CHAPTER 4

Discussion

FMS-like receptor tyrosine kinase 3 (FLT3), belongs to a group of class III receptor tyrosine kinase family, is a membrane-bound receptor with an intrinsic tyrosine kinase domain that promotes proliferation after activation. FLT3 protein expression is mostly found in early hematopoietic progenitor cells in normal bone marrow [20]. Moreover FLT3 is aberrantly expressed in human leukemia, including higher than 90% of AML and nearly 100% of B-lineage ALL [22, 23, 72, 88]. While FLT3 protein is usually overexpressed in blast cells in AML patients and played role in the pathogenesis of this disease [15]. The FLT3 protein has emerged as an important marker to determine prognostic of disease. The increased FLT3 protein expression in leukemic blast cells may be associated with a worse prognosis [27] and was correlated with increased relapse rate [28]. Moreover, evaluation of FLT3 protein overexpression at diagnosis can help identify the high risk group of patients which can be designed treatment plan [28]. Thus, this study specifically focuses on the optimization and validation of flow cytometry for detecting FLT3 protein expression using leukemic cell line model.

First, leukemic cell lines (e.g. EoL1, MV4-11, K562, Molt4, HL60, and U937 cells) were screened the FLT3 protein expression using flow cytometry and Western blotting to use as a leukemic cell model. The EoL1 cells showed the highest expression levels of FLT3 on cell surfaces, while K562 and U937 cells showed very low expression by flow cytometry. The immunoblotting assay was used to confirm the expression of FLT3 protein on each leukemic cell lines, and the results showed that HL60 cells expressed FLT3 protein levels higher than EoL1 cells due to the high level of cytoplasmic FLT3 protein when cytoplasmic and nuclear protein were separately extract to examine. However, HL60 cells could be detected both of cytoplasmic and

membrane FLT3 protein expression and showed a prominent band of FLT3 protein comparing to the other cells. K562 and U937 cells showed low levels of FLT3 protein expression. Consequently, EoL1 cells were selected as a positive control because they presented a strong expression of FLT3 when using flow cytometry, and K562 cells was also chosen as a negative cell line to set the experimental model for studying FLT3 protein expression in leukemia cells.

Additionally, FLT3 mRNA was determined to compare its result to those of flow cytometry and Western blotting. FLT3 mRNA level was studied by RT-PCR analysis. RT-PCR is normally used to qualitatively detect gene target expression by reversing mRNA to be cDNA. The results showed that EoL1 and MV4-11 cells had high levels of FLT3 mRNA and FLT3 mutant mRNA, followed in Molt4 as well as K562 and U937 cells, respectively.

To delineate types of leukemia cell lines which used in this study, EoL1, MV4-11, HL60, and U937 were AML-derived cell lines and the data showed relative expression of FLT3 protein except HL60 cells using flow cytometry and, Western blotting. The result showed that HL60 cells had a high level of cytoplasmic FLT3 protein as compared to that of FLT3 membrane protein level. Thus the FLT3 protein levels of HL60 cells were uncorrelated between Western blotting and flow cytometry. Moreover, the protein lysate was obtained from whole protein lysates and both cytoplasmic and membrane FLT3 proteins were detected by Western blotting while flow cytometry could detect only FLT3 protein on the cell membrane. The results of RT-PCR, MV4-11 cells showed FLT3 mutated band over the FLT3 wild type band (366 bp). The FLT3 mutations were identified as a band migrating in MV4-11 cells. These mutations are internal tandem duplication (ITD) which are duplications of 30 base pairs [129]. Therefore, MV4-11 cells were suitable to be used as a cell model of FLT3-ITD mutations using RT-PCR analysis. K562 cells (erythroleukemia, CML cell line) had a very low level of FLT3 protein and gene expression. Then it was used to be a negative control cell model in this experiment. Molt4 cells (T-cell ALL) was also found the relatively high level of *FLT3* gene expression but not in the expression of protein. In addition, the previous study reported that the FLT3 expression in hematopoietic cell lines of various origin revealed the presence of an FLT3-specific PCR product in most

pre-B-cell lines and promyeolocytic cell line, whereas erythroid and mature B-cell lines were negative. Interestingly, pre-B, pre-T, and T-cell leukemias were highly positive [22, 72].

