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RESEARCH ARTICLE

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FLT3, a prognostic biomarker for acute myeloid leukemia (AML): Quantitative monitoring with a simple anti-FLT3 interaction and flow cytometric method

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Background: Overexpression of fms-like tyrosine kinase 3 (FLT3) protein in leukemia is highly related to poor prognosis and reduced survival rate in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) patients. Simple but efficient quantification of FLT3 protein levels on the leukemic cell surface using flow cytometry had been developed for rapid determination of FLT3 on intact cell surface.

Methods: Quantitation protocol for FLT3 biomarker in clinical samples was developed and validated. Cell model selection for calibration curve construction was identified and evaluated. Selected antibody concentrations, cell density, and incubation time were evaluated for most appropriate conditions. Comparison of the developed FLT3 determination protocol with the conventional Western blot analysis was performed.

Results: EoL-1 cell line was selected for using as positive control cells. Calibration curve (20%-120% of FLT3 positive cells) and quality control (QC) levels were constructed and evaluated. The results demonstrated good linearity ($r^2 > 0.99$). The intra- and inter-day precision and accuracy, expressed as the coefficient of variation (%CV) and % recovery, were <20% and fell in 80%-120% in all cases. When compared with Western blotting results, FLT3 protein expression levels in leukemia patient's bone marrow samples were demonstrated in the same trend.

Conclusions: The effective, reliable, rapid, and economical analytical technique using the developed flow cytometric method was demonstrated for FLT3 protein determination on leukemic cell surface. This method provided a practical analysis of FLT-3 biomarker levels which is valuable for physician decision in acute leukemia treatment.

KEYWORDS
acute myeloid leukemia, flow cytometry, FLT3, leukemic cells, validation

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1 | INTRODUCTION

FMS-like receptor tyrosine kinase 3 (FLT3) belongs to the group of class III receptor tyrosine kinase family. This membrane-bound receptor comprises an intrinsic tyrosine kinase domain that promotes proliferation, survival, and differentiation of the early human hematopoietic precursor: stem cell.[1] In normal humans, CD34+ cells from bone marrow express low levels of FLT3.[2] This protein is also found in human leukemia, nearly 100% of B-lineage ALLs, 92% of AMLs, and 27% of T-ALLs.[3] Moreover, the overexpression of the FLT3 protein is usually found in leukemic blast cells of AML patients, and even in many cases of AML patients possessing an FLT3 mutation. The relationship between levels of FLT3 expression and mutation is an implication of poor prognosis of AML patients. The common mutation in approximately 25%-30% of patients with AML was reported to have the mutation at the internal tandem duplication (ITD), so called FLT3-ITD.[4] However, a related investigation reported the overexpression of the FLT3 protein could trigger downstream signaling cascades, resulting in apoptosis suppression, activation of the wild-type receptor in malignant cells and dysregulated cell proliferation. This is why patients with high percentages of bone marrow blasts and high leukocyte counts showed a high percentage of FLT3 on their cell surface.[5] At the molecular level, no differences in FLT3 expression levels were observed between AML with and without any FLT3 mutation.[6] Therefore, determining the wild-type FLT3 level may have greater validity as a prognostic biomarker than using only mutated FLT3 protein detection. In this study, the aberrant FLT3 protein on leukemic patient cell surfaces as one of the routine diagnoses that could be accomplished using the simple, rapid, and reliable screening flow cytometric method. The specific flow cytometry results can distinguish phenotypic differentiation of leukemia using a panel of dye-tagged antibodies with greater speed than Western blot analysis and also can identify aberrant marker expression on abnormal cells as well as FLT3 overexpressing cells. Thus, this study specifically focuses on optimizing and validating flow cytometry to detect FLT3 protein levels using the leukemic cell line model to conduct the performance, reliability, and economic analysis to detect FLT3 expression.

2 | MATERIALS AND METHODS

2.1 | Leukemic cell culture

All leukemic cells were cultured in a humidified incubator at 37°C with 5% CO_2. MV4-11 (human monocytic leukemia) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM). EoL-1 (human eosinophilic leukemia), Molt4 (human lymphoblastic leukemia), U937 (human monocytic leukemia), HL60 (human promyelocytic leukemia), and K562 (human chronic myelocytic leukemia) cells were cultured in RPMI 1640 medium. Peripheral blood mononuclear cells (normal PBMCs) were isolated and seeded in RPMI 1640 medium. The medium was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cell culture reagents were purchased from Invitrogen™ (Carlsbad, CA, USA).

