CHAPTER 1

Introductions

1.1 Historical background

Cancer is a public health problem that is a leading cause of death worldwide around 13% of all deaths (7.6 million deaths) in 2008. Deaths from cancer worldwide are extrapolated to rise, with an estimated 13.1 million deaths in 2030 [1]. The incidence and mortality rates of cancers are increasing in several economically developing and less developed countries [2]. From 2002 up to the present, cancer is the most common cause of death in Thailand [3]. Leukemia has the highest incidence rate of cancers in Thai children, and the mortality rates in Thai children aged 6-18 was approximately 2.15 per 100,000 during 2007-8 [4]. The four major types of leukemia are acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloblastic leukemia (AML), and chronic myeloblastic leukemia (CML). Moreover, AML is one of the most common hematologic malignancies associated with the greatest mortality and morbidity in adult populations in Thailand and other Southeast Asian countries [5, 6].

Acute myeloblastic leukemia (AML) is a broad range of disorders that are all characterized by an arrest of maturation along with uncontrollable proliferation of hematopoietic progenitor cells [7]. AML accounts for 30% of leukemia and 40% of leukemia-related deaths [8]. The overall 5-year survival rate for AML is still less than 50% in adults and significantly lower in the elderly [9]. Many biomarkers associated with a relatively poor prognosis of AML are present. For example, Wilms tumor 1 (WT1) [10], nucleophosmin 1 (NPM1) [11, 12], and FMS-like tyrosine kinase 3 (FLT3) [13, 14]. FLT3 is focused in this study, since an overexpressing FLT3 was reported in leukemic cells and played a role in the pathogenesis of this disease [15].

FMS-like tyrosine kinase 3 (FLT3), also known as fetal liver kinase 2 (FLK2) [16] or stem cell tyrosine kinase 1 (STK1) [17], belongs to the group of class III receptor tyrosine kinases. It is a membrane-bound receptor with an intrinsic tyrosine kinase domain [18]. The expression of FLT3 receptor was confined to early hematopoietic progenitor cells [19, 20] and mediated cell proliferation and differentiation in normal bone marrow [21]. Expression of FLT3 has been evaluated in hematologic malignancies. More than 90% of B-cell ALL and AML blasts expressed FLT3 protein at various levels, and the signaling promotes proliferation after activation [22, 23]. Consequently, FLT3 is increasingly recognized as a biomarker in the disease progression [15, 19]. The FLT3 protein is usually overexpressed in leukemic blast cells of AML patients, while the some cases in AML patients possessed mutant FLT3 [19]. Two types of activating mutations of FLT3 including internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations have been reported about 30 to 40% of patients with AML [24]. Both mutations caused constitutive activation of its kinase domain and downstream cell signaling [25, 26]. The previous studies found that increased FLT3 protein expression in leukemic blast cells may be related with a worse prognosis [27, 28]. Moreover, AML patients who carry the FLT3-ITD mutation appeared to have poorer clinical outcomes [7]. Therefore, FLT3 is an important prognostic biomarker used for determining the progression of AML patients. Determination of FLT3 expression levels on leukemic cell surface could be a supporting tool for physician treatment plan.

Different methods have been used to evaluate the expression of FLT3 levels. For FLT3 protein detections, a flow cytometry and Western blot analysis are the classical techniques to be used. Flow cytometry measures multiple characteristics of individual particles flowing in a single file in a stream of fluid, and light emitted from fluorescently labeled antibodies can identify a wide array of cell surface and cytoplasmic antigens [29]. Western blot analysis is a widely accepted analytical technique used for specific protein detection. Protein of interest is extracted and measured for normalized protein content. After protein separation by gel electrophoresis, all proteins are transferred to membrane and incubated with a chemically labeled antibody specifically bound to the targeted protein for protein

density detection [30]. In addition to protein analysis, determination of *FLT3* gene expression by Reverse Transcription–Polymerase Chain Reaction (RT-PCR) is a commonly used technique for studying gene of interest. This method is used for qualitative detection of a targeted gene through the creation of a cDNA transcripted from a mRNA sequence of interest using a reverse transcriptase enzyme with a specific primer [31].

A recent study demonstrated that the evaluation of FLT3 protein overexpression could identify disease severity and a treatment plan [28]. Flow cytometry is a sensitive and rapid tool for detection of a phenotypic marker expression [29]. It is also a current standard for hematopathologic diagnosis of acute leukemia. Immunophenotyping confirmation and identification of aberrant marker expression on abnormal cells can be accomplished by using this technique [32]. Thus, this study focused on evaluation of important parameters in sample pre-treatment and assay validation for the flow cytometry determination of FLT3 protein expression. It is essential to optimize and validate a developed method that will be used for the analysis of a biomarker. While the guidance documents from a variety of regulatory bodies were published but there are no specific regulations on bioanalytical method validations for flow cytometric method [33, 34]. Therefore, the aims of this study are the optimization and validation of the flow cytometric method for determination of FLT3 protein expression levels in leukemic cells. Leukemic cell lines were selected as leukemic cell models for the method validation. The reliable, reproducible, rapid and economical analytical technique for FLT3 expression evaluation is presented in this study.

1.2 Objectives

1.2.1 To validate flow cytometric method for detecting the expression of FLT3 protein levels in leukemic cells.

1.2.2 To evaluate FLT3 protein expression on leukemic cell surface detecting by flow cytometry and Western blot analysis.

1.3 Literature review

1.3.1 Cancer

Cancer is a large group of diseases that can affect any part of the body. The definition of cancer is the rapid creation of abnormal cells that grow out of control, and which can then invade the adjacent and spread to other organs *via* blood or lymphatic system [1]. This process is referred to metastasis, and it is the major cause of death from cancer. In 2013, about 580,350 Americans are expected to die of cancer, almost 1,600 people per day. Cancer is the second most common cause of death in the United States, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths [35]. WHO estimates that the number of deaths and new cases will continue to rise, to 12.4 and 21.4 million in 2030 [36]. The incidence and mortality rates are increasing in several less developed and economically transitioning countries because of adoption of unhealthy Western lifestyles such as smoking, physical inactivity and consumption of calorie-dense food [2]. The economic burden associated with those suffering from cancer was predicted it to be over 1,400 billion US dollars in 2020 [37]. There are over 200 different known cancers that affect humans. Lung, intestine, stomach, liver, and breast cancer cause the most cancer deaths each year [1]. From 2002 up to the present, cancer is the most common cause of death in Thailand [3].

