemicals and reagents used in this study are shown in Appendix.

2.2 Leukemic cell lines culture

MV4-11 (human monocytic leukemia) and EoL1 (eosinophilic leukemia) were kindly provided by Asst. Prof. Dr. Songyot Anuchapreeda, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. U937 (human monocytic leukemia) and Molt4 (human lymphoblastic leukemia) were kindly provided by Prof. Dr. Watchara Kasinrerk, Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. K562 (chronic myelocytic leukemia) was gift from Dr. Chaisuree Supawilai, Research Institute for Health Sciences, Chiang Mai, Thailand. HL60 (human promyelocytic leukemia) was provided by Prof. Dr. Haruo Sugiyama, Faculty of Medicine, Osaka University, Japan.

All cells were cultured in a humidified incubator at 37°C incubator with 5% CO_2. MV4-11 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM). All other cells including EoL1, Molt4, U937, HL60, and K562 cells were cultured in RPMI 1640 medium. Complete medium was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

2.3 Screening of FLT3 expression on leukemic cell lines

Six leukemic cell lines were used as model systems to optimize and validate the expression of FLT3. The expression of FLT3 protein were evaluated in EoL1, K562, MV4-11, Molt4, HL60, and U937 leukemic cell lines by flow cytometry and Western

blotting. Reverse transcription–polymerase chain reaction (RT-PCR) assay was used to evaluate FLT3 mRNA levels through creation of complementary DNA (cDNA) transcripts from RNA in each leukemic cell line.

2.3.1 Flow cytometric method

Flow cytometry provides a rapid quantitative analysis. Anti-FLT3 antibodies conjugated fluorescent dyes can bind specifically to FLT3 protein on cell surfaces. In this regard, Fluorescence-activated cell sorting calibur (FACSCalibur) flow cytometer (Becton Dickinson, NJ) and CELLQUEST^{TM} software were used for analysis of FLT3 protein expression. The flow rate was set at the position of low. The voltage, amps gain, and threshold were adjusted to ensure that the cells could be detected. Forward scatter (FSC) and side scatter (SSC) were collected in linear mode and fluorescence (FL2) in log mode. The criterion collection was set at 10,000 cells of event count. Acquisition of data could not start until the sample voltage became stable. In computer analysis, the cell population was selected from the FSC vs. SSC dot plot and FL2 histogram.

To analyze of FLT3 protein expression, leukemic cells were washed 3 times in phosphate-buffered saline (PBS). All cells were adjusted to be equal ($1x10^6$cells) in100 µl of staining volume and added with 25 µl of normal AB serum to block non–specific binding at 4°C for 8 min. Then, cells were reacted with 5 µl of anti-FLT3 monoclonal antibody R-PE (Invitrogen^{TM}, Carlsbad, CA) at 4°C for 45 min. Excess antibodies were removed by centrifugation at 3,000 rpm for 5 min, and washed 3 times with ice-cold 0.1% bovine serum albumin (BSA) in PBS. Finally, cells were fixed with 1% paraformaldehyde before analysis by flow cytometer.

2.3.2 Western blot analysis

1) Whole protein extraction

Cells were harvested and washed 3 times with PBS, pH 7.2. After that cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6,

150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors to block protein degradation for whole protein extraction. Cell suspension was vortexed every 10 min in an hour. Cell lysates were collected by centrifugation at 10,000 rpm for 10 min, at room temperature. The supernatant fraction containing whole protein was collected to a new sterile microcentrifuge tube. It was used for protein measurement. Whole protein lysates were kept at -20°C until analysis.

2) Measurement of protein concentration

The protein concentrations were measured by the Folin-Lowry method. The principle of this method is the combination between the reactions of copper II (Cu^{2+}) with the peptide bonds under alkaline conditions and the reduction of Folin-Ciocalteu reagent, a mixture of phosphotungstic acid and phosphomolybdic acid, to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic protein residues (tyrosine and tryptophan amino acid), obtained from the first reaction. The strong color of reduced Folin reagent results from the increase of protein concentration.

The stock solution of 1 mg/ml BSA protein was diluted with deionized distilled water to various concentrations for preparing the BSA protein standard curve, as shown in Table 2.1 to determine the protein concentration of unknown samples.