2.2 | Analysis of FLT3 expression with flow cytometry

FLT3 protein expression on cell surfaces was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with CELLQUEST™ software. Briefly, cells were prepared or collected and washed three times in phosphate buffer saline (PBS, pH 7.4) and adjusted to be equal (100 μL) in a staining volume, and then added normal AB serum to block nonspecific binding. After that, cells were reacted with anti-FLT3 monoclonal antibody conjugated with R-phycoerythrin (R-PE, Invitrogen™). Excess antibodies were removed by washing with an ice-cold 0.1% BSA in PBS. Finally, the labeled cells were then fixed with 1% paraformaldehyde solution and analyzed by flow cytometry using a FACSCalibur flow cytometer. The flow rate was set at the position of low. The voltage, amp gain, and threshold were adjusted to ensure that the cells could be appropriately detected. Control and sample cells were collected and adjusted to contain at least 5×10^5 cells each sample. Forward scatter (FSC) and side scatter (SSC) were collected in linear mode and fluorescence (FL2) in log mode. The collection criteria were set at 10 000 cells per an event count. In computer analysis, the cell population was selected from the FSC vs SSC dot plot and FL2 histogram.

To obtain reliable data, the flow cytometer calibration was regularly performed. Service and preventive maintenance were performed by a PCL-Holding (Bangkok, Thailand) service team every 3 months according to the instruction in the service maintenance handbook. Cleaning, lubricant, and leakage check were performed to prevent sample clogging, rust, and system leakage. The important parameters were checked. The sheath pressure was adjusted in the range of 4.00 ± 0.05 PSI. The laser power was 15.0 ± 0.1 mW. The sample event was adjusted for 150-250 event/s. Other parameters, including sample pressure and optical alignment, were also adjusted. In addition, daily startup procedures were performed before running the samples. Moreover, quality control materials were used for each QC process. The Flow-Check™ Fluoropheres were used for fluidics verification and laser alignment. The Flow-Set™ Fluorospheres were used for high voltage adjustment. The Immuno-Brite™ Fluoropheres were used to monitor the instrument linearity, and the absolute count was performed using the Flow-Count™ Fluorospheres.

2.3 | Immunoblotting for FLT3 protein expression

To select the cell lines model, immunoblotting assay was used to determine the level of FLT3 for each cell type. Cells (10×10^6 cells) were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors for whole protein extraction. The protein concentrations were measured using the Folin-Lowry method standardized with

BSA. Proteins were separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was incubated with anti-mouse FLT3 extracellular domain (Upstate Biotechnology, Lake Placid, NY, USA) or anti-GAPDH antibody (Santa Cruz, CA, USA) with dilutions of 1:500 and 1:1000, respectively. The secondary antibodies were goat-anti-mouse or rabbit-horseradish perdoxidase (Invitrogen™) with the dilution of 1:10 000. Protein bands were visualized using Luminata™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, USA) and quantified using Quantity One version 4.6.3 (BIO-RAD, Hercules, CA, USA).

2.4 | Optimization of flow cytometric method

Our method followed from our previous short report, leukemic cell line model selection experiments.[7] Briefly, leukemic cell lines including MV4-11, EoL-1, Molt4, U937, HL60, and K562 were verified for FLT3 expression levels by flow cytometry and Western blotting assay. Then, appropriate cell lines with high and low expressions of FLT3 protein were selected as positive and negative cell lines for constructing the FLT3 calibration curve. This cell model was titrated to serve as the calibrated FLT3 expression samples requiring reduced cell concentrations resulting in diminished primary antibody concentrations and incubation times. Dilutions of antibody concentrations (0.5, 1.0, and 2.0 μg/100 μL), a series of cells concentrations (2.5×10^5, 5×10^5, 7.5×10^5, and 1.0×10^6 cells/100 μL), and incubation times (15, 30, 45, and 60 minutes) were optimized. The optimal conditions were selected based on a high degree of Δ mean fluorescence intensity value (ΔMFI) that was produced upon changing cell concentrations and also considered cost effective and timely. All samples were prepared in triplicates and determined at three different times using a flow cytometer.

2.5 | Method validation

The developed method was validated using guideline documents or recommendations for the development of an analytical method for biomarker determination.[8-13]

Appropriate validation parameters were evaluated. Positive cells (EoL-1) at different ratios to negative cells (K562), 10%, 20%, 40%, 60%, 80%, 100%, and 120%, were prepared in PBS buffer pH 7.4 to construct the calibration curve. Quality control (QC) levels at 25%, 50%, and 75% of a positive cell line for low, medium, and high concentrations, respectively, were used for precise, accurate, and stabilized method evaluation. Samples were prepared in triplicate and determined at six independent experiments.