The causes of cancer were divided into two groups. Cancers are primarily an environmental disease with 90–95% of cases attributed to environmental factors and 5–10% due to genetics. The most common factors leading to cancer include unhealthy lifestyles such as smoking, eating an unbalanced diet, lack of physical activity, or drinking too much alcohol. The other cases include factors in an environment, such as infections, radiation, toxic chemicals, and environmental pollutants [38]. These environmental factors cause or enhance abnormalities in the genetic material of cells. Various cancers have been linked to genetic defects. Most cancers are caused by a gradual build-up of genetic damage in cells. In

general, cancer is highly occurred in older people. Many causes of cancer are still unknown. Inherited genes may also increase the risk of developing certain types of cancer [39].

The treatments for cancer generally consist of surgery, radiotherapy, and chemotherapy. The treatments are used depends upon the type, location and grade of the cancer. One or more of these treatments may be used for the best results. To remove a solid tumor, a surgical operation is the most common of cancer treatment. It offers the best chance of cure for many cancers that have not spread. When the cancer has spread, surgery is often used in combination with radiotherapy and/or chemotherapy. Radiotherapy can be used to reduce size of tumor before surgery, prevent the cancer from coming back after operation, and destroy the cancer cells in the treated area by damaging the DNA within these cells. In cancer treatment, chemotherapeutic or cytotoxic drugs can destroy the cancer cells which one chemotherapeutic drug or a combination of different chemotherapeutic drugs are used depend on type and stage of cancer. Chemotherapy may be used before surgery, and often used after surgery to eliminate any surviving cancer cells around tumor site. It can also help destroy cancer cells that have spread to other parts of the body. Finally, the goal of cancer treatment is to cure the disease or considerably prolong life while improving the patient's quality of life [40, 41].

1.3.2 Leukemia

Leukemia is a hematological malignancy, a type of cancer that affects the blood and bone marrow. The disease develops when hematopoietic cells produced in the bone marrow grow out of control. The cell in which the leukemic transformation occurs may be a precursor or hematopoietic stem cell that can differentiate into both myeloid and lymphoid cells. Myeloid leukemia can arise in a lineage-restricted cell, in a multipotential stem cell capable of differentiating into cell of erythrocytic, granulocytic, monocytic, and megakaryocytic lineages or in a pluripotential lymphoid-myeloid stem

cell [42]. This results in decreased production of normal blood cells. Leukemia can spread to other organs (lymph nodes, spleen, liver, and central nervous system) and cause pathogenesis in these organs.

About 48,610 new cases of leukemia are expected to be diagnosed in the United States in 2013 (Figure 1.1). Most cases of leukemia occur in older adults; the median age at diagnosis is 66 years and the most common types of leukemia in adults are AML and CLL. While the most common type of leukemia in children and adolescents younger than 20 years is ALL. Incidence rates for all types of leukemia are higher among males than among females; males are expected to account for approximately 57 percent of the new cases of leukemia in 2013. Approximately 23,720 deaths in the US are expected to be attributed to leukemia, and AML represents the highest death of leukemia in 2013 [43]. In Thailand, leukemia is the highest incidence rates of cancers in children [4]. The most common types of leukemia in adults are AML and CLL, followed by CML and ALL in the United States [44], whereas AML has the highest incidence in Thailand, followed by ALL, CML, and CLL [45].

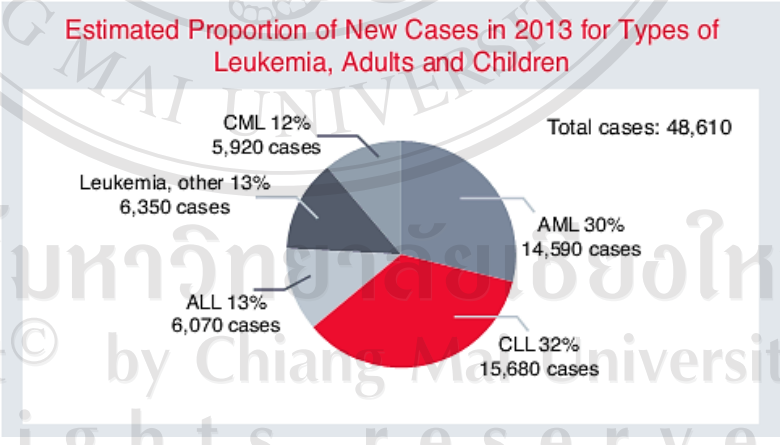

Figure 1.1 The new cases of leukemia are expected to be diagnosed in the United States in 2013 [43].

The causes for leukemia are unknown. Like other cancers, leukemia results from mutations in the DNA. Certain mutations can trigger leukemia

by activating oncogenes or deactivating tumor suppressor genes, and thereby disrupting the regulation of cell death, differentiation or division. Genetic alterations leading to leukemic transformation often result from major alterations in the chromosomes and other changes, such as point mutations or partial duplications. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances. [42, 44, 46]

1.3.3 Classifications of leukemia [42]

Leukemia is subdivided into acute and chronic leukemia. Acute leukemia is characterized by a rapid increasing of immature blood cells due to the rapid progression and accumulation of the malignant cells. The malignant cells invade to the bloodstream and spread to other organs of the body. If untreated, acute leukemia leads to death in weeks or months. Chronic leukemia is characterized by the excessive creation of abnormal mature blood cells which generally taking months or years to progress. Additionally, leukemia is subdivided according to a kind of blood cell that is affected. It is divided into lymphoblastic or lymphocytic leukemia and myeloblastic or myelocytic leukemia as shown in Table 1.1.

Table 1.1 Types of leukemia

Types of leukemia	**Definition**
Acute myeloblastic leukemia (AML)	- Mainly in adults. - Subtypes include promyelocytic, megakaryoblastic and myeloblastic leukemia.
Acute lymphoblastic leukemia (ALL)	- Most common type in children. - Subtypes include Burkitt's, precursor T or B lymphoblastic and biphenotypic leukemia.
Chronic myelocytic leukemia (CML)	- Commonly in adults.
Chronic lymphoblastic leukemia (CLL)	- Commonly in early adults. It sometimes occurs in younger adults.

1.3.4 Acute myeloblastic leukemia (AML)

Acute myeloblastic leukemia (AML) is a group of clonal hematopoietic stem cell disorders in which represents overproliferation and undifferentiation in the hematopoietic progenitor cells. This leads to an accumulation of nonfunctional immature myeloid cells in the bone marrow. In the World Health Organization (WHO) classification, the term of myeloid includes all cells belonging to the monocytic/macrophage, megakaryocytic, erythrocytic, granulocytic (neutrophil, basophil, and), and mast cell lineages. Common symptoms of untreated AML are fatigue, bleeding due to thrombocytopenia, organ infiltration and fatal infections due to neutropenia, all resulting from the suppression of normal bone marrow function, which in the absence of treatment, typically leads to death within weeks to months of its clinical presentation [47-49].