Table 2.1 Preparation of bovine serum albumin standard solution

BSA concentration (µg/ml)	Stock BSA 1mg/ml (µl)	Deionized distilled water (µl)
0	0	500
25	25	475
50	50	450
75	75	425
100	100	400
125	125	375
150	150	350
175	175	325
200	200	300

The protein sample concentration was compared to the bovine serum albumin (BSA) protein standard curve. Each protein sample was diluted with deionized distilled water (1:5 to 1:10) to achieve concentration within the linear range of standard curve. Then 2.5 ml of alkaline copper solution were added and mixed by using vortexing. After incubating at room temperature for 10 min, 250 µl of Folin-phenol reagent were added, mixed by vortex, and further incubated at room temperature for 30 min. Finally, the concentrations of standard and sample protein were determined by spectrophotometry at a wavelength of 750 nm.

3) Gel electrophoresis

SDS-PAGE was used to separate proteins according to their electrophoretic mobility, a function of length of polypeptide chain or molecular weight. The native form of protein, the fold into complex secondary, tertiary and quaternary structures of proteins is denatured by SDS. They also separate subunits from multimeric proteins by 2-

ME, which is a reducing agent to disrupt disulfide bonds through reduction. Moreover, SDS is an anionic detergent which applied a negative charge to each protein in proportion to its mass, giving a near uniform negative charge along the length of the polypeptide. Thus, when the protein was applied onto the SDS gel, the negatively charged protein could be moved toward the positively charged electrode at rate depending on its molecular weight. The distance of migration through the gel could be assumed to be directly related to only the size of the protein. Acrylamide is used to separate low and high molecular weight of proteins base on concentration. A low acrylamide concentration is used to separate high molecular weight of proteins, whereas a high acrylamide concentration is used to separate low molecular weight proteins.

In this study, SDS-PAGE was used for analyzing FLT3 protein and their molecular sizes. GAPDH (housekeeping protein) was used to quantify and normalize. The 7.5% separating gel monomer solution was prepared, and gel was allowed to polymerize for 20 min. After separating gel setting, the 4% stacking gel monomer solution was prepared and poured onto the top of the separating gel. A comb was inserted into the stacking gel solution, and the gel was allowed to polymerize for 15 min. After polymerization was complete, the comb was removed, and the wells were washed 3 times with deionized (DI) water. The gel was placed in the electrophoresis chamber and electrode buffer was added. Later, the pre-running step was performed at 100 volts for 30 min. After pre-running, protein samples were prepared by adding DI water to adjust volume of each sample to be equal and reducing buffer, and then all samples were loaded into well under electrode buffer. Electrophoresis was performed at 100 volts for 2 h 30 min. While proteins were running, a polyvinylidene fluoride (PVDF) was activated by absolute methanol for 5 min and soaked in transfer buffer with at 4˚C for 5 min. After that, the separated proteins

on the gel were transferred to the PVDF membrane in transfer buffer by using 30 volts of electricity overnight. After blotting, target proteins on the membrane were determined by Western blot analysis.

4) Western bloting for FLT3 and GAPDH protein detection

After blotting, the PVDF membrane with the transferred proteins was cut into 2 parts, which were used for the FLT3 protein (MW 130-160 kDa) and GAPDH protein (MW 37 kDa) detection. The membranes were shaken in PBS for 5 min to remove any residues of transfer buffer, and then incubated in 5% skim milk in PBS (blocking buffer) with shaking at room temperature for 2 h to blocking non-specific binding sites. After that the membranes were washed with 0.1% tween-PBS (washing buffer) every 5 min for 6 times. Each membrane was incubated with anti-mouse FLT3 extracellular domain (Upstate Biotechnology, Lake Placid, NY) with dilution of 1:500 to 1:1,000 at 4°C with shaking overnight and primary rabbit polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution for 2 h 30 min with shaking at 4°C. Membranes were washed 6 times for 5 min each with washing buffer to remove excess primary antibodies. The membranes were next incubated with anti-rabbit IgG HRP conjugate (Promega, Madision, WI) at a 1:10,000 dilution in blocking buffer for 1 h 30 min at room temperature. Then, the membranes were washed 6 times for 5 min each with washing buffer to remove excess antibodies. Finally, antibody-bound proteins were detected by enhanced chemiluminescence detection using Luminata™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA). The membranes were incubated in substrate solution for 5 min at room temperature, after that, the membranes were packed with clear thin plastic wrap. Each wrapped membrane was immediately placed onto a film cassette and then CL-XPosure ™ film (Thermo Scientific, Rockford, IL) was placed on the top of the

membrane in the appropriate time period for FLT3 (15-30 min) or GAPDH (2-5 min) detection. The film was removed from the cassette and the protein band signal was developed in the film developing solution for 1 min and then fixed in fixing solution for 1 min. Finally, the protein band signals were quantified by using Quantity One, Version 4.6.3 (BIO-RAD LABORATORIES, Hercules, CA).