2.6 | Application of the method to detect expression of FLT3 among leukemic patients

After optimization and validation of the developed analytical protocol by flow cytometry, three bone marrow samples from patients with newly diagnosed and untreated acute leukemia were included in this study to detect the expression of FLT3. Bone marrow specimens were collected from pre-diagnosed leukemic patients in heparinized tubes. The samples were prepared within 6 hours for triplicate analyses by centrifugation at 480 *g* and performing RBC lysis with hypotonic solution (0.083% NH_4Cl) for 8 minutes then washing the leukemic cell pellets with PBS (three times). When red cells were still present, the lysis process was repeated. Then, leukemic cell pellets were resuspended in PBS and divided into two parts for flow cytometry and Western blot analyses. Western blotting was repeated at least three times and one representative experiment was presented. This study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, where the guidelines are conformed with the Declaration of Helsinki.

2.7 | Statistical analysis

Data were collected as the difference in mean fluorescence intensity (ΔMFI) by subtracting the MFI value of the negative events (MFI of cells alone without primary antibody) from that of positive events (MFI of cells reacted with primary antibody). For quantification, the averages of three to six medians obtained from independent experiments and error bars showing standard deviations (SD) were calculated. Each sample was measured in triplicate. Statistical evaluation of data was performed using analysis of variance (one-way ANOVA). Newman-Keuls post hoc test was used to assess the interaction of significant difference, and a value of $P < 0.05$ was accepted as the level of significance.

3 | RESULTS

3.1 | FLT3 expression on leukemic cell lines

To complete the report, data from our previous study were included. The representative flow cytometry profiles are shown in the overlaid histogram (Figure S1). EoL-1 cells expressed a prominent degree of FLT3 protein on cell surfaces with the ΔMFI of 5.60 ± 0.72, compared to MV4-11, HL60, K562, Molt4, and U937 cells with 3.53 ± 0.93, 1.74 ± 0.10, 0.59 ± 0.57, 1.00 ± 0.64, and 0.66 ± 0.46, respectively. The immunoblotting assay showed that EoL-1 and HL60 cells expressed high levels of FLT3 protein compared with the other cells, while K562 cells showed the lowest level of FLT3 expression. Similarly to the K562, no different FLT3 levels from the negative control was observed from PBMCs (n = 3). Supporting the results from the flow cytometry, EoL-1 and K562 cells were selected as positive and negative cell lines, respectively, to design the model to study FLT3 expression on leukemic cells.

3.2 | Optimization of staining antibody concentration

EoL-1 cell as a positive control was used to determine the level of FLT3 protein expression, and the optimal antibody concentration was achieved by reacting fixed cells (5×10^5 cells) with serial anti-FLT3 antibody concentrations of 0.5, 1.0, and 2.0 μg in 100-μL