AML is the most common leukemia in adults, with a prevalence of 3.8 cases per 100,000 rising to 17.9 cases per 100,000 adults aged 65 years and older. The median at age at presentation is about 65-70 years, and three men are affected for every two women [50, 51]. The overall 5-year survival rate for AML is still less than 50% in adults and significantly lower in the elderly [51]. The median survival in patients over the age of 65 is less than one year and only 20% of these patients survive two years [52].

1.3.5 Classification of acute myeloblastic leukemia [49, 53-56]

Two systems have been used to classify AML into subtypes including; the French-American-British (FAB) classification and WHO classification. The diagnosis of AML is based on the demonstration of an accumulation of myeloid blasts in the bone marrow. According to the FAB cooperative group the diagnosis of AML requires at least 30% myeloid blasts in the bone marrow. FAB subtypes are based on how much the leukemic cells have matured and the type of blood cell the disease developed from. AML cells can have features of red cells, platelets, white cells (monocytes, eosinophils or, rarely, basophils or mast cells) and immature white cells

(myeloblasts or promyelocytes) and the Table 1.2 shows the FAB classification.

Table 1.2 The French-American-British (FAB) classification of AML [57]

Class	Cell Type Most Affected	Description
M0	Myeloblastic	Minimally differentiated AML
M1	Myeloblastic, with minimal maturation	Myeloblasts are the dominant leukemic cells in the marrow at the time of diagnosis
M2	Myeloblastic, with maturation	Many myeloblasts are present, but some cells are developing toward fully formed blood cells
M3	Promyelocytic (acute promyelocytic leukemia)	Leukemic cells have a translocation between chromosomes 15 and 17
M4	Myelomonocytic	Leukemic cells often have an inversion of chromosome 16
M5	Monocytic (acute monocytic leukemia)	Leukemic cells have features of developing monocytes (white cells)
M6	Erythroleukemic (acute erythroid leukemia)	Leukemic cells have features of developing red cells
M7	Megakaryocytic (acute megakaryocytic leukemia)	Leukemic cells have features of developing platelets

The more recent WHO classification defines AML as at least 20% leukemic blasts in the bone marrow, which includes different biologically distinct groups based on clinical features, cytogenetics, and molecular abnormalities in addition to morphology. The recent WHO classification reflects the fact that an increasing number of acute leukemias can be

categorized based upon their underlying cytogenetic or molecular genetic abnormalities, and describes in Table 1.3.

Table 1.3 WHO classification of AML [49, 53]

Acute myeloid leukemia with recurrent genetic abnormalities AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB -MYH11 APL with t(15;17)(q22;q12); PML -RARA AML with t(9;11)(p22;q23); MLLT3 -MLL AML with t(6;9)(p23;q34); DEK -NUP214 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1 -EVI1 AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15 -MKL1 Provisional entity: AML with mutated NPM1 Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes Following MDS or MDS/MPD Myelodysplasia-related cytogenetic abnormality Multilineage dysplasia
Acute myeloid leukemia and myelodysplastic syndromes, therapy related Alkylating agents; ionizing radiation therapy; topoisomerase II inhibitors; others.
Myeloid sarcoma Extramedullary myeloid tumor; granulocytic sarcoma; chloroma
Myeloid proliferations related to Down syndrome Transient abnormal myelopoiesis Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Table 1.3 WHO classification of AML (continued)

Acute myeloid leukemia, not otherwise categorized
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype leukemia with t(9;22)(q34;q11.2); BCR -ABL1
Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Provisional entity: NK cell lymphoblastic leukemia/lymphoma

1.3.6 Prognosis and genetics of AML

Remission rates with standard chemotherapy in AML patients range from 50% to 85% [58]. However, the majority of patients will relapse and die of their disease within 2 years of achieving a remission. Remission rates and overall survival depend on age of the patient, cytogenetics, other molecular changes within the malignant leukemia, previous bone marrow disorders (e.g. myelodysplasia disorder), and comorbid illnesses as well as others [48].

Prognosis and genetics of AML are tightly linked. Risk stratification based on cytogenetics divides patients into three main groups, those with favorable, intermediate, and unfavorable cytogenetics (Table 1.4) [59]. About 45% of acute leukemia has an abnormal karyotype with a recurrent chromosomal alteration, and about 15% has 3 or more cytogenetic abnormalities (complex karyotype) [60]. The cytogenetic abnormalities which are define as the group of unfavorable risk is known to associate with a poor prognosis, with patients rarely surviving beyond 1 year, and a high risk of relapse after treatment [61-63].

Table 1.4 Cytogenetic-based risk stratification

Risk	**Chromosomal aberration**
Favorable risk	t(15;17)(q22;q12-21), t(8:21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22)
Intermediate risk	Normal karyotype, t(9;11)(p22;q23), del(7q), del(9q), del(11q), del(20q), -Y, +8, +11, +13, +21
Unfavorable risk	Complex karyotype, inv(3)(q21q26)/t(3;3)(q21;q26), t(6;9)(p23;q34), t(6;11)(q27;q23), t(11;19)(q23;p13.1), del(5q), -5, -7

Cytogenetics and age have a close relationship. Adverse cytogenetic abnormalities increase with increasing age and, within each cytogenetic risk group, prognosis with standard treatment worsens with increasing age. Multidrug resistance occurred in 33% of AML patients below the age of 56 as compared to 57% in patients older than 75 years. The percentage of patients with favorable cytogenetics dropped from 17% in those younger than 56 to 4% in those older than 75 years, while the proportion of patients with unfavorable cytogenetics increased from 35% to 51% [51]. This cytogenetic information is the important tool to classify patients at their initial diagnosis. Currently, favorable risks AML patients are usually

treated with contemporary chemotherapy while poor risk AML patients receive allogeneic stem cell transplantation if suitable stem cell donors exist [7].

Moreover, the importance of gene mutations in leukemogenesis has also found in many cases of cytogenetically normal AML [64, 65]. In cytogenetically abnormal as well as in cytogenetically normal AML, these newly discovered genetic abnormalities may be associated with clinical, morphologic, and/or phenotypic features [66]. The mutations of genes such as *FLT3* that encode proteins involved in signal transduction pathways in leukemic cells may lead to worse prognosis. The prognosis of AML patients with absence of cytogenetic abnormalities is divided into favorable, intermediate and unfavorable subgroups (Table 1.5) [7, 67].