2.3.3 RT-PCR for *FLT3* gene expression

1) Total RNA extraction

Before RNA extraction, leukemic cells were washed 3 times with ice-cold sterile PBS, pH 7.2 and then total RNA was extracted by TRIzol® Reagent (Invitrogen™, Carlsbad, CA). Cell pellets (5-10x10^6cells) were lyzed by 1ml of TRIzol® Reagent and homogenized with 30 strokes on ice bath by homogenizer. The supernatant was collected and centrifuged at 12,000 rpm for 10 min, then incubated at room temperature for 5 min. Chloroform (200 µl) was added, mixed and centrifuged at 12,000 rpm for 10 min to separate the solution into aqueous phase organic phases. The aqueous phase containing fraction RNA was transferred to fresh microcentrifuge tube, the RNA was recovered by precipitation with isopropyl alcohol (500 µl), followed by centrifugation at 12,000 rpm for 10 min. The RNA precipitate was formed a gel-like pellet on the bottom of the tube. After centrifugation, the supernatant was removed, and pelleted RNA was washed with 75% ethanol, adding at least 1 mL of 75% ethanol per 1 ml of TRIzol® Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at 7,500 rpm for 5 min. The end of this procedure, the RNA pellet was dried (air-dry or vacuum-dry) for 5-10 min. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. RNA was dissolved in RNase-free water (50 µl). Finally, 1 µl of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™, Carlsbad, CA) was added for

RNA protection. Total RNA was used directly in RT-PCR or stored at -70°C for later analysis.

2) Measurement of purity and concentration of total RNA

Total RNA was determined for purity by measuring optical density (OD) *via* spectrophotometry at 260 nm/280 nm ratio. The RNA concentration was calculated from the following formula. Partially dissolved RNA samples would have an λ260/λ280 ratio less than 1.6.

Total RNA (µg/mL) =

Absorbance at λ260 nm x 40 µg/mL* x Dilution factor

* 1 OD = 40 µg/mL

3) cDNA synthesis and PCR reaction

After determination of total RNA concentration, cDNA was synthesized using SuperScript™ III one-step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen™, Carlsbad, CA) according to the provided protocol. Briefly, a master mixture was prepared as described in Table 2.2. Amplification conditions were: cDNA synthesis and predenaturation: perform 1 cycle (60°C for 30 min, 94°C for 2 min); PCR amplification: perform 34 cycles (denature, 94°C for 1 min, anneal, 60°C for 1 min, extend 72°C for 1 min/kb); final extension: 1 cycle (72°C for 3 min) with set of primers as described in Table 2.3 [24].

Table 2.2 The preparation of multiple reactions for cDNA synthesis

Component	Volume
2X Reaction Mix	10 µl
Template RNA	(0.4 µg) X µl
Sense primer (FLT3, β actin)	(10 µM) 0.2 µl
Anti-sense primer (FLT3, β actin)	(10 µM) 0.2 µl
SuperScript III RT/ Platinum *Taq* Mix	0.8 µl
Autoclaved distilled water	q.s. to 20 µl

Table 2.3 The set of FLT3 and β-actin primers

FLT3	Sense	5′-TGTCGAGCAGTACTCTAAACA-3′
	Anti-sense	5′ATCCTAGTACCTTCCCAAACTC-3′
β-actin	Sense	5′-ACAGGAAGTCCCTTGCCATC-3′
	Anti-sense	5′-GGGAGACCAAAAGCCTTCATAC-3′

All reagents were mixed carefully and kept on ice until the PCR cycling had started. The tube was placed in a block cycler with a heated lid to minimize evaporation, and then the PCR cycling for cDNA synthesis was started. Finally, the reaction was stopped by placing the tube on ice. The cDNA was kept at -20°C until analysis.

A total of 5 µl of each PCR product was electrophoresed on a 1% agarose gel with ethidium bromide (0.1 µg/ml) and 0.5X TAE buffer at 100 volts for 2 h 30 min. The PCR product bands were quantitated using Quantity One, Version 4.6.3 (BIO-RAD LABORATORIES, Hercules, CA).