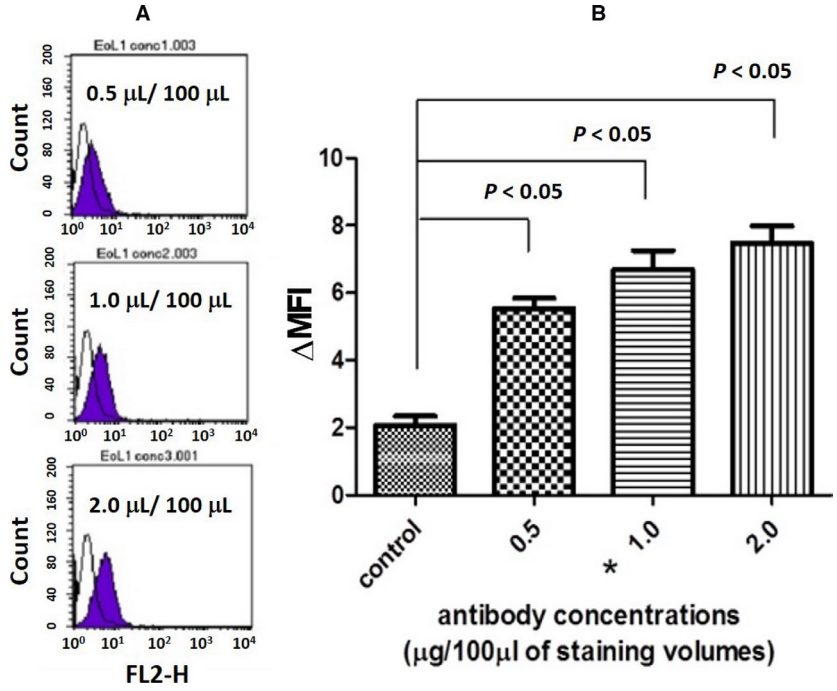

FIGURE 1 Optimization of primary antibody concentration. A, The histogram overlay of negative control and the EoL-1 cell that were reacted with anti-FLT3 antibody concentration in 0.5, 1.0, and 2.0 µg/100 µL. Filled histograms represent the mean fluorescence intensity of FLT3; open histograms represent the mean fluorescence intensity of the negative control. B, Data from flow cytometer was shown as the mean fluorescence intensity (MFI) level ± standard deviations (SD) of three independent experiments. Optimal concentration has been marked by an asterisk

staining volumes. The highest mean fluorescence intensity signal was obtained from the concentration of 2.0 µg of anti-FLT3 antibody with the value of 7.48 ± 0.50, followed by 1.0 and 0.5 µg with the value of 6.69 ± 0.57 and 5.33 ± 0.31, respectively, as shown in Figure 1. Significant difference was shown at three concentrations of anti-FLT3 antibody compared with the negative control.

3.3 | Optimization of cell concentration

The number of cells was determined to approximate the range of cell numbers. The EoL-1 cells were given a series of concentrations and were reacted with optimal primary antibody; after that, the samples were analyzed using flow cytometer. The Δ mean fluorescence intensity (ΔMFI) signals of 2.5×10^5, 5×10^5, 7.5×10^5, and 1.0×10^6 cells/mL were 4.7 ± 0.22, 5.0 ± 0.09, 5.24 ± 0.49, and 5.25 ± 0.94, respectively. The ΔMFI signals were increased by raising cell concentrations except the 1.0×10^6 cells/100 µL of cell concentration that showed saturated point, and maximum range of cell concentration was 7.5×10^5 cells (Figure 2A). The number of cells in the middle range of analysis as 5×10^5 cells was selected to ensure that FLT3 proteins on the cell surface were suited to the amount of optimal antibody.

3.4 | Optimization of staining time

Various staining times (15, 30, 45, and 60 minutes) for the reaction between the primary antibody and the FLT3 on cell surface were optimized. Different ΔMFI results indicated that the staining time

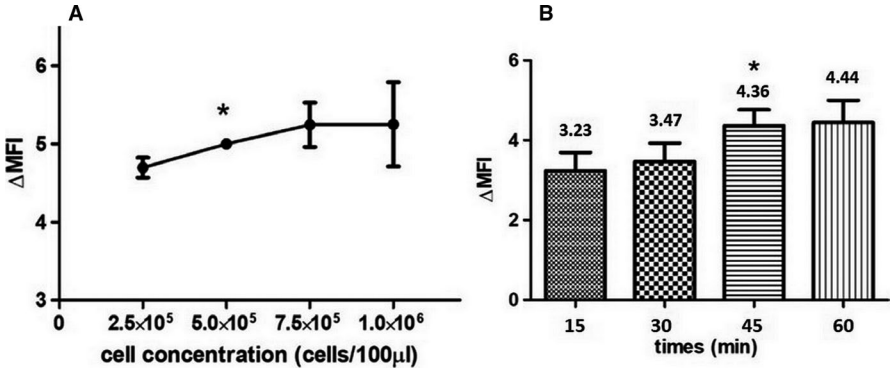

FIGURE 2 Optimization of cell concentration in the test mixture. The series of cell concentrations were tested to find the optimal cell number in the reaction mixture. The optimized cell concentration has been marked by an asterisk. Data were shown as the ΔMFI and error bars of SD and were the representative of three separate experiments (A), Optimization of staining time EoL-1 cells were fixed, and incubated with primary antibody for 15, 30, 45, and 60 minutes. Optimized staining time has been marked by an asterisk. The results were the representative of three separate experiments (B)

at 15 minutes produced a low level of ΔMFI while 60 minutes reaction produced the highest (Figure 2B). However, the difference was not significant between 45 and 60 minutes reaction time. Therefore, 45 minutes of antibody reaction was adopted to save the analysis time.

3.5 | Validation of flow cytometric analytical method

After all factors were optimized, the selected conditions for analyzing FLT3 expression on the leukemic cell surface were validated in the aspects of calibration curve, linearity and range, precision, accuracy, lower limit of quantification and stability.