Table 1.5 Genetic abnormalities in normal cytogenetic AML

Name	**Prognosis**	**Prevalence (%)**
NPM-1	Favorable	50–60
CEBPα	Favorable	15–20
FLT3-ITD	Unfavorable	30–40
FLT3-Asp835	Unclear	5–10
BAALC	Unfavorable	65.7
MN1	Unfavorable	50
MLL-PTD	Unfavorable	7.7
ERG-1	Unfavorable	25
AF1q	Unfavorable	75

For the important prognostic mutations and most frequent in cytogenetically normal AML are mutated nucleophosmin 1 (NPM1), FMS-like tyrosine kinase 3 (FLT3), and CCAAT/enhancer binding protein alpha (CEBPA). The NPM1 mutation is about 50 to 60% of cytogenetically normal AML. Mutated NPM1 is reported to be associated with approximately 50% survival at 4 years [66, 68]. FLT3 internal tandem duplications (ITD) has found nearly 30 to 40% of cytogenetically normal

AML [69]. FLT3-ITD is associated with a poor clinical outcome and a survival rate of only 20 to 25% at 4 years. CEBPA mutations are reported in around 15% of cytogenetically normal AML cases and 4 year survival rate in these patients are approximately 60%. When NPM1 and FLT3-ITD status are considered together, patients who have mutated NPM1 and absence of FLT3-ITD have a 4 year survival similar to that of the CEBPA-mutated cases at approximately 60%, whereas the remaining cases, being either NPM1 wild-type or FLT3- ITD–positive or both, have a dismal 30% survival rate at 4 years [66, 68]. Gene mutations studies have significant prognostic impact that can help identify genetic lesions associated with treatment failure, and allows discrimination of patients who may be candidates for alternative therapies [67]. Furthermore, some of the genetic abnormalities have also been found to be useful for minimum residual disease monitoring (MRD) and as potential therapeutic targets.

1.3.7 Standard treatment of AML

The treatment of AML has generally consisted of the combination of an anthracycline, such as daunorubicin or idarubicin, and cytarabine [47, 70]. Therapy consists of two phases. The first attempts to produce complete remission, defined as a marrow with less than 5% blasts, a neutrophil count greater than 1000, and a platelet count greater than 100,000. Complete remission is the only response that leads to cure or at the least to an extension in survival. The second phase of therapy aims to prolong the complete remission. Induction chemotherapy with 7 days of continuous intravenous infusion of cytarabine 100 mg/m^2 and 3 days of daunorubicin 45 to 60 mg/m^2, with a complete response (CR) rate between 45% and 60% [9, 48, 53].

1.3.8 FMS-like tyrosine kinase 3 (FLT3) receptor

FLT3, also known as fetal liver kinase-2 (FLK-2) and human stem cell kinase-1 (STK-1), was cloned independently by 2 groups in 1991 [16, 71]. FLT3 is a membrane-bound receptor and belongs to the class III subfamily

of receptor tyrosine kinase (RTK), which includes structurally similar members such as c-FMS, c-KIT, and platelet-derived growth factor (PDGF) receptor. FLT3 is consisted of an extracellular domain comprised of 5 immunoglobulinlike (Ig-like) domains, a transmembrane domain, a juxta-membrane domain, and a intracellular kinase domain interrupted by a kinase insert (Figure 1.2) [19, 23, 72, 73].

FLT3 is encoded by the *FLT3* gene located on chromosome 13q12 and comprises 24 exons [74, 75]. The protein encoded is a transmembrane receptor of 933 amino acids in the human and expressed in immature hematopoietic cells, placenta, gonads, and brain [76, 77]. The studies of FLT3 expression using immunoprecipitation demonstrate that FLT3 is expressed in 2 forms: a 160 kDa membrane-bound, fully N-glycosylated mature form and a 130-140 kDa incompletely glycosylated, mannose-rich immature form [19, 76, 78].

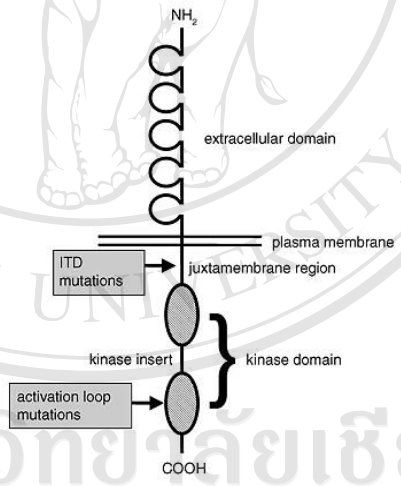

Figure 1.2 Schematic of the structure of the FLT3 receptor [79]

In normal tissues, FLT3 receptor is expressed commonly in CD34+ hematopoietic stem cells and immature hematopoietic progenitors, including the myeloid precursors and monocytes, B-lymphoid progenitors, and in dendritic cell progenitors in normal human bone marrow [17, 22, 73, 76, 80]. For the studies of FLT3 or FLT3-ligand in mice, FLT3 signaling appears to

be playing an important role in the regulating processes of early hematopoiesis [80, 81]. Moreover, FLT3 expression is also found in spleen, liver, thymus, lymph nodes and placenta, and blood-forming organs such as gonads and brain, where its function is unknown [76, 82].

In bone marrow, stromal cells and many haematopoietic cell types can produce FLT3-ligand (FL) in soluble and membrane-bound forms [83, 84]. Binding of ligand to FLT3 receptor induces dimerization of the receptor and activation of the kinase domains, which then autophosphorylate and catalyse phosphorylation of substrate proteins [85]. The activated signaling proteins by FL stimulation of the FLT3 receptor are including signal transducer and activator of transcription 5 (STAT5), phosphatidyl initosol-3-kinase (PI3K), phospholipase C-gamma (PLC-γ), RAS, mitogen activated protein kinase (MAPK), extracellular-signal regulated kinase (ERK1/2), SHC, Src-homology 2 containing protein tyrosine phosphate (SHP2), and Src-homology 2 containing inositol phosphatase (SHIP) [86, 87]. These pathways are involved in cellular proliferation, differentiation and survival. A schematic of some of the FLT3 signaling pathways is shown in Figure 1.3.

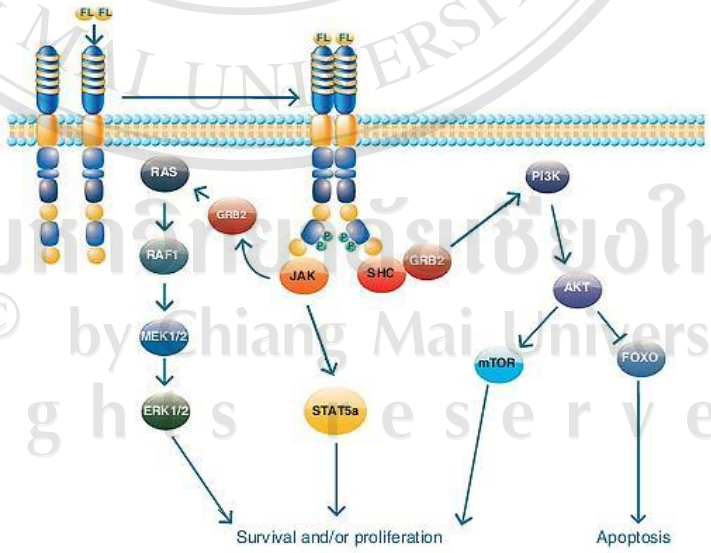

Figure 1.3 Signaling pathways initiated by FLT3 ligand (FL) activation of the FLT3 receptor [15].