2.4 Development of flow cytometric method

After MV4-11, EoL1, Molt4, U937, HL60, and K562 cells were verified FLT3 expression. Leukemic cell lines which had the highest and the lowest expression of FLT3 was used as positive and negative cell lines to establish the experimental model

for flow cytometric method development. First, leukemic cell lines were analyzed by flow cytometer for evaluating FLT3 expression, cells were adjusted to $1x10^6$ cells per 100 µl of staining volumes, then reacted with 5 µl (1.75 µg) of anti-FLT3 monoclonal antibody R-PE (Invitrogen™, Carlsbad, CA) according to manufacturer's protocol. To develop a flow cytometric method, the pilot study was performed.

2.4.1 Optimization of staining antibody concentration

The amounts of anti-FLT3 antibody and leukemic cells that used in this study were reduced to save cost of analysis and use very small amounts of samples. For optimizing antibody concentrations, cells were washed 3 times with PBS and fixed to $5x10^5$ cells per 100 µl of staining volumes, then added with normal AB serum (25 µl) and incubated at 4°C for 8 min to block non–specific binding. After that, cells were reacted with series of anti-FLT3 monoclonal antibody concentrations including 0.5, 1.0, and 2.0 µg/100 µl. Excess antibodies were removed by centrifugation at 3,000 rpm for 5 min, and washed three times with cold 0.1% BSA in PBS. Finally, cells were fixed with 1% paraformaldehyde before analysis. All samples were prepared in triplicates and determined at 3 independent experiments using flow cytometer.

2.4.2 Optimization of cell concentration

In order to determine the range of cell number which is appropriate for optimum primary antibody concentration, a series of cells concentrations including $2.5x10^5$, $5x10^5$, $7.5x10^5$, and $10x10^5$cells/100 µl were prepared. Cells were washed 3 times with PBS and adjusted to $2.5x10^5$, $5x10^5$, $7.5x10^5$, and $10x10^5$cells per 100 µl of staining volumes, then normal AB serum (25 µl) was added and incubated at 4°C for 8 min to block non–specific binding. After that, cells were probed by an optimal anti-FLT3 monoclonal antibody concentration as described in the section 2.4.1. Excess antibodies were removed by centrifugation at 3,000 rpm for 5 min, and washed three times with ice-cold 0.1% BSA in PBS. Finally, cells were fixed with 1% paraformaldehyde before analysis. All samples were

prepared in triplicates and determined in three independent experiments using flow cytometer.

2.4.3 Optimization of staining time

Staining time was examined to reduce time of analysis. Different incubation times including 15, 30, 45, and 60 min were tested for their ability. Cells were washed 3 times with PBS and adjusted to optimal number of cells as described in the section 2.4.2 per in 100 µl of staining volumes, then added with normal AB serum (25 µl) and incubated at 4°C for 8 min to block non–specific binding. After that, cells were probed with an optimal anti-FLT3 monoclonal antibody concentration according to section 2.4.1 for 15, 30, 45, and 60 min. Excess antibodies were removed by centrifugation at 3,000 rpm for 5 min, and washed three times with cold 0.1% BSA in PBS. Finally, cells were fixed with 1% paraformaldehyde before analysis. All samples were prepared in triplicates and determined at three independent experiments using flow cytometer. The optimal staining time bases on the highest degree of Δ mean fluorescence intensity value which shows in time saving.

2.5 Method validation of flow cytometric method

The developed method was validated as per the guidance document with respect to calibration curve, linearity and range, precision, accuracy, limit of quantification and stability [33, 34, 124-128].

2.5.1 Calibration curve

The calibration curve was used for the reliable assay evaluation. Serial dilutions of positive and negative cell lines with different ratio including 10, 20, 40, 60, 80, 100, and 120% of positive cell lines were prepared in 100 µl of PBS for a calibration curve production. Each dilution was prepared in triplicates and determined at 6 independent experiments. The expression of FLT3 protein was analyzed in all dilutions by flow cytometry as described in section 2.3.1. The Δ mean fluorescence intensity

signals from the assay were plotted with serial dilution to generate a calibration curve. The percentage coefficient of variation (%CV) as [(SD/average) x 100] was estimated for accepted criteria of calibration curve. The %CV at each concentration is not more than 20%. Moreover, QC levels were prepared at 25, 50, and 75% of positive cell lines for low, medium and high concentration, respectively. They were used to assess the reliability of the assay and would be performed for each validation run.

2.5.2 Linearity and range

The linearity is its ability to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The range is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. After 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 were analyzed. The linearity should be closed to 1 for good linearity ($r^2 > 0.995$).

2.5.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. In bio-analytical method validation, specificity could be defined by how well the antibody recognizes the antigenic target. For high specificity method, anti-FLT3 monoclonal antibody was used. Moreover, EoL1 and K562 cells (positive and negative control) were determined in all experiment runs for checking the specificity of this method.