3.5.1 | Calibration curve

To generate a calibration curve, EoL-1 and K562 cell lines were used as a positive reference and as a negative control for cells with and without FLT3 overexpression, respectively. A mixture of dilutions of EoL-1 and K562 cells were freshly prepared at different ratios to yield six standard cell mixtures; 20%, 40%, 60%, 80%, 100%, and 120% of EoL-1 cells (the optimal number of cells was 5×10^5 cells/100 μL). This was done in triplicate and measured for six independent experiments (N = 6). The ΔMFI signals from the assay were plotted with serial dilution of EoL-1 cells to generate a calibration curve (Figure 3). Moreover, quality control samples (QC) were prepared at 25%, 50%, and 75% of EoL-1 cells for low, medium, and high concentrations, respectively. The percentage of coefficient of variation (%CV) as [(SD/average) × 100] was estimated for the data integrity of the calibration curve. The data showed that %CV at each concentration was <20% in all cases.

3.5.2 | Linearity and range

Regarding the range of linearity, the correlation and regression analyses showed a strongly linear correlation between the Δ mean fluorescence intensity and the serial dilutions of positive cell lines (20%-120% EoL-1 cells). The ΔMFI range of the method was from 3.09 ± 0.44 to 5.10 ± 0.64 vs the series of positive cell ratios. The representative linear equation was $Y = 0.0203X + 2.72$ with the correlation coefficient (R^2) of 0.997 (Figure 3).

3.5.3 | Precision

Quality control samples were prepared at 25%, 50%, and 75% of EoL-1 cells (the optimal number of cells was 5×10^5 cells/100 μL) and analyzed against the same day calibration curve. Each run of the assay was performed on three separated preparations 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 was expressed as percentage coefficient of variation (%CV), and the results are presented in Table 1. The pooled repeatability and inter-day precision were 4.1% and 15.25%, respectively. The %CV levels of intra- and intra-day precision were <20% in all cases. The ΔMFI was calculated as described in Section 2. Each run of the assay was performed in triplicates for the same day precision (intra-assay repeatability) and six independent experiments on different days for inter-day precision evaluation.

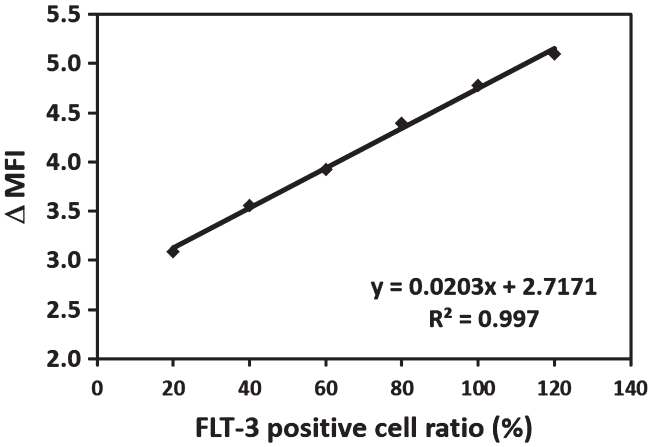

FIGURE 3 Calibration curve for FLT3 flow cytometric analysis. Serial dilutions of positive and negative cell lines with different ratios were prepared in PBS to create a calibration curve. Samples were prepared in triplicate and measured at six independent experiments (N = 6). The Δ mean fluorescence intensity (ΔMFI) was calculated as described in Section 2

3.5.4 | Accuracy

The accuracy reported as %recoveries for intra-day at 25%, 50%, and 75% of EoL-1 cell concentration were 97.74 ± 3.27%, 106.39 ± 3.58%, and 104.54 ± 5.83%, respectively. For the %recoveries of interday accuracy evaluation, their %recoveries were 99.85 ± 7.76%, 103.36 ± 5.99%, and 98.64 ± 5.88%, respectively. The %mean recoveries for all concentration of QC levels were between 80% and 120%. In addition, the percentage relative error (%RE) was reported. The %RE values of inter-day precision at each run were <15% in all cases. The results are exhibited in Table 2. Each run of the assay was performed in triplicate of QC levels at each concentration within the same day (intra-day assay) and on six independent days (inter-day variation).

3.5.5 | Lower limits of quantification (LLOQ)

The working range was defined by the calibration curve, and the lower limit of quantification was found to be 10% of EoL-1 cell concentration that could be quantitatively determined with acceptable precision and accuracy. Each run of the assay was performed in triplicate for six independent experiments (N = 6). The pooled %CV value of precision was 14.93%, and <15% in all cases. The %recovery assay was 99.67 ± 13.71% and the %RE was 10.66 ± 8.23%.