In human leukemia, FLT3 is expressed at various levels in most human leukemia, including nearly 100% of B-lineage ALL, greater than 90% of AML, and a smaller percentage of T-lineage ALL, CML in blast crisis and CLL [22, 88, 89]. FLT3 is also aberrantly expressed in a similar proportion of cell lines derived from human leukemia. Interestingly, co-expression of FLT3 and FLT3-ligand (FL) is demonstrated in almost leukemic cell lines, and also commonly in primary AML blasts [90]. Many data indicated that FLT3 signaling pathways might be important in some subtypes of human leukemia, and that lead to FLT3 expression may play a role in the proliferation and/or survival of leukemic blast cells [79]. Consequently, FLT3 is increasingly recognized as a biomarker in the disease progression. FLT3 protein is usually overexpressed in leukemic blast cells of AML patients, while in some cases of AML patients possessed FLT3 mutation [19]. Recently data suggest that very high levels of FLT3-wild-type expression may promote constitutive activation of the wild-type receptor in malignant cells, and may be associated with a worse prognosis [23, 27].

1.3.9 FLT3 mutations

While overexpression of FLT3 wild-type protein with its ligand is one potential mechanism of FLT3 involvement in leukemia, the FLT3 activating mutations have been found to be a stronger case for the importance of this gene. FLT3 is the most commonly mutated gene in AML with the mutation occurring in approximately 30 to 40% of AML patients [69]. These mutations are identified into two distinct mutations in juxtamembrane and kinase domains of the *FLT3* gene (Figure 1.2).

1) FLT3 internal tandem duplication (ITD) mutations

The first study reported FLT3-ITD in 1996, and suggested that these mutations might play an important role in pathogenesis of AML [24]. ITD occurs in the juxtamembrane region of the receptor. It is duplications of variable numbers of base pairs, and the most common

of these is on exon 14 of the *FLT3* gene, which varies from 3 to >400 base pairs [91]. This results in the insertion of repeated amino acid sequences in JM domain. FLT3-ITD disturbs the autoinhibitory function of the juxtamembrane domain, resulting in autophosphorylation of FLT3 and activation of its downstream effectors [19, 91]. FLT3-ITD in pediatric AML is around 10 to 15%, and in ALL, FLT3-ITD is rarely detected [24, 92, 93]. In addition, the frequency of FLT3-ITD appears to be higher in elderly patients with AML [94]. The presence of FLT3-ITD is approximately 20% in young adults, and >35% in AML patients older than 55 years [95]. FLT3-ITDs have been detected in all FAB subtypes of AML. FLT3 mutations are the most common in hematologic malignancies, occurring in CML (5-10%), MDS (5-10%), and AML (15-35%) patients [24, 93-96]. FLT3-ITDs have not been detected in limited numbers of patients with juvenile myelomonocytic leukemia (JMML) [93], non-Hodgkin lymphoma, adult T-cell ALL, CLL, or multiple myeloma [97].

In addition to the FLT3-ITD and mutant allele, the patients with FLT3-ITD and/or patients who lack the wild-type allele has been demonstrated to have worse prognosis significance [98, 99]. As FLT3-ITD is an adverse prognostic factor, it has been speculated that patients with this genetic abnormality should be considered for more intensive therapy.

2) FLT3 tyrosine kinase domain mutations (TKD)

The presence of point mutations in the activation loop of FLT3 were reported in patients with AML [26]. In generally, tyrosine kinase is in the "inactive" state, it functions to block access of adenosine triphosphate (ATP) and substrate to the kinase domain. When tyrosine kinase domain is activated by typically ligand binding, the loop converts to the "activated" form leading to phosphorylation within the loop in tyrosine kinase domain. FLT3-TKD mutations

cause substitutions of other amino acids (most commonly tyrosine) for the aspartate at position 835 or the isoleucine at position 836 [25, 26]. More recently, novel activation loop mutations such as insertion of a glycine and a serine residue between positions 840 and other have been reported [25, 26, 96, 100] and given in Table 1.6.

FLT3 kinase domain mutations have been found in patients with ALL (1-3%), MDS (2-5%), and AML (5-10%) [26, 95, 96]. In contrast to FLT3-ITDs, the prevalence of activation loop mutations seems to be constant across all age ranges [95]. Several studies indicate that the activation loop of FLT3 is also an adverse prognostic indicator while the clinical significance of activation loop FLT3 mutation is not clear [94, 101].

Table 1.6 Activation loop mutations of FLT3 kinase domain [79]

Designation	**Description**
D835Y	Asp-> Tyr
D835V	Asp-> Val
D835E	Asp-> Glu
D835H	Asp-> His
D835N	Asp-> Asn
D835G	Asp-> Gly
D835A	Asp-> Ala
D835L+K	Asp-> Leu+Lys
Δ835	Deletion of Asp
I836T	Ile-> Thr
I836S	Ile-> Ser
I836L+D	Ile-> Leu+Asp
I836M+R	Ile-> Met+Arg
Δ836	Deletion of Ile
840+GS	Insertion of Gly and Ser between Ser (840) and Asn (841)

For the mechanism of FLT3 activation by mutations, the juxtamembrane domain and the activation loop of the tyrosine kinase domain display an autoinhibitory regulation of FLT3 receptor. After activating, the autoinhibition is restricted, leading to activation. The hypothesis of FLT3-ITD and point mutations are abrogation of autoinhibitory function by autophosphorylation of FLT3 receptor, resulting in constitutive activation of the tyrosine kinase activity that is independent of ligand binding [19, 91]. Although, the pathway of FLT3 activating mutations leading to leukemia is not completely clear [87], the experimental data suggests that FLT3 signaling may contribute *via* enhancing proliferation, inhibiting apoptosis and blocking differentiation.

1.3.10 FLT3 signal transduction

1) Ras/MAPK pathway

Ras and MAPK display in FLT3 signaling pathway that involved in cell the proliferative pathway. Ba/F3 cells transfected with human wild-type FLT3 and activated with FL showed transient activation of MAPK, while Ba/F3 cells and 32D cells which transfected with FLT3-ITD show constitutive phosphorylation of MAPK [102]. MAPK is also demonstrated constitutively phosphorylation in many cases of AML blasts [103]. The Ras protein plays an important role by activation of intermediate kinases, resulting in the phosphorylation in FLT3-ITD–mediated transformation of 32D cells [104].