After the positive (EoL1) and negative (K562) cell controls were selected, the factors for flow cytometry development were changed to obtain the performance, reliability, and low-cost analysis for FLT3 protein levels detection in patients. The EoL1 and K562 cells were optimized and validated for flow cytometric method. For optimizing of flow cytometric method, the primary antibody concentrations, cell numbers, and incubation times were estimated. According to a manufacturer's protocol, this testing was performed by using 1.75 µg of antibody per $1x10^6$ cells in 100 µl staining volume. The protocol of this study required to reduce the sample obtained from leukemia patients for saving the specimens which were used to diagnose. A cell number that can be used to analyze the expression of FLT3 were determined. The optimal conditions were selected by based on a high degree of Δ mean fluorescence intensity value that produced an obviously assay and also considered the cost and time savings. Resulting of optimizing flow cytometric protocol demonstrated that optimal cell concentration was $5.0x10^5$ cells in 100 µl, the optimal primary antibody was 1.0 µg, then the incubation times were tested and showed that 45 min of staining time gave a clear result.

It is essential to validate the methods that were used in the analysis of a wide range for studying biomarker. The guidance document, from a variety of regulatory bodies were published but there are no specific regulations on bioanalytical method validations using flow cytometry [33, 34]. Many studies were published the four categories of bioanalytical assay (Table 4.1) with the performance validation characteristics that would be useful to explore in the validation of each category (Table 4.2) [127, 130]. The phenotypic biomarker assay was defined as quasi-quantitative group. Cummings *et al*. were reported a fit-for-purpose approach to biomarker method validation, and described how to get the procedures that assure the quality of every aspect of the trial should be implemented [127]. O'Hara *et al*. were announced the application of phenotypic biomarker validation using flow cytometry, and the basic validation parameters were accuracy, precision, specificity, sensitivity, reproducibility,

and stability. When there is not application in the regard of accuracy, specificity, and stability [128].

Table 4.1 Categories of bioanalytical methods [128].

Assay category	Definition
Quantitative	Uses calibration standard to determine the absolute quantitative values for unknown samples. The reference material is well defined and fully representative of the endogenous analyte. Example: pharmacokinetic assays
Relative quantitative	Uses a calibration standard to estimate the absolute quantitative values for unknown samples. The reference material is not fully representative of the endogenous analyte. Example: cytokine ligand binding assays
Quasi-quantitative	Does not use calibration standard, but has a continuous response. Numeric data is reported. Example: immunogenicity assays, phenotypic and functional biomarker assays, receptor occupancy assays
Qualitative	Lacks proportionality to the amount of analyte. Categorical data is reported. Example: immunohistochemical assays

Table 4.2 Recommended biomarker assay performance parameters [130].

Category	Quantitative	Relative quantitative	Quasi-quantitative	Qualitative
Accuracy	/	/		
Precision	/	/	/	
Sensitivity	/	/	/	/
Specificity	/	/	/	/
Linearity	/	/		
Parallelism	/	/		
Assay range	/	/	/	
Stability	/	/	/	/

Each laboratory should supply sufficient data to show that methods provide acceptable performance to meet their objectives. Several studies have attendance to standardize and validation of the methodology in their experiments to ensure the technical quality of the results. Green *et al*. reported flow cytometry analysis of leukemic cells for determining the effects of the novel protein kinase C (PKC) inhibitor enzastaurin on intracellular phosphoprotein signaling. The assay was done in validation with regarded to selectivity, sensitivity, and reproducibility, and the patient specimens were assayed for PKC activity using this approach [131]. Validation of a standardized method for enumerating circulating endothelial cells and progenitors has been pushed by Mancuso *et al*. They reported the experimental validation of a novel flow cytometry method that including reproducibility studies and recovery after sample freezing/thawing [132].