TABLE 1 Intra- and inter-day precision

QCs levels (%EoL-1)	Intra-day precision		Inter-day precision	
	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

%CV, percentage coefficient of variation; QC, quality control; SD, standard deviation; ΔMFI, mean fluorescence intensity difference of samples and control.

3.5.6 | Stability

The stability of the collected samples in the assay matrix stored at 2-8°C was evaluated by analyzing the concentration of the known QC levels prepared at 25%, 50%, and 75% of EoL-1. They were stored at 2-8°C and collected at the predetermined time intervals to analyze. Each run of the assay was performed on days 0, 1, 2, 7, and 14. The stability was monitored by observing the variation of ΔMFI values from the initial values and reported as coefficient of variation percentage (%CV). The %CV was <10% in all cases, and the data are demonstrated as in Table S1. The sample remained intact during 2 weeks at 2-8°C in the assay matrix.

3.6 | Application of the method to detect expression of FLT3 among leukemic patients

The expression of FLT3 on leukemic and EoL-1 cells was quantitatively assessed using flow cytometry and Western blot analysis. A calibration curve was constructed from the positive cells (EoL-1) at different ratios to negative cells (K562), 10%, 20%, 40%, 60%, 80%, 100%, and 120%, in PBS buffer pH 7.4 for the determination of FLT3 levels by flow cytometry. As shown in Figure 4, percentages of FLT3 from five patients' bone marrow samples were in an agreement with the results from Western blot analysis, but in the higher levels. In Western blot analysis, FLT3 protein expressions on these cells were analyzed and normalized with GAPDH housekeeping protein compared with the FLT3 expression on the EoL-1 cells (Figure 4). The compared %FLT3 from both flow cytometry and Western blot analysis are shown in Table 3 along with the patients' laboratory data and clinical status (Table 3).

4 | DISCUSSION

Researchers have extensively investigated the importance of FLT3 as a biomarker among leukemic patients. AML patients who carry the FLT3-ITD mutation were reported to have a poor prognosis[14] as well as patients with FLT3 protein overexpression in leukemic

TABLE 2 The percentage recovery and percentage relative error of intra- and inter-day assay

QC levels	Intra-day		Inter-day	
	%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

%RE, percentage of relative error; QC, quality control; SD, standard deviation.

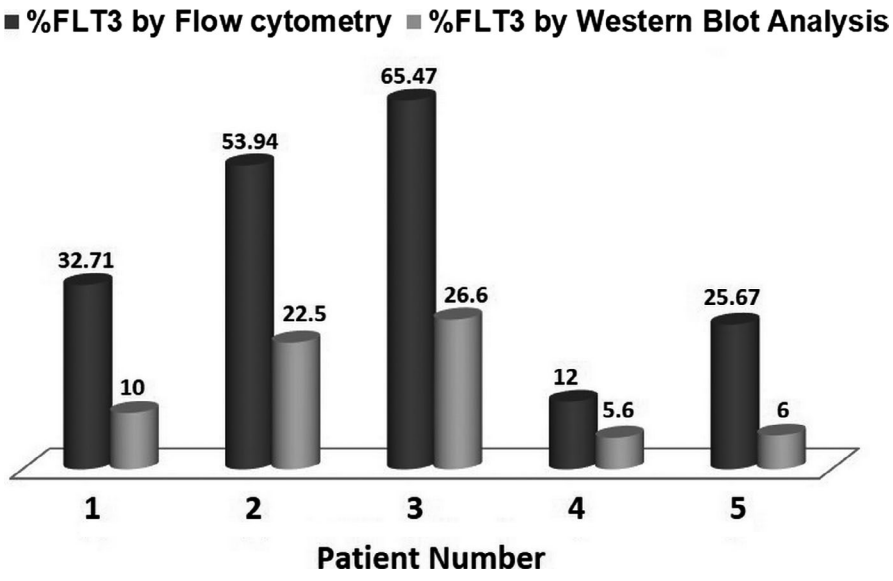

FIGURE 4 The expression of FLT3 protein on leukemic cell surface obtained from patients using flow cytometry and Western blotting analysis with each patient laboratory data and clinical status was presented. EoL-1 cells were used as the positive control cells and the results of FLT3 protein expression in both methods were calculated as the percentages of FLT3 protein expression compared with positive cell control (n = 3)