2) Jaks/STAT pathway

The Janus-activated kinases (Jaks) play an important role in signal transduction pathways of proliferation, differentiation, cell survival, and apoptosis. After activating of Jaks cause activating signal transducer and activator of transcription (STAT) proteins. STAT proteins are involved in signal transduction by cytokines,

hormones, and growth factors [87]. Many studies have reported the constitutive activation of STAT5 by ITD mutated FLT3 receptors, but not by ligand-stimulated FLT3-WT receptors, in myeloid and lymphoid cell lines and in primary murine bone marrow [102, 104-106] (Figure 1.4). The serine/threonine kinases PIM-1 and PIM-2 which is STAT5 target genes have also been found to be upregulated by FLT3-ITD, and have been played to be involved in proliferation and anti-apoptosis pathways in myeloid malignancies [107]. In contrast resulting to FLT3-ITD, STAT5 has shown very weakly activated by FLT3-TKD mutant receptors, and followed by a weak induction of STAT target genes [105, 108]. Although data are available for STAT5 in AML blasts, more study needs to determine the role of STAT5 in leukemic progenitor cells. The interpretation of STAT5 activation with FLT3-ITD may be an interesting new drug target in AML.

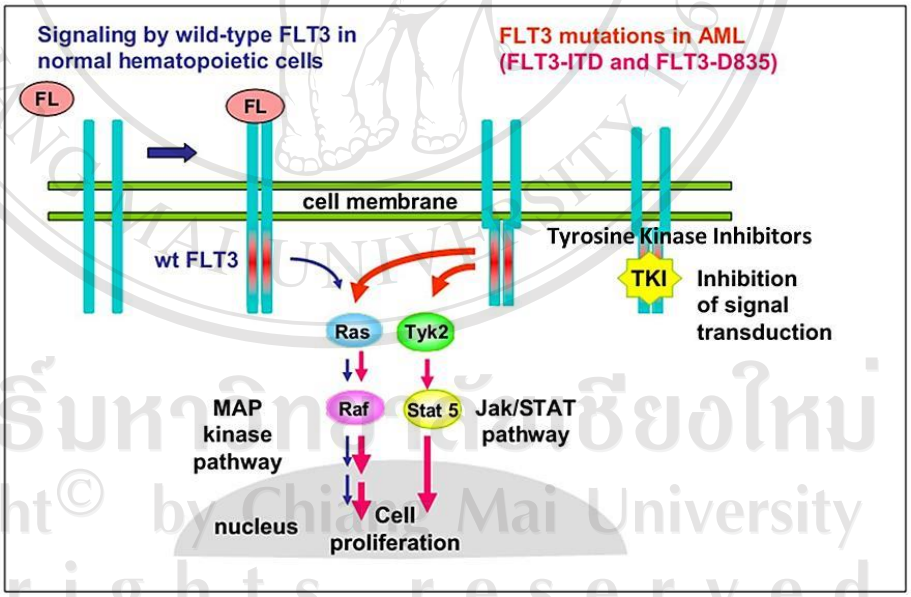

Figure 1.4 FLT3 signal transduction. Intracellular signaling is different in cells expressing the wild-type FLT3 versus mutant FLT3 [13].

3) Phosphoinositol 3 kinase (PI3K)

Phosphoinositol 3 kinases (PI3Ks) are involvement of the anti-apoptotic pathway in intracellular signal transduction through both FLT3 wild-type and ITD receptors [87]. The p85 subunit of PI3K also plays role in activation of FLT3 wild-type receptor by ligand, and relates to a complex of adaptor proteins, including SHIP, SHP-2, GAB2, and CBL [109]. Akt, a serine/threonine protein kinase down-stream of PI3K, has been shown to be constitutively activated in ITD-transfected 32D cells, and involved in multiple downstream biological functions. In one study demonstrated that FLT3 ITD receptors may retain the ability to be activated by FL [104].

4) Adaptor proteins [87]

Although, adaptor proteins (Gab1, Gab2, SHP-1, SHP-2, c-Cbl, VAV, GAP, PLCɤ, and SHIP) are reported to be involved in FLT3 signaling, the functions of these adaptor proteins and their contribution in leukemic cells with mutated FLT3 have not been studied.

1.3.11 Prognostic significance of *FLT3* gene

Several data indicate that both pediatric and adult AML patients with FLT3 mutations, particularly ITDs, have a worse prognosis than those without mutations. In pediatric studies, a poor prognosis was reported [110, 111]. In study that reported on 91 pediatric patients treated in the Children's Cooperative Group (CCG) presented about 17% of FLT3-ITD mutation. There was 40% versus 73% remission induction rate in FLT3-ITD patients compared to non FLT3-ITD patients (p = 0.005), and the 8-year event-free survival (EFS) was 7% versus 44%, respectively (p = 0.002) [111]. In adult AML, FLT3-ITD mutations also involve a poor prognosis. Several studies indicated that the patients with under the age of 60 have a worse prognosis [98, 99]. The data based on a study of 854 AML patients in the United Kingdom Medical Research Council demonstrated that FLT3-

ITD was 27%. The patients with FLT3-ITD had a 74% relapse rate versus 48% for ITD-negative group [99]. FLT3-ITD correlated with higher leukocyte and blast counts, with remission induction rates of 74% in the ITD-negative group and 40% in the ITD-positive group, 5-year overall survival (OS) rate was 32% for patients with FLT3-ITD AML versus 44% for patients with FLT3-wild-type AML [99]. The disease-free survival (DFS) was 20 versus 41 weeks in the ITD-negative and positive group, respectively ($p < 0.00001$) [112]. Similar results of DFS and OS were reported in a smaller study (82 AML patients with FLT3-ITD), and the study also played the evidence that FLT3-ITD patients with loss of the residual allele of FLT3 had a worse prognosis ($p = 0.008$) [98]. The studies elderly patients with AML reported that more elderly patients had a higher frequency of FLT3-ITD, and the patients over age 60 with FLT3-ITDs had a trend toward significantly worse prognosis [94, 113]. Further, the size of the internal tandem duplication has revealed significantly poor prognosis. The duplication can range in size from three to hundreds of nucleotides, and longer duplications correlate with a worse OS [114]. In addition to the mutant allele, AML patients with loss of wild-type allele have a worse prognosis [98, 99]. When the ratio of the mutant to wild-type level of FLT3-ITD has a greater than 0.78, the OS and DFS were significant shorter than those with a lower ratio. The OS and DFS in patients with a lower ratio were the same in the group of patients without FLT3 abnormalities [96]. In FLT3 activating point mutations, the significantly worse prognosis is not clear.