2.5.4 Precision

Precision is a measure of the reliability of the method to generate reproducible results. QC levels at 25, 50, and 75% of positive cells at the optimal number of cells for low, medium and high concentration were used

for precision experiments. Each run of the assay was performed on three separated occasions and assessed by three replicates of QC samples at each concentration within the same day (intra- assay repeatability) and on six independent experiments (inter-day variation). The precision is expressed as percentage coefficient of variation (%CV). The %CV at each concentration is not more than 20% for accepted criteria.

$$\text{The \% CV} = [(\text{SD/average}) \times 100]$$

2.5.5 Accuracy

The accuracy is assessed by the methodological recovery. QC levels (25, 50, and 75% of positive cell concentration) were determined for 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 % recovery =

[(ΔMFI of QC level recovered/ ΔMFI of QC level used) x 100]

The percentage mean recovery of intra-day and inter-day assay for each QC level was calculated and reported with its standard deviation. The percentage mean recoveries for all concentrations of QC levels should be between 80 to 120%.

Moreover, the percentage relative error (%RE) was also calculated. 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 %RE is the relative error expressed in terms of per 100.

$$\text{The \%RE} = \left| \frac{\Delta\text{MFI of QC level found - } \Delta\text{MFI of QC level theory) x 100}}{\Delta\text{MFI of QC level theory}} \right|$$

The %RE of intra-day and inter-day precision at each run were not more than 20%.

2.5.6 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). Dilution of 10% of positive cells concentration was determined on calibration curve. Each run of the assay was performed on triplicates for six independents experiments. The lowest concentration of positive cells can be quantitatively determined with acceptable precision and accuracy for accepted criteria as described in section 2.5.4 and 2.5.5.

2.5.7 Cell stability after fluorescence staining

Cell stability testing defines as period of times that 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 levels at 25, 50, and 75% of positive cells were used to assess the stability of assay. Samples were stored in 2-8°C for waiting to analyze on day 0, 1, 2, 7, and 14. The stability was monitored by percentage CV (%CV). The %CV of stability should be less than 20%.

2.6 Application for detecting expression of FLT3 in patients

After optimization and validation of flow cytometry, 21 bone marrows including AML (n=11) and ALL (n=10) were included in this study, and were analyzed to detect the expression of FLT3. This study was approved by Research Ethics Committee (No.107/2013), the Faculty of Medicine, Chiang Mai University. Patient leukemic cell samples were collected by centrifugation at 3,500 rpm, for 5 min and lyzed red blood cells by hypotonic solution (0.083% NH_4Cl) for 8 min. Then the leukemic cell pellets were washed by sterile PBS for 3 times. Leukemic cells were divided into two parts for flow cytometry and Western blotting.

The developed flow cytometric method in section 2.4 was applied for FLT3 determination in leukemic cell samples and compared to Western blotting. The optimal leukemic cell number (section 2.4.2) was blocked by 25 µl of normal AB serum at 4°C

for 8 min and incubated with the optimal anti-FLT3 antibody concentration (section 2.4.1) in optimal incubation time (section 2.4.3). Excess antibodies were removed by centrifugation at 3,000 rpm for 5 min, and washed three times with ice-cold 0.1% BSA in PBS. Finally, cells were fixed with 1% paraformaldehyde before analysis. All samples were prepared in triplicates and determined using flow cytometer.

Western blot analysis was also used for FLT3 protein level determination on each sample. Cells (5-10x10^7 cells) were extracted using RIPA buffer and measured protein concentrations by the Folin-Lowry method. The equal amount of protein samples were determined by Western blotting as described in section 2.3.2

Furthermore, RT-PCR was used for FLT3 mRNA level determination. Cell pellets (5-10x10^7 cells) were lyzed and extracted the total RNAs. RT-PCR was performed using 1 µg of total RNA with primers for amplification as described in section 2.3.3. Then PCR products were electrophoresed on 1% w/v of agarose gel.

2.7 Statistical analysis

For quantifications the averages of three independent experiments and error bars showing standard deviations (SD) were used. The Δ Mean fluorescence intensity (Δ MFI) was calculated subtracting the value of the mean fluorescence intensity (MFI) of the negative events (MFI of cells alone without primary antibody) from that of positive events (MFI of cells reacted with primary antibody). Statistical evaluation of data was performed using an Analysis of Variance (one-way ANOVA). Newman–Keuls post-hoc test was used to assess where the significance of differences occurred, and a value of $p < 0.05$ was accepted as the level of significance.