blasts.[14,15] An unfavorable clinical effect was found among 91 AML patients from 181 de novo acute myeloid leukemia (AML) cases with overexpression of the FLT3 even though no FLT3/ITD presented. The investigators concluded that the potent FLT3 kinase inhibitors could be used not only to target the mutated FLT3 but also AML cases overexpressing FLT3 even without mutations.[15] Another supported evidence from Thiede et al who analyzed the prevalence and the prognostic impact of FLT3 mutations in 979 AML patients. The results indicated that the wild-type FLT3 were more sensitive to treatment with AG1296, a potent FLT3 inhibitor than those with FLT3/ITD. Previous studies focused on the treatment targeted to the mutated FLT3 kinases, however; the AML cases overexpressing FLT3 even without mutations could be benefits to the inhibitors as well.[16] Therefore, simple FLT3 expression by flow cytometric determination could be more beneficial in this sense than the previous reverse transcription-polymerase chain reaction or RT-PCR technique where mutated FLT3 was identified. Recent studies also supported the benefit of rapid evaluation of FLT3 protein overexpression at the diagnosis step when a high-risk group of patients can be early identified.[17] The specifically designed treatment plan or the treatment progress of AML patients can be timely performed using an uncomplicated technique. In addition, regarding patient diagnostic and prognostic monitoring, the data of flow cytometry can be used to scan patients who have FLT3 overexpressed or do not respond to treatment as the result of gene abnormalities, and special techniques such as RT-PCR may be required later. The prompt response from physicians, obtaining sufficient data for decision making, is greatly beneficial for the high-risk patients.

This study specifically focused on optimizing and validating flow cytometric method to monitor FLT3 protein expression using a leukemic cell line model as a calibration tool. EoL-1 cells were selected as a positive control because they presented a prominent expression of FLT3 when using flow cytometry and Western blot analysis, and K562 cells having the lowest degree of FLT3 were chosen as the negative cell lines. Factors effecting to the FLT3 and anti-FLT3 interaction and flow cytometry were adjusted to obtain the performance, reliability, minimal cost, and time for the analysis of FLT3 protein expression. For optimizing the flow cytometric method, the primary antibody concentrations, number of cells, and incubation times were titrated. According to the manufacturer protocol, this testing requires 1.75 μg of antibody per 1×10^6 cells in a 100-μL staining volume. As mentioned above, attempts to use limited cell samples from leukemia patients were the intention in this study. Wide range of the calibration curve between ΔMFI value and EoL-1 cell percentage was adjusted to monitor the FLT3 expression in clinical samples. In addition, major influencing parameters were optimized with the consideration of cost and time savings as well. With the maximal cell density of 5×10^5 cells/100 μL, the ΔMFI was significantly different from the control, when the antibody concentration was 1.0 μg/100 μL. Non-significant increase was observed when the antibody concentration was raised to 2.0 μg/100 μL. In addition, the EoL-1 cells are the leukemic cell clone with FLT3 overexpression, sufficient antibody concentration should be reached when reacted with

TABLE 3 The patients' laboratory data, clinical response and %FLT3 expression from flow cytometry and Western blot analyses

AML patient No.	% FLT3 by Flow cytometry	% FLT3 by Western Blot	Case	% Blast in PB	% Blast in BM	Hemoglobin (g/dL)	Hematocrit (%)	Platelet (per cu.mm)	WBC (per cu.mm)	Response to treatment
1	32.71	10.00	New	65	90	7.8	23.9	200 000	8810	Palliative
2	53.94	22.50	New	60	100	9.9	30	45 000	24 660	Remission
3 APL	65.47	26.60	New	0	90	8.9	21.1	97 000	2300	Remission
4	12.00	5.60	Relapse	57	16	7.3	20	13 200	70 000	Dead
5 APL	25.67	6.00	New	10	90	7.1	21.3	50 000	31 640	Remission

BM, bone marrow; PB, peripheral blood; WBC, white blood cells.

the clinical samples, which contained various types of cells. Finally, the optimized flow cytometric protocol was obtained when using a cell concentration of 5.0×10^5 cells in 100 μL; the optimal primary antibody was 1.0 μg of antibody with 45 minutes of the incubation times.