1.3.12 Prognostic of FLT3 protein

The overexpression of FLT3 protein in the wild type and mutation has also been observed in AML patients. FLT3 overexpression in the absence of ITD has demonstrated an unfavorable prognostic factor for OS [27]. Recent study of FLT3 protein expression in 144 AML patients using flow cytometry, FLT3 overexpression was found in 62% of AML patients. The higher incidence of FLT3 overexpression in FLT3-ITD group was noted in

the age group of 15 to 59 years and in males. In FLT3-wild type group, significant higher incidence of FLT3 overexpression was indicated in WBC count > 11,000, and correlated with aggressive disease [28]. Two studies have shown a higher incidence of FLT3-ITD mutation was seen in FLT3 mRNA transcript and FLT3 overexpressing subgroups [27, 115]. Furthermore, high FLT3 mRNA expression level was reported in AML patients with FLT3 overexpression [88, 116]. FLT3 protein expression level was significance correlated with clinical outcome. The overexpression of FLT3 protein correlated with increasing of relapse rate with shorter DFS. Recently, FLT3 protein expression is equally important in predicting clinical outcome, and can be potential prognostic value in AML [27, 116].

1.3.13 FLT3 inhibitors

It has become clear that the presence of FLT3-ITD, which is a key indicator of worse prognosis, and recently studies found that increased FLT3 protein expression in leukemic blast cells may be related with a worse prognosis [27, 28]. Therefore, FLT3 is an important prognostic biomarker used for determining the progression of AML, and lead to identify the high risk group of patients which can be targeted by designing new therapies that include FLT3 inhibitor [28].

Several small-molecule inhibitors of FLT3 currently in development demonstrate several of clinical efficacies (Table 1.7). The affinity for FLT3 is use for classification of FLT3 inhibitor. The midostaurin, lestaurtinib (CEP701) and sorafenib are grouped as multitargeted tyrosine kinases, and other such as quizartinib (AC220) and tandutinib (MLN518) considered more specific for FLT3 with less clinically advanced agents. However, the efficacy of FLT3 inhibitors were disappointed in complete remissions [13, 15], causing drug-related factors such as potency for FLT3 inhibition, pharmacokinetic properties and the FLT3 ligand's ability to influence the activity of kinase inhibitors [15, 117].

The current focus on FLT3 inhibitor is in combination with chemotherapy. The preclinical data show synergy when FLT3 inhibitors are combined (but not used first) with chemotherapy [118]. Finally, preclinical data that will support the combination of FLT3 inhibitors with other relevant biologically targeted therapy are being developed.

Table 1.7 FLT3-targeted therapies currently in clinical development [15].

Agent	Efficacy
Midostaurin (PKC412) Phase 3	In combination with standard chemotherapy in AML with FLT3 mutant and WT. CR: 92% vs 74% ; similar rates of 1 year (85% vs 81%) and 2-year (62% vs 59%) OS in FLT3-ITD vs WT.
Lestaurtinib (CEP701) Phase 2	In combination with standard chemotherapy in AML with FLT3-mutant in first relapse: no difference in CR or OS compared with standard chemotherapy alone.
Sorafenib Phase 2	In combination with standard chemotherapy in AML with FLT3 mutant and WT: no differences in EFS, OS or rates of CR with sorafenib vs chemotherapy alone.
Quizartinib (AC220) Phase 2	Monotherapy in AML with relapsed/refractory FLT3-ITD: composite CR rate was 43%.
Tandutinib (MLN518) Phase 1	Monotherapy in AML or MDS: antileukemic response occurred in 2 of 8 AML with FLT3-ITD mutations.

1.3.14 Methods for detecting the expression of FLT3

Different methods have been used to evaluate the expression of FLT3 levels. For FLT3 protein detections, a flow cytometry and Western blot analysis are the classical techniques to be used. In addition to protein analysis, determination of *FLT3* gene expression by Reverse Transcription–Polymerase Chain Reaction or RT-PCR assay is commonly used in research methods for studying the genome.

1) Western blot analysis [30, 119]

Western blot analysis is a widely accepted technique used for specific protein detection or immunodetection. The procedures of Western blotting are formed of four steps (Figure 1.5).

For samples preparation, interested protein is extracted from different kind of samples, such as tissue or cells. Cells can be extracted by lysis buffers. Protease inhibitors are often added to prevent the digestion of protein by enzymes. Sample preparation would be done in cold temperatures to avoid protein denaturing and degradation. Then the extracted proteins are measured for normalized proteins content.

The proteins are separated using sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). Separation of proteins depends on the nature of the gel and sample treatment. SDS-PAGE maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are separated according to size. Acrylamide is used to separate low and high molecular weight of proteins base on concentration. The concentration of acrylamide determines the resolution of the gel. 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. A marker or ladder, a commercially available mixture of proteins, typically stained so as to form visible, colored bands is used for defined molecular weights.

The proteins within the gel are moved onto the membrane by electric current to make them accessible to antibody detection. As a result of blotting process, the proteins are exposed on membrane for detection. Blocking of non-specific binding is achieved by soaking the membrane in 3-5% bovine serum albumin (BSA) or non-fat dry milk (skim milk) in phosphate-buffered saline (PBS). The protein in the dilute solution attaches to the membrane in all places except the specific proteins. Thus, when the antibody is added, there is no non-specific binding on the membrane. This process reduces "noise" in Western blotting, leading to eliminate false positives, and clearing results.

During the detection process the membrane, the targeted protein is incubated with specific monoclonal antibody, and then exposed to the labeled antibody, usually with an enzyme such as horseradish peroxidase (HRP). The signal is produced by the enhanced chemiluminescence detection corresponding to the position of the targeted protein. This signal is captured on a film which is usually developed in a dark room. Finally, protein bands are visualized and analyzed by densitometer.

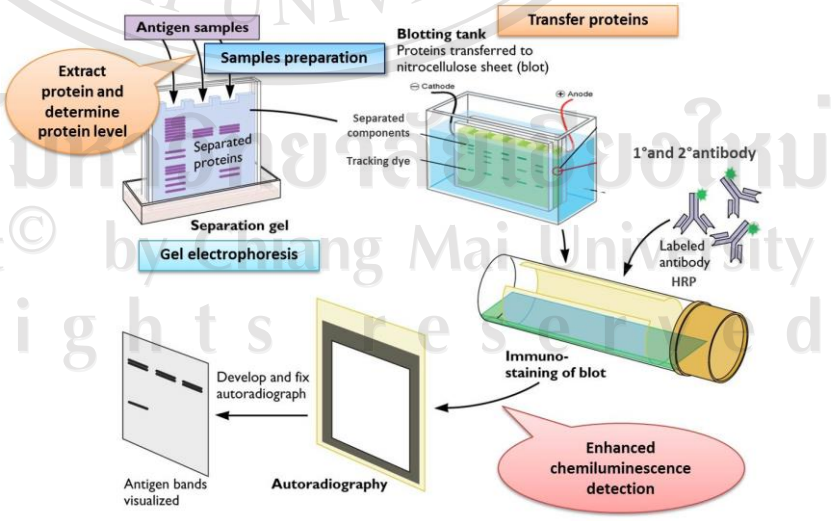

Figure 1.5 Schematic of a Western blot analysis [120].