Thus, this study desired to validate the developed flow cytometric method for detecting FLT3 protein expression levels according to the guidance documents [33, 34, 124-128]. The availability of the developed method was validated by serial dilution of EoL1 cells as a reference standard, and they were mixed with negative control for creating a calibration curve. The calibration curve should consist of at least six concentrations. For the curve within a run, the precision value for at least 75% of the calibration standards should lie within 20% [34]. The calibration curve demonstrated good linearity of the assay ($r^2 > 0.995$). The percentage coefficient of variation (%CV) was estimated for accepted criteria of calibration curve. The results showed that %CV at each concentration were less than 20% in all cases. The saturated point of the calibration curve presented when increasing the concentration of positive control dilution.

QC levels would be performed for each validation run, and were prepared at 25, 50, and 75% of EoL1 cells for low, medium, and high concentrations, respectively and they were used to assess the ability of the assay to measure the biomarker of interest for precision, accuracy, and stability experiments. The QCs levels are expected with 25% CV (30% at the LLOQ) acceptable for both intra-day and inter-day precision to validate phenotypic biomarker assays [128] when the validation of quantitative as pharmaco-kinetic assays that uses a calibration curve to estimate the protein expression has upper

acceptance criteria within <15 to 20% CV (25% at the LLOQ) [34]. This study showed that %CV of precision was less than 20% CV in all cases. The acceptance criteria of accuracy express as relative error, ±20% RE is the default value (30% at the LLOQ) [128]. During validation, the results played to achieve acceptable accuracy. The stability was monitored by %CV of QC levels, that analysis on day 0, 1, 2, 7, and 14. Their %CV values were within 10% for all concentrations at each run and the pooled %CV of QC levels. Therefore, the samples could be kept for analysis within two weeks under temperature of 2-8°C in the assay matrix. Whereby the sensitivity of the method is based on the LLOQ, the lowest concentration of FLT3 protein expression that can be quantitatively determined with acceptable precision and accuracy was done in this study (within 30% CV and 30% ER at the LLOQ). Hence, this study was done in validation of flow cytometric method with acceptable criteria. However, the signal of fluorescence intensity can be variable depending on the analyst, instrument, and instrument settings [128]. The signal of fluorescence intensity is recommended to quantify using fluorescence calibration [133]. FACSCalibur flow cytometer (Becton Dickinson, NJ) which be used in this study was calibrated following manufacturer's guideline.

After validation, this method was applied for detecting FLT3 expression on leukemic cells obtained from patients and confirmed the results by Western blotting. Although Western blotting could not detect the expression of FLT3 protein in some cases of patient samples but the positive and negative cell lines showed the expected results. This may be due to the sensitivity of Western blotting. It could not analyze and distinguish the low levels of FLT3 protein expression. To compare the results from flow cytometry and Western blotting, the tendency of FLT3 expression levels were in line with each other. Nevertheless, the method here described to gain preliminary data, and expected to be possible to apply this method to measure FLT3 protein expression on leukemic cells from patients in routine analysis. However, this obviously remains to be proved with further experiments in acceptable numbers of samples to detect the FLT3 expression on leukemic cell surface based procedures.

When considering the advantages and disadvantages of each method were used to analyze, by observing the variance using flow cytometry was less than the variance using Western blotting. The Western blot analysis has its advantage when information

about molecular weight is needed. Many advantages of single cell analysis like flow cytometry are that less time consuming and use small samples than other methods. For quantification of protein levels on the cell surface using flow cytometry, commercial kits are now available to establish calibration curves [134]. While RT-PCR technique shows many advantages including high specificity, sensitivity, and speed in genomic analysis. The RT-PCR assay for *FLT3* gene expression can be used to identify the FLT3 mutations, especially FLT3-ITD mRNA level in this study. However, this technique is more expensive than other methods.

The method analysis is important to estimate the validation for supporting biomarker studies because the impact of biomarker studies is directly related to the quality of the underlying data. Method validation remains an essential determinant in the successful of biomarker qualification, while the failure of a biomarker in the clinic is a low-quality of assay and lack of validation [127]. Application of this method validation protocols will provide a model to validate the assay of biomarkers expression by flow cytometry in other studies, further expanding the utility of the method validation.