The flow cytometric method is categorized as a high complexity laboratory test by the Centers for Disease Control and Prevention (CDC, USA).[18] All newly developed protocols should be validated and documented to maintain the laboratory's performance standards. Due to the wide varieties and cross existence of molecular biomarkers in and on the cells, validating the methods that were used in the analysis is essential. The guidance documents from a variety of regulatory bodies were published but no specific regulations exist on bioanalytical method validations using flow cytometry.[10,19] Each laboratory is accountable for providing sufficient data to show that methods provide acceptable performance to meet their objectives, and several studies have endeavored to standardize and validate the methods used in their experiments to ensure the technical quality of the results.[20,21] In this regard, the availability of the developed method was validated by serial dilution of EoL-1 cells as a reference standard, and they were mixed with the negative control (K562) to create a calibration curve. The calibration curve should consist of at least six concentrations. For within-run, the precision value (%CV) for at least 75% of the calibration standards should lie within 20%.[13] The results demonstrated good linearity from the assay by flow cytometer ($r^2 = 0.997$). The percentage coefficient of variation (%CV) was estimated following the accepted criteria of the calibration curve. The data showed that %CV at each concentration was <20% in all cases. The saturated point of the calibration curve was presented when the concentration of positive control dilution increased.

Quality control levels should be included in every analytical run and were prepared at 25%, 50%, and 75% of EoL-1 cells for low, medium, and high concentrations, respectively, to measure the FLT3 biomarker for precise, accurate, and stable experiments. The acceptable variation of data from the analysis of QCs samples should be <25% CV (30% at the LLOQ) for both intra- and inter-day precision, recommended in the phenotypic biomarker assay validation.[13] Validating quantitative pharmacokinetic assays that use a calibration curve to estimate the protein expression, the criteria have an upper acceptance within <15% to 20% CV (25% at the LLOQ).[12] This study showed that %CV values were <20% CV in all cases. The acceptance criteria of accuracy expressed as relative error, ±20% RE is the default value (30% at the LLOQ). In this method validation, the results fell within the acceptable precision and accuracy recommendation.[13] The stability was monitored by %CV of QC levels, and their %CV values were within 10% for all concentrations at each run. Therefore, the sample could be kept for analysis within 2 weeks at 2-8°C in the assay matrix. The sensitivity of the method was at 10% EoL-1 which is the lowest concentration of FLT3 protein expression (LLOQ), that could be quantitatively determined with acceptable precision and accuracy (within 30% CV and 30% RE at the LLOQ). Hence, this study was conducted to validate the flow cytometric method for FLT3 analysis which all validate topics were in the agreement of the recommended criteria.[13,22] However, the signal of fluorescence intensity could vary depending on the analyst, instrument and instrument settings. Consequently, the standard calibration and QC samples should be included in the method validation and daily routine analysis.

This method was applied to determine FLT3 expression on five bone marrow specimens from AML patients and the results were confirmed by comparing to the Western blotting assay. The results demonstrated FLT3 expression percentages analyzed by flow cytometry higher than those from Western blotting. However, similar trends of FLT3 expression were observed (Figure 4). Most of the bone marrow samples included in this study were the samples from AML patients with new diagnosis and relapse status, whose samples consisted of blast cells (>90%) as shown in the patients' clinical data except the sample from patient no. 4 (Table 3). All flow cytometric parameters were set for gating of the blast cells. Therefore, the cells determined for their cell surface FLT3 in this study were mainly blast cell population. The high levels of FLT3 protein expression in both flow cytometry and Western blotting in patients' samples are highly related to the high level of %blast in bone marrow (90%-100%). Further study for factors affecting FLT3 expression levels such as disease status, leukemic type, or laboratory parameters would be beneficial for physician. Nevertheless, the method here described allowed us to gain preliminary data, and we expect to apply this method to measure FLT3 protein expression on leukemic cells from patients in a routine analysis. However, this obviously remains to be studied with further experiments using a greater number of samples to detect FLT3 expression on leukemic cell surface based on similar procedures. Flow cytometry had been recommended as an ideal platform for determination of receptor on cell surface or receptor occupancy.[13] Patients with a high expression of *FLT3*, consecutively FLT3 protein expression potentially have higher risk in leukemia.[15,23] Clear diagnosis consisted of biomarker determination and more intensive drug regimen plan are highly valuable for this group of patients. In the guideline, a well-defined reference material to be a fully representative of the endogenous analyte was recommended for the quantitative biomarker determination. Not only using normal cells as a negative cell control, EoL-1 was selected and shown to be appropriate cell-based marker in this aspect. Application of this method's validation protocols provides a model to validate the assay of biomarker expression by flow cytometry in other studies, further expanding the utility of this method. In conclusion, this study reported on optimizing and validating flow cytometry to detect FLT3 protein expression using an EoL-1 leukemic cell line model. The reliability, validity and applicability of the determination of FLT3 protein on cell surface are warranted.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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