2) Flow cytometry [29, 121]

In cell biology, flow cytometry is a biophysical technology that has many applications in basic research, clinical practice and clinical trials including cell counting, cell sorting, biomarker detection and protein engineering. Recently, flow cytometry has been used in routine diagnosis of disorders, especially blood cancers. Flow cytometry measures optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, and chromosome preparations) by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. Physical properties, such as size and internal complexity are represented by forward angle light scatter, and right-angle scatter, respectively. Additionally, antibodies conjugated to fluorescent dyes can bind to specific proteins on extracellular cell membranes or inside cells such as cytoplasmic protein, and DNA or RNA. The fluorescein, propidium iodide (PI), and phycoerythrin (PE) dyes are common used for labeling cells or molecules. When cells were analyzed by flow cytometer, cells in suspension are drawn into a stream created by isotonic sheath fluid that creates laminar flow. The cells allow in an interrogation point, and a beam of monochromatic light (usually from a laser) intersects the cells. Emitted light is given off in all directions and is collected *via* optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals from fluorescently labeled antibodies are detected by photomultiplier tubes and digitized for computer analysis (Figure 1.6), and the resulting information is usually displayed in histogram or dot-plot formats.

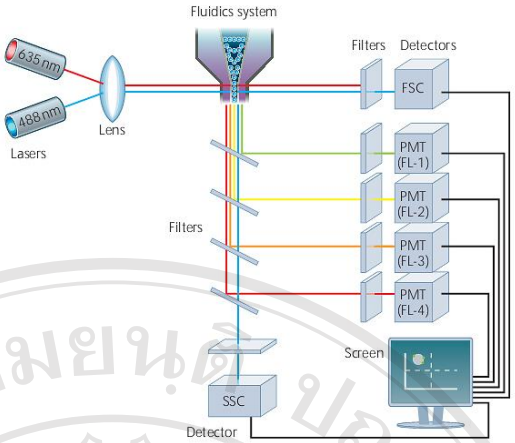

Figure 1.6 A schematic overview of a flow cytometer diagram [121]

3) Polymerase Chain Reaction (PCR) [31, 122]

The polymerase chain reaction (PCR) is the most common technique that is used for nucleic acid analysis with highly specific and sensitive. This method relies on the exponential amplification of specific DNA fragments, resulting in millions of copies of templates for using in analysis. PCR is the enzymatic amplification of a specific DNA sequence, and this process uses multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences. It is an exponential process since the amplified products from each previous cycle serve as templates for the next cycle of amplification, thus making it a highly sensitive technique for the detection of specific nucleic acids sequences, and using very small amounts of protein samples for analysis.

A single DNA strand is used as template for the synthesis of new complementary chains using DNA polymerase enzyme. Taq DNA polymerase is common used, which is able to bind the nucleotides present in reaction to the template. Additionally, the one of many variants of PCR is reverse transcription polymerase chain reaction (RT-PCR). For analyzing gene expression, RT-PCR technique is commonly used to detect RNA expression levels through

creation of cDNA transcripts from RNA using a reverse transcriptase (Figure 1.7).

In amplification procedure, there are three steps in each cycle including annealing of primers to the DNA template, extension of the DNA, and protein denaturation. First, a polymerase enzyme will copy a DNA strand starting from the 3′ end of the primer and synthesizing new DNA in a 5′ to 3′ direction. DNA extension is stopped by raising the temperature (usually to 94ºC), causing separation of all DNA strands. Cooling to temperatures of 37-60ºC causes annealing of the primer and DNA template. Raising the temperature again (typically to 72ºC) stimulates the Taq polymerase to copy the DNA template in a further extension step. Typically amplified product is about 20 to 40 cycles of PCR. After amplification, the products are size-separated by electrophoresis on agarose or polyacrylamide gel stained with ethidium bromide. Products appear as bands corresponding to the sizes as measured in base pairs (bp).

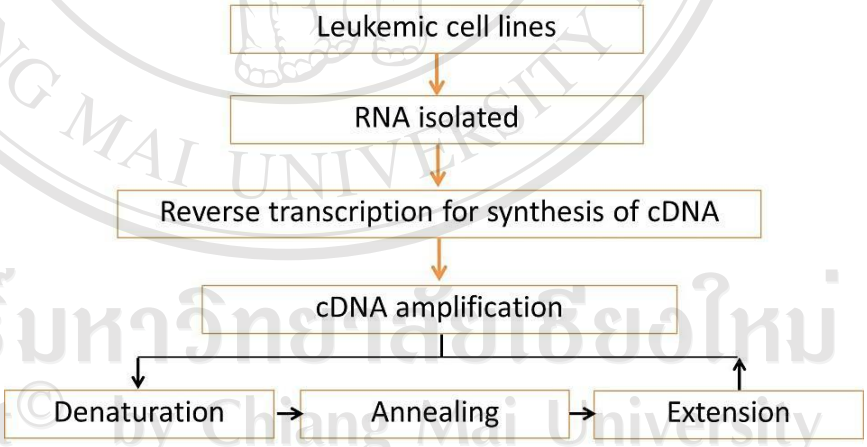

Figure 1.7 Schematic diagram of RT-PCR [31]

1.3.15 Method validation [123]

Method validation is the process used to confirm that the analytical procedure for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, and reliability of analytical results. Analytical methods need to be validated before their introduction into routine use, the changed conditions in the method (e.g. an instrument with different characteristics or samples with a different matrix), and when the method is changed and the change is outside the original scope of the method.

Bioanalytical methods are used for the quantitative determination of drugs and/or their metabolites in biological matrices and biomarkers. The reliability of analytical findings is a matter of great importance in clinical researches. The results from unreliable method could also lead to wrong treatment of the patients. It is essential to validate bioanalytical methods to yield reliable results. Validating bioanalytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum, or urine) is reliable and reproducible for the intended use. The validation of bioanalytical method was published in many regulatory bodies. The United State Food and Drug Administration (U.S.FDA) was published the performance validation that would be applied to bioanalytical procedures. Typical bioanalytical validation parameters which should be considered are listed following:

1) Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

2) Precision is a measure of the reliability of the method to generate reproducible results. The samples should be showed the closeness of their replicated measurement results.

3) Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

4) Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. It is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision (Lower limit of quantitation; LLOQ).

5) Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

6) 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.